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Molecular and Genetic Analysis of *disconnected*, a Gene Required for Proper Visual System Development in *Drosophila*

by

Kevin J. Lee

B. S., Cellular and Molecular Biology
University of Michigan, 1984

Submitted to the Department of Biology in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
in Biology

at the

Massachusetts Institute of Technology
February, 1994

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Signature of Author___
Department of Biology
December 9, 1993

Certified by___
Hermann Steller
Associate Professor of Biology
Thesis supervisor

Accepted by___
Frank Solomon
Chairman of the Graduate Committee
Department of Biology

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Abstract

The work presented in this thesis addresses the molecular mechanisms which underlie the formation of ordered axonal pathways and neuronal connections during nervous system development. These studies are focused on the development of a simple neuronal circuit, the *Drosophila* larval visual system. This thesis describes a molecular genetic analysis of the *Drosophila disconnected (disco)* gene aimed at exploring its role in the development of the larval visual pathway.

Mutations in the *disco* gene prevent the larval optic nerve from forming appropriate connections with its target cells in the brain. Several lines of evidence suggest that the *disco* gene product is a regulator of gene expression, possibly a transcription factor. *disco* encodes a nuclear protein with two zinc finger sequences, a structural motif found in a variety of nucleic acid-binding regulatory proteins. The importance of these zinc finger domains to *disco* protein function is indicated by the evolutionary conservation of these sequences and the observation that *disco* loss-of-function alleles have alterations in these zinc finger sequences. Moreover, a portion of the *disco* protein including these zinc finger sequences has sequence-specific DNA binding activity *in vitro.*

The *disco* gene is expressed in a highly complex pattern in a subset of tissues throughout the life cycle. Of particular relevance to visual system development is expression of the *disco* gene in the target region of the larval optic nerve. Interestingly, the *disco* gene product regulates its own mRNA expression in this target region in a highly cell type-specific manner. High affinity *disco* protein binding sites are found in the *disco* promoter region, suggesting that the autoregulation of *disco* expression may occur by a direct interaction of the *disco* protein with cis-acting regulatory elements in the *disco* promoter. Furthermore, ectopic expression of the *disco* gene during embryogenesis results in developmental defects and embryonic lethality. These defects include severe perturbations in axonal pathways and connectivity throughout the nervous system.

In wild type *Drosophila,* the projection of larval optic nerve develops in a series of discrete steps. During embryonic development of the visual system, the larval optic nerve contacts a group of identified neurons and a number of glia. The *disco* gene is expressed in these cells and *disco* function is required for their proper differentiation. These results suggest that the defects in the larval optic nerve development in *disco* mutants may result from the absence of critical cellular interactions which direct neuronal connectivity.

Thesis Supervisor: Dr. Hermann Steller
Title: Associate Professor of Biology

Acknowledgments

I sincerely thank Hermann Steller for his enthusiasm, his support and for being a wonderful and good-hearted mentor. I also thank the members of my thesis committee, Ruth Lehmann, Richard Hynes, Michael Rosbash and Terry Orr-Weaver, for their comments and their interest. Special commendations go to Ruth Lehmann for a week of thorough Stellerization.

I especially thank Ana Regina Campos for almost everything - for being a collaborator, a teacher and a great friend.

I also thank my other roommates, Clive Wilson and Megan Grether, for exceedingly valuable advice and discussions, for moral and emotional support and for many happy days. I thank Clive, Megan and Deborah Goberdhan for loyal friendship, and for tea and sympathy.

I thank other members of the Steller lab, and my friends and colleagues everywhere. In particular, I thank Ben, Ljuba, Kei, Rob and Katie, Anna and Stefan, especially for their help in this last rather harrowing year.

I thank former my former mentors and teachers, especially Lily Jan, who took me in off the streets, Tom Schwartz, who first introduced me to the wonders of molecular biology, and Ethan Bier, a great collaborator and a genuine friend.

I thank Kathleen for many great times. I thank Delphine for unwavering loyalty.

Finally, I thank my family for their undying love, support and patience.

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Chapter 1.
Introduction

One characteristic feature of the nervous system which has long captivated biologists is the remarkable complexity and precision with which neurons and their target cells are connected in vast networks. The accuracy of these connections is, of course, critical for proper functioning of the nervous system. Understanding how the patterns of neural connectivity are controlled and specified is a major goal in developmental neurobiology.

It is now widely accepted that the final pattern of cellular interconnections in a nervous system is the product of growth and development prior to the onset of neural activity as well as "editing" and modification occurring after neural activity commences. To what extent each of these aspects of cellular modeling contributes to the formation of precise networks of neural connections has been, for many years, the subject of considerable debate [for example, (Easter et al., 1985; Sperry, 1963; reviewed in Goodman and Schatz, 1993)]. However, it has been shown that in numerous vertebrate and invertebrate contexts, the initial connections formed by developing neurons are extremely accurate. Moreover, when neural activity is blocked by pharmacological agents or by mutation, patterned neural connections can be formed (Harris, 1984; Westerfield et al., 1990). Thus at least part of the specification of neuronal connectivity is determined by interactions between growing neurons and their environment during development.

The formation of patterned neuronal connections depends on a number of different phenomena occurring at various times during development. These different phenomena are often interrelated, and may or may not occur sequentially. First, neurons and target cells are generated and acquire specific identities. Second, these cells may migrate over considerable distances to reach their final location. Third, axonal processes which extend from developing neurons are influenced by guidance cues to choose specific pathways. Fourth, neuronal processes find and recognize defined targets and synaptic connections are formed between specific cells. Finally, programmed cell death and synaptic remodeling may further refine patterns of cellular interconnections. Molecular mechanisms which are responsible for these phenomena have been the subject of considerable study; a wealth of information has accumulated, but, to a great extent, much of this evidence is circumstantial. In addition, it has been difficult to synthesize the often contradictory details into a coherent whole. It can be argued that much of the difficulty in the study of neuronal patterning relates to the large number of interrelated developmental phenomena which influence the final organization of the nervous system.

The studies presented in this thesis rely on a genetic approach to identify molecular mechanisms which regulate the formation of appropriate connections in a single, well-defined neural circuit. The power of genetic screens to identify functionally important molecules is that such methods do not rely on prior assumptions about the biochemical nature or cellular distribution of the relevant factors. Of course, the genetic approach is not without its own biases and limitations [see also (Stent, 1981)]. As an example, functions which are absolutely required earlier in development or which are partially or wholly redundant are not uncovered by this approach. However, genetics, in combination with biochemical and/or immunohistochemical methodologies, should provide a powerful means to identify many of the molecular players involved in the development of patterned neuronal connectivity.

It should be noted that genetic screens can uncover molecules which act at any step in the process leading to the final pattern of neuronal connections. Thus, neuronal connectivity can be affected by mutations in genes that encode molecules which regulate cell identity and cellular differentiation programs, cell migration, axon guidance and target cell recognition. In principle, a concerted effort concentrated on a simple system should uncover molecules acting in numerous steps in a regulatory hierarchy.

This introductory chapter will first review the current ideas about the formation of patterned neuronal connections that have arisen from studies on various invertebrate and vertebrate systems. Mechanisms and molecules responsible for the guidance of axons along specific pathways and the recognition of targets will be addressed. The molecules which have been identified in genetic screens in the two invertebrate organisms which are most amenable to classical genetic methods, *Drosophila melanogaster* and *Caenorhabditis elegans*, will then be described. The organization and the development of the Drosophila visual system, a model system which is well suited to a study of neuronal connectivity, will be discussed. Finally, the isolation and initial characterization of the *Drosophila disconnected (disco)* gene, which is required for proper neuronal connectivity in the larval visual system, will be reviewed.

One theme which will emerge from this introduction, as well as from the studies presented in subsequent chapters of this thesis, is that many of the developmental mechanisms involved in the patterning of neuronal connections are remarkably similar in both vertebrates and invertebrates. Mechanisms which were initially identified in invertebrate nervous system development have been subsequently observed in vertebrate systems. Likewise, neural development in invertebrates has been found to share many features which were first described in vertebrates. For example, recent studies on the development of the Drosophila visual system (Kunes et al., 1993) have demonstrated that this system may have more in common with its vertebrate counterpart than was previously appreciated.

Neuronal specificity and the formation of precise connections

Two classical vertebrate systems which have been used to demonstrate that neuronal connections are specified during development are the ordered projection of retinal fibers to the optic tectum (Sperry, 1963), and the selective innervation of muscles by motor neurons (Lance-Jones and Landmesser, 1981). Numerous studies on these two systems have uncovered some of the underlying mechanisms by which neuronal pathways and connections are specified.

In a series of experiments on the regeneration of the optic nerve in lower vertebrates, Sperry and co-workers demonstrated that a robust organizational mechanism specifies the orderly projection of axonal fibers from the retina to the optic tectum. The relaying of visual information from the eye to the brain follows a precise and orderly topographical map: axons from the nasal side of the retina project to the rostral tectum, axons from the ventral retina to the lateral tectum and so on. This topographical map is fundamental to the proper interpretation of visual information in the brain. Sperry showed that when the optic nerve was cut and the eye was rotated by 180°, retinal axons regenerated and projected to the position on the tectum appropriate for their orientation prior to operation. Additionally, in experiments on regeneration of retinal fragments, it was found that "fibers arising from different parts of the retina preferentially select separate central pathways as they grow to the brain, and that they eventually find, and connect with, specific predesignated target zones in the midbrain tectum." [reviewed in (Sperry, 1963)]. The results of these experiments led to the "chemoaffinity hypothesis", that some type of molecular mechanism specifies the identities of retinal axons along the nasotemporal and dorsoventral axes and this mechanism determines the recognition and selection of corresponding targets in the tectum. Sperry envisaged that similar, or perhaps even identical, chemoaffinity factors could determine the selection not only of synaptic targets in the tectum, but also the pathways or routes used to reach those targets.

The strictest version of the chemoaffinity hypothesis proposed a point-to-point mapping of retinal positions and tectal targets, in which individual points in the retina, perhaps even individual neurons, were uniquely specified to recognize a precise pathway to the tectum and a precise target position. Sperry himself recognized that such a strict interpretation would require an enormous number of distinct chemical labels to distinguish individual neurons in the nervous system. He admitted that it would be difficult to build a brain on this plan, considering the finite amount of "'bits of information' within the zygote" that would direct this development. This objection was countered by models which proposed that gradients of identity information in the retina and tectum would mark each cellular position and specify the interconnection of matching values. Similar versions of this model are still viable today and the search for gradients of positional information in the vertebrate visual system is an active area of research [for example, (Baier and Bonhoeffer, 1992; Stahl et al., 1990); reviewed in (Stirling, 1991)].

The idea that growing axons accurately select specific pathways and targets has also arisen from studies of muscle innervation by vertebrate motor neurons and from experiments on the development of identified neurons in the invertebrate nervous system (discussed in more detail below; Bate, 1976; Bentley and Keshishian, 1982; Goodman et al., 1984).

The predisposition of growing motor neurons for specific pathways and target muscles has been best demonstrated in the chick (Lance-Jones and Landmesser, 1981) and the zebrafish (Eisen et al., 1986) embryo. In the chick, groups of motor neurons located in particular positions in the spinal cord extend axons along specific nerve routes to particular muscle groups in the limbs. These axons make pathway decisions in the plexuses found at the base of the developing limbs. When segments of the spinal cord are surgically manipulated, motor neuron axons frequently project to their correct plexus from a new starting point (Lance-Jones and Landmesser, 1981). This finding demonstrates a specificity of motor neurons for particular pathways. When the surgical manipulations are drastic, however, motor neuron axons may enter inappropriate plexuses. However, even in these cases, the axons are occasionally able to find their correct target muscles without passing through the normal plexus (Lance-Jones and Landmesser, 1981). These observations suggest that motor neurons are specified for both particular nerve plexuses and target muscles.

In the zebrafish embryo, axons from primary motor neurons select specific pathways and innervate particular muscles in a highly stereotyped fashion (Eisen et al., 1986). Additionally, it has been shown that these axons are capable of making cell type-specific selections independently (Pike and Eisen, 1990).

In both the chick and the zebrafish, motor neuron axons traverse specific "choice points", where these axons select from a number possible pathways and targets. The development of an axonal projection from neuron to target unfolds in a series of discrete steps and at each step, a mechanism, which acts specifically for each neuronal type, determines axonal behavior. In at least some cases, the mechanisms operating at various steps are independent; appropriate pathways can be chosen independent of the target, as for example, when the limb bud is ablated (Tosney and Landmesser, 1984), and appropriate targets can be recognized without migrating along the normal pathway (Lance-Jones and Landmesser, 1981).

The labeled pathways hypothesis and cellular guidance cues

The grasshopper embryo is well suited to anatomical studies of axonal pathways at the level of single cells. Analysis of the formation of axonal pathways in the central nervous system (CNS) and peripheral nervous system (PNS) has revealed that individual axons in the invertebrate embryo make specific pathway choices in a stereotyped fashion (Bate, 1976; Goodman et al., 1984). These studies have led to two fundamental proposals for mechanisms that guide axons along specific pathways: first, that axons select and associate with pathways that are labeled with specific guidance cues and second, that specific intermediate targets or "guidepost" cells act to steer and orient developing axons. Although these ideas were originally formulated to explain observations made in invertebrate embryos, other studies have shown that similar mechanisms are likely to operate during vertebrate development as well.

The insect CNS is an ordered array of nerve fiber tracts in which particular axons fasciculate together in a highly invariant pattern (Goodman et al., 1984). This ordered pattern arises from the ability of individual CNS neuron growth cones to discriminate between different fascicles and make selective choices during outgrowth. For example, the axon from the G neuron has a stereotyped growth pattern: this axon extends across the CNS midline and contacts the A/P fascicle, where it turn anteriorly. In doing so, the G axon selects this fascicle in preference to numerous other longitudinal fascicles. Within the A/P fascicle, the G axon associates with the P axons and avoids the A axons. If the P axons are ablated by laser treatment, the G axon stops short and shows little affinity for the other fascicles; if instead the A axons are ablated, the G axons associates with and extends along the P axons normally (Raper et al., 1984).

These and other similar observations are the basis of the "labeled pathways" hypothesis, which predicts that specific neighboring fascicles in the embryo are differentially labeled with recognition molecules. These molecules allow growth cones to distinguish between different fascicles and to select and associate with appropriate axonal pathways (Goodman et al., 1984). Later studies have identified cell surface molecules which are expressed on specific fascicles and are therefore candidates for such recognition molecules (Bastiani et al., 1987; Elkins et al., 1990; Grenningloh et al., 1991; Harrelson and Goodman, 1988; Snow et al., 1988; Zinn et al., 1988). In some instances (Grenningloh et al., 1991), genetic analysis has supported these predictions and has demonstrated the functional significance of these molecules.

Studies on the embryonic development of the zebrafish CNS have shown that similar mechanisms may operate in vertebrates as well (Kuwada, 1986). The labeled pathways hypothesis may also explain the ability of axons from chick motor neurons to select appropriate nerve routes and plexuses as described above (Lance-Jones and Landmesser, 1981). This general principle may apply even to development of the mammalian cerebral cortex. In this system, early developing axons from cortical subplate cells pioneer the pathway from the cortex to the thalamus and other subcortical targets (McConnell et al., 1989). Later, follower axons from cortical neurons associate with these subplate fibers and may be guided along these pathways to their appropriate targets.

In the grasshopper limb, sensory neurons are born in the periphery and extend afferent axons towards the CNS. The first neurons which differentiate in the limb send out axons along a defined pathway and the pathway navigated by these pioneer fibers provides a framework for later follower axons (Bate, 1976). At specific points, the growing pioneer axons make abrupt transitions in orientation; at some of these points, the pioneer axons contact specific cells which are likely to act as intermediate targets and guidance cues. The route taken by the pioneer axons always passes over these so-called "guidepost" cells, which are immature neurons, and transient gap junctions between the pioneer fibers and the guidepost cells are observed. Laser ablation of the guidepost cells leads to misdirected growth of the pioneer axons (Bentley and Caudy, 1983).

Cells with characteristics which may be analogous to guidepost cells are also likely to act in the patterning of axonal pathways in the vertebrate nervous system. The first post-mitotic neurons of the mammalian forebrain are a transient population of cells called subplate neurons. As mentioned above, these cells pioneer the pathway from the cortex to the thalamus (McConnell et al., 1989), but they are likely to play a role in the development of the ascending pathway, from the thalamus to the cortex, as well. Axons from the thalamus arrive in the subplate area beneath their appropriate cortical target before the cortex neurons are born. The thalamic axons wait in this subplate for several weeks before invading the overlying cortical plate. Ablation of the subplate neurons, by kainate injection, prevents the ascending thalamic axons from recognizing and innervating the appropriate cortical target (Ghosh et al., 1990). In the absence of subplate cells, thalamic axons grow past the cortical region they normally invade, despite the presence of their synaptic targets in the overlying cortex. Thus, subplate cells may provide critical guidance cues which help thalamic axons to locate and recognize their correct target.

Many other types of non-neuronal cells have been implicated in the patterning of axonal pathways and in the specification of connections in various other parts of the vertebrate and invertebrate nervous systems. Glial cells located at the midline of the developing *Drosophila* CNS are thought to play a role in establishing the scaffold of CNS axonal commissures and connectives (Klämbt et al., 1991). The widespread occurrence of glia along axonal pathways in the vertebrate nervous system, and results of *in vitro* and *in vivo* studies which show that growing axons interact with glial surfaces (Neugebauer et al., 1988; Schwab and Schnell, 1991), suggests that glia may also help specify axonal pathways during vertebrate development as well. Studies on the development of motor neuron projections to limb muscles in the chick embryo have indicated that mesenchyme and connective tissue can directly or indirectly pattern nerve pathways in the nerve plexus region and in the limb (Lance-Jones and Dias, 1991; Tosney and Landmesser, 1984). In addition, somitic tissue can also specify axonal pathways, as demonstrated by the fact that axons from motor neurons will grow only through the anterior portion of the adjacent somite (Keynes and Stern, 1984).

The patterning of neuronal pathways and connections may also be influenced by distinct boundaries of specific cell groups. For example, growing axons from the pioneer neurons in the grasshopper limb make stereotyped turns at the trochanter-coxa limb segment boundary (Caudy and Bentley, 1987; Kolodkin et al., 1992). In the vertebrate spinal cord, commissural axons cross the ventral midline by passing through a specialized group of cells called the floor plate. On the contralateral side of the floor plate, they turn anteriorly and grow along the floor plate margin (Bovolenta and Dodd, 1990). The importance of the floor plate in directing this stereotyped pathway has been demonstrated by ablation studies and by analysis of mutations which affect floor plate development (Bernhardt et al., 1992; Bovolenta and Dodd, 1991).

Molecules involved in the specification of axonal pathways and neuronal connections

In recent years, the number of molecules which have been proposed to mediate mechanisms of axon guidance and target recognition has grown enormously [reviewed in (Bixby and Harris, 1991; Dodd and Jessell, 1988; Goodman and Schatz, 1993; Hortsch and Goodman, 1991; Hynes and Lander, 1992; Jessell, 1988)]. The majority of these molecules have been identified by *in vitro* assays and biochemical approaches and on the basis of distribution patterns which suggest a role in axonal patterning and specification. Interestingly, it seems that many of these molecules can be grouped into several families based on their sequence, structure and localization. In addition, similarities between molecules identified in both vertebrate and invertebrate studies are evident. This conservation suggests that the mechanisms acting in neuronal development in vertebrates and invertebrates may be similar even at the molecular level.

It has been proposed [see for example, (Letourneau, 1975)], largely on the basis of tissue culture studies, that the chemoaffinity phenomenon, and the specificity and ordering of axon pathways could be explained by differential adhesion. In this view, growth cones would be directed and contacts and connections would be formed on the basis of differences in adhesion to the substrate and the surfaces of other cells. These differences in adhesion would arise from the differential expression of various adhesion molecules and their receptors. Many molecules which may act to direct neuronal connectivity and patterning have been isolated in cell adhesion assays [for example, N-CAM (Cunningham et al., 1987)]. Other molecules identified by distribution patterns have been shown to mediate cell adhesion by a homophilic or heterophilic mechanism [for example, (Grenningloh et al., 1991; Kolodkin et al., 1992; Nose et al., 1992; Snow et al., 1989)].

Many of the molecules which might mediate selective associations between axons and the recognition of cellular guidance cues and synaptic targets are members of the immunoglobulin and cadherin superfamilies. Some of these molecules, for example N-CAM (Cunningham et al., 1987) and N-cadherin (Takeichi, 1988), are widely distributed in the nervous system and thus probably serve a very general role in neurite outgrowth and adhesion. Other molecules are much more selectively distributed and might contribute to specific recognition of axonal pathways and targets (Bastiani et al., 1987; Dodd et al., 1988; Grenningloh et al., 1991; Kolodkin et al., 1992; Nose et al., 1992). Interestingly, some of these molecules are expressed along specific segments of axonal pathways and axons can sequentially change their patterns of gene expression as they extend into different environments (Bastiani et al., 1987; Dodd et al., 1988).

Other guidance and recognition cues may be provided by extracellular matrix proteins, such as laminin, fibronectin and thrombospondin. Receptors for these extracellular cues include members of the integrin family (Hynes, 1992).

Recent experiments have indicated that the activity of cell-surface and extracellular matrix molecules on axon guidance and target recognition may involve more than simple adhesiveness. For example, in at least some contexts, the *in vitro* growth promoting and neurite guiding activity of laminin does not correlate with cell adhesion at all (Gunderson, 1987). In fact, it may be misleading to characterize these molecules as adhesion molecules; instead, it seems likely that many of them may act as signalling molecules, which provide or transduce cues that reorient the growth cone or induce other cellular changes (Lemmon et al., 1992; Schuch et al., 1989). Experiments that show *in vitro* cellular aggregation mediated by binding of cell surface molecules should be interpreted with caution. The *Drosophila* sevenless and bride-of-sevenless molecules, a cell surface receptor tyrosine kinase and a membrane bound ligand, respectively, also mediate heterophilic cell adhesion in an *in vitro* transfection assay (Krämer et al., 1991). Although they might be thus classified as adhesion molecules, genetic and cell biological data suggest that the interaction of these two molecules is responsible for the cell-cell signalling that induces R7 photoreceptor cell differentiation.

Axons might also be guided during development by gradients of diffusible chemoattractant molecules (Tessier-Lavigne and Placzek, 1991). This phenomenon was first demonstrated by *in vitro* experiments in which growth cones from chick dorsal root ganglion neurons reorient in response to soluble NGF diffusing from a micropipette (Gunderson and Barrett, 1980). More recent experiments have lent support to the idea that chemotropic guidance of axons is a physiologically relevant phenomenon. *In vitro* studies have shown that various embryonic tissues are capable of secreting factors which can attract and orient the growth of axons from neuronal populations which normally innervate these tissues *in vivo* (Heffner et al., 1990; Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988).

Molecules might also direct the formation of patterned axonal pathways and specific connections by inhibitory or repulsive signals. This phenomenon, too, was first observed in *in vitro* studies and later work has indicated that the mechanism of repulsion may play an important role *in vivo.* Inhibitory signals have been found on the surface of CNS axons (Kapfhammer and Raper, 1987), oligodendrocytes (Schwab and Schnell, 1991) and on the posterior portion of somites (Davies et al., 1990). Recently, a candidate repulsive guidance factor called collapsin has been characterized and cloned (Luo et al., 1993). This factor, a glycoprotein isolated from chick CNS, induces the collapse of sensory ganglion growth cones *in vitro.* Interestingly, the predicted protein sequence of collapsin shows significant similarity to grasshopper *fasciclin IV* (Kolodkin, et al., 1992). This finding again suggests that axon guidance mechanisms are conserved between vertebrates and invertebrates. The topographic guidance of retinal axons in the vertebrate tectum may also depend on repulsive cues. Recent experiments in this system have shown that temporal axons can respond to a gradient of a repulsive activity isolated from the tectum (Baier and Bonhoeffer, 1992). The purification of this activity has indicated that is mediated by a glycoprotein component of tectal cell membranes (Stahl et al., 1990).

Molecules and cues may act in a combinatorial fashion to specify pathways and connections

As described above, one difficulty which is inherent in some formulations of the chemoaffinity and labeled pathways hypotheses is that the number of discrete chemical labels which would be required to specify all of the different neuronal pathways and targets which can be selectively recognized is prohibitively large. One way to generate greater specificity from a limited number of labels is to use a combination of chemical "affinities" to distinguish functionally different recognition states. This idea is inherent in Sperry's intersecting gradients of positional information which specify different retinotectal mapping points (Sperry, 1963).

Both genetic data and the results from antibody blocking experiments suggest that multiple molecular mechanisms may act in combination to direct axonal growth and targeting. Single gene mutations in many of the *Drosophila* molecules which might be expected to regulate axonal fasciculation produce relatively minor, if any, phenotypic defects (Bieber et al., 1989; Elkins et al., 1990). Embryos which are mutant for both *fasI* and *abl*, the *Drosophila* homologue of the Abelson tyrosine kinase gene, display severe CNS fasciculation defects, although embryos mutant for either of these genes alone are grossly normal (Elkins et al., 1990). Similar results are obtained by vertebrate studies in which the function of adhesion molecules and ECM proteins is disrupted by antibody treatments. In these studies, much more dramatic effects on axonal outgrowth were seen when antibodies directed against several different types of molecules were applied (Bixby et al., 1987; Neugebauer et al., 1988). These results could indicate that there is a synergistic interaction between different molecular cues and mechanisms which regulate axonal growth and patterning.

Further support for the idea that combinatorial cues specify cellular interactions in nervous system development comes from the observation that many different molecules with distinct, restricted distributions on axonal tracts are expressed in overlapping patterns. For example, the fas I, fas II and fas IV surface glycoproteins label distinct but overlapping subsets of axon pathways in the grasshopper CNS (Kolodkin et al., 1992).

Finally, the existence of multiple cues which direct and specify axonal growth is suggested by the variability of growth cone behavior in response to perturbation by mutation, laser ablation or antibody application (Bernhardt et al., 1992; Harrelson and Goodman, 1988; Kolodkin et al., 1992; Raper et al., 1984). The relatively stochastic and variable projection of grasshopper sensory axons following treatment with antibodies directed against fasciclin IV, or the range of aberrant pathways taken by spinal neurons in zebrafish mutants which lack midline floor plate cells contrasts with the extremely precise behavior of these axons in untreated or wild type animals. These results imply that multiple cellular cues as well as multiple molecular cues are operating to insure fidelity of axonal patterning. Inhibiting the action of one of these cues does not prevent growth altogether or lead to consistently inaccurate growth. Instead, these alterations simply increase the frequency with which pathfinding and targeting errors occur.

Genetic approaches to neuronal pathway formation and connectivity

It is clear from the previous discussion that numerous cellular and molecular mechanisms which may be directing the formation of precise neuronal pathways and connections have been described in a variety of systems. However, it has been difficult to determine the *in vivo* role of these factors involved in neuronal pathway formation in any system. Many of the molecules which have been isolated are likely to act as general factors which promote axon outgrowth and neuronal adhesion throughout the nervous system. Other molecules are expressed on subsets of axonal pathways or in gradients across a target field, which suggests a role in specifying neuronal interactions, but further evidence to demonstrate such hypotheses is lacking.

Furthermore, the fundamental mechanism of action of these putative cellular and molecular determinants is unclear. For example, does the floor plate, which has been shown to direct the patterned growth of commissural axons (Bernhardt et al., 1992; Bovolenta and Dodd, 1990; Bovolenta and Dodd, 1991; Dodd et al., 1988), provide instructional or inductive cues to growing commissural axons, or does it exert its action by a simple adhesive mechanism? What is the importance of contact guidance or mechanical guidance in these and other systems? Cell surface molecules of the immunoglobulin superfamily appear in some cases at least, to specify selective fasciculation as proposed in the labeled pathways hypothesis (Grenningloh et al., 1991; Kolodkin et al., 1992). Are these molecules simple adhesive factors or are they signalling molecules? If they are signalling molecules, do they act as ligands, receptors or both? How is the signal transduced? What intracellular changes are induced by activation of this signalling apparatus?

One powerful approach which can be used to address these questions is a genetic analysis in organisms which are suited to this type of study. At the present time, there are four main systems which can used for genetic studies: *C. elegans, Drosophila*, the zebrafish and the mouse. The first two are suitable for classical genetic methods, including mutational screens to identify functionally relevant molecules and searches for interacting genes. The third, zebrafish, has the potential to be used for such classical approaches, but relevant studies in this system have just begun. Finally, the last system, the mouse, is amenable to a "reverse" genetic approach, where the function of previously identified molecules can be tested by loss of function and misexpression studies.

Analysis of connectivity and pathway formation in *C. elegans* and *Drosophila* have also been advanced by the relative simplicity of their nervous systems and the availability of reagents to specifically identify individual cells and classes of cells (Bellen et al., 1989; Bier et al., 1989; Jan and Jan, 1982; McIntire et al., 1992; White et al., 1986; Wilson et al., 1989; Zipursky et al., 1984). This ability to reliably identify individual cells is fundamental for testing the function of mechanisms which direct the development of specific pathways and connections.

Mutations affecting axonal patterning and connectivity in C. elegans

In *C. elegans*, analysis of mutants which were identified in behavioral screens has identified a number of genes which affect axonal patterning in the nervous system (McIntire et al., 1992). These genes fall into several distinct groups based on the aspects of axonal development they direct. Mutations in the genes *unc-5*, *unc-6* and *unc-40* affect circumferential elongation of axons along the body wall. Mutations in the genes *unc-34*, *unc-71* and *unc-76* affect fasciculation and elongation along the nerve cords. Mutations in a third class of genes affected multiple aspects of axonal outgrowth.

Of the genes which affect circumferential elongation, *unc-40* affects only dorsally-directed growth, while *unc-5* affects only ventrally- and *unc-6* affects both dorsally- and ventrally-directed growth. These genes disrupt the migration of mesodermal cells as well as pioneer axons. Recent isolation of the *unc-5* and *unc-6* genes has indicated that circumferential growth along the body wall may depend on interactions between neurons and the extracellular matrix. The gene *unc-5* encodes a transmembrane protein with immunoglobulin and thrombospondin domains (Leung-Hagesteijn et al., 1992). Mosaic analysis suggests that *unc-5* is required cell-autonomously in neurons and migrating cells for their guided navigation. The gene *unc-6* encodes a putative extracellular matrix protein that is related to laminin (Ishii et al. 1992). These results have led to a model in which *unc-6*, perhaps distributed in a graded fashion in the basal lamina, acts as a guidance cue for directed growth. This signal is received and transduced by the *unc-5* receptor, which directs dorsal migration. Another receptor, perhaps the product of the *unc-40* gene, also interacts with *unc-6*, but it directs ventral navigation. Further analysis of these gene products should provide information about the validity of this model and should indicate how the guidance signal is regulated and transduced, and what intracellular changes result from receptor activation.

Another nematode gene which was identified by a mutation resulting in an uncoördinated behavior is the gene *unc-4*. Ultrastructural analysis of *unc-4* mutant animals has shown that the locomotor defect is correlated with specific changes in synaptic connections on identified motor neurons (White et al., 1992). Although the synaptic inputs on these motor neurons are altered, their synaptic outputs are unchanged. The motor neurons which are affected in the mutation all arise in development by a similar lineage and the alteration in synaptic input is such that the affected motor neurons receive input like their lineal sisters. Thus, the gene affects a certain aspect of the lineage decision: the specification of synaptic input. Other aspects, axonal projection, synaptic output and neuronal morphology are unaffected. The gene encodes a homeodomain protein which likely acts as a transcription factor (Miller et al., 1992). The relatively limited effect of *unc-4* loss of function may suggest that *unc-4* regulates the expression of a small number of target genes and that some of these genes are directly involved in synaptic specification. These data also indicate that different aspects of neuronal determination, such as morphology, axonal projection and pre- and post-synaptic connectivity may be independently controlled by distinct regulators of gene expression.

Genetic analysis of axon guidance and connectivity in Drosophila

Genes which are required for proper axonal patterning and connectivity in Drosophila have been identified in behavioral screens (Thomas and Wyman,

1984) and in anatomical screens based on histological analysis (Heisenberg and Böhl, 1979; Seeger et al., 1993; Van Vactor et al., 1993). In addition, many other candidates for genes involved in this process have been identified by specific expression patterns [see, for example, (Elkins et al., 1990; Grenningloh et al., 1991; Nose et al., 1992). Finally, genes which affect these developmental processes are also found among the genes previously identified for other phenotypic effects (Doe et al., 1988a; Giniger et al., 1993; Schmucker et al., 1992).

In many cases, genes which are implicated in axon guidance and connectivity have been cloned and characterized. These genes have been found to encode numerous different types of molecules, including nuclear proteins which specify programs of gene expression (Crews et al., 1988; Doe et al., 1991; Smouse et al., 1988; Vaessin et al., 1991), adhesion or recognition molecules of the immunoglobulin superfamily (Bieber et al., 1989; Grenningloh et al., 1991; Seeger et al., 1988; Snow et al., 1989) and cell surface glycoproteins of other types (Kolodkin et al., 1992; Krishnan et al., 1993; Nose et al., 1992; Zinn et al., 1988). Others, such as *bendless*, which encodes a protein likely to function as a ubiquitin-conjugating enzyme (Muralidhar and Thomas, 1993), are involved in cellular processes which were not previously thought to be involved in the establishment of neuronal connectivity.

Although many molecules have been identified, the exact nature of the mechanisms of neuronal specificity, axon guidance, and target recognition are unknown. The adhesive activity of molecules like fasciclin II and connectin, cell surface molecules which label specific axonal pathways (Grenningloh et al., 1991) and a subset of motor neurons and their synaptic targets (Nose et al., 1992), respectively, supports some of the predictions of the "labeled pathways" and "chemoaffinity" hypotheses. Genetic data which is necessary to firmly establish the validity of these models is accumulating.

The identification of transcription factors and nuclear proteins that are involved in axonal pathway formation and target recognition indicates that these processes may be controlled in part by the regulation of cell identity and cell fate. In some cases, it seems that pathway and target recognition is essentially a function of the cell fate or identity of neurons and cells with which they interact. For example, the segmental identity of sensory neurons and their environment alters their projection and connectivity in a predictable way (Ghysen et al., 1983). Likewise, ectopic expression of the paired box gene, *pox neuro*, which specifies sensory organ identity, transforms axonal morphology and connectivity in a manner consistent with neuronal identity (Nottebohm et al., 1992).

In other cases, the relationship between cellular identity and pathway and target recognition is unclear [see also, (Miller et al., 1992), discussed above]. The gene *cut*, which encodes a homeodomain protein (Blochlinger et al., 1988), also regulates the identity of sensory neurons in the Drosophila PNS. Mutations in the gene transform sensory organs of one class, the external sensory (es) organ, into sensory organs of another class, the chordotonal (ch) organ (Bodmer et al., 1987). In these *cut* mutant embryos, both neurons and support cells of the es organ adopt the external morphology and antigenic properties of ch organ neurons and support cells. However, the transformed es sensory neurons in *cut* mutant embryos show rather variable projections in the CNS. Some project like wild type es neurons, others project like ch neurons and yet others have novel projection patterns. These data are difficult to interpret, since the projection of the transformed es cells may be influenced by their novel position in the embryo or the lack of appropriate central cues required for ch neuron-appropriate projection. Nevertheless, these data suggest that cell identity and neuronal connectivity are experimentally separable. In addition, the loss of *fushi tarazu* and *even skipped* activity changes the projection of identified CNS neurons (Doe et al., 1988a; Doe et al., 1988b), but the new projections of these neurons are quite variable, and it is difficult to conclude whether cell identity *per se* has been altered in these mutants.

Analysis of the gene *prospero* has also demonstrated the difficulty in sorting out the relationship between cellular identity and neuronal projection patterns. The *prospero* gene product is a nuclear protein with multiple homopolymeric amino acid stretches which is widely expressed in precursors of CNS and sensory neurons, but not in differentiated neurons. Mutations in the gene drastically affect CNS and PNS axonal pathfinding (Doe et al., 1991; Vaessin et al., 1991). Although some evidence indicates that the identity of CNS neurons is affected by *pros* mutations (Doe et al., 1991), analysis of the PNS, where individual neurons are more easily identified and characterized, shows that neuronal identity is unaffected by loss of *pros* activity (Vaessin et al., 1991). These findings support the idea that regulators of gene expression, in this case, a gene which is specifically expressed in neuronal precursors and not in mature neurons, control different aspects of neuronal differentiation, including axon outgrowth and connectivity, independent of cell identity.

Structure and development of the Drosophila visual system: a model system for axon guidance and connectivity studies

The ultimate aim of studies of axon guidance and connectivity specification is to identify all of the molecular players acting at each step in the regulatory hierarchy: all of the guidance cues and receptors, genetic regulators and effectors, signal transduction machinery and so on. In order to approach this goal, genetic, cell biological, biochemical and molecular biology approaches are being utilized. In addition, advances in this field will depend on studies in systems where many of these approaches are applicable and where individual cells can be reliably identified and analyzed.

The visual system of *Drosophila* offers a particularly suitable system for genetic studies of the molecular basis of axon guidance and connectivity [see also (Kunes et al., 1993)]. It is composed of a limited number of cell types, which are organized in a repeated structure. The advantages of this system also include numerous molecular markers which can be used to identify and analyze individual cells (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989; Zipursky et al., 1984), the availability of behavioral tests (phototaxis and optomotor behavior) to assay function, advanced molecular biology and gene transfer technology and the techniques for generating genetic mosaics. Finally, under laboratory conditions, the visual system is not essential for viability of the organism, which facilitates the analysis of genes required for visual system function and development.

Recent studies on the development of cell identity in the Drosophila visual system have greatly advanced understanding of cell-cell interactions in the determination of cell fate [reviewed in (Hafen and Basler, 1991)]. The success of this work has depended on the same features and advantages listed above.

The visual system of Drosophila, a holometabolous insect, includes both larval and adult forms, which mediate the phototactic and visual behaviors of the two stages of the life cycle. By comparison, the adult visual system is much more complex, which is in keeping with the more sophisticated behavior of this stage. Ultrastructural as well as genetic studies indicate that the larval visual system and the adult visual system are related, both spatially and in terms of their development (Green et al., 1993; Meinertzhagen, 1973; Melamed and Trujillo-Cenóz, 1975; Steller et al., 1987; Tix et al., 1989).

The adult visual system consists of the compound eyes and the optic lobes, the portion of the brain which receives and processes visual information from the eyes (Meinertzhagen, 1973). The compound eyes are composed of approximately 800 repeated units, called ommatidia (Ready et al., 1976). Each ommatidium consists of eight photoreceptor cells which have axonal projections that synapse with interneurons in the first and second ganglion layers of the optic lobe, the lamina and medulla.

The photoreceptor cells of the compound eye differentiate from the eye disc during the third instar larval stage (Ready et al., 1976) and extend axons to the optic lobe primordia through an epithelial tube called the optic stalk. These retinal axons navigate to target positions in the brain that correspond to the position of the photoreceptor cell bodies in the retina (i. e., dorsal to dorsal, anterior to anterior) (Meinertzhagen, 1973). These retinal axons grow a large distance and make a number of specific choices to reach their appropriate targets. An examination of the projection of retinal axons in mutant animals with a reduced number of photoreceptors or with small patches of wild type axons in a mutant background has shown that these axons can be independently guided to their correct retinotopic targets (Kunes et al., 1993). These findings suggest a model similar to Sperry's chemoaffinity hypothesis (Sperry, 1963), where positional guidance and recognition cues label pathways and synaptic targets.

Although the compound eye and optic lobes arise somewhat independently, the two tissues interact with one another extensively during development. Proper development of the adult optic lobe depends on innervation from the compound eye (Fischbach and Technau, 1984; Meinertzhagen, 1973; Meyerowitz and Kankel, 1978; Power, 1943). The terminal cell divisions that generate lamina neurons are induced by ingrowth of retinal axons (Selleck and Steller, 1991). Lamina glia are also induced to differentiate by retinal ingrowth (Winberg et al., 1992). Moreover, retrograde interactions between the brain and the eye are evidenced by the fact that long-term survival of photoreceptor cells requires connections with the optic ganglia (Campos et al., 1992). The molecular mechanisms of these trophic and inductive interactions between the compound eyes and optic ganglia have not yet been elucidated.

The adult visual system is preceded in development by the much simpler larval visual system. Fly larvae respond to light by negative phototaxis and this response is mediated by a pair of sense organs in the anterior end of the larva, adjacent to the cephalopharyngeal skeleton (Bolwig, 1946). In *Drosophila*, these larval photoreceptor organs, also known as Bolwig's organs, consist of approximately twelve photoreceptor cells arranged in a loose cluster (Steller et al., 1987). These twelve cells have axonal projections which are fasciculated together to form the larval optic nerve. This nerve extends from the photoreceptor cell bodies through the optic stalk to the ventrolateral side of the brain (Melamed and Trujillo-Cenóz, 1975; Steller et al., 1987; Zipursky et al., 1984). In the brain, it projects through the primordia of the adult optic lobes and forms synaptic connections with identified interneurons at the medial margin of the presumptive medulla. These synaptic partners can be visualized by anterograde filling of the larval optic nerve with cobalt chloride (Steller et al., 1987).

The three components of the *Drosophila* visual system, the larval photoreceptor organ, the eye disc and the optic lobe originate from a similar location in the ectoderm during embryogenesis [figure 1.1; see also Chapter 6; (Green et al., 1993; Steller et al., 1987)]. These components develop in a manner that is unique in *Drosophila* nervous system development. The eye discs develop from two pouches of the anterior ectoderm during late embryogenesis. Each primordium of the adult optic lobes develops from a mass of tissue that invaginates from the posterior portion of the procephalic lobe during stage 12 of embryogenesis [about 8-10 hr after fertilization; staging according to (Campos-Ortega and Hartenstein, 1985)]. This invagination moves as a mass around the developing larval brain hemisphere. This optic lobe primordium remains attached to the developing brain and by late embryogenesis, it is completely incorporated into the brain hemisphere. The optic lobe primordium remains mostly undifferentiated until larval life, at which time it divides and differentiates to form the optic ganglia (Meinertzhagen, 1973; White and Kankel, 1978).

The larval photoreceptors begin differentiation in the ectoderm just adjacent to the invaginating optic lobe primordium. They differentiate asynchronously and develop very short axonal projections which contact the cells of the neighboring optic lobe invagination [see also Chapter 6; (Green et al., 1993; Schmucker et al., 1992; Steller et al., 1987). This early connection between the larval photoreceptors and the optic lobe primordium prefigures the arrangement of the larval visual system later in development. Subsequent morphogenetic movements called head involution shape the larval head. This process of head involution and the movement of the optic lobe invagination around the brain hemispheres separates the larval photoreceptor organs and the optic lobe primordium. Meanwhile, contact between the terminus of the larval optic nerve and the optic lobe primordium is maintained, and the optic nerve undergoes extensive elongation. At the end of embryogenesis, the terminus of the optic nerve extends deeper into the brain, past the optic lobe primordium where it makes synaptic connections with a group of interneurons at the margin of the optic lobe primordium. Thus, the projection of the larval optic nerve develops not by long range pathfinding, but by establishment of contact early in development when cell bodies and target region are in close proximity, then maintenance of this contact during separation, followed by target recognition and synaptogenesis (diagrammed schematically in figure 1.1).

During larval life, the optic lobe primordium consists largely of undifferentiated and dividing cells. However, a cluster of roughly three neurons differentiate early in this primordium and occupy a central location close to the base of the optic stalk (Tix et al., 1989). These cells, termed the optic lobe pioneers (OLPs), can be identified in the third instar larval brain by HRP injection of embryos in the syncytial blastoderm stage. Following this injection, HRP is distributed equally throughout the nervous system, but during massive proliferation of the optic lobe anlagen in the third instar, HRP is diluted. Activity staining at this stage specifically labels cells which have undergone few divisions, that is, cells which have differentiated during embryonic development. The OLPs are closely associated with the larval optic nerve and they have axonal projections which fasciculate with this nerve and project along with it to the central brain. Although this association with the optic nerve may suggest that the OLPs represent synaptic targets of the larval photoreceptors, the OLPs are not visualized by anterograde filling of the optic nerve in the third instar larva (Steller et al., 1987).

Isolation and characterization of *disconnected*, a mutation which affects pathway formation and connectivity in the visual system

The *disconnected (disco)* mutation was first isolated in a screen for X-linked mutations affecting brain organization and anatomy (Heisenberg and

Böhl, 1979). Two alleles, *disco*^{1} and *disco*^{1656}, were independently isolated in such screens. The two alleles exhibit the same range and severity of phenotypes. Both alleles have a somewhat reduced viability with most of the mortality occurring in the pupal stage. In the majority of adult *disco* mutant flies the compound eyes fail to innervate the brain and the optic lobes are greatly reduced in size. This phenotype is termed the "unconnected " phenotype (Steller et al., 1987). In such flies, the mutant optic lobe typically contains just a rudiment of the medulla and lobula complex of the optic lobes and frequently, disorganized tissue resembling muscle occupies the space where the optic lobe is normally found. In other adult *disco* flies, retinal innervation of the optic ganglia does occur and the optic ganglia in these animals are roughly normal in size although still grossly disorganized. This phenotype is the so-called "connected" phenotype (Steller et al., 1987).

As described above, numerous studies have demonstrated the importance of retinal innervation to proper development of the optic ganglia. Thus, the reduced optic ganglia in unconnected *disco* mutants is at least partly due to the epigenetic consequences of the lack of retinal innervation. However, a genetic mosaic analysis of the *disco* gene revealed that the loss of *disco* activity in the compound eyes has no effect on the ability of retinal fibers to innervate the brain. The requirement for *disco* gene activity to direct proper optic lobe development must therefore lie outside the compound eye.

Analysis of the visual system in third instar larvae showed that here again, there are two distinct phenotypes found in *disco* mutant animals (Steller et al., 1987). The two phenotypes are distinguished by the presence or absence of the optic stalk. Individual larvae can lack just one or both optic stalks. When the optic stalk is missing, retinal axons from the eye disc form a disorganized plexus at the posterior of the eye disc. Retinal axons from an eye disc which is attached to the brain by an optic stalk, however, innervate the optic lobe primordium in an apparently normal manner.

Examination of the larval visual system in *disco* mutants reveals a consistent failure of the larval optic nerve to form appropriate connections with its target cells in the brain (Steller et al., 1987). In some third instar larvae, those which lack optic stalks ("unconnected"), the larval optic nerve bypasses the eye disc and projects to a variable, ectopic location. In the instances where an optic stalk is present, the larval optic nerve projects through the stalk to the optic lobe primordium. Even in these instances, however, the nerve shows no synaptic connections with its normal target cells and instead exhibits extensive, apparently undirected, axonal growth in the brain hemispheres.

The failure of the *disco* mutant larval optic nerve to establish normal connections with its target cells in the brain can be first observed during embryonic development [diagrammed schematically in figure 1.2; (Steller et al., 1987). In *disco* mutant embryos, both the larval photoreceptors and the optic lobe invagination are formed normally and initial contact between the two is established as in wild type. During subsequent separation of the photoreceptor cell bodies and the optic lobe invagination during head morphogenesis, contact between the terminus of the optic nerve and the optic lobe primordium is frequently lost. In these cases, the optic nerve projects in a variable way in any of a number of ectopic locations. In some embryos, contact between the optic nerve and the optic lobe primordium is maintained. Even in these cases, the nerve fails to project into the brain and form normal synaptic connections with its target interneurons. Thus, the defect in connectivity of the larval optic nerve results not from a failure in axon guidance or pathfinding, but from a failure in target recognition (Steller et al., 1987).

In addition to defects in visual system connectivity and reduced viability, the *disco* mutation also results in other anatomical abnormalities during embryogenesis, albeit with lower frequency. First, the mutation leads to defects in clustering and placement of the larval photoreceptor organs. In many *disco* embryos, larval photoreceptor cells are scattered and/or photoreceptor clusters are found in an abnormal, posterior location. Moreover, defects in neuronal cell body placement and axonal projection throughout the PNS are also observed in *disco* mutant embryos.

As described above, both alleles of *disco* show the same spectrum and severity of phenotypic abnormalities. In addition, the same range and severity of anatomical defects are seen in embryos hemi- or homozygous for the Df(1)l9 chromosome, a large genetic deletion which completely removes the *disco* gene. Although many genes in addition to *disco* are deleted in these embryos, the Df(1)l9 embryos develop to late embryogenesis and are anatomically indistinguishable from *disco*^{1} or *disco*^{1656} embryos. Thus, it is likely that the EMS-induced *disco* alleles represent complete loss-of-function alleles.

One interpretation for the origin of the adult visual system defects in *disco* mutants is a requirement for a pioneering function of the larval optic nerve (Steller et al., 1987). The presence of an optic stalk in *disco* mutants is always correlated with the projection of the larval optic nerve through the eye disc, and this fact suggests there may be a direct or indirect causal relationship between the two structures. Such a pioneering activity of the larval optic nerve was originally proposed on the basis of ultrastructural studies (Meinertzhagen, 1973; Melamed and Trujillo-Cenóz, 1975). However, analysis of animals mutant for the gene *glass (gl)*, a zinc finger gene required for photoreceptor-specific development, suggests that the larval optic nerve is not an obligate pioneer. In *gl*^{60J} animals, a strong allele of *gl*, the larval photoreceptor cells fail to differentiate normally, yet the optic stalk is present (Moses et al., 1989). In fact, mosaic analysis of *gl* demonstrates that *gl* activity is not required in the larval optic nerve for correct retinotopic projection of the adult photoreceptor axons (Kunes et al., 1993). Analysis of the earliest stages of larval photoreceptor development in *gl*^{60J} embryos (Schmucker et al., 1992) indicates that larval optic nerve development fails very early, well before optic nerve defects are observed in *disco* embryos. On the basis of these data, it seems likely that the larval optic nerve is not required for the formation of the optic stalk.

Where is the *disco* gene required for the proper development of the visual system? The failure in connectivity of the larval optic nerve in *disco* mutants may result from requirement for *disco* function in the nerve, in the targets or in other intervening cells. The defects in the adult optic lobe may result from an additional autonomous requirement of *disco* function in post-embryonic development. A genetic mosaic analysis using the unstable ring-X chromosome was used to determine which structures must be mutant for the *disco* gene in order for the defects in the compound eyes and adult optic lobes to be manifest (Steller et al., 1987). This analysis revealed a single discrete focus of the *disco* mutation on the blastoderm fate map. The focus was mapped relative to external cuticle markers as assayed by presence or absence of the *yellow* and *singed* genes. Comparing the position of this focus to the positions previously reported for the origins of the adult nervous system and other adult internal tissues (Kankel and Hall, 1976) suggested that the *disco* focus was not located in either the compound eye or the optic ganglia, but in some unknown structure. However, other more recent analyses (Technau and Campos Ortega, 1985; Hartenstein, et al., 1985) have placed the origin of the optic ganglia in a more dorsal position in the blastoderm fate map than was given by Kankel and Hall (1976). This more dorsal position is more consistent with the location of the optic lobe primordia in the developing embryo (Campos-Ortega and

Hartenstein, 1985). This position for the origin of the optic ganglia is also much closer to the focus of the *disco* mutation as determined by genetic mosaic analysis. Thus, the data available at present do not exclude the possibility that *disco* acts in the optic ganglia to direct proper visual system development. In order to accurately and unequivocally determine the focus of the *disco* mutation, however, it is necessary to generate genetic mosaics and assay the genotype of internal structures directly.

The *disco* mutation and circadian rhythms

Analysis of the *disco* mutation may also contribute to the study of the biology of circadian behavioral rhythms. Adult *Drosophila* show circadian rhythms in locomotor and eclosion behavior [reviewed in (Rosbash and Hall, 1989)]. Little is known about the molecular and cellular mechanisms that are responsible for the generation of these rhythms. The behavioral rhythms are thought to depend on an endogenous biological pacemaker, which is free-running and can be entrained or set by environmental stimuli such as light. The location of the pacemaker as well as the cells which mediate sensory input to the pacemaker are for the most part unknown. In holometabolous insects, the known photoreceptors, the eyes and ocelli, are not essential for pacemaker entrainment and function (Dushay et al., 1989).

Mutations in the *period (per)* gene lengthen, shorten or abolish circadian rhythms in eclosion and locomotor behavior (Konopka and Benzer, 1971; Rosbash and Hall, 1989). These observations suggest that the product of the per gene is directly involved in pacemaker function. The per protein is predominately nuclear (Ewer et al., 1992; Liu et al., 1992) and shows sequence similarity to known or suspected transcription factors (Hardin et al., 1992b). These facts suggest that per may act as a transcriptional regulator. Interestingly, per protein and mRNA show circadian fluctuations in abundance in various neurons and glia in the brain and optic lobes and in the adult photoreceptors (Ewer et al., 1992; Hardin et al., 1990; Hardin et al., 1992b; Zerr et al., 1990). Moreover, *per* mutations which lengthen or shorten behavioral circadian rhythms also affect cycling of *per* mRNA and protein in a similar manner (Hardin et al., 1990; Zerr et al., 1990). The circadian fluctuation in per mRNA is primarily controlled at the level of transcription and 5' flanking sequences which are sufficient to drive circadian cycling of heterologous genes have been identified (Hardin et al., 1992b). These various observations have led to a model in which the mechanism of the circadian pacemaker involves a daily oscillation of per activity produced by negative feedback loop of per protein on *per* transcription (Hardin et al., 1992b).

Mosaic analysis aimed at identifying the cells where *per* function is required for normal rhythmicity was undertaken in an attempt to locate the circadian pacemaker (Ewer et al., 1992). The *per* product is expressed in a group of neurons located at the medial/anterior margin of the medulla (termed lateral cells or lateral neurons) (Ewer et al., 1992; Zerr et al., 1990). Based on their size and position, these cells are likely to be medulla tangential neurons (Fischbach and Dittrich, 1989). In addition, *per* is expressed in a smaller neurons in the dorsal posterior central brain and in numerous glia scattered throughout the central brain and optic ganglia. The results of mosaic analysis showed that *per* expression in one side of the brain is sufficient for normal rhythms. Also, these studies revealed that a tiny patch of *per* expression in glial cells of the brain can be sufficient to drive rhythmic behavior, although the rhythms in these cases are rather weak. Expression of *per* in the lateral neurons is not required for some weak rhythmicity, although expression in these cells is necessary for normal robust rhythmicity. Thus these data are consistent with a diffuse focus of the per mutation. The circadian pacemaker may therefore be located in a number of cells throughout the brain, any of which are capable of directing some rhythmicity.

