

**TOWARD IDENTIFICATION OF
DISCO REGULATORY PROTEINS**

TOWARD IDENTIFICATION OF
DISCO REGULATORY PROTEINS
VIA GENETIC INTERACTION SCREENS

By

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ABSTRACT

The Disco protein has been identified as a transcription factor involved in the development of the *Drosophila melanogaster* nervous system (Steller et al., 1987; Heilig et al., 1991; Lee et al., 1991). Although, its loss of function phenotype is well characterized, the specific function of Disco in development remains unknown. One method of elucidating the role of *disconnected* is to identify additional signaling components with which it interacts. The GAL4-UAS system (Brand and Perrimon, 1993) was used to create gain of function mutant phenotypes of the embryonic central nervous system, larval visual system and adult compound eye. These dominant phenotypes had in common perturbations in cell differentiation and axonogenesis. Thus, these dominant phenotypes have provided an opportunity for further genetic interaction screens that would lead to the identification of proteins that interact with Disco.

A putative binding site for Disco itself, called s120, has been identified in the 5' flanking region of the *disco* locus (Lee et al., 1999). To discern whether tissue-specific Disco expression is regulated by this sequence, we utilized a promoter-*lacZ* fusion that included this regulatory sequence. The germ-line transformant lines carrying this construct showed β -galactosidase expression patterns that resembled wildtype *disco* expression, both in embryos and in the larval central nervous system (previously described by Lee et al., 1991). The expression of β -galactosidase remained like wildtype in the presence of excess Disco protein. Thus, this s120 regulatory sequence and the 5' flanking region studied were not sufficient to regulate Disco expression in

the optic lobe primordium. Upon sequencing and analysis of this region, several transcription factor binding site consensus sequences were identified. These sequences, as well as others, may be required for interaction with Disco to confer tissue-specific autoregulation. Further sequencing and expression from deletion constructs is necessary to identify additional *cis*-acting regulatory regions of Disco.

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LIST OF ABBREVIATIONS

BN	Bolwig's Nerve
BO	Bolwig's Organ
β -gal	β -galactosidase
bp	base pair
CNS	central nervous system
DAB	diaminobenzidene
ddH ₂ O	deionized, distilled water
DNA	deoxyribonucleic acid
ESEM	Environmental Scanning Electron Microscope
G _x	generation x
GMC	ganglion mother cell
HRP	horseradish peroxidase
ISN	intersegmental nerve
kb	kilobase or kilobase pair
kDa	kiloDalton
LON	larval optic nerve
LVS	larval visual system
MAb	monoclonal antibody
MF	morphogenetic furrow
MG	midline glial cell
MT	metallothionein
NB	neuroblast
NGS	normal goat serum
ORF	open reading frame
OLP	optic lobe pioneer cell
PBS	phosphate-buffered saline
PBT	phosphate-buffered saline with Triton X-100
PNS	peripheral nervous system
R	retinula or photoreceptor cell
S2	Schneider Line 2 cells

SO	sensory organ
SOP	sensory organ precursor
T _x	transformant line <i>x</i>
UAS	Upstream Activating Sequence
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER 1
Introduction

1.1 VISUAL AND NERVOUS SYSTEMS OF *D. MELANOGASTER*

Drosophila melanogaster (*D. melanogaster*) is a model organism for the study of the development of the nervous system, because it is a species readily amenable to genetic, cellular and molecular analyses. Such analyses are used to decipher genetic information and processes underlying neuronal development and pattern formation. The embryonic central and peripheral nervous systems, as well as the adult compound eye, all develop in stereotypical manners, thus, any perturbation in development is easily identifiable.

The Embryonic Central Nervous System

The main component of the *Drosophila* central nervous system is the ventral nerve cord. It consists of a ladder-like array of axons that follow the segmental pattern of the embryo from anterior to posterior. Each segment of the ventral nerve cord, called a neuromere, is comprised of two axon bundles that extend across the midline: the anterior and posterior commissures (ac and pc) (reviewed by Goodman and Doe, 1993). The commissures and neighbouring neuromeres are connected by axonal tracts on either side of the midline, called the longitudinal connectives (lc) (reviewed by Goodman and Doe, 1993).

The central nervous system (CNS) arises from the embryonic mesectoderm and neurectoderm (reviewed by Goodman and Doe, 1993). The

majority of neurons and glia of the CNS arise from the neurectoderm, while the midline neurons and glia, which form the ventral nerve cord, arise from the mesectoderm (reviewed by Goodman and Doe, 1993). Neurogenesis begins at stage 9 of embryo development, when specific cells, the neuroblasts (NBs), enlarge and delaminate from the ectoderm. Through a series of asymmetric cell divisions each NB gives rise to several ganglion mother cells (GMCs), which in turn, each divide once to give rise to two daughter cells that differentiate into neurons (reviewed by Goodman and Doe, 1993). For example, division of neuroblast NB4-2 gives rise to GMCs 1 through 5; the GMC-1 divides into the RP2 neuron and the RP2 sib (undefined neuron), and the GMC-2 divides to form neurons RP1 and RP3 (reviewed by Goodman and Doe, 1993).

Neuroblasts also give rise to the segmentally repeated pioneer neurons, pCC, MP1, dMP2 and vMP2, whose growth cones establish the axon scaffold of the ventral nerve cord (Jacobs and Goodman, 1989b). The pioneer neuron growth cones cross the midline, initiating the commissural pathways and on contralateral sides, these growth cones establish the longitudinal pathways. The pCC and vMP2 axons extend together anteriorly to form the pCC/vMP2 longitudinal pathway, and the MP1 and dMP2 axons extend together posteriorly, for the MP1/dMP2 longitudinal pathway (Jacobs and Goodman, 1989b; Hidalgo and Brand, 1997). Follower neurons project growth cones along the established axon scaffold to form the commissural bundles and the three longitudinal connectives, the pCC/MP2, MP1 and FN3 fascicles (Jacobs and Goodman, 1989b; Hidalgo and Booth, 2000).

The axon scaffold is partially prefigured by a glial scaffold (Jacobs and Goodman, 1989a). The glial scaffold, along which the longitudinal axonal tracts pioneer, is formed by longitudinal glia that arise from lateral glioblasts, the glial cell precursors (Jacobs and Goodman, 1989a). The six midline glial (MG) cells (3 pairs of anterior, middle and posterior midline glia, MGA, MGM and MGP, respectively) guide neuronal growth cones across the midline to the contralateral sides (Klamt et al., 1991). Once the axon scaffold has been well established, the MG cells migrate, and in doing so, they separate the fused commissural bundles into distinct anterior and posterior commissures (Klamt et al., 1991). Thus, the midline and longitudinal glia play a vital role in the establishment of the ventral nerve cord.

Genes Involved in CNS Development

The development of the CNS begins with the establishment of the embryonic co-ordinates by the early prepatter genes. In the undifferentiated ectoderm, the ventral cells express the gene *twist* (*twi*) to establish the mesoderm, and the gene *snail* to establish the midline cells (Leptin, 1991). In the lateral cells, expression of the gene *short gastrulation* (*sog*) identifies the neuroectoderm (Francois et al., 1994). In these newly formed domains of equivalent cells, proneural genes of the *achaete-scute* complex (Skeath and Carroll, 1994) and the gene *atonal* (*ato*) control a cell's competency to become a neuroblast. Neurogenic genes, such as *Notch* (*N*) and *Delta* (*D*), prevent more than one cell from the proneural cluster from becoming a NB

(reviewed by Beatus and Lendahl, 1998). Cells that are destined to become NBs enlarge and delaminate from the ectoderm. The neural precursor genes *deadpan* and *asense* are expressed in all NB, but their function is unclear (reviewed by Doe and Skeath, 1996). Each CNS NB is specified autonomously by neuroblast identity genes (reviewed by Doe and Skeath, 1996). These are often genes that are expressed in narrow stripes of ectodermal cells along the antero-posterior axis, commonly referred to as the segmentation or segment polarity genes (reviewed by Doe and Skeath, 1996). Examples of neuroblast identity genes are *runt* (Dormand and Brand, 1998) and *ming* (Cui and Doe, 1992), which are expressed in specific neuroblasts and their progeny. A mutation in either of these genes leads to changes in GMC and neuronal identities (Cui and Doe, 1992; Dormand and Brand, 1998). Further specification occurs within individual GMCs – genes such as *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) are expressed in specific subsets of GMCs and neurons (Doe et al., 1988). For example, GMC-1 of NB4-2 and its daughter neuron RP2 express *ftz* which is required to activate *eve* expression (Doe et al., 1988). An adjacent GMC gives rise to RP1/RP3 neurons and only expresses *ftz* (Doe et al., 1988). Loss of *ftz* expression leads to a loss of *eve* expression in GMC-1 and switches the fate of the RP2 neuron to RP1/RP3 (Doe et al., 1988). This example shows that individual GMCs express distinct subsets of genes that govern their specificity (Doe et al., 1988).

In the CNS the first cells to be specified are the midline cells that arise from the mesectoderm (Crews et al., 1988). These cells are the 3 pairs of midline glia (MG), the median neuroblast (MNB), 6 ventral unpaired neurons (VUMs), a pair of midline precursor 1 neurons (MP1) and two UMI

neurons (Jacobs and Goodman, 1989b; Klambt et al., 1991; Bossing and Technau, 1994). They are distinguished from neighbouring cells when they begin to express the genes *single-minded* (*sim*) and *slit* (reviewed by Goodman and Doe, 1993). Once differentiation of the midline cells begins, only the MG continue to express *sim* and *slit* (reviewed by Goodman and Doe, 1993). Other genes involved in the specification of midline cell fates are the “*spitz* group” genes (*spitz*, *rhomboid*, *Star* and *pointed*) (Mayer and Nüsslein-Volhard, 1988), *orthodenticle* (*otd*) (Finkelstein et al., 1990) and others (reviewed by Goodman and Doe, 1993). In *spitz* mutants, the NBs delaminate and neurons form, but the anterior and posterior commissures remain fused, indicating a perturbation specific to the development of midline glia (Klambt et al., 1991). In *sim* mutants, NBs do not delaminate from the mesectoderm, midline neurons do not form and therefore commissural and longitudinal tracts fail to form properly (Thomas et al., 1988; Nambu et al., 1991). Thus, *sim* seems to play the ‘master control gene’ role in the CNS by regulating midline cell development (Thomas et al., 1988; Nambu et al., 1991).

The role of ‘master regulator’ of lateral glial cell development is fulfilled by the gene *glial cells missing* (*gcm*) (Schreiber et al., 1997). Loss of function of *gcm* leads to a lack of all glia, except the MG and the glioblast progeny cells adopting a neuronal fate (Schreiber et al., 1997). Ectopic expression of *gcm* to non-neural cells alters their fate to that of mesenchymal cells (Akiyama-Oda et al., 1998). However, once a glial cell fate has been established, other genes participate in its differentiation, for example *repo* (Halter et al., 1995) and *pointed* (*ptd*) (Klambt, 1993; Klaes et al., 1994). Perturbations in the development of glia, are reflected in the development of

either the axon scaffold, or in the survival of midline neurons (Jacobs, 1993; Booth et al., 2000). The development of the midline and its lateral components depends on the function of many genes, which are involved in various regulatory pathways.

The CNS does not develop independently from the peripheral nervous system (PNS). Motor neurons from within the CNS project their axons dorsally to innervate the peripheral muscles, while the sensory neurons from the PNS extend their axons ventrally towards the CNS (Ghysen et al., 1986). In each abdominal segment motor and sensory axons fasciculate along two major axonal tracts: the intersegmental nerve (ISN) which is formed by axons of the peripheral neuronal cell clusters (the dorsal and lateral clusters), and the segmental nerve, formed by ventral neuron axons (Ghysen et al., 1986). The ISN and segmental nerve connect the PNS from each abdominal segment, to each of the corresponding CNS neuromeres.

The Embryonic Peripheral Nervous System

At the end of embryogenesis, the *D. melanogaster* peripheral nervous system consists of approximately 650 neurons arranged in a highly invariant and reproducible pattern (Ghysen et al., 1986). Peripheral neurogenesis begins at germ band retraction (stage 12) and is complete after dorsal closure (stage 14/15), with the neurons arranged identically in all seven of the abdominal segments (A1 to A7) and in two of the thoracic segments (T2 and T3) (Ghysen et al., 1986). In each of the abdominal segments, 44 neurons are organized

into four distinct clusters along the dorsal-ventral axis (Ghysen et al., 1986). Starting from the dorsal side, there are: 12 neurons in the first cluster (dorsal cluster, d), 12 neurons in the lateral cluster (l), and 9 and 11 neurons in the two ventral clusters (v and v') (Figure 8A) (Ghysen et al., 1986). The neurons are of three different types : i) external sense neurons (es neurons) which innervate mechanoreceptors and chemoreceptors; ii) internal sense neurons (ch neurons) which innervate internal stretch receptors; and iii) multiple dendritic neurons (md neurons) of unknown function (Bodmer and Jan, 1987). The es and the ch neurons are each a part of a sensory organ that consists of one or two neurons and three support cells, all of which arise from a single cell, called the sensory organ precursor (SOP) (Bodmer et al., 1989).

Genes Involved in the Development of the PNS

The SOP arises in a similar manner as the neuroblasts of the CNS. It is designated to a neuronal fate from a proneural cluster – a domain of equivalent epidermal cells (Bodmer et al., 1989). Once committed to its fate, the SOP delaminates from the epidermis and divides twice to form all the necessary cells of the sensory organ (SO) (reviewed by Ghysen and Dambly-Chaudiere, 1989). The specification of the SOP and its subsequent development requires numerous genes. First, early prepatter genes establish the embryonic coordinates (reviewed by Ghysen and Dambly-Chaudiere, 1989). From the undifferentiated ectoderm, proneural genes such as those of the *achaete-scute* complex (Skeath and Carroll, 1994) and the gene *atonal* (*ato*)

define domains of equivalent cells, the proneural clusters (reviewed by Jan and Jan, 1993). The ensuing neurogenic genes, such as *Notch* (*N*) and *Delta* (*D*), prevent other cells from becoming SOPs by inhibitory signaling (reviewed by Beatus and Lendahl, 1998). Early neuronal characteristics are given to the SOPs by neuronal precursor genes like *asense* (*ase*) (Brand et al., 1993) and *prospero* (*pros*) (Doe et al., 1991). Once it has been established which cell of the proneural cluster is to be the designated SOP, the *cut* (*ct*) gene plays a role in deciding which type of sensory organ, es or ch, the SOP shall become (Bodmer et al., 1987). Expression of the gene *numb* and other genes, further defines the individual SO cell identities (Uemura et al., 1989), after which each SO cell differentiates along its own developmental pathway, requiring genes for growth cone formation, axon guidance, fasciculation and other developmental processes.

The Larval Visual System

During embryogenesis, not only do the embryonic central and peripheral systems develop, but the larval visual system is also established. The larval visual system consists of the larval eyes, called Bolwig's Organs (BO), the larval optic nerves (LON) (often called Bolwig's Nerves (BN)) (Bolwig, 1946) and their targets, the optic lobe primordia. The larval eyes are photosensory organs, located in the embryonic head on either side of the midline (in a dorsomedial position). Each BO is composed of 12 photoreceptor neurons, arranged in a rosette of seven superficial and five

deeper neurons (Green et al., 1993). These neurons express three of the four rhodopsins found in the adult photoreceptors, but differ from adult photoreceptors in that they are not surrounded by any accessory cells (Pollock and Benzer, 1988). The larval photoreceptor axons fasciculate to form the LON, which enters the optic lobes and synapses with targets in the central brain (Meinertzhagen, 1973; Trujillo-Cenoz and Melamed, 1973).

The optic lobe primordium and BO develop from the same region of the embryonic head, the posterior procephalic region (Green et al., 1993). During germ band retraction (stage 12), the optic lobe placode of approximately 85 cells invaginates, forming a V-shaped structure with anterior and dorsal lips (Green et al., 1993). This placode then loses contact with the epidermis, attaches to the developing brain eventually forming the optic lobe (Green et al., 1993). After the completion of dorsal closure (stage 15), four to six cells segregate from the ventral-most part of the dorsal lip of the invaginating pouch to form an irregular cell cluster (Schmucker et al., 1997). These cells then divide and differentiate to 12 photoreceptors on each side of the midline – the BOs (Steller et al., 1987). Throughout head involution and subsequent stages, BO remains in contact with the optic lobe primordium via the photoreceptor axons (Steller et al., 1987). After the completion of head involution, the LON follows an invariant path from BO toward the optic lobe, where it turns ventrally to curve around the optic lobe, and then turns 90° to penetrate the cortex of the brain (Campos et al., 1995). En route to the brain, the LON encounters three distinct neurons and fasciculates with their processes (Tix et al., 1989; Campos et al., 1995). These neurons are the first cells to differentiate in the prospective optic lobe region

and are thus called the optic lobe pioneers (OLPs) (Tix et al., 1989). At the end of embryogenesis, the development of larval visual system (LVS) by coordination of gene expression and morphogenetic movements is complete.

Gene Expression Patterns During Larval Visual System Development

Several genes are expressed in the optic lobe placode prior to its invagination: *sine oculis* (*so*) (Cheyette et al., 1994), *Notch* (*N*) (Green et al., 1993) and *tailless* (*tll*) (Daniel et al., 1999). In *so* null mutants, the optic lobe placode fails to invaginate and BOs fail to form, thus assigning *so* a prominent role in LVS development (Cheyette et al., 1994). *Notch* expression in the developing LVS is within the invaginating optic lobe placode (Green et al., 1993). Mutants in *N* have an increased number of cells in BOs and in the optic lobes (Green et al., 1993). In the LVS, *N* plays a similar role as it does in the CNS and PNS – it appears to inhibit cells from a neural fate (Green et al., 1993). Loss of *tll* function mutants also exhibit increased numbers of cells in BO, but without any development of the optic lobes (Daniel et al., 1999). The gain of function *tll* phenotype consists of optic lobes, but no BOs (Daniel et al., 1999). During germ band retraction, *tailless* expression is upregulated in the anterior lip of the optic lobe primordium, is turned on in posterior lip and remains strong in the optic lobe through further development stages (Daniel et al., 1999). However, expression is never seen in BOs (Daniel et al., 1999). Thus, *tll* functions to drive cells towards an optic lobe fate, rather than a larval photoreceptor fate (Daniel et al., 1999).

By restricting the cells from a BO fate, *tll* function counteracts that of *atonal*. *ato* is also expressed in the optic lobe invagination at stage 11: in six to eight cells at the posterior boundary (Daniel et al., 1999). At stage 12, the expression becomes restricted to three or four cells called the BO founder cells (Daniel et al., 1999). In *ato* mutants, there is a complete absence of all BO photoreceptors, thus it appears that expression of *ato* in a small subset of cells, the BO founders, may signal neighbouring cells to become secondary BO cells (Daniel et al., 1999). This *atonal* signal drives cells to a BO fate – the opposite of *tll* function.

Another set of counteracting genes expressed in the invaginating optic lobe primordium is *beaten path* (*beat*) and *fasciclin II* (*fasII*) (Holmes and Heilig, 1999). *fasII* is expressed in the optic lobe primordium prior to the time when BO becomes a separate entity (Holmes and Heilig, 1999). Later in LVS development, it is expressed in the optic lobe, LON and larval photoreceptors (Holmes and Heilig, 1999). *beat* is also expressed in the invaginating optic lobe (stage 12), in the subset of cells that are destined to become BO (Holmes and Heilig, 1999). The phenotypes of *beat* and *fasII* mutants are exact opposites: in *beat* mutants excess larval photoreceptors develop and in *fasII* mutants the BOs are reduced in size (Holmes and Heilig, 1999). In both mutants, at later stages, the photoreceptor cells are seen scattered in the area of BO, perhaps due to a loss of cell adhesiveness (Holmes and Heilig, 1999).

The phenotype of loosely associated BO cells is also seen in *Krüppel* (*Kr*) and *glass* (*gl*) mutants (Schmucker et al., 1992). Both *Kr* and *gl* are transcription factors that are required independently of each other and most likely are not directly responsible for the loosely-associated BO cells

phenotype (Schmucker et al., 1992). *Kr* expression begins, in the presumptive BO, in a single cell that has already attained a neuronal fate, and continues through all stages of development of BO (Schmucker et al., 1992). Strong loss of function *Kr* alleles do not allow development of BO and LON, but with weaker *Kr* alleles rudimentary organs develop with defective LON projections (Schmucker et al., 1992). These mutants resemble those of *glass*: normal BOs are replaced with loosely clustered neurons with abnormal or missing projections (Moses et al., 1989). *glass* is normally expressed in the rosette-like pattern of the BOs and, later in development, in the presumptive brain (Ellis et al., 1993).

Another gene that is involved in the final stages of LON development, is *disconnected* (*disco*). *disco* is expressed in the ventral lip of the optic lobe primordium at stage 13, and then in a group of immature neurons just anterior to the optic lobe primordium (Lee et al., 1991; Campos et al., 1995). *disco* loss of function does not affect BO development, nor does it affect initial LON formation (Steller et al., 1987). Initial LON contact with the optic lobe primordium is normal, but as development progresses the LON becomes disconnected from the optic lobe, similar to the *Kr* phenotype, suggesting that *disco* too may function in guidance or target recognition of the LON (Steller et al., 1987).

All of the above mentioned genes are also expressed in various other tissues during development. Recently, a newly identified homeobox-containing gene *Munster* (*Mu*) has been identified, and it is exclusively expressed in BOs (Goriely et al., 1999). In the adult, it is also expressed in the

medulla, lamina of the optic lobes and in all photoreceptors of the retina (Goriely et al., 1999). However, the function of *Mu* has yet to be determined.

The Adult Visual System – Structure of the Compound Eye

The compound eye develops during the third instar and pupal stages by reorganization of existing structures, addition of new neurons and the degeneration of others. The crystal-like structure of the compound eye is due to the arrangement of the 800 single facets, called ommatidia (reviewed by Freeman, 1997). Each ommatidium consists of 19 radially-arranged cells: six photoreceptors, also called retinula cells or R cells (R1-R6), surround two central receptors (R7 and R8); overlying the photoreceptors are four cone cells, and surrounding these cone cells are two primary pigment cells (reviewed by Freeman, 1997). The six secondary and three tertiary pigment cells are shared between neighbouring ommatidia, as are the small mechanosensory bristles that project from alternate vertices (reviewed by Freeman, 1997). The retinula cells are unipolar neurons that project their axons from the eye disc, down the optic stalk along the LON, to the optic lobes (Meinertzhagen and Hanson, 1993). R1-R6 axons terminate in the first optic ganglion, the lamina, whereas R7 and R8 project through to the underlying second optic ganglion, the medulla (Meinertzhagen and Hanson, 1993).