Mutations in the *disco* gene also greatly impair behavioral rhythmicity (Dushay et al., 1989; Hardin et al., 1992a). Under free-running conditions, *disco* adults show no rhythms of locomotor activity. They can however be "forced" to display behavior rhythms by light entrainment. In double mutant combinations with an allele of *per* that shortens the circadian rhythm, *disco* flies exhibit shortened behavioral rhythms. Moreover, in *disco* flies, *per* mRNA and protein cycles under both free-running and light entrainment conditions (Hardin et al., 1992a; Zerr et al., 1990). Thus, there is a free-running circadian oscillator in *disco* mutant flies, despite the absence of apparent behavioral rhythms. On the basis of these observations, it has been suggested that the *disco* mutation disrupts the output pathway which mediates the effects of the circadian pacemaker on behavioral activity. Interestingly, *per* expression in the lateral neurons is largely missing in *disco* mutant adults. As described above, mosaic analysis indicates that *per* expression in the lateral neurons is required for robust behavioral rhythms. These data taken together suggest that the lateral neurons may be important for normal rhythmicity and for mediating the effects of the endogenous rhythm on behavioral activity. It is presently unknown whether the lateral neurons are present and properly connected in *disco* mutants. Previous studies have demonstrated that medulla tangential neurons arise early in development of the optic lobe and are relatively autonomous in their development (Fischbach and Technau, 1984; Meinertzhagen, 1973). Indeed, some data has indicated that the rudimentary optic lobes in *disco* mutants still contain these tangential neurons (Fischbach and Heisenberg, 1984).

Questions and aims of this thesis

The goal of this work is to uncover some of the molecular mechanisms which underlie the development of neuronal pathways and cellular connections by focusing on the development of the *Drosophila* larval visual system, a simple model pathway which is amenable to molecular genetic and cell biological studies. In particular, I have sought to analyze and characterize the product of the *disco* gene, which is required for the development of appropriate connectivity in the visual system. I have chiefly examined the activity of *disco* during embryonic development, but many of these studies have a bearing on the function of *disco* during post-embryonic development as well. The questions which have guided these studies are: What type of molecule is encoded by the *disco* gene? What biochemical activities does the gene product demonstrate? Which parts of the molecule are essential to function? What are the cellular and molecular mechanisms involved in the establishment of larval visual nerve projection and connectivity? Where in this developmental process is the *disco* gene product acting to direct larval visual system patterning? The expectation is that many of the answers derived from this analysis will be generally applicable to understanding different aspects of neural development in a variety of contexts.

Chapter 2 of this thesis describes the isolation and characterization of the *disco* gene. The predicted amino acid sequence of the gene product contains two "zinc finger" domains, a sequence motif found in a number of nucleic-acid binding regulatory proteins. The significance of this region of the protein is highlighted by the fact that the two *disco* point mutant alleles show alterations in the conserved cysteine residues in these zinc finger domains.

Chapter 3 presents an analysis of *disco* mRNA and protein expression during development. The *disco* protein is localized to the cell nucleus, which further suggests that it acts as a regulator of gene expression. The *disco* gene is expressed in a complex and dynamic pattern throughout the life cycle. During visual system development, it is expressed in the optic lobe region in cells near the terminus of the larval optic nerve.

Chapter 4 describes the phenotypic consequences of ectopic expression of the *disco* gene during embryonic development. Axonal patterning in the CNS and PNS is severely disrupted by ubiquitous expression of *disco* early in development. These defects are not easily explained by changes in cell fate or cell identity in specific nervous system components.

Chapter 5 is an analysis of the activity of the *disco* protein to regulate its own mRNA expression in the developing visual system. This activity, which is highly cell type-specific, may result from a direct binding of the *disco* protein to its own promoter. This proposal is supported by a demonstration that *disco* protein binds specific DNA sequences located near the *disco* transcription unit *in vitro.*

Finally, Chapter 6 presents a study of cell interactions which occur during embryonic development of the larval visual system. During visual system development, the larval optic nerve contacts a group of early-differentiating neurons and a number of glial cells. The *disco* gene is expressed in these neurons and glia and wild type gene function is required for their proper differentiation. Thus, these cells may represent an obligate guidance cue required for proper larval optic nerve development. If so, the *disco* gene may be acting in these cells to direct the larval optic nerve projection and connectivity.

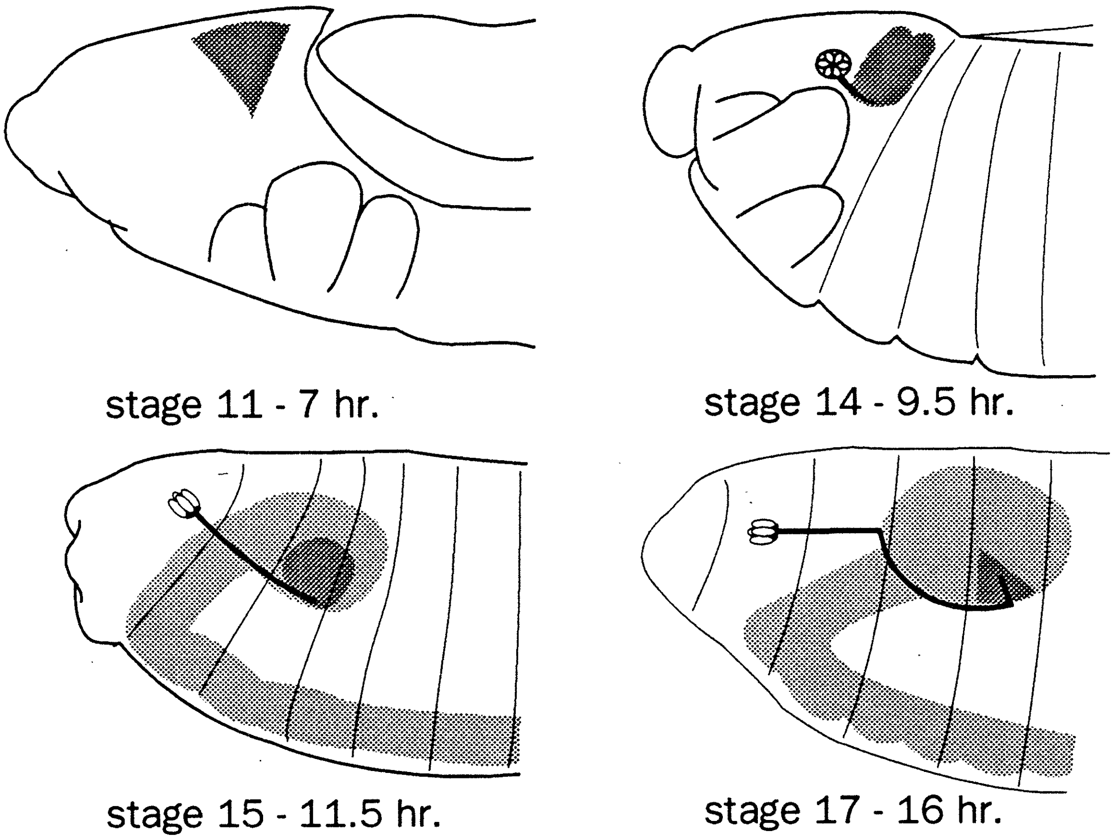
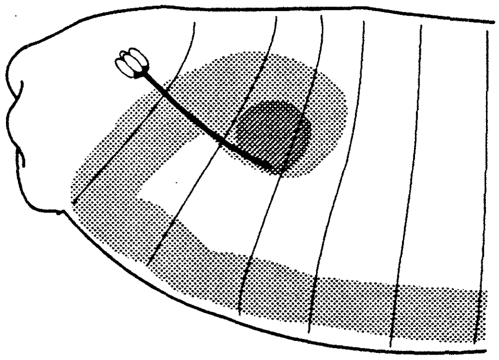
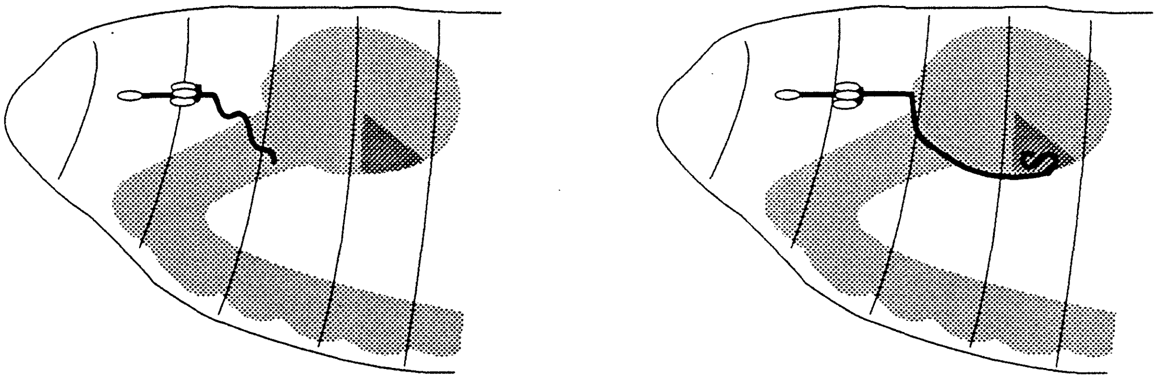


Figure 1.1 Embryonic development of the visual system. This diagram illustrates the development of the larval optic nerve and the optic lobe primordium during embryogenesis. Lateral views of the anterior half of embryos in successive stages in development are shown. Both the larval photoreceptor cells and the optic lobe primordium arise from ectoderm of the posterior procephalic lobe (shaded in gray in stage 11 embryo at upper left). During embryonic stages 12 and 13, the optic lobe primordium invaginates (shaded region) while larval photoreceptors remain at the surface and differentiate. Axonal projections from the larval photoreceptors, which give rise to the larval optic nerve, contact the optic lobe primordium at this stage, when these groups of cells are adjacent to one another. Subsequently, during separation of the larval photoreceptor cell clusters and the optic lobe primordium in stages 14-16, the larval optic nerve elongates while maintaining this contact with the optic lobe primordium. Finally, by stage 17, the larval optic nerve has acquired its characteristic projection and its terminus has extended through the optic lobe primordium toward the central brain.

disconnected



stage 15 - 11.5 hr.



stage 17 - "unconnected"

stage 17 - "connected"

Figure 1.2 Defects in larval visual system development in *disco* mutants.

This diagram illustrates the visual system defects observed in *disco* mutant embryos and the manner by which these defects arise. Compare this illustration to figure 1.1. In *disco* embryos, initial contact between the larval optic nerve and the optic lobe primordium is established normally. During subsequent development, however, contact with the invaginating optic lobe primordium is frequently lost. In these instances, the larval optic nerve projects in a variable way to an ectopic location ("unconnected"). In other individuals, contact with the optic lobe primordium is maintained, but the larval optic nerve fails to establish normal connections with its synaptic targets deeper in the brain ("connected").

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Chapter 2.
Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster*

Preface

The work presented in this chapter was initiated by Dr. Hermann Steller as a postdoctoral fellow in the laboratory of Dr. Gerald Rubin at the University of California, Berkeley. After Dr. Steller's arrival at MIT, the work was carried out as a collaboration between the Steller and Rubin laboratories. My contribution to this project is in sequence analysis of wild type and mutant *disco* alleles and the identification and characterization of the *disco* zinc finger motifs.

The EMBO Journal vol.10 no.4 pp.809 815, 1991

Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster*

Joseph S.Heilig[1,3], Matthew Freeman[1], Todd Laverty[1], Kevin J.Lee[2], Ana R.Campos[2], Gerald M.Rubin[1] and Hermann Steller[2]

[1]Department of Molecular and Cell Biology and Howard Hughes Medical Institute, 539 Life Sciences Addition, University of California, Berkeley, CA 94720 and [2]Howard Hughes Medical Institute, Department of Brain and Cognitive Sciences and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[3]Corresponding author

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Mutations in the *disco* (*disconnected*) gene prevent the establishment of stable connections between the larval optic nerves, the Bolwig's nerves, and their target cells in the brain during embryonic development. The failure of this initial connection is associated with aberrant development of the optic lobes which are largely degenerate in the mutant adult fly. In order to understand the role of *disco* in establishing this connection, we isolated and characterized the *disco* gene. A 22 kb DNA fragment can completely rescue the mutant phenotype. A single transcript, 2.9 kb in length, is found in this region and is expressed throughout development of the fly. We determined the nucleotide sequence of the *disco* gene to be unique when compared with sequences in a number of databases. The predicted amino acid sequence contains a region with similarity to the consensus established for the zinc finger motif. Mobilization of a P-element inserted near the gene resulted in the deletion of the 5′ end of the gene and produced flies indistinguishable from those carrying the *disco*^{1} allele.

Key words: Bolwig's nerve/*disconnected* gene/*Drosophila melanogaster*/neuronal connectivity

Introduction

Elaboration of a complex nervous system requires establishment and maintenance of connections between peripheral sensory organs and the brain. These connections are maintained through gross morphological changes ranging from vast increases in body size during normal growth to metamorphosis. It has been determined that, at least in insects, single neurons, referred to as pioneer neurons, grow out in early stages of development to form the initial connections between peripheral structures and the brain (Bate, 1976; Bentley and Keshishian, 1982a,b). Subsequent establishment of the extremely complex patterns of innervation found in the adult organism follows the pathways defined by these pioneering connections. Complete loss of sensory function and degeneration of organ or brain tissue or both often results from improper innervation of these tissues (see for example Power, 1943). Because of the extreme complexity of these connections and the severe consequences of their failure, understanding the cellular and biochemical mechanisms by which neurons navigate their proper paths, and establish and maintain their initial contacts has been difficult.

In traversing the paths to their targets, neurons contact and grow over a large number of cells of a variety of types. Some of these cells serve as guideposts (Bastiani *et al.*, 1985; Bentley and Keshishian, 1982a,b; Goodman *et al.*, 1984) and appear to provide signals to the growing neuron to direct its path. The nature of the signals provided by the guidepost cells to influence direction of growth and by the target cells to cause growth to cease and innervation to begin are unknown. We have addressed these questions by studying the pioneer neurons of the visual system of *Drosophila melanogaster*.

Larvae of dipteran insects are negatively phototactic and have two light sensitive organs, Bolwig's organs (Bolwig, 1946) located symmetrically on either side of the midline in the anterior region of the larva. In *Drosophila*, these organs can first be identified at ~9 h of embryonic development (Steller *et al.*, 1987). Each Bolwig organ is a cluster of photoreceptor cells from which a nerve, Bolwig's nerve, extends and, following a characteristic path, establishes the first connection between the light sensitive organ and the embryonic brain hemisphere (Trujillo-Cenóz and Melamed, 1973). It has been proposed that the successful establishment of this connection is required for subsequent development of the adult visual system including both the compound eye and the optic lobes (Meinertzhagen, 1973; Melamed and Trujillo-Cenóz, 1975). The initial stages of development of these adult structures, however, do not begin until the third larval instar, ~110 h after outgrowth of Bolwig's nerve is first detected in *Drosophila*. The thorough characterization of the anatomy of the Bolwig nerves (Melamed and Trujillo-Cenóz, 1975; Steller *et al.*, 1987), the relative simplicity of the initial stages of their outgrowth and association with the embryonic brain (Steller *et al.*, 1987), and the role these initial connections play in subsequent development of the visual system make the Bolwig nerve an attractive model for the study of neuronal connectivity. The Bolwig nerves can be readily observed in the embryo and larva with a variety of reagents and traced along their entire length from the cluster of cell bodies from which they arise to their growth cones terminating in the optic lobe anlagen (Steller *et al.*, 1987). The mechanism by which the Bolwig nerves recognize their targets and synapse with them is unknown but the importance of this process is strikingly demonstrated by mutations in the *disconnected* (*disco*) gene which result in the failure of these initial connections (Steller *et al.*, 1987). In *disco* mutant embryos, the nerves can often be seen to extend in the proper direction and follow paths very similar to those observed in wild-type embryos. Upon reaching the proper region of the brain anlagen, however, the Bolwig nerves in *disco* mutants continue to grow and appear to be unable to recognize or adhere to their targets. A thorough

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description of the *disco* mutant phenotype has been presented previously (Steller *et al.*, 1987). The availability of this apparently specific mutation disrupting the connection of the larval visual organs and brain and the proposal that the mutation specifically affects target recognition by the Bolwig nerves make it an appealing starting point for a molecular analysis of neuronal connectivity.

We have studied the *disco* gene at the molecular and genetic levels. Although ~70% of the neurons in the head of the adult fly are related to the visual system, it is expendable and under laboratory conditions flies are fully viable in the absence of any innervation of the optic lobes by retinula axons. This facilitated genetic analysis of the *disco* mutation. We have mapped the *disco* gene relative to a number of chromosomal aberrations, isolated the gene by chromosomal walking, and analyzed the structure of its transcript. Excision of a P-element inserted very close to the 5′ end of the transcript demonstrated that deletion of DNA encoding this transcript results in the mutant phenotype. We have also completely rescued the mutant optic lobe phenotype by P-element mediated germline transformation. We have sequenced the gene and found it to contain no extensive similarity to any genes available for comparison in a number of nucleic acid and protein databases. The sequence did reveal a region of similarity to the consensus sequence for the zinc finger motif found in a variety of DNA-binding proteins. The *disco* gene is expressed throughout development. Description of the expression patterns of the *disco* gene transcript and protein are reported in the accompanying paper (Lee *et al.*, 1991).

Results and Discussion

Genomic location of the disco gene

The *disco* mutation was isolated in a screen for structural brain mutations mapping to the X chromosome (Fischbach and Heisenberg, 1984) and further localized by recombination analysis to 53 ± 0.8 cM or polytene band 14 (Steller *et al.*, 1987). The *disco* gene was mapped more precisely by analyzing a collection of chromosomal aberrations (see Materials and methods). The deletion Df(1)81f20a was found to uncover *disco* while Df(1)81j6e complements *disco*. This indicated that at least a portion of the *disco* gene is located in the 50 kb region between the breakpoints of these deletions (see Figure 1).

Chromosomal walk and identification of transcripts in the region of the disco gene

Starting with a clone, no. 548 (Levy *et al.*, 1982) that maps to polytene chromosome region 14B5-10 and a lambda phage library, we obtained overlapping clones of genomic DNA from Canton S flies spanning ~150 kb of the X chromosome, the relevant region of which is depicted in Figure 1.

Because defects in the Bolwig nerve can be detected in mutants as early as 12 h of embryogenesis, and there is no evidence of a maternal effect, transcripts of the *disco* gene must be present by that time. To identify regions of the genome covered in the walk that contain transcripts corresponding to *disco* gene candidates, we performed RNA blot analyses of embryonic and adult RNA using as probes 1–5 kb DNA fragments covering the cloned region shown in Figure 1. This identified a single candidate gene encoding a 2.9 kb mRNA detected by two fragments within 5 kb of each other and noted in Figure 1. Only two other transcripts reflecting single copy sequences, both present in adults but not embryos, were detected in the ~150 kb of genomic DNA screened. Several lines of evidence discussed below demonstrate that the 2.9 kb transcript is the *disco* mRNA.

The *disco* gene transcript is present throughout the life cycle of the fly (Figure 2). The transcript can be detected at least as early as 6 h of embryonic development and is readily detected throughout the embryonic and larval stages, increasing in abundance at each larval molt. The level remains high during pupal life but decreases to low levels

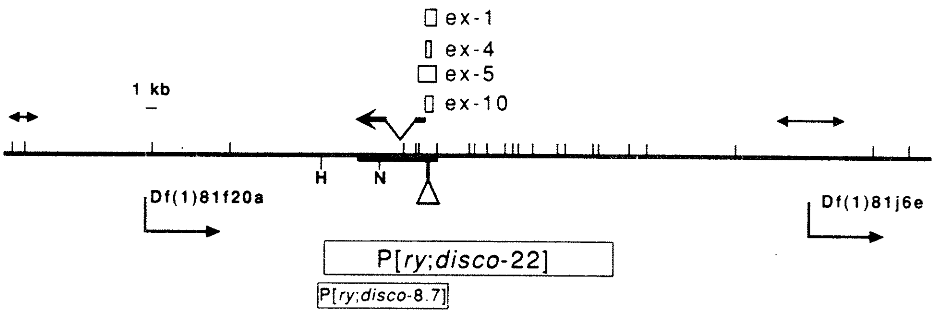

Fig. 1. Schematic diagram of the X chromosome around polytene region 14B3-4. The centromere is to the right. Arrows below the line mark the approximate break points of the deficiencies relevant to locating the *disco* gene; the arrows point in the direction of the deleted material which extends into polytene region 15A (Falk *et al.*, 1984). The arrows above the line represent transcripts detected by RNA blot analysis. The single-headed arrow represents the *disco* transcript and the direction of transcription, the approximate locations of the axons are indicated by the solid lines, the intron by the V-shaped line The double-headed arrows denote approximate locations of other transcripts for which the structure and the direction of transcription were not determined The thicker line demarcates the genomic region for which the entire nucleotide sequence was determined. The boxes below the line indicate the genomic regions used for germline transformation. P[*ry:disco*-22] was cloned in two fragments, a *Hinc*II–*Not*I fragment noted by the H and N on the figure, and a large *Not*I fragment extending rightward from the N of the figure to a *Not*I site in the vector λGEM12 including the genomic region above the box labeled P[*ry:disco*-22]. P[*ry:disco*-8.7] was constructed from a single genomic *Cla*I fragment. The vertical lines extending above the line represent *Eco*RI sites Most of the probes used for the RNA blot analysis of the region were these *Eco*RI fragments, the larger fragments were further divided by other restriction enzymes such that no probe was greater than ~5 kb in length. The site of insertion of the enhancer trap P-element rH219 is indicated by the triangle The extents of the deletions generated by excision of this P-element are indicated by the open boxes above the line and labeled ex-1, ex-4, ex-5, or ex-10 to indicate the excision allele represented.

in the adult head and very low, but detectable, levels in the body (not shown).

Rescue of the disco mutant phenotype

In order to demonstrate that the entire *disco* gene was contained within the region identified, we rescued the mutant phenotype by P-element mediated germline transformation. A region of the chromosome ~22 kb in length and indicated in Figure 1 was cloned into the plasmid transformation vector pDM30 (Mismer and Rubin, 1987). From the several hundred adult flies produced from the injected embryos, a single transformant was obtained, P[*ry*:*disco*-22]-14B; this low efficiency may be due to the large size of the construct. The rescuing plasmid in the one transformant had transposed onto the X chromosome. It was necessary to mobilize this X-linked transposon in order to obtain transformants carrying the rescuing element on an autosome. Ten such lines were obtained and males of these lines were crossed to virgin females of the genotype *disco*^{1};*ry*$^-$. Of the ten lines, four completely rescued the *disco*^{1} phenotype as shown by the wild type appearance of the optic lobes (Figure 3). Of the six remaining lines, two produced no progeny and could not therefore be tested and four failed to rescue. The failure to rescue may be attributable to insertion of the transposon in regions of the genome in which transcriptional activity is reduced or absent, or the large rescuing construct may have undergone rearrangement or deletion in the process of transposition. The cause of the failure of some of these transformants to rescue was not pursued since the ability of others to provide *disco* gene function while present on either the second or third chromosome demonstrates that the *disco* gene is located within the region of DNA contained in P[*ry*:*disco*-22].

A smaller region of DNA was cloned into pDM30 and tested for its ability to rescue the *disco*^{1} phenotype. This construct, designated P[*ry*:*disco*-8.7], contained sequences extending ~5 kb upstream of the start of transcription (Figure 1). Nine lines carrying this transposon failed to

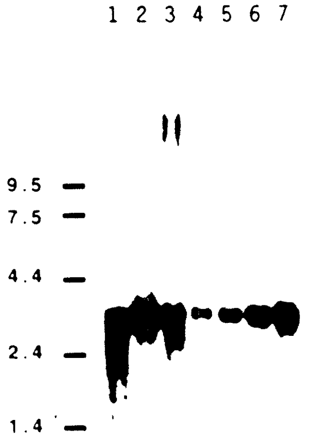

Fig. 2. Transcription of the *disco* gene throughout development PolyA$^+$ RNA isolated from different stages of development was probed with a fragment of genomic DNA from within the coding region of the gene. Positions of size markers (Promega) are indicated. 1, 0–6 h embryos; 2, 0–24 h embryos; 3, first instar larvae; 4, second instar larvae; 5, early third instar larvae; 6, late third instar larvae; 7, pupae.

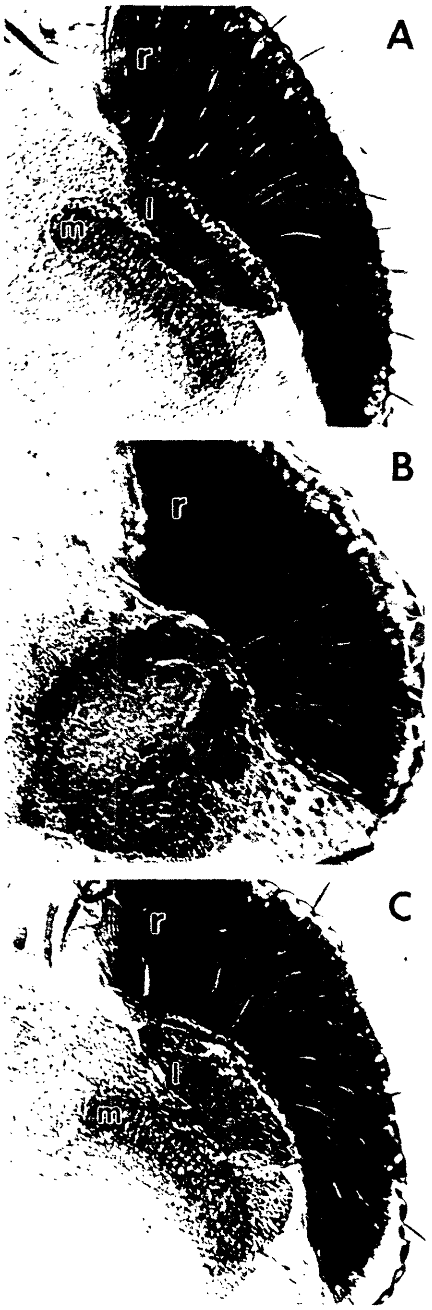

Fig. 3. Horizontal sections through heads of *disco*^{1} mutant flies and their brothers carrying the P[*ry*:*disco*-22] construct stained with Mab 24B10. Panel **A** is a section through the optic lobes of a wild type fly. Panel **B** is a section through the head of a male *disco*^{1};*ry*^{506} fly, note the characteristic lack of order in the optic lobes. Panel **C** is a section through the head of a brother of the fly in panel B but carrying the P[*ry*:*disco*-22]-49A construct. The three other transformant lines, P[*ry*:*disco*-22]-49F, P[*ry*:*disco*-22]-92A, and P[*ry*:*disco*-22]-66F, yielded flies (not shown) with optic lobe morphology indistinguishable from that shown in panel C. The regions of the optic lobe are labeled: r, retina; l, lamina, m, medulla

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                                            1→                                     ← PE       PCR →
TCCCTGCTCTCCGCACATCCGCATCTTCGTCGCTACTTCGTTTGCCACTCCGCATATTCACATATTCGCATCCGCATATTCGCGTCCAGTCCACTCTTCTCTCTGCCGCGCGACCCAGTG   76

CATAAGTGTGCGTGCATACGAGCGTGTGTGTGTGTGTGTGTGTGTCATCGTCAACTGTGAACGACCAACTTTTTTAATATCTCAGCAAAATGCAACAAAAATAAATGCACCACGGGCATG   196

AAAACAACAACAGCACACAGCTCCAACAGTGAATTCTACCATCTGAATTTATCAACTTTTTACGCAACAAAATATCAAAACAAAACAAAGCGAAAGAAACCCTTTTTTTAAATCTCAAAC   316
                                                                                           2927 |
CTGTGACAAAGTGAGAAAATCTAAAACAAAAAAAAAAAAACAGCAATAAACCAAAATACAATAAAATATCTACAACTCGCTGCTAAATTAGCAAAAGGCACCACCAGTCATCGCCATCGCC   436

ATCGCCAGCGCGACCATAGAAACAAAACCCACCACAGACGACATGGAGCACATAATGAATCCGTTCATGTCACCGGCTTATCTGCTGGGCCATGGCCCACATTCCCACCAGCATGTGCAC   556
                                          M  E  H  I  M  N  P  F  M  S  P  A  Y  L  L  G  H  G  P  H  S  H  Q  H  V  H

                                                        ← PCR
TCCCATCTACCGTCACACCCGCAGCCAAATGCCGCCTCTCCGGCCAGCTCACCCGGCGGATCCAGTGGCTCTGGATCGGGATCGGCAGCGGGATCGGGCACTGGATCAGGTTCATCCTTA   676
S  H  L  P  S  H  P  Q  P  N  A  A  S  P  A  S  S  P  G  G  S  S  G  S  G  S  G  S  A  A  G  S  G  T  G  S  G  S  S  L
AAGCCTCGTCGCTGGGGCTCACCGCCCATCAATCTGGCCGGTCAGTTCATCAATCCAGCCACGGGAAAGAAACGAGTACAGTGCTCCATTTGCTTCAAGACCTTCTGCGACAAGGGCGCC   796
K  P  R  R  W  G  S  P  P  I  N  L  A  G  Q  F  I  N  P  A  T  G  K  K  R  V  Q  C  S  I  C  F  K  T  F  C  D  K  G  A
CTTAAGATTCACTTTTCGGCCGTGCATCTTCGCGAGATGCACAAGTGCACGGTGGAGGGCTGCAATATGGTGTTCAGTTCGAGGCGTTCGAGGAATCGCCATAGCGCCAATCCGAATCCC   916
L  K  I  H  F  S  A  V  H  L  R  E  M  H  K  C  T  V  E  G  C  N  M  V  F  S  S  R  R  S  R  N  R  H  S  A  N  P  N  P
AAACTCCATTCGCCACACATCCGGCGCAAGATCTCGCCGCACGATGGCCGCACTGCCCAGCAGTTTCCGGTTTTCTCGCCCGGAACAGCCGCAGCAGCGGCCGCTGTCGCCGGACGACTT   1036
K  L  H  S  P  H  I  R  R  K  I  S  P  H  D  G  R  T  A  Q  Q  F  P  V  F  S  P  G  T  A  A  A  A  A  A  V  A  G  R  L
CCGGTTGCCTTTCCCGGACTTCTGCCACCGCCGCCGCCGCATCATGGTCACCATCCGTACGTCATGTTCGGCGGACAGGCGGGTCTACATGGCTTGGGCCTGCTTTCCACCGGCTGTCAG   1156
P  V  A  F  P  G  L  L  P  P  P  P  P  H  H  G  H  H  P  Y  V  M  F  G  G  Q  A  G  L  H  G  L  G  L  L  S  T  G  C  Q
GATCCCGATTCTGGATCGGTGGACAATGAACAGGATGCTGATCCCGAAGATGACAACGACTTTGTGTACGTGGACATGCAGGCCAATAGCTCTAGTCCGGCGGCCTCCAGCGAGGATCAG   1276
D  P  D  S  G  S  V  D  N  E  Q  D  A  D  P  E  D  D  N  D  F  V  Y  V  D  M  Q  A  N  S  S  S  P  A  A  S  S  E  D  Q
GAGGAACACGAACGGGATAATGAGCAGGATGAGGAGATGCACTGCAGCTTGAGCCTGGCCAGCAGTAGTAGCATTGCCGCCGATGAGGAGCGGGCAGCTGACCAGCCATTGGATTTCAGC   1396
E  E  H  E  R  D  N  E  Q  D  E  E  M  H  C  S  L  S  L  A  S  S  S  S  I  A  A  D  E  E  R  A  A  D  Q  P  L  D  F  S
CTTCACAAGCGTCGCAAGTCGGAGCAGGATCGGGAACAGGAGCAGGAGCAGGAGCAGGAACGGGAGCGGGAGGCGGAAAAGGAGCAGGAACAGGATGTGGAATCCGACAAGGAGCACGAA   1516
L  H  K  R  R  K  S  E  Q  D  R  E  Q  E  Q  E  Q  E  Q  E  R  E  R  E  A  E  K  E  Q  E  Q  D  V  E  S  D  K  E  H  E
CCCGAACAGGAGCATGAGCTGGAGCGTGAGAAGCGCTCGCCCAGCGATGCCTTCTCCATGGATCAGTTGCTGGGCAAGCGTAAGCGTCACGACAGCACCGCCTCCAGCTCCGCCTGTTCC   1636
P  E  Q  E  H  E  L  E  R  E  K  R  S  P  S  D  A  F  S  M  D  Q  L  L  G  K  R  K  R  H  D  S  T  A  S  S  S  A  C  S
ACGGCAGCCGCCTCCTCCGCCTCTAGTTCCTCCGCCAGCGCTAGCGCCAATCCACCACAGACGAGCATCAAAATGGAACTGGACCCGGATAGCGACAGTGCCTACATGACCAGCCGGCGG   1756
T  A  A  A  S  S  A  S  S  S  S  A  S  A  S  A  N  P  P  Q  T  S  I  K  M  E  L  D  P  D  S  D  S  A  Y  M  T  S  R  R
CAAATGCTCCCACTGCCCGTTCTGGACCTCGAGGAGCATCATCATCTGCGCCTGCTGCAGACCCAGATGTTTGCCGCCGCAGCGGCTGCCGCTGCCACCAGCCAGGCACCGCCCACCGCC   1876
Q  M  L  P  L  P  V  L  D  L  E  E  H  H  H  L  R  L  L  Q  T  Q  M  F  A  A  A  A  A  A  A  A  T  S  Q  A  P  P  T  A
TTCCTGCCGGCGGGATCACCCGTGGATCTGGCCAAGGACTCGCCGCCCATGTGGAGTCTCCTCTCCGAAATGTACCGCTCCATGCTGCTGAAGACACAGCACCAGCAATACAACCACCAT   1996
F  L  P  A  G  S  P  V  D  L  A  K  D  S  P  P  M  W  S  L  L  S  E  M  Y  R  S  M  L  L  K  T  Q  H  Q  Q  Y  N  H  H
CACCAGCTGCAGCAGCAACACCAGCAGGAGCAGCATCATCATCTGACCCTGAGCCACCACCATCAGGAGCAGCACCATCATCTGGGTCATCACCACATGGGTCACCACCACCATCATCAC   2116
H  Q  L  Q  Q  Q  H  Q  Q  E  Q  H  H  H  L  T  L  S  H  H  H  Q  E  Q  H  H  H  L  G  H  H  H  M  G  H  H  H  H  H  H
CATCAGCACCACCAACAGCAGCCGCAACAGCAATCGCCGGCGGCCACGAATGCGCCGATTTCCGTCTAATGTCCAGACTGCTGATCCATGGCGATGATTTCTAGATGATTTCCTTAGTCA   2236
H  Q  H  H  Q  Q  Q  P  Q  Q  Q  S  P  A  A  T  N  A  P  I  S  V  *
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ACCATTAAATGGCTGGCACTCCTCCTCAGGCGCGACCCCGCAACACCCGCAACCAATGCCACTAAGTACCCCACCCCCCCTCTGAAAACTGGAATTGCTCCGAAACTAAGCTTTTTCTGC   2476

TCGTACATGTGTTATATGGATTTTTTTTTTTTTGGAAACCTATGTATAATGTATAATGTATACTGTAGTGCGACATCTAAGTGTATAGTATTCAATCCGTTCCTCAGTGTGAATGTGCAG   2596

ATGCATGAATGAGAGTGTGTGTGTGTGTGTGTGGGATCCCAGGATCGGATCCAAAAAAAAAAAAAAAATTAAAGTCTGTCATCTGAAAAGCGGGCTGGGCAGTGCAGAAGCGGGGATT   2716

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CCAATCCCTAAAGAAAAAAAATAAACCCAAAAAAAAAAAAAA   2998
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Fig. 4. Nucleotide sequence of the *disco* gene transcript. The sequence represents a composite of sequences determined by cDNA and genomic clones, PCR and primer extension. The primary transcript begins at the C residue below the numeral 1 on the figure and extends in the direction indicated by the arrow. A single intron of 2927 nucleotides (not shown) separates nucleotides 411 and 412 of the mature message and is indicated by the vertical line beside the number 2927. The precise 3′ end of the transcript has not been identified and several possible polyA addition sites are present. The predicted amino acid sequence of a 568 residue polypeptide is indicated in one-letter code beginning at the first methionine residue preceding a long open reading frame and terminating at the asterisk beneath the *ochre* codon. The codons for the two cysteine residues altered in the two mutant alleles (see text) are doubly underlined. In *disco*^{1} the Cys127 codon TGC is changed to a serine residue AGC and in *disco*^{1656} the Cys94 codon TGC is a tyrosine codon TAC. The singly underlined regions indicate the sequences used to make synthetic oligonucleotides for primer extension (PE) to determine the 5′ end, or for PCR to determine the intron–exon boundaries; the arrows indicate the direction of extension allowed by the primer. These sequence data are available from EMBL/GenBank/DDBJ under accession number X56232.

rescue the mutant phenotype. This result may indicate that sequences necessary for proper expression of the *disco* gene extend beyond the limits of the P[*ry*;*disco*-8.7] construct.

Molecular characterization of the disco gene

Complementary DNA clones corresponding to the *disco* gene transcript were isolated from a cDNA library made from mRNA isolated from Canton S embryos (gift of K.Zinn and C.Goodman, see Materials and methods). The nucleotide sequence of the longest of these was determined. The genomic region from which the gene is transcribed was also sequenced. The sequence of the gene is presented in Figure 4. The genomic region from which this sequence derives is indicated in Figure 1. Because the cDNA sequences did not contain the complete 5′ end of the gene, this and the intron–exon boundaries were determined through a combination of RNase protection, primer extension and PCR analyses and are indicated in Figure 4 (the fragments and primers used for these procedures are indicated in Figure 4 and its legend). Evaluation of these data revealed the presence of a single intron of 2.9 kb separating a 412 bp 5′ exon of non-coding sequence from a 2.5 kb exon containing a single large open reading frame capable of encoding a polypeptide of 568 amino acids (Figure 4).

The nucleic acid and the predicted amino acid sequences of the *disco* gene were used to search a number of databases and revealed no extensive similarity to any sequence available for comparison. Analysis of the predicted amino acid sequence indicates that the disco protein has an estimated molecular weight of 62 385 daltons and is generally hydrophilic. No hydrophobic regions of the protein are predicted that satisfy the requirements of a signal sequence of a secreted protein or a membrane spanning domain. The middle of the protein contains a region of 40 amino acids in which glutamate residues (E) alternate with other amino acids, many of which are glutamine (Q). The nucleic acid sequence encoding this repetitive region contains a large number of CAX codons, tandem copies of which define the *opa* class of repetitive DNA (Wharton *et al.*, 1985). Although no striking similarities to previously identified proteins were discovered in the sequence databases, careful examination reveals that amino acids 89–149 of the predicted sequence resemble two repeats of the consensus established for the zinc finger motif (Gibson *et al.*, 1988; Klug and Rhodes, 1987) (Figure 5). The zinc finger sequence is found in a number of proteins known to regulate transcription by direct binding to promoters of specific genes and the zinc finger region itself has been demonstrated to contact DNA.

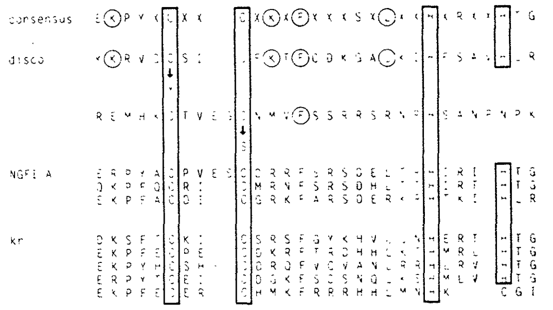

Fig. 5. The amino acid sequence surrounding the cysteine residues mutated in the EMS-induced *disco* mutant alleles compared with the zinc finger consensus and two zinc finger proteins. The consensus sequence was determined by the comparison of 148 zinc finger domains (Gibson *et al.*, 1988). Those amino acids present in >50% of the sequences are indicated by one-letter amino acid code. X indicates a position at which no amino acid predominates. The invariant amino acids are enclosed by the boxes and amino acids shared by the *disco* sequence and the consensus are circled. The amino acids altered in the alleles *disco*^{1} and *disco*^{1656} are indicated: in *disco*^{1} Cys127 is changed to a Ser, in *disco*^{1656}Cys94 is changed to a Tyr. The sequences encoding the zinc fingers of NGFI-A (Milbrandt, 1987) and *kr* (Schuh *et al.*, 1986) are presented for comparison and as examples of divergence from the consensus, no functional analogy with *disco* is implied. A region between the C and H residues containing multiple S and R residues is found in the second finger-like region of *disco* as well as in the NGFI-A fingers.

The zinc finger domain of the disco protein, however, differs from these more thoroughly studied proteins in a number of ways. The first zinc finger (amino acids 89–119) agrees with the consensus sequence very well, whereas the second (amino acids 117–149) lacks some of the consensus residues including the final histidine residue present in all reported zinc finger sequences. Another region well conserved in the zinc finger class of proteins is the linker region between repeats, the H–C link. This link region is seven or eight amino acids and is the most highly conserved region of the motif (Berg, 1990). The region linking the two zinc finger-like domains in the disco protein is only six amino acids and lacks some of the conserved amino acids. In modeling the structure of the TFIIIA protein and its interaction with DNA, Berg posits that the sixth finger (of nine), in which the linker regions are shorter than the normal seven amino acids, does not contact the DNA. A final characteristic of the zinc finger resembling region of the disco protein is that only two putative fingers are found, one of which is incomplete. Most zinc finger proteins carrying the C-C-H-H motif contain at least three, and often more, tandem repeats of this pattern (Gibson *et al.*, 1988; Klug and Rhodes, 1987). However, a protein from *Neurospora* with apparent transcriptional regulatory activity has been identified that has only a single zinc finger, corresponding to a different motif (Fu and Marzluf, 1990; Scazzochio, 1990). In addition to their role in regulating gene transcription, proteins containing metal binding domains similar to zinc fingers have been implicated in activities such as RNA processing (Chang *et al.*, 1988), protein–protein interactions (Frankel *et al.*, 1988) and enzyme catalysis (Aronson *et al.*, 1989). The disco protein is also predicted to contain stretches of amino acids with a net negative charge. Such acidic regions have frequently been associated with the activation function of transcriptional regulatory proteins such as GCN4 and GAL4 of yeast (Hope and Struhl, 1986; Ma and Ptashne, 1987; Ptashne, 1988).

Enhancer trap elements located in 14B3-4 and mutagenesis of the disco gene

In an independent screen, three enhancer trap lines (Bier *et al.*, 1989; Wilson *et al.*, 1989) were recovered in which a *lacZ*-containing P-element was inserted in polytene band 14B3-4; rL219, rH222 and rH875. Expression of the *lacZ* gene, as determined by β-galactosidase activity, in flies carrying these enhancer traps was detected in the region of the Bolwig nerves and brain anlagen in embryos, and in the presumptive optic lobes of the larval brain. Expression was also seen in other larval tissues as described by Cohen and co-workers (1991). Although there is some variation in expression pattern between the individual lines, in general the expression of these enhancer trap elements reflects the expression of the *disco* gene and gene product as described by Lee *et al.* (1991) (accompanying paper).

We located the points of insertion of the enhancer trap transposable elements and found one of them, rL219, to be inserted within 200 nucleotides of the beginning of the *disco* transcript. The approximate location is indicated in Figure 1. We mobilized this P-element (see Materials and methods) in order to obtain flies in which imprecise excision had occurred and DNA bordering the P-element had been deleted. By this method we recovered four mutant alleles of *disco* (ex-1, ex-4, ex-5 and ex-10), all of which are viable and fail to complement *disco*^{1}. DNA blotting analysis of these excision alleles indicates that in each a small region of DNA has been deleted including or immediately preceding the transcription start site (Figure 1). In at least one of these excision alleles, ex-5, the entire 5′ exon of the *disco* transcript has been removed. These excision alleles provide additional evidence for the identity of the *disco* gene.

Characterization of EMS-induced mutant alleles

The original *disco* allele, *disco*^{1}, was obtained by EMS mutagenesis (Fischbach and Heisenberg, 1984). A second allele, *disco*^{1656}, was obtained in a similar screen conducted by M.Heisenberg (Heisenberg and Böhl, 1979). Both alleles were sequenced and each was found to contain a point mutation when compared with its parental strain, as indicated in Figure 4. Both EMS-induced alleles contain single base changes resulting in mis-sense mutations changing a cysteine residue to another amino acid; as shown, they alter different cysteines in the predicted sequence. In *disco*^{1}, Cys127 is replaced by a Ser residue and in *disco*^{1656}, Cys94 is replaced by Tyr. No change in the size or abundance of the *disco* transcript, as determined by RNA blot analysis, was detected in the mutants (not shown) and, as is discussed elsewhere (Lee *et al.*, 1991), the tissue distribution of the mRNA is largely unaltered in the mutants. It is interesting that these point mutations result in alterations of cysteine residues located in the potential metal-binding domain of the disco protein. In addition to the potential role of this region discussed above, cysteine residues are often crucial to the tertiary or quaternary structure of proteins through their role in intrachain or interchain disulfide bonds and it is possible that disruption of these sorts of interactions is responsible for the phenotype of the EMS-induced alleles. Generation of a more extensive array of mutant *disco* alleles and associating the region affected in them with the phenotype expressed will permit us to identify the regions of the gene necessary for proper function. A thorough biochemical analysis of the *disco* gene product may reveal the role of other molecules with which it interacts.

Effects of multiple copies of the disco gene

Changing gene dosage can reveal phenotypes that may be informative about the function of the gene (Muller, 1932). To determine whether additional copies of the *disco* gene have effects on development, we generated flies carrying six copies of the *disco* gene as described in Materials and methods. These flies, which have normal X chromosomes and are homozygous for second and third chromosomes carrying functional copies of the P[*ry*;*disco*-22] transposon, show no obvious defects. Assuming that increasing the copy number of the *disco* gene is reflected in increased levels of expression, as has been shown for other genes (Muller, 1950; Seecof *et al.*, 1969), we conclude that increasing the number of copies of the *disco* gene has no major effects on development.

Possible role of the disco gene

The larval visual system of *Drosophila* provides a model for the study of neuronal connectivity. The simplicity of the network and the thoroughly defined anatomy facilitate analysis of the few, identifiable components of the circuit from mid-embryogenesis to larval development. Mutations in the *disco* gene provide an opportunity to study the consequences of a very specific disruption in this circuit: the failure of the Bolwig nerves to establish stable contacts in the developing brain. In an attempt to understand the *disco* mutant phenotype, we analyzed the *disco* gene molecularly and genetically. The presence of the Bolwig nerves in *disco* mutant animals indicates that the gene is not necessary for establishment of the nerve (Steller *et al.*, 1987). Because of the apparent defect in the projection of the Bolwig nerves in *disco* mutant embryos, it was plausible that the *disco* gene encoded a cell surface molecule. The nucleotide and predicted amino acid sequences of the gene, however, reveal none of the hallmarks of cell surface molecules such as a signal sequence or trans-membrane domain. If the *disco* gene does not specify a cell surface molecule, perhaps its function is required upstream of the actual intercellular recognition step; for example, regulating the expression of genes encoding cell recognition or adhesion molecules. Alternatively *disco* could be required for processes occurring subsequent to recognition; for example, signaling that contact with the appropriate target has been established. The sequence of the *disco* gene provided no insight into the possible role of the gene because no similarities were found with any sequences available for comparison. The presence of a region of the predicted polypeptide resembling the zinc finger motif as well as regions of net negative charge permit speculation that the *disco* gene is a transcriptional regulator of genes more directly involved in the recognition process. Further studies are necessary to determine the validity of this speculation. Cellular localization studies reported in the accompanying paper (Lee *et al.*, 1991) demonstrate that the *disco* gene product is localized to the nucleus, as would be expected of a transcriptional regulatory molecule.

The localization studies demonstrate that the *disco* gene product is expressed in many cells (including cells not related to the larval visual system). It is conceivable that the focus of the larval visual system defects observed in *disco* mutants is in the target cells of the Bolwig nerves rather than in the nerves themselves. The *disco* gene product may be involved in the establishment of the identity of these target cells or regulating in them expression of molecules necessary for recognition by the Bolwig nerves. While the cells with which the Bolwig nerves synapse in the embryonic brain have not been well characterized, some have been identified (Steller *et al.*, 1987). Additional candidates for these targets have been reported. Tix and co-workers identified a cluster of cells, which they suggest originate at approximately the same time as those of the Bolwig nerves, that pioneer a path through the optic lobes, eventually reaching the central brain (Tix *et al.*, 1989). They called these cells the optic lobe pioneers (OLPs). These workers propose that the ocellar bundle, described by Meinertzhagen (Meinertzhagen, 1973) as critical in the establishment of optic neuropils, is composed of axons of both the OLPs and Bolwig nerves. Although the OLPs are not identifiable in the embryo, preliminary experiments suggest that the OLPs are not present in *disco* mutant larvae (S.Tix and K.-F.Fischbach, personal communication). It has not yet been determined whether the OLPs fail to develop in *disco* mutant embryos or if they fail to thrive in the absence of fasciculation with the Bolwig nerves. It will be interesting to investigate the possibility that the *disco* gene is expressed in the OLPs. The answer to this question as well as continued characterization of the *disco* gene, the *disco* gene product and the consequences of mutations in the gene will be necessary for understanding the role of the *disco* gene in the establishment of the connection of the Bolwig nerves with their targets.

Materials and methods

Drosophila stocks

Genetic nomenclature is consistent with Lindsley and Zimm (Lindsley and Zimm, 1985). Mapping of the *disco* gene was accomplished using a collection of chromosomes in which a segment of the X chromosome containing the region 14A1-2 to 16A1-2 is translocated to the fourth chromosome, Dp(1;4)r$^+$f$^-$ (Falk *et al.*, 1984). We screened a series of derivatives of Dp(1;4)r$^+$f$^+$ in which a portion of the duplicated region of the X chromosome is deleted (Falk *et al.*, 1984). Male flies carrying a deficiency in Dp(1;4) were mated to virgin females of the genotype *disco*^{1} *para*^{ts} *f*/FM3. Male flies of the F1 generation were selected which were *f*$^-$, indicating that they carried the Dp(1;4) chromosome on which the deletions were generated, and screened for *disco* phenotype. The mutant alleles *disco*^{1} and *disco*^{1656} were isolated by K.-F.Fischbach and M.Heisenberg as described (Fischbach and Heisenberg, 1984; Heisenberg and Böhl, 1979) by EMS mutagenesis of the Berlin strain. The enhancer trap P-element lines were made using the PZ element (Y.Hiromi, unpublished). Excision of the lines was accomplished by matings to flies carrying a stable source of transposase (Robertson *et al.*, 1988). Dosage effects of the *disco* gene were studied by making stocks homozygous for X, second and third chromosomes each carrying a functional copy of the *disco* gene using the P[*ry*;*disco*-22] stocks described below. Flies homozygous for P[*ry*;*disco*-22]-92A were crossed to *CyO*; TM3 and virgin females of the genotype +/*CyO*; *P*[*ry*;*disco*-22]-92A/TM3 were obtained. These were mated to P[*ry*;*disco*-22]-49A/*Sp*;*ry*^{506}/TM6 males obtained from the cross of P[*ry*;*disco*-22]-49A; *ry*^{506} to *CyO*/*Sp*;*Ki*/TM6. The resulting flies heterozygous for both P[*ry*;*disco*-22] chromosomes, P[*ry*;*disco*-22]-49A/*CyO*; P[*ry*;*disco*-22]-92A/TM6, were mated to yield flies homozygous for both P[*ry*;*disco*-22]-49A and P[*ry*;*disco*-22]-92A.

Molecular biology

Standard procedures [for example Ausubel *et al.* (1987); Sambrook *et al* (1989)] were used to prepare nucleic acids and for RNA and DNA blots, primer extension, RNAse protection and PCR analyses. The probes and primers used in determining the 5′ end and the intron–exon boundaries are indicated in the legend to Figure 4. Genomic libraries were constructed from Canton S DNA partially digested with *Sau*3AI and ligated into arms of λEMBL3 (Frischauf *et al.*, 1983) or λGEM12 (Promega). The cDNA library used was prepared by K.Zinn from mRNA isolated from 9–12 h Canton S embryos (Zinn *et al.*, 1988). Sequencing was performed using Sequenase (USB). All sections of coding region sequence were sequenced at least three times.

Rescuing construct and transformation

A 22 kb *Not*I fragment and an 8.7 kb *Not*I – *Hinc*II fragment were obtained from different λGEM12 clones spanning the region indicated in Figure 1. These were ligated in two steps into transformation vector pDM30 (Mismer and Rubin, 1987) which had been cut with *Sal*I, the ends filled with T4 DNA polymerase, and then cut with *Not*I. DNA of the final rescuing construct, P[*ry*:*disco*-22], was combined with DNA from pUChsπΔ2-3 (D.Rio, unpublished) at a ratio of 3:1 and injected into 1 – 2 h *ry*^{506} embryos as described (Spradling and Rubin, 1982). The single transformant obtained, P[*ry*:*disco*-22]-14B, carried the transposon on the X chromosome. The transposon was mobilized as described above. Two lines were obtained carrying a functional P[*ry*:*disco*-22] element on the second chromosome, P[*ry*:*disco*-22]-49F and P[*ry*:*disco*-22]-49A and two were obtained carrying a functional element on the third, P[*ry*:*disco*-22]-92A and P[*ry*:*disco*-22]-66F. Males carrying P[*ry*:*disco*-22] were then crossed to *disco*^{1};;*ry*^{506} females and *ry*$^-$ males were inspected for optic lobe morphology. The *disco*^{1}; P[*ry*:*disco*-22] lines were maintained by crossing females to C(1) *y f*;;*ry*^{506} females allowing direct comparison of *ry*$^+$ and *ry*$^-$ brothers.