The Development of the Compound Eye

The compound eye develops from a sac of undifferentiated cells, called the eye imaginal disc, during third larval instar and pupal stages. The eye imaginal disc develops during embryogenesis from a domain of 6 to 23 cells located in the dorsal ectoderm (reviewed by Wolff and Ready, 1993). This ectoderm folds inward to form the monolayer of epithelial cells (reviewed by Wolff and Ready, 1993). At the first larval instar, the disc is small consisting of approximately 130 cells that multiply to about 1600 cells by the third larval instar stage (reviewed by Wolff and Ready, 1993). During the third larval instar stage, a first mitotic wave of proliferation increases the number of undifferentiated cells to about 9700 (reviewed by Wolff and Ready, 1993). Shortly afterward, an invagination called the morphogenetic furrow traverses the eye imaginal disc from posterior to anterior, changing the disc from a proliferative epithelium to a differentiating entity (Tomlinson and Ready, 1987). Posterior to the furrow, cell recruitment and differentiation occur, with R8 being the first photoreceptor cell to differentiate (Tomlinson and Ready, 1987). R2/R5 and R3/R4 are then recruited to form a five cell precluster immediately posterior to the furrow (Tomlinson and Ready, 1987). As the furrow progresses, R1/R6 join the precluster and the last photoreceptor to differentiate is R7 (Tomlinson and Ready, 1987). A second round of mitosis, posterior to the furrow, gives rise to the cone cells, and during pupal development the primary, secondary and tertiary cells are added to the cluster to create the final ommatidial structures (Tomlinson and Ready, 1987).

Genes Governing Cell Differentiation in the Eye Imaginal Disc

In the eye imaginal disc, development begins with the *eyeless (ey)* gene. *eyeless* is expressed in the undifferentiated cells of the eye imaginal disc anterior to the morphogenetic furrow (Quiring et al., 1994). Since the loss of function of *ey* leads to no eye development, and the gain of function to the development of ectopic eyes, the *ey* gene has been called the 'master control gene' for eye development (Quiring et al., 1994). However, other loss of function studies have shown that additional genes, including *eyes absent (eya)* (Bonini et al., 1993), *sine oculis (so)* (Cheyette et al., 1994) and *dachshund (dac)* (Mardon et al., 1994) also lead to the complete loss of eyes. Targeted expression of both *eya* (Bonini et al., 1997) and *dac* (Shen and Mardon, 1997), as well as a third gene, *teashirt (tsh)* (Pan and Rubin, 1998) induces ectopic eye development. Thus, *ey*, *eya*, *dac* and *tsh* form a regulatory network that specifies eye fate. Once eye development has begun, the progression of the morphogenetic furrow is controlled by the expression of *decapentaplegic (dpp)* which is maintained by expression of the gene *hedgehog (hh)* (reviewed by Bonini and Choi, 1995). As the furrow progresses, early pattern formation occurs in the furrow – *ato* expression establishes rosettes of 5 to 10 cells (Dokucu et al., 1996). The *scabrous (sca)* gene is also expressed within the furrow and functions in the equal spacing of these early rosettes (Mlodzik et al., 1990). The rosettes transform into arcs by the loss of cells, the arcs close forming 5 cell preclusters of R8, R2/R5 and R3/R4 (reviewed by Wolff and Ready, 1993) and *sca* expression becomes restricted to the R8 (Mlodzik et al.,

1990). *Notch* and *Delta* refine the neurogenic pattern conferred by *scabrous* (reviewed by Beatus and Lendahl, 1998). R8, R2 and R5 all produce Spitz which diffuses to neighbouring cells and activates its receptor, the Drosophila EGF Receptor (DER) (reviewed by Freeman, 1997). Activation of DER recruits R3/R4, R1/R6 and R7 to the precluster and they, in turn, begin to express *argos* (reviewed by Freeman, 1997). *Argos* diffuses out to the neighbouring cells, and inhibits Spitz-DER signaling in these cells (reviewed by Freeman, 1997). As the ommatidia mature, *rhomboid* (*rho*) and *Star* (*S*) expression expand, thus expanding the expression of *spitz*, the ligand they regulate (reviewed by Freeman, 1997). Greater *spitz* expression levels, overcome the *argos* block in the next concentric ring of cells, and the development of ommatidia continues (reviewed by Freeman, 1997).

R7 development differs from the recruitment and development of the outer photoreceptors, R1 to R6, because its differentiation is triggered by two rounds of Ras signaling (reviewed by Klambt, 1997). R8 expresses *Bride-of-sevenless* (*Boss*), the ligand for the *sevenless* (*sev*) receptor that is expressed by all other R cells (reviewed by Basler and Hafen, 1991; Wolff and Ready, 1993). In all but the R7 cells, *seven-up* (*svp*) inhibits *sev* function, and thus only the R7 is affected by the *Boss-sev* activated Ras pathway (reviewed by Basler and Hafen, 1991). The second activation of the Ras pathway, is by *spitz*/DER and proceeds as in R1-R6 (reviewed by Klambt, 1997).

The development of the embryonic PNS and CNS, and the adult compound eye, occurs in a similar manner, beginning with the expression of proneural genes, which define regions of equivalent cells. From these

domains, neural precursor cells are identified and their differentiation continues by the expression of many different genes and the activation of various signaling pathways.

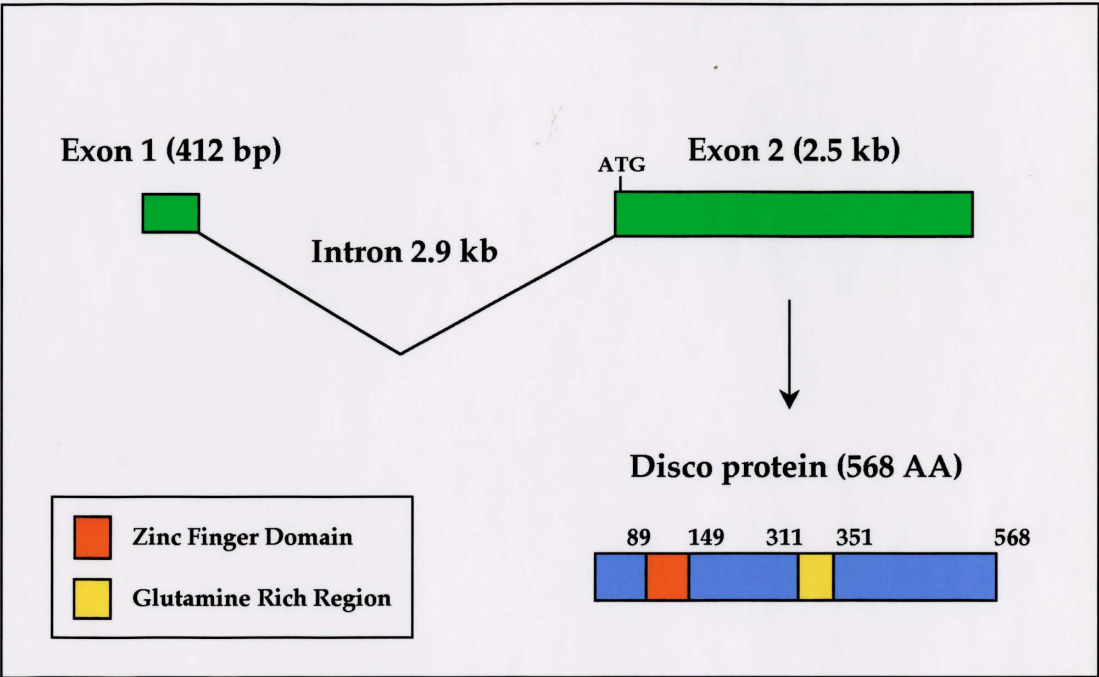
1.2 DISCO

The *disconnected* Locus

The *disco* locus has been localized to the X chromosome, polytene band 14 (Steller et al., 1987). Initial sequencing of complementary deoxyribonucleic acid (cDNA) and genomic clones, showed that the *disco* locus consists of two exons (412 bp and 2.5 kb) separated by a single intron (Figure 1) (Heilig et al., 1991). The first exon remains untranslated and the entire 1.7 kb open reading frame (ORF), is carried in the second exon, which encodes a 568 amino acid (AA) protein (Figure 1) (Heilig et al., 1991). Within this ORF are two zinc finger motifs located near the N-terminus, in the regions of amino acids 89-119 and 117-149, as well as a glutamine rich domain at AA 311-351 (Figure 1) (Heilig et al., 1991). These domains were characterized to be functional transactivation domains by their ability to activate transcription of a heterologous promoter in yeast (Lee et al., 1999).

Figure 1. Organization of the *disco* Transcription Unit and Protein.

The *disco* transcription unit consists of two exons. The first exon (412 bp) is separated from the second exon (2.5 kb) by an intron of 2.9 kb (Heilig et al., 1991). The first exon remains untranslated and the entire 1.7 kb ORF is within the second exon (Heilig et al., 1991). It encodes a 568 AA protein (Heilig et al., 1991).



The *disconnected* Phenotype

Adult *disco* mutants have rough-looking compound eyes, with older adults bearing dark patches due to progressive degeneration of their photoreceptors (Campos et al., 1992). These photoreceptors degenerate due to abnormal innervation of the optic lobes by their axons, which terminate in a plexus at the posterior margin of the eye disc (Campos et al., 1992). Because retinal innervation of the optic lobe primordium during late larval stages is required for the proper development of the optic lobes (Selleck and Steller, 1991), “unconnected” *disco* mutants have only rudimentary optic lobes, without the lamina (Steller et al., 1987). In a few “connected” phenotype mutants, the retinula axons innervate the normal sized optic lobes, but the lobes are of a disorganized nature (Steller et al., 1987).

Besides the compound eye phenotype and malformation of the optic lobes, *disco* mutants also display subtle defects in the embryonic PNS (Steller et al., 1987) and CNS (Glossop and Shepherd, 1998). In embryonic abdominal and thoracic segments, although the general organization is like wildtype, some subtle defects are found (Steller et al., 1987). Neuronal clusters are not arranged in an orderly fashion and axons are found crossing segmental boundaries (Steller et al., 1987). In the larval nervous system severe disruptions in the central projections were found, including axons that meander at the midline, axon tangling and excessive axon growth (Glossop and Shepherd, 1998). Similar defects are observed in the pupal central projections, indicating a general failure in axon growth/guidance in *disco* mutant embryos (Glossop and Shepherd, 1998).

In the larval visual system, the LON of *disco* mutants either projects to an ectopic location, is twice to three times its normal length, or simply fails to establish the appropriate synaptic contact in the central brain (Campos et al., 1995). The glial cells of the optic nerve are present in *disco* mutants, but they are loosely associated with the LON and fail to show the typical elongated morphology (Campos et al., 1995). Thus, either the defects in glial morphology and their association with the LON lead to the aberrant LON phenotype, or the misplaced LON causes the glia to dissociate and to change their morphology (Campos et al., 1995).

The described *disco* mutant phenotypes may be explained by examining the wildtype pattern of expression.

Disco Expression Patterns

Disco protein is localized to the nucleus of both neural and non-neural cells (Lee et al., 1991). Both the protein and mRNA are widely distributed in the developing embryo, including in the CNS, PNS and primordia of adult optic lobes (Lee et al., 1991). Expression begins at stage 5 in the posterior end of the embryo and persists throughout embryogenesis (Lee et al., 1991). At stage 6, Disco expression is seen at the anterior end, as two stripes around the cephalic furrow (Lee et al., 1991). Widespread expression begins at stage 11, when Disco is seen not only in the gnathal and antennal segments, and the procephalic region of the head, but also in the thoracic segments and the visceral mesoderm (Lee et al., 1991). As the gut develops, expression is seen

surrounding the gut, scattered throughout the CNS and in the cardioblasts of the dorsal vessel (Lee et al., 1991). In the CNS, *disco* is expressed on both sides of the midline in a segmental pattern along the ventral nerve cord (Lee et al., 1991). In each neuromere, *disco*-expressing cells are located in two pairs in a dorsal position, and in clusters of 12 cells located more ventrally and laterally (Lee et al., 1991). Expression is also seen in the lateral ectoderm, in several neurons and support cells of the PNS (Lee et al., 1991).

At the end of embryogenesis, the larval visual system has been established and *disco* expression is seen in a large region at the terminal end of the LON in the brain lobe (Lee et al., 1991; Campos et al., 1995). Although, no expression of *disco* is seen in the BOs, there is expression in the atrium adjacent to the BO neuronal bodies (Lee et al., 1991; Campos et al., 1995).

In third instar larval brains, the pattern of Disco expression is a band that wraps around each of the lobes from near the optic stalk, around to the dorsal surface of the lobe (Figure 16A) (Lee et al., 1991). Expression of *disco* is also seen in cells scattered across the lamina, in two cells at the base of the optic stalk and in cells along the ventral nerve cord (Lee et al., 1991). The leg imaginal discs and the antennal discs also express Disco, but no expression is seen in the eye imaginal disc (Lee et al., 1991).

The *disco* expression patterns have been found to be recapitulated in several enhancer trap lines (Cohen et al., 1991). In the C50.1S1 enhancer trap the P-element is not located adjacent to the *disco* promoter, but it confers the wildtype *disco* expression pattern in its entirety (Cohen et al., 1991).

Regulation of Disco Expression

In *disco* mutant embryos, *disco* expression is like wildtype, except at stage 12, when no expression is seen in the optic lobe region, indicating that maintenance of *disco* expression in this region requires wildtype Disco activity (Lee et al., 1999). Similarly, ubiquitous expression of *disco* from a heat-shock promoter increases endogenous *disco* expression in the optic lobe primordium of *disco* mutants (Lee et al., 1999). Together, these results indicate that *disco* expression in the optic lobe primordium is positively autoregulated. In addition, Disco binds to its own promoter *in vitro*, as well as to a site within its intron (Lee et al., 1999). One of the Disco binding sites lies approximately 2.5 kb upstream of its transcription start site and is 120 bp long; the other site is within the intron and is 280 bp long (Lee et al., 1999). The presence of two functional transactivation domains (Lee et al., 1999), Disco's localization to the nucleus (Heilig et al., 1991), its ability to autoregulate its own expression in the optic lobe primordium (Lee et al., 1999) and the presence of two putative Disco binding sites within the locus (Lee et al., 1999) all suggest that Disco is a transcription factor that may be involved in its own transcriptional regulation.

Disco expression has been found to be regulated in embryonic tissues. In the leg primordia, a homeodomain containing protein *Distal-less* (*Dll*), is expressed. The onset of *Dll* expression in the leg primordia is at germ band extension, which is followed by *disco* expression (Cohen et al., 1991). In *Dll* mutants *disco* expression remains like wildtype in all tissues, but is abolished in the leg primordia (Cohen et al., 1991). Thus, it is believed that *Dll* exerts its

function on *disco* in the leg primordia (Cohen et al., 1991). A second gene was found to tissue-specifically regulate *disco* expression. In wildtype embryos, *disco* is expressed in heart precursors, the cardioblasts (Lee et al., 1991), as is the *tinman (tin)* gene (Bodmer, 1993). In *tin* mutants, *disco* expression in the cardiogenic tissue is absent (Bodmer, 1993). Neither the leg primordia, nor the cardioblasts develop abnormally in *disco* mutants, suggesting a redundancy in gene function. *Dll* regulates *disco* expression in leg primordia, *tin* regulates *disco* expression in the heart primordia and in the optic lobe primordium, *Disco* regulates its own expression. Thus, the widespread *disco* expression pattern is specifically regulated in several tissues indicating that there may be other transcription factors that interact with and regulate *Disco* expression.

1.3 AN APPROACH TO IDENTIFYING INTERACTING PROTEINS

In *Drosophila*, the function of a gene can be dissected in several different ways: examining the phenotype caused by a loss of function mutation, looking at the gene's wildtype expression pattern, comparing sequences between species and basing probable function on sequence similarity, or by examining the consequences of misexpressing the gene. All are good strategies, however, the loss of gene function may not provide an obvious phenotype; the expression pattern may be too widespread; and sequences for comparison may not be available. By expressing a single gene

either in a tissue where it is not normally expressed or at a time when it is not normally expressed, a dominant phenotype may be generated, providing additional information regarding the gene's function. This dominant phenotype may be used in a second site mutagenesis screen to isolate enhancers or suppressors of the phenotype, thus providing an opportunity to identify probable interacting proteins. These enhancers and/or suppressors have mutations in specific regions of the *Drosophila* genome. Genes that reside in these specific regions may code for the proteins which regulate expression.

Ectopic Expression as a Means of Identifying the Function of a Gene

Ectopic expression can be accomplished using a variety of techniques, one of which is to fuse the gene of interest to a promoter such as *tubulin* or *actin* (reviewed by D'Avino and Thummel, 1999). These promoters provide both constitutive and ubiquitous expression, however the levels of transcription are relatively low and expression is not uniform (reviewed by D'Avino and Thummel, 1999). Heat-shock promoters are often used to achieve temporally regulated expression, but low basal transcription under non-heat-shock conditions, the possibility of phenocopies and ubiquitous expression do not make this an ideal system (reviewed by D'Avino and Thummel, 1999).

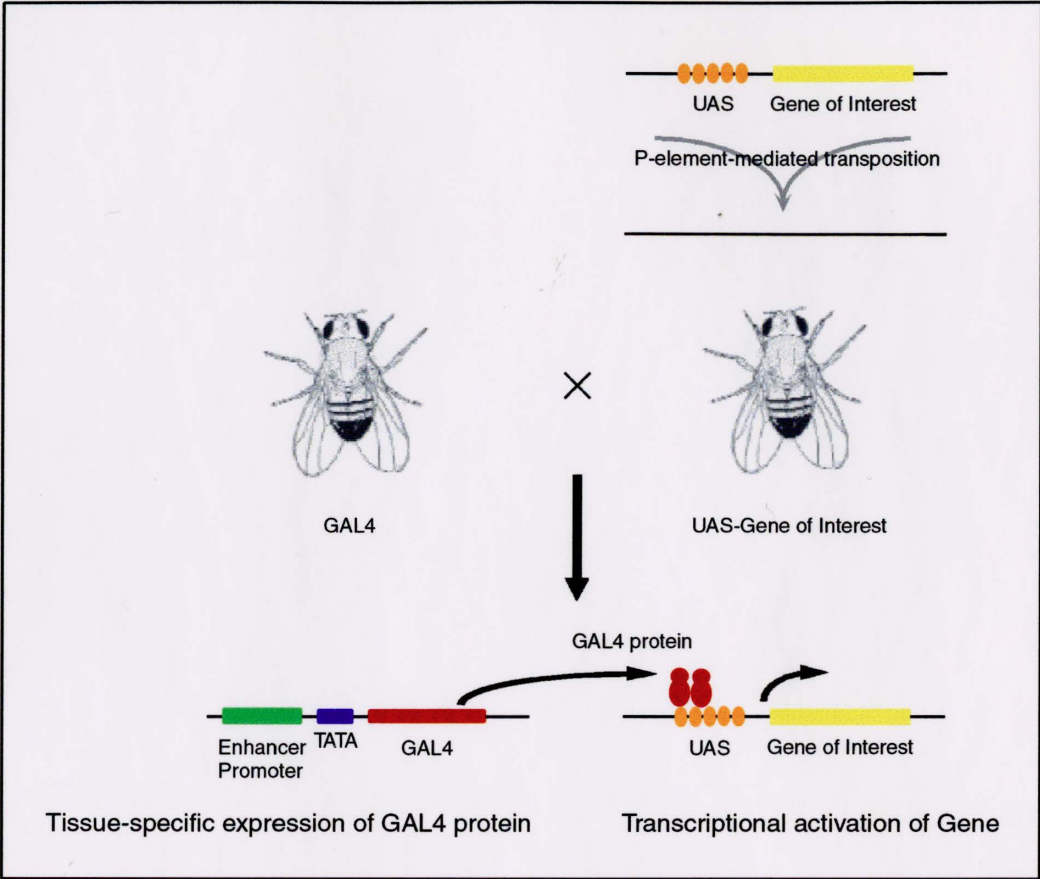
Both spatial and temporal regulation can be achieved by a technique that takes advantage of the yeast transcriptional activator, GAL4 and its

binding site, the Upstream Activating Sequence (UAS). In this system, the gene of interest is placed under the control of the UAS and the expression of GAL4 is controlled either by flanking genomic enhancer elements or by specific promoters (Brand and Perrimon, 1993). The driver-GAL4 and the UAS-target gene constructs are kept in separate fly lines, such that the target gene remains silent in the absence of the activating GAL4. Once the two fly lines are crossed, the resulting progeny have one copy of the driver-GAL4 and one copy of the UAS-target gene (Figure 2). The GAL4 protein binds to the UAS, thereby directing expression of the target gene in specific tissues. The advantages of this system are that ectopic expression can be directed to a specific tissue or cell type and a library of various GAL4 lines can be used for ectopic expression. Although this GAL4-UAS system is a good way to ectopically express genes of interest, it has the drawback of delayed effects due to earlier expression of GAL4 or variable levels of GAL4-mediated transcription from cell to cell (reviewed by D'Avino and Thummel, 1999).