Histology

Heads were removed from Canton S flies, flies carrying the P[*ry*:*disco*-22] construct, or their untransformed brothers. The proboscides were removed and the tissue fixed for 60 – 90 min in 2% formaldehyde in PBS at 4°C. The tissue was washed in PBS and transferred to 12% sucrose in PBS at 4°C and allowed to equilibrate for ~ 16 h. The heads were removed from the sucrose solution and allowed to equilibrate with OCT (Miles) at room temperature for 10 – 30 min and then embedded in OCT medium and frozen in an ethanol – dry ice bath. Nine μm horizontal sections were cut, dried briefly onto freshly gelatinized microscope slides and immediately fixed in 0.5% formaldehyde in PBS for 20 – 60 min at room temperature. The fixative was washed away with PBS, and the slides blocked for 30 min with PBSG (0.2% BSA, 1% goat serum, 0.01% saponin in PBS) and washed with PBS/0.01% saponin before incubating for 30 min with Mab24B10 (Fujita *et al.*, 1982) diluted 1:1 in PBSG. After washing in several changes of PBS/0.01% saponin the slides were incubated with HRP-conjugated goat-anti-mouse Ig (Bio-Rad) diluted 1:200 in PBSG, for 30 min. The slides were washed again and then incubated with 0.5mg/ml DAB in PBS containing 0.003% H_2O_2 and 1.5 mM each $CoCl_2$ and $NiCl_2$. The reaction was monitored under a microscope and when the signal was adequate it was stopped by washing in PBS. The sections were then dehydrated in a 30%, 50%, 70%, 80%, 90%, 100% ethanol series, washed in xylene, and coverslips mounted with DPX mountant (Fluka).

Acknowledgements

We are particularly grateful to U.Gaul and S.Cohen for bringing the enhancer-trap lines to our attention. We thank: M.Mullins for plasmid DNA and valuable suggestions; M.Mlodzik and R.Carthew for RNA and comments on the manuscript; I.Hariharan for tips on PCR and, along with K.Moses and T.Xu for comments on the manuscript; U.Heberlein for hints on primer extension; K.-F.Fischbach and M.Heisenberg for providing the *disco*^{1} and *disco*^{1656} alleles, K.-F.Fischbach and S.Tix for communicating preliminary results; K.Zinn and C.Goodman for providing the cDNA library; M.Goebel and G.Barnes for searches performed on private sequence databases. We thank L.Pillus for comments on the manuscript and, along with J.Rine, for valuable discussions. J.S.H. was a Fellow of the Jane Coffin Childs Fund for Medical Research; M.F. received a SERC(UK) NATO postdoctoral fellowship; K.L. a NSF predoctoral fellowship; A.R.C. a fellowship from the Muscular Dystrophy Association of America. H.S. is the recipient of N.I.H. grant RO1-NS26451, a Searle Scholars Award and, during the initial phase of this work, a long-term EMBO postdoctoral Fellowship.

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Received on December 13, 1990.

Chapter 3.

Expression of the *disconnected* gene during development of *Drosophila melanogaster*

Preface

The work presented in this chapter was published as: Lee, K. J., Freeman, M., and Steller, H. S. (1991). Expression of the *disconnected* gene during development of *Drosophila melanogaster*. EMBO J. *10,* 817-826.

Abstract

Proper development of the larval visual nerve, Bolwig's nerve, of *Drosophila melanogaster* requires the wild type function of the *disconnected (disco)* gene. In *disco* mutants, the nerve does not make stable connections with its targets in the larval brain. We have begun to explore the role of *disco* in the formation of the nervous system by examining the distribution of *disco* mRNA and protein in embryos and third instar larvae using *in situ* hybridization and antibody staining respectively. No differences between the distribution patterns of the two products are detected; *disco* is expressed in many tissues including both neural and non-neural cells. Many of the cells which express *disco* undergo extensive movement during development as they participate in major morphogenetic movements. Antibody staining shows that the protein is found in the cell nucleus. Products of the *disco* gene are detected in cells near the terminus of the growing Bolwig's nerve. In embryos homozygous for either of two mutant alleles of *disco*, the *disco* protein is absent near the nerve terminus, although protein distribution elsewhere is indistinguishable from wild type.

Introduction

Development of the nervous system poses a unique problem of pattern formation for the ontogeny of higher animals. Many cells in the nervous system extend axonal processes which traverse large distances following defined paths to form connections with other selected cells. Understanding the molecular mechanisms which play a role in the development of the nervous system is a goal which is being approached through the combined use of different methods. One such method is the application of classical genetic techniques to isolate genes which act to direct development of the nervous system. The development of the larval visual system of *Drosophila* is a simple model well suited to a study using this approach.

Mutations in the *disconnected (disco)* gene result in striking and relatively specific defects in neural cell patterning in the *Drosophila* visual system. Embryos homozygous for mutant alleles of this gene show defects in the projection of the larval visual nerve, Bolwig's nerve (Steller et al., 1987). In these mutant embryos, Bolwig's nerve consistently fails to form stable connections with its synaptic partners and, in most cases, loses contact with the larval brain. Subsequent innervation of the optic lobes by the adult photoreceptors is also absent in the majority of *disco* mutants, due to an apparent pioneering function of Bolwig's nerve. In addition, the optic lobes of these mutants are extremely reduced in size. Since the development of the optic lobes is dependent on input from the developing eye [see, for example, (Fischbach and Technau, 1984; Meinertzhagen, 1973; Meyerowitz and Kankel, 1978)], the phenotype of the optic lobes in *disco* mutants can be partly explained by the lack of retinal innervation. However, the degree of disruption in *disco* mutant optic lobes is more severe than that observed when eye innervation alone is absent (Fischbach et al., 1989; Steller et al., 1987). This difference could result from an organizing activity of Bolwig's nerve or from an autonomous requirement for *disco* in the optic lobes.

Mutations in the *disco* gene also give rise to defects in axonal patterning throughout the larval peripheral nervous system (PNS) (Steller et al., 1987). These defects, although significant, are much more variable than the conspicuous abnormalities observed in the visual system of *disco* mutant larvae and adults.

We have begun a characterization of the *disco* gene product in order to explore its role in the development of the *Drosophila* nervous system. In the previous chapter, the cloning of the *disco* gene and its sequence are presented. Here we describe the spatial and temporal pattern of expression of the *disco* transcript and protein as revealed by *in situ* hybridization and immunohistochemistry. The *disco* protein and mRNA are widely distributed; the gene products are detected in both neural and non-neural tissues. Immunohistochemistry shows that the protein is localized to the cell nucleus. Interestingly, many of the cells which express *disco* are participating in morphogenetic movements such as dorsal closure, head involution and formation of the gut. The *disco* protein is expressed in a subset of cells in the central nervous system (CNS) and the PNS and in at least a portion of the primordium of the adult optic lobes, near the terminus of Bolwig's nerve. We observe that the expression of the protein in the region of the optic lobe primordium is largely absent in *disco* mutant embryos. These data are consistent with the proposal that the wild type *disco* gene product is required in cells near the terminus of Bolwig's nerve to direct the proper formation of the larval visual pathway.

Results

Expression of disco mRNA during embryonic development

Previous analysis (Steller et al., 1987) showed that nervous system defects resulting from *disco* mutations can be detected after 10-12 hr of embryonic development. We therefore expected to find some level of *disco* gene expression in the embryo. This prediction was confirmed by Northern blotting experiments (Heilig et al., 1991) which demonstrate that *disco* transcripts are present throughout much of embryogenesis. Transcripts of the same size are also found in larvae and, at somewhat lower levels, in adult heads and bodies. As phenotypic analysis suggested that the embryonic defects in Bolwig's nerve are responsible for later defects in the adult visual system, we initially focused on the embryonic expression. We sought to determine the spatial and temporal pattern of *disco* transcript accumulation by examining a collection of progressively older embryos hybridized with DNA probes from the *disco* gene.

This analysis was initiated using radioactively labeled DNA probes to detect mRNA in paraffin sections (data not shown). A complex and dynamic expression pattern was detected; spatially restricted expression was apparent in many tissues. Subsequently a whole-mount *in situ* hybridization technique using non-radioactive probes (Tautz and Pfeifle, 1989) was adopted. The results obtained with both techniques are virtually identical; some domains of expression are more clearly detected with the non-radioactive probes due to the greater resolution of this technique. Evidence that the signals detected result from products of the *disco* gene is provided by hybridization experiments using the deletion mutation Df(1)l9 (Steller et al., 1987). This deficiency lacks a portion of the X-chromosome containing the *disco* gene, and as expected, embryos hemizygous for the deletion show no staining (data not shown).

The results of whole-mount *in situ* hybridization to wild type embryos are shown in Figure 3.1. Expression of the *disco* transcript is first seen as a cap over the posterior pole of cellular blastoderm embryos (Fig. 3.1A). Soon thereafter staining develops in the anterior region as well. After head segmentation becomes evident, staining is detected in the gnathal buds, the clypeolabrum, the antennal segment and a posterior region of the procephalic lobe. Additional staining is observed in the ectoderm of the thoracic segments and in the visceral mesoderm.

Many of the stained cells, especially those in the head region and the visceral mesoderm, participate in extensive morphogenetic movements. These movements are illustrated by Figures 3.1D-F. During head involution the gnathal segments move forward and into the anterior orifice, the atrium. At the same time, the staining cells in the posterior region of the procephalic lobe invaginate as a whole and move to join the brain lobe. It seems likely that this group includes the primordium of the optic lobe as described by (Poulson, 1950) [see also (Campos-Ortega and Hartenstein, 1985; Hartenstein et al., 1985)]. Meanwhile, the cells of the visceral mesoderm elongate and contact the midgut primordia. They spread out, eventually flattening to form a sheet around the gut.

After retraction of the germ band, staining is detected in many parts of the nervous system (Fig. 3.1F). Groups of stained cells are seen in a regular array along the ventral nerve cord. There are a large number of these cells, approximately 30 in each segment. The pattern in each segment is invariant, with two pairs of cells located in a dorsal position on either side of the midline and two clusters of roughly 12 cells located more ventrally and laterally. In addition, there are many staining cells scattered throughout the brain lobes. A mass of stained cells are seen in the ventral portion of the brain lobes; this group includes the primordium of the optic lobes (see also above).

The pattern of staining remains largely constant after dorsal closure is complete. From that point until formation of the cuticle which prevents further hybridization experiments, staining is detected in the atrium, surrounding the gut and in cells scattered throughout the central nervous system.

Production of antisera specific for disco protein

We produced *disco* polypeptide fusion proteins in *E. coli* and used these bacterial products to generate *disco*-specific polyclonal antisera. A number of different expression systems were used to synthesize fusion proteins containing a 183 amino acid fragment from the N-terminal region of *disco*. This fragment includes a sequence with similarity to the "zinc-finger" motif consensus (see

Chapter 2). By first immunizing rabbits with one fusion protein, boosting with a second fusion protein and affinity purifying the antibody preparations with a third, we produced antisera which are specific for the *disco* portion of the fusion proteins.

We tested the specificity of the antisera for the *disco* gene product by immunohistochemical staining of embryos hemizygous for Df(1)I9 which deletes the *disco* gene (see above). Most of the batches of antiserum we used showed a complete absence of staining in embryos which lack the *disco* gene. One preparation did show some slight staining in the deficiency mutant embryos (Figure 3.2J). In this case staining was seen in the lumen of the salivary gland and in all experiments using this antiserum, this signal was regarded as cross-reactivity with antigens not related to the *disco* gene.

Spatial and temporal expression of disco protein in wild type embryos

Expression of the *disco* protein was detected in wild type embryos of the Berlin strain using antisera drawn from three rabbits. Sera from all three rabbits gave qualitatively identical staining patterns with the exception of variable non-specific staining in salivary glands described above and shown in Figure 3.2J. Incubation of samples with the secondary antibody alone gave no staining (data not shown).

Results of whole-mount immunohistochemistry experiments with wild type embryos are shown in Figure 3.2. The pattern of *disco* protein distribution as detected by the antibodies was very similar if not identical to the mRNA distribution pattern revealed by *in situ* hybridization (compare Figures 3.1 and 3.2). The *disco* protein, like the mRNA, is first detected at the posterior pole of cellular blastoderm embryos. Later it is found in the gnathal segments and the antennal segment, in clusters in the thoracic segments, in the visceral mesoderm, in the central nervous system and in the region of the optic lobe primordium.

As can be seen in Figure 3.2, the *disco* protein is generally localized to the nucleus. In some tissues where nuclei occupy most of the cellular volume it is difficult to rule out cytoplasmic staining as well. In cells where a distinction can be made, such as in the nervous system and the visceral mesoderm, the staining is strictly nuclear.

Some structures not stained by *in situ* hybridization are recognized by *disco*-specific antisera. As these structures are not stained in *disco*- deficiency embryos and are recognized by the sera of all three rabbits we believe the staining results from antibody binding to the *disco* gene product in these tissues. The differences between the two staining results are likely due to lack of adequate resolution with the *in situ* hybridization technique. At the dorsal margin of the lateral epidermis there are two longitudinal rows of conspicuously stained cells (Figure 3.2E, 3.2F). During the period of dorsal closure they move dorsally, eventually meeting at the midline (Figure 3.2G, 3.2H). These cells are the cardioblasts (Campos-Ortega and Hartenstein, 1985; Poulson, 1950), mesodermal derivatives which give rise to the dorsal vessel. In the lateral ectoderm there are also scattered stained cells arranged in a segmental array (Figure 3.2E, 3.2G). Double labeling experiments (see below) show that some of these cells are closely apposed to parts of the peripheral nervous system. In a few cases PNS-specific antibodies stain some of these *disco* containing cells (data not shown). Therefore, a subset of the stained cells may be neurons or support cells of the PNS, but due to technical limitations of the double labeling techniques (discussed below), more definitive identification has proven difficult.

disco expression during development of the larval visual system

Staining with *disco*-specific antisera shows that the gene is expressed in many parts of the embryonic nervous system. We wished to examine which of these expression domains could have a bearing on the Bolwig's nerve defects of *disco* mutants. To help resolve this question we used double-labeling techniques to study the relationship between domains of *disco* expression and elements of the developing visual system.

Wild type embryos were stained with antisera to the *disco* gene product and either a monoclonal antibody which labels the embryonic PNS, MAb 22C10 (Zipursky et al., 1984), or a monoclonal antibody which labels all photoreceptors including the cells of Bolwig's nerve, MAb 24B10 (Zipursky et al., 1984). Since we were unable to generate strong enough signals using fluorochrome-conjugated secondary antibodies, we used more sensitive horseradish peroxidase-conjugated secondary antibodies and employed cobalt-nickel enhancement to distinguish between the two antibody labels. The drawback of this method is that co-localization of both signals to a single cell may not be possible.

Figures 3.3A and 3.3B show double-labeling of an embryo with anti-Disco antiserum and MAb22C10. This monoclonal antibody labels the embryonic PNS and a subset of CNS neurons and it stains Bolwig's nerve after about 10 hrs of embryonic development. The terminal region of Bolwig's nerve is adjacent to a large domain of *disco* expression in the brain lobe. This domain of expression originates as an invagination in the procephalic lobe and includes the optic lobe primordium (see above).

Results of double-labeling experiments with a *disco*-specific antiserum and MAb 24B10 are shown in Figures 3.3C and 3.3D. This monoclonal antibody clearly labels Bolwig's nerve from the cell bodies to the terminus. In older embryos, such as that shown in Figures 3.3C and 3.3D, the *disco* expression around the terminus of Bolwig's nerve has become more diffuse and is ring shaped. Several smaller clusters of staining cells are seen as well and many of these are only a short distance from the nerve terminus. However, at this level of resolution we cannot determine whether any of these cells are the primary targets of Bolwig's nerve.

Expression of disco gene products in third instar larvae

The tissue distribution of the gene product in third instar larvae was examined by whole mount immunohistochemistry with *disco*-specific antisera and the results are presented in Figures 3.4B and 3.4C. It was impossible to use Df(1)l9 hemizygotes to verify that the staining in larvae results from *disco* protein as was done for the embryonic analysis above. Individuals hemizygous for this large deletion die as embryos. However we believe that our staining results reflect the true distribution of the gene product since identical staining patterns are produced using sera drawn from three different rabbits. Moreover, *in situ* hybridization of *disco* gene probes to larval tissues produces staining patterns that are very similar if not identical to the immunohistochemical staining patterns (compare Figures 3.4A and 3.4B). A few scattered elements are stained by *disco*-specific antibodies but not by *in situ* hybridization (see, for example, Figure 3.4C). Presumably these differences result from the lower sensitivity of *in situ* hybridization for the detection of isolated positive cells.

Figure 3.4 shows that some regions stained by *disco*-specific antibodies in embryos are also stained in larvae. For example, mesodermal layers around the larval gut and some scattered groups of cells in the larval central nervous system are stained. Most conspicuous in the larval nervous system however, is a band of staining that wraps around each brain lobe (Figure 3.4A, 3.4B). This band extends from a point on the lateral surface posterior to the optic stalk (see Figure 3.4C) to the dorsal surface of the brain lobe. It is located in or near the superficial layers of the brain. This staining domain does not correspond exactly to any previously described brain structure. It is found near the boundary between central brain and optic lobes and is at least partially contained within the primordia of the inner medulla and lobula complex of the optic lobes. This staining is not seen in second instar larvae (not shown) but develops during the third larval instar. In addition, *disco* gene product is detected in sparsely distributed nuclei in two other areas of the larval visual system (Figure 3.4C). Firstly, a very small number of stained nuclei are found scattered across the developing lamina. This structure is easily distinguished as a crescent shape demarcated by furrows in the brain surface. The number of these stained nuclei varies from one to seven in different brains, perhaps in an age-related manner. Secondly, two large stained nuclei are found at the base of the optic stalk near its connection with the eye disc.

We also examined the imaginal discs of third instar larvae for *disco* gene products. Figures 3.4B and 3.4C show the extensive staining detected in the antennal disc. Staining was also detected in the leg discs. The eye discs, however, are unstained. The latter result is consistent with mosaic studies which showed that wild type *disco* function is not required in the eye for its wild type development (Steller et al., 1987).

Expression of disco protein in disco mutant embryos

Two point mutant alleles of the *disco* gene have been isolated and sequenced. Both of these are missense mutations altering codons that specify cysteine residues within the putative "zinc finger" domain. Northern analysis reveals that wild type amounts of *disco* mRNA are transcribed in embryos carrying these mutant alleles (Chapter 2). These mutations could however affect stability or processing of the protein product. We wished to determine whether the abundance, subcellular localization and tissue distribution of *disco* protein in these mutants differs from that in wild type embryos.

Collections of homozygous *disco*^{1} and *disco*^{1656} embryos were stained with *disco*-specific antiserum. The results, shown in Figure 3.5, are identical for the two mutant alleles. Neither mutant differs significantly from wild type in terms of overall level or in terms of subcellular localization of *disco* protein. We do find one small but nonetheless important difference in the tissue distribution of the protein in *disco* mutant embryos. The mutant embryos consistently lacked the domain of staining which originates from the posterior portion of the procephalic lobe (Figures 3.5B and 3.5D). As mentioned above, we believe this structure corresponds to the optic lobe primordium. Some mutant individuals do exhibit residual traces of staining in this area (Figure 3.5F). The absence of staining in this region represents the only difference in *disco* protein distribution between the wild type and *disco* mutant embryos; in all other tissues, including other parts of the nervous system, the staining patterns are perfectly identical.

Discussion

In the present study we have examined the spatial and temporal distribution of the *disco* mRNA and protein. We have found no significant differences between the distribution of the two gene products. Both are present in many different tissues throughout the embryo and in these tissues the distribution pattern of the gene products is complex and dynamic. Much of the expression is in groups of cells that are involved in major morphogenetic events such as gastrulation, head involution, dorsal closure and formation of the gut. The gene products are found in a subset of cells throughout the nervous system, in both the CNS and PNS.

The pattern of *disco* expression in embryos is more widespread than might be predicted from the relatively specific phenotype of the existing *disco* mutant alleles. Mutant embryos exhibit defects in placement and projection of Bolwig's nerve and variable abnormalities in the patterning of the peripheral nervous system, but appear otherwise anatomically normal. Although the mutant alleles we have examined here may not be nulls, the results of complete loss of *disco* gene function can be determined by examining embryos hemizygous for Df(1)I9, which removes a portion of the X-chromosome containing the *disco* locus. These embryos lack between 5-10% of the X-chromosome, but they develop until late in embryogenesis and show nervous system defects no more severe than the *disco* point mutants (Steller et al., 1987). Moreover, the Df(1)I9 mutant embryos do not show consistent gross defects in head involution or dorsal closure although significant amounts of *disco* gene product are found in cells participating in these processes. Finally, P-element excision has been used to generate small deletions removing the first exon of the *disco* gene. Flies homozygous for these small deletions are viable and have a phenotype like the existing point mutants (Chapter 2).

Results from a number of studies in different organisms indicate that many of the molecules involved in development of axonal pathways and cellular connections have more general roles in development. The majority of molecules shown by *in vitro* studies to affect axonal growth and connectivity can be found in non-neural tissues. Examples include laminin, N-CAM, NG-CAM, L1, and tenascin [see reviews by (Jessell, 1988; Lander, 1989). Molecules that are involved in these processes in *Drosophila*, for example, amalgam (Seeger et al., 1988), fasciclin I (Elkins et al., 1990; Zinn et al., 1988), fasciclin II (Harrelson and Goodman, 1988; Snow et al., 1988), and neuroglian (Bieber et al., 1989) are similarly expressed in many parts of the embryo outside the nervous system. Only a small subset, if any, of the tissues that express these genes show discernible defects in the corresponding mutants. Likewise, many of the tissues that normally express *disco* are not phenotypically abnormal in *disco* mutants. One possible explanation for the lack of obvious abnormalities in these regions is that their morphogenesis is mediated by functionally redundant systems.

We have begun to address the role of the *disco* gene product in the development of the larval visual system by relating the gene expression to the time and location of the outgrowth of Bolwig's nerve. Previous mosaic studies located the focus of the *disco* mutant phenotype in an anterior dorsal position (Steller et al., 1987). This location is far removed from the site of origin of the optic lobes as determined by a previous fate mapping study (Kankel and Hall, 1976). Steller et al. (1987) also observed that the cell bodies of the neurons of Bolwig's nerve are frequently dispersed and abnormally positioned in *disco* mutants. This fact together with the results of mosaic analysis led to the proposal that the requirement for wild type *disco* function is in the neurons of Bolwig's nerve. Consistent with this hypothesis, we find expression of the *disco* gene in the posterior procephalic region which is the likely site of origin of the cells of Bolwig's nerve (see also Chapter 6). However, we have not identified significant *disco* expression in the cells of Bolwig's nerve after axonal outgrowth.

We have also found significant *disco* expression in the primordium of the adult optic lobes near the terminal region of Bolwig's nerve. Therefore, there could be non-autonomous requirements for *disco* gene function to direct normal Bolwig's nerve connectivity. Whereas expression of *disco* in the cells of Bolwig's nerve may be transient, expression in the optic lobe primordia persists well beyond the period of Bolwig's nerve outgrowth. This domain of expression is altered in *disco* mutant embryos (see discussion below). In addition, significant expression is detected in the cells of the atrium. These cells are adjacent to the Bolwig's nerve cell bodies in their final position (see Figure 3.3D). Requirements for wild type *disco* function in the atrium and in cells near the terminus of Bolwig's nerve could explain both the cell body and axon positioning defects seen in *disco* mutants. At present we cannot determine in which cells *disco* function is required for proper connectivity and positioning of the larval photoreceptor cells. Mapping the *disco* focus with a marker for the genotype of internal tissues is needed to resolve this question.

We have observed that the *disco* protein is found in the cell nucleus in embryonic and larval tissue. Analysis of the sequence of the *disco* gene (Chapter 2) reveals a short region with similarity to the zinc finger class of nucleic acid binding proteins, many of which are transcription factors. These findings raise the possibility that the *disco* gene product may be a regulatory protein. If so, *disco* could, for example, be directing the expression of molecules necessary for recognition of synaptic partners. More generally it could affect the development or differentiation of cells that are required for some step in the patterning process.

We have found that overall levels of *disco* gene expression in embryos homozygous for either mutant *disco* allele are normal. Subcellular localization of the gene product in these embryos is also indistinguishable from wild type. However, a single domain of gene expression, namely, expression in the optic lobe primordium near the Bolwig's nerve terminus, is missing in the mutant embryos. The absence of staining in this single region in mutant embryos demonstrates that this tissue has a different requirement for wild type gene function than the other tissues which show no obvious alteration in staining.

This indicates that, at some level, the gene is influencing either directly or indirectly its own expression in this region. Given the nuclear localization and the previously described sequence similarity to a motif found in transcriptional regulators, it is possible that the alteration in staining represents a direct autoregulatory function. An alternate explanation is that the cells which normally express *disco* in this region are absent or are ectopically located. However, there is no evidence of morphological abnormalities in the optic lobe primordium in *disco* mutant embryos.

Tix et al. (1989) have identified a cluster of cells (named OLPs) in the optic lobes which differentiate early, probably during embryonic development. Their location and projection pattern, together with their birthdate, suggest they might interact with Bolwig's nerve during embryonic development or larval life. Preliminary evidence indicates that these cells are missing in *disco* mutant larvae (S. Tix and K.-F. Fischbach, personal communication; see also Chapter 6). One question raised by these data is whether *disco* is expressed in the OLPs during formation of the larval visual system. The experiments described here do not show expression of *disco* in these cells in third instar larvae. As the OLPs can currently be identified only during late larval stages, it is unknown whether *disco* gene products are found in these cells earlier (see Chapter 6).

Finally, we have found that *disco* expression continues beyond embryogenesis in many tissues. The gene is expressed in late third instar larvae in or near the developing imaginal optic lobes. These expression data indicate a need for further experiments to ascertain whether this later *disco* expression plays an autonomous role in development of the optic lobes.

Materials and Methods

In situ hybridization

Whole mount *in situ* hybridization to embryos and larvae was performed essentially as previously described (Tautz and Pfeifle, 1989). In all experiments the probe was an embryonic cDNA isolate (Chapter 2) which contains most of the *disco* open reading frame. This DNA was labeled using methods and reagents supplied by Boehringer Mannheim Biochemicals.

For embryonic staining we used a slightly modified protocol provided by L. Dickinson and R. Lehmann. This protocol differed from the method of (Tautz and Pfeifle, 1989) only in the hybridization solution [which was 50% formamide, 1X salts (from a 10x solution: 3 M NaCl, 100 mM Tris, 100 mM sodium phosphate, 2% polyvinylpyrrolidone, 2% Ficoll 400, 50 mM EDTA, pH6.8), 10% dextran sulfate, 1 mg/ml yeast tRNA, 0.1% Tween 20] and the inclusion of an additional 1 hr, 45° washing step in hybridization solution after the overnight hybridization.

For larval staining, we used a slightly modified protocol provided by S. Campuzano and J. Modolell. This protocol differed from the method of (Tautz and Pfeifle, 1989) chiefly in the initial fixation step. Larvae were dissected and fixed in 4% paraformaldehyde in PBS for 20', washed in PBS, then fixed in 0.5% glutaraldehyde in PBS for 2' on ice.

Production of bacterial fusion proteins

Briefly, a 541 bp Bam HI fragment, corresponding to nucleotides 615-1156 of the mature *disco* message (Chapter 2), was ligated into the Bam HI site of the expression vectors pRIT2 (Nilsson et al., 1985), pUR291 (Rüther and Müller-Hill, 1983) and pET-3a (Studier et al., 1990). After transformation into the appropriate bacterial hosts, cultures were grown and fusion proteins were induced as previously described. In the case of the pET derivative, it was necessary to use the BL21(DE3) host carrying the pLysS plasmid (Studier et al., 1990) in order to reduce loss of the expression vector during culture. The pRIT fusion protein was purified by affinity chromatography over a rabbit IgG agarose (Sigma) column. The pUR and pET fusion proteins were partially purified by preparative SDS-polyacrylamide gel electrophoresis.

Generation of antisera

Typically, standard procedures were used for preparation and purification of antisera [see, for example, (Harlow and Lane, 1988)]. The purified pRIT fusion protein (100 μg) was mixed 1:1 with MPL+TDM+CWS adjuvant (RIBI Immunochem) and injected subcutaneously into three female New Zealand White rabbits. These rabbits were boosted several times at four week intervals with the same immunogen. The same rabbits were then boosted at four week intervals with the partially purified pET product (125 μg) mixed 1:1 with MPL+TDM+CWS adjuvant. Serum was collected two weeks after each boost and stored at -20°. The sera were purified by chromatography over a column containing the partially purified pUR fusion protein coupled to Affigel 10 (Bio-

Rad). Antibodies were eluted from the column with 50mM glycine pH 2.5, 150mM NaCl, then neutralized, dialyzed against PBS and stored at 4°.

Immunohistochemistry

Staining of embryos and larvae was performed essentially as previously described (Steller et al., 1987) with the following modifications: Tissues were fixed in PLP fixative (McLean and Nakane, 1974) for 20-30 min at room temperature. Subsequent washing steps were done in BSS(Ashburner, 1989) and antibody incubations and blocking steps were in BSN (0.9X BSS, 0.2% Triton-X100, 10% filtered goat serum). These antibody incubation steps were for 4-6 hr at room temperature or overnight at 4°. Monoclonal antibodies were used at a dilution of 1:1, affinity purified anti-Disco antibodies at a dilution of 1:5 and HRP-conjugated secondary antibodies (Bio-Rad) at a dilution of 1:100 for embryonic tissues, 1:500 for larval tissue. Double-labelling experiments were performed using the method of (Lawrence et al., 1987). Briefly, embryos were incubated simultaneously with polyclonal anti-Disco sera and monoclonal antibodies, followed by washing, blocking, incubation with HRP-conjugated goat anti-rabbit IgG and reaction with DAB as before. This was followed by washing, blocking and another incubation with HRP-conjugated goat anti-mouse IgG. After this incubation, the samples were reacted with DAB in the presence of 0.03% nickel sulfate and 0.03% cobalt chloride, then dehydrated and mounted as before.

Acknowledgments

We are grateful to Deborah Goberdhan-Vigle for the larval *in situ* hybridizations. We thank R. Lehmann, L. Dickinson, S. Campuzano and J. Modolell for whole-mount *in situ* hybridization protocols, D. Rio, P. Kaufman, D. Hartley and A. Lander for help with antibody preparation and purification and F. W. Studier for bacterial expression systems. We thank K.-F. Fischbach and M. Heisenberg for mutant alleles and S. Benzer for providing monoclonal antibodies. We acknowledge A. R. Campos and other members of the Steller lab for valuable discussions and for their critical comments on this manuscript. This work was supported by NIH grant RO1-NS26451 and a Searle Scholars Award to H. S. M. F. received a SERC(UK)-NATO postdoctoral fellowship and K. L. a N.S.F. predoctoral fellowship.

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Figure 3.1 Tissue distribution of *disco* mRNA during embryonic development.

The *disco* transcript was detected in whole mount embryos by *in situ* hybridization using digoxigenin labeled probes. (A) and (C-F) are lateral views, (B) is a dorsolateral view. Anterior is to the left and dorsal is up. Staging follows the convention of Campos-Ortega and Hartenstein (1985). (**A**) cellular blastoderm embryo (stage 5). *disco* mRNA is found in a cap at the posterior pole surrounding the pole cells. (**B**) gastrulation (late stage 6). *disco* mRNA is found in the amnioproctodeal invagination (api) and in two dorsal transversal stripes (arrowheads) in and just anterior to the cephalic furrow. (**C**) extended germ band embryo (stage 11). *disco* mRNA is detected in the proctodeum (pr), in small clusters of cells in the ectoderm of abdominal segments, in larger clusters in the ectoderm of thoracic segments (t), in the three gnathal segments (gn), in the clypeolabrum (cl) and in parts of the procephalic lobe. Staining in the procephalic lobe comprises two domains: one group in the antennal segment (an) and one in and around the invaginating optic lobe primordium (arrowhead). (**D**) embryo during germ band retraction (stage 12). *disco* mRNA is distributed as in the previous panel with additional staining in the visceral mesoderm (vm). (**E**) stage 14 embryo. *disco* mRNA is found in the thoracic and gnathal segments as before. The gnathal segments are moving forward through the process of head involution. Staining persists in the antennal segment and in the optic lobe primordium (arrowhead). This latter domain has at this stage been internalized and is in contact with the brain lobe. The visceral mesoderm, which also continues to stain, is spreading out around the gut. (**F**) late stage 15 embryo. *disco* mRNA is found as before in the gnathal segments. By this time these segments are almost entirely contained within the anterior opening (atrium). Staining persists in the visceral mesoderm which has become a thin covering surrounding the gut (g). Staining is found in many parts of the nervous system including the ventral nerve cord (vnc) and the optic lobe primordium (arrowhead), now incorporated within the brain lobe. Bar = 50 µm.

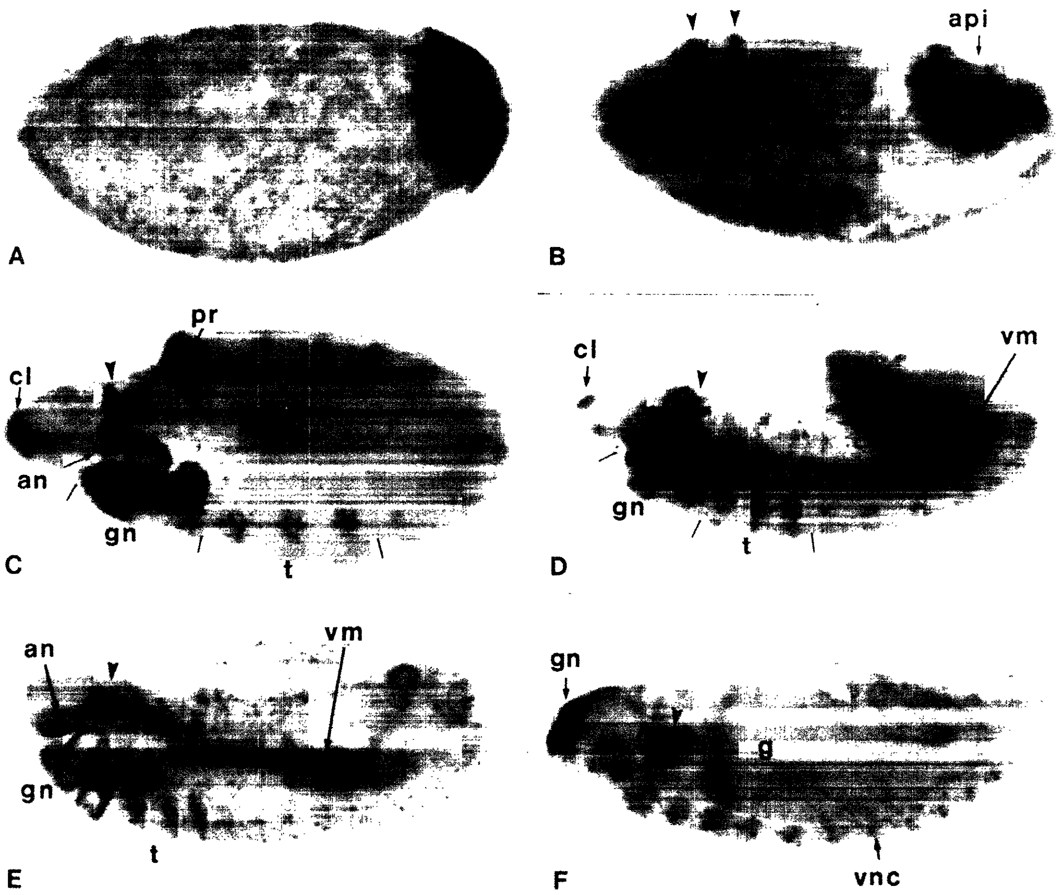
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Figure 3.2. Tissue distribution of the *disco* protein during embryonic development.

Embryos were stained with an affinity purified *disco*-specific polyclonal antiserum. Note the similarities between the staining shown here and that shown in Fig. 1. (A-F, I, J) show lateral views, (G) a dorsolateral and (H) a dorsal view. Abbreviations are as in the preceding figure. (A) embryonic stage 5. anti-Disco staining is present around the posterior pole. (B) stage 6. Staining can be seen in the amnioproctodeal invagination and in a transverse stripe (arrowhead) anterior to the cephalic furrow. (C) stage 10. *disco* protein is detected here in the proctodeum, in the clypeolabrum and ventrally on either side of the cephalic furrow in the primordia of the gnathal segments. (D) early stage 12. Staining can be seen in the gnathal segments, in the visceral mesoderm and in two parts of the procephalic lobe: the antennal segment and in and around the optic lobe primordium (arrowhead). This latter structure is clearly visible here as a groove in the posterior procephalon. (E) and (F) two focal planes of a late stage 13 embryo. *disco* protein is detected in the gnathal segments, in ventrolateral groups of cells in the thoracic segments and in and around the optic lobe primordium (arrowhead). (E) shows segmentally repeated staining in the lateral ectoderm (small arrows) which is near and likely includes a subset of the peripheral nervous system. (F) shows staining in the visceral mesoderm and in the cardioblasts, a longitudinal row of cells (large arrow) near the amnioserosa. (G) and (H) embryos during the process of dorsal closure. *disco* protein is detected in the rows of cardioblasts (large arrows) which meet at the dorsal midline and eventually give rise to the larval heart, the dorsal vessel. (I) late stage 15. Staining is seen in the gnathal segments, in the visceral mesoderm surrounding the gut, in the cardioblasts (large arrow) and in many parts of the nervous system including the optic lobe primordia (arrowhead). (J) embryo hemizygous for the deficiency Df(1)l9 which deletes the *disco* gene. The embryo was incubated with *disco*-specific antiserum as above and shows none of the previously described staining. In this case, non-specific staining is detected in the lumen of the salivary gland (arrow). Bar = 50 μm.

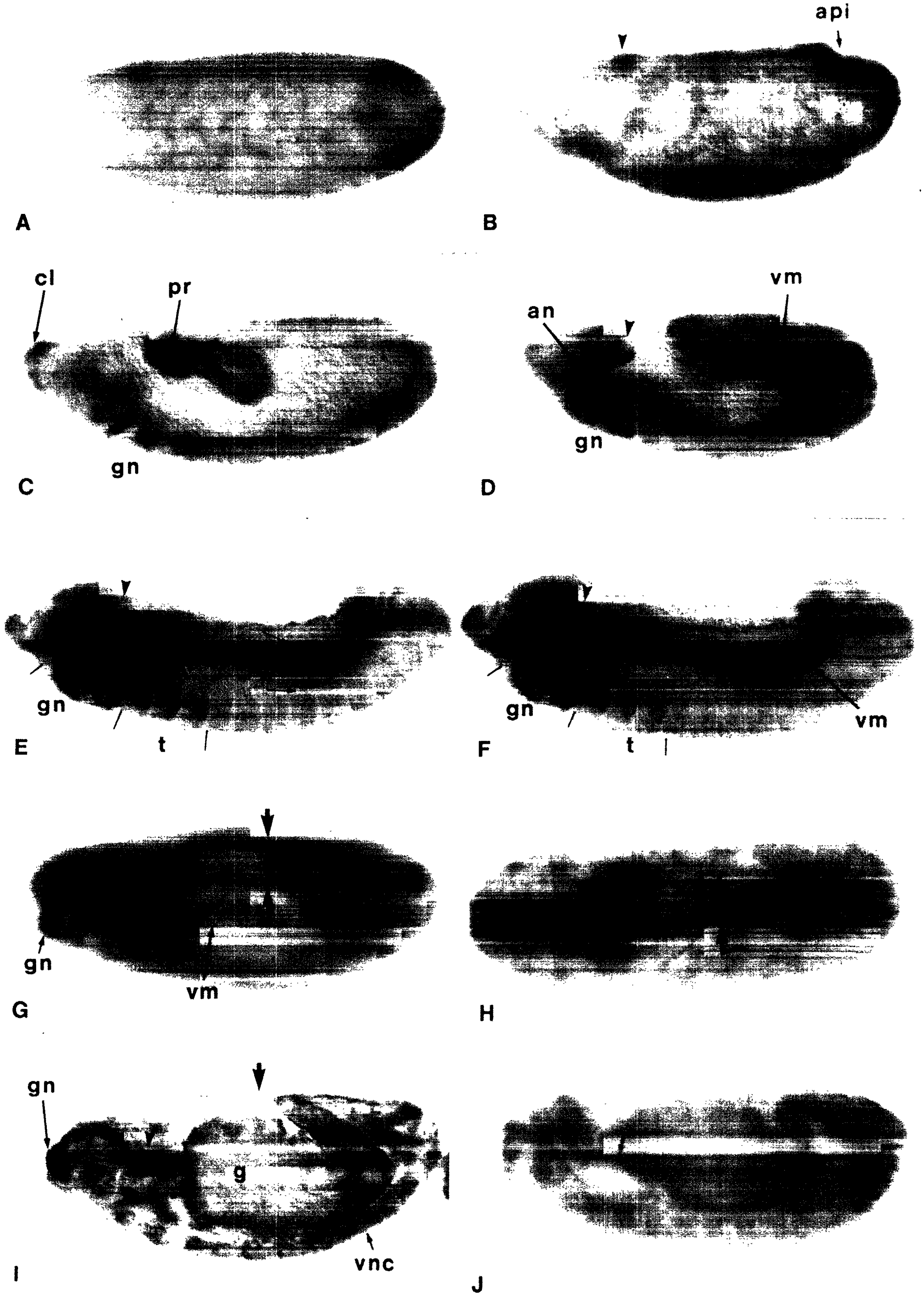
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Figure 3.3 Tissue distribution of the *disco* protein during development of the larval visual system.

Embryos were stained with an affinity purified *disco*-specific polyclonal antiserum and either a PNS-specific monoclonal antibody, MAb22C10 (A, B), or a photoreceptor-specific monoclonal antibody, MAb24B10 (C, D). In both cases anti-Disco staining is indicated by a brown reaction product and monoclonal antibody staining by a black reaction product. (**A**) and (**B**) two focal planes of the anterior region of a stage 14 embryo. At this stage of embryonic development, the neuronal cell bodies of Bolwig's nerve are only a short distance from the target region in the developing brain lobes. In (A) the position of these cell bodies (cb) is indicated and a short process is seen extending from them posteriorly and ventrally to the brain lobe surface. The terminal region of the nerve (te), shown in (B), is located at the edge of a large triangular domain of *disco* expression. This domain includes the primordium of the imaginal optic lobes. (**C**) and (**D**) two focal planes of the anterior region of a stage 17 embryo. In the period between the stage shown in the two previous panels and this stage, head involution has moved the neuronal cell bodies of Bolwig's nerve to their final position away from the brain lobes. These cell bodies are found in contact with the atrium (at) at a point near the larval mouthhooks. Bolwig's nerve extends back and penetrates the brain lobes. The domain of *disco* expression near the Bolwig's nerve terminal region is at this stage more dispersed and roughly ring shaped. Note also the extensive staining in the atrium near the Bolwig's nerve cell bodies. Bar = 19.5 μm in (A, B), 25 μm in (C, D).

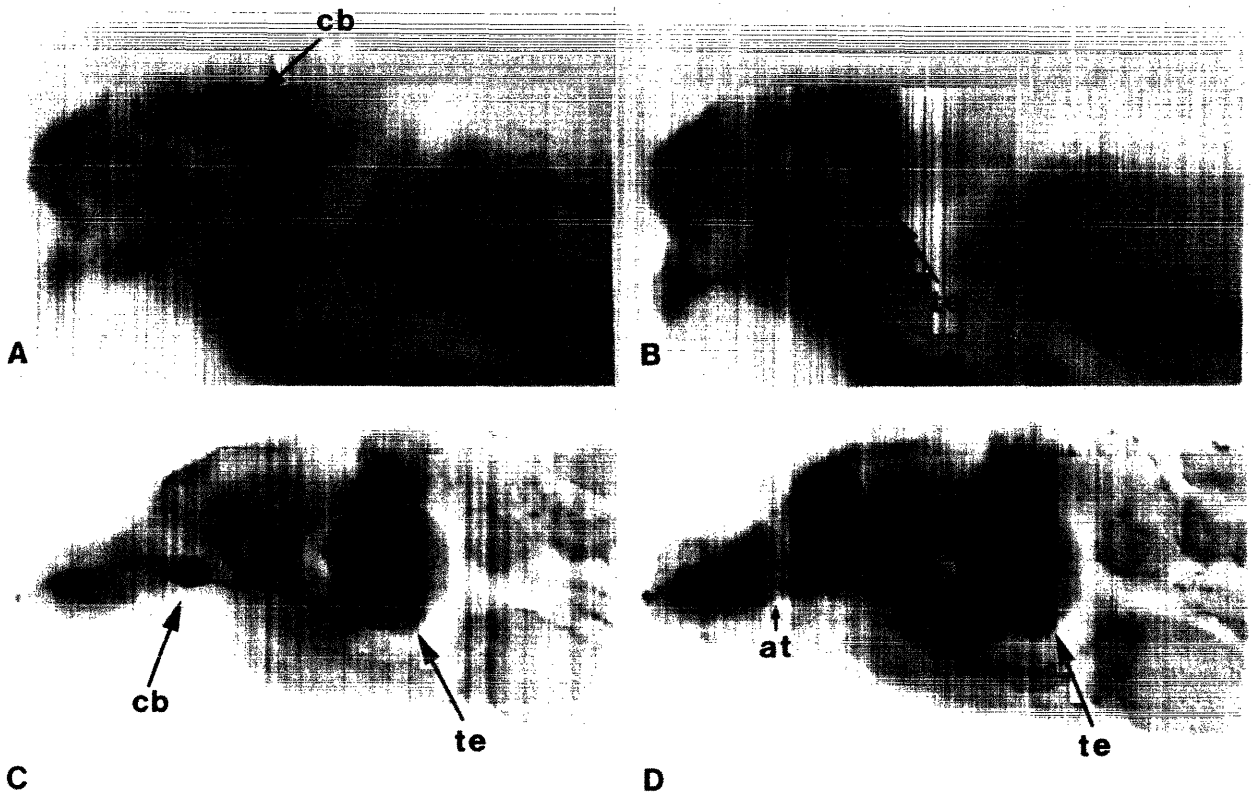
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Figure 3.4 Tissue distribution of *disco* mRNA and protein in third instar larvae.

Third instar larvae were dissected and the head regions, imaginal discs and CNS were used for *in situ* hybridization (A) or stained with an affinity purified *disco*-specific polyclonal antiserum (B, C). (A, B) are dorsal views; (C) is a ventrolateral view. In all panels anterior is to the left. (**A**) the distribution of *disco* mRNA in third instar larvae. *disco* transcripts are seen in several imaginal discs and in parts of the larval brain lobe. A band of stained cells (arrowhead) is found on the surface of each brain lobe. This band extends from a ventrolateral position near the optic stalk to the dorsal surface. (**B**) the distribution of *disco* protein in third instar larvae. Staining is found in the antennal discs (ad), the leg discs (ld) and in parts of the CNS. As in (A), a band of staining is found wrapping around each brain lobe (large arrowheads). A regular array of stained cells is found in the ventral nerve cord (vnc). A small number of stained cells are found in the lamina primordium (small arrowhead shows one in this plane of focus). (**C**) distribution of the *disco* protein in the antennal-eye disc and brain lobe of a third instar larva. The antennal disc portion is extensively stained whereas the eye disc (ed) is unstained. Two large stained cells are found in the optic stalk (os), one of which is visible in this focal plane. At the base of the optic stalk is the primordium of the lamina, a crescent shape demarcated by furrows (between small arrows). In this individual seven stained cells are found extending across this crescent. The large band of stained cells described above (arrowhead) begins just posterior to this region and extends around the surface of the brain, out of the focal plane. Bar = 62.5 μm in (A), 50 μm in (B, C).

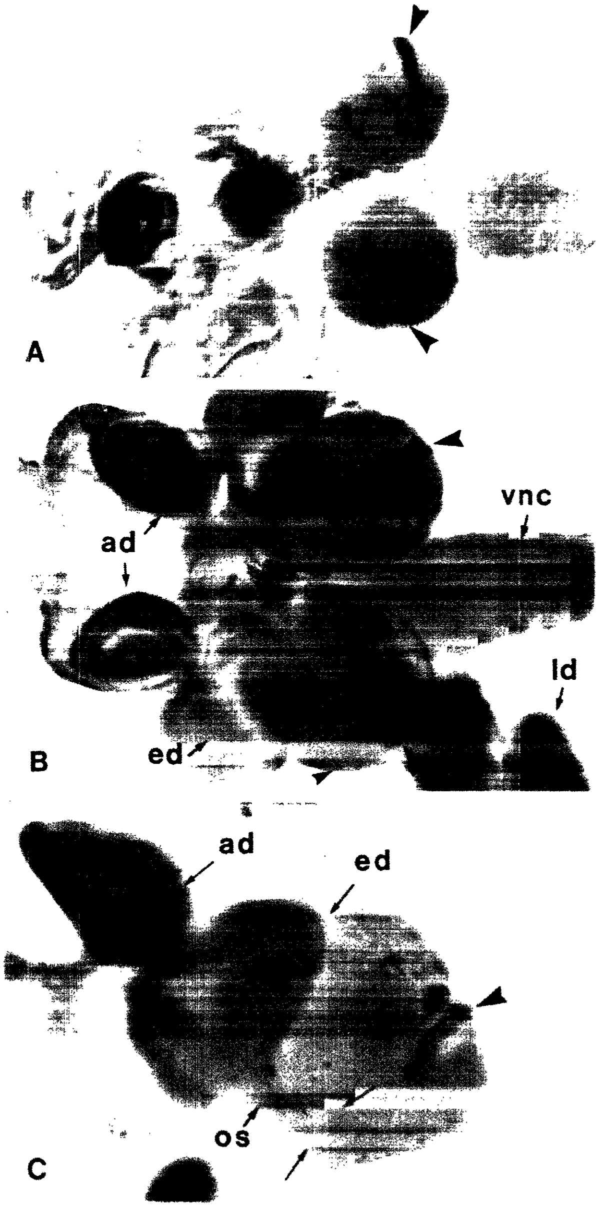
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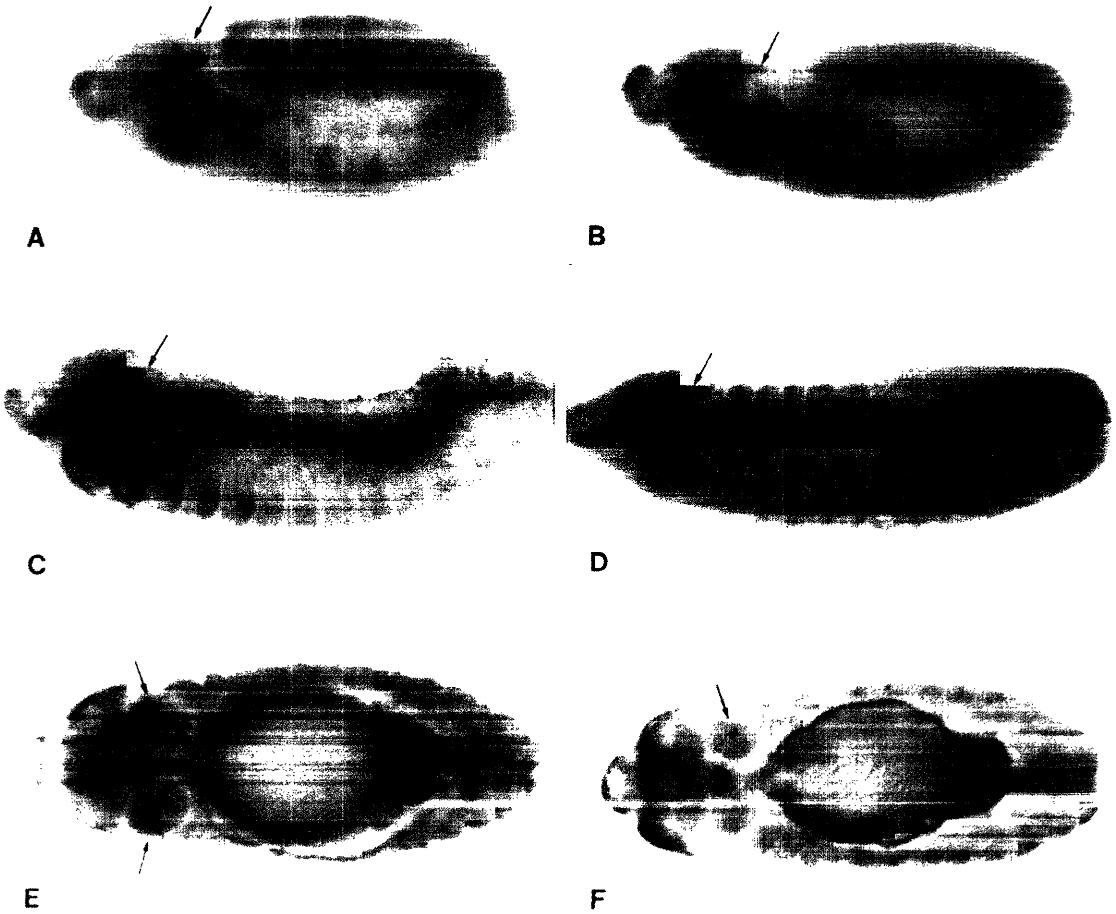
Figure 3.5 Tissue distribution of the *disco* protein in wild type and *disco* mutant embryos.

Wild type (A, C, E), *disco*^{1} homozygous (B, F) and *disco*^{1656} homozygous (D) embryos were stained with an affinity purified *disco*-specific polyclonal antiserum. (A-D) show lateral views; (E, F) show dorsal views. (**A**) and (**B**) stage 11 embryos. In the wild type embryo *disco* expression is found in the region of the optic lobe primordium (arrow). In the mutant staining is absent in this region. In all other tissues staining in the mutant is identical to that observed in the wild type . (**C**) and (**D**) stage 13 embryos. Again the only difference in staining between the wild type and mutant is a lack of staining in the region of the optic lobe primordium (arrow) in the mutant. (**E**) and (**F**) stage 14 embryos. Staining in the two embryos again differs only in the region of the optic lobe primordia (arrows). In this case the mutant embryo shows a tiny rudiment of staining in the right brain lobe (arrow). Bar = 50 µm.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Chapter 4.

Ectopic expression of the *Drosophila disconnected* gene causes nervous system defects

Preface

The work presented in this chapter is part of a very fruitful collaboration with Dr. Ana Regina Campos, a former postdoctoral fellow in the Steller laboratory, now an independent researcher at McMaster University, Hamilton, Ont. My contribution to the studies in this chapter is the analysis of *disco* expression in the *hs-disco* strain. These studies have also been submitted for publication as: Campos, A. R., Lee, K. J., and Steller, H. S. Ectopic expression of the *Drosophila disconnected* gene causes nervous system defects.