The GAL4-UAS system has been successfully used to drive ectopic expression of the *eyeless* gene. Previously, various approaches had been used to identify the function of the *eyeless* gene. The phenotypes for the loss of function of the *eyeless* gene are characterized by markedly reduced or missing compound eyes (Quiring et al., 1994). The sequence of the *eyeless* locus shows extensive sequence similarity with the mouse *Small eye* and the human *Aniridia* genes, and it contains a paired domain and homeodomain, suggesting that it functions as a transcriptional regulator (Quiring et al., 1994). The *eyeless* gene is expressed embryonically in the ventral nervous system, in parts of the brain and in the optic lobe primordia (Quiring et al., 1994). In the

Figure 2. The GAL4-UAS System

The GAL4-UAS system directs tissue-specific expression of a gene of interest (Brand and Perrimon, 1993). P-element mediated germ-line transformation (Rubin and Spradling, 1982) is used to insert the gene of interest under the control of UAS, into the *Drosophila* genome. This fly line is crossed with one carrying the UAS activator, GAL4, under the control of an enhancer or a specific promoter. In the progeny of this cross, the UAS-gene of interest is activated in the same cells that the GAL4 is expressed.



larva, it is expressed in the undifferentiated cells of the eye imaginal disc anterior to the morphogenetic furrow (Quiring et al., 1994). These results provide a partial understanding of the function of *eyeless*. By ectopically expressing *eyeless* using the GAL4-UAS system, ectopic eye structures developed on antenna, legs, wings and halteres (Halder et al., 1995). This suggests that *ey* acts as a 'master control gene' for eye development. Thus, by ectopically expressing *ey*, its function in eye development has been suggested to be the regulation of expression of various downstream eye-specific genes.

Regulation of Gene Expression

Regulation of gene expression may be accomplished at the transcriptional level by binding of transcription factors to *cis*-regulatory sequences. These regulatory proteins may bind the DNA alone or in transcription factor complexes, to either upregulate or downregulate transcription. Furthermore, regulation may be of a tissue-specific manner and autoregulatory. Promoters and enhancers of many genes have been studied extensively. For example, it has been found that *sevenless* expression is positively regulated and *glass* expression is negatively regulated, as well as autoregulated.

A method of determining which sequences are necessary and sufficient for the transcriptional regulation of a gene, is to create a fusion between the sequence in question and a reporter gene, for example *lacZ*. It was previously shown that a 16.3 kb genomic fragment that included 967 bp located upstream

of the transcription start site, could rescue the *sevenless* phenotype (Bowtell et al., 1988). Thus, either the region upstream of the transcriptional start site or the sequences within the locus are important in the regulation of *sev* expression. Two different transcriptional fusions were created with the *sevenless* promoter and *lacZ*, each differing in the length of upstream sequence it contained (Bowtell et al., 1989). One transcriptional fusion, *967lacZ*, included bp -967 to +89, fused to a *lacZ* reporter cassette, while the other, *3300lacZ*, included a much longer upstream sequence, from bp -3300 to bp +89, was fused to the reporter cassette (Bowtell et al., 1989). Both constructs were germ-line transformed and most of the transformants failed to show any β -galactosidase activity in the eye imaginal discs, except for two lines which showed weak, but reproducible β -galactosidase expression (Bowtell et al., 1989). Previous work with *sev* cDNA constructs lacking introns 2 to 7 also induced weak reporter gene expression (Bowtell et al., 1988). The transcriptional constructs *967lacZ* and *3300lacZ*, were also missing these introns (Bowtell et al., 1989). From these experiments, it was concluded that sequences within the body of the *sevenless* gene, namely introns 2 to 7, were required for expression of *sev* (Bowtell et al., 1989).

The *glass* gene encodes a zinc finger protein (Moses et al., 1989), which has site-specific DNA binding ability, and localizes to all nuclei in the third instar eye imaginal discs (Moses and Rubin, 1991). Using an enhancer trap line B172, which confers a *gl* expression pattern on the *lacZ* reporter gene, it was shown that Glass positively autoregulates its own expression (Moses and Rubin, 1991). The B172 enhancer trap recapitulates *gl* expression in BO and in some cells of embryonic and third instar brain. β -gal expression was also

seen in the eye imaginal disc posterior to the morphogenetic furrow, similar to that of wildtype *glass* expression. In a wildtype background, a mutant B172*lacZ* line showed a normal expression pattern, while in a *gl* mutant background, B172*lacZ* expression was greatly reduced or eliminated in BO and in the eye imaginal disc (Moses and Rubin, 1991). However, in the mutant background expression in the embryonic brain was neither reduced nor abolished, indicating tissue-specific autoregulation (Moses and Rubin, 1991).

By creating promoter-reporter fusion constructs, one can study promoter regions, isolating sequences necessary for the regulation of a particular gene. These sequences would provide a starting point for the isolation and identification of regulatory proteins.

1.4 THIS WORK

The Disco protein has been shown to regulate its own mRNA expression. In *disco* mutants, *disco* expression is downregulated in the optic lobe primordium, but in the presence of excess Disco, endogenous *disco* expression in the optic lobe primordium is upregulated (Lee et al., 1999). Two putative binding sites for Disco protein have been found within the *disco* locus, one upstream of the transcriptional start site, the other within the intron (Lee et al., 1999). One or both of these putative binding sites may be responsible for the regulation of *disco* expression in the optic lobe

primordium. It is also possible that this tissue-specific regulation may be due to other *cis*-binding transcription factors, or a combination of both, tissue specific transcription factors and Disco binding. Thus, the tissue specificity of Disco autoregulation may be due to tissue specific interacting proteins and/or the two putative Disco binding sites.

The first part of this thesis characterises *disco* gain of function mutants. These mutants were created by misexpressing *disco* at different stages in development and in various tissues using the GAL4-UAS system (Brand and Perrimon, 1993). The dominant phenotypes induced by ectopic expression of *disco* were in the embryonic peripheral and central nervous systems, as well as the larval and adult visual systems. Although these dominant phenotypes were not sufficient to suggest a function for Disco, they can be used in a second site mutagenesis screen to uncover enhancers or suppressors of the *disco* misexpression phenotypes. These enhancers and/or suppressors can then be used to identify proteins that may interact with *disco*.

In order to identify the transcription factors that may complex with Disco, it is important to identify *cis*-acting regulatory sequences responsible for *disco* expression. The 5' flanking sequence to the *disco* locus, which includes the putative Disco binding site, s120 (Lee et al., 1999) was studied. We wished to determine whether the s120 region was necessary or sufficient to drive Disco expression *in vivo* and whether ubiquitous expression of Disco protein, by way of a heat-shock promoter, would regulate *disco* expression in the optic lobe primordium. This was accomplished by fusing 7.2 kb of *disco* upstream sequence to a *lacZ* reporter and germ-line transforming the construct for *in vivo* study. Previous studies with a translational fusion

containing the s280 putative Disco binding site fused to a reporter gene suggested that this region alone was not sufficient to direct expression in the proper tissues (Lee, 1994). The expression patterns of the 7.8 kb promoter-reporter gene fusion in a wildtype background recapitulated that of wildtype *disco*. However, in the presence of excess Disco protein, the expression patterns were not dramatically different from those of the *disco* enhancer trap, indicating that the s120 putative Disco binding site alone, is not sufficient to confer tissue-specific autoregulation. Sequencing and analysis of the upstream region, confirmed the presence of the identified s120 binding site and uncovered several transcription factor binding site motifs. Thus, evidence suggests that tissue-specific autoregulation of *disco* expression requires binding by other transcription factors, other than Disco.

1.5 TWO OBJECTIVES

The work for this thesis was divided into two parts. The objective for the first part, was to establish whether Disco overexpression mutants would provide clues to its function. The overexpression mutants were created using the GAL4-UAS system and the mutant phenotypes characterised. The second objective was to determine whether the s120 putative Disco binding site and the surrounding upstream sequence were sufficient for tissue-specific autoregulation. A promoter-reporter construct was germ-line transformed and the expression patterns characterised. Results from both parts of the thesis are important for future studies identifying Disco regulatory proteins.

CHAPTER 2
Materials and Methods

2.1 FLY WORK

Drosophila melanogaster Stocks

All *D. melanogaster* fly stocks were maintained at room temperature on standard medium containing inactivated yeast, sucrose and agar, supplemented with fresh active yeast (Fleischmann Active Dry Yeast). Ten percent Tegosept M (methyl-*p*-hydroxybenzoate) in ethanol and propionic acid were added to the medium to reduce the growth of molds.

Fly Strains

Canton Special (CS): Wildtype line.

disconnected^{CS0.1S1} (C50.1S1): A *disco* enhancer trap line, in which the expression pattern of the β -galactosidase protein mimics that of the *disconnected* phenotype, due to the insertion of a P-element near regulatory elements of the *disco* locus (Cohen et al., 1991).

heat-shock disco/heat-shock disco (D/D): A homozygous line with a P-element insertion containing *disco* cDNA under transcriptional control of the heat inducible hsp70 promoter that forces the ubiquitous nuclear expression of *disco*.

yellow, white, disconnected², forked / XX (y, w, disco², f/XX): A line with an ethylmethane sulfonate induced allele of *disco*; also carries visible mutations for the genes *yellow* (*y*), *white* (*w*) and *forked* (*f*), which confer yellow bodies, white eyes and bristle morphologies.

yellow, white (yw): A line with visible mutations for the genes *yellow* (*y*) and *white* (*w*). *y* controls the melanotic pigment pattern of the cuticle of the adult fly and the pigmented mouth parts and denticle belts of the larval cuticle and *w* is involved in the production and distribution of pigments in the compound eyes and ocelli of adult flies, as well as the pigments in adult testis sheaths and larval Malpighian tubules.

yellow, white; scute/Curly of Oster, engrailed (yw; sc/CyO,en): A second chromosome balancer stock, in a *yw* background. Description of balancer and mutations can be found in Lindsley and Zimm (1992).

yellow, white; ; Stubble/Third Multiple 3, Serrate (yw; ; Sb/TM3,Ser): A third chromosome balancer stock, in a *yw* background. Description of balancer and mutations can be found in Lindsley and Zimm (1992).

A62-GAL4/TM3: An enhancer trap that directs the yeast GAL4 transcription activator expression to the posterior population of follicle cells of stage 10 oocytes (Gustafson and Boulianne, 1996). This line has a floating TM3 balancer.

A90-GAL4/TM3: An enhancer trap that directs GAL4 expression to nurse cell associated follicle cells of stage 10 oocytes (Gustafson and Boulianne, 1996). This line carries a floating TM3 balancer.

T80-GAL4/*Curly of Oster*: The T80 enhancer trap line directs GAL4 expression to third instar tissues: the brains, all imaginal discs and the salivary glands (Brand and Perrimon, 1993; Rorth, 1996). This particular line carries a floating second chromosome balancer.

***engrailed*-GAL4 (P[*en*-GAL4]):** A line in which the GAL4 transactivator is expressed under the control of the *en* promoter and is directed to the posterior of each of the 14 parasegments of older embryos (stages 12 to 16); also expressed in the developing ventral nerve cord, in specific subsets of neurons. Embryonic *engrailed* expression is extensive and complex due to its function as a segmentation and homeotic gene. Its expression begins very early in embryogenesis, just prior to gastrulation (stage 4/5) (Karr et al., 1985), and continues to the end of embryogenesis (Kornberg et al., 1985). The earliest ectodermal expression pattern consists of single cell wide stripes that transect the embryo and span from the anterior to the posterior of the embryo (DiNardo et al., 1985). These stripes, at germ band retraction (stage 12) become two to three cells wide and demarcate the anterior compartment of each parasegment (Figure 3) (DiNardo et al., 1985). Near the end of embryogenesis, *en* is also expressed in the labral, intercalary, ocular and antennal segments (DiNardo et al., 1985). In the CNS, early at stage 6/7 the ectoderm flanking the ventral midline expresses the engrailed protein, and by stage 14/15 (dorsal

Figure 3. Schematic Representation of GAL4 Patterns of Expression in the Embryo.

Several promoters were chosen to drive expression embryonically, at different stages of development. The diagrams are schematic representations of the onset of expression (first embryo) and the final pattern of expression (second embryo) (modeled after Hartenstein, 1993).

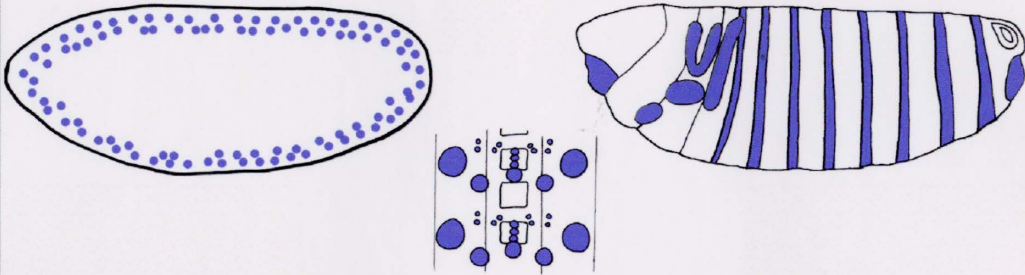
A. *en* expression is first encountered just prior to gastrulation (stage 4/5) (Karr et al., 1985). At the end of embryogenesis (stage 15/16), *en* is expressed in 14 stripes at the posterior margin of each parasegment and in the labral, intercalary, ocular and antennal segments, and (DiNardo et al., 1985). In the ventral nerve cord, *en* is expressed in specific subsets of cells per neuromere.

B. *sca* expression begins early in development (stage 5) in the neurogenic regions (Mlodzik et al., 1990). Eventually expression becomes restricted to neurons of the CNS and PNS (stage 15) (Mlodzik et al., 1990).

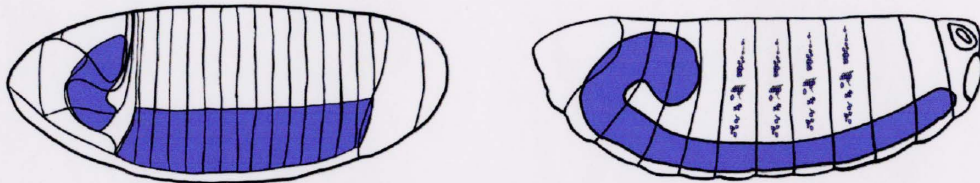
C. *elav* expression begins late in embryogenesis (stage 12) in all postmitotic neurons of the CNS and PNS and persists to the end of development (stage 15) (Robinow and White, 1988).

D. *ey* expression is detected in the CNS in groups of cells lateral to the midline, the optic primordia and the brain (Quiring et al., 1994; Hauck et al., 1999).

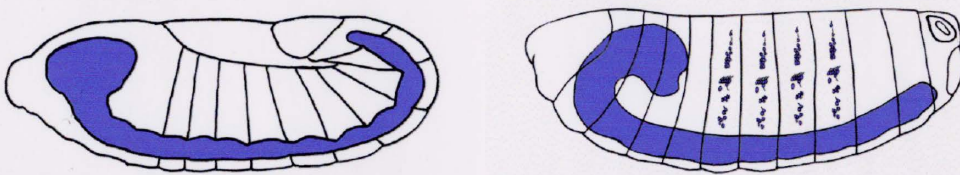
A en



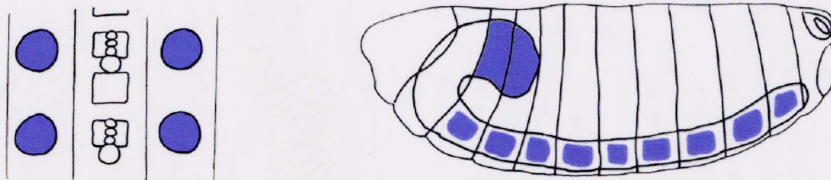
B sca



C elav



D ey



closure) a definite neuronal pattern is evident (Cui and Doe, 1992). Within each neuromere, *engrailed* is detected in several cell clusters: in bilateral posterior lateral (PL) groups and in the posterior intermediate (PI) groups (Figure 10C) (Cui and Doe, 1992). Also, two pairs of neurons located on either side of the midline and cells in the anterior region of the neuromere (NH cells) express the engrailed protein (Cui and Doe, 1992). In the midline, *en* is detected in three ventral unpaired midline cells (VUMs), the dorsal median cells (DM) and in two pairs of non-neuronal median support cells (MS) (Cui and Doe, 1992) (Figure 3, 10C).

***scabrous-GAL4* (P[*sca-GAL4*]):** The *scabrous* enhancer elements direct GAL4 expression to various neurogenic regions. Scabrous protein expression is first detected early in embryogenesis, in the neurogenic ectoderm and by early extended germ band stage (stage 8/9), expression is restricted to smaller neurogenic regions (Mlodzik et al., 1990). Neuroblasts (neuronal precursors) delaminate from the neuroectoderm and continue to express Scabrous through their differentiation to neurons of the CNS and PNS (Figure 3) (Mlodzik et al., 1990). In the third instar larval CNS, expression is seen in the brain lobes and in scattered cells along the ventral nerve cord (Mlodzik et al., 1990). Also, all imaginal discs contain *scabrous* expressing regions, neurogenic clusters, in which expression is later restricted to neural precursors (Mlodzik et al., 1990). Specifically, in the eye imaginal disc, expression is first detected anterior to the morphogenetic furrow, in groups of 7 to 10 cells. Posterior to the furrow, expression is restricted to R8 (Figure 4) (Mlodzik et al., 1990).

Figure 4. Schematic Representation of GAL4 Expression Patterns in the Eye Imaginal Disc.

A. Diagrammed is an eye-antenna imaginal disc from a third instar larva (modeled after Warren, 1993). Anterior (mouth hooks) is left, posterior (optic lobe anlagen) is right. MF indicates the position of the morphogenetic furrow. Columns indicates the number of ommatidial cluster columns between the morphogenetic furrow and the posterior end of the eye disc. Diagram is not to scale. Each colour shows the pattern of expression of a specific promoter-GAL4.

eyeless (green) is expressed in proliferating cells anterior to the morphogenetic furrow (Quiring et al., 1994).

scabrous (red) is expressed at the anterior edge of the furrow; posterior to the furrow *sca* expression is restricted to a single cell per cluster, the R8 precursor (Mlodzik et al., 1990).

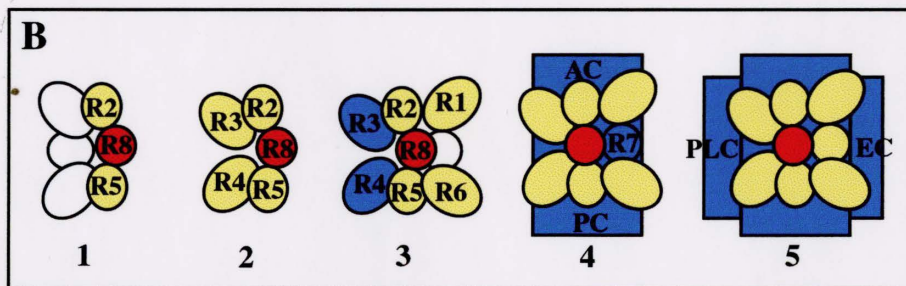
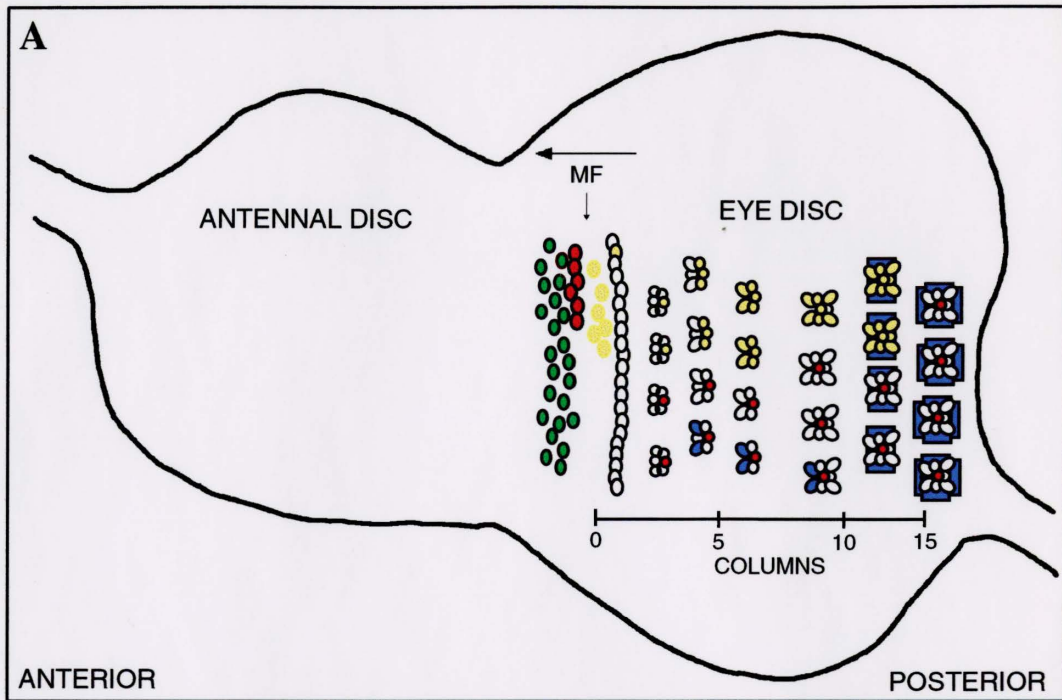
glass (*gmr*) (yellow) expression begins in the furrow, in all cells (Moses et al., 1989). Later expression persists in all cells, but is prevented from functioning in non-photoreceptor cells (Ellis et al., 1993).

sevenless (blue) is expressed posterior to the furrow in R3, R4 and R7, and in non-neural cone cells (Tomlinson et al., 1987). Expression begins in the fourth column of *sca* expressing cells.

Rh1 is expressed in the eye imaginal disc, but not until late pupal stages (Kumar and Ready, 1995).

B. Enlarged diagrams of a single ommatidial cluster as it differentiates (modeled after Warren, 1993).

1. 5 cell precluster: R8, R2/R5 have differentiated. *Sca* is expressed in R8. All cells are *Glass* expressing, but only R8, R2/R5 express active *Glass* protein.
2. 5 cell precluster: R3/R4 have differentiated and become *Glass* expressing cells. R8 remains the only *Sca* expressing cell. *Sev* protein is expressed in R3 and R4 and begins in the fourth column of *sca*-expressing cells (not shown).
3. 8 cell cluster: R1/R6 begin to express active *Glass* protein.
4. R7 has differentiated and become a *Glass* expressing cell. The anterior and posterior cone cells (AC, PC) have differentiated and are expressing the *Sev* protein.
5. At the final stage of differentiation, all photoreceptors are expressing *Glass*, including the *Sca*-expressing R8, and the polar and equatorial cone cells (PLC, EC) have begun to express *Sev* protein.



embryonic lethal, abnormal visual system-GAL4 (P[elav-GAL4]): In this line the GAL4 gene is expressed under the control of the *elav* enhancer, and is directed to all post-mitotic neurons of the CNS and the PNS (Figure 3). More specifically, *elav* expression begins early during embryogenesis, when the first neurons are born from the GMCs (stage 11), persists through the end of stage 16, and continues into both the larval and adult stages (Robinow and White, 1991). Larval expression consists of cell clusters in the PNS, the antennomaxillary complex, the frontal ganglia, BO and at the third instar stage, in all cells of the developing eye disc, posterior to the morphogenetic furrow (Robinow and White, 1991). In the embryonic optic lobe primordia, a single neuron close to the first optic chiasm (perhaps the OLP) produces the *elav* antigen, as do several other cells near the second optic chiasm (Robinow and White, 1991).

eyeless-GAL4 /Curly of Oster (P[ey-GAL4/CyO): The expression of the transcriptional activator GAL4 is directed in the *eyeless* pattern of expression. The *eyeless* gene is expressed during all three stages of *D. melanogaster* development. Embryonically, it is expressed in the brain, ventral nerve cord (Figure 3), eye-antennal disc precursor cells (Quiring et al., 1994; Hauck et al., 1999) and in BO (Sheng et al., 1997), although there is some dispute about expression in BO and the optic lobes (Daniel et al., 1999). In the larva, *eyeless* is expressed in the optic lobes, in spots along the ventral ganglion and most importantly, in the undifferentiated cells (anterior to the morphogenetic furrow) of the eye imaginal disc (Figure 4) (Quiring et al., 1994; Hauck et al.,

1999). *Eyeless* is also expressed in the differentiated photoreceptors of the adult head (Sheng et al., 1997).

***sevenless-GAL4/Curly of Oster* (P[*sev-GAL4*]/*CyO*):** The *sevenless* enhancer drives expression of GAL4 in differentiating photoreceptors R3, R4 and R7, and in lens-secreting cone cells of the eye imaginal discs (Figure 4) (Tomlinson et al., 1987; Bowtell et al., 1989). Expression of *sevenless* in the larval brain and in the adult retina has also been reported (Banerjee et al., 1987).

***Rh1-GAL4* (P[*Rh1-GAL4*):** A line with GAL4 expression directed to the photoreceptors, due to germ-line transformation with a fusion vector of Rh1-GAL4. The Rh1 rhodopsin gene, *ninaE*, is a terminal photoreceptor-specific gene expressed late during the pupal stage in the developing eye, in photoreceptors R1-R6 (O'Tousa et al., 1985; Zuker et al., 1985; Kumar and Ready, 1995).

***glass multimer reporter-GAL4* (P[*gmr-GAL4*):** This line contains a fusion vector in which the yeast GAL4 transcription factor is under the control of a pentamer of truncated *glass*-binding sites derived from the promoter of the *Drosophila* gene coding for *Rh1* (a photoreceptor specific opsin) (Hay et al., 1997). This *gmr-GAL4* confers a *glass* expression pattern, that is, the promoter drives expression in BOs, in BN growth cones, to a small group of cells in the embryonic brain and in the larval eye imaginal disc (Moses et al., 1989; Hay et al., 1997). The pattern of expression in the eye imaginal disc is restricted to

cells within the morphogenetic furrow and to all neural and non-neural cells posterior to the furrow (Figure 4) (Moses and Rubin, 1991). This line is maintained at 18°C to prevent a background eye phenotype.

***glass multimer reporter-P35* (P[*gmr-P35*]):** This line contains a fusion vector in which the baculovirus P35 protein is expressed in all photoreceptors in and posterior to the morphogenetic furrow, due to the multimerized glass binding sites derived from the *Drosophila* Rh1 promoter (Hay et al., 1994).

Upstream Activating Sequence-*tau-lacZ* (UAS-*tau-lacZ*): The Upstream Activating Sequence is bound by several GAL4 proteins thereby allowing transcription of the *lacZ* reporter construct (Hidalgo et al., 1995). Tau is a microtubule-associated protein, and fusing it to β -gal binds the reporter protein to microtubules for visualization of entire cells, including neurons and their axons (Callahan and Thomas, 1994). This particular line has multiple copies of the insert on both chromosomes two and three.

Genetics

To determine the chromosomal location of the UAS-*disco* insert in germ-line transformants, males from UAS-*disco* transformant lines were crossed to virgins from each balancer stock: *yw;Sc/CyO,en* and *yw;;Sb/TM3,Ser*. Individual progeny males and progeny virgin females with pigmented eyes that showed the balancer phenotype were crossed to *yw*

virgin females and males, respectively. In the progeny, the second and third chromosome inserts were identified because the genetic marker, *miniwhite*, segregated with the *CyO* and *Ser* balancers respectively.

GAL4 expression patterns were determined by crossing males from each insert line to UAS-*tau-lacZ* reporter virgins. Progeny embryos were analysed for β -galactosidase expression using anti- β -galactosidase antibodies.

Disco misexpression was done by crossing virgin females from each GAL4 line to males from the UAS-*disco* transformant lines. Progeny embryos, larva and adults were analysed for mutant phenotypes.

P35 rescue experiments were conducted by crossing males heterozygous for *gmr*-GAL4 or *ey*-GAL4 and UAS-*disco* to virgins homozygous for *gmr*-P35. 25% of progeny have one copy of each of the GAL4 driver, the UAS-*disco* responder and *gmr*-P35.

Embryo Collection and Staging

Adults aged from one to seven days were housed and allowed to lay eggs on fresh apple juice agar plates (60 g agar, 2 L deionized distilled water (ddH₂O), 66.6 g sucrose, 666 ml apple juice; 60 x 15 mm plates, Becton Dickinson, Catalog No. 35-3002). Embryos were collected on plates at room temperature from 9:00 am to 5:00 pm or overnight, from 5:00 pm to 9:00 am. For late stage embryos, plates with embryos were incubated at 18°C for at least 8 hours. At this temperature the rate of development slowed to half the normal rate. For younger embryos, collections were immediately placed at

4°C, a temperature at which development arrests. All embryo collections were stored at 4°C, for no more than 72 hours. Before fixation, the plates with embryos were removed from 4°C and left at room temperature for at least one hour. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Embryo Fixation

At 16 to 18 hours after egg lay (AEL) (stages 12 to 17) the chorion was removed from the embryos on the apple juice agar plates, by immersion in 50% sodium hypochlorite (bleach) for 3 to 5 minutes. The dechorionated embryos were washed from the apple juice agar plates with ddH₂O into open-ended scintillation vials, which had a nitex membrane at one end, and the embryos were then thoroughly rinsed. The embryos were collected off the nitex membrane and fixed in scintillation vials with 4.5 ml of 1X Phosphate Buffered Saline (1X PBS), 0.5 ml of 37% formaldehyde and 5 ml of heptane for 20 minutes at room temperature. To remove the vitelline membrane, the aqueous layer was replaced with methanol, and the embryos were aggressively shaken in this 1:1 heptane/methanol mixture for 30 to 60 seconds. Embryos that had not been dechorionated nor devitellinized, as well as the vitelline membranes, remained at the heptane/methanol interface and were removed along with the heptane. The fixed embryos, which had dropped to the bottom of the vial, were further dehydrated with several changes of methanol. Fixed embryos were transferred in methanol to

borosilicate glass culture tubes (Fisher Scientific, Catalog No. 14-961-26) and were either prepared for reacting with antibodies (see below) or stored in the methanol at -20°C for later use.

Heat-shock Induction

Embryos were collected on apple juice agar plates for 3 hours at room temperature and aged for 2 hours at 25°C . These embryos were subjected to a 45 minute heat-shock in a 37°C incubator and allowed to recover for 1 to 2 hours at 25°C before fixation or overnight aging at 18°C .

Wandering third instar larvae were collected into borosilicate glass culture tubes and subjected to a 45 minute heat-shock in a 37°C incubator. These larvae were then allowed to recover for one hour at 25°C , after which the brain lobes, attached eye-antennal imaginal discs and ventral nerve cords were removed.

Embryo Immunohistochemistry and X-gal Staining

Embryos stored in methanol were rehydrated with 1X PBT (0.2% Triton X-100 in PBS) with three 5 minute washes and a single 30 minute wash. Non-specific antibody binding was blocked by incubating fixed embryos in a 1:15 (vol/vol) mixture of PBT and Normal Goat Serum (NGS) (Cedar Lane Laboratories Ltd., Catalog No. CL-1200) for 30 minutes at room temperature.

Table 1. Antibodies Used For Immunolabeling.

Antibody and Source	Specificity	Location of Binding Antigen and Reference	Dilution
22C10 (S. Benzer)	mouse monoclonal	Axon cytoskeletal antigen of CNS and PNS (Fujita et al., 1982; Goodman et al., 1984; Zipursky et al., 1984).	1:10
24B10 (anti-CHAOPTIN) (H. Steller)	mouse monoclonal	Binds to a photoreceptor specific adhesion molecule, Chaoptin (Zipursky et al., 1984).	1:100
BP102 (C.S. Goodman)	mouse monoclonal	Binds to a surface antigen on most or all CNS axons (Seeger et al., 1993).	1:10
BP104 (anti-NEUROGLIAN) (C.S. Goodman)	mouse monoclonal	Binds to all neurons and axon tracts in the CNS and PNS (Hortsch et al., 1990).	1:2
anti-ENGRAILED (4D9) (C.S. Goodman)	mouse monoclonal	Binds to Engrailed expressed in the posterior of the 14 parasegments, and in the CNS in the (DiNardo et al., 1985; Patel et al., 1989).	1:1.5
anti-FASCICLIN II (1D4) (C.S. Goodman)	mouse monoclonal	Binds to axon fascicles of longitudinal connective axons, optic lobe and retina (Bastiani et al., 1987; Grenningloh et al., 1991).	1:20
anti-β-galactosidase (Cappel, Catalog No. 55976)	rabbit	Binds to β -galactosidase (<i>E. coli</i>).	1:200

anti-V5 (Invitrogen, Catalog No. R960-25)	mouse monoclonal	An antibody that binds to recombinant proteins containing the V5 epitope (14 amino acids).	1:5,000 recom.; 1:17000 used
Alexa488-conjugated Goat Anti-Rabbit IgG (Molecular Probes, Catalog No. A11008)	rabbit	Fluorophore with an excitation peak at 495nm and emission peak at 519nm.	1:300
Texas Red-conjugated Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc., Catalog No. 115-075-147)	rabbit	Fluorophore with an excitation peak at 596nm and emission peak at 620nm.	1:200
Horseradish Peroxidase (HRP) - conjugated Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Catalog No. 111-035-003)	rabbit		1:100

Horseradish Peroxidase (HRP) - mouse
conjugated Goat Anti-Mouse
IgG
(Jackson ImmunoResearch
Laboratories, Inc., Catalog No.
111-035-020)

1:100

Primary antibodies were added at the appropriate concentration (Table 1) and adsorbed for 4 to 6 hours at room temperature, or overnight at 4°C. The primary antibodies were washed out with three 5 minute, and four 30 minute washes with PBT (0.2% Triton), and nonspecific antibody binding was blocked again with goat serum. Secondary antibodies were added at the appropriate concentrations (Table 1) and incubations lasted for 2 to 4 hours at room temperature, after which the secondary antibodies washed out 3x 5 minutes, 4x 30 minutes with PBT (0.2% Triton).

Embryos labeled with fluorescent polyclonal secondary antibodies were mounted in 70% glycerol in PBS containing *p*-phenylenediamine (Sigma, Catalog No. P-6001). Horseradish peroxidase (HRP)-conjugated secondary antibodies were enzymatically reacted by immersing the embryos in 500 μ l of 0.5 mg/ml diaminobenzidine (DAB) for two minutes and adding 6 μ l of 3% hydrogen peroxide. The reaction was neutralized with the addition of 1X PBS and the embryos were then washed for 10 minutes with three consecutive changes of 1X PBS. Before immersing in methyl salicylate (Sigma, Catalog No. M-6752), the reacted embryos were dehydrated through an ethanol series (50%, 70%, 90%, 95% and two absolute ethanol washes). The reacted, dehydrated embryos were then mounted on microscope slides (VWR Carlab, Catalog No. 48323-185) in non-aqueous mounting medium Permount (Fisher Scientific, Catalog No. SP15-100) and covered with a micro cover slip (VWR Scientific, Catalog No. 48366 067 or 48366 205).

Embryos that were collected for reacting with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining were dechlorinated in 50% sodium hypochlorite for 3 to 5 minutes, washed with ddH₂O, transferred to

borosilicate tubes and fixed for 15 minutes in 2.5 ml of heptane and 2.5 ml of 2% paraformaldehyde. All heptane and fixative were removed and residual solutions washed away with PBT (0.2% Triton). The PBT was removed and the embryos were immersed in a solution (warmed to 65°C) containing 10 mM $\text{Na}_2\text{HPO}_4\cdot\text{NaH}_2\text{PO}_4$ (pH 7.2), 150 mM NaCl, 1 mM MgCl_2 , 3.1 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 3.1 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.3% Triton X-100 and 2% X-gal in dimethylsulfoxide (DMSO). After several hours of incubation at 37°C, the staining solution was rinsed away with PBT (0.2% Triton) and the embryos were mounted on microscope slides in 70% glycerol in 1X PBS.

Larval Brain Immunohistochemistry and X-gal Staining

The larval central nervous systems with attached eye-antennal discs were dissected from wandering third instar larvae in chilled 1X PBS and kept on ice. The specimens were fixed in 4% paraformaldehyde (pH 7.4) in PBS for 1 hour at room temperature, followed by an hour of PBT (0.1% Triton) washes with changes every ten minutes. Subsequently the specimens were blocked for non-specific binding for 1 hour in 1:15 of NGS:PBT. After blocking, primary antibody was added and adsorbed overnight at 4°C. The following day, brains were rinsed in PBT for 2 hours, with PBT changes every ten minutes, and blocked for nonspecific binding a second time. Incubations with HRP-conjugated secondary antibody were carried out for 6 to 8 hours at room temperature or overnight at 4°C. After washing the specimens for four hours in PBT, the HRP enzymatic reaction was carried out with DAB and 3%

hydrogen peroxide. The enzymatic reaction was neutralized with the addition of 1X PBS. For ten minutes the brains were washed with consecutive changes of 1X PBS and then mounted on microscope slides in 70% glycerol.

Brains to be stained for β -galactosidase activity were fixed for one hour in 4% paraformaldehyde pH 7.4. After the fixative was removed, the brains were rinsed several times with PBT (0.2% Triton) and incubated overnight at 37°C in X-gal solution. Brains with blue staining were rinsed several times with 1X PBS and mounted in 70% glycerol.

Detection of Cell Death

Larval eye-antennal imaginal discs were dissected, under minimal light, in a 2.5 μ g/ml solution of acridine orange dissolved in 1X PBS. Each dissected disc was immediately mounted on a microscope slide, viewed and photographed with Nomarski optics in a Zeiss Axioscope microscope.

Photomicroscopy

Tissues were viewed with Nomarski optics on a Zeiss Axioscope microscope and photographs were taken with a Zeiss MC100 camera using either TMAX100 black and white film (ISO 100) or Kodak Ektachrome 64T RTP colour film (ISO 64). Confocal microscopy was done using a Bio-Rad

MRC600 laser scanning confocal microscope equipped with a krypton/argon laser. Images were digitized and panels were assembled using Adobe Photoshop 5.0 software.

Environmental Scanning Electron Microscopy

Environmental Scanning Electron Microscopy (ESEM) of whole adult eyes was done by immobilizing etherized adult flies in a water-based colloidal carbon glue on an ESEM mount. The electroscan was performed at 20 kV and 3.0 Torr using the ESEM model 2020, manufactured by ElectroScan Corp (now Philips Electroscan). Micrographs were printed out on Polaroid Polapan 53 (ISO 800) film and saved as digital images.

2.2 DNA MANIPULATION

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in these studies are listed in Table 2. Bacterial strains were stored as permanent stocks in 80% glycerol at -80°C. Frozen stocks were recovered by dipping a sterile inoculating loop into a semi-thawed sample and by streaking it out on solid medium (described below). All plasmids were stored in sterile ddH₂O at -20°C.

Table 2. Bacterial Strains and Plasmids.

Strain or Plasmid	Genotype	Use and Source or Reference
<i>Escherichia coli</i>		
DH5 α	<i>SupE44 ΔlacU169 (ϕ80lacZΔM15) <i>hsdR17</i> <i>recA1 endA1 gyrA96 thi-1 relA1</i></i>	Wildtype, general use: transformations and plasmid preparations (Sambrook et al., 1989).
Plasmids		
pUAST	pUC8, amp ^r , SV40 and <i>miniwhite</i>	Vector for subcloning genes behind the GAL UAS (Ashburner, 1989).
pUAS- <i>disco</i>	pUAST containing 1.7 kb EcoR1 <i>disco</i> PCR fragment	This work.
p π 25.1	P element, region 17C of X chromosome, pBR322	Helper plasmid containing a P-element, used as a source of transposase (O'Hare and Rubin, 1983).
pCaSpeRAUG β galB K7.8	pUC8, amp ^r , SV40, <i>lacZ</i> , AUG, <i>white</i> gene, contains <i>disco</i> upstream 7.8 kb <i>Bam</i> HI- <i>Kpn</i> I fragment	(Lee, 1994)
pCaSpeR β galCN6.5	pUC8, amp ^r , <i>lacZ</i> , SV40, <i>white</i> gene, contains <i>disco</i> 6.5 kb <i>Cla</i> I- <i>Not</i> I fragment	(Lee, 1994)
pMT/V5-HisB	Metallothionein promoter, C-terminal V5 epitope tag	Invitrogen (Catalog No. V4120-20)

pMT/V5-HisB-
disco

pMT/V5-HisB containing 1.7 kb *disco* *EcoR*I
fragment

P. Pelka

Growth Conditions and Media

E. coli strains were grown in Luria-Bertani medium (LB) (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, 1 L deionized H₂O, pH 7.0) with the appropriate antibiotic. The antibiotics used for selection were: ampicillin (100 µg/ml) or kanamycin (25 µg/ml). For growth of single colonies on solid media, 12 g/ml of agar was mixed with the LB and poured into plates (100 x 15mm, Becton Dickinson, Catalog No. 35-1029). Bacterial strains were streaked out on LB-antibiotic plates. From these plates single bacterial colonies were used to inoculate small and large liquid cultures. Small cultures (3 to 5 ml), for small scale DNA isolations, were inoculated in 15 ml polypropylene tubes and larger cultures (500 ml to 1000ml), were inoculated in 2 L flasks. To ensure optimum growth and proper aeration, cultures were placed on a shaker at 37°C and grown for 16 to 24 hours. Bacteriophage P1 clones were inoculated in LB medium containing kanamycin (25 µg/ml) and isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM), which induces the lytic replicon to amplify the plasmid copy number, and grown for 16 to 24 hours at 37°C.

DNA Methodology

Small scale DNA isolations were performed using a mini alkaline-lysis procedure (Applied Biosystems, Inc.) without polyethylene glycol (PEG) precipitation. Large scale DNA preparations for sequencing purposes were

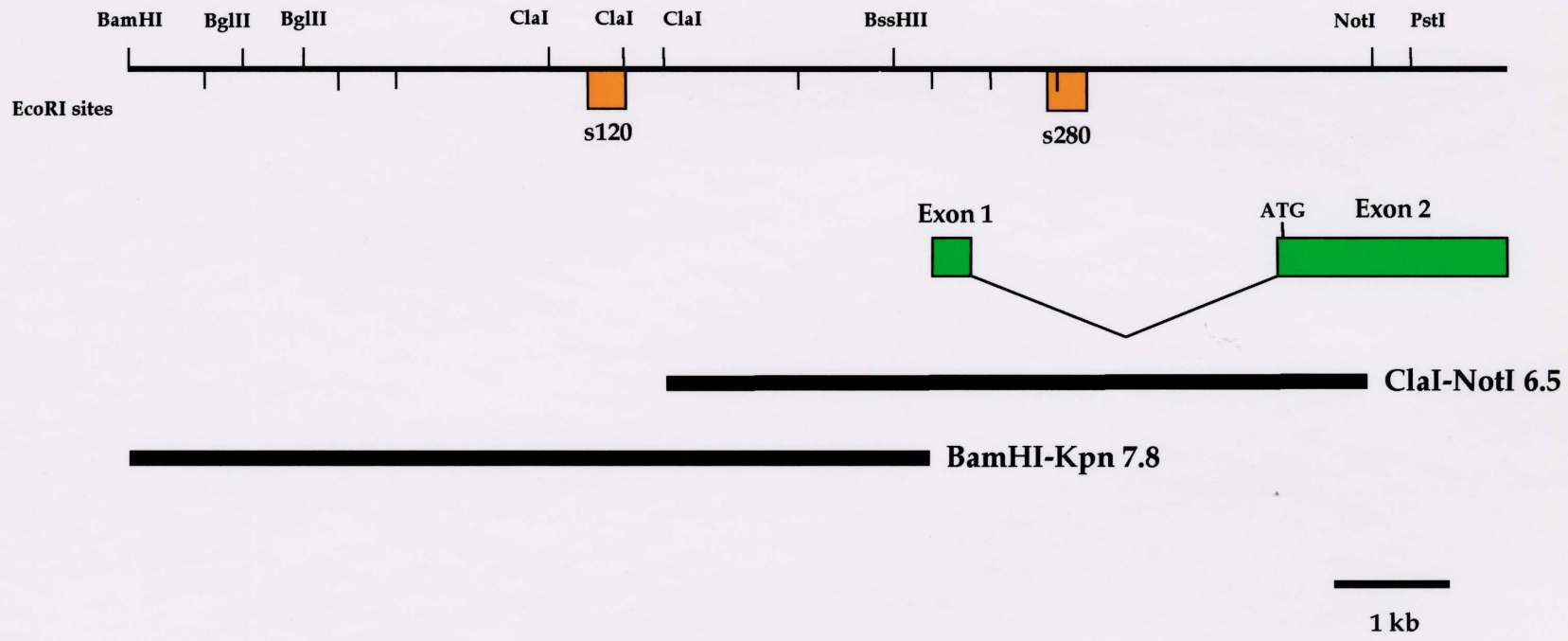
performed using the Qiagen QIAfilter Plasmid Maxi Kit (Catalog No. 12263) and the isolated DNA was resuspended in sterile ddH₂O. Endotoxin-free DNA for embryo microinjection was isolated using the QIAGEN EndoFree Plasmid Maxi Kit (Catalog No. 12362). Restriction enzymes and other DNA modifying enzymes were used according to manufacturers recommendations. Digested and/or modified DNA was electrophoresed in 0.7% agarose gels in Tris-acetate (TAE) buffer, containing ethidium bromide (0.5 µg/ml), and visualized with ultraviolet illumination on a FisherBiotech Variable Intensity Transilluminator FBTIV-816. DNA required for subcloning was extracted from agarose gels using the Qiagen QIAquick Gel Extraction Kit (Catalog No. 28704). DNA concentrations were calculated from optical density readings obtained using a UVIKON 930 Spectrophotometer at 260 nm.