Abstract

The *disconnected* (*disco*) gene is required for the correct development of the larval visual system. Here, we describe the consequence of ectopic expression of the *disco* gene during embryogenesis. Ectopic expression disrupts the pattern of axonal projections in both the central and the peripheral nervous systems. Additionally, it causes aberrant projections of the larval optic nerve. Analysis of this phenotype suggests that the defect in the projection pattern of the larval visual system is due to the abnormal positioning of the optic lobe primordium, thought to contain the synaptic partners of the larval photoreceptor cells.

Introduction

The proper interaction of growing axons with the surrounding environment is essential for the assembly of a functional nervous system. The larval optic nerve of Diptera, Bolwig's nerve (Bolwig, 1949), is an attractive model system to study the molecular basis of neuronal specificity, since it represents a rather simple, well described neuronal circuit (Steller et al., 1987). During *Drosophila* embryogenesis, each of the two clusters of larval photoreceptor neurons projects a bundle of axons, forming Bolwig's nerve, to the optic lobe primordium of the developing brain where the synaptic partners are thought to reside. We have previously identified a gene, *disconnected (disco)*, which is required for the establishment of stable connections between the larval optic nerve and its target cells in the embryonic brain (Steller et al., 1987). We have cloned the *disco* gene and determined its structure, nucleotide sequence and pattern of expression (Heilig et al., 1991, Lee et al., 1991). These studies indicate that *disco* may encode a transcription factor, since the conceptually translated *disco* protein contains two putative zinc binding fingers, a nucleic acid binding motif found in many transcriptional regulators. Consistent with this hypothesis, the *disco* protein is located within the nucleus (Lee et al., 1991), and it shows a sequence-specific DNA binding activity *in vitro* (Chapter 5).

In addition to connectivity defects in the larval visual system, *disco* embryos also display projection defects in the peripheral nervous system (PNS). These phenotypes are more subtle and variable, involving the abnormal fasciculation of some axons which can be seen occasionally to cross segmental boundaries (Steller et al., 1987). Furthermore, *disco* mutants lack locomotor activity rhythms, probably due to disruption in the output pathway of circadian rhythms (Dushay et al., 1989, Hardin et al., 1992). Finally, *disco* may be involved in limb development, since its expression in the leg primordia is under the control of the *Distal-less* gene (Cohen et al., 1991).

The *disco* gene displays a complex pattern of expression throughout development (Lee et al., 1991). Of particular interest with regard to the role of the *disco* gene in pathway formation is its expression in the larval visual system. The *disco* protein is detected in an invagination of the embryonic epidermis, the optic lobe placodes, which comprises the primordium of the imaginal optic lobes. Reduced *disco* protein is detected once the optic lobe placode has been incorporated into the brain hemispheres (Lee et al., 1991). Additionally, the *disco* protein has also been detected in cells situated along Bolwig's nerve which have been provisionally identified as glial cells (Chapter 6). No *disco* expression has been detected in the larval photoreceptor cells at times when they can be unequivocally identified, i.e. after the onset of axonal outgrowth. However, given the presence of *disco* protein in the ectodermal region from which the larval photoreceptor neurons are thought to derive, it is possible that *disco* is expressed in these cells or their precursors at earlier stages.

The analysis of the *disco* mutant phenotype and its pattern of expression during visual system development suggest a role of the *disco* gene product in axon guidance and/or target cell recognition. Here we describe experiments aimed at investigating the consequence of ectopic expression of the *disco* gene for the development of the nervous system. By expressing *disco* under the control of the heat inducible heat shock protein 70 gene (*hsp70*) promoter we sought to determine whether *disco* serves merely a permissive role or, alternatively, a more active, instructive role during development. In the first case, we would expect that expression of the *hs-disco* transgene may lead to phenotypic rescue of *disco* mutants but, in analogy to previous studies with other genes (e.g. Steller and Pirrotta, 1985, Basler and Hafen, 1989), ectopic expression would have no detrimental effects on development. On the other hand, if *disco* played an instructive role, one might expect that its ubiquitous expression would interfere with normal development, leading to a dominant mutant effect of the transgene (e.g. Schneuwly et al., 1987, Kuziora and McGinnis, 1988, Basler et al., 1990). In this study we report that the ectopic expression of *disco* during embryogenesis causes striking developmental abnormalities, including severe projection defects in both the central and peripheral nervous system. These results indicate that *disco* expression is sufficient to interfere with a cell's normal developmental program, suggesting an instructive role of this gene during nervous system development.

Results

Expression of disco protein under the control of the heat shock promoter

In order to alter the expression of the *disco* gene, we constructed a chimeric gene containing the *disco* coding sequence downstream from the promoter of the heat shock protein gene 70 (*hsp70*). Similar constructs have been previously used to induce ectopic expression of several genes in *Drosophila* (e.g. Steller and Pirotta, 1985, Schneuwly et al., 1987, Kuziora and McGinnis, 1988, Blochlinger et al., 1991). The fusion gene was inserted into the P-element transformation vector CaSpeR (Pirrotta, 1988) and used to transform a *white* mutant strain as described in Materials and Methods. Fig. 4.1 depicts a diagram of the construct. The chimeric gene is referred to as the *hs-disco* fusion gene and the flies harboring this construct as *hs-disco* strains. For this study, two independent lines homozygous for autosomal insertions were used. These lines are fully viable, fertile and display no detectable morphological defects in the absence of heat shock (see below).

To test the expression of the *hs-disco* fusion gene, embryos from the hs-*disco* strains were heat shocked for 45 min between four to six hours of development, and stained with the anti-*disco* antibody after varying periods of recovery. As a control, Canton S strains were subjected to the same treatment. Fig. 4.2 shows photomicrographs of such embryos in two different planes of focus. Thirty minutes after heat shock the *disco* protein is found throughout the embryo (Fig. 4.2B). Interestingly however, two hours after the heat shock, the ectopic expression of *disco* becomes restricted to the developing central nervous system (Fig. 4.2 E-F). After four hours of recovery, *disco* protein is no longer detected in ectopic sites (data not shown).

Misexpression of the disco gene product causes nervous system defects

Misexpression of the *disco* gene product under the control of the *hsp70* promoter during embryogenesis resulted in high mortality. The highest incidence of lethality (199 out of 210 embryos, corresponding to 95%) was observed when the heat pulses were given between four to six hours after egg laying (AEL), during a period where neurogenesis and the initial steps in pattern formation of the nervous system occur (reviewed by Campos-Ortega and Hartenstein, 1985). Under the same conditions, the rate of lethality of the non-transformed y w host strain was less than 0.3% (2 out of 706 embryos) upon heat shock. In the absence of heat shock, the mortality of hs-disco embryos was less than 3.5% (7 out of 204 embryos). It is not clear whether this is due to a low basal level of transgene expression in the absence of heat shock. Alternatively, the presence of background mutations and the isogenization of the transgene-bearing autosomes may be responsible for the slightly elevated lethality rate.

Fig. 4.3 shows photomicrographs of stage 16 (according to Campos-Ortega and Hartenstein, 1985) wild type and *hs-disco* embryos which were heat shocked for 45 min between four to six hours of embryogenesis. These embryos were stained with an anti-HRP antibody (Jan and Jan, 1982) which recognizes an epitope present in the membrane of all *Drosophila* neurons, and the monoclonal antibody 22C10 (Zipursky et al., 1984) which binds to an antigen in neurons of the peripheral nervous system (PNS). Several hundred embryos were analyzed in this manner (for details see Materials and Methods). Under these heat shock conditions the overall morphology of the embryo was not drastically altered. However, approximately 50% of these embryos (among 301 counted) displayed striking nervous system defects, such as interruption or malformation of the neuropil region of the central nervous system. In contrast, less than 1% (3/338) of the embryos from the non-transformed *y w* host strain displayed notable CNS defects upon heat shock. In the peripheral nervous system (PNS) a general disorganization with respect to the location of cells and routing of axon fascicles was visible (Fig. 4.3D). Although later heat shocks caused a marked decrease in the frequency of nervous system defects they still produced relatively high lethality. Heat shocks given between 4 to 6 hours AEL disrupted the nervous system in roughly half of all the embryos examined and caused lethality in more than 90%. In contrast, heat shocks applied 6 to 8 hours AEL caused nervous system defects in only ~5% of the embryos, but resulted in a lethality rate of ~30%. Therefore, it is possible that the lethality caused by ectopic *disco* expression is not exclusively due to nervous system defects, but may result from defects in other tissues as well. The effect of ectopic expression of the disco gene outside the nervous system is exemplified by impairment of germ band retraction observed in some of the heat shocked embryos. We conclude that the aberrant expression of *disco* has pleiotropic effects on embryogenesis, with particularly dramatic consequences for the proper development of the nervous system.

Defects in the CNS

The development of the nervous system in *Drosophila* occurs over a period of several hours. It begins during stage 9 (3 hours and 40 min AEL) with segregation of the neuroblasts lasting for approximately three hours. The first neural fibers can be seen at the beginning of stage 12 (7:20-9:20 hr). By the end of stage 12 the definitive pattern of neuropil can already be distinguished (reviewed in Campos-Ortega and Hartenstein, 1985).

It is possible that the ectopic expression of the *disco* gene disrupts early steps in the assembly of the nervous system, for example, segregation of neuroblasts from the ectoderm. However, as we observed persistent presence of the *disco* protein in the developing nervous system for up to two hours after heat shock, it is possible that the ectopic expression of the *disco* gene disrupts early events in pathway formation. In an attempt to distinguish between these alternatives, we examined the nervous system defect in more detail.

Here, we sought to determine whether disruption of the CNS neuropil due to ectopic expression of the *disco* gene 4 to 6 hours AEL was also associated with a loss of the corresponding cell bodies in the cortex. To that end, double labeling experiments were performed where the neuropil and the adjacent cell body layer were visualized by staining with anti-HRP antibody and mAb44C11, which recognizes the neuron-specific *elav* protein (Bier et al. 1988, Robinow, 1989, Robinow and White, 1991).

In *hs-disco* embryos, the CNS neuropil was frequently seen to be interrupted at the level of commissures and connectives (Fig. 4.4B and C). Often, we observed fusion between the anterior and posterior commissures. Interruption of the neuropil was not accompanied by significant reduction in the number of neuronal cell bodies, since staining of the cortex layer with 44C11 monoclonal was essentially normal.

In order to assess the effect of *disco* ectopic expression on the overall organization of the CNS we investigated the presence and distribution of particular cell types. We examined the pattern of neurons expressing the *even* *skipped* (*eve*) gene by immunohistochemistry of whole mount embryos. Fig. 4.5 shows the ventral nerve cord of such embryos. *eve* protein is found in distinct clusters per hemisegment (Frasch et al., 1987). In embryos carrying the *hs-disco* construct induction of this chimeric gene caused some disruption in the pattern of the *eve*-expressing cell clusters, characterized by reduction in the number of cells in some but not all clusters. Nevertheless, the general organization was still retained, suggesting that the differentiation of at least some specific cell types is not severely affected.

These observations suggest that aberrant expression of the *disco* gene in the CNS affects the development of the neuropil layer. However, it is not clear whether the observed disruptions are due to a direct effect on axon growth or absence of cells with a crucial role in pathway formation.

Defects in the PNS

The results described above indicate that the aberrant expression of the *disco* gene during the development of the CNS disrupts the formation of the regular array of axons that form the CNS neuropil. The structure of the PNS in *Drosophila* embryos has been described in detail (Campos-Ortega and Hartenstein, 1985, Ghysen et al., 1986). The neurons of the PNS are stereotypically distributed in ventral, lateral and dorsal clusters within each segment (Fig. 4.6A). The PNS is well suited to address the consequences of ectopic *disco* expression at the level of individual cells, as all peripheral neurons can be readily identified upon staining with the monoclonal antibody 22C10. The *disco* gene appears to be required for the proper development of the PNS, since mutant embryos display subtle but significant abnormalities in the projections and position of peripheral neurons (Steller et al., 1987). We wanted to examine whether misexpression of the *disco* gene product between four to six hours AEL disrupts early determinative steps in the development of the PNS, or whether it affects the PNS at the level of axonal projection.

We observed various PNS defects upon heat shocking embryos carrying the *hs-disco* construct. The peripheral neurons displayed their distinct morphology and were organized into three clusters per hemisegment. There was a general disorganization in the positioning and number of cells (Fig. 4.6B and C). Neurons in all three clusters were affected equally. Incomplete migration of the lch1 cell to its final position dorsal and lateral to the lateral cluster, was often observed (Fig. 4.6B open arrow). Of particular interest was the abnormal projection pattern of peripheral nerves in approximately 10 to 20% of the heat shocked embryos. Single axons were observed straying from their normal route. In addition, abnormal fasciculation of axons from one segment with axons from the adjacent segment was seen (Fig. 4.6E and F). Frequently, such aberrant projection patterns were observed in regions where the arrangement of PNS cell body clusters appeared normal.

In summary, the PNS phenotypes caused by ectopic expression of the *disco* gene involve the disruption of the neural projection patterns and the misplacement of neuronal cell bodies. In general, the cytomorphology of peripheral neurons appeared normal, indicating that their general differentiation was not perturbed.

Defects in the projection pattern of the larval visual system

In order to investigate the effects of ectopic disco expression on the development of the larval visual system, we analyzed the projection pattern of the larval optic nerve in *hs-disco* embryos which had been heat shocked four to six hours AEL.

In a stage 16 wild type embryo the cell bodies of Bolwig's organ are located anterior to the brain lobes, apposed to the antennal nerve. These cells send their projections posteriorly around the brain hemisphere and terminate in a ventro-lateral area of the brain where the primordium of the imaginal optic lobe is located (Poulson, 1950, White and Kankel, 1978, Fig. 4.7A, 4.8A, B, and 4.9A). Induction of the *hs-disco* chimeric gene caused abnormal positioning of photoreceptor cell bodies and abnormal projection of the optic nerve, as seen by staining with mAb22C10 and mAb24B10. In these embryos, the larval optic often took a more dorsal path and frequently did not terminate in the region of the brain hemisphere normally occupied by the optic lobe primordium (Fig. 4.7C-F). A few embryos however, showed optic nerves that, in spite of the larval optic nerve dorsal route, terminated roughly in the correct region of the brain (Fig. 4.7B).

One interpretation of these results is that the projection defect is due to the inability of the optic nerve to properly recognize the surrounding environment. Alternatively, it is possible that this phenotype is caused by defects in other structures, for example the optic lobe primordium. In order to investigate this further, we determined the position of the optic lobe primordium relative to the larval optic nerve in *hs-disco* embryos using anti-HRP, 22C10 and *fas II* antibodies.

In a stage 16 embryo the optic lobe primordium in the brain hemisphere can be easily identified, using confocal microscopy, by the lack of the neural antigens recognized by the antibodies anti-HRP and anti-*elav* (Campos and Steller, unpublished results). Since the larval photoreceptor cells stain only weakly with anti-HRP antibody, it is necessary to perform double labeling with anti-HRP and 22C10 antibodies in order to visualize both the optic lobe primordium and the larval optic nerve.

Fig. 4.8 shows wild type (A and B) and *hs-disco* embryos (C-F) that have been stained with both 22C10 and anti-HRP antibodies. In wild type embryos the larval optic nerve enters the optic lobe primordium, here defined as the domain devoid of anti-HRP staining in the most ventral portion of the brain. The larval optic nerve terminates in a group of anti-HRP positive cells located more medially within this domain (Fig. 4.8B, open arrow). In all *hs-disco* embryos analyzed the larval optic nerve terminated within the optic lobe primordium. In the vast majority of cases where the nerve projected dorsally, the optic lobe primordium was located in the dorsal area of the brain hemispheres (Fig. 4.8C and D). However, we also observed a few instances where the larval optic nerve took a somewhat dorsal path but terminated in an optic lobe primordium that was found in the approximately correct area of the brain (Fig. 4.8E and F).

Similar results were obtained when *fas II* antibodies were used (Bastiani et al., 1987, Grenningloh et al., 1991). The *fas II* antigen can be found in the optic lobe primordium and along the distal segment of the larval optic nerve (Fig. 4.9A); the photoreceptor cells bodies are only weakly stained by *fas II* antibodies. In *hs-disco* embryos the larval optic nerve was always seen terminating in a *fasII*-expressing domain, even when located in the dorsal part of the brain (Fig. 4.9B and C).

The results show that the aberrant dorsal pathway of the larval optic nerve found in *hs-disco* embryos is strongly correlated with abnormal location of the optic lobe primordium. Thus, it appears that the primary cause for the larval optic nerve misrouting is the dorsal incorporation of the optic lobe placodes into the brain hemispheres. However, the existence of cases where misrouting of the larval optic nerve does not correlate with an obvious displacement of the optic lobe primordium suggests that the interaction of Bolwig's nerve with other cells may also be impaired by ectopic expression of the *disco* gene.

Discussion

Mutations in the *disco* gene prevent the establishment of stable connections between the larval optic nerve and target cells in the optic lobe primordium. A role for the *disco* gene in the development of the PNS is implied by the subtle disturbance in the pattern of PNS projections in mutant embryos. Additional roles are inferred from the pattern of expression of the *disco* gene product during development and reduced viability of mutant strains (Steller et al., 1987, Lee et al., 1991). Here, we attempted to investigate the role of the *disco* gene in the development of the nervous system by analyzing the effect of ectopic *disco* expression under the control of the *hsp70* heat shock promoter.

Our results show that aberrant expression of the *disco* protein disrupts the ordered arrangement of axonal projections throughout the nervous system. Ectopic expression of the *disco* protein persists longer in the nervous system than in other ectopic locations. It is possible that the continued expression of the *disco* protein is necessary for the induction of the observed developmental defects. Whether transcriptional or post-transcriptional mechanisms are responsible for the longer persistence of the *disco* protein in the nervous system has not been investigated. The phenotype caused by ectopic expression of *disco* does not require an endogenous wild type *disco* gene (data not shown).

On the other hand, the *hs-disco* construct is able to provide wild type *disco* function. We observed that in *disco* mutant strains carrying one to two copies of this fusion gene there is a significant increase of flies displaying a less severe mutant phenotype (data not shown). In addition, like the endogenous *disco* gene product, the *hs-disco* construct is capable of regulating *disco* expression in the optic lobe (Chapter 5). These data argue against a novel deleterious activity of the protein produced by the *hs-disco* construct.

The disruption in the connectivity pattern of the CNS that is associated with ectopic expression of the *disco* gene product does not seem to be accompanied by equally significant reduction in the number of CNS neurons. Therefore, it is possible that aberrant *disco* expression primarily affects axonal patterning. However, we cannot rule out direct effects on cells that are crucial for the control of axon growth. Similar results were obtained from the analysis of the PNS phenotype. The PNS phenotype following induction of the *hs-disco* construct suggests that the *disco* protein interferes with processes required for proper axon navigation and the correct positioning of cell bodies. The PNS phenotype caused by ectopic expression of the *disco* gene is similar but much more severe than that caused by the complete lack of *disco* protein, as observed in *Df(1)l9* deletion embryos (Steller et al., 1987, Lee et al., 1991).

We have failed to observe widespread cellular degeneration upon ectopic expression of *disco* . In addition, it appears that cellular differentiation in general was not affected, as evidenced by the presence and morphological appearance of particular classes of neurons in the CNS and PNS. For example, peripheral neurons still displayed their characteristic cell shapes, and expressed various differentiation markers, (such as *elav*, mAb22C10, mAb24B10, fasII). Therefore, we believe that the phenotypes observed do not result from a trivial cytotoxic action of ectopic *disco* expression, but from a more specific effect on cell movements and neuronal pathway formation.

In *hs-disco* embryos, Bolwig's nerve often displayed a dorsal projection towards the posterior part of the brain. This was correlated with a correspondingly aberrant location of the nerve's target region, the optic lobe primordium. It is likely that the position of the optic lobe primordium determines the initial orientation of larval optic nerve outgrowth and that contact with the optic lobe invagination helps to direct the normal optic nerve projection (see Chapter 6; Green, et al., 1993). Thus, the optic nerve pathway defects displayed in *hs-disco* embryos are probably due to impairment in the morphogenetic movements that lead to incorporation of the optic lobe placodes in the brain hemispheres. No abnormality in the projection pattern of other anterior sensory organs (antennal maxillary complex) was observed (data not shown), suggesting that the effect on the larval visual system is related to the role of the *disco* gene during the normal development of this particular structure, rather than a non-specific effect on any sensory structure that contacts the brain.

What is the relevance of these phenotypes for the function of the *disco* gene in a wild type situation? Among the various structures affected by ubiquitous expression of the *disco* gene, the larval visual system is of particular interest, since it is severely affected by loss-of-function *disco* mutations. Neither excess expression of *disco* (*hs-disco* embryos after heat shock) not the lack thereof (embryos carrying a chromosomal deletion encompassing the *disco* gene) seem to affect early steps in the determination of the larval photoreceptor cells. In both situations the projection pattern of the larval optic nerve is severely abnormal. However there are fundamental differences. In *disco* mutants the larval optic nerve fails to establish stable connections with cells in the optic lobe primordium. In contrast, in hs-*disco* embryos the nerve always terminates in this structure, but the target region is frequently misplaced. Apparently, this misplacement accounts for the pathway defect observed in these instances. However, we also observed cases where Bolwig's nerve projected via an aberrant route to an optic lobe primordium that appeared properly located. Therefore, it is possible that the effect of ectopic *disco* expression in the larval visual system is not restricted to the target region, but may also affect the interaction of the larval optic nerve with other cells outside the optic lobe primordium.

The results from this study are not easily reconciled with a simply permissive role of *disco* in neuronal pathway formation. The results presented in this chapter, together with the similarity of *disco* to known transcription regulators (Heilig et al., 1991, Lee et al., 1991, Chapter 5), are consistent with the idea that *disco* controls the expression of genes involved in the correct patterning of the nervous system.

Acknowledgments

We thank our colleagues in the Steller laboratory for comments on this manuscript. We are grateful to Vincenzo Pirrotta for the CaSpeR vector, Seymour Benzer for the mAb22C10, Manfred Frasch for the *eve* antibody, Corey Goodman for the *fas II* monoclonal antibody, and Lily and Yuh Nung Jan for mAb44C11. We would like to acknowledge Dave Smith for his help with the confocal microscopy. This work was supported in part by NIH grant RO1-NS26451 and a Searle Scholars Award to H. S. K. J. L. was supported by a National Science Foundation Fellowship. During the initial phase of this work A. R. C. was supported by a postdoctoral Fellowship from the Muscular Dystrophy Association of America. A. R. C. is a postdoctoral associate, and H.S. is an Assistant Investigator of the Howard Hughes Medical Institute.

Materials and Methods

hs-disco construct

A three-way ligation was done using the P element-containing CaSpeR (Pirrotta, 1988) vector cut with EcoRI and XbaI, an EcoRI/ SalI 456 bp fragment containing the upstream regulatory sequences of the *hsp70* gene (Ingolia et al., 1980) and a SalI/SpeI cDNA fragment containing all of the coding region of the *disco* gene (Heilig et al., 1991).

DNA from the *hs-disco* construct was combined with DNA from the helper plasmid pπ25.7wc (Karess and Rubin, 1984) at a ratio of 10 to 1 and injected into preblastoderm *y* w^{67c23} embryos. Only one transformant which carried a lethal insertion on the X chromosome was obtained. The transposon was mobilized by crossing the transformant strain to flies carrying a stable source of transposase (Robertson et al., 1988). Several non-lethal insertions were obtained. The present work utilized either a 2nd or 3rd chromosome insertion strain, both of which are viable as homozygotes.

Heat shock treatments

Wild type and *hs-disco* embryos were collected on molasses agar plates for two hours at 25° C, aged for four hours at 25° C and transferred to a 37° C incubator for 45 min. The embryos were then allowed to develop for an additional nine hours at 25° C or 18 hr at 19° C before being processed for antibody staining.

Histology

Aged embryos were dechorionated in bleach and fixed in 2% paraformaldehyde and octane (1:1) for 30' at room temperature for all stainings except for the *disco* protein where heptane was used instead of octane. The vitelline membrane was removed by the octane-methanol method (Mitchison and Sedat, 1983). This was followed by washes in BSS (Ashburner, 1989). The blocking step and antibody incubations were done in BSN (BSS, 0.3% triton X-100, 10% filtered goat serum), for four to six hours at room temperature or overnight at 4° C. The monoclonal antibodies 22C10 (Zipursky et al., 1984), 44C11 (Bier et al., 1988) and *fas II* (Bastiani et al., 1987) were used at a dilution of 1:3, affinity purified *disco* antibodies at 1:3 (Lee et al., 1991), the anti-HRP FITC conjugated antibody at 1:100 (Jan and Jan, 1982), and the anti-*eve* antibody at 1:500 (Frasch et al., 1987). The secondary antibodies were used at 1:50 for FITC conjugated (Cappel), 1:100 for Rhodamine conjugated (Boehringer) and 1:100 for HRP conjugated (Bio-Rad). Specimens were viewed on a Zeiss Axiophot or on a MRC600 confocal microscope, and the data interpreted using the manufacture's software (Biorad).

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Figure 4.1 The *hs-disco* construct

The terminal boxes represent the P element ends that mediate the transposition event. The arrows indicate the direction of transcription of the marker gene, *white* (*w*), that confers eye color, and of the *disco* cDNA. The heat shock promoter element is contained in a 456 bp fragment (Ingolia et al., 1980, Steller and Pirrotta, 1984). Transcription of the *disco* cDNA starts 204 bp within this fragment.

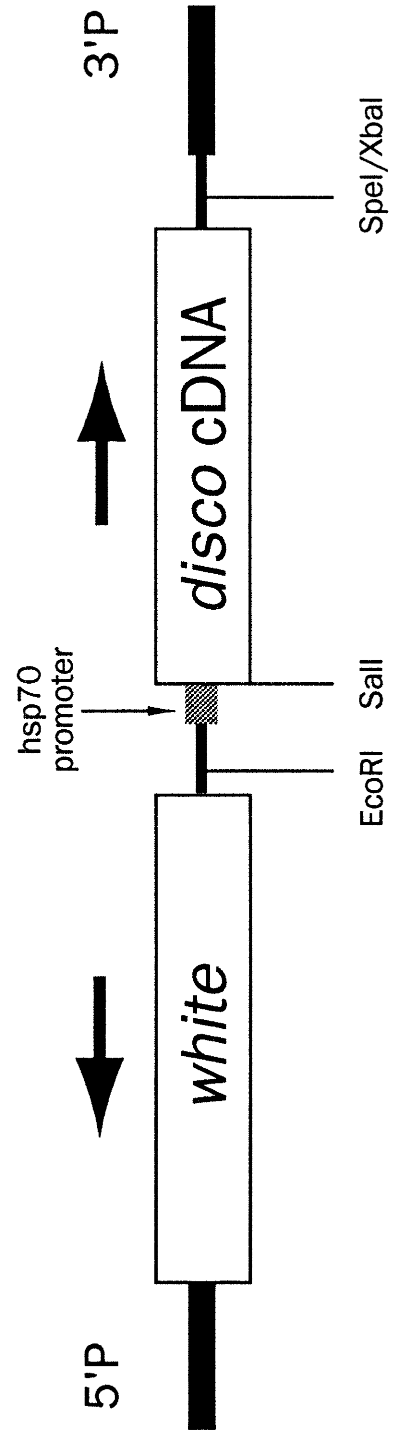
5'P
white
EcoRI
hsp70 promoter
SalI
disco cDNA
SpeI/XbaI
3'P

Figure 4.2 Expression of the *hs-disco* construct upon heat shock

Confocal photomicrographs of wild type (A, C, E, G) and *hs-disco* (B, D, F, H) embryos following heat shock and staining with anti-*disco* antiserum. In all cases, four to six hour embryos (developmental time at 25° C) were subjected to a 45 min heat shock at 37° C and allowed to recover at 25° C. In this and all subsequent figures, dorsal is up and, where applicable, anterior is to the left. (A) Lateral and (C) more medial optical sections of a wild type embryo fixed and stained 30 min after heat shock. In (A) characteristic anti-disco immunoreactivity is detected in the gnathal segments (arrow). (B) Lateral and (D) more medial optical sections of *hs-disco* embryo fixed and stained 30 min after heat shock. Compare anti-*disco* staining in gnathal segments (arrow) with ectopic staining in other tissues. Immunoreactivity is found in most, if not all, tissues and appears to be restricted to the nucleus. (E) Lateral and (G) more medial optical sections of wild type embryos fixed and stained 2 hr after heat shock. (F) Lateral and (H) more medial optical sections of *hs-disco* embryos fixed and stained 2 hr after heat shock. In addition to the wild type pattern of immunoreactivity, staining is detected in dorsal patches in the lateral ectoderm (F) and in the CNS neurogenic region (arrow in H).

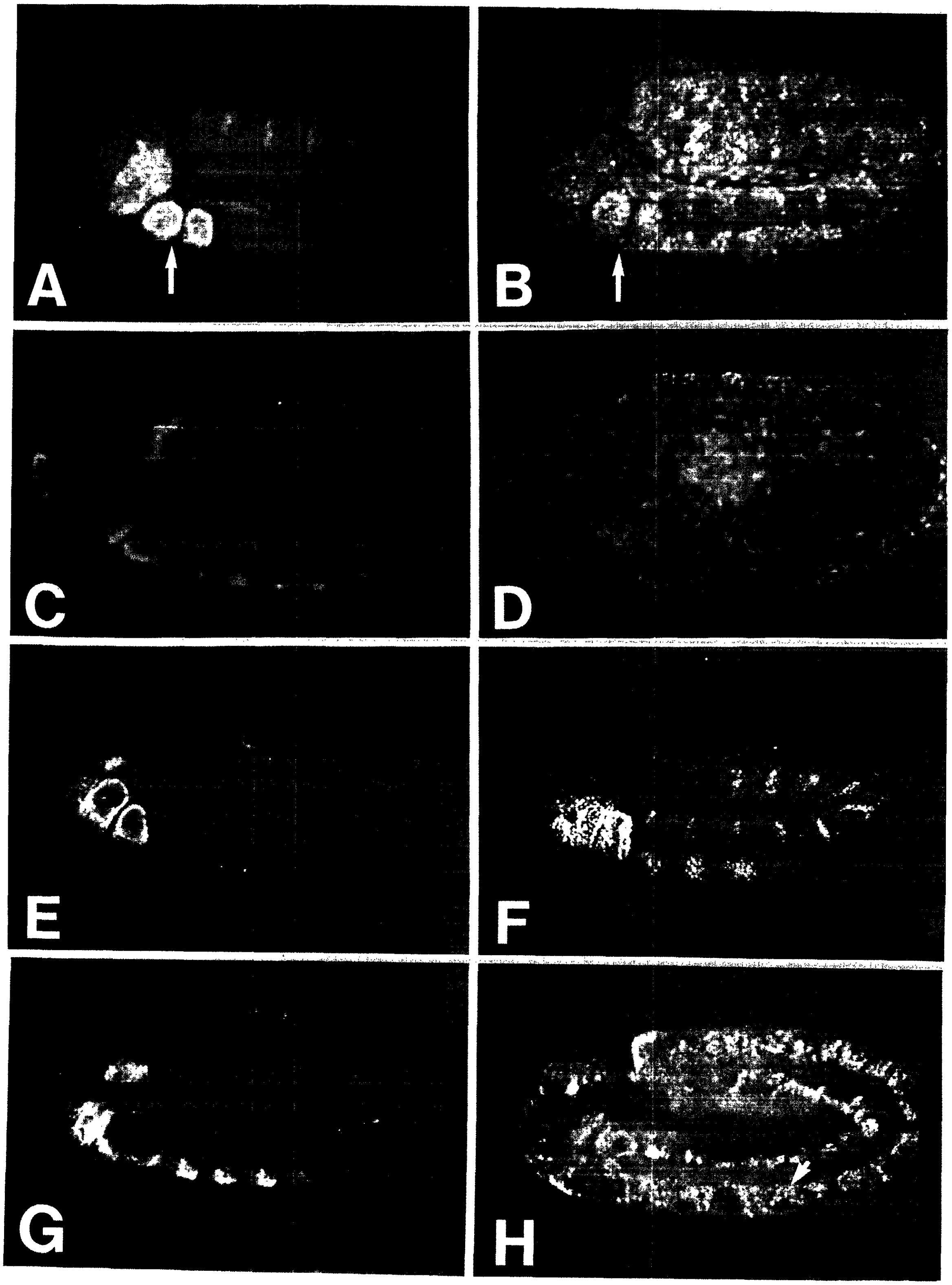
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Figure 4.3 Misexpression of the *disco* gene causes nervous system defects

Fluorescence photomicrographs of embryos heat shocked between four to six hours AEL stained as whole mounts with anti-HRP antibody and the mAb22C10 (Zipursky et al., 1984). Dorsal view of a late stage 16 wild type embryo (A), a *hs-disco* embryo (B), both stained with anti-HRP antibody. Misexpression of the *disco* gene does not impair the development of major features of the CNS, except for completion of ventral nerve cord condensation (arrow towards the anterior). Lateral views of an early stage 16 wild type embryo (A), and a *hs-disco* embryo (B), both stained with the mAb22C10. In heat-shocked transgenic animals the general pattern of the PNS with its three distinct clusters can be recognized, however a widespread disorganization as well as defects in pathway formation can be observed (arrow).

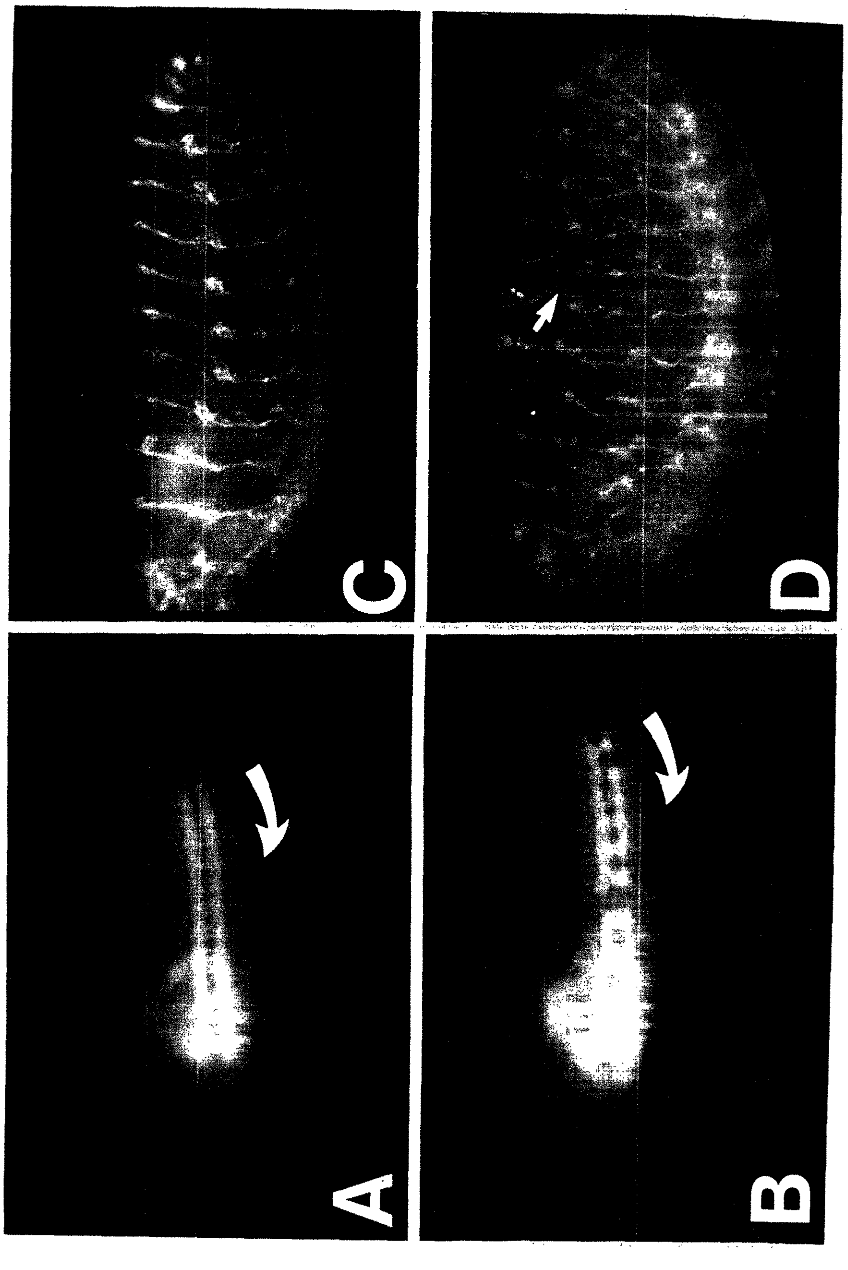
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Figure 4.4 Central nervous system defects in *hs-disco* embryos

Confocal images of the ventral nerve cord of heat shocked wild type and *hs-disco* embryos stained as whole mounts with anti-HRP and the mAb44C11. The anti-HRP antibody recognizes an epitope present in the membrane of all neurons in the CNS and is used here to visualize the neuropil region. mAb44C11 recognizes the product of the *elav* gene (Bier et al., 1988, Robinow, 1989) which is expressed in the nuclei of all neurons of the CNS (Robinow and White, 1991). It serves as an index of the integrity of the cell body layer located ventrally from the neuropil layer. The images were taken in two planes and in two channels. The top micrographs show the neuropil layer stained with anti-HRP while the bottom show the underlying cell body layer as detected by the mAb44C11. (A) and (D) show two focal planes of the same wild type embryo. (B) and (C), (E) and (F) show two examples of*hs-disco* embryos. Heat shock induction of the transgene disrupts the pattern of CNS projections, as seen by the interruption of the connectives (arrow in B) and commissures (arrow in C). Scale bar is 25 μm.

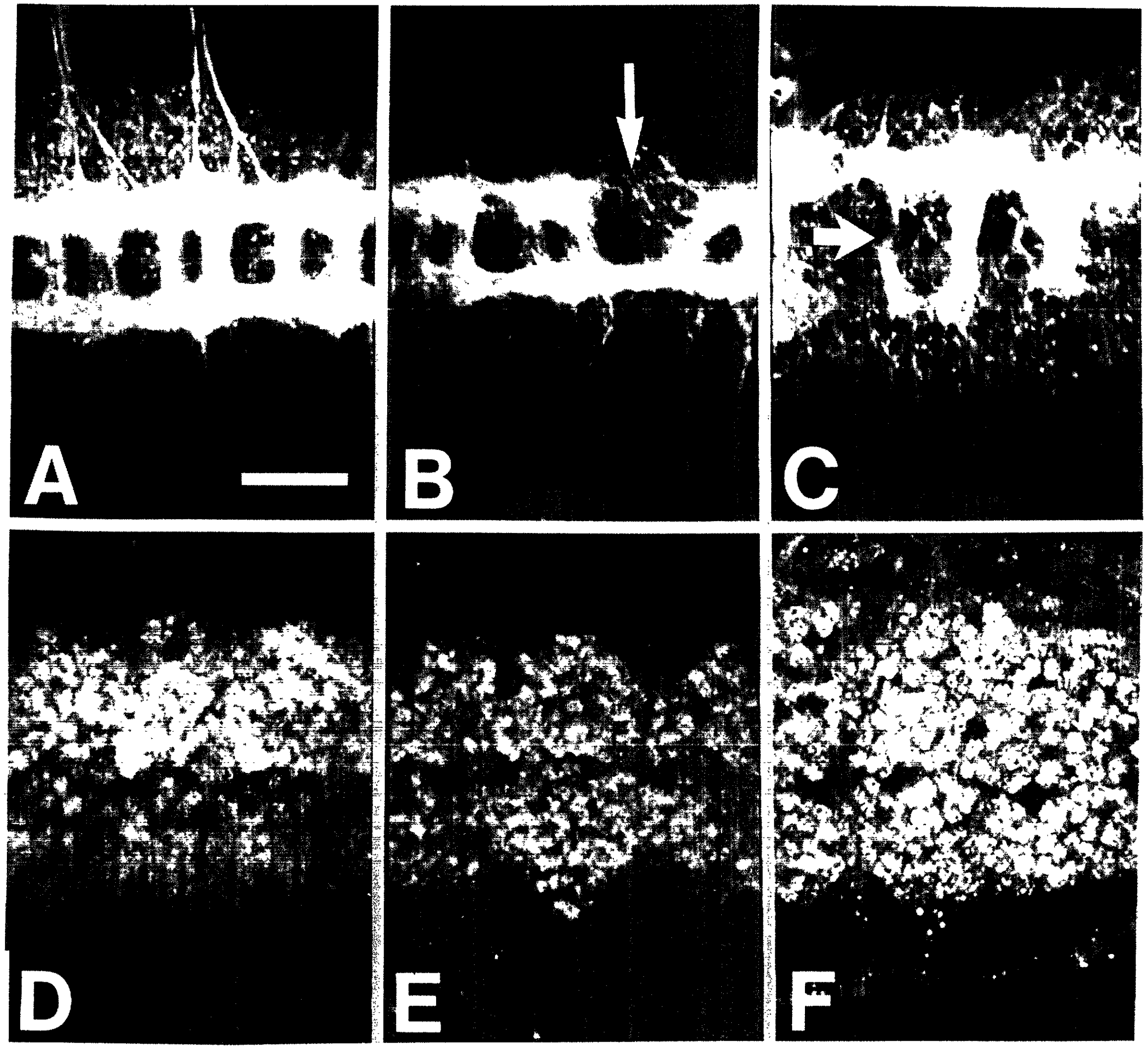
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Figure 4.5 Organization of *eve*-expressing cells in *hs-disco* embryos

Ventral nerve cords of heat-shocked embryos stained as whole mounts with an anti-eve antibody. (A) Wild type (B) *hs-disco*. After expressing the transgene the general organization of the *eve*-expressing clusters is similar to wild type. However, some clusters have fewer cells than in wild type (compare arrow in A with arrow in B).

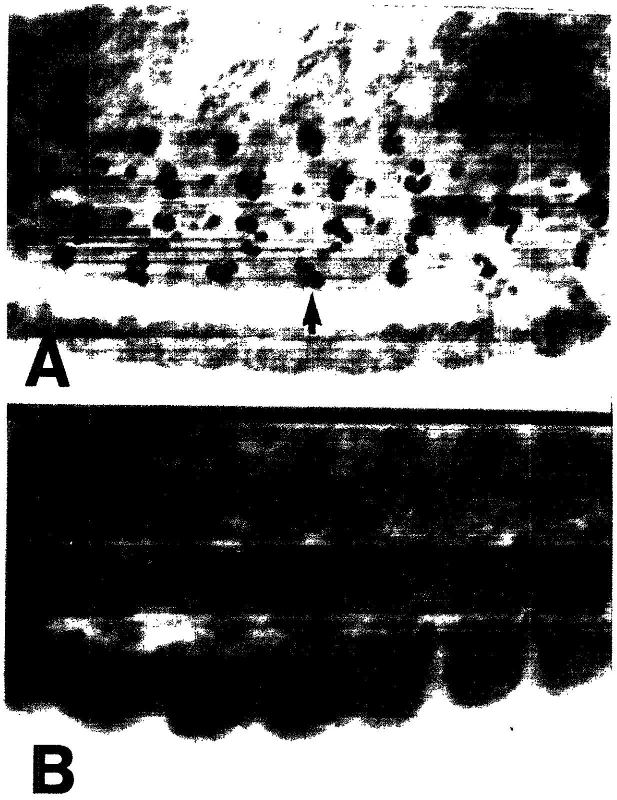
A
B

Figure 4.6 Peripheral nervous system defects in *hs-disco* embryos

Confocal images of the PNS of heat-shocked wild type and *hs-disco* embryos labeled with mAb22C10. Images were taken in two planes in order to visualize the cell bodies (top panels) and the anterior fascicles of the same embryo (bottom panels). The wild type pattern is shown in A and D. Two examples of *hs-disco* embryos are shown in panels B, E and in panels C, F. Misexpression of the *disco* gene leads to a general disorganization of the PNS cell clusters. However, cellular differentiation has occurred somewhat normally as can be determined from the morphology and location of the various cell types. The arrows indicates abnormal fascicles in *hs-disco* embryos (B, E, F). Scale bar is 25 µm.

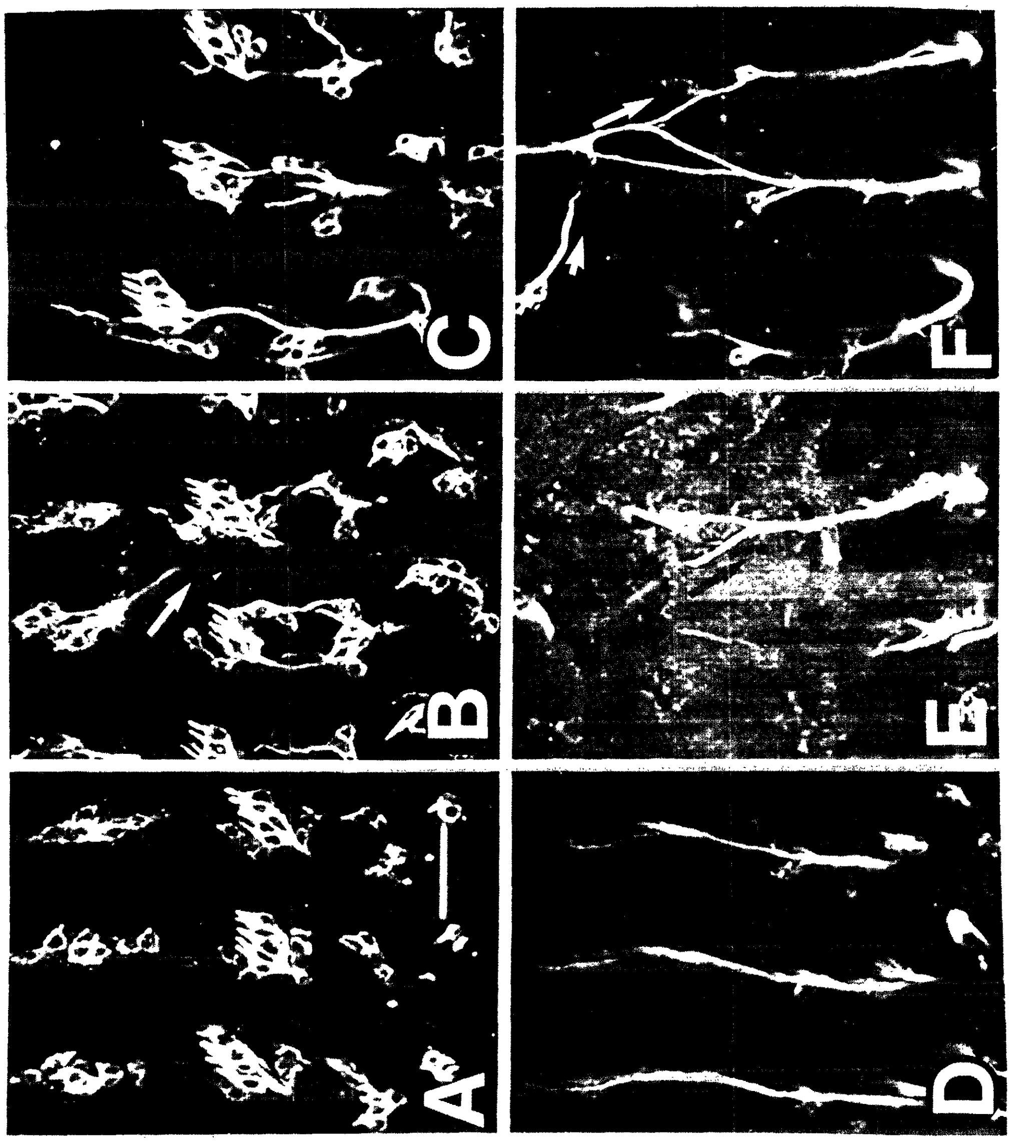
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Figure 4.7 Defects in the larval visual system of *hs-disco* embryos

Larval photoreceptor cells of heat shocked wild type and *hs-disco* embryos visualized by mAb22C10 antibody and confocal microscopy. (A) In wild type embryos (stage 16), the larval photoreceptor cell bodies are located anterior to the brain hemisphere and apposed to the antennal nerve (solid arrows). The antenno-maxillary complex is indicated by the solid triangle. The larval optic nerve projects posterior and around the brain (curved arrow). It terminates in the optic lobe primordium located in the ventral region of the brain hemisphere (open arrow) . Ubiquitous expression of the *disco* gene disrupts the organization of the larval visual system. (B-F) Three examples of hs-*disco* embryos. The phenotype of *hs-disco* embryos is characterized by a dorsal routing of the larval optic nerve. The lower four panels show the photoreceptor cell bodies (solid arrows in C and E) and the corresponding nerve termini (open arrows in D and F) in two *hs-disco* embryos.
Scale bar is 50 µm.

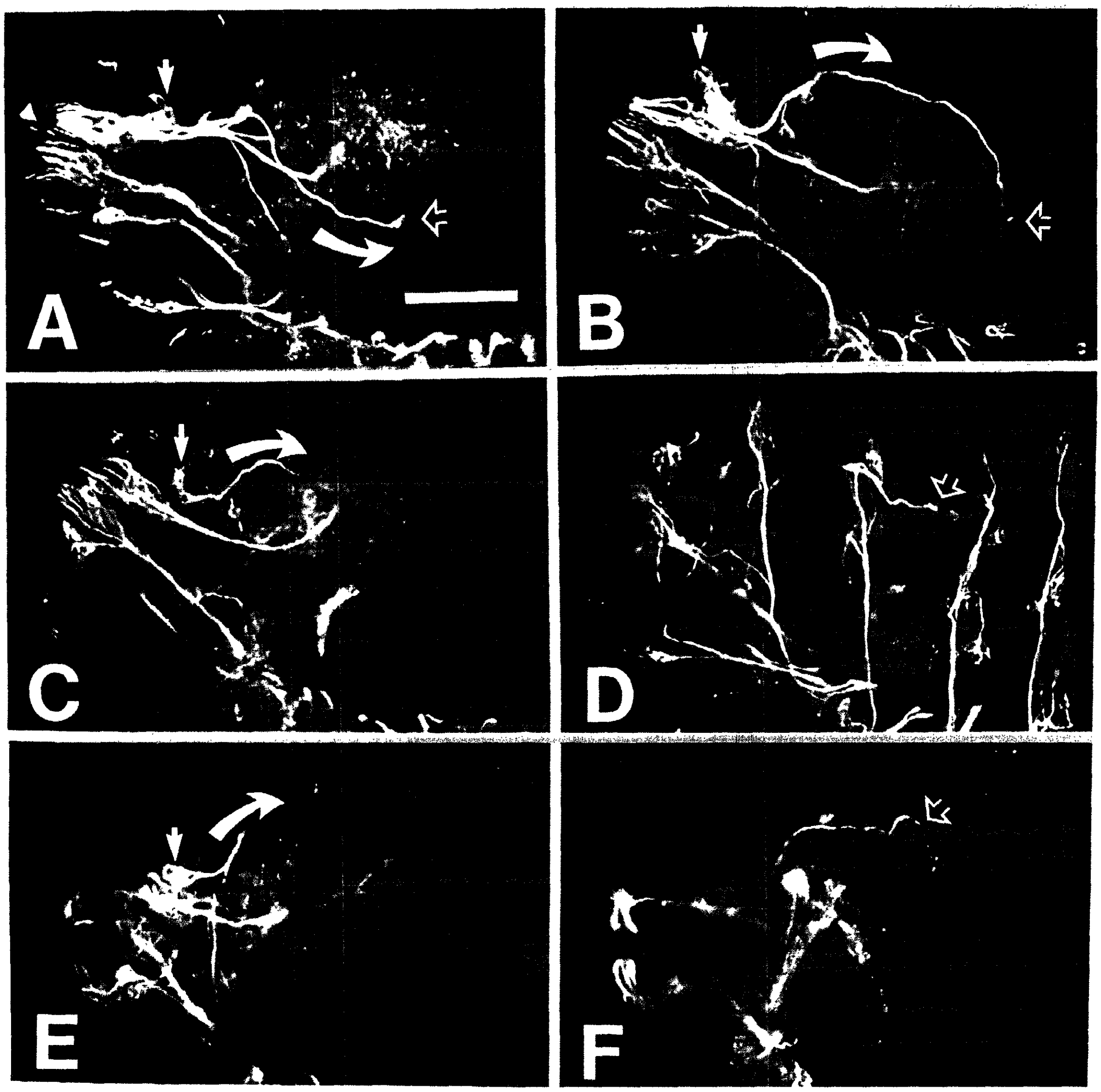
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Figure 4.8 Defects in the localization of the optic lobe primordium of *hs-disco* embryos shown by anti-HRP and mAb22C10 double labeling

Heat-shocked wild type and *hs-disco* embryos were double labeled with mAb22C10 and the anti-HRP antibody and analyzed under the confocal microscope. Adjacent panels depict two focal planes of the same specimen. In this experiment, the optic lobe primordium is identified as a domain devoid of anti-HRP staining, which in wild type embryos (A and B) is located in the ventral part of the brain hemisphere (open arrows). The larval photoreceptor cell bodies lie near the antennal sensory organ apposed to the antennal nerve (solid arrows). Upon ectopic expression of the *disco* gene the larval optic nerve takes an abnormal dorsal route, but always terminates in the optic lobe primordium (C-F).
Scale bar is 50 μm.

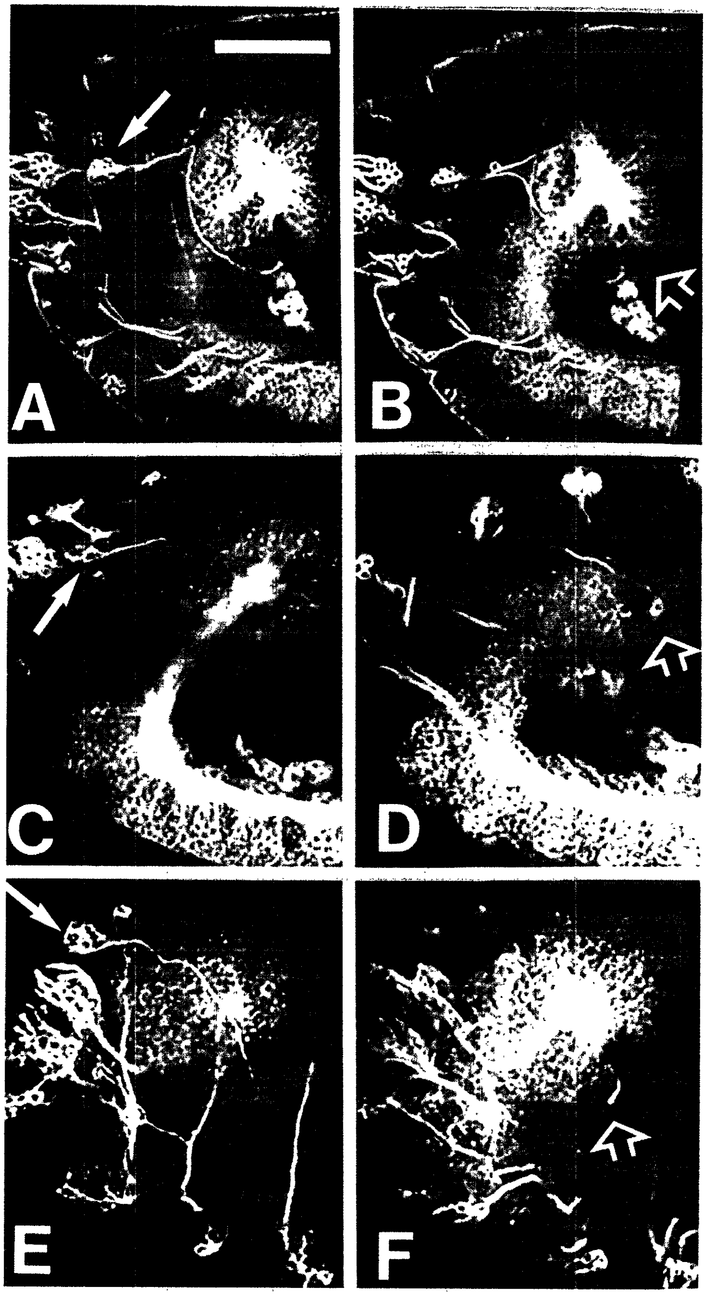
A
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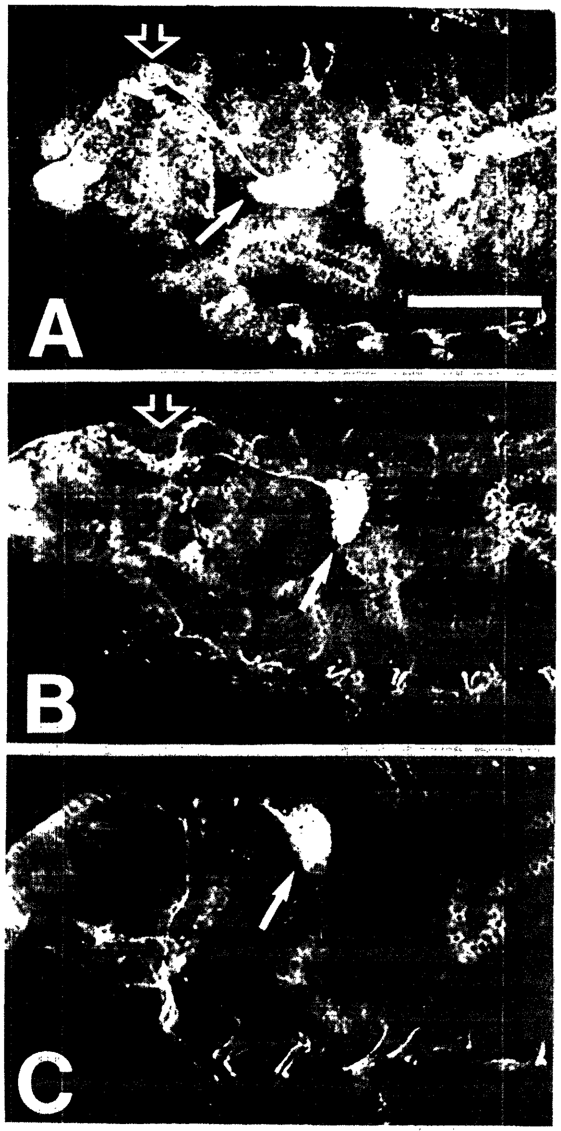
Figure 4.9 Defects in the localization of the optic lobe primordium of *hs-disco* embryos shown by anti-*fas II* staining

Heat-shocked wild type and *hs-disco* embryos were stained with the anti-*fas II* monoclonal antibody and viewed under confocal microscopy. (A) Wild type. (B and C) Two examples of *hs-disco* embryos. The *fas II* antigen is easily detected in the most distal part of the larval optic nerve and in the primordium of the optic lobe (solid arrows). In A and B the photoreceptor cell bodies are also stained and are indicated by open arrows. Ectopic expression of the *disco* gene affects the localization of the optic lobe primordium as well as the path of the larval optic nerve.

Scale bar is 50 μm.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Chapter 5.

Tissue-specific autoregulation of the *Drosophila disconnected* gene

Preface

The work presented in this chapter is being submitted for publication as: Lee, K. J., and Steller, H. S. Tissue-specific autoregulation of the *Drosophila disconnected* gene.

Abstract

The *Drosophila disconnected (disco)* gene is required for the formation of appropriate connections between the larval optic nerve and its target cells in the brain. The gene encodes a nuclear protein with two zinc fingers, which suggests that the gene product may be a transcription factor. Here, we present data supporting this notion. We find that *disco* expression in the optic lobe primordium, a group of cells which is contacted by the developing optic nerve, depends on an autoregulatory feedback loop. We show that wild type *disco* function is required for maintenance of *disco* mRNA and protein expression specifically in this tissue. In addition, we demonstrate that ubiquitous *disco* protein activity supplied by a heat-inducible gene construct activates expression from the endogenous *disco* promoter specifically in the optic lobe primordium. Consistent with a role of *disco* as a transcriptional regulatory protein, we find that the zinc finger portion of *disco* has sequence-specific DNA binding activity *in vitro* and that high affinity binding sites are found near the *disco* transcription unit. These data suggest that *disco* may autoregulate its transcription in the optic lobe primordium by direct binding to an upstream regulatory region in its own promoter.

Introduction

The proper development of a functional nervous system involves the formation of a precisely patterned network of axonal pathways and neuronal connections. Interactions between developing neurons and their surrounding environment are responsible for the establishment and maintenance of specific pathways and connections. These interactions require that the molecules which mediate axon guidance and cell recognition are expressed with temporal and spatial specificity.

The *Drosophila disconnected* (*disco*) gene is essential for the establishment of proper neuronal connections in the larval visual system. *Drosophila* larvae have a pair of visual organs, each consisting of twelve photoreceptor cells, which have axons that fasciculate together to form the larval optic nerve. This nerve projects directly to the brain via a defined pathway (Bolwig, 1946; Steller et al., 1987). In the brain, the axonal termini of the photoreceptor cells make synaptic connections with specific interneurons.

This larval optic nerve pathway is formed in a series of discrete steps during embryogenesis (Chapter 6; Green et al., 1993; Steller et al., 1987). Early in their development, the photoreceptor cells send out axons to contact the adjacent invaginating primordia of the optic lobes. This contact is maintained during subsequent incorporation of the optic lobe primordia into the larval brain and movement of the photoreceptor cells to their final location during head involution. In late embryogenesis, the photoreceptor axons penetrate into the larval brain and specific synaptic connections are established. In *disco* mutant embryos, the initial steps in the establishment of the optic nerve pathway are normal. Contact between the photoreceptor cells and the optic lobe primordia is formed as in wild type, but this contact is frequently lost later in development. As a result, the optic nerve is frequently found growing in an inappropriate environment. In the cases where contact with the brain is not lost, synaptic connections are never properly formed in *disco* mutants. Thus, the *disco* gene appears to be required during visual system development for the recognition of the appropriate target by the axons of the larval photoreceptor cells (Steller et al., 1987).

The *disco* gene has been cloned and its sequence was shown to contain two zinc-finger motifs (Heilig et al., 1991), a feature shared by many transcription factors (Harrison, 1991; Berg, 1990). This fact, along with the localization of the gene product in the nucleus (Lee et al., 1991), has led to the suggestion that *disco* encodes a nucleic acid-binding regulatory protein. Recently, a human gene that encodes a putative DNA-binding protein with similarity to *disco* has been identified (Tseng and Green, 1993). This protein, basonuclin, contains three repeats, each of which is related to the pair of zinc fingers found in *disco*. The striking conservation of these zinc finger sequences in evolution suggests an important role in protein function. Thus, *disco* may act in visual system development either to regulate the expression of molecules involved in cell adhesion and/or cell recognition, or to direct the differentiation of specific cells which act as guidance or recognition cues.

Earlier immunohistochemical studies with *disco*-specific antisera (Lee et al., 1991) showed that *disco* is expressed in a specific and highly dynamic pattern throughout the life cycle. Among other tissues, the *disco* gene is expressed during embryogenesis in the primordia of the imaginal leg discs (Cohen et al., 1991) and in the precursors of the larval heart, the cardioblasts (Bodmer, 1993). In the leg disc primordia, *disco* gene expression is dependent on the homeodomain-containing *Distalless (Dll)* gene product (Cohen et al., 1991). *disco* expression in the cardioblasts is dependent on the homeodomain protein encoded by *tinman (tin)* (Bodmer, 1993)

Of particular importance with regard to visual system development is the expression of *disco* in the primordia of the optic lobes, a group of cells contacted by the axonal projections of the larval photoreceptors (Lee et al., 1991). Two mutant alleles of the *disco* gene show alterations in the highly conserved cysteines of the zinc-finger motifs, leading to the production of a apparently non-functional protein. Embryos carrying either of these two alleles lack *disco* protein in the optic lobe region, although expression in other embryonic tissues is normal. One interpretation of this result is that wild type *disco* activity is required for proper *disco* expression in the optic lobe region. Moreover, given that *disco* shows sequence similarity to known transcriptional regulatory proteins, the dependence of *disco* expression on *disco* function may indicate an autoregulatory activity at the level of transcription.

Here we present data which supports the notion that the *disco* protein is a transcription factor with a direct, tissue-specific autoregulatory activity in the developing optic lobe primordium. Using markers which selectively label cells of the optic lobe, we show that the lack of *disco* expression in the mutant optic lobe region cannot be attributed to cell loss or mislocalization. In addition, we demonstrate that *disco* activity is required for maintenance, as opposed to initiation, of *disco* mRNA expression in this tissue. We have also used a transgenic strain of flies carrying the *disco* gene under the control of a heat-inducible promoter (Chapter 5) to examine the proposed autoregulatory activity. We demonstrate that wild type *disco* activity supplied by the transgene construct can activate expression from the endogenous *disco* locus specifically in the optic lobe region in mutant embryos. The tissue specificity of the autoactivation suggests that other factors must interact with the *disco* protein to restrict the autoregulatory activity to the optic lobe primordium. We have also assayed the *in vitro* DNA binding activity of the *disco* protein using genomic DNA from the *disco* locus. We find that the protein has sequence-specific DNA binding activity and that two high affinity binding sites are located in or near the *disco* transcription unit. Taken together, these data strongly support the hypothesis that *disco* positively regulates its expression by transcriptional regulation, mediated by direct binding to its own promoter region.

Results

Characterization of disco-expressing cells in the optic lobe region

In order to explore the putative autoregulatory activity of *disco* in the developing visual system, we first sought to characterize the wild type *disco* expression in the relevant tissue, the optic lobe primordium, in greater detail. Our previous studies (Lee et al., 1991) indicated that *disco* is expressed in a portion of the primordia of the adult optic lobes. These primordia develop by invagination from each lateral surface of the posterior procephalic lobe (Campos-Ortega and Hartenstein, 1985; Green et al., 1993). They move to the ventral side of the developing brain hemispheres where they are eventually incorporated. They remain largely non-neuronal until larval life, at which time they divide and differentiate to form the adult optic lobes.

Wild type embryos were double-labeled with an anti-Disco polyclonal antiserum (Lee et al., 1991) and various other antisera to characterize the *disco*-expressing cells. The embryos were then analyzed using confocal laser fluorescence microscopy. Figure 5.1B shows an optical section from a stage 13 embryo [staging based on (Campos-Ortega and Hartenstein, 1985)] stained with anti-Disco and the monoclonal antibody 44C11 (Bier et al., 1988). This antiserum recognizes the product of the *elav* gene, a nuclear antigen which is specifically expressed in all *Drosophila* neurons (Robinow and White, 1988; Robinow and White, 1991). The developing brain hemisphere consists at this stage of many immature neurons which express the *elav* antigen. The optic lobe primordium, which has invaginated and is in contact with the developing brain, can be identified in optical sections of anti-Elav stained preparations as a mass of unstained cells, surrounded by stained cells of the developing brain hemisphere. We observe that a ventral portion of the optic lobe primordium and a group of immature neurons just anterior to the optic lobe primordium express the *disco* antigen. The dorsal half of the optic lobe primordium does not express *disco*. Double-labeling with anti-Disco and anti-HRP (Jan and Jan, 1982), which, like 44C11, stains developing neurons of the brain hemisphere, confirms the identification described above (data not shown).

Lack of disco activity does not result in gross defects in the optic lobe region

Two point mutant alleles of the *disco* gene show alterations in the conserved cysteines of the zinc finger domain (Heilig et al., 1991). These mutants produce normal amounts of *disco* mRNA as assayed by northern analysis, but the protein appears to lack wild type activity. In fact, embryos carrying either of these *disco* mutant alleles have a morphological phenotype indistinguishable from embryos hemizygous for a deletion which completely removes the *disco* gene. These point mutations are therefore likely to represent complete loss-of-function alleles.

We have previously determined that in embryos carrying either of these mutant alleles, *disco* protein is mostly or completely absent in the optic lobe region (Lee et al., 1991). In contrast, distribution of the *disco* protein in all other tissues in the mutants is normal.

One trivial explanation for the loss of *disco* protein in the optic lobe region in *disco* mutant embryos is that the cells which normally express *disco* degenerate or are ectopically located. This interpretation seems unlikely because the optic lobe primordium in *disco* mutant embryos appears morphologically normal and roughly comparable to wild type in size (Lee et al., 1991; Steller et al., 1987). Nonetheless, we sought to further study the fate and morphology of the optic lobe region in *disco* mutants using other markers which selectively label this tissue.

We examined the distribution of two markers which are selectively expressed in the optic lobe primordium in wild type, *disco*^{1} and *disco*^{1656} mutant embryos. The expression of the two markers, fasciclin II (Grenningloh et al., 1991) and beta-galactosidase in the enhancer trap strain A6-2-45 (Bier et al., 1989), at least partially overlaps the expression of *disco* in this region. The expression patterns of all three proteins indicate that the optic lobe primordium is composed of several distinct cell types: *disco* is expressed in the ventral half of the optic lobe primordium, fasciclin II in the posterior half, and in A6-2-45, beta-galactosidase is expressed in the entire optic lobe primordium (see also Chapter 4, Chapter 6).

Figure 5.2 shows confocal photomicrographs of wild type and *disco* mutant embryos double-labeled with anti-Disco antiserum and with a monoclonal antibody which recognizes the *Drosophila fasciclin II (fasII)* protein. Identical results were obtained when embryos homozygous for either the *disco*^{1} or the *disco*^{1656} mutation were examined. FasII is a cell surface molecule implicated in axonal patterning and neuronal recognition in the central nervous system (CNS) (Grenningloh et al., 1991). The molecule is expressed in a specific subset of cells in the CNS, in other neural tissues and in non-neural tissues. In wild type embryos, *disco* and *fasII* are co-expressed in the posterior ventral portion of the optic lobe primordium (Figure 5.2A, 5.2C) In *disco* mutant embryos, *fasII* is expressed in the optic lobe region in a manner indistinguishable from wild type (Figure 5.2D). Thus, expression of *fasII* in the optic lobe primordium is not dependent on wild type *disco* activity.

We have also used the expression of beta-galactosidase in the enhancer trap A6-2-45 (Bier et al., 1989) as a marker for the optic lobe primordium. Beta-galactosidase expression in embryos carrying this enhancer trap insertion is found in most, if not all, of the optic lobe primordium as well as in some neighboring cells, including the larval photoreceptors, and in groups of ectodermal cells near the germ band segment boundaries. Many of the beta-galactosidase expressing cells in the optic lobe primordium co-express *disco*. As is found with *fasII*, expression of beta-galactosidase in A6-2-45 embryos is not changed in a *disco* mutant background (data not shown).

These observations indicate that the optic lobe primordium cells which normally express *disco* are not ectopically located or degenerating in the *disco* mutant embryo. Moreover, they indicate that the loss of *disco* activity has no gross effect on the cellular identity or differentiation of the majority of the cells in the optic lobe primordium.

disco activity is required for tissue-specific maintenance of disco mRNA expression

As described above, embryos carrying either of two mutant *disco* alleles show a specific loss of *disco* protein expression in the optic lobe region (see also Figure 5.2B). We sought to determine whether this alteration is also found in the *disco* mRNA distribution. We examined the expression of *disco* mRNA in wild type and *disco* mutant embryos of various stages in order to learn at what point during development any differences appear.

Non-radioactive DNA probes were used to detect *disco* mRNA by whole mount *in situ* hybridization (Tautz and Pfeifle, 1989) in wild type, $disco^{1}$ and $disco^{1656}$ embryos. Distribution of the *disco* mRNA in wild type and *disco* mutant embryos is identical until roughly stage 9 or stage 10 (Figure 5.3A, 5.3B). This includes expression in a dorsolateral band just anterior to the cephalic furrow, a region of the blastoderm embryo fated to become the optic lobe primordia (Green et al., 1993). In wild type embryos, *disco* staining in this region can be followed into the optic lobe anlagen. A fraction of embryos carrying either mutant *disco* allele have fewer *disco* expressing cells in this area by stage 10 or 11 (Figure 5.3, compare panels C and D). By stage 12, all *disco* mutant embryos show no or a greatly reduced number of *disco*-expressing cells in the optic lobe region (Figure 5.3F). Wild type embryos, in contrast, show *disco* mRNA expression in the optic lobe region throughout embryogenesis (Figure 5.3E, 5.3G). At all stages, *disco* mRNA distribution is at least qualitatively similar in wild type and mutant embryos in tissues other than the optic lobe region. Thus, wild type *disco* activity is required for proper expression of *disco* mRNA as well as protein in a specific tissue. This activity is not required for the initiation of expression in the posterior procephalic region, but it is required for maintenance of expression after stage 9 or 10.

heat shock-inducible disco specifically activates expression of the endogenous disco gene in the optic lobe region

The results described above suggest that *disco* positively autoregulates its expression in the optic lobe primordium. We tested this hypothesis using a P-element construct containing the *disco* cDNA under the transcriptional control of the *hsp70* gene promoter (*hs-disco*). Heat shock treatment of embryos carrying the *hs-disco* P-element leads to nuclear expression of *disco* in virtually all cells (Chapter 4). The ectopic expression of *disco* results in gross morphological abnormalities in later embryogenesis, including defects in the central and peripheral nervous systems and aberrant development of the optic lobe primordia. The most pronounced defects were observed when *hs-disco* embryos were heat-shocked between 4 to 8 hr of development (Chapter 4).

We sought to determine whether the ubiquitous expression of *disco* protein in embryos carrying the *hs-disco* construct could affect expression of the endogenous gene. For these experiments, it was necessary to distinguish the transcript produced by the endogenous *disco* gene from the transcript produced by the *hs-disco* transgene. The cDNA contained in the *hs-disco* construct does not include the entire 5' untranslated region of the full length *disco* mRNA. Therefore, we could specifically detect expression from the endogenous *disco* gene in *in situ* hybridization experiments by using a probe corresponding to the first 275 base pairs of the *disco* 5' untranslated region.

We assessed the effects of ectopic expression of the transgene in *hs disco* embryos which were given a 45 min heat-shock between 4 to 6 hr of development. The treated embryos were allowed to age a further 1.5 hr at 25°C before fixation and hybridization with endogenous *disco* gene-specific probes.

In a wild type background, the ectopic expression of *disco* under the heat shock promoter has no detectable effect on endogenous *disco* expression (data not shown). In the *disco* mutant background, however, the transgene has a pronounced effect on endogenous gene expression in the optic lobe region (Figure 5.4). In the absence of heat shock, mutant embryos carrying two copies of the transgene exhibit greater *disco* expression in the optic lobe primordium than mutant embryos without the transgene (Figure 5.4C, 5.4E). We attribute this result to a low basal activity of the *hsp70* promoter. Upon heat shock, *disco* expression in the optic lobe primordium is even more pronounced (Figure 5.4D, 5.4F). In some cases, expression in the optic lobe region is restored to apparently wild type levels.

We conclude that *disco* expression in the optic lobe primordium is postively regulated by *disco* activity in a feedback loop. The feedback of *disco* activity on *disco* expression is not functional in other embryonic cell types, since we observe this autoactivation specifically in the optic lobe region. In tissues where the expression of the endogenous *disco* gene is not affected by the *disco* loss-of-function mutation, however, it is difficult to determine whether the autoactivation is also functional. In these cells, any effect of the transgene may be undetectable, since slight quantitative differences in expression are not revealed by *in situ* hybridization analysis.

in vitro DNA binding activity of the disco protein

Given the possible function of the *disco* gene product as a transcriptional regulatory protein, the autoactivation of *disco* expression we have observed might result from a direct binding of *disco* protein to its own promoter region. We sought to test this hypothesis by examining the sequence-specific *in vitro* binding of *disco* protein to genomic DNA sequences from the *disco* locus.

We used a modified immunoprecipitation assay (Desplan et al., 1985; McKay, 1981) to search for *disco* protein-binding sites in some 35 kb of DNA encompassing the *disco* transcription unit. End-labeled DNA was mixed with a fusion protein containing the zinc finger portion of the *disco* protein and protein-DNA complexes were isolated by binding to magnetic beads (see Materials and methods for experimental details). Bound DNA species were then analyzed by polyacrylamide gel electrophoresis. By increasing the amount of unlabeled competitor DNA included in the binding reactions, we identified fragments with the greatest affinity of binding to *disco* protein (Figure 5.5A). Two fragments were preferentially bound by the *disco* fusion protein in our assay. One of these fragments corresponds to a region located about 2.5 kb upstream of the *disco* transcription start site. The second is located in the intron of the *disco* transcription unit (Figure 5.5B). Other DNA fragments, including fragments of the bacteriophage lambda vector which were present in all the binding reactions, were also bound with varying, but lower, affinities. This binding could result from chance occurrences of sequences which resemble the preferred recognition site of the *disco* protein. The sequences of the two fragments bound by *disco* protein (s120 and s280) are shown in Figure 5.6.

Binding of *disco* protein to the 120 bp DNA fragment (s120) located 2.5 kb upstream of the *disco* transcript start site was also analyzed by electrophoretic mobility shift assays (EMSAs). The formation of shifted complexes was observed when the labeled DNA fragment was incubated with a fusion of the *disco* zinc finger region to *S. aureus* protein A (Figure 5.7, lanes 2,3) or when the fragment was incubated with a fusion of the *disco* zinc finger region to a short bacteriophage T7 polypeptide (Figure 5.7, lanes 4,5). The relative shifts observed in the assay reflect the predicted sizes of the two different complexes: the larger *disco*-protein A fusion produced a larger mobility shift. These complexes were not formed when the DNA fragment was incubated with a control bacterial extract (Figure 5.7, lanes 6,7). In addition, the shifted complex was not formed when the DNA fragment was incubated with a *disco*-T7 protein fusion which contains the *disco*^{1656} mutation (Figure 5.7, lanes 8,9). This mutation is a missense alteration of the first Cys residue in the first zinc finger (Heilig et al., 1991). Thus, both the autoregulatory function of *disco* protein *in vivo* and the specific binding of *disco* protein to upstream DNA sequences *in vitro* are abolished by this same change in one of the conserved residues of the zinc finger motif.

Discussion

The *Drosophila disco* gene is required for the proper development of neuronal connectivity in the larval visual system. The gene encodes a protein with two zinc fingers, a structural motif which has been found in numerous nucleic acid binding regulatory proteins [reviewed in (Harrison, 1991; Berg, 1990)]. Here, we have presented evidence that *disco* feeds back on its own level of expression in the optic lobe primordium. Furthermore, we have demonstrated that the *disco* protein has sequence-specific DNA binding activity *in vitro* and that this activity requires an intact zinc finger region. These observations, along with the localization of the gene product in the nucleus, implicate the *disco* protein in the regulation of gene expression, most likely at the level of transcription.

Significance of the disco zinc finger domain

Several lines of evidence indicate that the zinc finger domain of the *disco* protein is a key part of the molecule, crucial to protein function. We have previously reported that two point mutant alleles of *disco* show changes in the conserved cysteines in one of the two zinc fingers (Heilig et al., 1991). These two mutant alleles have an identical mutant phenotype and this phenotype is comparable to the phenotype which results from a deletion of the *disco* gene. Thus, changes in critical residues in either zinc finger appear to result in a complete loss of function. This loss of function is not the result of protein instability, as embryos carrying these mutant alleles have normal amounts of *disco* protein when assayed at the level of whole animals(Lee et al., 1991). Here we have shown that the mutation of one of the critical zinc finger residues also leads to a loss of DNA binding activity assayed *in vitro*. By comparison, similar mutations in one or the other zinc finger of the yeast ADR1 protein also result in a complete loss of function (Blumberg et al., 1987). Likewise, it has been demonstrated that two of the five zinc fingers found in the PRDI-BF1 protein are necessary and sufficient for proper sequence-specific DNA binding *in vitro* (Keller and Maniatis, 1992). Therefore, in the context of these proteins at least, a minimum of two intact zinc fingers is required for protein function, assayed either genetically or biochemically.

Recently, a human keratinocyte protein with striking sequence similarity to *disco* has been described (Tseng and Green, 1992). This protein, basonuclin, contains six zinc fingers, arranged in three separate pairs. Each of these pairs of zinc fingers is very closely related in sequence to the pair of zinc fingers found in *disco*. Outside the zinc finger sequences, *disco* and basonuclin share very little sequence similarity. Thus, the evolutionary conservation of the zinc finger sequences indicates that this region plays an important role in the function of the two proteins. It also suggests that the two proteins may have similar activities; for example, they might bind nucleic acids with similar binding specificity.

disco has an autoregulatory activity in the developing visual system

In wild type embryos, the *disco* gene is expressed in a portion of the optic lobe primordium and in some adjacent immature neurons. We have demonstrated that *disco* expression in this region is lacking in embryos homozygous for the *disco* zinc finger point mutations. In this one specific tissue, maintenance of *disco* mRNA and protein expression beyond stage 9 or 10 depends on wild type *disco* function. We find that the altered *disco* expression in the mutants is not the result of cell absence, degeneration or mislocalization. Moreover, we observe that the optic lobe primordium expresses appropriate cell-specific markers in a mutant background. Thus, the loss of *disco* activity does not result in large-scale changes in cellular identity or differentiation in the developing optic lobe. Using a P-element construct to direct the ubiquitous expression of wild type *disco* protein in a *disco* mutant background, we show that functional *disco* protein supplied by the gene construct activates expression from the endogenous gene specifically in the optic lobe primordium.

Taken together, our data suggest a model wherein *disco* expression in the optic lobe region is initiated by other transcription factors. This initiation of *disco* expression is independent of *disco* activity. Later, an autoregulatory feedback mechanism is required for the subsequent maintenance of *disco* expression in this tissue. In other regions of the embryo, in the the leg disc primordia and in the cardioblasts, gene functions upstream of *disco* in a transcriptional regulatory cascade have been identified. In the leg disc primordia, the homeotic gene *Ultrabithorax (Ubx)* regulates the expression of *Distalless (Dll)*, which in turn regulates the expression of *disco* (Cohen et al., 1991). In the larval heart precursor cells, the cardioblasts, *disco* expression depends on the product of homeodomain-containing gene, *tinman (tin)* (Bodmer, 1993).

Given the similarity of *disco* to known transcription factors, we hypothesized that the autoregulation of *disco* might involve a transcriptional activation induced by direct binding of the *disco* protein to its own promoter region. Consistent with this hypothesis, we find that the *disco* protein has a sequence-specific DNA binding activity *in vitro* and that high affinity *disco* binding sites are found in the genomic DNA near the *disco* transcription unit. The location of these binding sites is reminiscent of the autoregulatory enhancers previously described in *Drosophila* (Bergson and McGinnis, 1990; Pick et al., 1990). Further experiments will be needed to determine whether these binding sites are relevant to the normal regulation of *disco* expression *in vivo.*

Similarities to previously described autoregulatory mechanisms

Autoregulatory mechanisms have been observed in the expression of many of the transcription factors involved in *Drosophila* development. This includes segmentation and homeotic genes (Bienz and Tremml, 1988; Hiromi and Gehring, 1987; Kuziora and McGinnis, 1988) and genes required for the development of particular neuronal cell types (Blochinger et al., 1991; Moses and Rubin, 1991). In the case of *fushi tarazu*, it has been demonstrated that the autoregulation is clearly mediated by a direct interaction with a transcriptional regulatory element (Schier and Gehring, 1992). The autoregulation of the *Deformed (Dfd)* gene is also likely to be direct, since high affinity binding sites for the *Dfd* protein have been shown to be required for the function of a *Dfd* autoregulatory enhancer element (Regulski et al., 1991).

It has been proposed that the autoregulatory function of homeotic "selector" genes is required to maintain a determined state after cellular identity has been established [see, for example, (Kuziora and McGinnis, 1988)]. The importance of the autoregulatory activity in the normal function of *disco* remains unclear. We have detected no change in the mutant optic lobe primordium indicative of a change in cellular identity. Nonetheless, the persistent expression of *disco* might be required to maintain a "determined" state in terms of the expression of a limited number of as yet unidentified genes.

The autoregulatory activity of disco is restricted to the optic lobe primordium

We have observed that the autoregulation of *disco* is highly tissue-specific. Some degree of tissue specificity has also been found in the other previously described examples of autoregulatory activity of *Drosophila* genes [for example, (Bienz and Tremml, 1988; Blochinger et al., 1991; Kuziora and McGinnis, 1988)]. We infer that the tissue specificity of *disco* autoregulation must involve interaction with another factor or factors which are spatially restricted. Such factors may prevent autoregulation in tissues other than the optic lobe primordium or they may promote autoregulation specifically in the optic lobe primordium. The proposed interaction could influence the DNA binding affinity of *disco* or alternately, it could affect the activation of the *disco* promoter upon binding.

Although the autoregulatory activity of *disco* is restricted to the optic lobe primordium, it is likely that the protein can and does function in other tissues. The *disco* gene is normally expressed in a subset of cells in the peripheral nervous system (PNS) (Lee et al., 1991) and a role for the gene in the development of the PNS is indicated by the abnormalities in PNS patterning observed in *disco* mutant embryos (Steller et al., 1987). Moreover, ectopic expression of *disco* in *hs-disco* embryos produces striking defects throughout the nervous system (Chapter 4). As we show here, however, this ectopic *disco* activity does not autoactivate *disco* expression in this tissue. Therefore, these other activities of the *disco* gene, which might be expected to involve the regulation of the expression of other genes, are not dependent on the same tissue-specific factors as is *disco* autoregulation.

Possible roles of the disco gene in the development of the visual system during embryogenesis

We have observed that the *disco* gene product has several features characteristic of transcriptional regulatory proteins and that it autoregulates its expression in the optic lobe primordium. What is its function in the development of the larval visual system during embryogenesis?

In light of the phenotypic consequences of the loss of *disco* function on proper interaction between the larval photoreceptors and the optic lobe primordium, it is reasonable to propose that *disco* is directly regulating the expression of certain critical molecules involved in this interaction. These putative targets of *disco* regulatory activity remain to be determined, but they might include genes involved in cell recognition or cell adhesion.

Alternately, *disco* may be more generally affecting the differentiation of a small number of cells which play an important role in the patterning of the visual system. Possible candidates for such cells are the so-called optic lobe pioneers (OLPs), a group of three neurons which are among the first cells of the optic lobe to differentiate (Tix et al., 1989). These neurons are located near the point where the larval optic nerve enters the optic lobe primordium and they extend processes which fasciculate with the nerve. These features have led to the suggestion that the OLPs may play a role in guiding the larval visual nerve or promoting the formation of stable contacts between the nerve and the brain (Tix et al., 1989). Present evidence suggests that they are missing or develop abnormally in *disco* mutant brains (Chapter 6; Tix et al., 1989). Therefore, the improper development of these cells in *disco* mutant embryos may be the direct cause of the failure of the larval optic nerve to form normal connections with the brain. Further experiments will be needed to determine whether the *disco* gene is indeed required in the OLPs for proper neuronal pathway formation in the *Drosophila* visual system.

Materials and methods

Immunohistochemistry

Immunohistochemical staining of embryos was performed essentially as previously described (Lee et al., 1991; Steller et al., 1987). For detection using HRP-conjugated secondary antibodies, embryos were fixed in a 1:1 mixture of heptane and PLP fixative (McLean and Nakane, 1974). For detection using fluorescent secondary antibodies, embryos were fixed in a 1:1 mixture of heptane and 4% paraformaldehyde in 0.1 M phosphate buffer. In either case, fixation was for 30 min. at room temperature. The affinity purified anti-Disco antibody (Lee et al., 1991), the anti-Elav monoclonal antibody, 44C11 (Bier et al., 1988) and the anti-FasII monoclonal antibody (Bastiani et al., 1987) were used at a dilution of 1:3. Secondary antibodies (HRP-conjugated anti-rabbit 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. All antibody incubations were done overnight at 4°C. Stained specimens were analyzed using either a Zeiss axiophot microscope or 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.

In situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (Tautz and Pfeifle, 1989) with modifications as described in (Lee et al., 1991). The probes used in hybridization were either generated from a cDNA isolate of the *disco* gene and used as previously described (Lee et al., 1991), or, for specific detection of the endogenous *disco* transcript in heat shock experiments, generated using the polymerase chain reaction (PCR) as follows: Two separate fragments, corresponding to bases 26-142 and bases 122-241 of the *disco* gene (Heilig et al., 1991) were generated by PCR using total *Drosophila* genomic DNA as a template. Products of the appropriate size were isolated by preparative gel electrophoresis using low melting temperature agarose. Digoxigenin-labeled probes were prepared from these fragments in a 50 µl reaction containing 100 ng template DNA, 1X standard PCR buffer (Perkin-Elmer Cetus), 0.2 mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.13 mM TTP, 0.07mM digoxigenin-11-dUTP (Boehringer Mannheim), 0.3 µg oligonucleotide primer (one of the primers used in the initial PCR reaction) and 2.5 U Taq polymerase (Perkin-Elmer Cetus). The reaction was subjected to 30 cycles of 95°C for 45 sec, 55°C for 30 sec, 72°C for 1 min, followed by an additional incubation at 72°C for 10 min. After this cycling, the reaction was ethanol precipitated twice and resuspended in 500 µl hybridization buffer (Lee et al., 1991). For *in situ* hybridization, a 1:1 mixture of the probes generated from each PCR fragment was diluted 1:5 in the hybridization reaction. The alkaline phosphate detection reaction following hybridization with the PCR-generated probe required a 8 hr incubation at room temperature.

Heat shock induction of disco expression

Construction of the *hs-disco* P-element and generation of a strain of flies harboring this element is described in Chapter 4. For the experiments described in this chapter, a strain carrying a homozygous viable third chromosome insertion of the P-element was used. A strain of flies homozygous for the third chromosome *hs-disco* insertion and the *disco*^{1} mutation was generated by crossing. For these studies, *disco*^{1} and *disco*^{1}*; hs-disco* embryos were collected on molasses-agar plates for 2 hr at 25°C, aged for 4 hr at 25°C, then heat-shocked by immersion in a 37°C water bath for 45 min. After a further 1.5 hr incubation at 25°C, embryos were fixed and processed for *in situ* hybridization. Control, non-heat-shocked embryos (*disco*^{1} and *disco*^{1}*; hs-disco*) were collected as above (2 hr at 25°C), but were maintained at 25°C for 6 hr 15 min and then fixed and processed as above. Several hundred embryos were analyzed in three separate experiments; representative results are shown in Figure 5.4.

Fusion proteins

Generation of *disco* fusion proteins is described in (Lee et al., 1991). Fusions to *S. aureus* protein A [using expression vector pRIT2 (Nilsson et al., 1985)] and a short bacteriophage T7 polypeptide [using the expression vector pET-3a (Studier et al., 1990)] were used in these studies. These expression plasmids contain a portion of the *disco* gene corresponding to bases 615-1156 of the mature transcript (Heilig et al., 1991), which includes the entire zinc finger region. A pET derivative which directs the expression of a mutant *disco*-T7 fusion protein containing the *disco*^{1656} mutation was constructed by the following means: A 305 bp Bst XI-NotI fragment from the *disco* gene sequence contained in the pET-3a vector was excised and replaced with the corresponding fragment obtained by PCR amplification of genomic DNA isolated from *disco*^{1656} flies. The presence of the mutation was confirmed by restriction analysis. The expression levels and solubility of the mutant protein was found to be comparable to that of the wild type fusion protein. The pRIT fusion protein was purified by affinity chromatography as previously described (Lee et al., 1991). The pET fusion proteins used in DNA binding studies were bacterial extracts made by lysis in 10 mM Tris•HCl pH 8, 300 mM NaCl, 1 mg / ml lysozyme. Extracts were sonicated and Tween-20 was added to 0.5% before centrifugation to remove insoluble material.

DNA binding assays

Immunoprecipitation reactions to identify *disco* protein binding sites in genomic DNA were based on methods previously decribed (Treisman and Desplan, 1989; Desplan et al., 1985). Briefly, DNA from bacteriophage lambda isolates containing inserts of *Drosophila* genomic DNA from the *disco* locus (Heilig et al., 1991) was digested with Sau3A, dephosphorylated and end-labeled with ^{32}P by kinasing. Labeled DNA (approximately 30 ng) was incubated with an affinity-purified *disco*-protein A fusion protein (approximately 0.5 µg) on ice for 30 min in a 50 µl reaction containing 20 mM Tris•HCl pH 7.5, 170 mM NaCl, 0.2mM NaEDTA, 1mM DTT, 0.5 mM $ZnCl_2$, 50 µg / ml BSA, 10% glycerol and varying amounts of competitor DNA . Protein-DNA complexes were isolated by binding to rabbit IgG coupled to magnetic beads (Advanced Magnetics). Magnetic beads were extensively washed prior to use in the above binding buffer. Protein-DNA complexes bound to the magnetic beads were subjected to three washes in binding buffer, followed by extraction in formamide loading buffer and resolution by electrophoresis in 5% denaturing polyacrylamide gels.

The genomic locations of the binding sites were mapped by first using overlapping phage isolates in the binding assays, followed by assaying binding to plasmid subclones of these phage isolates.
Electrophoretic mobility shift assays (EMSAs) were performed essentially as previously described (Fried and Crothers, 1981; Garner and Revzin, 1981). DNA fragments used in these assays were end-labeled by fill-in reaction with the Klenow fragment of DNA polymerase. Labeled DNA (roughly 0.3 ng) was incubated with fusion proteins (50-250 ng of affinity-purified *disco*-protein A fusion, 0.6-3 µg of bacterial extracts containing the *disco*-T7 fusions) in a 20 µl reaction containing 10 mM HEPES pH 7.9, 50 mM KCl, 0.5 mM NaEDTA, 0.5 mM DTT, 0.5 mM $ZnCl_2$, 0.25 mg / ml BSA, 300 ng of poly (dI-dC) and 10% glycerol. Reactions were incubated on ice for 30 min and resolved in 4% non-denaturing polyacrylamide gels using 1X Tris-glycine buffer.

Acknowledgments

We wish to thank Ana Regina Campos, Clive Wilson and Megan Grether for valuable discussions and critical reviews of this manuscript. We are grateful to Lily and Yuh Nung Jan for the 44C11 antibody and the A6-2-45 enhancer trap and to Corey Goodman for the anti-FasII antibody. We acknowledge Dave Smith for help with laser scanning confocal microscopy. We thank Claude Desplan, Benjamin Shykind and Joel Pomerantz for advice and protocols for DNA binding assays, and Nipam Patel for providing a protocol for PCR-based generation of digoxigenin-labeled probes for *in situ* hybridization. This work was supported in part by NIH grant RO1-NS26451 and a Searle Scholars award to H.S. Confocal microscopy was performed in a facility supported by the Lucille P. Markey Charitable Trust. During the initial phase of this work, K.J.L. was supported by a National Science Foundation Fellowship. H.S. is an Associate Investigator of the Howard Hughes Medical Institute.

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Figure 5.1 Expression of *disco* protein in the optic lobe primordia of wild type embryos.

Embryos were labeled with affinity-purified anti-Disco antiserum (A) or double-labeled with anti-Disco serum and the anti-Elav monoclonal antibody, 44C11 (B). The *elav* gene encodes a nuclear protein specifically expressed in all neurons. In this figure and all following figures, lateral views of embryos are shown, with anterior oriented to the left and dorsal up. Staging of embryos follows the convention of Campos-Ortega and Hartenstein (1985). **(A)** In the stage 13 embryo, *disco* protein is expressed in a characteristic pattern with abundant expression in the gnathal segments (open triangle) and in the invaginating optic lobe placodes (arrowhead). **(B)** A confocal image of the head region of an embryo of roughly the same stage as in (A) is shown. Expression of *disco* protein (green) and *elav* protein (red) are superimposed in this image. Cells which express both antigens appear yellow. The positions of the optic lobe placode (arrowhead) and the gnathal segments (open triangle) are indicated as in (A). The *disco* protein is detected in the ventral portion of the optic lobe primordium and in immature neurons just anterior to the optic lobe primoridium. Bar = 50 μm (in A), 25μm (in B).

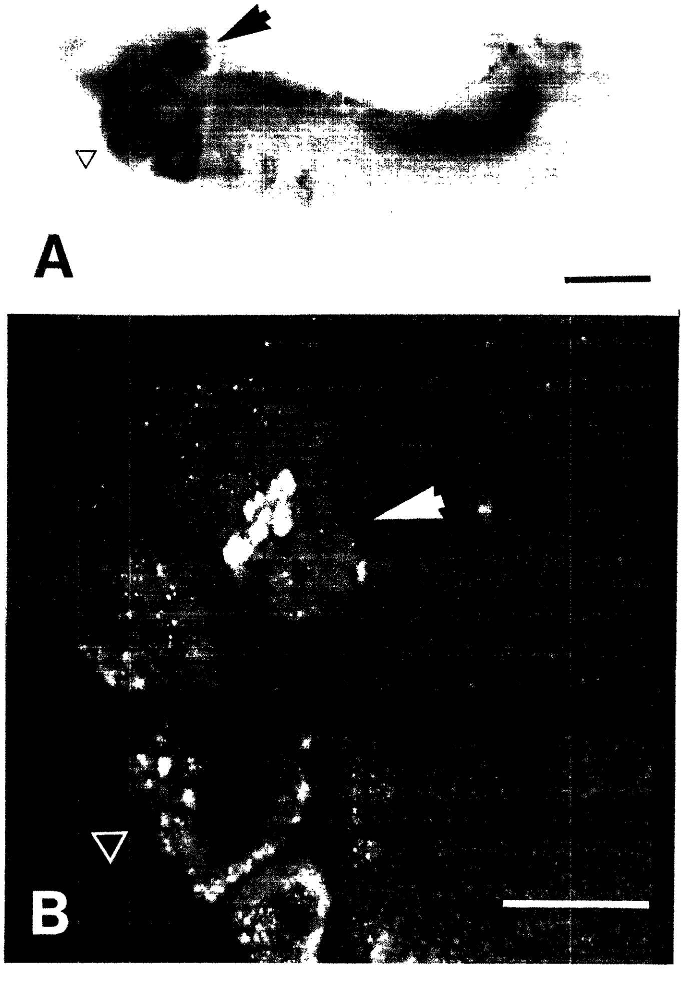

A
B

Figure 5.2 Expression of *disco* and fasciclin II in wild type and *disco* mutant embryos.

Confocal images of the anterior half of a stage 13 wild type (A, C) and a stage 13 *disco*^{1} mutant embryo (B, D). The embryos have been double-labeled with anti-Disco antiserum and an anti-FasII monoclonal antibody. Panels (A, B) show expression of *disco* protein in an optical section at the level of the invaginated optic lobe placode. Panels (C, D) show expression of *fasII* protein in the same field as in panels (A, B), respectively. **(A)** In the wild type embryo, abundant *disco* expression is detected in the region of the optic lobe primordium (arrowhead). **(B)** In *disco* mutants, expression in this region is largely absent (arrowhead). In contrast, the expression of fasciclin II, which is found in the posterior half of the optic lobe primordium (arrowheads), is the same in wild type **(C)** and *disco* mutant embryos **(D)**. Thus, in the *disco* mutant embryo, the cells of the optic lobe primordium are not absent. These cells fail to express *disco*, but they still express *fasII*, a characteristic marker. Bar = 50 µm.

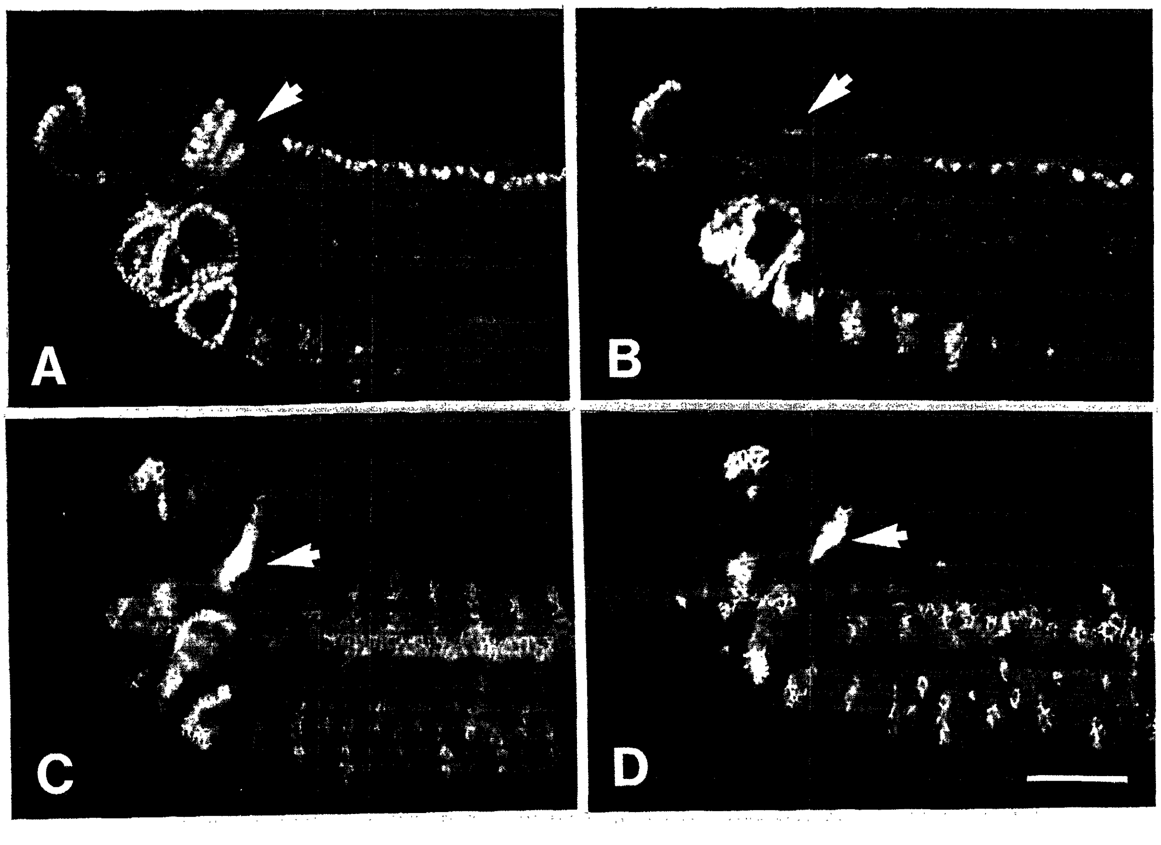

A
B
C
D

Figure 5.3 Distribution of *disco* mRNA in wild type and *disco* mutant embryos.

The *disco* transcript was detected in embryos of varying stages by whole-mount *in situ* hybridization using digoxigenin-labeled probes. Panels (A, C, E, G) show wild type embryos; Panels (B, D, F, H) are *disco*^{1} mutant embryos. In all panels, the position of the optic lobe primordium is indicated with an arrowhead. In late stage 5 wild type (A) and *disco* mutant (B) embryos, *disco* mRNA is found in the dorsal anterior region fated to become the optic lobe primordium. By stage 11, *disco* mRNA expression in the optic lobe primordium in *disco* mutants (D) is reduced in comparison to wild type (C). In stage 12 and later embryonic stages, expression of *disco* mRNA persists in the wild type optic lobe primordium (E, G), whereas expression in the mutant optic lobe primordium is mostly or completely absent (F, H). Thus, wild type *disco* activity is required for the maintenance, but not the initiation, of *disco* mRNA expression in the optic lobe region. Bar = 50µm.

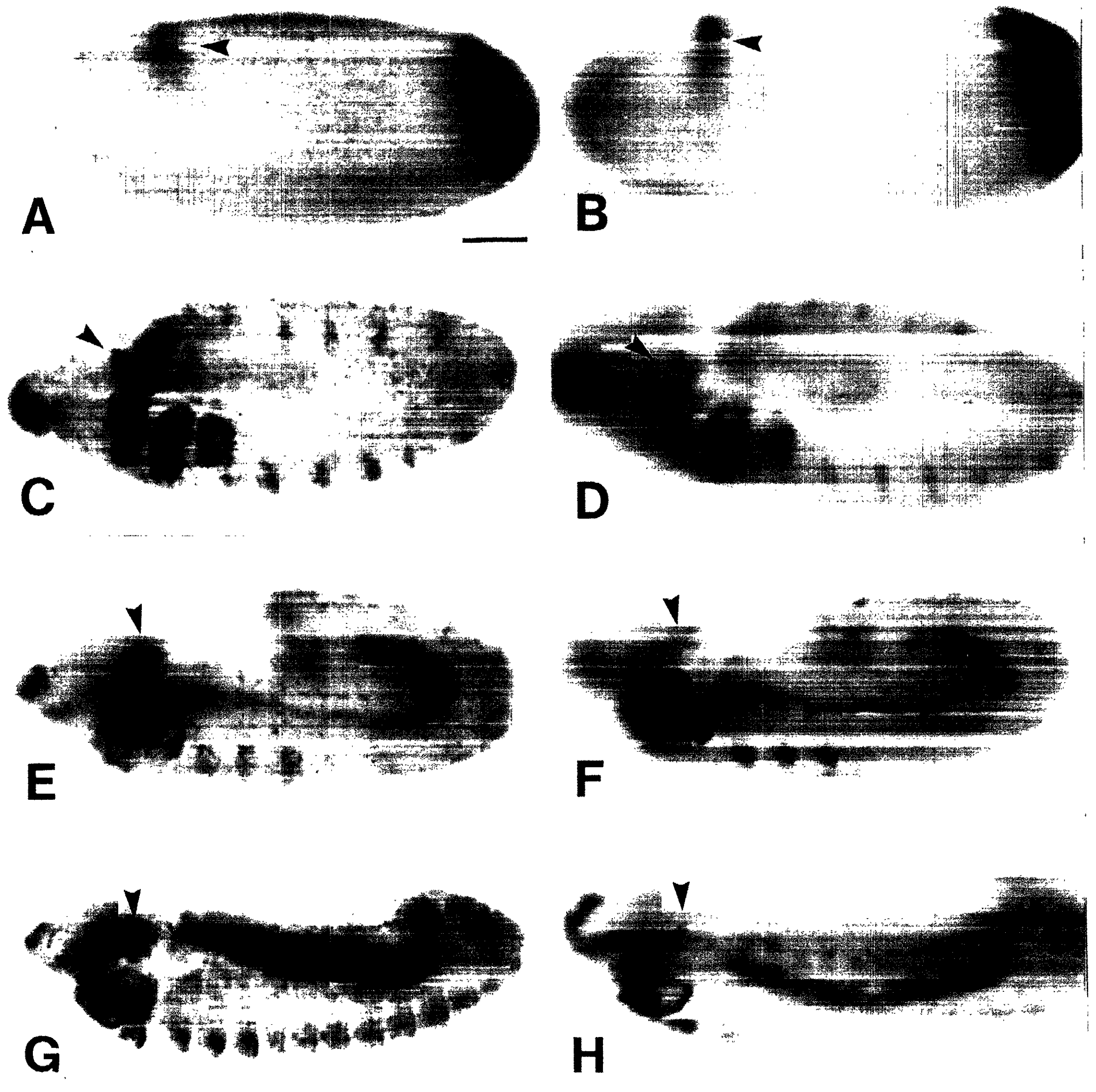

A
B
C
D
E
F
G
H

Figure 5.4 Tissue-specific activation of *disco* gene expression by a heat shock-inducible *disco* gene carried by a P-element construct.

A P-element construct which contains the *disco* gene coding region under the control of the *hsp70* promoter (*hs-disco*) was made and a strain of flies harboring this construct was generated by P-element-mediated transformation. This *hs-disco* P-element was introduced into the *disco*^{1} mutant background by crossing. This figure shows embryos which were subjected to whole-mount *in situ* hybridization using a digoxigenin-labeled probe which hybridizes to sequences found in the endogenous *disco* transcript and not present in the ubiquitously expressed *hs-disco* transcript. Stage 12 control embryos [*disco*^{1} mutant embryos; panels (A, B)] and similarly staged experimental embryos [*disco*^{1} mutant embryos carrying two copies of a second chromosome insertion of the *hs-disco* P-element; panels (C-F)] are shown. In panels (B, D, F), embryos were subjected to a 45 min. heat shock at 37° between 4 to 6 hr. of development. The region of the optic lobe primordium is indicated in all panels with an arrowhead. **(A)** *disco*^{1} mutant embryos show little *disco* mRNA expression in the optic lobe primordium. **(B)** After heat shock treatment, *disco* embryos show no increase in *disco* expression in this tissue. **(C, E)** The presence of two copies of the *hs-disco* gene has a noticeable effect on expression of the endogenous *disco* gene in the *disco* mutant optic lobe primordium, even without heat shock treatment. We attribute this effect to a low basal level of expression from the *hsp70* promoter. **(D, F)** Heat shock treatment of *disco* mutant embryos carrying two copies of the *hs-disco* transgene greatly increases endogenous *disco* gene expression in the optic lobe primordium. In some cases, expression of the endogenous *disco* gene in this tissue reaches levels indistinguishable from wild type (F). Note that although the *hs-disco* gene is expressed ubiquitously, activation of endogenous *disco* gene expression is observed exclusively in the optic lobe primordium. Bar = 50μm.