Source and Construction of Plasmids

The *disco* promoter constructs were a generous gift from H. Steller. The pCaSpeR β -galCN6.5 plasmid is a “translational fusion” containing the *disco* transcription start site, the first exon, the intron and a portion of the second exon (Table 2, Figure 5) (Lee, 1994). The construct contains about 2 kilobases (kb) of upstream DNA sequence and the s280 *disco* binding site (Lee, 1994). The pCaSpeRAUG β -galBK7.8 promoter-*lacZ* fusion construct, is a “transcriptional fusion” (Lee, 1994). It contains 7.2 kb of upstream DNA,

Figure 5. Promoter-*lacZ* Fusion Constructs.

The horizontal line represents the genomic region surrounding the *disco* locus. The *EcoRI* sites are indicated by short lines below the genomic map. Other restriction sites are indicated by short lines above the map and are labeled. The two putative Disco binding sites, the s120 and s280, are indicated by boxes. The position of the *disco* transcription unit is also indicated. The solid boxes below the genomic map indicate the portion of the *disco* locus included in the two promoter-*lacZ* fusion constructs. The construct pCasSpeRAUG- β gal7.8 is a "transcriptional fusion" containing a *Bam-Kpn* 7 kb fragment of upstream sequence, including the s120 Disco binding site. The pCaSpeR β gal6.5 contains a *Cla-Not* fragment that includes the first non-coding exon and the intron. This "translational fusion" has only the s280 Disco binding site. (modeled after Lee, 1994)



which contains the *disco* promoter and the s120 *disco* binding site (Table 2, Figure 5) (Lee, 1994).

The UAS-*disco* plasmid was constructed for germ-line transformation. Two primers used to amplify a 1.7 kb fragment of the *disconnected* gene, were designed to contain an *EcoRI* site (underlined): primer AB6530 5'-CCC ACC ACA GAA TTC ATG GAG CAC-3' and primer AB6531 5'-TCG CCA TGG ATC AGA ATT CTG GAC-3' (Table 3). The 1.7 kb fragment, starting at the translation start site, was amplified from cDNA using the polymerase chain reaction (PCR), electrophoresed on a 0.7% agarose gel and extracted. The fragment was subcloned into *EcoRI* digested pUAST (Brand et al., 1994), to create the UAS-*disco* plasmid (Figure 2). Correct orientation of the *disco* insert was verified by restriction digests with *EcoRI* and *XhoI*.

Cell Transformation

100 μ l of thawed *E. coli* DH5 α competent cells (Hanahan, 1983) were added to a pre-chilled polypropylene tube. The cells were incubated for 30 minutes, on ice, with 10 μ l of a ligation mixture (a completed DNA ligation reaction mixture containing vector DNA, insert DNA, T4 DNA ligase and ATP). The cells were heat-shocked by incubation in a 42°C water bath for 90 seconds, and the tube was returned to the ice for an additional 2 minutes. 900 μ l of 1X LB was then added to the cells, and the mixture was incubated in a 37°C water bath for 45 minutes. 100 μ l of newly transformed cells were plated

Mannheim, Catalog NO. 1004 /00) and the 1.7 kb *disco* cDNA fragment extracted from an agarose gel. This probe was used to screen 13 bacteriophage P1's (from the Berkley Drosophila Genome Project, www.fruitfly.org) containing cloned genomic *D. melanogaster* DNA from the 14B and 14C regions.

on LB-amp plates and grown overnight at 37°C. From these plates, single bacterial colonies were used to inoculate liquid cultures.

Electrotransformation

40 μ l of electro-competent DH5 α bacterial cells (Table 2) were mixed with 2 μ l of UAS-*disco* DNA and added to 500 μ l of SOC medium (Sambrook, 1989) in a prechilled 0.1 cm electroporation cuvette (Bio-Rad, Catalog No. 165-2083). Using a Bio-Rad *E. coli* Pulser, a voltage pulse of 2.5 kV was applied to the cuvette containing the cells. The cells and DNA were subsequently incubated in a 37°C water bath for 45 minutes. 100 μ l of electroporated cells were plated on LB-amp plates and grown overnight at 37°C. Single colonies from the plates were used to inoculate liquid cultures.

P1 Clone Screening

A radiolabeled *disco* probe was created using the random-primed DNA labeling method (Random Primed DNA Labeling Kit, Boehringer Mannheim, Catalog No. 1004 760) and the 1.7 kb *disco* cDNA fragment extracted from an agarose gel. This probe was used to screen 13 bacteriophage P1's (from the Berkley Drosophila Genome Project, www.fruitfly.org) containing cloned genomic *D. melanogaster* DNA from the 14B and 14C regions.

DNA Sequencing

Sequencing of approximately 7 kb upstream genomic *disco* region and the *disco* intron was accomplished by bi-directional primer walking (single and double stranded). For efficient priming, most of the sequencing oligonucleotides were designed to be 18 to 24 nucleotides long with a melting temperature higher than 50°C and with a 3' GC clamp. Primers were generated by the MOBIX facility (The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University) and resuspended in sterile ddH₂O to a concentration of 50 pmoles/μl. Primer names, sequences and complement regions are listed in Table 3. 5 μl of 200 ng/μl template DNA was used per sequencing reaction and primers were used at a concentration of 1.0 pmoles/μl. Sequencing was performed by the MOBIX facility using an ABI 373 Stretch Automatic Sequencer with dye terminator chemistry and cycle sequencing. Sequences were assembled using the SEQUENCHER program, version 3.0 and restriction site analyzed using DNASTrider1.1. The BLAST 2.0 program was used to compare nucleotide and amino acid sequences against those already in the database at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

Table 3. Nucleotide Sequences of Primers.

These primers were used to bi-directionally sequence 7 kb of genomic DNA upstream of the *disconnected* locus, exon 1, the intron and part of exon 2.

Primer Name	Nucleotide Sequence (5' to 3')	Complement Region
AB14910	CTA ATT TAG CAG CGA GTT GTA	Complements to bp -404 to -384 from the predicted <i>disco</i> transcriptional start site
AB15129	CAT GTC GTC TGT GGT GGG TTT*	Complements to bp 3382 to 3362 from the predicted <i>disco</i> transcriptional start site
AB15710	CCA ACT AAT CGC TGT AAT CCA CTG	Complements to bp 2944 to 2967 from the predicted <i>disco</i> transcriptional start site
AB15960	CAG CAA TAA ACC AAA ATA CAA TAA AAT	Complements to bp 354 to 380 from the predicted <i>disco</i> transcriptional start site
AB16079	TGC GGA TGT GCG GAG AGC	Complements to bp -22 to -39 from the predicted <i>disco</i> transcriptional start site
AB16188	CGC TGC GTT CTC TTT GGC	Complements to bp -679 to -662 from the predicted <i>disco</i> transcriptional start site
AB16281	GAT GTC CGG ACC ATC GGC	Complements to bp 906 to 923 from the predicted <i>disco</i> transcriptional start site
AB16282	ATT GAT GCG CAA TTA GAG TGC	Complements to bp 3059 to 3039 from the predicted <i>disco</i> transcriptional start site
AB16431	TTC GAA GAA GTG GAT CTC TGC	Complements to bp -47 to -67 from the predicted <i>disco</i> transcriptional start site
AB16760	TGT GTT ATG AAA TAG GAA CGC	Complements to bp 563 to 583 from the predicted <i>disco</i> transcriptional start site
AB16761	CTT GGA AGA TAA AAA GGT ATA GGC	Complements to bp 1521 to 1544 from the predicted <i>disco</i> transcriptional start site

AB16762	TTA TCA TTT TGG TTC CTA TGC	Complements to bp 2425 to 2445 from the predicted <i>disco</i> transcriptional start site
AB16977	GGT CGC TTT TTA TGG GGC	Complements to bp 1825 to 1808 from the predicted <i>disco</i> transcriptional start site
AB16978	GCT CAC AAT TAC GGT AGC	Complements to bp 2126 to 2143 from the predicted <i>disco</i> transcriptional start site
AB16979	AAC GTT TCG TCC GAG AGC	Complements to bp 1215 to 1232 from the predicted <i>disco</i> transcriptional start site
AB17074	CAT ACA TAC TAG AAT TCG AGC	Primer designed for multiple cloning site of pCaSpeRAUG- β gal (Thummel et al., 1988), complements to bp 7715 to 7736 of this plasmid
AB17159	ACG TTT TTT GAT TAT CGG C	Complements to bp 1201 to 1219 from the predicted <i>disco</i> transcriptional start site
AB17160	CIT TTG GCG TGG CAA TGC	Complements to bp 2749 to 2766 from the predicted <i>disco</i> transcriptional start site
AB17161	CIT CCT GCA ACT GTG TGC	Complements to bp 1835 to 1852 from the predicted <i>disco</i> transcriptional start site
AB17250	<u>TCG</u> ACA GAA ATT GGG TGC**	Complements to bp 2409 to 2426 from the predicted <i>disco</i> transcriptional start site
AB17251	CCC CGA AAG GAT TCA AGC	Complements to bp -6372 to -6355 from the predicted <i>disco</i> transcriptional start site
AB17365	CTA ATC CCA TTG CCA TGC	Complements to bp -5806 to -5789 from the predicted <i>disco</i> transcriptional start site
AB17366	ACC GTC TTG ACT TTT CGC	Complements to bp 3023 to 3040 from the predicted <i>disco</i> transcriptional start site
AB17667	TTG ACA CAT GCG ACA TGC	Complements to bp -591 to -608 from the predicted <i>disco</i> transcriptional start site
AB17667	TTG ACA CAT GCG ACA TGC	Complements to bp -591 to -608 from the predicted <i>disco</i> transcriptional start site

AB17738	TTT GCG AGT ACG CAA AGC	Primer designed for multiple cloning site of pCaSpeRβgal (Thummel et al., 1988); genomic read started at bp -2344 and continued to bp -1669
AB17743	CTC AAC GCA ATT TAT GGT AGC	Complements to bp -5249 to -5229 from the predicted <i>disco</i> transcriptional start site
AB17897	AGA AGC CTT AAA GCT TAA AGC	Complements to bp -1880 to -1860 from the predicted <i>disco</i> transcriptional start site
AB17898	GCA CCA ATG GAA TTC GAG C	Complements to bp -1157 to -1175 from the predicted <i>disco</i> transcriptional start site
AB17899	TGT GCA ATA TCC AAG GGC	Complements to bp -4604 to -4587 from the predicted <i>disco</i> transcriptional start site
AB18060	TCA GTT AAA GTT AGG TCA AGC	Complements to bp -3993 to -3973 from the predicted <i>disco</i> transcriptional start site
AB18075	CAA GGT GTC CTC AAA TTG C	Complements to bp -2270 to -2288 from the predicted <i>disco</i> transcriptional start site
AB18145	TGG ATA ACT TCC CAT GGC	Complements to bp -3416 to -3399 from the predicted <i>disco</i> transcriptional start site
AB18559	GGT CCG AGA TCC GAG ACG	Complements to bp -2870 to -2887 from the predicted <i>disco</i> transcriptional start site
AB18560	CGG ATC TCG GAC CTC G	complements to bp -2867 to -2882 from the predicted <i>disco</i> transcriptional start site

*, ** underlined nucleotide corresponds to a single base pair mismatch, in each case, should be A

2.3 EMBRYO MICROINJECTION (GERM-LINE TRANSFORMATIONS)

DNA Preparation

DNA for embryo microinjection was isolated using the QIAGEN EndoFree Plasmid Maxi Kit and was resuspended in injection buffer (5 mM KCl, 0.1 mM PO₄ pH 7.8, (Ashburner, 1989)). The helper plasmid p π 25.1 was resuspended to a concentration of 100 μ g/ml and the defective P-element vector was resuspended to a concentration of 600 μ g/ml in one injection cocktail.

Needle Preparation

Microinjection needles were pulled from borosilicate glass capillary tubing (FHC, Catalog No. 30-30-0) using the Narishige PB-7 Micropipette Puller. Needles were pulled using the two stage pull method (described in Narishige Instruction Manual), and the points beveled by breaking the tip against another needle while viewing under the microscope.

Egg Collection

Synchronous egg collections from the host *yw* strain were done under dark and calm conditions, on grape juice agar plates (8 ml grape juice, 45 ml

water, 1.8 g agar (Sigma); 60 x 15 mm Becton Dickinson plates), with a dab of yeast paste in the center. Embryos were collected for 30 minutes, dechorionated in 50% sodium hypochlorite, washed with water from the grape juice agar plates into open ended scintillation vials, which had nitex membrane at one end, and then thoroughly rinsed. The eggs were lined up on a slab of 1% agar with a paint brush, such that the tapered anterior pole with the micropyle was closest to the right edge of the agar. The eggs were then transferred to a strip of double-sided sticky tape on microscope slide. To make room for DNA suspended in injection buffer, the eggs were desiccated by placing the slide in a petri dish filled with Drierite (anhydrous calcium sulfate) for 5 to 15 minutes. After desiccation the eggs were covered with a layer of Halocarbon oil, Series 700, to prevent the injected DNA from leaking out, as well as to keep the eggs from further dessication.

Injection and Post Operative Care

Injection of DNA into the posterior end of the eggs was done before pole cell formation ensued. This was done under an inverted microscope, Leitz DM IL, and using the Leitz Micromanipulator M and a needle holder. An air pressure system was used to force the injection buffer carrying the DNA out of the needle and into the posterior end of the egg.

After injection, the eggs/embryos were kept in a moist environment, under halocarbon oil, and allowed to develop at 18°C. Surviving embryos hatched and the larvae were picked from the slides two and three days

following injection. They were placed 10 to a vial containing standard medium and allowed to reach adulthood (at 25°C). Any adults that emerged with coloured eyes were presumed to have undergone germ-line transformation and were immediately crossed back to the host *yw* strain. To establish isogenized lines, progeny with coloured eyes were once again mated with the *yw* strain. Then brother and sister crosses were done to establish homozygous lines.

Determination of chromosomal location of the P-element inserts was previously described under the heading "Genetics".

CHAPTER 3

Results

3.1 ECTOPIC EXPRESSION OF DISCO

Construction of p[UAS-*disco*]

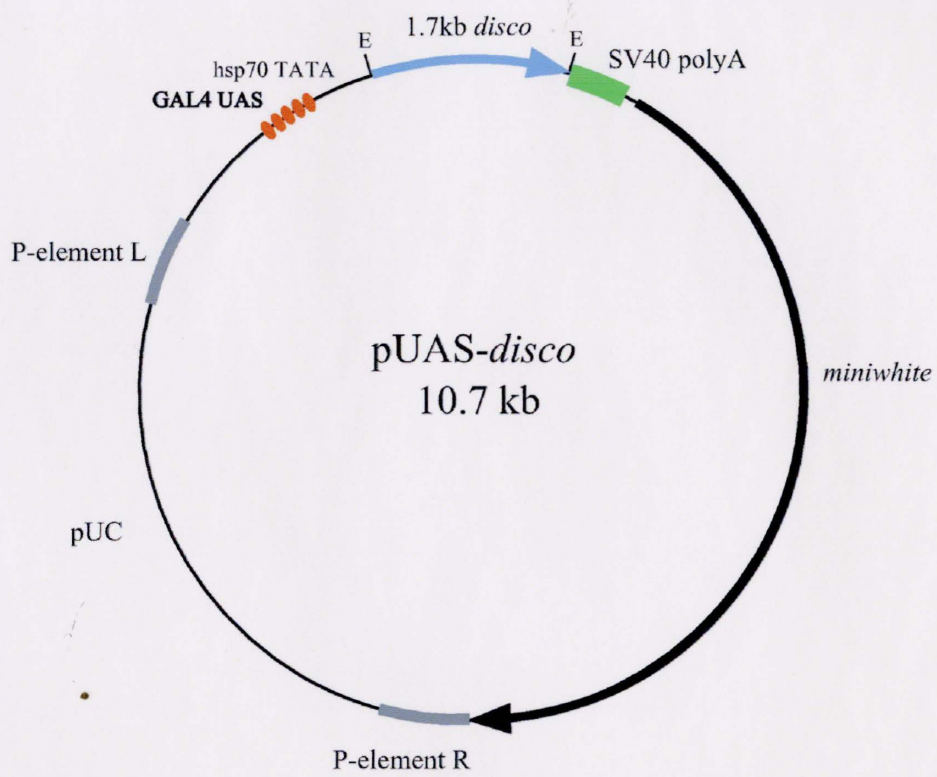
A 1.7 kb fragment spanning the 5' end of the *disconnected* locus was amplified by PCR from cDNA. The fragment, containing the complete ORF but omitting the untranslated region (UTR), was cloned into the *EcoRI* site of the polylinker in pUAST (Brand et al., 1994), creating the plasmid pUAS-*disco* (Figure 6). pUAST is a P-element defective vector into which sequences are subcloned behind the Upstream Activating Sequences (Brand et al., 1994), a tandem array of five optimized GAL4 binding sites. By sequence analysis it was concluded that the 1.7 kb fragment began at the *disco* translational start site and included the entire protein coding sequence (568 amino acids), without any mutations.

P-element Mediated Transformation

Incorporation of UAS-*disco* into the *Drosophila* genome was done by P-element mediated germ-line transformation as described by Rubin and Spradling (1982). The pUAS-*disco* construct was injected into the host fly strain *yw* and the defective P-element was mobilized with the transposase provided by the co-injected helper plasmid $\text{p}\pi 25.1$ (Table 2, O'Hare and Rubin, 1983). These endotoxin-free DNAs were microinjected into 2157 embryos.

Figure 6. Structure of pUAS-*disco*.

The 1.7 kb coding sequence of the *disco* gene was cloned into the *EcoRI* site of the multiple cloning site in the vector pUAST. pUAST contains five optimized GAL4 binding sites, a *hsp70* TATA box and transcriptional start site. An SV40 small t intron and polyadenylation sites are also present (Brand et al., 1994). This construct was used for P-element mediated germ-line transformation.



From the injected embryos, 124 fertile adults (G_0 generation) were obtained, which when backcrossed to the host strain, gave rise to 8 red-eyed flies – germ-line transformants (G_1). The estimated transformation frequency was 8 adults of 2157 injected eggs (0.4%) or 6.5% of fertile adults (Table 4). Each of the 8 red-eyed G_1 transformants was crossed again to yw to generate 26 red-eyed G_2 flies. These red-eyed G_2 flies were once again crossed back to yw and the progeny (G_3) mated amongst themselves in brother-sister crosses. To homozygote each of the 26 lines, they were inbred for several generations. The sequence of steps followed to create isogenized lines is shown in Figure 7.

Table 4. Transformation with pUAS-*disco*.

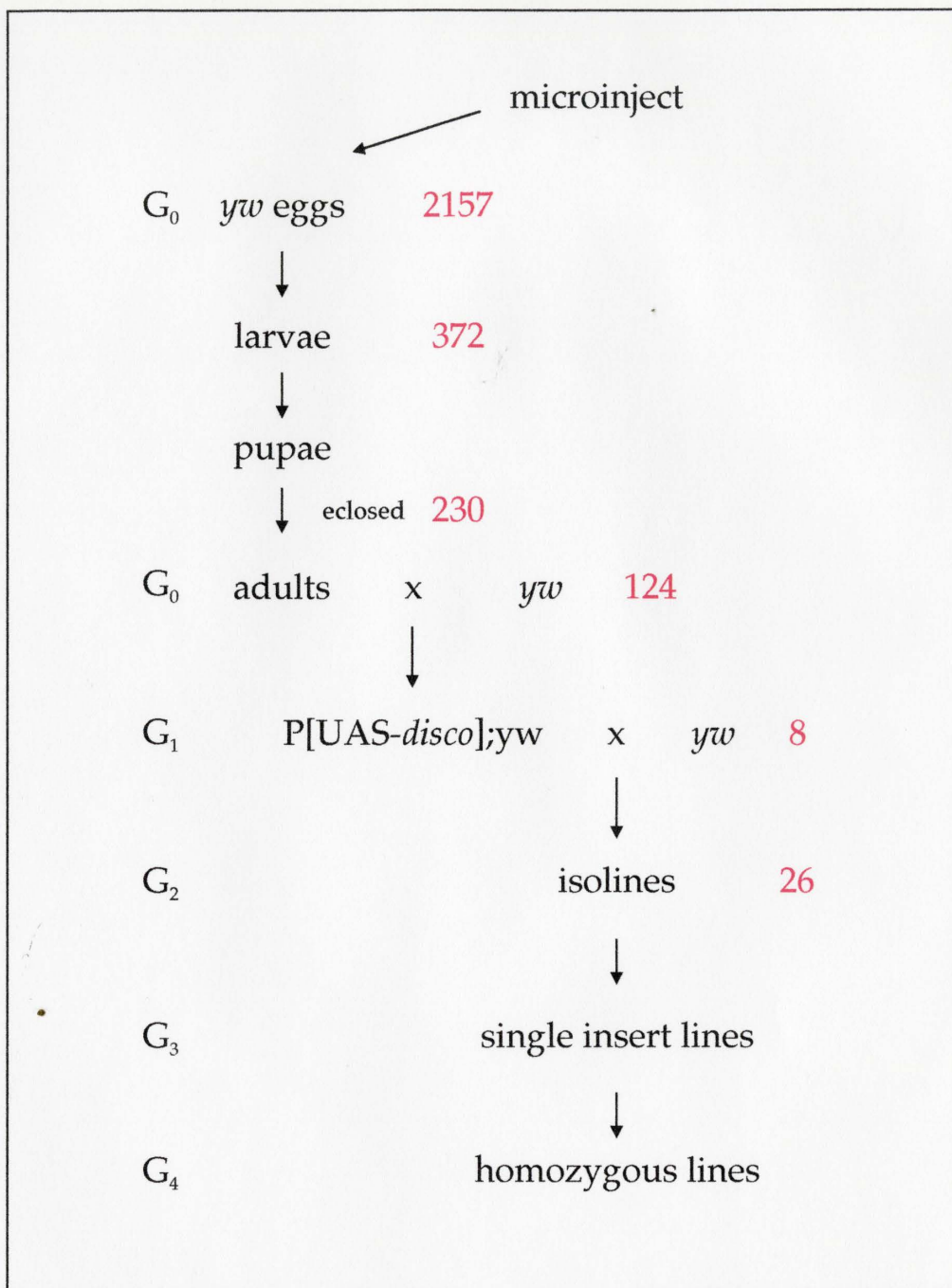
Injected	Hatched	Eclosed (G_0)	Fertile	G_1	Transformed(G_2)	Transformation Frequency
2157	372	230	124	8	26	6.5% (26/124)

The UAS-*disco* Transformant Lines

Chromosomal location of P-element insertions was determined using balancer chromosomes. Of the 8 parental lines, 10 resulted in independent transformation events (Appendix A), none of which were lethal insertions. Visual inspection of each transformant line and several ESEM micrographs (data not shown), indicated that size, shape and overall structure of the

Figure 7. Sequence of Steps Followed to Isogenize Transformant Lines.