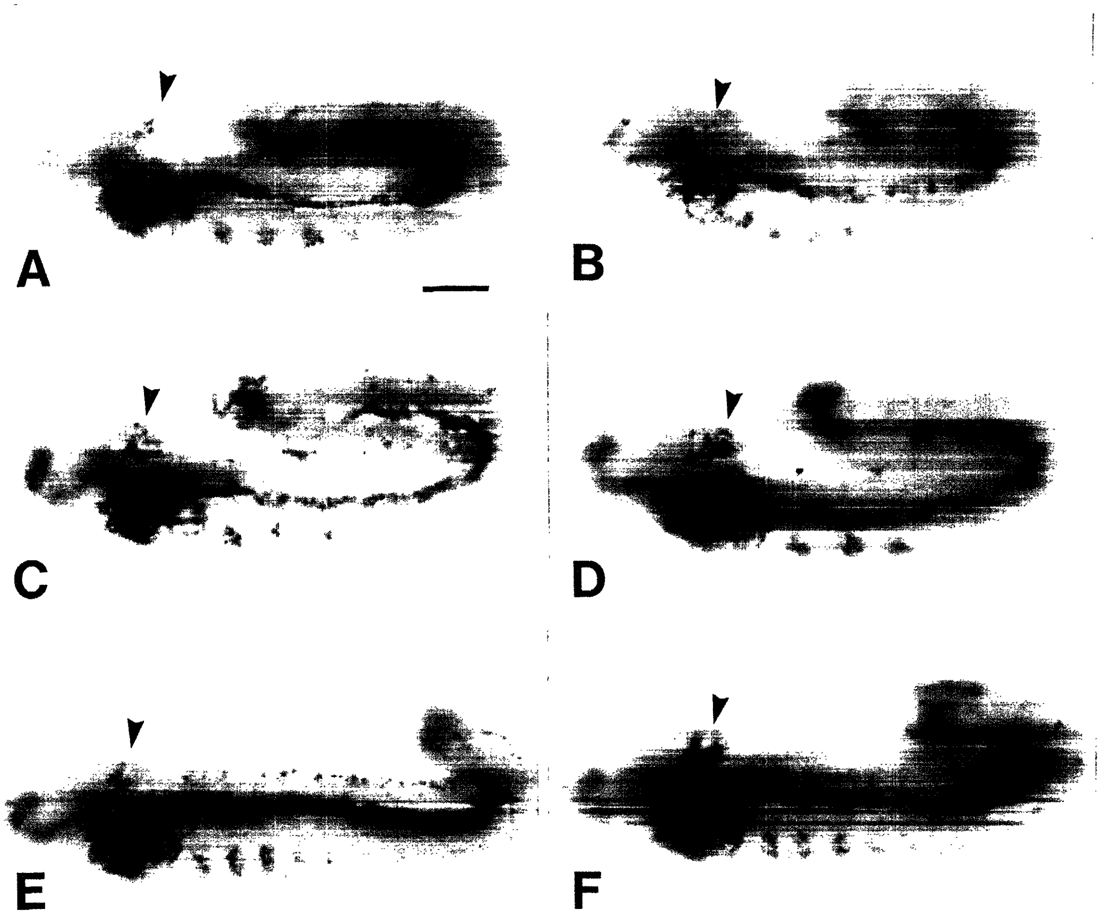
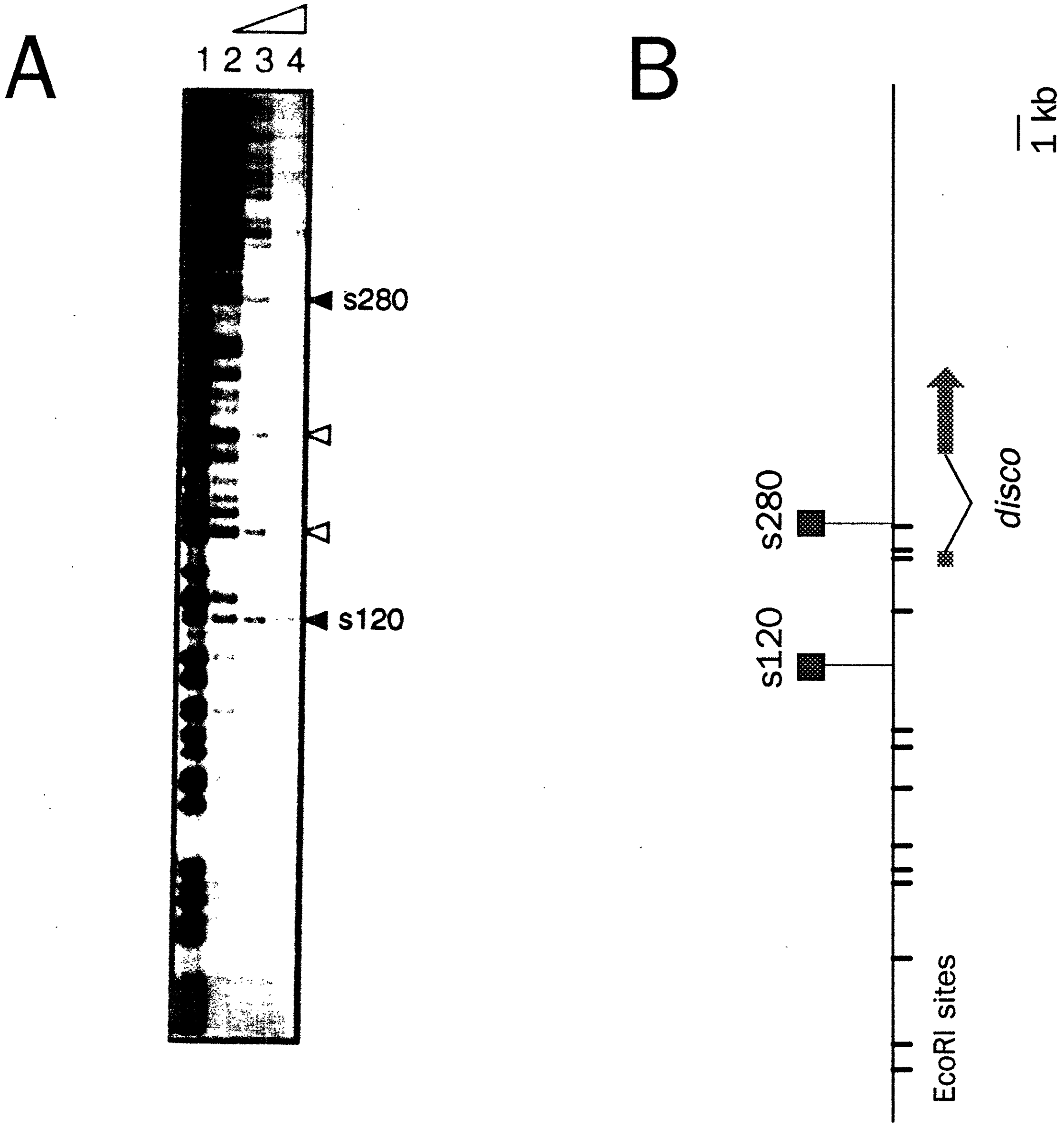

A
B
C
D
E
F

Figure 5.5 Identification of genomic DNA sequences near *disco* transcription unit bound by *disco* protein *in vitro*.

(A) A modified immunoprecipitation protocol was used to assay sequence-specific binding of *disco* protein to fragments of cloned genomic DNA. Preparation of the *disco* fusion protein in bacteria, DNA binding and immunoprecipitation reactions are described in Materials and methods. A representative assay using a Sau3A-digested, end-labeled bacteriophage lambda vector containing a roughly 15 kb segment of the *disco* locus is shown. Lane 1 shows the input DNA used in the immunoprecipitation assay. Lane 2 shows DNA fragments which are bound by *disco* protein and immunoprecipitated in the absence of competitor DNA. Lanes 3 and 4 show fragments which are bound in the presence of increasing amounts of competitor DNA (lane 3: 1 μg poly(dI-dC) and 200 ng Sau-3A digested bacteriophage lambda DNA; lane 4: 1 μg poly(dI-dC) and 1 μg Sau-3A digested bacteriophage lambda DNA). Two fragments, one of approximately 120 bp (marked s120) and one of approximately 280 bp (s280), are preferentially bound in this assay. Other fragments, marked by open triangles, represent portions of the bacteriophage lambda vector sequences which are also immunoprecipitated, albeit somewhat less efficiently. The immunoprecipitation of these fragments could reflect chance occurrences of sequences which resemble the specific sequence recognized by *disco* protein. **(B)** The location of binding sites in the genomic DNA surrounding the *disco* transcription unit is shown. A region of roughly 35 kb of DNA (represented by the horizontal line) was assayed for sequence-specific binding of *disco* protein as shown in (A). The short vertical lines represent the positions of EcoRI sites in the genome. Two fragments (shaded boxes) were consistently and tightly bound. One fragment of 120 bp (s120) is located about 2.5 kb upstream of the start of the *disco* transcript. The second fragment, of 280 bp (s280), is located within the single intron of the *disco* transcript, about 1 kb downstream of the transcription start site.


A
1 2 3 4
s280
s120
B
1 kb
s280
s120
disco
EcoRI sites

A

```
  1  GATCAATCAA TTTTTCACAC TTTGTCGTCC AGGTTCTCAT TCTCCATCCC
 51  ATTGCCATTA CGATTACCAT TACCATTCTC CGACCTCTAT CCTCTATCCT
101  CATTCCCATA CCCATACCCA TACCCATCCA GATC
```

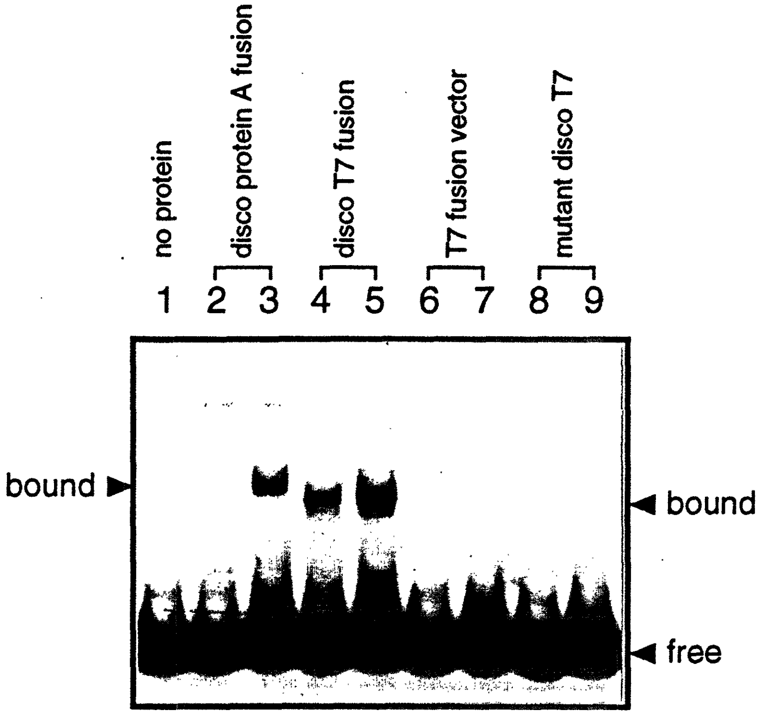
B

```
  1  GATCTACCAT CTACCATCTA AAGCGTCTCC CAACTCTCGG AATTCTTCAA
 51  TCTTGGTCGT TTCTTGTCTT GGGACTTTCA ACATTTTGCT GCCTTTGCCA
101  CTGCCGATAA TCAAAAAACG TTTCGTCCGA GAGCCATCAA AAGTTGTGAT
151  TGGTTTTTCC CCTACCCCCC AGCTGCCCAA CATCACCCCC CCTTTCACCA
201  TCGGTGGCTT AAGACATTTT AAGCGTTTGA CAAAAATAAT TACCCATGTA
251  CATGCATACT CACCTCGATC
```

Figure 5.6 DNA sequence of the *disco* protein binding sites. The sequence of two fragments specifically bound by *disco* protein in *in vitro* binding assays is shown. **(A)** The sequence of the s120 Sau3AI fragment . This fragment is located roughly 2.5 kb upstream of the *disco* transcription start. Note the highly repetitive sequences (underlined). This portion of the s120 fragment includes multiple repeats of the sequence C/ACCATA/T. (B) The sequence of the s280 Sau3AI fragment. This fragment is located within the *disco* intron, about 1 kb downstream of the transcript start site. This sequence includes two repeats (underlined) of the sequence ACCATCT, which is related to the sequence repeated in s120.

Figure 5.7 Comparison of DNA binding activities of wild type and mutant *disco* proteins.

Fusion proteins containing the wild type and the *disco*^{1656} mutant zinc finger regions were prepared and used in an electrophoretic mobility shift assay (EMSA) as described in Materials and methods. The *disco*^{1656} mutation is a change of Cys94, the first conserved Cys residue of the the first zinc finger in the *disco* sequence, to a Tyr residue. In *Drosophila*, this *disco* mutation behaves like a complete loss-of-function allele. The probe used in this EMSA is the 120 bp fragment (s120), which is specifically bound by *disco* protein in the immunoprecipitation assay shown in Fig.5. Lane 1 shows free probe without protein addition. Lanes 2 and 3 show the formation of a complex upon addition of 50 and 250 ng, respectively, of an affinity purified *disco*-protein A fusion. Lanes 4 and 5 show the formation of a slightly smaller complex upon addition of 600 ng and 3µg of an extract from bacteria producing a *disco*-bacteriophage T7 protein fusion. This complex is not formed upon addition of similar amounts of an extract from bacteria producing the T7 polypeptide without the fused *disco* sequence (lanes 6 and 7). Moreover, the complex is not formed upon addition of equivalent amounts of an extract containing a *disco*-T7 fusion protein with the *disco*^{1656} mutation. This mutation, therefore, eliminates the high affinity DNA binding activity exhibited by the *disco* protein *in vitro*.


no protein
disco protein A fusion
disco T7 fusion
T7 fusion vector
mutant disco T7
1
2
3
4
5
6
7
8
9
bound
bound
free

Chapter 6.

Establishment of neuronal connectivity during development of the *Drosophila* larval visual system.

Preface

The work presented in this chapter is also part of a collaboration with Dr. Ana Regina Campos, a former postdoctoral fellow in the Steller laboratory, now an independent researcher at McMaster University, Hamilton, Ont. My contributions to these studies are the analysis of A6-2-45 reporter gene expression, *bsh* expression, *disco* expression, and RK2 antigen expression in wild type and *disco* mutant embryos. Dr. Campos contributed the A31 (*fasII* enhancer trap) expression studes and did the analysis of *glass* mutant embryos.

Abstract

We used confocal microscopy in conjunction with specific antibodies and enhancer trap strains to identify and characterize the cellular interactions occurring during larval visual system development in wild type *Drosophila*. We also examined visual system development in embryos mutant for the *disconnected* (*disco*) and *glass* (*gl*) genes. We show that the projection of the larval optic nerve develops in a series of discrete steps. During embryogenesis, the nerve contacts several different cell types: neurons located near the anterior margin of the optic lobe primordium, a more centrally located neuron and a number of glial cells. The neurons we observe in contact with the larval optic nerve are the embryonic precursors of the previously-described optic lobe pioneers (OLPs). We demonstrate that the OLP neurons do not depend on interaction with the larval optic nerve for differentiation and proper axonal projection. These cells are present and project normally in *gl* embryos in which the larval optic nerve fails to develop. In *disco* mutant embryos, where appropriate connections between the larval optic nerve and its targets in the brain are not formed, the OLPs fail to differentiate properly. We show that the *disco* gene is expressed in the OLPs and may act autonomously to direct the differentiation of these cells. Taken together, our results suggest that the OLPs act as an intermediate target required for the establishment of normal optic nerve projection and connectivity.

Introduction

A fundamental unresolved question in developmental neurobiology is how neurons form appropriate connections with specific target cells during the elaboration of a complex nervous system. The proper development of both vertebrate and invertebrate nervous systems depends on mechanisms which guide growth cones, direct the selection of specific axonal pathways and regulate the recognition of correct synaptic targets [for reviews, see (Dodd and Jessell, 1988; Goodman and Schatz, 1993)]. Among the mechanisms which have been proposed to account for the precision and specificity of neuronal connectivity are localized cell-adhesion and -repulsion molecules, diffusible chemotropic factors, and molecules which mediate the recognition of labeled pathways, pioneer neurons, cellular guidance cues and synaptic targets.

The visual system of lower vertebrates has long been a favored model for the study of the specification of neuronal connections (Sperry, 1963). Recent studies have demonstrated that *Drosophila* visual system development may utilize cellular mechanisms which are quite analogous to mechanisms operating during vertebrate visual system development (Kunes et al., 1993). Analysis of visual system development in *Drosophila* is facilitated by the relative simplicity of the invertebrate nervous system and the availability of powerful molecular genetic and cell biological techniques.

Drosophila have separate visual systems in the adult and larval stages. The adult visual system consists of the compound eyes, the ocelli and the optic lobes, the portion of the brain which receives and processes visual information. In comparison, the larval visual system is extremely simple. It consists of a pair of bilateral visual organs, the lateral ocelli or Bolwig's organs (Bolwig, 1946), located in the larval head. Each of these visual organs consists of roughly twelve photoreceptor cells, which have axons that fasciculate together to form the larval optic nerve or Bolwig's nerve. Each larval optic nerve shows an highly invariant projection pattern in wild type individuals: it extends posteriorly from the photoreceptor organ to the ipsilateral larval brain hemisphere, where it turns ventrally to curve around the brain. On the ventral side of the brain hemisphere, the larval optic nerve turns and extends toward the central brain, where it synapses with specific interneurons.

It has been proposed, both on the basis of ultrastructural studies and on the phenotype of mutations which affect the visual system, that the development of the adult visual system is directed and organized by components of the pre-existing larval visual system (Fischbach et al., 1989; Meinertzhagen, 1973; Melamed and Trujillo-Cenóz, 1975; Steller et al., 1987). The larval optic nerves turn and enter the brain hemispheres in the vicinity of the optic lobe primordia, groups of cells which arise by invagination from the head ectoderm and proliferate and differentiate during late larval and pupal development to form the adult optic lobes. The axons of the adult photoreceptor cells which grow out from the eye imaginal disc during larval and pupal development follow the larval optic nerve through the optic stalk to their targets in the brain. The innervation of the optic lobe primordia by adult photoreceptor axons is a critical inductive event which coordinates the development of pre- and post-synaptic elements in the visual system (Fischbach and Technau, 1984; Meyerowitz and Kankel, 1978; Power, 1943; Selleck and Steller, 1991).

The embryonic development of the larval photoreceptor organs and the optic lobe primordia is related both temporally and spatially. These structures arise from the same location in the ectoderm of the embryonic head region (Green et al., 1993). Projections from the larval photoreceptors contact the optic lobe primordia early in development, when these cell groups are adjacent to one another (Green et al., 1993; Schmucker et al., 1992; Steller et al., 1987). It is likely that this early contact is part of the process by which the normal pathway and connectivity of the larval optic nerve is established.

Although the early development, proliferation patterns and migration of the larval photoreceptors and the optic lobe primordia have been described in some detail (Green et al., 1993), little is known about the cell types which are contacted by the larval optic nerve and the role these cell interactions might play in establishing neuronal connectivity in the larval visual system.

Here, we have sought to address these questions by using laser scanning confocal microscopy and antibody and enhancer trap markers to identify and characterize the cellular components of the larval visual system. We have examined the origin and interactions of the larval photoreceptor cells and neurons and glia which are contacted by the larval optic nerve during wild type embryonic development. In addition, we have examined the formation of these cell types in *disco* and *glass* mutant embryos. These studies show that discrete elements of the visual system interact in a series of defined steps to direct the orderly connection of the larval optic nerve to the brain hemisphere.

Results

Morphology of the larval visual system at the end of embryogenesis

At the time of hatching, the larval visual system consists of two photoreceptor organs located bilaterally, apposed to the cephalopharyngeal skeleton (Bolwig, 1946; Steller et al., 1987). Each organ is made up of roughly twelve photoreceptor cells whose axons bundle together to form the larval optic nerve (or Bolwig's nerve). Each larval optic nerve projects posteriorly and ventrally around the ipsilateral brain hemisphere, terminating in an area of the brain destined to become the adult optic ganglia (Green et al., 1993; Meinertzhagen, 1973; Steller et al., 1987) (Figure 6.2A, diagrammed schematically in Figure 6.1). Using a combination of monoclonal antibodies and enhancer trap strains, we examined the structure of the larval visual system at the end of embryogenesis and characterized some of the different cell types in contact with the larval optic nerve. Figure 6.1 illustrates these cell types schematically and Table 6.1 lists the markers used in our analyses.

The monoclonal antibody (mAb) 22C10 recognizes a cytoplasmic antigen found in all peripheral neurons and in a subset of central nervous system (CNS) neurons (Zipursky et al., 1984). It labels the larval photoreceptor neurons beginning around stage 13 of embryogenesis [staging based on (Campos-Ortega and Hartenstein, 1985)]. In late embryos, mAb22C10 immunoreactivity is also found in three cells associated with the larval optic nerve in the optic lobe primordium (Figure 6.2). We provisionally identify these cells as neurons, based on their morphology and expression of *elav* (Figure 6.2F), a neuron-specific nuclear protein recognized by mAb44C11 (Bier et al., 1988; Robinow and White, 1988; Robinow and White, 1991). The optic lobe primordium in late embryos consists chiefly of undifferentiated cells, which are quiescent until larval and pupal stages, when they will divide and form the imaginal optic lobes. No other cells in the optic lobe primordium express *elav* at this stage, which suggests that this group of cells are the first neurons to differentiate in the optic lobe anlage.

The early differentiation of these cells, along with their position and association with the larval optic nerve suggests that these neurons are the embryonic precursors of the previously-described optic lobe pioneers (OLPs) (Tix et al., 1989). These cells were previously identified in the third instar larval brain by HRP injection into syncytial blastoderm stage embryos, which selectively labels cells that stop dividing and differentiate during embryogenesis. In the larval brain, the OLPs are located in the distal optic lobe anlage near the base of the optic stalk. They extend axons which fasciculate with the larval optic nerve and project straight through the optic lobe toward the central brain. We confirmed that the neurons we observe in the late embryonic brain give rise to the OLPs by examination of mAb22C10-labeled brains of successively older animals. The identification is further strengthened by analysis of mutants in which the OLPs, and the corresponding embryonic neurons, are abnormal (see below).

The three embryonic OLP precursors can be divided into two groups by virtue of their position and origin (Figure 6.2B). Two of the OLP precursors, which we refer to as the corner OLPs, are located at the anterior margin of the optic lobe primordium in late stage 16 embryos. The third OLP precursor, here designated the central OLP, is separated from the other two in a position deeper in the brain hemisphere. By stage 17 of embryogenesis, all three OLPs have axons which fasciculate with the larval optic nerve and project along with this nerve toward the central brain (Figure 6.2C-E).

We identified a number of markers which label components of the larval visual system during embryogenesis. In the enhancer trap strain A6-2-45, ß-galactosidase (ßgal) is selectively expressed in virtually the entire visual system (Figure 6.3). It is expressed in the larval photoreceptor cells, most, if not all, of the cells of the optic lobe primordium, including the OLP precursors, and a number of cells with elongated nuclei distributed along the larval optic nerve. The A6-2-45 enhancer trap is an insertion near the *sine oculis (so)* gene, a homeodomain-containing gene required for the proper development of both the larval and adult visual systems (L. Zipursky, V. Hartenstein, personal communication). In the enhancer trap strain A31, an insertion near the *fasciclinII (fasII)* gene (Grenningloh et al., 1991), ßgal is expressed in the larval photoreceptors, in the posterior portion of the optic lobe invagination, in the corner OLPs and in the elongated cells distributed along the larval optic nerve (Figure 6.5). We conclude that the elongated cells distributed along the optic nerve are glia by virtue of their morphology and their RK2 immunoreactivity (Figure 6.9A). The RK2 antibody recognizes a homeodomain protein specifically expressed in all glia (Andrew Tomlinson, personal communication). Finally, the more posterior corner OLP expresses the *brain-specific homeobox (bsh)* gene (Jones and McGinnis, 1993) beginning at about stage 15 of embryogenesis (Figure 6.4). The *bsh* gene product is an extremely specific marker: in the mature embryo, its expression is restricted to roughly 30 cells in each brain hemisphere. In the optic lobe primordium, *bsh* is expressed exclusively in the posterior corner OLP.

Origin and differentiation of components of the larval visual system

In the *fasII* enhancer trap strain A31, the reporter gene ßgal is expressed in cells of the larval visual system beginning relatively early in embryogenesis. Thus, we were able to follow the early development of these cells by examining A31 strain embryos immunolabelled with mAb22C10 and anti-ßgal. These experiments indicate that components of the visual system have diverse origins and undergo an invariant pattern of differentiation during formation of the larval visual system (Figure 6.5).

The larval photoreceptor cells differentiate asynchronously. In stage 13 embryos, a cluster of approximately twelve ßgal-expressing cells are located in the posterior procephalic lobe, just adjacent to the ventral lip of the invaginating optic lobe primordium (Figure 6.5A). Initially, a single cell within this cluster expresses the mAb22C10 antigen; subsequently, mAb22C10 immunoreactivity appears in the other cells of this cluster. The precursors of these photoreceptor cells originate either from the optic lobe placode *per se* or from cells very close to it (Green et al., 1993). As the optic lobe primordium invaginates and detaches from the superficial ectoderm, the photoreceptor cells remain in the head epidermis.

We also followed the differentiation of the OLP precursors in the context of the optic lobe primordium development. Among the OLPs, the central neuron is the first to express the mAb22C10 antigen, beginning roughly in stage 14. At this time, the central OLP precursor is located in the developing brain, medial to the optic lobe primordium (Figure 6.5F). This neuron is therefore likely to derive not from the optic lobe primordium itself, but from CNS neuroblasts which have delaminated from the ectoderm earlier in development. The corner OLP precursors are first labeled with mAb22C10 in late stage 16 embryos (Figure 6.5G). These OLPs arise from the ventral, later the superficial, lip of the optic lobe primordium. Thus, the origin and timing of differentiation of the two types of OLPs are distinct. By the time of hatching, however, the three cells are in close proximity and share a common axonal tract with the larval optic nerve.

Establishment of the larval optic nerve projection pattern.

The projection pattern of the larval visual system is established in a series of distinct, stereotyped steps. Based on our observations, we defined three stages which are outlined in a diagram shown in Figure 6.6.

In the first stage, axonal processes from the larval photoreceptor cells contact the adjacent optic lobe invagination (Figure 6.5A-C; Figure 6.6A). Coincident with the appearance of mAb22C10 immunoreactivity, the photoreceptor cells develop short processes which extend to the underlying cells of the invaginating optic lobe primordium (Figure 6.5C). The processes later fasciculate to form the larval optic nerve. Initially, the distal end of the nerve is diffuse and numerous cells of the optic lobe invagination are contacted. Based on their position and relation to the larval optic nerve, some of the optic lobe primordium cells contacted by the nerve are likely to be the precursors of the corner OLPs.

In a second stage of larval visual system development, during stage 14 and 15 of embryogenesis, the larval optic nerve elongates as the larval photoreceptors and the cells of the optic lobe primordium are separated from one another (Figure 6.5D-F; Figure 6.6B). The photoreceptor cell bodies move to a more anterior position during the process of head involution. At the same time, cells of the optic lobe primordium move ventrally around the developing brain hemisphere, where they are gradually incorporated. During these morphogenetic movements, the contact between the larval optic nerve terminus and the optic lobe primordium is maintained. At this stage, the distal end of the larval optic nerve is well defined and wraps around the outer edge of the invaginating optic lobe primordium (Figure 6.5E). The optic nerve terminus is in contact with two cells which give rise to the corner OLPs. The more posterior of these OLPs, which begins to express *bsh* at the end of this stage, is a large cell in the center of a cup of optic lobe primordium cells. The tip of the optic nerve is invariably found between this *bsh*-expressing cell and the surrounding cells (Figure 6.3A). The anterior OLP cell is tightly associated with the nerve at the anterior edge of the cup of optic lobe primordium cells. During this stage of visual system development, the central OLP cell, which can be visualized with mAb22C10, is located deeper in the brain and is not yet contacted by the larval optic nerve (Figure 6.5F). It develops a centripetal projection which extends toward the central brain neuropil.

A third stage of visual system development occurs after the photoreceptor cells and the optic lobe primordium have reached their final positions and the optic lobe primordium has been incorporated into the brain hemisphere (Figure 6.5G-I; Figure 6.6C). In this stage, the distal tip of the larval optic nerve extends beyond the cell bodies of the corner OLP cells and projects toward the central brain. At the same time, the corner OLP cells begin expressing mAb22C10 immunoreactivity and extend axonal projections which fasciculate and project with the larval optic nerve. It is not clear whether the photoreceptor axons or the OLP axons pioneer this path to the central brain. The photoreceptor and corner OLP axons contact the central OLP and fasciculate with its centripetal projection. The central OLP gradually descends toward the corner OLPs. In very late embryos, the three cells are in close proximity. In such embryos it is difficult to determine the exact endpoint of the different fasciculated axons. Together, the processes extend toward the central brain neuropil.

Development of the larval visual system in glass mutant embryos

By analogy to the requirement for retinal innervation to induce development of the first optic ganglion of the adult (Meinertzhagen, 1973; Meyerowitz and Kankel, 1978; Power, 1943; Selleck and Steller, 1991), we wondered whether the development of these OLP neurons was likewise dependent on innervation from the larval photoreceptors. In addition, we sought to determine whether the projections of the OLPs toward the central brain might require a pioneering function of the larval optic nerve. The central OLP precursor, at least, appears to differentiate and project independently of the larval optic nerve. This neuron expresses the mAb22C10 antigen and extends an axonal projection toward the central brain prior to its contact with the larval optic nerve. In order to determine whether the corner OLPs are capable of differentiating and projecting properly without photoreceptor contact, we examined their development in embryos mutant for the *glass (gl)* gene. The *gl* gene encodes a homeodomain protein required for the differentiation of both larval and adult photoreceptor cells (Moses et al., 1989). The *gl* protein is thought to regulate the expression of photoreceptor-specific genes (Moses and Rubin, 1991). In *gl* mutant embryos, a rudimentary photoreceptor organ is formed, but the larval optic nerve fails to develop contact with the optic lobe primordium (Moses et al., 1989; Schmucker et al., 1992).

In stage 17 *gl* mutant embryos, both the central OLP neuron and the later-differentiating corner OLPs are present and positioned normally. All three OLP cells develop mAb22C10 immunoreactivity in the same temporal sequence as in wild type (Figure 6.7). Moreover, the corner OLPs in the *gl* mutant embryo extend centripetal projections toward the central neuropil in a manner indistinguishable from wild type (Figure 6.7A,B). These observations demonstrate that the corner neurons do not depend on contact with the larval optic nerve for proper development. Our findings are consistent with a previous report which indicated that *bsh* immunoreactivity is also normal in the brain of *gl* mutant embryos (Jones and McGinnis, 1993). Thus, the proper differentiation and projection of all three OLP neurons does not appear to require interaction with the larval optic nerve.

Larval visual system development in disco mutant embryos

The results presented above indicate that the OLP neurons differentiate and project properly in the absence of interactions with the larval optic nerve. We wished to address whether the larval optic nerve, on the other hand, might require interactions with the OLP neurons for the establishment of its projection to brain targets. To that end, we analyzed the structure of the larval visual system in *disconnected (disco)* mutant embryos. In *disco* embryos, the larval optic nerve fails to establish appropriate connections with its targets in the brain (Steller et al., 1987). We sought to determine whether this defect is correlated with changes in the differentiation or position of any of the various cells contacted by the larval optic nerve.

Two different types of aberrant larval visual system phenotypes are found in homozygous *disco* mutant embryos (Steller et al., 1987). In one class of *disco* embryos (the "unconnected " class), the larval optic nerve projects to a ectopic location, often terminating far from the optic lobe primordium. In a second class of *disco* mutants (the "connected" class), the larval optic nerve contacts the optic lobe primordium, but fails to recognize and connect with its appropriate synaptic target cells. Examination of the early stages of visual system development in *disco* embryos shows that initial contact between the larval optic nerve and the optic lobe primordium is normal (Steller et al., 1987). However, the maintenance of this contact during head morphogenesis, as well as the recognition of synaptic targets by the larval optic nerve, fail to occur properly in *disco* mutants.

In stage 17 *disco* embryos, the corner OLPs cannot be visualized either by mAb22C10 or anti-Bsh immunoreactivity (Figure 6.8B-D). From these data alone, we cannot determine whether these cells fail to differentiate, degenerate or undergo a transformation in cell fate. Given the highly specific pattern of *bsh* expression, it seems unlikely that the defect is simply due to mispositioning of the corner OLPs. The central OLP cell, on the other hand, appears to differentiate and project roughly normally in a fraction of *disco* mutant embryos (~30%, Figure 6.8F). Interestingly, the differentiation and placement of this central OLP cell seemed to have no influence on connection of the larval optic nerve to the optic lobe primordium. Embryos of the "unconnected," as well as "connected ," class were equally likely to have normal central OLP cells. In fact, the presence or absence of the central neuron is not correlated with ability of the larval optic nerve to project to the central brain neuropil. In some embryos, the larval optic nerve failed to recognize and fasciculate with an apparently normal central OLP cell, even when the nerve was within filipodial reach of this cell (Figure 6.8E,F).

The differentiation of other visual system components is less dramatically and consistently affected by the *disco* mutation. The optic lobe primordium is appropriately positioned and incorporated into the brain hemispheres in a normal fashion in *disco* mutants. Moreover, expression of markers specific to optic lobe primordium cells, including reporter gene expression in the enhancer trap strains A31 and A6-2-45, is equally unaffected by *disco* mutation (see Chapter 6.5). In many *disco* mutant embryos, cells expressing the RK2 glial marker are distributed along the larval optic nerve (Figure 6.9), even when the projection of the nerve is quite abnormal. In some *disco* embryos, however, fewer glia are found associated with the larval optic nerve. In addition, the nuclei of the optic nerve glia in *disco* mutants fail to show the characteristic elongated morphology. Moreover, they appear to be not closely associated with the larval optic nerve as in wild type (Figure 6.9B,C).

Taken together, these observations demonstrate that the defects in larval optic nerve development in *disco* mutants is not the result of gross abnormalities in the morphogenesis of the optic lobe primordium. Additionally, these results suggest that proper differentiation of the corner OLPs and may have an important role in the establishment of the larval optic nerve projection pattern and connectivity. Whether the larval optic nerve phenotype in *disco* mutants is directly caused by aberrant differentiation of the corner OLPs, glial cells and/or photoreceptor cells themselves can not be distinguished by these results.

Expression of the disco gene during development of the larval visual system

Defects in the differentiation of the OLP cells in *disco* mutant embryos could be attributed to an autonomous requirement for *disco* gene function in these cells. Previous studies demonstrated that the *disco* gene product is expressed early in embryogenesis in a portion of the optic lobe invagination (Lee et al., 1991). Later in embryogenesis, after larval photoreceptors have differentiated and the larval optic nerve extends to the optic lobe primordium, *disco* gene expression is detected in cells near the terminus of the larval optic nerve. Here, we describe a more detailed analysis of *disco* expression as it relates to the development of the larval optic nerve projection pattern. To that end, we used laser scanning confocal microscopy to analyze embryos stained with a *disco*-specific antiserum (Lee et al., 1991), as well as ßgal expression in embryos of the *disco* enhancer trap strain C50.1S1 (Cohen et al., 1991; Heilig et al., 1991). In either case, the expression patterns observed were spatially and temporally identical. As ßgal immunoreactivity in the enhancer trap strain C50.1S1 is quite robust, in some cases, we present these results here.

Given that the larval photoreceptors originate from an ectodermal position immediately adjacent to the optic lobe invagination, early expression of *disco* in the vicinity of this optic lobe primordium may include expression in the immature photoreceptor cells or their immediate precursors. However, *disco* protein is not found in the earliest mAb22C10-immunoreactive photoreceptor precursors (Figure 6.10A-C). In contrast, *disco* expression is detected in cells of the optic lobe primordium just medial to these early photoreceptor cells. These *disco*-expressing cells include cells contacted by neuronal processes from the developing larval photoreceptor cells (Figure 6.10D-F).

Examination of *disco* protein expression in later embryonic stages yields similar results. Expression of *disco* protein is not found in larval photoreceptor cells, whereas cells of the optic lobe primordium, including cells in contact with photoreceptor axons, continue to show *disco* immunoreactivity. In late embryos, *disco* protein expression is found in the glial cells associated with the optic nerve and in both types of OLP cells (Figure 6.11; Figure 6.12). These data raise the possibility that *disco* protein acts in these different cell types which contact the larval optic nerve. If so, *disco* may be autonomously required in these cells to direct their proper differentiation.

Discussion

In the studies described in this chapter, we have explored the mechanisms responsible for the formation of a simple, stereotyped neuronal pathway, the *Drosophila* larval visual system. Our observations of larval optic nerve development in wild type and mutant embryos suggest that the formation of this pathway depends on an series of specific cell interactions occurring in a defined sequence of steps. During embryonic development, larval photoreceptor axons contact a group of early-differentiating neurons in the optic lobe primordium. In addition, they are associated with a number of glial cells, which, by late embryogenesis, are distributed along the length of the larval optic nerve.

We found that some of the cells contacted by the larval optic nerve are a group of early-differentiating neurons known as the optic lobe pioneers (OLPs). These neurons were previously identified in the third instar larval brain (Tix et al., 1989). We have determined the embryonic origin of the OLPs and have found that these cells develop in two different ways. Two of the OLPs, which we call the corner OLPs, arise from the optic lobe primordium itself. It is likely that these cells are contacted by the larval photoreceptors during the very initial stages of axon outgrowth. A second type of OLP, which we call the central OLP cell, originates from outside the optic lobe primordium. This cell is contacted by the larval optic nerve in the final stage of nerve pathway formation. At the time of contact, the central OLP has a centripetal axonal projection extending into the central neuropil. By late embryogenesis, however, all three OLPs have axons which share a common tract with the larval optic nerve and project toward the central brain.

It is likely the OLPs are not the final synaptic targets of the larval optic nerve. Cobalt filling of the larval optic nerve in the third instar stage allows visualization of photoreceptor axon endings on target cells at the medial margin of the prospective medulla, much deeper within the brain (Steller et al., 1987). Instead, the OLPs may act as an intermediate target of the larval optic nerve. As such, they may provide a cellular guidance cue which directs the development of the larval optic nerve pathway. Alternately, or in addition, they may pioneer the route taken by the larval optic nerve toward the central brain. Among these cells, the central OLP cell is especially likely to serve such a pioneer function, as its centripetal projection exists prior to fasciculation with the photoreceptor or corner OLP axons.

Development of the larval visual system: a stepwise process

We have observed three distinct phases of visual system development, during which different mechanisms are likely to regulate the stereotyped movement of cells and establishment of precise cellular contacts. In the first phase of this development, larval photoreceptors develop short neuronal processes which contact an intermediate target, the immature corner OLP cells in the adjacent optic lobe primordium. During a second phase of development, the larval photoreceptors and this intermediate target are separated by head morphogenesis and the incorporation of the optic lobe primordium into the ventral brain. Concomitant to these cell movements, the neuronal processes between the larval photoreceptors and the optic lobe primordium elongate to form the larval optic nerve. Finally, in a third phase, the distal end of the larval optic nerve progresses beyond the corner OLP cells to extend toward the central neuropil, where synaptic connections are formed. Thus, we observe that the formation of this stereotyped nerve pathway occurs not by a long-range pathfinding process, but by neuronal recognition of specific targets located within filipodial reach.

In the initial phase of larval optic nerve formation, when contacts between the nerve and an intermediate target in the brain are established, cellular recognition may occur even before axon outgrowth. The first larval photoreceptors and their intermediate target cells may be immediately adjacent at the time of the commitment to these cell fates. If so, inductive interactions between the two cell types may play a role in their early differentiation, as, for example, interactions between neighboring cells in the developing eye imaginal disc regulate adult photoreceptor cell fate [for review, see, (Hafen and Basler, 1991)]. In both of these contexts, mutations in the *Notch* gene affect cell fate decisions (Cagan and Ready, 1989; Green et al., 1993), which suggests that similar mechanisms may regulate these processes. Early larval photoreceptor cells extend diffuse processes which contact the underlying optic lobe primordium (see Figure 6.5C). These filipodial processes may probe the neighboring epithelium of the optic lobe primordium before recognizing a specific target. From the outset, however, these early processes are directed toward the optic lobe primordium. Some directional cue, a chemotropic factor, for example, may orient this initial extension of neuronal processes. At this stage in development, the cells which surround the photoreceptors do not express neuronal markers. Therefore, any cellular guidance cues would be provided by non-neuronal cells. As judged by the expression of markers like the mAb22C10 antigen, the photoreceptor cells develop asynchronously. It is possible that one particular cell among the differentiating photoreceptors is required to "pioneer" the initial contact with the optic lobe primordium. The systematic analysis of additional mutant strains with larval visual system defects may help elucidate the mechanisms which regulate larval optic nerve development at this stage.

During a second phase of visual system development, the larval optic nerve elongates as the optic lobe primordium moves around to the ventral side of the brain hemisphere. The processes of nerve elongation and optic lobe invagination occur independently. In *disco* mutant embryos, the larval optic nerve often loses contact with the invaginating optic lobe primordium, yet optic nerve outgrowth is unaffected (Steller et al., 1987). Likewise, in *glass* mutant embryos, the larval optic nerve fails to develop, yet the optic lobe invaginates and is incorporated into the brain hemispheres normally.

As cell movements separate the larval photoreceptors and the optic lobe primordium, the distal end of the larval optic nerve normally maintains a defined connection with the corner OLPs in the optic lobe invagination. This contact may involve a simple cell adhesive mechanism or a more active process of cell signaling and cell recognition. By either mechanism, the corner OLPs might stabilize the terminus of the larval optic nerve while larval head morphogenesis and optic lobe incorporation takes place. In addition, this stabilization may allow time for the central OLP and the final synaptic targets of the larval optic nerve to differentiate. The idea that the corner OLPs are required to stabilize the optic nerve terminus during morphogenetic movements at this stage is supported by the analysis of visual system development in *disco* mutants. Previous reports suggested that the OLPs are missing or abnormally located in *disco* mutant third instar larvae (Tix et al., 1989). Here we show that in *disco* mutant embryos, the corner OLPs, at least, fail to differentiate. The frequent detachment of the larval optic nerve in *disco* mutant embryos could therefore result from the absence of these corner OLPs.

Finally, in a third phase in the establishment of the larval optic nerve pathway, the distal end of the optic nerve grows past the corner OLPs towards the central brain. This phase is similar to the first phase, as it involves movement of the nerve terminus and target recognition. At this stage of development, the nerve recognizes and fasciculates with a process from the central OLP neuron. This centripetal projection may act as a pioneer pathway which guides the optic nerve toward the central brain. The corner OLPs also extend axons which fasciculate with the central OLP projection. It is not clear from these studies whether the photoreceptor axons or the corner OLP axons develop this central projection first. We examined the development of the larval visual system in *gl* and *disco* mutant embryos to assess the role interactions between these cells might play in guiding the axonal projections toward the central brain. Our analysis of *gl* mutant embryos indicates that the corner OLPs are capable of developing this central projection without the accompanying photoreceptor axons. However, in *disco* mutant embryos, the corner OLPs fail to differentiate and the larval photoreceptors fail to develop their central projections. Therefore, the larval photoreceptor axons may require interactions with the corner OLPs to project properly. Interestingly, when the OLP cells are missing in *disco* mutant embryos, the larval photoreceptor axons do not fasciculate with the central OLP projection even when they are immediately adjacent to it. This observation suggests that the corner OLP cells may provide an instructive signal to the ingrowing photoreceptor axons, as opposed to simply providing a passive substrate which guides these axons toward the central OLP cell.

Our analysis of visual system development in *gl* mutant embryos suggests that the OLP neurons can differentiate without inductive cues from the larval photoreceptor axons. The apparently independent development of these neurons contrasts with the differentiation of neurons in the first optic ganglion of the adult, which require innervation from the adult photoreceptors for their appropriate development (Fischbach and Technau, 1984; Meyerowitz and Kankel, 1978; Power, 1943; Selleck and Steller, 1991). However, the conclusion that components of the larval visual system develop independently must be viewed with some caution. It is possible that larval photoreceptor precursors interact with adjacent cells in the optic lobe primordium very early in visual system development. Recent studies have shown that *gl* mutant embryos have rudimentary photoreceptor organs which exhibit some aspects of normal differentiation. In *gl* embryos, the first photoreceptors to differentiate establish neuronal processes that contact the neighboring optic lobe primordium (Schmucker et al., 1992). In principle, these early interactions might be sufficient to induce or affect OLP development.

Role of glial cells in larval visual system patterning

We have also identified a number of glial cells distributed along the larval optic nerve. Our identification of these cells as glia is based on their flattened, elongated morphology and their expression of the homeodomain protein recognized by the RK2 antibody. In addition, they express several enhancer trap strains (data not shown), which are thought to be specific markers of glial components of the PNS and CNS (C. Klämbt, personal communication). From the studies presented here, it appears that the origin of these glia is related, both temporally and spatially, to the origin of the other components of the visual system. Like the larval photoreceptor cells, the corner OLPs and other cells of the optic lobe invagination, these glia express ßgal in the A31 (*fasII*) and A6-2-45 (*so*) enhancer traps. This expression pattern may indicate that these glia share a common lineage with other components of the larval visual system. It suggests that all these cells may be derived from a common structure, perhaps the so-called optic placode (Green et al., 1993), which may represent a head segment or "compartment".

When the larval optic nerve is first detected, it lies along a path of *fasII* positive cells (see figure 6.5B). It is unclear whether these cells are interspersed between the photoreceptor cells and the optic lobe invagination prior to this stage, or whether they may be recruited from adjoining areas. Later, as the optic nerve elongates, these cells spread out along the nerve, express the RK2 antigen and finally a flattened morphology.

From this sequence of events, it is difficult to conclude whether the position of these glia is determined by the larval optic nerve, or whether the pathway of the nerve may be fixed by the orientation of the glia. A proposed role for glia in guiding and patterning axonal outgrowth has precedent in the development of both vertebrate and invertebrate nervous systems, as well as in tissue culture studies [see, for example, (Klämbt et al., 1991; Neugebauer et al., 1988; Schwab and Schnell, 1991)]. In *disco* mutant embryos, where the larval optic nerve is ectopically located, these glial cells are present and are still at least loosely associated with the nerve. This finding could, in theory, support a glial role in nerve guidance, in which case, the nerve mislocation in *disco* would result from an altered behavior of the glia. Alternately, the *disco* visual system phenotype might suggest that glia take up a novel location in response to misplacement of the larval optic nerve. An examination of differentiation and positioning of these glia in *gl* mutant embryos may help sort out the relationship between glial and nerve development. In addition, an analysis of larval optic nerve projection in mutants in which glial differentiation is specifically disrupted may indicate whether these glia may direct positioning of the photoreceptor axons.

Expression of the disco gene in visual system development and etiology of the visual system defects in disco

The studies described in this chapter provide some clues regarding the causes of larval optic nerve defects in *disco.* We have used confocal microscopy to analyze *disco* expression in the context of larval visual system development. We find that as soon as larval photoreceptor cells can be visualized by staining with specific markers, these cells do not express *disco.* The *disco* protein is expressed in several of the different cell types contacted by the larval optic nerve, including the optic nerve glia and the OLPs. Although we cannot exclude the possibility that *disco* may be expressed and functional in photoreceptor cell precursors, it seems likely that *disco* is directing larval optic nerve development non-autonomously. *disco* gene activity may be required in the optic nerve glia, in

OLPs or in both to direct proper nerve patterning. Interestingly, we find different effects of the loss of *disco* function in these different cell types: In the corner OLP cells, loss of *disco* function significantly disrupts cell differentiation, as judged by the loss of expression of several markers. The differentiation of central OLP cell and the glial cells, in contrast, is less frequently and/or less significantly affected.

The *disco* gene encodes a nuclear protein with zinc finger domains (Heilig et al., 1991) This gene product is thus likely to act as a regulator of gene expression, possibly a transcription factor, and this proposal is supported by the finding that *disco* has sequence-specific DNA binding activity *in vitro* (Chapter 5). Here we have shown that a small subset of the cells which express the *disco* gene do not differentiate properly in *disco* mutants. The specificity of the *disco* mutation is remarkable: *disco* is expressed in numerous tissues, including the leg primordia and the larval heart (Bodmer, 1993; Cohen et al., 1991; Lee et al., 1991), yet cell differentiation is noticeably affected in only these few cells in the embryonic visual system.

Which genes might be regulated by the *disco* protein? The loss of *disco* function leads to defects in cell interactions between the larval optic nerve and the cells it contacts during pathway formation, including optic nerve glia and OLP cells. As only certain nerve-target and nerve-glia interactions are affected, the *disco* gene may regulate molecular guidance and recognition cues which are specific to the development of the larval optic nerve pathway and connectivity. In addition, we have demonstrated here that several markers are not expressed in the corner OLP cells in *disco* mutants. The genes which encode some of these markers, for example, the *bsh* gene, are also candidates for regulation by the *disco* protein. However, these genes are expressed in other cells during development, and their expression in these other cells does not depend on *disco* function. Thus, the regulation of these genes by *disco*, whether direct or indirect, must be highly cell type-specific.

Role of the larval visual system in patterning the adult visual system

During larval and pupal stages, adult photoreceptors travel through the optic stalk to their targets in the optic lobe anlagen. At the time of retinal fiber ingrowth, the optic stalk already contains the larval optic nerve (Meinertzhagen, 1973; Melamed and Trujillo-Cenóz, 1975). This observation, along with the correlation between larval optic nerve misplacement and the failure of retinal fibers to innervate their targets in *disco* mutants (Steller et al., 1987), suggested an obligate pioneering function of the larval optic nerve. In *gl* mutants, however, the larval optic nerve fails to develop, yet retinal innervation of the optic lobes can proceed normally (Kunes et al., 1993; Moses et al., 1989). Thus, the larval optic nerve is likely to be dispensable for proper development of the adult visual system. In contrast, some of the other components of the larval visual system identified here might play a later role in organizing adult visual system development. Such an organizing function has been previously proposed for the OLPs (Tix et al., 1989) and their early differentiation is consistent with this proposal. The severe optic lobe defects in *disco* adults might then be attributed to the failure of these OLPs to differentiate. However, *disco* is more widely expressed in the optic lobe anlage, both in embryogenesis and during larval and pupal stages, and might thus be more directly required for adult optic lobe development. The glial cells described in these studies might also act to direct later visual system development. For example, they might be required for optic stalk formation, which is frequently absent in *disco* mutants. Analysis of the development of other visual system mutants may help resolve these questions.

General mechanisms for directing neural pathways and connectivity

Some of the cellular mechanisms we observe acting in visual system development may be analogous to phenomena which direct neuronal pathway formation in both invertebrate and vertebrate systems. We have observed that the formation of the larval optic nerve involves establishment of connections early in development, when the neuronal cell bodies and their targets are in close proximity. This might be expected to represent a very general strategy in nervous system development, as embryonic growth and remodeling can drastically alter the relationship between a neuron and its surroundings.

Our analysis of larval visual system development suggests that the establishment of the optic nerve pathway and connectivity follows an obligate sequence of cell interactions. In initial steps, an intermediate target is contacted and this interaction is correlated with changes in axonal behavior, including a reorientation in nerve growth. The mechanisms operating here might be very analogous to the guidepost cell phenomenon, which is most clearly defined in sensory neuron ingrowth in the grasshopper limb [(Bate, 1976; Bentley and Keshishian, 1982); reviewed in (Palka et al., 1992)]. As in the grasshopper limb, the intermediate targets described here are immature neurons. The larval optic nerve defects observed in *disco* may suggest that the OLPs are an necessary intermediate target, just as certain grasshopper limb guidepost cells are necessary for directing sensory neuron ingrowth (Bentley and Caudy, 1983). In both of these systems, loss of these intermediate targets is correlated with misdirected nerve growth, as opposed to a failure of axons to grow altogether. Similar intermediate targets acting during vertebrate nervous system development might include the floorplate of the neural tube, which seems to be required for proper orientation of commissural axons (Bernhardt et al., 1992; Bovolenta and Dodd, 1991), and the cortical subplate, where a transient population of neuronal cells may be required for proper development of thalamocortical projections (Ghosh et al., 1990) [see also reviews by (Dodd and Jessell, 1988; Goodman and Schatz, 1993; Hynes and Lander, 1992)].

Although the cellular mechanisms at work in these different systems may be conserved, little is known about the molecules responsible for the recognition and guidance function of any of these intermediate targets, although a few candidates have emerged (Klar et al., 1992; Kolodkin et al., 1992). A systematic analysis of mutants which affect larval visual system in *Drosophila* might provide insights into this phenomenon as well as offering potential approaches for identifying the relevant molecular players.

Materials and methods

Fly Stocks

All fly strains were maintained on standard cornmeal medium at 18°C or 25°C. The *fasII* enhancer trap, A31, was provided by C. Goodman. The enhancer trap A6-2-45 was provided by V. Hartenstein and Y. N. Jan. The *disco* enhancer trap strain C50.1S1 was provided by G. Gibson. All three enhancer trap strains were maintained and analyzed as homozygous stocks.

Immunohistochemistry

Immunohistochemical staining of embryos was performed essentially as previously described (Steller et al., 1987; Lee et al., 1991). Embryos were fixed in a 1:1 mixture of heptane and 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. at room temperature. The mAb22C10 (gift of S. Benzer) and the anti-Elav mAb44C11 (gift of Y. N. Jan) were used at a dilution of 1:3. The rat glial-specific antiserum RK2 (gift of A. Tomlinson) was used at a dilution of 1:200.