To create homozygous transformant lines, a series of post injection crosses were done. The G_0 generation are the injected yw embryos. Those that survived to adulthood were backcrossed to the yw host strain. Progeny from these crosses, G_1 , which had red-eyes were germ-line transformants and were crossed to yw to generate more red-eyed progeny, G_2 . These G_2 transformants were considered isolines. Isolines were crossed one last time to the yw strain to generate siblings for inbreeding (G_3). By G_4 the single insert lines may be homozygous stocks. (Spradling, 1986)



compound eyes are similar to those in wildtype, although pigmentation is variable amongst the transformant lines. All other adult structures observed were like wildtype. Transformant line number 46 (T₄₆) was used most often, with UAS-*disco* inserted on the third chromosome. T₄₆ was examined for PNS, CNS and adult eye phenotypes, which were all found to resemble wildtype. Random sampling of the transformant lines did not provide evidence of background phenotypes. Therefore, based on normalcy of the UAS-*disco* transformant line T₄₆ and observations of several other lines, it was concluded that none of the transformant lines have a background phenotype.

The Expression Patterns of the GAL4 lines

A library of GAL4 lines (Table 5) was assembled such that ectopic expression of the *disconnected* gene would be directed to a variety of tissues at various times in development using the GAL4-UAS system (Brand and Perrimon, 1993). The specific embryonic expression patterns of several of the GAL4 drivers were characterized by crossing the GAL4 line to a UAS-reporter gene line (UAS-*tau-lacZ*) and visualizing β -galactosidase expression using an anti- β -galactosidase antibody. Other promoter-GAL4 expression patterns were assumed to be similar to the expression pattern of the genes themselves. In total, ten different enhancer/promoter-GAL4 carrying fly lines (Table 5) were crossed to transgenic UAS-*disco* lines and a detailed description of each GAL4 expression pattern was previously given.

Table 5. GAL4 Line Library.

Each GAL4 line was used to ectopically express Disco protein using the GAL4-UAS System.

GAL4 LINE	Chromosome	Expression Pattern	Reference	Source
A62/TM3	III	Posterior follicle cells of st.10 oocyte.	(Gustafson and Boulianne, 1996)	G. Boulianne
A90/TM3	III	Nurse cell associated follicle cells.	(Gustafson and Boulianne, 1996)	G. Boulianne
T80/CyO	II	Embryonic expression; All discs, larval brain, salivary glands.	(Brand and Perrimon, 1993; Rorth, 1996)	Bloomington Drosophila Stock Center
<i>engrailed</i>	II	Neuroblasts of st. 8-10, continues in posterior of each hemisegment; expression in cells of the CNS.	(DiNardo et al., 1985)	C.S. Goodman
<i>scabrous</i>	II	Neurogenic regions, neurons and glia; R8 cells in eye disc.	(Baker et al., 1990; Mlodzik et al., 1990)	C.S. Goodman
<i>elav</i>	III	All postmitotic neurons of CNS and PNS.	(Robinow and White, 1988; Lin and Goodman, 1994)	C.S. Goodman
<i>eyeless/ CyO</i>	II	Embryonic ventral nervous system; anterior portion of eye disc.	(Quiring et al., 1994)	Novartis Corp.
<i>gmr</i>	II	All neural and non-neural cells posterior to furrow of eye disc.	(Ellis et al., 1993; Hay et al., 1997)	H. Steller
<i>sevenless/ CyO</i>	II	Punctate embryonic expression; R3, R4, R7 and nonneural cone cells.	Battye, R. pers. comm.; (Tomlinson et al., 1987; Bowtell et al., 1989)	Novartis Corp.
<i>Rh1</i>	II	R1-R6 at late pupal stage.	(Zuker et al., 1985; Kumar and Ready, 1995)	C. Desplan

i) T80-GAL4

Males from the T80-GAL4 enhancer trap line were crossed to UAS-*tau-lacZ* virgin females to observe the GAL4 embryonic pattern of expression. When reacted with X-gal, embryonic β -galactosidase expression was observed, however the precise pattern of expression was not determined.

ii) *en*-GAL4

The *en*-GAL4 line crossed to UAS-*tau-lacZ* directed β -galactosidase expression to ectodermal cells in the anterior compartment of each of the 14 parasegments (Figure 8A). The pattern of expression was visualized with anti- β -galactosidase antibody.

iii) *sca*-GAL4

The *scabrous*-GAL4 directed β -galactosidase expression from the UAS-*tau-lacZ*, visualized with anti- β -galactosidase antibody, to the intersegmental boundaries of stage 15/16 embryos (Figure 8B). This is pattern of expression similar to that of *en*-GAL4 crossed to UAS-*tau-lacZ*.

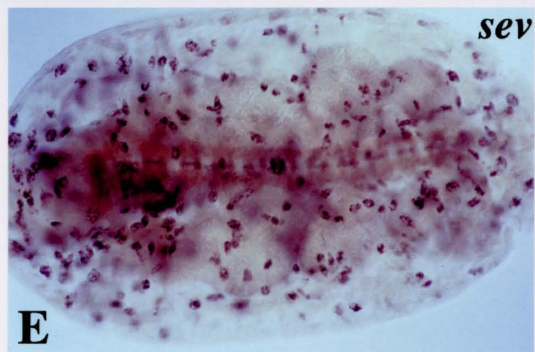
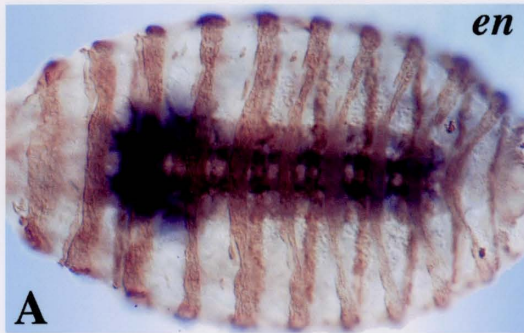
iv) *elav*-GAL4

Anti- β -galactosidase antibody staining shows that the *elav*-GAL4 directs β -galactosidase expression to all post-mitotic neurons in both the PNS and CNS (Figure 8C).

Figure 8. Embryonic Expression Patterns of GAL4 Lines.

Whole mount preparations of stage 15/16 embryos. GAL4 expression patterns were visualized by antibody labeling of β -gal expression (and subsequent HRP immunohistochemistry) in progeny of the GAL4 line crossed to UAS-*tau-lacZ*. Anterior is left. Work done by Robin Battye.

- A. *en*-GAL4 is expressed in the posterior of each parasegment.
- B. *sca*-GAL4 is expressed similarly to *en*-GAL4, at the intersegmental boundaries.
- C. *elav*-GAL4 drives expression to all postmitotic neurons of the PNS and CNS.
- D. *ey*-GAL4 expression is seen in groups of cells lateral to the ventral nerve cord and in the brain lobes.
- E. embryonic expression of *sev*-GAL4 is punctate and not in any specific cell type.



v) *ey*-GAL4

The *eyeless*-GAL4 driver used, is located on the second chromosome and balanced with *CyO*. When crossed to UAS-*tau-lacZ*, embryonic β -galactosidase expression was visualized with anti- β -galactosidase antibody in the brain and in the CNS in groups of cells lateral to the ventral nerve cord (Figure 8D).

vi) *sevenless*-GAL4

From the *sev*-GAL4, embryonic β -galactosidase expression was characterised to be punctate throughout the late stage embryo (Figure 8E), as visualized by the anti- β -galactosidase antibody.

Generalities About the GAL4 Lines

Each GAL4 line was observed for background phenotypes. Embryonic phenotypes were characterized by the organization of the peripheral and central nervous systems at stage 15/16 of development. Monoclonal antibodies (MAb) 22C10 (Fujita et al., 1982; Goodman et al., 1984) and MAb BP104 (Hortsch et al., 1990) were used to label PNS neurons, dendrites and axons. CNS axon tracts were labeled with MAb BP102 (Seeger et al., 1993). Both the PNS and CNS phenotypes of the following GAL4 lines were confirmed to be indistinguishable from wildtype: T80, *en*, *elav*, *sca* and *sev*

(Table 6). The *ey*-GAL4 is a lethal insertion that is balanced with a second chromosome balancer, *CyO*. Some mild background embryonic phenotypes were seen (Table 6), but were attributed to embryos homozygous for the balancer chromosome. Larval brains and eye-antennal imaginal discs of *sca*-GAL4 and *sev*-GAL4 were labeled with MAb 24B10 (Zipursky et al., 1984). Both GAL4 lines showed normal photoreceptor development. The adult phenotypes of all lines, except *gmr*-GAL4, resembled that of wildtype. The compound eyes of *gmr*-GAL4 adults raised at room temperature were found to have a background phenotype of rough eyes. However, this phenotype was absent when the line was reared at 18°C.

Each GAL4 line was crossed to UAS-*disco* transformant line T46 and at least one other transformant line, to verify that the observed phenotypes were not a result of the UAS-*disco* insertion disrupting a required developmental gene. Effects of *disco* overexpression on the development of the embryonic PNS, CNS and adult compound eye were observed. Effects on viability were also noted and are described.

Table 6. Mutant Phenotypes of Various GAL4 Lines and UAS-*disco* Combinations (Penetrance).

GAL4 Line	ANTIBODY	CONTROL %* (phenotypes/n §)	x UAS- <i>disco</i> %** (phenotypes/n §)
T80	22C10	0 (0/16)	55.1 (27/49)
	BP104	0 (0/15)	73.3 (11/15)
	BP102	0 (0/16)	72.7 (16/22)
	24B10	0 (0/3)	0 (0/23)
<i>en</i>	22C10	ND	75 (9/12)
	BP104	5.9 (1/17)	66.7 (14/21)
	BP102	6.3 (1/16)	30.8 (8/26)
	4D9 PNS	ND	22.2 (6/27)
	4D9 CNS	ND	26.3 (5/19)
<i>sca</i>	22C10	0 (0/13)	87.5 (21/24)
	BP104	ND	63.5 (33/52)
	BP102	0 (0/15)	81.3 (26/32)
	24B10	0 (0/5)	0 (0/16)
<i>elav</i>	22C10	0 (0/13)	44.4 (8/18)
	BP104	18.8 (3/16)	42.9 (27/63)
	BP102	0 (0/16)	15.0 (9/60)
<i>ey/CyO</i>	22C10	31.3 (5/16)	35.7 (10/28)
	BP104	23.5 (4/17)	48.1 (13/27)
	BP102	13.3 (4/30)	20.3 (13/64)
	24B10	ND	83.3 (10/12)
<i>gmr</i>	24B10	ND	23.0 (3/13)
<i>sev/CyO</i>	22C10	7.1 (1/14)	50.0 (8/16)
	BP104	ND	50.0 (20/40)
	BP102	0 (0/15)	34.5 (10/29)

* GAL4 line alone scored for background phenotypes

** Progeny of GAL4 line crossed to UAS-*disco* scored for mutant phenotypes

§ Percentage of embryos with nervous system defect, followed by the number of mutant embryos/total number scored in brackets.

ND Not Determined

Effects of Disco Ectopic Expression on CNS Development

The ventral nerve cord consists of axonal tracts arranged in a ladder-like array. The 'rungs' of the ladder are the anterior and posterior commissures, which are joined together by contralateral longitudinal connectives (Figure 9A). This arrangement and any perturbances to it, were visualized by labeling with MAb BP102.

i) T80-GAL4

Embryos in which the enhancer trap T80-GAL4 was driving embryonic expression of Disco were scored for CNS defects. Figure 9B shows a representative CNS phenotype of the T80-GAL4;UAS-*disco* progeny. 16 of the 22 embryos examined (72.7%, Table 6) had a defect in at least one neuromere. In general, the defects consisted of weakened or absent longitudinal tracts and defasciculation of axons from commissures into the intercommissural space. The defects encountered were visible either within a single neuromere, between neuromeres or in many neuromeres. On average, four neuromeres were affected per embryo, although the number of neuromeres affected differed between embryos.

ii) *en*-GAL4

Defects in the embryonic CNS of *en*-GAL4 driving UAS-*disco* to *en*-expressing neuroblasts were not completely penetrant. The majority of embryos developed with a typically wildtype ventral nerve cord (data not

Figure 9. Ectopic Expression of Disco Affects CNS Development.

Frontal views of whole mount preparations of stage 15/16 embryos. CNS tracts were labeled using MAb BP102 and subsequent HRP immunohistochemistry. Anterior is up. Scale bar, 31.7 μm .

A. The ladder-like array of axonal tracts in the CNS in wildtype embryos consists of the anterior and posterior commissures (ac and pc). The ac, pc and neighbouring neuromeres are joined together by the longitudinal connectives (lc).

B. T80-GAL4 directed Disco expression. Aberrant axons are observed in the intercommissural space. The longitudinal connectives are weakened and occasionally missing.

C. A minority of embryos showed CNS defects when Disco protein was expressed in the *en* pattern. Commissures and connectives are weakened and missing, and they are laterally displaced from the midline.

D. *sca*-GAL4 driving Disco expression caused difficulty in the formation of commissures and connectives. Growth cones avoided crossing the midline and weak longitudinal connectives formed. There is also lateral displacement from the midline.

E. Embryos where Disco had been misexpressed to all postmitotic neurons, by *elav*-GAL4, did not develop properly leading to perturbances in the development of the CNS. In this specific example, the posterior part of the VNC did not develop.

F. *ey* directed expression of Disco affected development, consequently perturbing the development of the CNS. The growth cones that extended seem to have no direction.

G. An overall reduction in axonal growth was seen in embryos expressing Disco in a *sev* expression pattern. Weak commissures and connectives formed.

shown). Only 8 of 26 scored embryos (30.8%, Table 6) had a malformed ventral nerve cord with lateral displacement from the midline in several neuromeres, and deterred growth of commissures and connectives (Figure 9C). Of 38 embryos observed for *engrailed* expression using MAb 4D9, 9 embryos (24%, Table 6) suffered severe developmental defects – these embryos also appeared twisted (data not shown). The majority of embryos showed wildtype *engrailed* patterns of expression, both in the ectoderm and in the CNS (Figure 10B, D).

iii) *sca-GAL4*

A high penetrance of mutant CNS phenotypes occurred in embryos from the *sca-GAL4* UAS-*disco* cross. *Disco* was misexpressed in neuroblasts and neurons of the embryonic CNS. Deformities in the CNS were seen in 26 of 32 embryos (81.3%, Table 6). Figure 9E shows the observed mutant phenotypes: fused and/or missing commissures, weak or missing longitudinal connectives and their lateral displacement from the midline. The occurrence of the displacement phenotype was quite common, for example 17 of the 26 mutant phenotype embryos (65%) suffered displacement from the midline at a single or several neuromeres.

iv) *elav-GAL4*

elav-GAL4 directed *Disco* expression to post-mitotic neurons of the CNS. The development of the CNS proceeded undisturbed, with embryos of late stage 16 displaying a typical wildtype CNS (data not shown). A small

Figure 10. Expression of Engrailed Protein Remains Like Wildtype.

(A and B) Whole mounts of stage 15/16 embryos labeled with MAb 4D9 (anti-engrailed) with subsequent HRP immunohistochemistry. Anterior is left, dorsal is up. Scale bar, 50 μm .

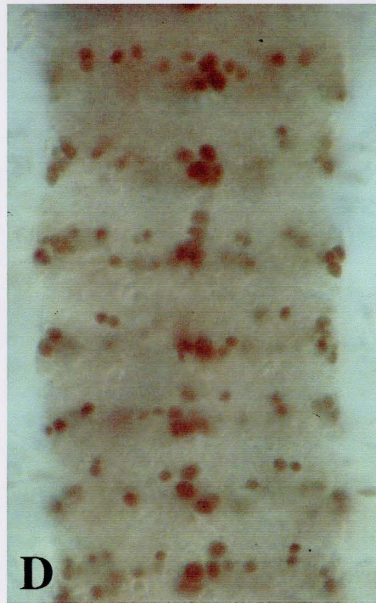
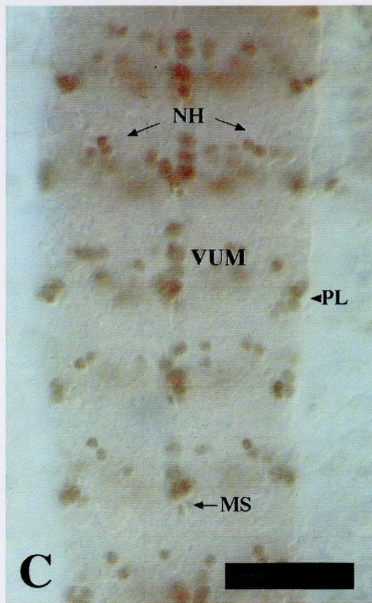
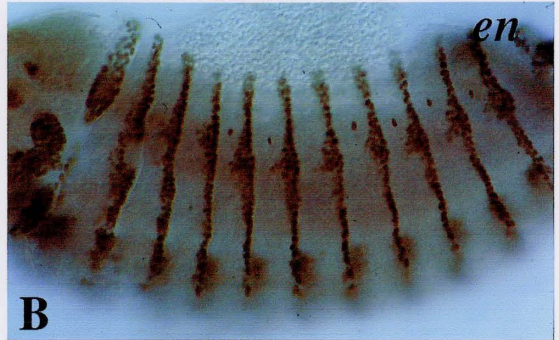
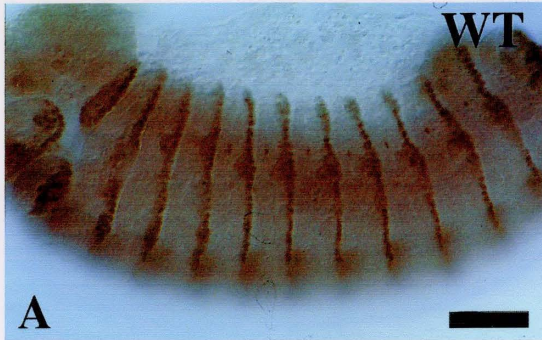
A. Wildtype embryo shows the Engrailed pattern of expression, one to two cells wide, in the posterior of each parasegment.

B. In embryos where Disco was expressed in the *engrailed* pattern of expression, in the majority Engrailed expression resembles that of wildtype.

(C and D) Frontal view of stage 15/16 embryos labeled with MAb 4D9 (anti-engrailed). Anterior is up. Scale bar, 31.7 μm .

C. Specific cells in the CNS express Engrailed: two pairs of NH neurons, two pairs of MS cells, three VUMs, PL and PI groups (Cui and Doe, 1992).

D. All Engrailed-expressing cells seem to be present in embryos where Disco was expressed under the *engrailed* promoter.



percentage, 15% (n=60, Table 6), showed some defects in the general anatomy of the ventral nerve cord (Figure 9E). While one embryo had a slight commissure formation defect in a single neuromere, others showed lateral displacement of the CNS from the midline and still others were contorted embryos with only partial CNS formation (data not shown).

v) *ey-GAL4*

Driving *disco* expression to groups of cells lateral to the ventral nerve cord using the *eyeless* promoter caused some CNS defects. The majority of 64 scored embryos developed like wildtype (data not shown) and only 13 (20.3%, Table 6) showed a secondary phenotype of malformed ventral nerve cords. Of these 13, several embryos were twisted and the others developed ventral nerve cords with severely misrouted axon tracts. An array of axon tracts developed resembling a web and not a ladder (Figure 9F).

vi) *sev-GAL4*

Disco misexpression by *sev-GAL4* to random cells in the embryo had a marked effect on CNS development. Of the 29 embryos scored, 10 (34.5%, Table 6) were found with defects in the CNS (Figure 9G). The phenotypes observed ranged from deformed embryos with a minimal CNS, to embryos with some semblance of a ventral nerve cord, consisting of neuromeres with partial commissures and some longitudinal connectives. However, most ventral nerve cords lacked both the commissures and connectives.

Expression of Disco, driven by T80, *sev* and *sca*-GAL4s, resulted in mutant CNS phenotypes, consisting of ventral nerve cords with absent axonal tracts and lateral displacement from the midline. Low penetrance of mutant CNS phenotypes occurred when *en*, *elav* and *ey*-GAL4s directed Disco overexpression. These CNS mutant phenotypes were attributed to the embryos having difficulty developing.

Effects of Disco Ectopic Expression on the Development of the PNS

The embryonic peripheral nervous system develops in a stereotypical manner that is repeated in all segments. Neurons are arranged into dorsal, lateral and ventral clusters (Figure 11A). This cluster pattern and any perturbances to it were visualized with MABs 22C10 and BP104, which bind to specific antigens present on the surface of PNS neurons.

i) T80-GAL4

Disco expression driven by the enhancer trap T80-GAL4 had profound effects on PNS development. 27 of 49 embryos (55.1%, Table 6) with neurons labeled with MAb 22C10 showed defects in the PNS, and 11 of 15 embryos (73.3%, Table 6) labeled with MAb BP104 showed similar PNS defects. Taken together, of 64 embryos scored for PNS perturbances, 38 (59.4%, Table 6) displayed a mutant phenotype. A variety of phenotypes was observed in T80;UAS-*disco* peripheral nervous systems, and included: i) meandering axon tracts between clusters within a segment, ii) misrouting of the ISN to

Figure 11. Ectopic Expression of Disco Alters PNS Development.

Whole mount preparations of stage 15/16 embryos, stained for the presence of 22C10 or BP104 antigens with subsequent HRP immunohistochemistry. Lateral views, anterior to the left. Scale bar, 31.7 μm .

A. In a wildtype (WT) embryo, PNS neurons are arranged in a highly organised manner. They are arranged into three clusters: the dorsal cluster (d) of 12 neurons, the lateral cluster (l) also of 12 neurons and the ventral clusters (v and v') of 9 and 11 neurons, respectively.

B. T80-GAL4 driving embryonic Disco expression leads to a PNS phenotype where the ISNs are misrouted, not only within segments but also between segments.

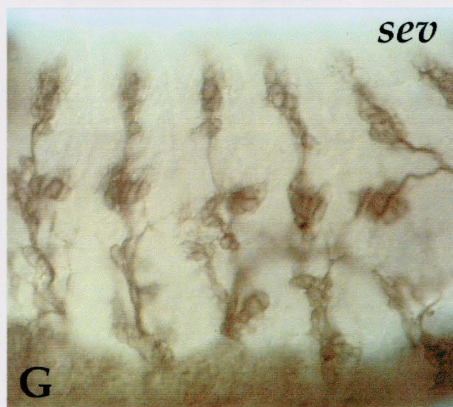
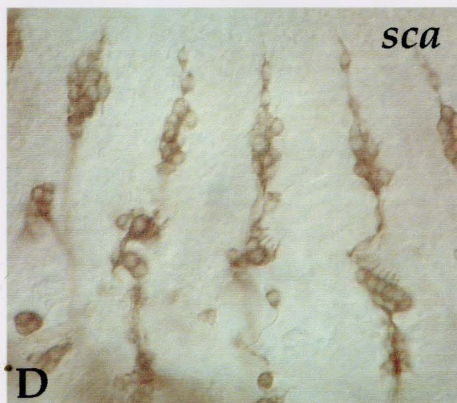
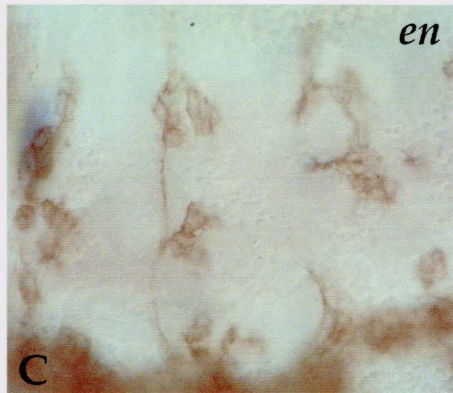
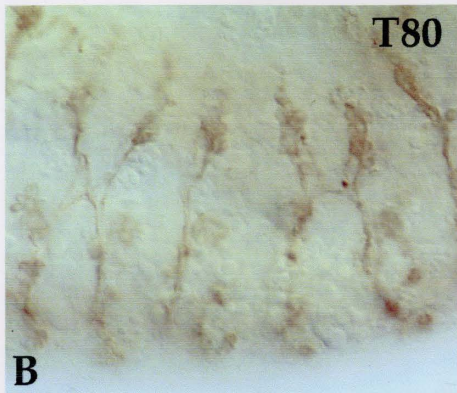
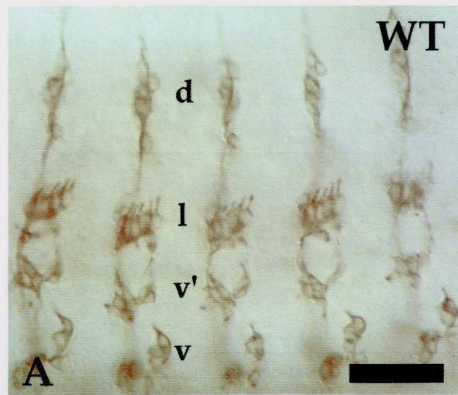
C. Driving Disco early in development (stage 4/5) using the *en*-GAL4 caused morphological defects, and consequent perturbations in the PNS: neurons developed but not in the appropriate places or numbers.

D. Expression of Disco protein in the *sca* pattern had mild effects on the PNS: some detouring of ISNs and slight disorganization of clusters.

E. ISN pathfinding difficulties and misplaced neuron clusters were the consequences of expressing Disco protein with the *elav* driver.

F. Embryos where *ey*-GAL4 was driving UAS-*disco* showed general developmental difficulties, leading to PNS perturbations.

G. Disco protein expressed in a *sev* expression pattern caused PNS abnormalities, such as cluster mislocation and aberrant ISNs.



clusters in neighbouring segments, iii) misplacement of entire neuronal clusters and iv) misplacement of single neurons. Figure 11B shows the ISNs of neighbouring segments criss-cross between the dorsal and lateral clusters. No obvious lack or overabundance of peripheral neurons was observed and the neurons displayed normal morphology in all embryos.

ii) *en-GAL4*

Flies from the *en-GAL4* line were crossed with those of a UAS-*disco* transformant line and the collected embryos expressed *disco* under the control of the *en* promoter. Thus, Disco expression was directed to ectodermal cells in the anterior compartment of every parasegment. 33 embryos were scored for PNS defects and 23 (~70%, Table 6) had developed deformed and twisted. In these embryos, the basic segmentally-repeated PNS pattern was not recognizable. Only fragments of the PNS developed with a concomitant decrease in the number of neurons (Figure 11C).

iii) *sca-GAL4*

Several different UAS-*disco* transformant lines were crossed to the *sca-GAL4* line, in order to direct Disco expression to neuroblasts and neurons of the PNS. Of the 66 examined *sca-GAL4*;UAS-*disco* embryos, 54 (81.3%, Table 6) displayed a perturbed PNS, with phenotypes ranging from mispositioned single neurons and whole clusters, to ISN pathfinding problems, between clusters within a segment and between segments (Figure 11D). There was no obvious lack or abundance of neurons and their morphology was normal.

iv) *elav-GAL4*

Overexpression of the *disco* gene panneurally by the *elav-GAL4* driver caused an assortment of phenotypes in the embryonic PNS. These mutant phenotypes were not completely penetrant; only 44.4% of embryos (n=81) had PNS defects (Table 6). The observed phenotypes ranged from mild to severe; some embryos had subtle pathfinding defects and in others, entire neuronal clusters were not situated in the appropriate locations. The neurons themselves seemed morphologically normal and in wildtype numbers. Figure 11C provides an example of an *elav-GAL4;UAS-disco* embryo where axons are shown leaving their fascicle and crossing segment boundaries.

v) *ey-GAL4*

The *eyeless* promoter driving *disco* expression caused minor PNS defects. These were not observed in 50% of the embryos as one may expect by Mendelian predictions, but in only 41.9% (n=55, Table 6). Some embryos had mild misrouting of the ISN (Figure 11F), but others (7 of 16 phenotype embryos) showed extreme developmental problems and had a twisted appearance (data not shown). These phenotypes differed from those encountered as background in the *ey-GAL4* line.

vi) *sev-GAL4*

Embryonic punctate misexpression of Disco protein using the *sev-GAL4* driver resulted in embryos with defective PNSs. The number of

embryos with phenotypes coincided with the Mendelian expectation; 50% of examined *sev*-GAL4;UAS-*disco* embryos had a mutant PNS phenotype (Table 6). Figure 11G shows an example of the encountered phenotypes – an ISN crosses segmental boundaries to fasciculate with axons from the wrong cluster and one of the lateral clusters did not develop like wildtype. Other embryos showed simple pathfinding difficulties (detouring ISNs), as well as misplaced neuronal clusters. Morphologically, the neurons resemble wildtype.

In summary, misexpression of Disco in various tissues at different times in development did not directly affect the development of the PNS. The overexpression with each GAL4 line caused general developmental problems and secondary PNS phenotypes. The mutant PNS phenotypes predominantly consisted of misrouted ISNs and misplacement of single neurons or neuronal clusters.

Overexpression of Disco in the Eye Imaginal Disc

The development of photoreceptors and their retinal projections can be visualized by MAb 24B10, which recognizes a photoreceptor-specific antigen. Normal development consists of organised ommatidial clusters in the eye disc, retinal projections R1-R6 synapsing in the lamina, and R7 and R8 synapsing in the medulla (Figure 12A). The LON traverses the eye-antenna disc to the OL, and makes a synaptic connection deep in the central brain. A

Figure 12. Effects of Disco Ectopic Expression in the Eye Imaginal Disc.

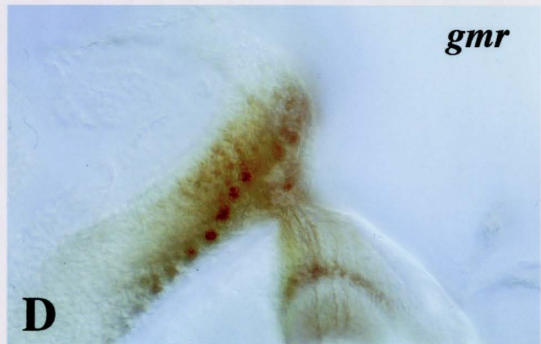
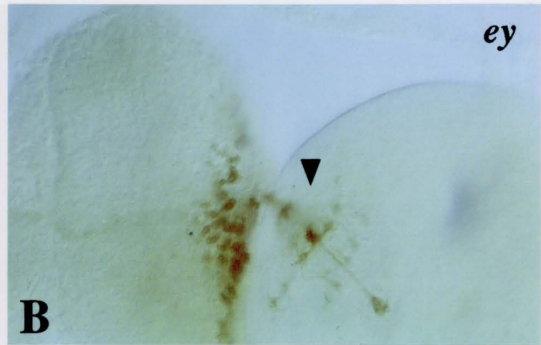
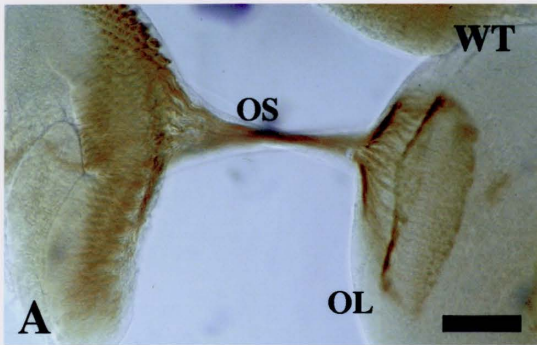
Whole mounts of late third instar eye discs, optic stalks and optic lobe anlagen labeled for the Chaoptin antigen (MAb 24B10) with subsequent HRP immunohistochemistry. Scale bar, 50 μ m.

A. Wildtype expression of Chaoptin shows the ordered arrangement of developing photoreceptors, their axons fasciculating down the optic stalk (OS) and innervating the first two optic ganglia of the optic lobe (OL). R1 to R6 terminate in the first ganglion, the lamina, and R7 and R8 terminate in the second ganglion, the medulla.

B. Ectopic expression of *disco* by the *ey* promoter to the proliferating cells ahead of the morphogenetic furrow, reduced the number of developing photoreceptor cells, with a concomitant reduction in the number of axons synapsing in the optic lobe.

C. *sca* directing Disco protein expression to the eye imaginal disc had no effect on the development of the photoreceptor neurons and their axons.

D. *gl* driven Disco expression to the photoreceptor cells had no effect on the general development of the photoreceptor cells, although not clear in the specimen of the figure.



disturbance in the development of the compound eye can be easily visualized.

i) T80-GAL4

The T80-GAL4 enhancer trap is reported to drive expression to all imaginal discs. Brains and the attached eye-antennal imaginal discs from T80-GAL4;UAS-*disco* third instar larvae were dissected and stained with MAb 24B10. All 23 specimens showed normal development of the visual system: BN was present, photoreceptors were developing as a regular array, retinal projections followed the same pathway as the LON and synaptic connections were made in the appropriate ganglia in the optic lobes (data not shown). However, no conclusions can be drawn, as some or all of the dissected larvae may not have been of the T80-GAL4;UAS-*disco* genotype.

ii) *ey*-GAL4

ey-GAL4 directed *Disco* expression to the undifferentiated cells anterior to the morphogenetic furrow. Each of the surviving adult flies displayed an eye phenotype, which ranged from slightly rough and smaller eyes (Figure 13B, F), to a complete absence of one or both eyes (data not shown). The most frequently occurring phenotype was smaller and slightly rough eyes (63 to 100% of phenotype progeny), whereas absence of eyes occurred only in none to 37% of the phenotype progeny. However, in all the mutant eyes the overall structure of individual ommatidia remained like the wildtype (compare E, F in Figure 13).

Figure 13. Compound Eye Phenotypes Due to Ectopic Disco Expression.

Environmental Scanning Electron Micrographs show the compound eye phenotypes when *disco* was ectopically expressed in the eye imaginal disc.

(A, B, C and D) Live whole fly mounts. Anterior is up, dorsal is to the left. Scale bar, 250 μm .

A. Wildtype eye with an organized, smooth ommatidial array.

B. Disco expression driven to the undifferentiated cells of the eye imaginal disc by *ey-GAL4*, leads to eyes reduced size or completely absent.

C. *gmr* driving Disco expression to the eye imaginal disc, within and posterior to the furrow, causes a rough eye phenotype. Eye size is normal, but pigmentation is lost. Bristles are misplaced and lenses are fused.

D. Expression of Disco in *sca* expressing cells leads to a rough eye phenotype similar to *gmr;UAS-disco*. Size of the eye remains like wildtype but pigmentation is lost, bristles are misplaced and lenses are fused.

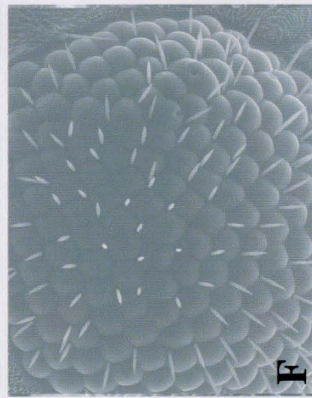
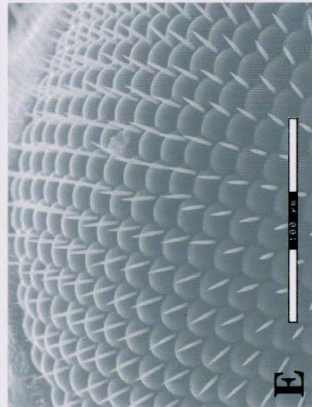
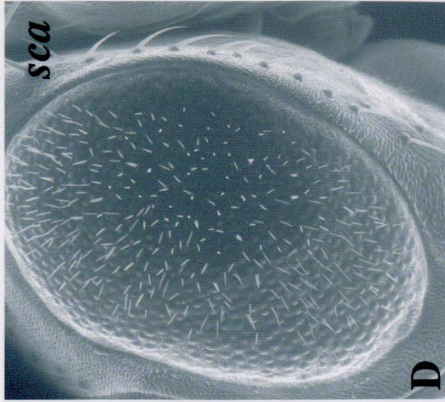
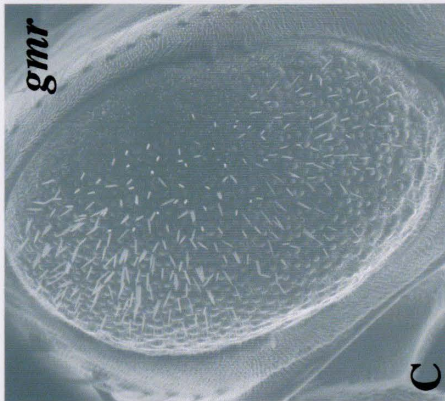
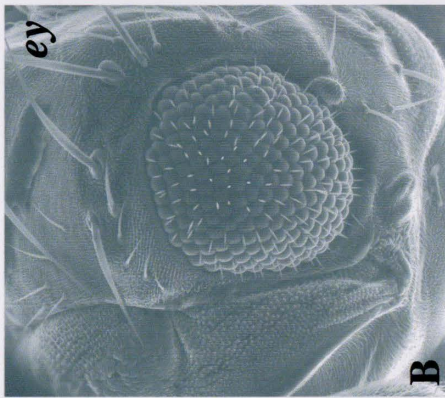
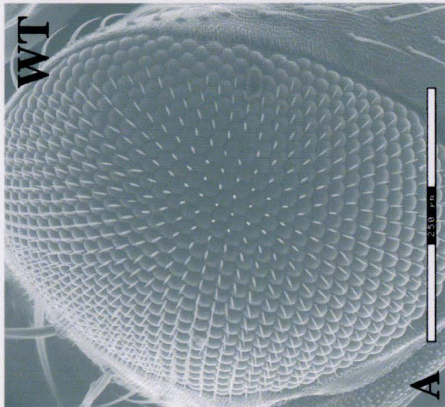
(E, F, G and H) Magnifications of A, B, C and D. Scale bar, 100 μm .

E. Smooth, organized wildtype ommatidial array.

F. Disco expressed anterior to the MF by the *eyeless* promoter, has no effect on the organization of individual ommatidia which resemble wildtype.

G. *gmr;UAS-disco* compound eyes are rough and disorganized.

H. *sca;UAS-disco* compound eyes are also rough and disorganized.



The phenotype of small or missing eyes suggested that *disco* expression anterior to the morphogenetic furrow caused cell death in the eye imaginal disc. When cells die, they fragment into small pieces, which can be identified by staining with acridine orange (AO). Thus, acridine orange staining of the eye-antennal discs was conducted to assess cell death. All dissected eye-antennal discs (n=60) appeared to be of normal size and shape. Results from AO staining of eye imaginal discs were inconclusive, since the amount of cell death was not obviously excessive. Labeling the eye-brain complexes with MAb 24B10 showed that BN was present and connected to the brain lobes in all specimens (n=12). However, BN appeared to be longer than its required length in some instances. In the eye discs very few photoreceptor neurons developed (Figure 12B), but of those that did, their axons followed the LON and synapsed in the optic lobe. These defects in the developing adult visual system were encountered in 10 of 12 examined brains (Table 6).

iii) *sca-GAL4*

Disco misexpression to the eye imaginal disc by *sca-GAL4* resulted in adults, all with the same mutant phenotype – a rough compound eye (Figure 13D). From the magnified micrograph (Figure 13H) several things are obvious: interommatidial sensory bristles are displaced and/or missing, many smaller bristles of unknown origin are present and the lenses secreted by cone cells appear to be fused. However, the eye itself remains similar in size and shape to wildtype. There is also a loss in pigmentation of the eyes that can be seen with the naked eye, but is not seen in the micrographs. All other adult

appendages and bristle patterns appear normal, from the wings to the thorax, to the bristles surrounding the eyes.

MAB 24B10 labeling of *sca*-GAL4;UAS-*disco* CNSs with attached eye-antennal imaginal discs revealed that the photoreceptors, their axons and BNs had all developed like wildtype. Thus, ectopic expression of *disco* in the neurogenic regions in the eye disc did not affect the overall organization of the photoreceptors or their retinal projections.

iv) *gmr*-GAL4

A similar to *sca*-GAL4;UAS-*disco* compound eye phenotype was seen in adults where *gmr*-GAL4 directed *Disco* expression in the eye disc. Adult progeny with misexpression of *disco* to cells posterior to the morphogenetic furrow developed with rough compound eyes. The eyes were colourless and rough due to displaced or missing sensory bristles and fused lenses (compare D, H and C, G in Figure 13). MAB 24B10 staining (Figure 12D) revealed that in 10 of 13 specimens the larval eye disc and retinal projections to the optic lobe developed like that in wildtype (Table 6). In the remaining 3 specimens, BN traversed the eye-antennal disc, entered the optic lobe and meandered around its target in the central brain before synapsing (data not shown).

v) *Rh1*-GAL4

This GAL4 line directed *Disco* expression to photoreceptors of the eye disc during pupal development. All adult progeny that emerged from *Rh1*-GAL4 crossed to UAS-*disco*, displayed an overall wildtype phenotype.

Lethality Due to Disco Misexpression

i) A62-GAL4

Disco expression driven by the A62 enhancer trap resulted in progeny that did not survive to the adult stage, as only first or second larval instars were observed (Table 7). Supporting evidence of lethality was provided from a cross of A62-GAL4 and UAS-*disco* T₁₉, where only 5 adults with a *serrate* wing phenotype emerged, indicating that progeny with A62-GAL4 do not survive (Table 7).

Table 7. Lethality of Various GAL4 Lines and UAS-*disco* Combinations.