The rabbit polyclonal anti-Bsh antibody (gift of W. McGinnis) was preabsorbed at a dilution of 1:10 overnight at 4°C against 0-3 hr embryos, then used in staining reactions at a final dilution of 1:500. Affinity purified anti-Disco antibody (Lee et al., 1991)(Chapter 3) was used at a dilution of 1:3. ß-galactosidase expression was detected using a mouse anti-ßgal monoclonal antibody (Promega) at a dilution of 1:200 or an affinity purified rabbit anti-ßgal serum (Cappel) at a dilution of 1:500. Secondary antibodies (Rhodamine- and FITC-conjugated anti-rabbit Ig from Boehringer Mannheim and FITC-conjugated anti-mouse Ig from Cappel) were used at a dilution of 1:100. Cy3-conjugated anti-rat Ig (Jackson Immuno.) was used at a dilution of 1:200. All antibody incubations were done overnight at 4°C. Stained specimens were analyzed using a BioRad MRC600 laser scanning confocal microscope equipped with a krypton/argon laser. Data generated by confocal microscopy was processed using manufacturer's software and instructions.

Acknowledgments

We wish to thank Megan Grether and other members of the Steller lab for valuable discussions and critical reviews of this manuscript. We acknowledge Y. N. Jan, L. Jan, S. Benzer, C. Goodman, V. Hartenstein, A. Tomlinson, B. Jones, W. McGinnis and G. Gibson for antibodies and enhancer trap strains. We are grateful to Dave Smith for help with laser scanning confocal microscopy. This work was supported in part by NIH grant RO1-NS26451 and a Searle Scholars award to H.S. Confocal microscopy was performed in a facility supported by the Lucille P. Markey Charitable Trust. During the initial phase of this work, K.J.L. was supported by a National Science Foundation Fellowship. H.S. is an Associate Investigator of the Howard Hughes Medical Institute.

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Figure 6.1. Structure of the larval visual system at the end of embryogenesis.
The components of the larval visual system and their positions at the end of embryogenesis are illustrated schematically. The diagram shows a lateral view during embryonic stage 17 [staging based on (Campos-Ortega and Hartenstein, 1985)]. The larval photoreceptor cells are organized into two clusters on either side of the embryo and have axonal projections which constitute the larval optic nerve. Each nerve projects from the photoreceptor cluster straight back to the ipsilateral brain hemisphere, then turns ventrally to curve around the brain. As it follows the curvature of the brain, the nerve is associated with several glial cells. On the ventral side of the brain, in the region of the optic lobe primordium (show in gray), the nerve turns and enters the brain. Here, the nerve contacts the corner and central optic lobe pioneers (OLPs). These OLPs have axonal projections which fasciculate with the larval optic nerve. Together, these axons extend toward the central brain neuropil.

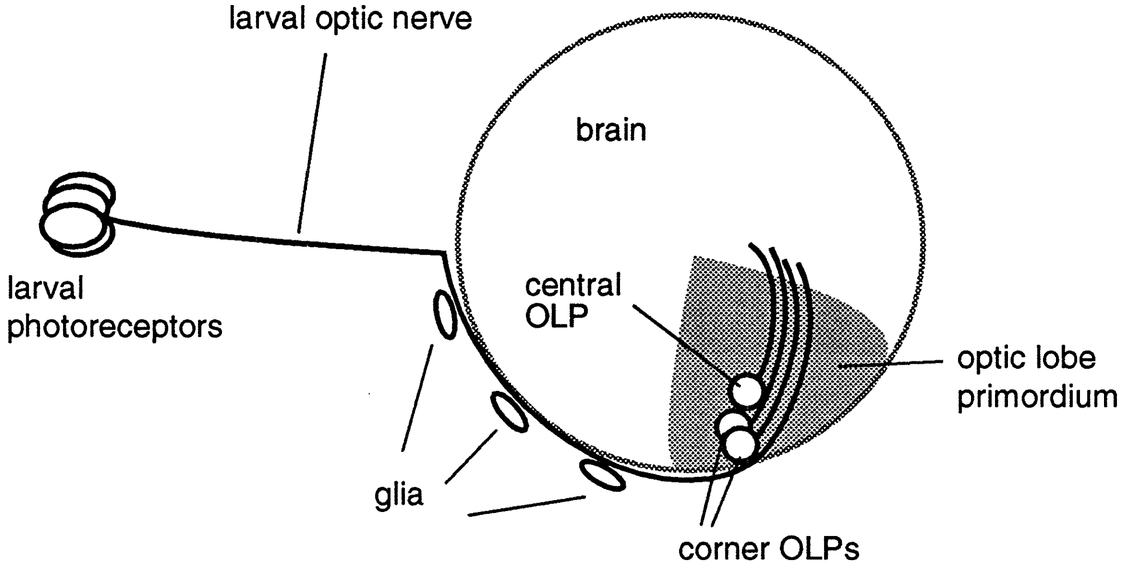

larval optic nerve
brain
larval
photoreceptors
central
OLP
optic lobe
primordium
glia
corner OLPs

Table 6.1. Markers used to identify components of the larval visual system. This table lists the markers used in this study and their expression in various components of the larval visual system. The cytoplasmic antigen recognized by mAb22C10 (Zipursky et al., 1984) is found in PNS neurons and a subset of CNS neurons. The enhancer trap A6-2-45 (Bier et al., 1989; Green et al., 1993) is an insertion near the *sine oculis (so)* gene (L. Zipursky, V. Hartenstein, personal communication), a homeodomain protein required for visual system development. The A31 enhancer trap is an insertion near the *fasciclinII (fasII)* gene (Grenningloh et al., 1991), which encodes a cell surface protein expressed on a subset of axonal pathways and in various CNS and PNS cells, as well as in a variety of different non-nervous tissues. The *disconnected (disco)* gene encodes a zinc-finger protein (Heilig et al., 1991), which is also expressed in a subset of CNS and PNS components, as well as in several non-neural tissues (Lee et al., 1991). The RK2 antibody recognizes a homeodomain protein specifically expressed in glia (A. Tomlinson, personal communication). The *elav* protein, recognized by the mAb44C11, is a nuclear protein specifically expressed in all neurons (Bier et al., 1988; Robinow and White, 1988; Robinow and White, 1991). The *brain specific homeobox (bsh)* protein is a homeodomain protein specifically expressed in a small subset of cells in the embryonic brain (Jones and McGinnis, 1993).

	larval photoreceptors	glia	central OLP	corner OLPs	optic lobe primordium
22C10	X		X	X	
A6-2-45 (so)	X	X	?	X	X
fas II	X	X		X	X (posterior)
disco		X	X	X	X (ventral)
RK2 (glial marker)		X			
elav (neuronal marker)	X		X	X	
bsh				X (1 of 2)	

Figure 6.2. The group of optic lobe pioneer (OLP) neurons is contacted by the larval optic nerve in late embryos.
Confocal photomicrographs of a late stage 16 wild type embryo stained with mAb22C10 (panels A-E), or an *A31* (*fasII* enhancer trap) embryo, of similar age, stained to detect *elav* (green) and ß-galactosidase (ßgal, red) expression (panel F). Cells which express both *elav* and ßgal in (F) are yellow. In this and all subsequent figures, anterior is oriented to the left and dorsal is up. (A) The larval photoreceptors (ph) extend axons which project back to the brain (br) and around to the ventral side where they turn and project through the optic lobe primordium (arrowhead). Panels (B-D) show higher magnification views of the optic lobe region in the embryo shown in (A). (B) In the optic lobe primordium, the photoreceptor axons contact the corner OLPs (solid arrows) and the central OLP cell (open arrow). (C-E) A series of focal planes, proceeding from superficial to medial. Each corner OLP cell (solid arrows) has a distinct axonal projection (arrowhead) which fasciculates with the bundle of photoreceptor axons (ph). This group of axons contacts the central OLP cell and fasciculates with its centripetal projection (arrowhead in E). All these fibers extend toward the central brain neuropil. (F) The optic lobe primordium, on the ventral side of the brain (br), is marked by ßgal expression in the A31 enhancer trap strain. In the optic lobe primordium, the OLPs (arrowhead) are the first neurons to differentiate, as indicated here by the appearance of *elav* immunoreactivity.

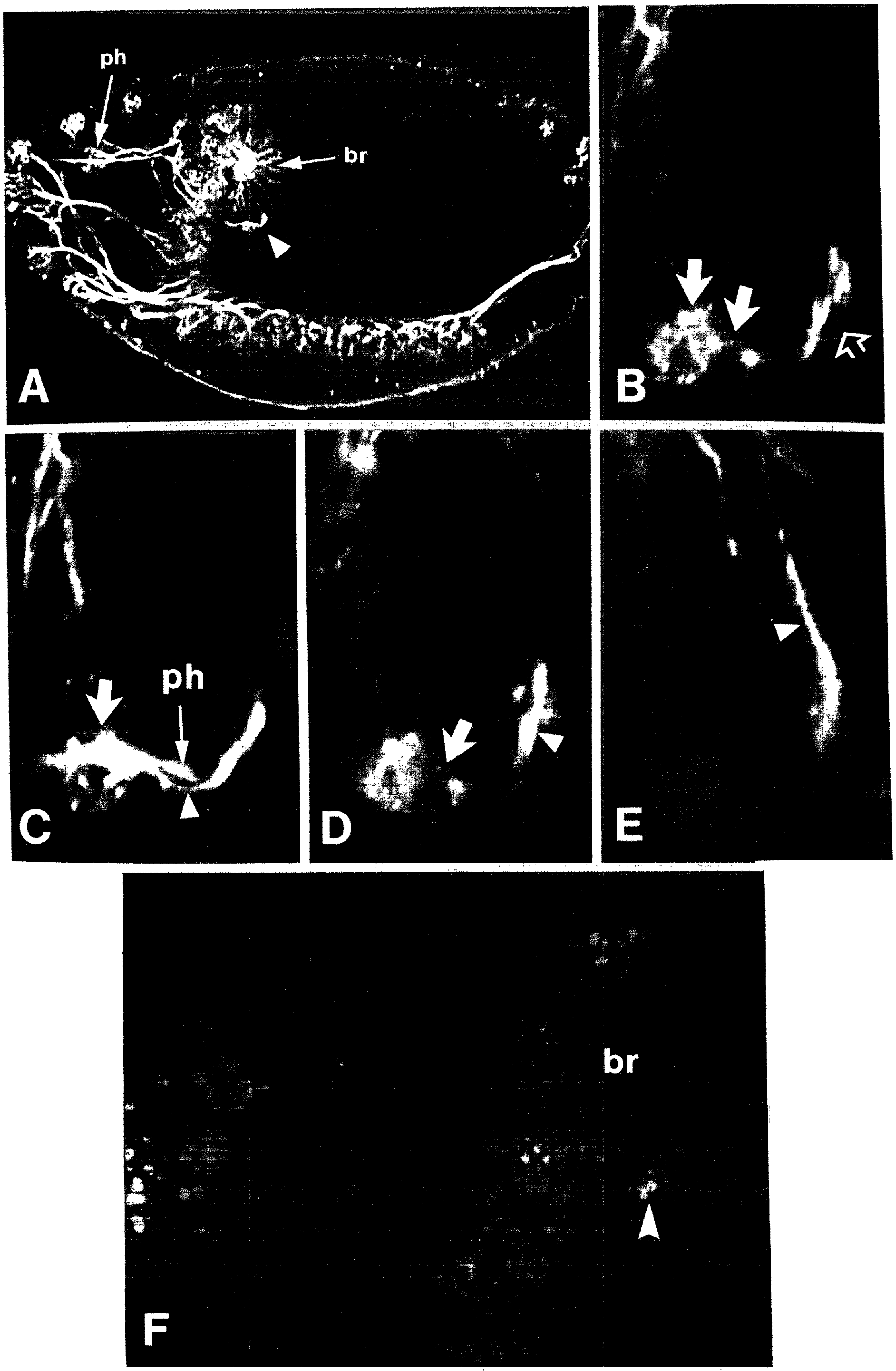

ph
br
A
B
ph
C
D
E
br
F

Figure 6.3. Cells of the larval visual system are marked by ßgal expression in the A6-2-45 (*so*) enhancer trap strain.

Confocal images of the anterior end of a stage 16 A6-2-45 embryo, stained with mAb22C10 (green) and anti-ßgal (red). Two focal planes are shown. The larval optic nerve extends from the photoreceptor cell cluster (ph) to the brain (br), where it curves around to the optic lobe primordium on the ventral side. As it extends around the brain, the nerve is associated with a number of glia (open triangles). The larval photoreceptors, the optic nerve glia and the cells of the optic lobe primordium, including the corner OLPs, all show reporter gene expression. At this stage, the terminus of the optic nerve is located in a cup of optic lobe primordium cells which surround a single large cell (arrowhead in A). This large cell is the *bsh*-expressing corner OLP (see Figure 6.4C,D).

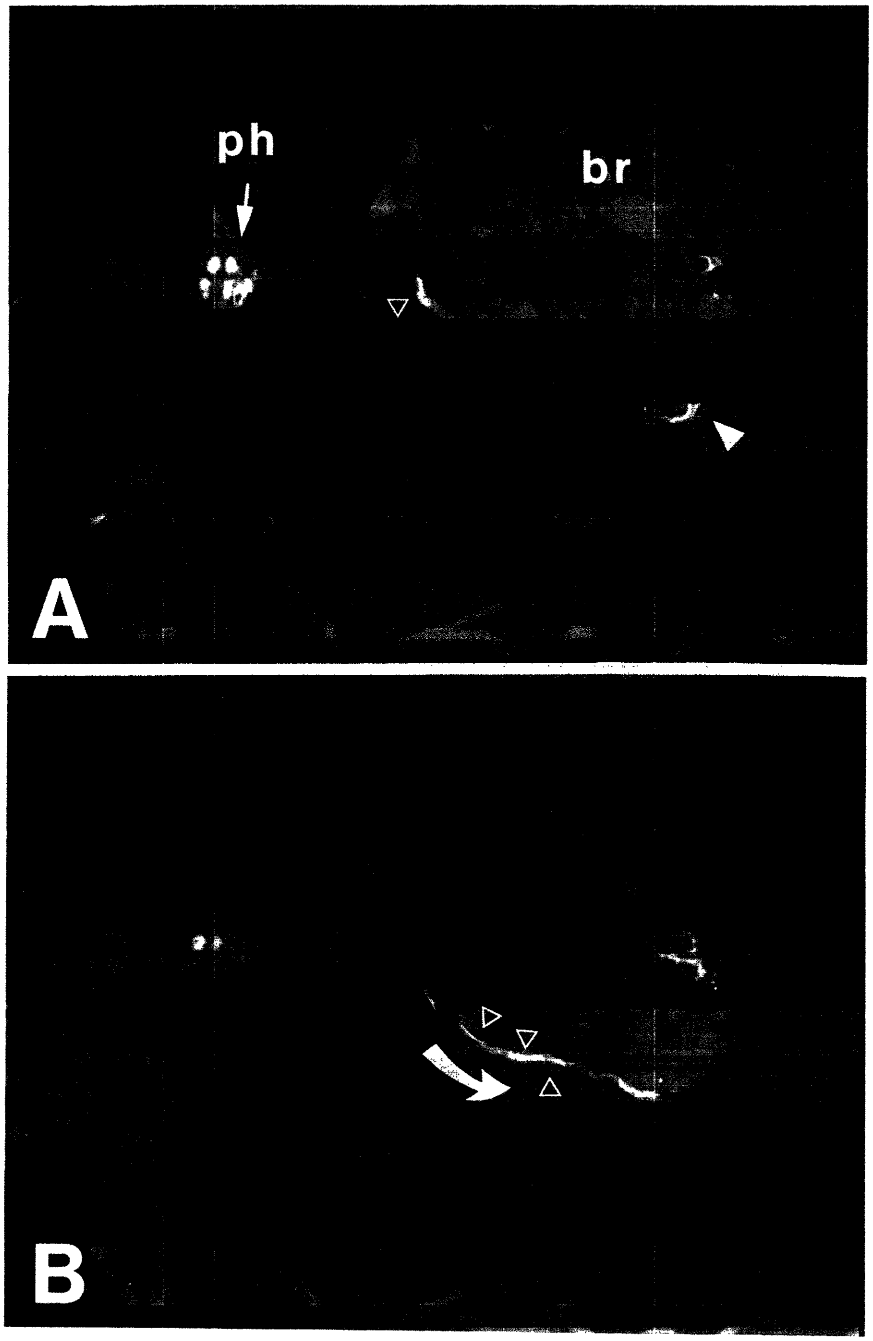

ph
br
A
B

Figure 6.4. The *bsh* gene is expressed in one of the two corner OLP cells. Confocal photomicrographs of a late stage 16 wild type embryo (A,B) and a slightly younger A31 (*fas II* enhancer trap) embryo (C,D). Panels (B) and (D) show higher magnification views of the optic lobe primordium region in the embryos shown in panels (A) and (C), respectively. In (A,B), mAb22C10 immunoreactivity is green, anti-Bsh staining is red. In panels (C,D), ßgal expression is shown in green, anti-Bsh is again red. Overlap of the two signals appears yellow. The larval photoreceptors are indicated in (A) with an open arrow. In the ventral side of the brain (br), the larval optic nerve contacts the two corner OLPs. The more posterior of these two neurons expresses *bsh* (arrows in A-D). This *bsh*-expressing cell is a characteristically large cell surrounded by a cup of optic lobe primordium cells (which show reporter gene expression in D). The *bsh* gene is also expressed in some clusters of cells in the more dorsal half of the brain hemisphere.

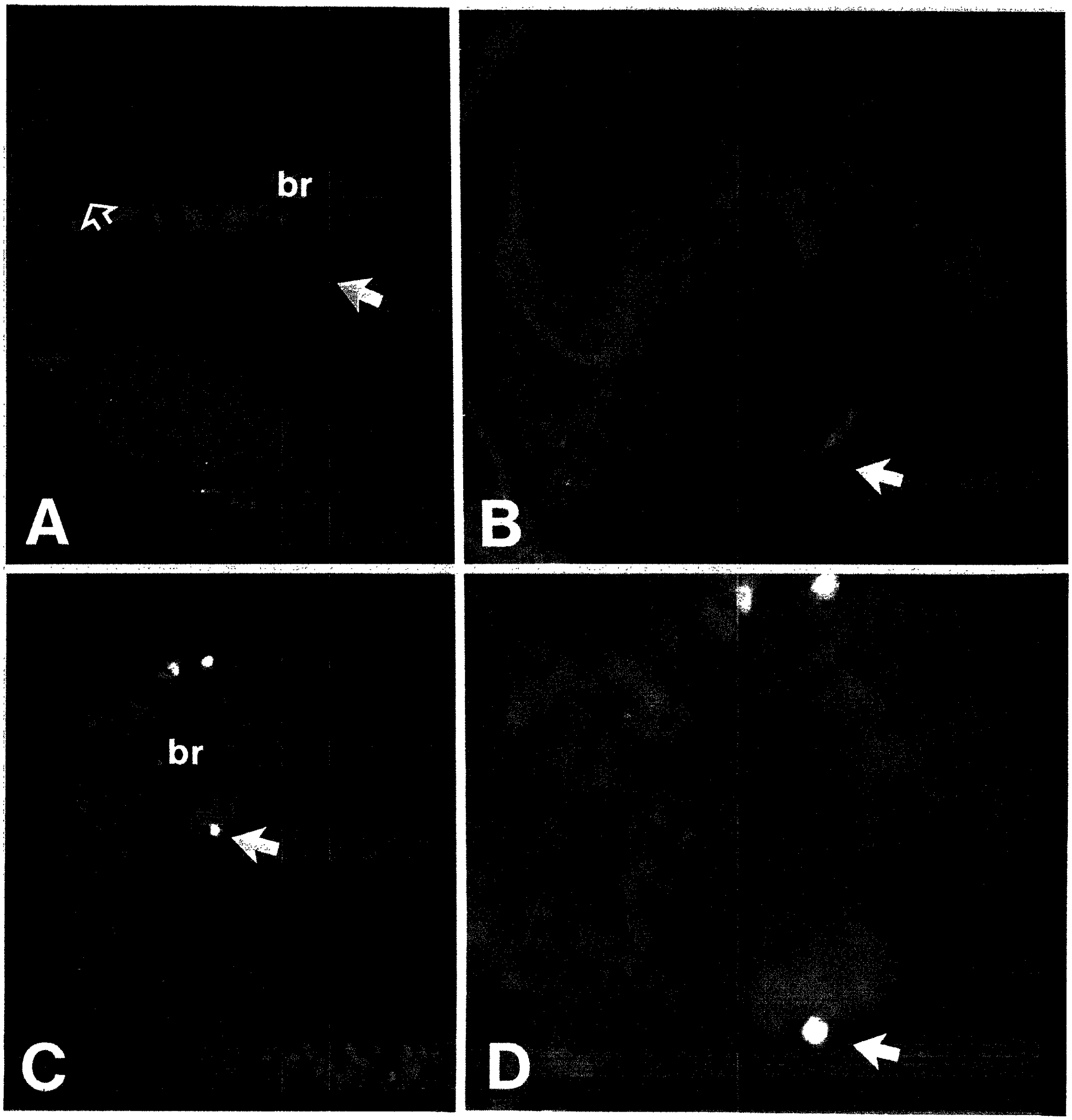

br
A
B
br
C
D

Figure 6.5. Embryonic development of the larval visual system. Confocal photomicrographs of A31 (*fasII* enhancer trap strain) embryos stained with mAb22C10 (green) and anti-ßgal (red). Panels (A-C) show three focal planes of a stage 13 embryo; Panels (D-F), three focal planes of a stage 15 embryo; Panels (G-I), three focal planes of a stage 16 embryo. The focal planes are arranged from superficial to more medial. In panels (A, D, H), the cluster of larval photoreceptors (ph) are indicated. The larval optic nerve (on), which extends from this cell cluster, is associated with a number of ßgal-expressing cells (arrowheads in B). Later in embryogenesis, ßgal-expressing cells which lie along the nerve show a characteristic glial cell morphology (arrowheads in D, G, H). The early optic nerve has a diffuse terminal region, which contacts adjacent cells in the optic lobe primordium (arrow in C). As the larval photoreceptor clusters and the optic lobe primordia separate during later development, the optic nerve terminus maintains contact with the optic lobe primordium (arrows in E, H, I). In (F), the central OLP cell is indicated. This cell lies outside the optic lobe primordium. The corner OLPs (small arrows in G) begin expressing mAb22C10 immunoreactivity during embryonic stage 16. During stage 16, the terminus of the larval optic nerve (arrow in I) projects deeper in the optic lobe primordium beyond the corner OLP cell bodies.

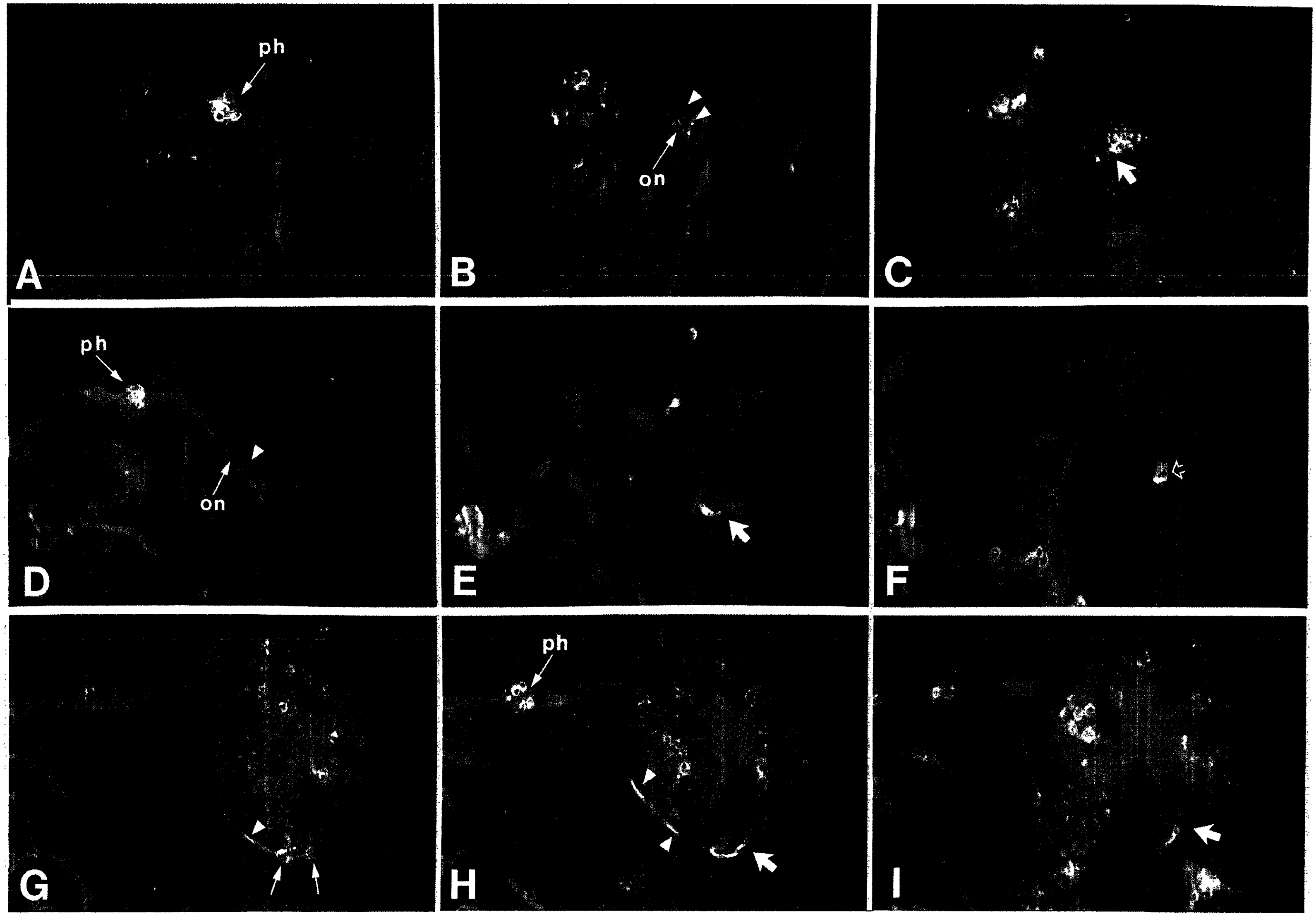
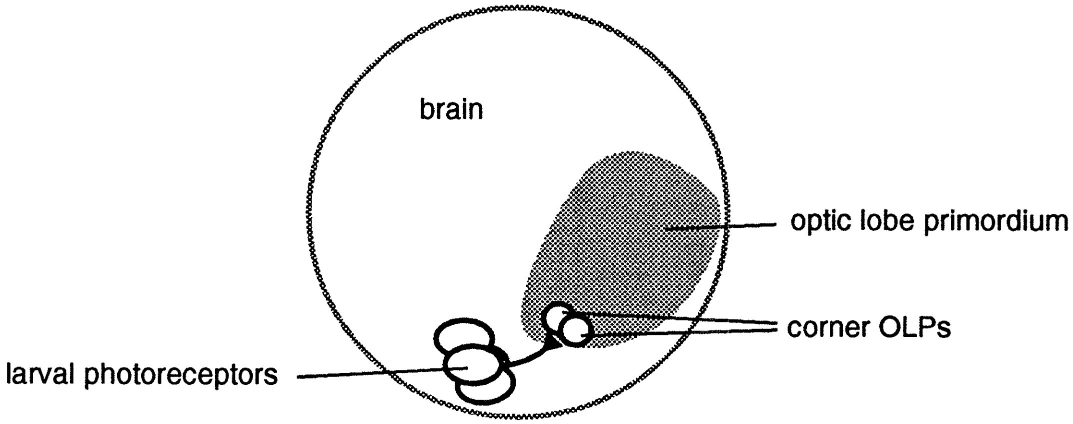
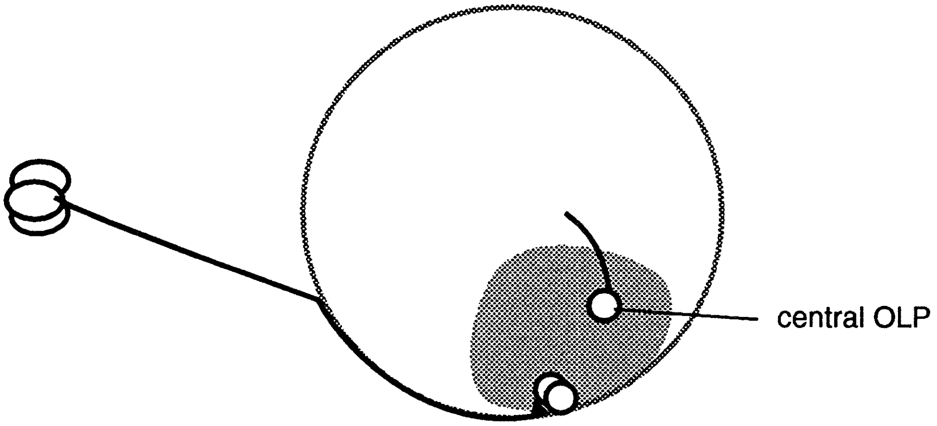

ph
A
on
B
C
ph
on
D
E
F
G
ph
H
I

Figure 6.6. Schematic diagram illustrating the sequence of events which occur during development of the larval visual system.
In an initial phase of development (embryonic stage 12 - 13), neuronal projections from the larval photoreceptors (which give rise to the larval optic nerve) contact cells in the adjacent optic lobe primordium. As illustrated here, these cells include the precursors of the corner OLPs. Later, the optic lobe primordium moves around and becomes incorporated in the ventral side of the brain hemisphere. Concomitantly, the larval photoreceptor cluster moves to a more anterior position. During these morphogenetic movements, the larval optic nerve elongates and its terminus maintains contact with the corner OLPs in the optic lobe primordium (stage 14 - 15). The central OLP cell differentiates outside the optic lobe primordium, and develops a centripetal projection toward the central brain neuropil. During later development, this cell descends into the optic lobe primordium. Finally (late stage 16 - 17), the photoreceptor axons and projections from the corner OLPs extend and contact the central OLP. These axons fasciculate with the centripetal projection of the central OLP and extend toward the central brain.

Phase 1 - initial contact


brain
optic lobe primordium
corner OLPs
larval photoreceptors

Phase 2 - axon elongation


central OLP

Phase 3 - final patterning - synaptic connection

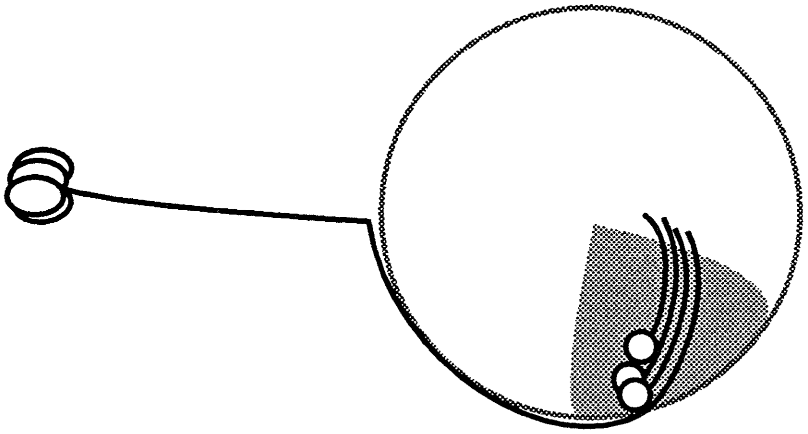


Figure 6.7. The corner and central OLPs are present and project normally in *glass* mutant embryos.

Confocal images of the optic lobe primordium in a gl^{60J} embryo (stage 17), stained with mAb22C10. Three focal planes, arranged from superficial to more medial, are shown. As before, anterior is to the left, dorsal is up. Although the larval optic nerve is not formed in *gl* mutant embryos, the corner OLPs (arrows in A) and the central OLP (open arrow in C) are positioned normally. In addition, the corner OLPs extend axons which project normally (curved arrows in A,B) and fasciculate with the centripetal process of the central OLP.

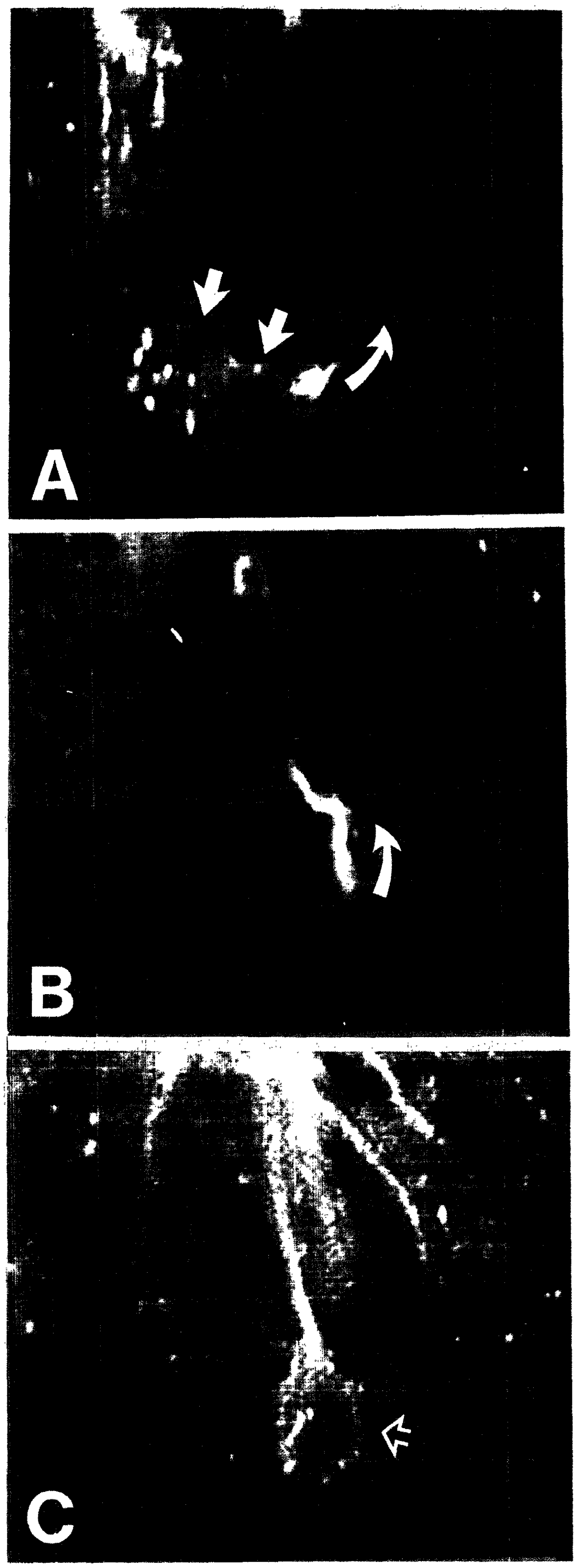
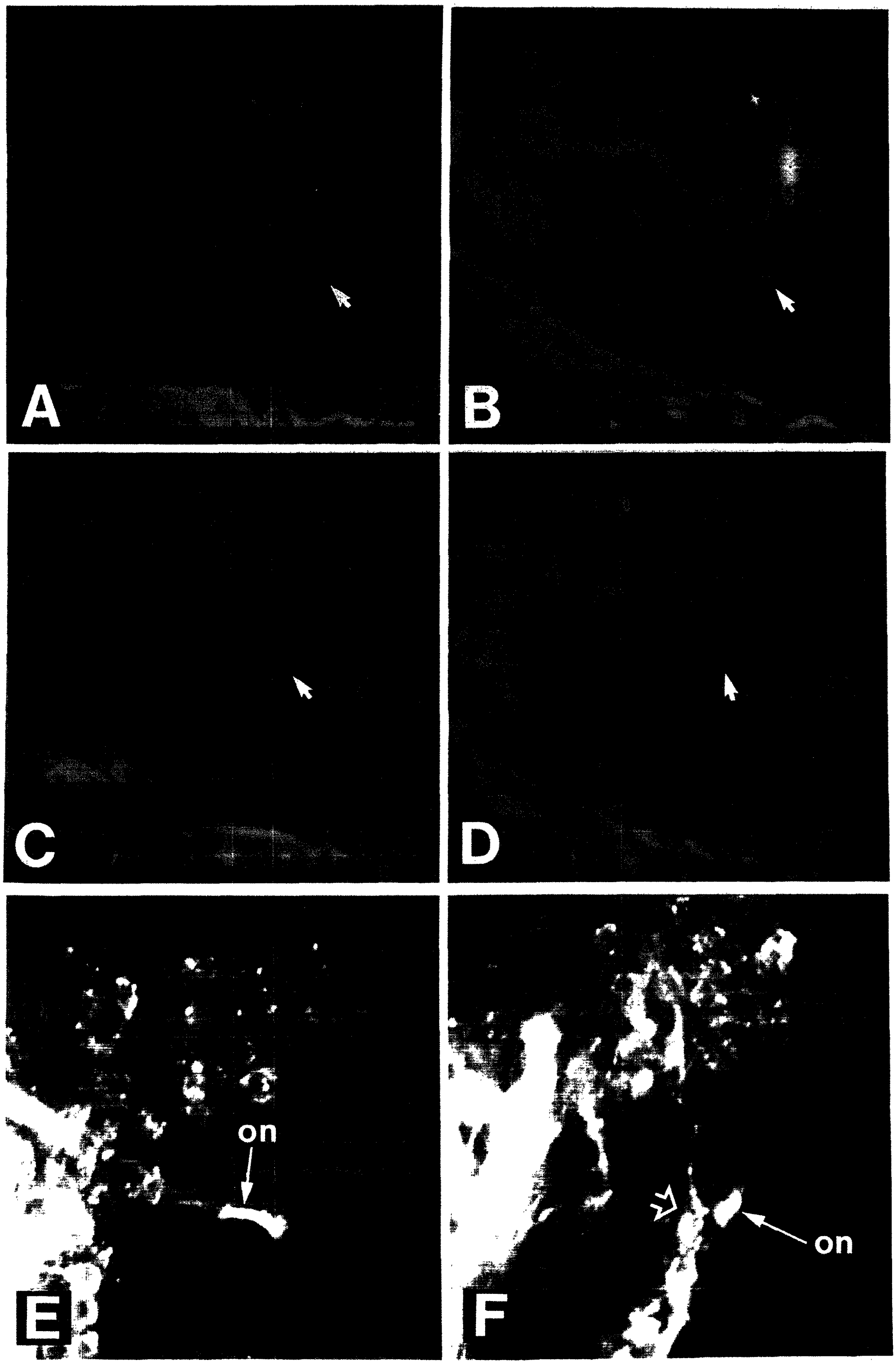

A
B
C

Figure 6.8. The corner OLPs fail to differentiate in *disco* mutant embryos. Confocal images of the anterior end of late stage 16 wild type (A) and *disco*^{1} (B-D) embryos, stained with mAb22C10 (green) and anti-Bsh (red). In wild type, *bsh* protein is found in the corner OLP cell contacted by the larval optic nerve (arrow in A). In *disco* mutant embryos, *bsh* expression is detected in clusters of cells in dorsal brain, but *bsh* expression is not found at optic nerve terminus whether the nerve contacts the optic lobe region (arrow in B) or projects ectopically (arrow in C). Irrespective of nerve positioning, *bsh* expression is never found in normal target location in ventral brain (arrow in D). In addition, staining with mAb22C10 does not detect either of the corner cells in *disco* mutants. We conclude that the corner OLPs fail to differentiate properly in the *disco* mutant. The central OLP cell, in contrast, is present in roughly 30% of *disco* mutant embryos. (E,F) Confocal images of the brain and optic lobe region of stage 17 *disco* embryo, stained with mAb22C10. E is a more superficial focal plane. In this embryo, the larval optic nerve (on) contacts the optic lobe primordium, and although the central OLP cell is present (open arrow in F), the axons of the larval optic nerve fail to fasciculate with this cell.


A
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on
E
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F

Figure 6.9. Larval optic nerve glia are present but display altered morphology in *disco* mutant embryos.

Confocal images of wild type (A) and *disco* mutant (B,C) embryos, stained with mAb22C10 (green) and the glial-specific antibody RK2 (red). In (A), the larval photoreceptor cell cluster is indicated with an arrow. The larval optic nerve in wild type embryos is associated with a number of glial cells (arrowheads) as it curves around the brain hemisphere. In *disco* embryos, these glia are present and still express the RK2 antigen, but they are less tightly associated with the optic nerve and they do not exhibit the characteristic elongated glial morphology (arrowheads in B,C).

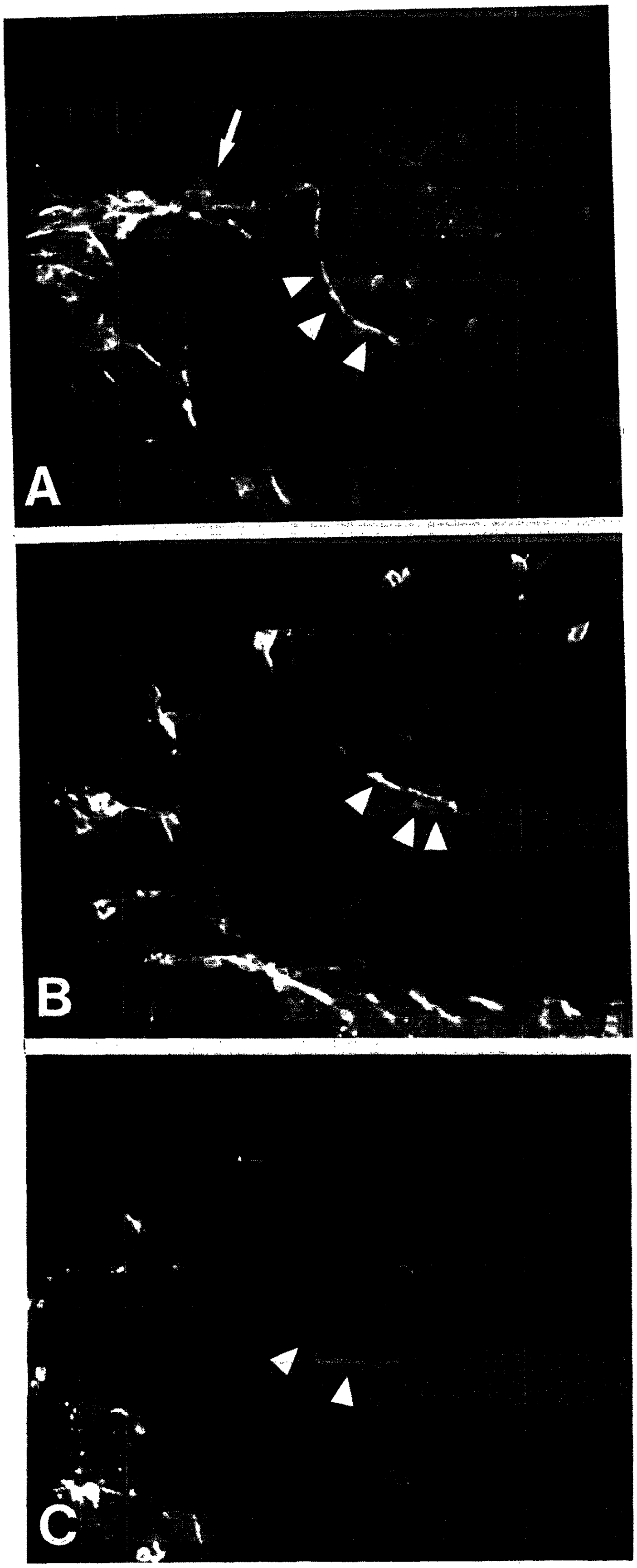
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Figure 6.10. Expression of *disco* in the early embryonic visual system. Confocal images of the anterior region of a wild type embryo. (A) mAb22C10 immunostaining in a superficial focal plane. The developing larval photoreceptors are indicated by the arrow. (B) *disco* expression in the same focal plane. Disco protein is not detected in the photoreceptor cell cluster. (C) Superimposition of the preceding images (mAb22C10 staining in green; anti-Disco in red). (D) mAb22C10 immunoreactivity in a more medial section, showing neuronal processes of the larval photoreceptors (arrow). (E) *disco* expression in the same focal plane. (F) Superimposition of the two images. Larval photoreceptor processes contact *disco*-expressing cells of the underlying optic lobe primordium (arrow).

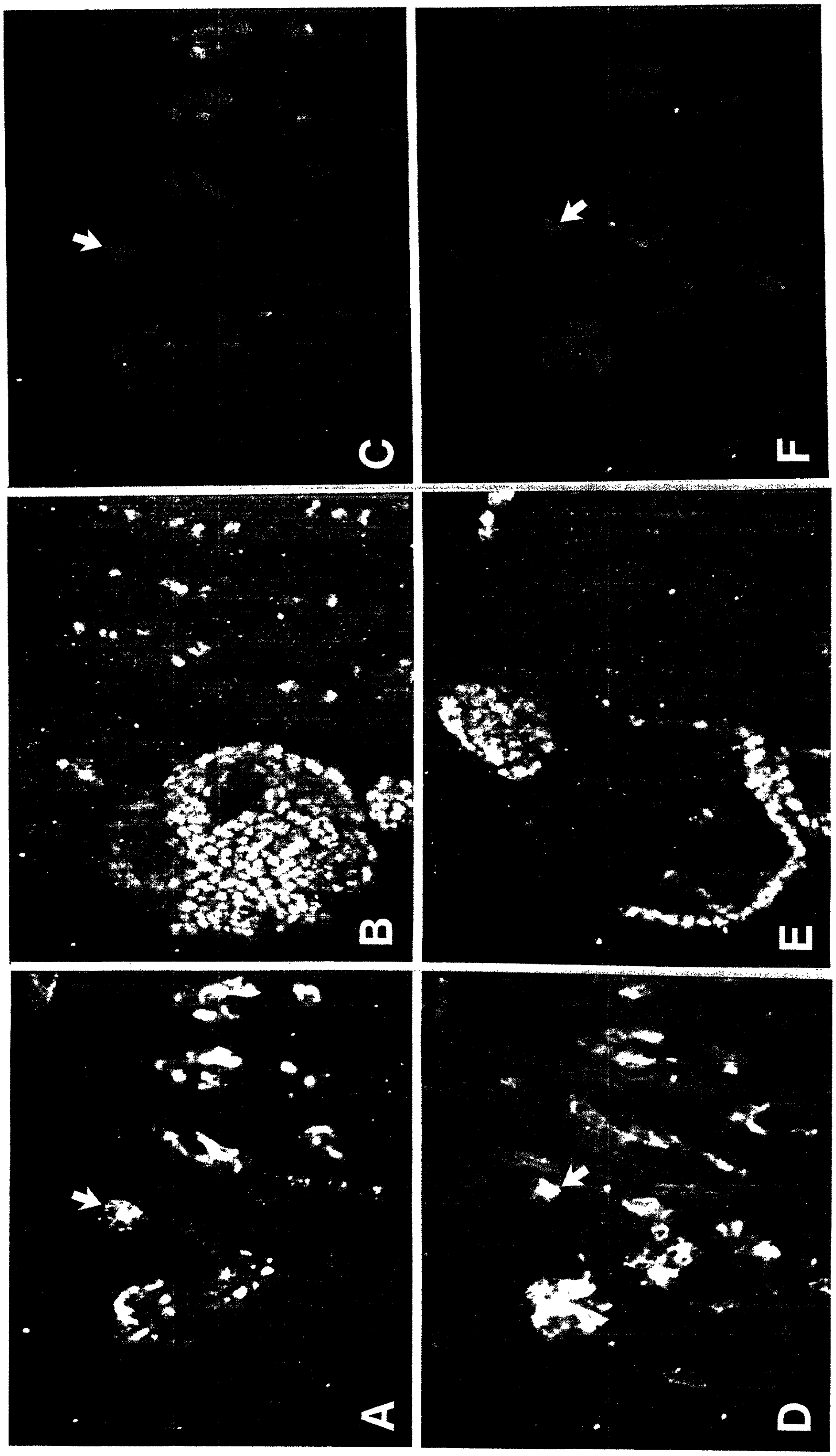
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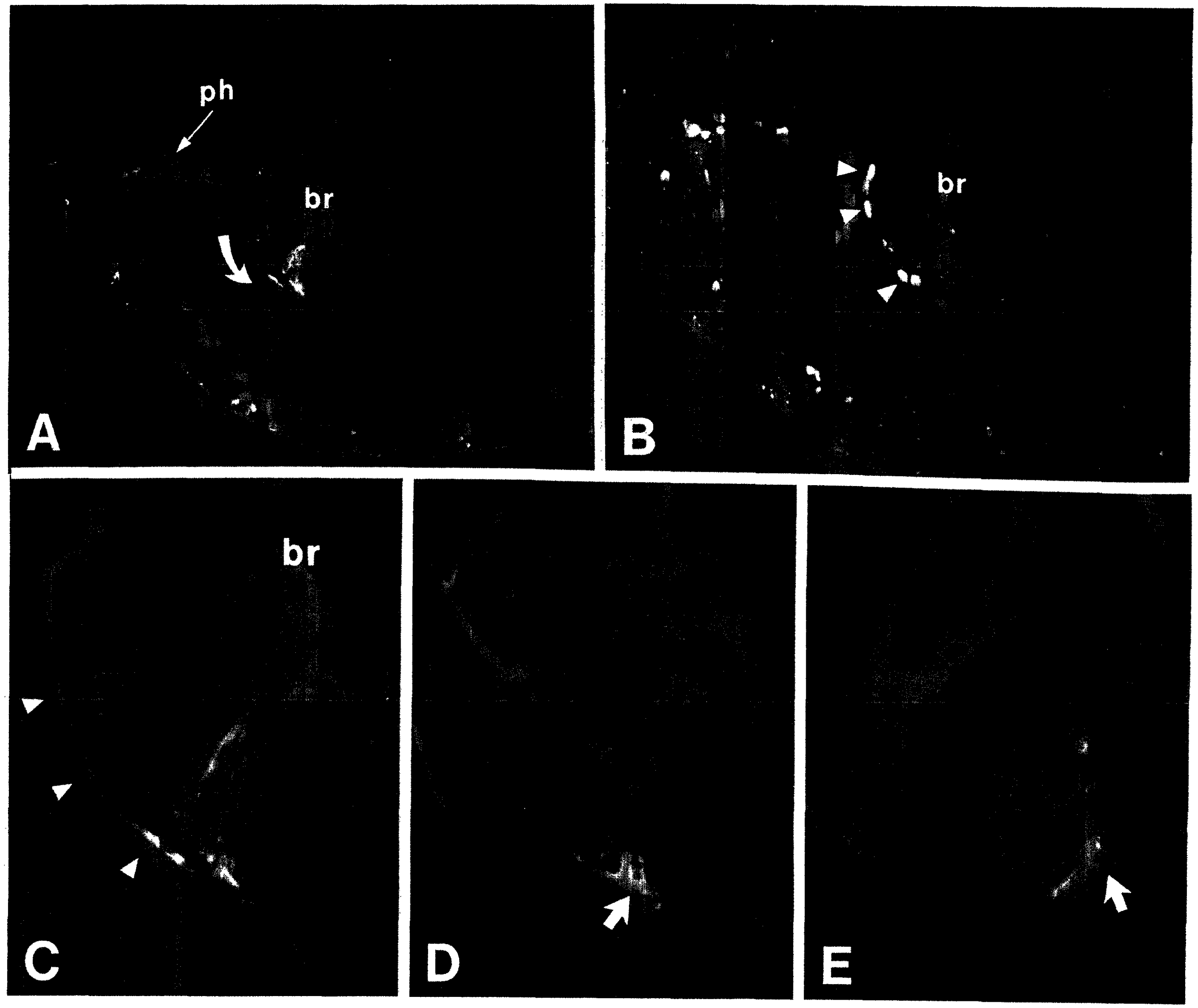
Figure 6.11. Expression of *disco* in the late embryonic visual system. (A) Confocal image of a C50.1S1 (*disco* enhancer trap strain) embryo (late stage 16), stained with mAb22C10 (green) and anti-ßgal (red). In this enhancer trap strain, ßgal is expressed in a pattern that is temporally and spatially identical to the pattern of *disco* expression (Cohen et al., 1991; Heilig et al., 1991). The reporter gene is not expressed in the larval photoreceptor cell clusters (ph). It is expressed in numerous cells contacted by the optic nerve, including optic nerve glia and cells in the optic lobe primordium. (B) Confocal image of a similarly aged C50.1S1 embryo, stained with the glial-specific antibody RK2 (green) and anti-ßgal (red). The RK2 antigen and *disco* are co-expressed in the glia which lie along the optic nerve pathway (arrowheads). (C-E) Higher magnification confocal images of the optic lobe primordium region in the embryo shown in (A). As above, mAb22C10 immunoreactivity appears green, anti-ßgal is red. (C) Reporter gene expression in glia (arrowheads) along the nerve pathway. (D) A more medial focal plane showing reporter gene expression in the corner OLPs (arrow). (E) A further medial focal plane, showing reporter gene expression in the central OLP (arrow).


ph
br
A
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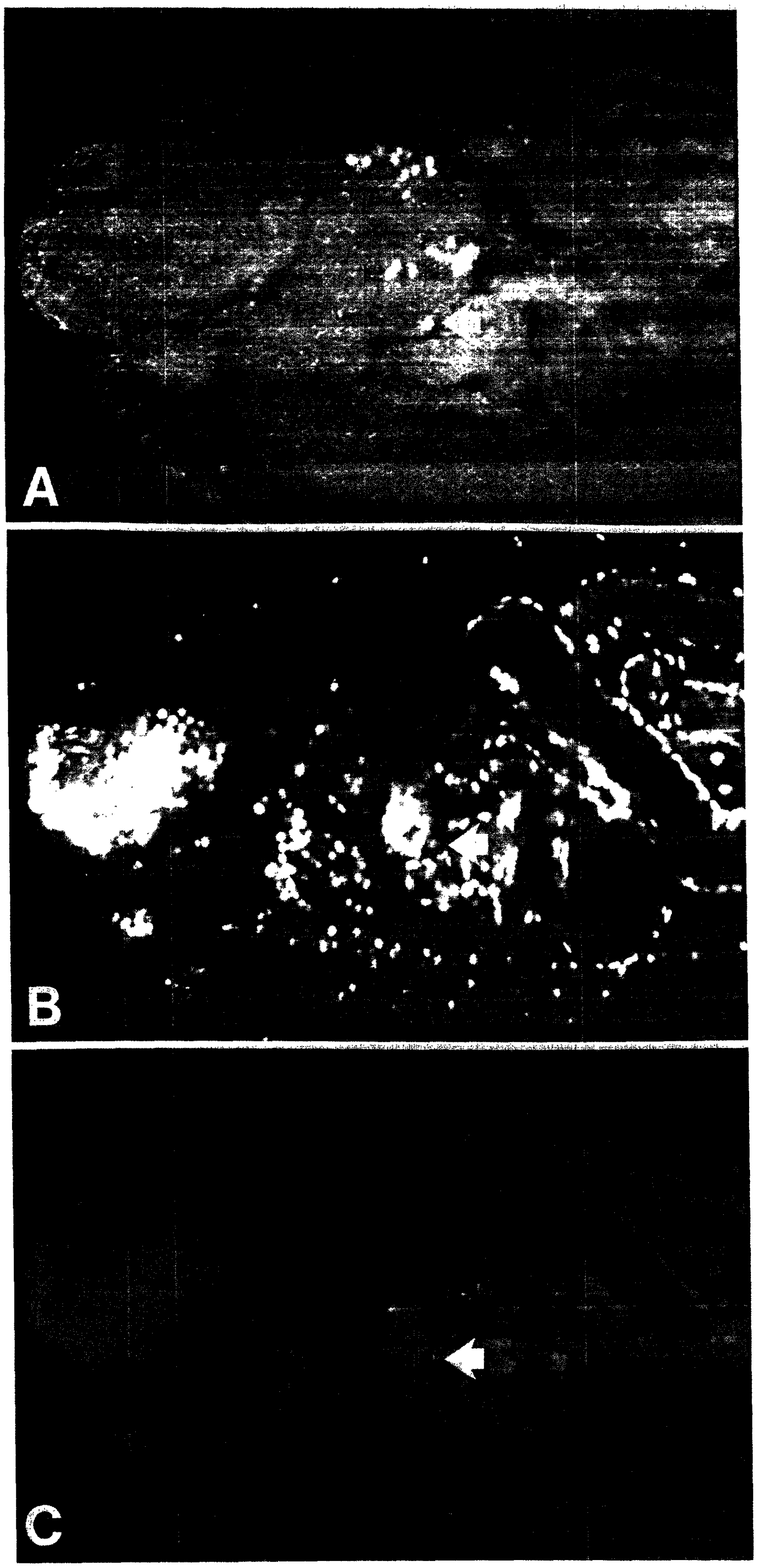
E

Figure 6.12. *Disco* and *bsh* are co-expressed in one of the corner OLPs. Confocal images of a late stage 16 C50.1S1 (*disco* enhancer trap) embryo, stained with anti-Bsh and anti-ßgal. (A) Anti-Bsh immunoreactivity is detected in a single cell in the ventral brain, the posterior corner OLP (arrow). (B) reporter gene expression in the same focal plane. (C) Superimposition of the preceding images (anti-ßgal in green, anti-Bsh in red). Both *disco* and *bsh* are expressed in the posterior corner OLP cell (arrow).


A
B
C

Chapter 7.
Conclusions and Future Prospects

The studies presented in this thesis have addressed the role of the *disco* gene product in the establishment of neuronal connectivity in the *Drosophila* visual system. This analysis has demonstrated that *disco* encodes a nuclear protein with zinc finger motifs. The significance of this zinc finger domain is demonstrated by the conservation of these sequences in evolution and the observation that mutations in critical zinc finger residues result in loss of *disco* function. These observations, taken together, suggest that the *disco* gene product is a protein which acts as a regulator of gene expression, possibly a transcription factor. Consistent with this hypothesis, these studies show that the *disco* protein has sequence-specific DNA binding activity in vitro. Moreover, *disco* function regulates *disco* expression in an autoregulatory feedback loop. The data presented here also suggest that this autoactivation may occur by direct interaction of the *disco* protein with putative cis-acting elements in the *disco* promoter.

As a transcriptional regulatory protein, the *disco* protein may regulate molecules involved in pathway and target recognition during larval optic nerve development. At present, these putative targets of *disco* regulation remain unknown. In principle, it may be possible to identify these targets by genetic screens, for example, in screens for enhancers of the *disco* mutant phenotype. Alternately, biochemical methods, employing immunoprecipitation or similar techniques [see, for example, (Gould et al., 1990; Payre and Vincent, 1991)], may be used. Additional approaches include expressing *disco* in the larval salivary gland and examining protein binding to sites along the polytene chromosome (Zink and Paro, 1989), or identifying enhancer trap strains (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989) with *disco*-dependent reporter gene expression.

The studies presented in this thesis have identified a few markers whose expression depends on *disco* activity. One such marker is the product of the *bsh* gene, which is expressed in an identified neuron contacted by the developing larval optic nerve. Expression of *bsh* in this cell depends on *disco* function. This dependence on *disco* is specific to this cell; *bsh* expression in some 30 other cells in the brain hemisphere appears to be unaffected by *disco* mutation. Is *disco* directly regulating *bsh* in this cell? This question may be approached by analyzing the *bsh* promoter to identify cis-acting elements which mediate *disco*-dependent gene expression. These cis-acting sequences could be tested for *disco* protein binding *in vitro*. In addition, it is possible to determine whether ectopic expression of *disco* might affect *bsh* expression. This question can be addressed by examining *bsh* expression in *hs-disco* (Chapter 4) embryos.

Both autoregulation of *disco* expression and the putative activation of *bsh* expression by *disco* activity are highly cell-type specific. This observation suggests that other factors which are restricted to certain cell types interact with the *disco* gene product to promote this regulatory activity. The interaction with these other factors may affect the DNA binding specificity or affinity of the *disco* protein or it may influence promoter activation following binding. Possible approaches which may be used to identify interacting factors include genetic screens, immunoprecipitation assays and so-called "interaction trap" methodologies (Fields and Song, 1989; Zervos et al., 1993).

The data presented in this thesis are consistent with a model in which *disco* acts in cells contacted by the developing larval optic nerve to direct neuronal connectivity and pathway formation. These cells include the group of optic lobe pioneer neurons (OLPs) and a small number of glia. In order to determine whether the focus of the *disco* mutation indeed corresponds to these cells, a high resolution genetic mosaic analysis might be undertaken. These studies would rely on a cell-autonomous marker to assay cell genotype directly and a method to reliably generate mosaics early in development. Another approach which might prove useful is to express the *disco* gene in identified cells using heterologous promoters [see, for example, (Brand and Perrimon, 1993)]. The *disco* gene may be specifically expressed in the larval photoreceptors, for example, using an identified element from the *Krüppel (Kr)* promoter (Schmucker et al., 1992). Another potentially useful approach is to attempt to phenocopy the *disco* mutation by selective ablation of identified cells, for example, the OLPs or the larval optic nerve glia. This might be achieved by using cell-type specific promoters to drive toxin gene expression (Kunes and Steller, 1991). These proposed studies may also serve to determine whether *disco* is required in the optic lobe post-embryonically for proper visual system development. In addition, these experiments may elucidate the causes of defects in optic stalk formation and adult photoreceptor innervation found in *disco* mutants.

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Appendix A. Evolutionary conservation of the *disco* gene: Analysis of the *Drosophila virilis disco* homolog

Summary

The conservation of amino acid sequences in homologous proteins of distantly related species is likely indicate the presence of functionally significant domains. To determine which regions of the *disco* protein are highly conserved and are thus candidates for functionally significant domains, we cloned the *disco* gene from *Drosophila virilis.* This fly species is separated from *Drosophila melanogaster* by roughly 65 million years of evolution (Beverley and Wilson, 1984). An examination of the *D. virilis* gene shows that the *disco* zinc finger region, together with sequences located just on N-terminal side of the zinc fingers, are extremely highly conserved. Over a stretch of 84 amino acids in this region of the protein, all but two residues are identical.

Materials and Methods

Pairs of oligonucleotide primers corresponding to sequences separated by roughly 300 nt in the *D. melanogaster disco* gene were synthesized. Approximately 0.5 μg of *D. virilis* genomic DNA was amplified by PCR using various pairs of these primers (0.5 μg of each) in a 100 μl reaction containing 1X standard PCR buffer (Perkin-Elmer Cetus), 0.2 mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.2 mM TTP, and 2.5 U Taq polymerase (Perkin-Elmer Cetus). The reaction was subjected to 30 cycles of 95°C for 30 sec, 54°C for 1 min, 72°C for 45 sec (increased by 2 sec after each cycle), followed by an additional incubation at 72°C for 10 min. Of the several pairs of primers tested, only one set gave a reaction product of the expected size. These primers were (see also Figure A.1):

disco5B: 5'-GGGAATTCACCGGCTTATCTGCTGGG-3'
disco3B: 5'-GGGAATTCTTGAAGCAAATGGAGCACTG-3'

The reaction product was isolated by preparative gel electrophoresis, cloned into M13 and sequenced with the ^{35}S dideoxy chain termination method using Sequenase (US Biochem.).

Additional flanking sequences were obtained by "inverse PCR" as follows: Roughly 2 μg of *D. virilis* genomic DNA was digested to completion with PvuII and HincII, extracted with phenol/chloroform-isoamyl alcohol and precipitated with ethanol. The digested DNA was circularized by incubation at

14°C for 18 hr in a 1 ml reaction containing 1600 U T4 DNA ligase (New England Biolabs) and 1X ligase reaction buffer (New England Biolabs). This reaction was then precipitated with ethanol and one-third was amplified using PCR. The PCR was performed exactly as described above, using 1 μg of each of the following primers:

virkev1: 5'-GAATTCAATTTAGCGGGTCAGTTCATCA-3'
virinv: 5'-GGGAATTCGGATGGGCATGCGGGTGTC-3'

This reaction yielded a DNA species of approximately 550nt with an internal EcoRI site. A 150nt fragment of this product (from the internal EcoRI site to the virinv primer) was cloned into Bluescript and sequenced using Sequenase as above. Additional sequence corresponding to the region of this PCR product extending from the virkev1 primer was obtained by direct sequencing of the PCR product using materials and protocols supplied in the Circumvent kit (New England Biolabs). For this reaction, the following primer (labelled by kinase reaction with ^{33}P-ATP) was used:

virdisco5A: 5'-GGGAATTCCGGCGACGGGCAAGAAGCG-3'

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Figure A.1 Partial sequence of the *D.virilis disco* gene.
The DNA sequence shown here is a composite of fragments obtained by PCR and "inverse PCR". The positions of primers used in PCR reactions are indicated by lines above (those which hybridize to the sense strand) and below (those which hybridize to the antisense strand) the DNA sequence. Refer to the text for details of these PCR reactions. The predicted amino acid sequence which corresponds to this DNA sequence is also given (in single letter code). The predicted translation start is the methionine residue which is specified by the codon beginning with nucleotide 87. On the 5' side of this codon, there is little similarity between the *D. virilis* and *D. melanogaster* sequences.

```
  1  GAATTCCTTAGTATTTCACACACACATGCAGACAATTTTCTAATCAGCGACTTTTTCCAT  60
      I  P  *  Y  F  T  H  T  C  R  Q  F  S  N  Q  R  L  F  P  F

 61  TTCGTTTGTAGCATTTGCCCAAGACAATGGAGCACATTATGAATCCGTTCATGCGCCGTT  120
      R  L  *  H  L  P  K  T  M  E  H  I  M  N  P  F  M  R  R  Y

     disco5B  ______virinv________                                      .
121  ATCTGCTGGGACACCCGCATGCCCATCCGCATTCGCACATGGGCCAGACCGCCAAATCGC  180
      L  L  G  H  P  H  A  H  P  H  S  H  M  G  Q  T  A  K  S  P

181  CAGCCAGCTCGCCGGGCACAGCGGGTGCAACAGGAACTGGCACTGGAGCTGGAGCTGGAA  240
      A  S  S  P  G  T  A  G  A  T  G  T  G  T  G  A  G  A  G  T

241  CTGGCTGCGGCACGGGTAAGGGCAGCTCCAGTTCACTTGCGAGCCATGCGAAGCCAAAGC  300
      G  C  G  T  G  K  G  S  S  S  S  L  A  S  H  A  K  P  K  R

                             virkev1                 virdisco5A
301  GCTGGGGCTCGCCGCCGATCAATTTAGCGGTCAGTTCATCAATCCGGCGACGGGCAAGA  360
      W  G  S  P  P  I  N  L  A  G  Q  F  I  N  P  A  T  G  K  K

             ______disco3B_________                                     .
361  AGCGTGTCCAGTGCTCGATTTGCTTCAAGACCTTCTGCGACAAGGGCGCGCTCAAGATCC  420
      R  V  Q  C  S  I  C  F  K  T  F  C  D  K  G  A  L  K  I  H

421  ATTTCTCGGCGGTGCATCTGCGCGAGATGCACAAGTGCACCGTGGATGGCTGCAACATGG  480
      F  S  A  V  H  L  R  E  M  H  K  C  T  V  D  G  C  N  M  V

481  TGTTCAGCTCAAGACGTTCACGGAATCGGCACAGCGCCAATCCGAATCCAAAGCTCCATT  540
      F  S  S  R  R  S  R  N  R  H  S  A  N  P  N  P  K  L  H  S

541  CG  542
```

Figure A.2 Comparison of the *D. virilis* and *D. melanogaster disco* sequences.

The predicted amino acid sequences of the *D. melanogaster* and *D. virilis disco* genes were aligned using the programs supplied with the University of Wisconsin GCG sequence analysis package. Residues which are identical are indicated by a vertical line; conservative substitutions are indicated with a dot. The zinc finger domains are indicated by the unshaded boxes. Both zinc finger domains, the intervening amino acids and a stretch of 27 residues which lie on the N-terminal side of the zinc fingers are extremely well conserved. Note also the highly conserved Spaß-box at positions 49-54 (shaded box).

```
                      10               30               50
                           .         .         .         .         .
D. melanogaster   MEHIMNPFMSPAYLLGHGPHSHQHVHSHLPSHPQPNAASPASSPGGSSGS
                  |||||||||   ||||   | | | |||.       | |||||||  ..
D. virilis        MEHIMNPFMRR.YLLG...HPHAHPHSHMG....QTAKSPASSPGTAGAT

                                    70                  90
                           .         .         .         .         .
D. melanogaster   GSGSAAGSGTGSG......SSL....KPRRWGSPPINLAGQFINPATGKK
                  | | .|| |||.|      |||    ||.|||||||||||||||||||||
D. virilis        GTGTGAGAGTGCGTGKGSSSSLASHAKPKRWGSPPINLAGQFINPATGKK

                         110                 130                 150
                           .         .         .         .         .
D. melanogaster   RVQCSICFKTFCDKGALKIHFSAVHLREMHKCTVEGCNMVFSSRRSRNRH
                  ||||||||||||||||||||||||||||||||||.|||||||||||||||
D. virilis        RVQCSICFKTFCDKGALKIHFSAVHLREMHKCTVDGCNMVFSSRRSRNRH

                                   170                 190
                           .         .         .         .         .
D. melanogaster   SANPNPKLHSPHIRRKISPHDGRTAQQFPVFSPGTAAAAAAVAGRLPVAF
                  ||||||||||
D. virilis        SANPNPKLHS
```

Appendix B. Expression of the *disco* gene in the adult *Drosophila* brain

Summary

As described in Chapter 2, the *disco* gene is transcribed at a low, but detectable level in the adult head. Chapter 3 examines *disco* expression in embryonic and larval stages. Here we analyze reporter gene expression in a *disco* enhancer trap strain in order to characterize the spatial pattern of *disco* expression in the adult head. We find that the gene is expressed in portions of the lobula complex and in a region at the medial margin of the medulla cortex. Expression of *disco* in this latter region may provide some clues as to the etiology of circadian rhythm defects in *disco* mutants. In addition, these results suggest that some of the defects in the *disco* adult optic lobe may result from an autonomous requirement for the gene in these tissues.

Results

Attempts to detect *disco* expression in the adult head by immunohistochemical staining of cryostat sections with a anti-Disco rabbit polyclonal serum (see Chapter 3) were unsuccessful. Immunohistochemical staining of cryostat-sectioned larval tissue was similarly negative, but as whole mount staining of larvae demonstrates that *disco* is expressed in numerous larval tissues, including the brain, we reasoned that the *disco* epitope may not be recognized by our antiserum following cryostat treatment. Whole-mount staining of adult brains with the anti-disco antiserum suggested that *disco* was expressed in some portions of the optic lobes, but exact details of the expression pattern were difficult to determine in these preparations. For these reasons, we analyzed the pattern of *disco* expression in the head using the enhancer trap line C50.1S1 [(Cohen et al., 1991); see also Chapters 2, 3 and 6]. During embryonic and larval stages, the spatial and temporal pattern of expression of the C50.1S1 reporter gene is identical to the *disco* expression pattern. Thus, it is likely that the pattern of reporter gene expression in the adult head is reflective of the *disco* expression pattern in this tissue. This proposal is supported by the similarity between the *disco* expression pattern in the larval brain (Chapter 3) and the pattern of reporter gene expression in the adult brain.

Figure B.1 shows expression of the C50.1S1 reporter gene in horizontal cryostat sections of the adult head. The ßgal reporter gene is expressed in parts of the lobula complex and in cells at the medial margin of the medulla, at the border with the central brain. In more dorsal sections, the reporter gene is expressed in cells just beneath the retina. These cells may comprise the dorsal-most portion of the medulla cortex. Finally we also detect expression in the antennae and in three clusters beneath the dorsal ocelli. Reporter gene expression is not found in the compound eye or the lamina.

Discussion

Typically, flies which are mutant for the *disco* gene show drastically reduced optic lobes. These optic lobe defects result, at least in part, from the failure of retinal fibers to innervate the optic lobe anlagen during larval and pupal life. However, in some *disco* animals (the "connected" class) (Steller et al., 1987), in which retinal innervation is not disrupted, optic lobe stucture is still quite disorganized. This disorganization may result from an additional autonomous requirement for *disco* gene function during development of the adult optic lobes. The finding that *disco* is expressed in a portion of the optic lobes during embryonic, larval and even adult stages, lends support to this proposal.

We have detected reporter gene expression at the medial margin of the medulla, a region which contains the medulla tangential neurons (Fischbach and Dittrich, 1989). Interestingly, some reports have indicated that these medulla tangential neurons are still present in the rudimentary optic lobe found in *disco* mutant adults (Fischbach and Heisenberg, 1984). However, identification of these cells is made difficult by the disorganized structure of the mutant brain and the lack of normal landmarks.

This region of the brain and optic lobe at the medial margin of the medulla may be important for the control of circadian rhythms. The gene *period (per)*, which is thought to play a central role in the functioning of the circadian pacemaker [reviewed in (Rosbash and Hall, 1989)], is expressed in a subset of glia and neurons in the adult brain (Ewer et al., 1992; Zerr et al., 1990). Expression of *per* in neurons at the medial margin of the medulla is required for normal circadian rhythms, although mosaic animals which lack *per* expression in this region exhibit some weak rhythmicity (Ewer et al., 1992). In addition, *per* expression in this tissue is largely absent in *disco* mutants (Zerr et al., 1990), which are also defective for circadian rhythmicity (Dushay et al., 1989; Hardin et al., 1992). It is at present unclear whether the change in *per* expression in *disco* mutants results from a lack or mispositioning of the relevant cells.

Expression of *per* in other regions of the *disco* brain is roughly normal. In addition, *per* mRNA and protein undergo daily fluctuations in *disco* brains as in wild type (Hardin, et al., 1992). This result indicates that the circadian pacemaker itself is not disrupted in *disco* mutants; rather, the rhythm defects of *disco* mutants result from a failure in the circadian pacemaker output pathway. The *per*-expressing neurons at the medial margin of the medulla might therefore relay information from the circadian pacemaker to effector systems which control behavior and locomotor activity in response to this pacemaker output.

What is the nature of the change in *per* expression in *disco* mutants? The identification of appropriate cell markers would facilitate studies to determine whether the cells which normally express *per* are missing or mislocated in the *disco* brain. In this regard, a recent report which describes the distribution of pigment dispersing hormone (PDH) -immunoreactive neurons in wild type and mutant *Drosophila* brains may be informative (Helfrich-Förster and Homberg, 1993). Increasing evidence from a variety of arthropods suggests that PDH-immunoreactive neurons may play an important role in circadian rhythms [reviewed in (Helfrich-Förster and Homberg, 1993)]. In wild type *Drosophila*, PDH immunoreactivity is found in cells at the medial margin of the medulla. Interestingly, this immunoreactivity is not found in *disco* mutant brains, suggesting that the relevant cells, which may correspond to the *per*-expressing cells, are missing.

Materials and methods

Heads from freshly eclosed adult flies homozygous for the PLacZ (Bellen et al., 1989; Wilson et al., 1989) insertion C50.1S1 were embedded in OCT medium and 10μm sections were cut by cryostat sectioning. Sections were collected on subbed slides and fixed for 10 min in 1% glutaraldehyde, 0.1M PIPES, 1 mM $MgSO_4$. Fixed tissue was then rinsed in PBS (four rinses of 5 min each) and stained with X-gal for 18 hr at 37°C. The X-gal staining solution contained 10mM $NaPO_4$ pH7.2, 150 mM NaCl, 1mM $MgCl_2$, 3.1 mM $K_4[Fe(II)(CN)_6]$, 3.1 mM $K_3[Fe(III)(CN)_6]$, 0.3% Triton x-100, 0.2% X-gal. Stained sections were rinsed in PBS and mounted in 70% glycerol.

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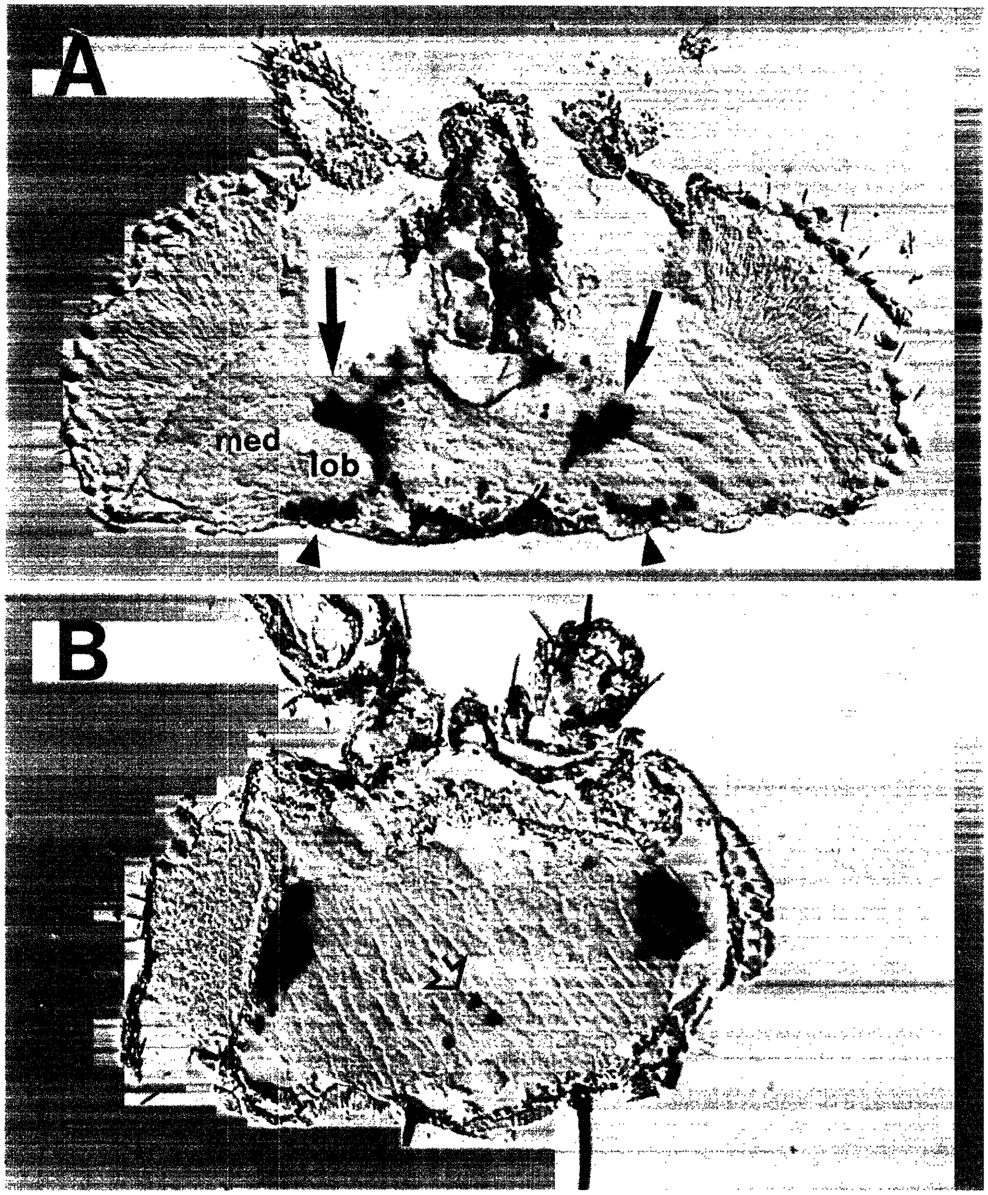
Figure B.1 ***disco*** **enhancer trap expression in the adult head.** Horizontal sections of heads from freshly eclosed adult flies are shown. Anterior is oriented up. (A) A section through the optic lobes shows reporter gene expression in the posterior cortex (arrowheads) of the lobula (lob) complex, as well as prominent expression at the medial margin of the medulla (med) cortex (large arrows). (B) In a more dorsally located section, reporter gene expression is located just beneath the retina, in a region which is likely to include the dorsal-most medulla cortex. In addition, reporter gene expression is found in three clusters underneath the ocelli (open arrow).


A
med
lob
B

Appendix C. *In vivo* analysis of the *disco* promoter

Summary

In order to identify cis-acting elements which direct proper expression of the *disco* gene, and to test the *in vivo* relevance of sequences in the *disco* promoter bound by disco protein *in vitro*, we constructed a series of *disco* promoter-lacZ fusion genes. Two of these constructs were introduced into the genome by P-element mediated transformation. Neither of these transformant lines shows a pattern of reporter gene expression that corresponds to the *disco* expression pattern. From these results we can conclude that sequences in the first 2 kb of upstream DNA, together with the first *disco* exon and the *disco* intron are not sufficient alone to drive proper *disco* gene expression.

Introduction

Previous studies have shown that *disco* gene function positively regulates its own mRNA expression in the embryonic optic lobe primordium. As the *disco* gene product is likely to be a transcriptional regulatory protein, this autoregulation may occur by direct binding of the disco protein to an upstream regulatory region in its own promoter. This proposal is supported by data, presented in Chapter 5, which show that the disco protein binds specific DNA sequences in its own promoter region *in vitro*. We wished to determine whether the sequences to which disco binds *in vitro* are necessary or sufficient to drive *disco*-dependent gene expression in optic lobe primordium cells *in vivo*, as would be expected for a cis-acting autoregulatory control element.

Results

Four different *disco* promoter constructs were made. The portions of the *disco* gene included in these constructs is diagrammed in Figure C.1. Two of these constructs, pCaß-CN6.5 and pCaß-CN7.5, are in-frame "translational fusions" of varying portions of the *disco* promoter to a *LacZ* reporter gene. Both of these constructs contain the endogenous *disco* transcription start site, the first, non-coding exon, the *disco* intron and a portion of the second exon including the first 180 codons of the *disco* open reading frame. The two constructs differ in the extent of upstream DNA they include: 2 and 3 kb, respectively. As shown in figure C.1, the 1 kb Cla fragment found in pCaß-

CN7.5, but not in pCaß-CN6.5, contains the s120 upstream disco binding site. A third construct, pHZ-C1.0, is an "enhancer fusion", in which the 1 kb ClaI fragment containing the s120 disco binding site is fused to a heterologous basal promoter from the *Drosophila hsp70* gene (Hiromi and Gehring, 1987). Finally, a fourth construct, pCaAß-BK7.4, is a "transcriptional fusion" of some 7.2 kb of upstream DNA, together with the *disco* promoter, to the *LacZ* reporter gene. All but 45 bp of the *disco* locus DNA included in this last construct is non-coding sequences.

Two of the four constructs have been successfully introduced into the genome by P-element mediated transformation. Five independent insertions of the HZ-C1.0 element were obtained. In all of these HZ-C1.0 transformant lines, the ßgal expression pattern in embryos is qualitatively, if not quantiatively, identical. The reporter gene expression pattern in embryos heterozygous for one of these insertion is shown in Figure C.2. ßgal expression is detected in several groups of cells in the gnathal segments, in bands of cells near the gnathal, thoracic and abdominal segment boundaries and in a band of endodermal cells in the midgut. However, as shown in Figure C.2, reporter gene expression in HZ-C1.0 transformant embryos shows little overlap with the expression of the endogenous *disco* gene. Most importantly, the HZ-C1.0 reporter gene is not expressed in the developing optic lobe primordium.

Two independent insertions of the Caß-CN6.5 element were obtained. The reporter gene expression patterns in embryos of these two Caß-CN6.5 transformant lines are quite different. This observation suggests that "position effects" are modulating ßgal expression in these two lines. The embryonic reporter gene expression patterns of the two Caß-CN6.5 transformant lines are shown in Figures C.3 and C.4. In both of the Caß-CN6.5 lines, the ßgal expression pattern shows little similarity to the endogenous *disco* expression pattern. Moreover, neither line shows ßgal expression in the developing optic lobe primordium.

Discussion

From the results presented here, we are unable to identify elements in the *disco* promoter that mediate *disco* gene expression in the appropriate tissues. Our data are also inadequate to determine whether the identified *disco* protein binding sites mediate autoregulation of *disco* gene expression. We can conclude that certain portions of the promoter are not sufficient for proper *disco* expression and for the autoregulatory response. Thus, upstream sequences within 2 kb of the *disco* transcript start, as well as sequences located within the first exon and in the intron, are not capable, by themselves, of directing the appropriate *disco* expression pattern. As indicated in Figure C.1, the downstream disco protein binding site, s280, is located within the *disco* intron. Thus, this sequence alone is not sufficient to direct expression in the proper tissues, nor to confer the response of gene expression to *disco* function.

The 1.0 kb Cla fragment, which contains the s120 upstream disco protein binding site, does not act as a *disco* autoregulatory control element in the "enhancer fusion" construct HZ-C1.0. This negative result may indicate that other sequences are responsible for the autoregulatory response of the endogenous *disco* promoter, or that this element is incapable of interacting with the basal *hsp70* promoter in the HZ50PL vector. Alternately, the spacing between the putative regulatory element and the *hsp70* promoter in the HZ-C1.0 element may not allow the proper response.

In order to determine which elements in the genomic DNA surrounding the *disco* locus are responsible to direct proper *disco* expression, further transformants are needed. The remaining two constructs which were not successfully transformed into the genome to date may include the necessary cis-acting elements. If not, the construction of even larger promoter fusions is warranted.

Materials and methods

Construction of disco promoter-ßgal fusions

A *disco* region genomic DNA isolate (λ51) in the bacteriophage lambda vector EMBL4 was digested with PstI, filled in with T4 DNA Polymerase and digested with ClaI. The resulting 6.4 kb fragment, spanning from the first upstream ClaI site, roughly 2 kb from the *disco* transcript start [see Figure C.1], to the Pst site roughly 4.3 kb downstream from the transcript start, was then cloned into ClaI/HincII-digested pBluescriptII (Stratagene) to yield pBS-CP6.5. The 1 kb ClaI fragment, located between 2 and 3 kb upstream of the *disco* transcript start, was cloned into the ClaI site of pBS-CP6.5 to yield pBS-CP7.5. This contruct was restriction mapped to verify that the relative orientation of this 1 kb ClaI fragment is the same as in the genome. A BamHI-NotI adaptor oligonucleotide (5'-GATCCACAGCGGCCGCTGTG-3') was self-annealed and cloned into BamHI-digested pCaSpeR-ßgal (Thummel, et al., 1988) to give pCaSpeR-ßgal-Not. The 6.5 and 7.5 kb NotI fragments from the constructs pBS-CP6.5 and pBS-CP7.5, respectively, were then cloned into the NotI site of pCaSpeR-ßgal-Not to yield the corresponding translational fusion constructs pCaß-CN6.5 and pCaß-CN7.5. The proper orientation of these insertions was verified by restriction mapping and the *disco*-ßgal fusion junction was checked by double-stranded sequencing using Sequenase (USB).

The 1 kb ClaI fragment, located between 2 and 3 kb upstream of the *disco* transcript start, was also cloned into the ClaI site of pBluscriptII and then re-isolated by digesting with XbaI and KpnI. This fragment was then cloned into XbaI/KpnI-digested HZ50PL (Hiromi and Gehring, 1987) to yield pHZ-C1.0. Again, the orientation of the ClaI fragment relative to the direction of transcription was mapped to verify that it was the same as in the genome.

A 425 bp DNA fragment representing the genomic DNA segment spanning from -366 to +45 relative to the *disco* transcript start site was generated by PCR using the following synthetic primers:

+40BgK : 5'-CGGGGTACC AGATCTGGACGCGAATATGCGG-3'
-365B : 5'-CGCGGATCCATCGACGACAATGTGAC-3'

These primers were designed to provide suitable KpnI, BglII and BamHI restriction sites (underlined above) for subsequent cloning. The PCR product was digested with BamHI and KpnI and cloned into BamHI/KpnI digested pBS(+) (Stratagene). This construct was then digested with BamHI and BssHII and a 7.4kb BamHI-BssHII DNA fragment, representing the genomic segment located just upsteam of the *disco* transcript start site, was inserted to yield pBS-BK7.4. The 7.4 kb BamHI-KpnI fragment from pBS-BK7.4 was then inserted into BamHI/KpnI-digested pCaSpeR-AUG-ßgal (Thummel, et al., 1988) to give the transcriptional fusion construct pCaAß-BK7.4.

P-element mediated germline transformation of promoter constructs

P-element mediated germline transformation and establishment of transformant stocks was carried out using standard procedures (Rubin and Spradling, 1982). A mixture of P element vector DNA (prepared using materials and protocols supplied by Qiagen), at a concentration of 1μg/μl, and pπ25.7wc (Karess and Rubin, 1984), at a concentration of 0.15 μg/μl, was injected into either *ry*^{506} or *yw*^{67c23} strain host embryos. Four different constructs were injected: The construct pCaß-CN6.5 was injected into 1042 embryos, from which 118 fertile G_0 adults were recovered. In the progeny of these adults, two transformant lines were obtained. These two lines (called E2-57 and E2-123) contained independent autosomal insertions of the Caß-CN6.5 P-element. The construct pHZ-C1.0 was injected into 460 embryos, from which 58 fertile adults were recovered. These G_0 flies gave five transformant lines. The five lines each contained independent autosomal insertions of the HZ-C1.0 P-element. The remaining constructs, pCaß-CN7.5 and pCaAß-BK7.4, were injected into 1042 and 1248 embryos, respectively, from which 118 and 128 fertile adults were recovered. None of these G_0 adults, however, produced transformant progeny.

Immunohistochemistry

Immunohistochemical staining of embryos was performed essentially as previously described (Steller, et al., 1987; Lee, et al., 1991). Embryos were fixed in a 1:1 mixture of heptane and 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. at room temperature. Affinity purified anti-disco antibody (Chapter 3) was used at a dilution of 1:3. ß-galactosidase expression was detected using a mouse anti-ßgal monoclonal antibody (Promega) at a dilution of 1:200. Secondary antibodies (Rhodamine-conjugated anti-rabbit Ig from Boehringer Mannheim and FITC-conjugated anti-mouse Ig from Cappel) were used at a dilution of 1:100. All antibody incubations were done overnight at 4°C. Stained specimens were analyzed using a BioRad MRC600 laser scanning confocal microscope equipped with a krypton/argon laser. Data generated by confocal microscopy was processed using manufacturer's software and instructions.

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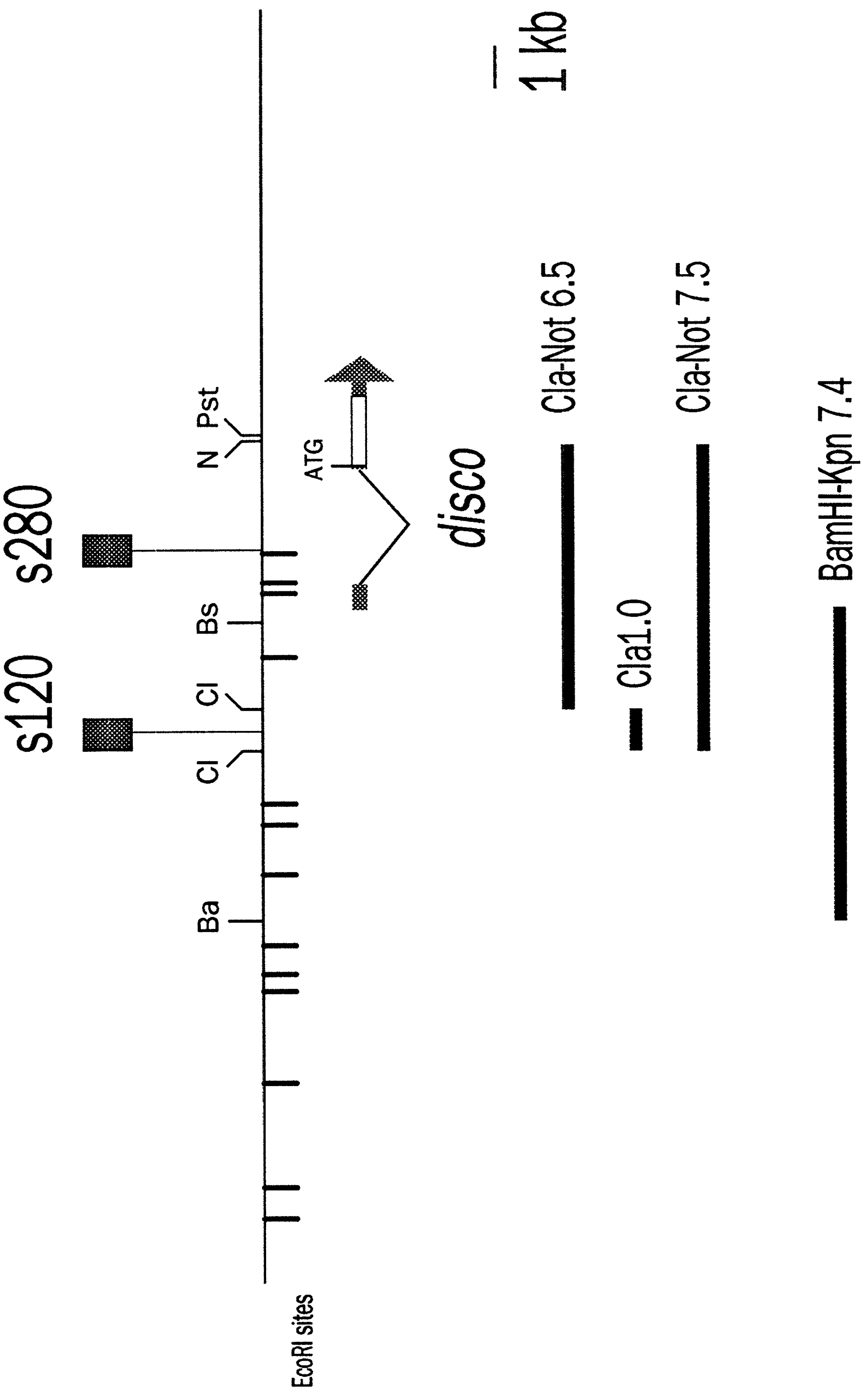
Figure C.1. *disco* promoter-LacZ fusion constructs.
The genomic region surrounding the *disco* transcription unit is indicated by the horizontal line. The positions of EcoRI sites in the genome are indicated by the short lines below this genomic map. Other relevant restriction enzyme sites are indicated by lines above the map (Ba=BamHI, Bs=BssHII, Cl=ClaI, N=NotI, Pst=PstI). The location of the two disco protein binding sites, s120 and s280 (see Chapter 5), is indicated by the shaded boxes. The position and structure of the *disco* transcription unit is indicated by the shaded arrow. The open box within this arrow indicates the position of the translated region, wholly contained within the second exon. The portions of the *disco* promoter included in promoter-LacZ fusion constructs are indicated by the solid boxes at the bottom of the diagram. Four different constructs were made. The 6.5 kb Cla-Not fragment and the 7.5 kb Cla-Not fragment were each inserted as a "translational fusion" in the vector pCaSpeR-ßgal. The 1.0 kb Cla fragment was inserted as an "enhancer fusion" into the vector pHZ50-PL. Finally, the 7.4 kb Bam-Kpn fragment, in which an artificial Kpn site was created just downstream of the *disco* transcript start site by PCR, was cloned as a "transcriptional fusion" into the vector pCaSpeR-AUG-ßgal.



s120
s280
Ba
Cl
Cl
Bs
N
Pst
ATG
disco
EcoRI sites
Cla-Not 6.5
Cla1.0
Cla-Not 7.5
BamHI-Kpn 7.4
1 kb

Figure C.2. Reporter gene expression in embryos containing the HZ-C1.0 element.
Confocal photomicrographs showing endogenous *disco* expression (green) and ßgal expression (red) in an embryo heterozygous for one of the HZ-C1.0 insertions. Cells which express both *disco* and ßgal appear yellow. As described in the text, five transformant lines carrying independent insertions of the HZ-C1.0 element were obtained. Reporter gene expression in all of these lines is identical. Four focal planes, arranged from lateral to medial, of a stage 13 embryo are shown. ßgal is expressed in clusters of cells in the gnathal segments (A, B), which also express *disco*, in groups of cells at segment boundaries, and in a band of cells in the midgut [arrowhead in (D)]. This expression of ßgal in the midgut is restricted to the endoderm, and does not include the overlying visceral mesoderm (vm), where *disco* expressed. ßgal is not expressed in the developing optic lobe primordium [arrows in (C, D)], where *disco* activity has been shown to regulate its own gene expression.

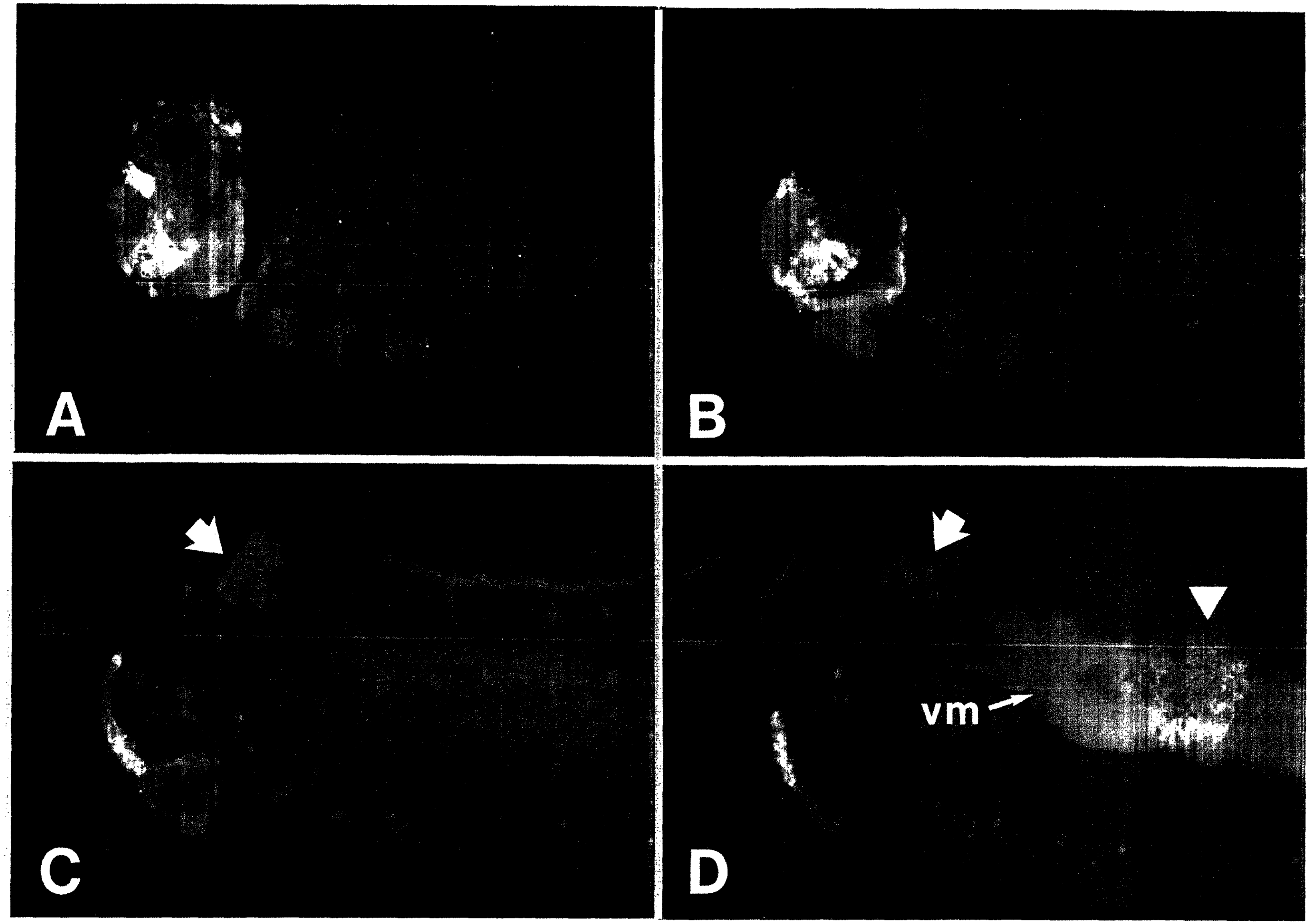

A
B
C
vm
D

Figure C.3. Reporter gene expression in embryos carrying the Caß-CN6.5 insertion E2-57.

Confocal photomicrographs showing endogenous *disco* expression (green) and ßgal expression (red) in an embryo heterozygous for one of the two insertions of the Caß-CN6.5 promoter fusion. Cells which express both *disco* and ßgal appear yellow. Lateral (A) and more medial (B) focal planes of a stage 14 embryo. ßgal is expressed throughout the nervous system, in both the peripheral nervous system (pns) and in the ventral nerve cord (vnc). ßgal is not expressed in the optic lobe primordium (arrow in B). (C) Reporter gene expression in a late stage 15 embryo. ßgal is expressed in the brain hemisphere (br), but not in the optic lobe primordium (arrow). Some cells just anterior to the optic lobe primordium express both *disco* and ßgal.

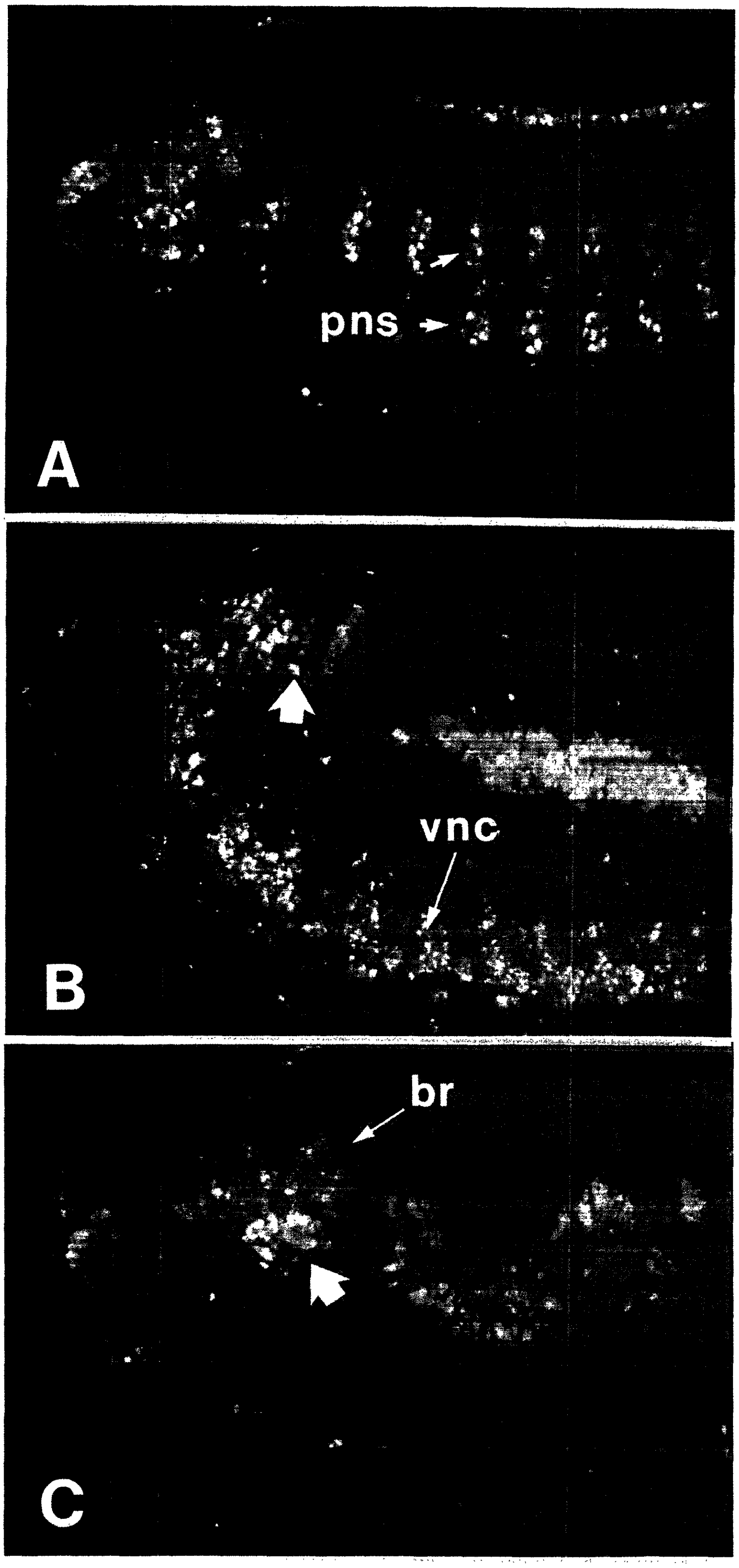
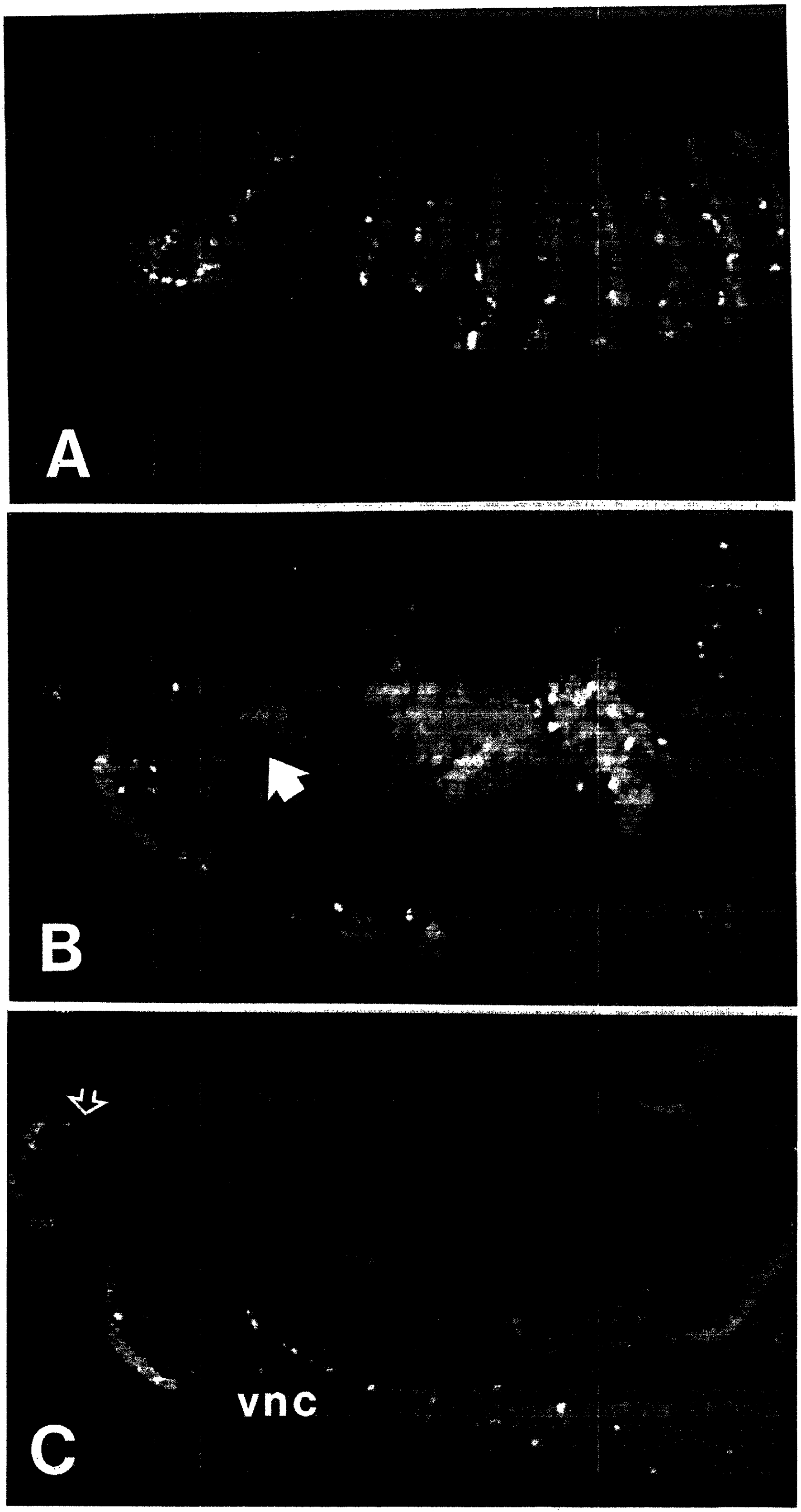

pns
A
vnc
B
br
C

Figure C.4. Reporter gene expression in embryos carrying the Caß-CN6.5 insertion E2-123.

Confocal photomicrograph showing endogenous *disco* expression (green) and ßgal expression (red) in an embryo heterozygous for one of the two insertions of the Caß-CN6.5 promoter fusion. Cells which express both *disco* and ßgal appear yellow. Three focal planes of a stage 15 embryo, arranged from more lateral to more medial, are shown. ßgal is expressed in the ectoderm at the posterior margin of each segment (A), in cells along the dorsal surface of the ventral nerve cord (vnc) (C), and in a number of large cells dispersed throughout the embryo. These large cells are likely to be circulating macrophages. Note the abundance of these cells in the clypeolabrum (open arrow in C). ßgal is not expressed in the optic lobe primordium (arrow in B).


A
B
C
vnc