GAL4 Line	x UAS- <i>disco</i> , T ₄₆	x UAS- <i>disco</i> *
A62	larval lethal	T ₁₉ - larval lethal
A90	larval lethal	T ₃₂ - larval lethal
T80	lethal	T ₄ - lethal
<i>en</i>	embryonic lethal	T ₂₉ - pupal lethal
<i>sca</i>	lethal/eye phenotype	T ₃₆ - lethal/eye phenotype
<i>elav</i>	embryonic lethal	T ₄ - adult escapers
<i>ey</i>	lethal/eye phenotype	T ₁₆ - lethal/eye phenotype
<i>gmr</i>	eye phenotype	T ₃₂ - eye phenotype
<i>sev</i>	lethal	T ₁₆ - lethal
<i>Rh1</i>	no phenotype	T ₃₆ - no phenotype

* random UAS-*disco* line

ii) A90-GAL4

Progeny of the A90-GAL4 enhancer trap driving *disco* expression from UAS-*disco* T₄₆ did not survive past the first or second larval instar stage (Table 7). In a cross with UAS-*disco* T₃₂, only 4 adults with a *serrate* phenotype emerged (Table 7). This result provides supporting evidence that a single copy of the A90-GAL4 driving *disco* expression is lethal.

iii) T80-GAL4

All adult progeny that emerged from the T80-GAL4 UAS-*disco* cross had the curly wing phenotype. This indicates that progeny carrying a single copy of the T80 enhancer trap and a single copy of UAS-*disco* did not survive to the adult stage (Table 7). However, due to the inability of distinguishing progeny of the T80-GAL4;UAS-*disco* genotype from the *CyO*;UAS-*disco*, it could not be established at which stage lethality was caused.

iv) *en*-GAL4

Disco expression driven by *en*-GAL4 from UAS-*disco* lines T₄₆ and T₄₃ led to lethality (Table 7). No larvae were observed, therefore lethality was suggested to occur at the embryonic stage (Table 7). Driving *disco* expression from UAS-*disco* T₂₉, yielded progeny that survived to the pupal stage, but did not eclose (Table 7).

v) *sca*-GAL4

Directing *disco* expression during neurogenesis with the *sca*-GAL4 driver, resulted in progeny that, in general, did not survive to adulthood (Table 7). Some adult escapers were observed, all of which developed with mutant compound eyes (Table 7).

vi) *elav*-GAL4

When *disco* expression from UAS-*disco* T₄₆ was directed by the *elav*-GAL4 driver, it was observed that progeny did not survive past the first or second larval instar stage of development (Table 7). However, upon crossing this GAL4 driver to the UAS-*disco* T₄ line, five pupae formed from which three adults without a mutant phenotype emerged (Table 7).

vii) *ey*-GAL4

Approximately 70% of progeny from this cross did not carry the *ey*-GAL4 driver, but the balancer chromosome, and developed into adults with curly wings. The other 30% that survived to adulthood, developed with mutant compound eyes (Table 7). According to Mendelian predictions, 50% of the progeny should have had curly wings and 50% should have had the mutant eye phenotype. These results suggest that some progeny where *ey*-GAL4 directed *disco* expression, did not survive to adulthood, thus characterizing the emergent mutant eye flies as escapers.

viii) *gmr*-GAL4

A sufficient number of adult flies emerged from crosses of *gmr*-GAL4 and UAS-*disco*. Therefore, it was assumed that misexpression of *disco* in the eye imaginal disc using the *gmr*-GAL4 driver did not cause lethality (Table 7).

ix) *sev*-GAL4

From the cross of *sev*-GAL4 and UAS-*disco*, the only progeny to survive to adulthood are those carrying the balancer chromosome, not *sev*-GAL4, and UAS-*disco*. This suggests that *disco* expression driven embryonically by *sev*-GAL4 is lethal (Table 7).

x) *Rh1*-GAL4

It was observed that a sufficient number of progeny survived to the adult stage from crosses of *Rh1*-GAL4 to UAS-*disco*. This suggests that driving *disco* expression to differentiated photoreceptors in the pupal eye is not lethal (Table 7).

The various GAL4 lines directed Disco expression to different tissues at different times in development. Of the ten different GAL4 lines used to ectopically express Disco, eight resulted in lethality. Both enhancer traps chosen to direct expression to somatic follicle cells in ovaries, A62 and A90, led to lethality at the embryonic and early larval stages. The T80 enhancer trap and other GAL4 lines that expressed Disco early in development, also caused lethality. *sca*-GAL4 and *ey*-GAL4 lines had reduced viability with

adult escapers displaying mutant eye phenotypes. The two GAL4 lines that directed expression specifically to the eye imaginal disc, *gmr* and *Rh1*, had no effect on viability.

Anti-Apoptotic P35 and Cell Death

The baculovirus P35 gene is a viral anti-cell death protein and has been shown to prevent cell death in a variety of systems, including *Drosophila* (Hay et al., 1997; Davidson and Steller, 1998; Warrick et al., 1998). To determine whether the phenotype caused by expression of *disco* in all GLASS expressing cells using *gmr*-GAL4 was due to cell death, a fly line transformed with a fusion vector containing the five truncated tandem *glass* binding sites (GMR), and the baculovirus P35 gene (*gmr*-P35), was crossed to flies heterozygous for both UAS-*disco* and *gmr*-GAL4. Of the resulting progeny, 25% should carry all three genes of interest. The baculovirus P35 was expressed in all cells in which *disco* was expressed and it appeared that P35 expression did not suppress the rough eye phenotype (Figure 14B). 236 flies (68.2%, n=346) emerged with wildtype eyes, and 110 with a mutant eye phenotype similar to that of *gmr*-GAL4;UAS-*disco* (without P35). However, of the flies with mutant phenotypes, three were found to have a tiny patch of bright red pigment in one eye. This patch seemed to have developed wildtype ommatidia - perhaps an indication of an attempt at rescuing the mutant phenotype (Figure 14B, arrowhead). Heterozygous flies expressing *disco* in the *eyeless* pattern of expression were also crossed to the homozygous

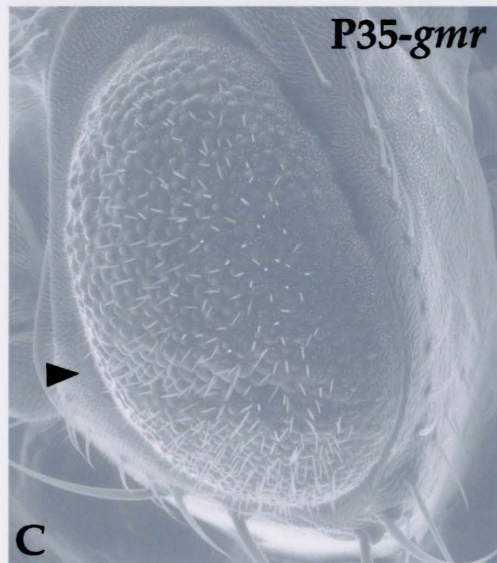
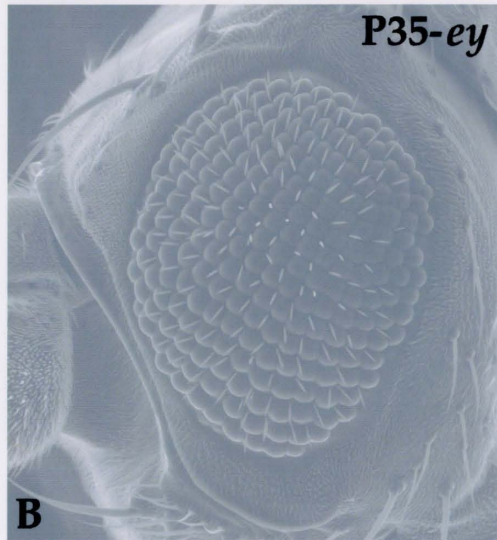
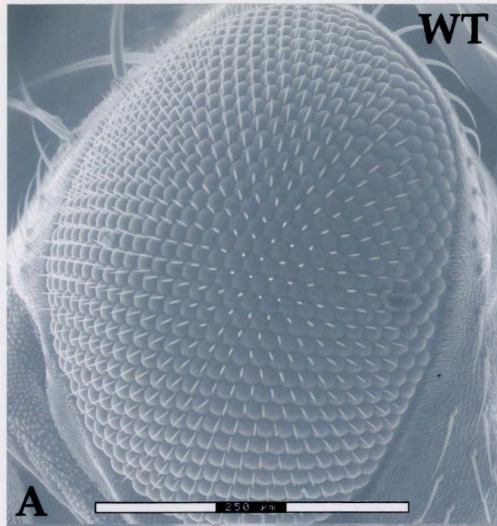
Figure 14. Compound Eye Phenotypes of P35 Expressing Disco Misexpression Mutants.

Environmental Scanning Electron Micrographs show the compound eye phenotypes when the baculovirus P35 protein was expressed in GLASS expressing cells. Live whole fly mounts. Anterior is up, dorsal is left. Scale bar, 100 μ m.

A. Wildtype eye with an organized, smooth ommatidial array.

B. Disco was expressed anterior to the morphogenetic furrow by *ey*-GAL4 and anti-apoptotic baculovirus P35 protein was expressed posterior to furrow in all Glass expressing cells. All flies developed both eyes, but eye size was smaller than wildtype.

C. Both Disco and baculovirus P35 protein are expressed in Glass expressing cells posterior to the furrow, by *gmr*. Phenotype was similar to that of only Disco misexpression to cells posterior to furrow. Three flies showed tiny patches of red pigmentation; arrowhead points to the patch that was red. This patch seems to have been rescued – it shows well-organized ommatidia.



gmr-P35 line. Thus, if cell death was occurring in the furrow or posterior to it, P35 would have a chance to rescue those cells. Preliminary results do not indicate rescue of the eye phenotype. In all progeny (n=90), both eyes developed and the mutant eye phenotypes (15 mutants, 16.7%) encountered were not as severe as those observed in flies without the anti-apoptotic P35. However, in these P35;*ey*-GAL4;UAS-*disco* flies the compound eyes developed to a smaller size and were more rough than those of wildtype (Figure 14C).

Summary of Disco Misexpression Mutants

The dominant mutant phenotypes caused by the misexpression of Disco to various tissues at different times in development included lethality, CNS and PNS defects and disruption in the development of the compound eye.

3.2 PROMOTER-LACZ FUSION EXPRESSION

P-element Mediated Transformation

The *disco* promoter-*lacZ* construct pCaSpeRAUG β -galBK7.8 (Figure 3) was microinjected into *yw* host strain embryos, along with intact transposon DNA, p π 25.1, for germ-line transformation. Of the injected embryos, 237

hatched (G_0) and were allowed to develop to adulthood. From surviving fertile adults which were backcrossed to the host strain, three germ-line transformants (G_1) were recovered. Each of these were again backcrossed to *yw* and 8 red-eyed G_2 flies were recovered. The eight transformants were established as separate transgenic lines by crossing back to the host strain, and then crossing the progeny in brother and sister matings. Three of the pCaSpeRAUG β -gal7.8 (*7.8lacZ*) transgenic lines were maintained as homozygous lines. Determination of chromosomal linkage of the P-element insertions was not possible due to the similarity of eye colour of germ-line transformants with balancer lines.

Expression Pattern of the Promoter-*lacZ* Fusion

A. Expression in the Embryonic Visual System

In wildtype embryos, Disco and Fasciclin II (FasII) are co-expressed in the posterior lip of the invaginating optic lobe placode. In *disco* mutants, the optic lobe primordium develops like normal, as indicated by the unchanged from wildtype expression of *fasII* (Lee et al., 1999). In this study, embryos homozygous for the promoter-*lacZ* fusion, *7.8lacZ*, were double-labeled with anti- β -gal and anti-*fasII* antibodies. *fasII* expression is seen in the optic lobe anlagen (Figure 15A, B) and in the gnathal segments (Figure 15C). The β -galactosidase reporter gene expression was seen in the optic lobe primordium and the gnathal segments (Figure 15). β -gal expression was also seen along

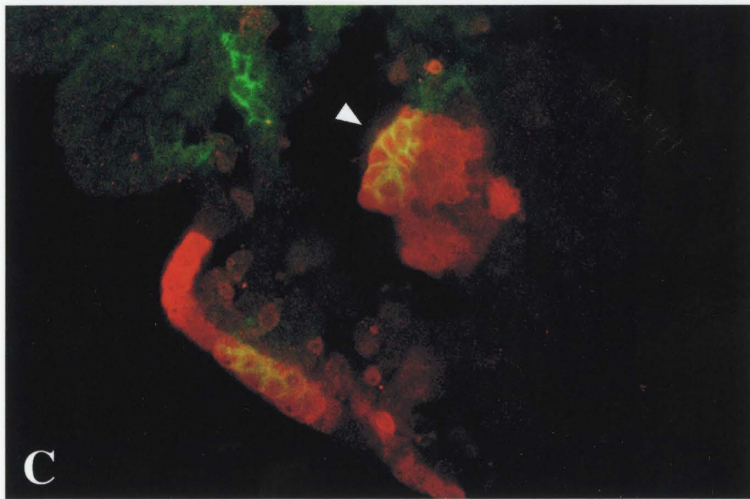
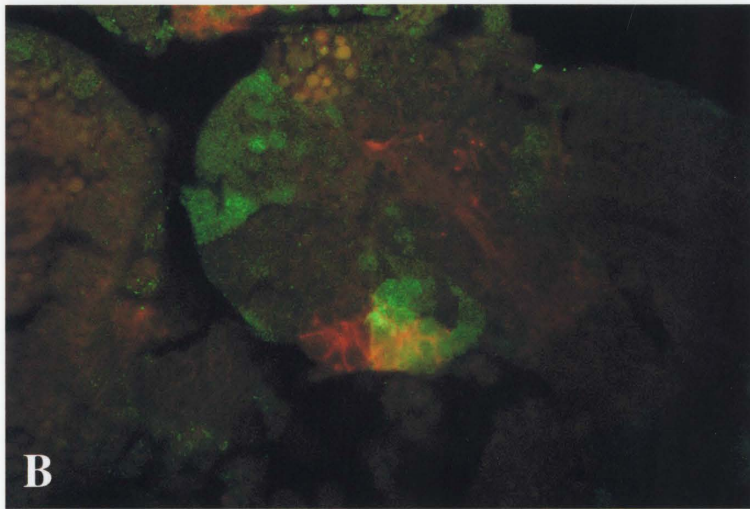
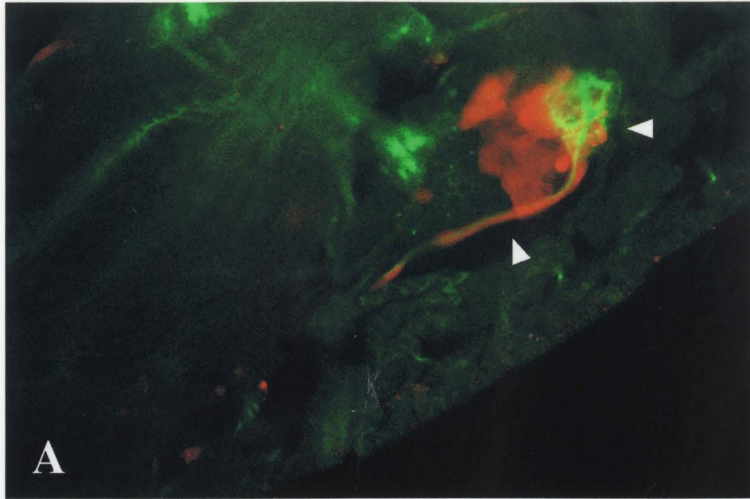
Figure 15. Confocal Images Show Overlapping Expression of the Promoter-lacZ Fusion with *fasII* Expression.

Whole mounts of stage 15/16 embryos double-labeled with anti- β -gal and anti-*fasII*. Red indicates *lacZ* expression; green indicates *fasII* expression; yellow shows overlapping expression of *lacZ* and *fasII*.

A. Horizontal view. Anterior is to the bottom left corner, posterior top right. Posterior end of optic lobe is labeled with *fasII* with some overlap of β -gal, (yellow; arrowhead). β -galactosidase expression is also seen along Bolwig's Nerve, in the glial cells (arrowhead).

B. Ventral is toward the bottom, dorsal to top. Anterior to right, Posterior to the left. Next to the gut (at left) the optic lobe is labeled with *fasII* at the posterior end, with some overlap of β -galactosidase expression (yellow).

C. Ventral view. Anterior to bottom right. *fasII* expression indicates the invaginating optic lobe placode (at center) and a gnathal segment. β -galactosidase expression is shown to overlap in the anterior region of the optic lobe placode (arrowhead) and in the gnathal segment.



the larval optic nerve (Figure 15A) in what may be glial cells, based on their appearance. *fasII* and β -gal were co-expressed in the posterior region of the optic lobe primordium (Figure 15A, B) and in the invaginating optic lobe placode (Figure 15C). In the embryonic visual system and its anlagen, the pattern of expression of the *7.8lacZ* promoter-reporter fusion mimics the wildtype *disco* expression pattern.

B. Reporter Expression Pattern in Embryos

Previous *disco* promoter-*lacZ* fusions have not shown an expression pattern similar to that of the *disco* expression pattern (Lee, 1994). In this study, it was shown that embryos germ-line transformed with the *7.8lacZ* construct have a β -gal expression pattern that is similar to the *disco* expression pattern. Young embryos, stage 5/6, showed strong β -gal expression at the posterior pole and in a dorsal band at the anterior end (Figure 16C), similar to the *disco* enhancer trap, C50.1S1 (Figure 16A). In older embryos, the pattern of expression also resembles that of the *disco* enhancer trap, but is much weaker. Expression of β -gal was seen in the gnathal and antennomaxillary segments, Bolwig's Organs and the optic lobe anlagen (Figure 16D). No expression was seen in cardioblasts, and weak expression was seen in the visceral mesoderm. Embryos homozygous for the insert were heat-shock treated and no change in the β -gal expression pattern was observed in either early or late stage embryos (Figure 16E, F). Although there was no expression in the cardioblasts and weak expression in the visceral

Figure 16. Embryonic Expression of the Promoter-*lacZ* Fusion.

Whole mount preparations of early and late stage embryos showing β -galactosidase expression with chromogen X-gal staining. Views are ventral, anterior to the left. Scale bar, 200 μ m.

(A, B) The enhancer trap, C50.1S1, expresses β -galactosidase in all the same tissues that *disco* expressed, both at early and late stages of embryogenesis (described by Lee et al., 1991). Both A and B also represent the expression patterns of heat shocked C50.1S1 embryos and of progeny of C50.1S1 crossed to heat shock *disco* (D/D), heat shocked and not heat shocked. In A, expression is seen as a dorsal band at the anterior end. In B, at stage 15/16, strong expression is seen in the gnathal and antennal segments, the OL primordia, BOs and in the visceral mesoderm.

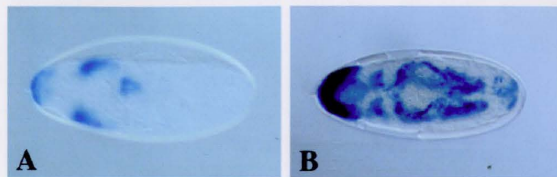
(C, D) β -galactosidase expression pattern of the *disco* promoter-*lacZ* fusion in non-heat shocked embryos. In young embryos, C, the pattern of expression resembles wildtype – a dorsal band at the anterior end. Older embryos, D, show strong expression in the gnathal segments, BOs and in the OL primordia. Weaker or no expression was seen in the visceral mesoderm.

(E, F) Heat shocked embryos carrying the promoter-*lacZ* fusion exhibit the same expression patterns as the non-heat shocked embryos. Young embryos, E, have a dorsal band of expression and older embryos, F, show expression in the gnathal segments, BOs and OL primordia.

(G, H) Young progeny of the promoter-*lacZ* fusion crossed to D/D show strong expression in a dorsal band, G, and in H, an older embryo shows β -galactosidase expression in the *disco* expression pattern: gnathal segments, BOs, and in the optic lobe primordia.

(I, J) Heat shocked progeny carrying the heat shock *disco* construct and the promoter-*lacZ* fusion display wildtype expression patterns. The dorsal band is still present in young embryos, I, and in J, β -gal is expressed in the gnathal segments, BOs and OL primordia, like the *disco* expression pattern.

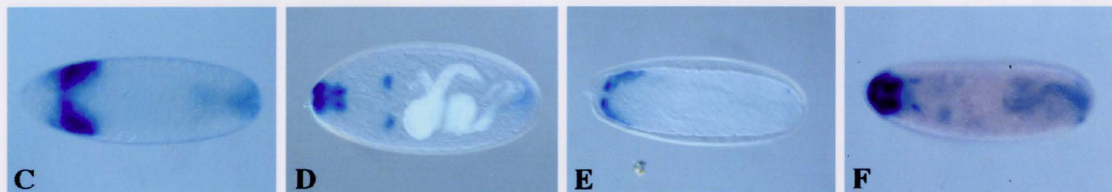
C50.1S1



7.8lacZ

not heat shocked

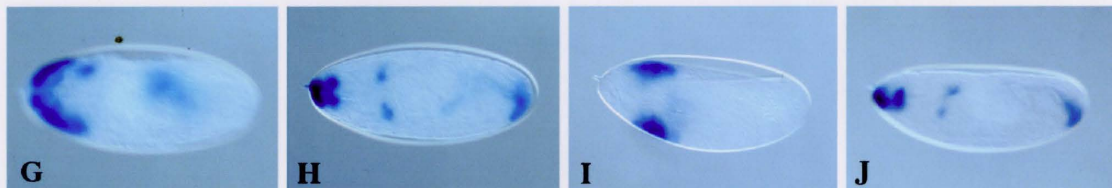
heat shocked



D/D x 7.8lacZ

not heat shocked

heat shocked



mesoderm, it was concluded that there was no difference between the *disco* enhancer trap expression pattern and the promoter-*lacZ* fusion insert. The expression pattern conferred by this promoter-*lacZ* fusion indicates that the *cis*-acting regulatory sequences contained upstream of the *disco* locus are sufficient to drive *disco* expression in tissues where wildtype expression occurs, although to a lesser degree.

When Disco was ubiquitously expressed from a heat-shock promoter in *disco* mutants, significant upregulation of endogenous Disco expression in the optic lobe primordium was observed (Lee et al., 1999). In order to determine whether the s120 binding region contained in the promoter-*lacZ* fusion 7.8*lacZ* was sufficient for the upregulation of Disco expression, transformants homozygous for the *disco* promoter insert were crossed to a line homozygous for the heat-shock *disco* construct (D/D). β -gal expression patterns in both non-heat-shocked and heat-shocked embryos, were found to resemble those of the enhancer trap C50.1S1 and of the insert alone. In a young heat-shock treated embryo (Figure 16G), expression of β -galactosidase was observed in a dorsal band, as it was in non-heat-shocked young embryos (Figure 16I). Gnathal segments, BOs and optic lobe primordia all show expression of the reporter gene in both non-heat-shock (Figure 16H) and heat-shock (Figure 16J) treated late stage embryos. Thus, ubiquitous expression of Disco protein had no effect on the expression of the *lacZ* reporter gene, suggesting that the s120 binding site is not sufficient to autoregulate *disco* expression.

C. Embryonic Expression in a *disco* Mutant Background

To test whether the 7 kb of upstream sequence was sufficient to rescue *disco* expression in the optic lobe primordium, female flies transformed with the promoter- β -gal fusion, were crossed to a heterozygous *disco* mutant line (*y, w, disco², f/XX*). From this cross, 50% of embryos should carry the promoter insert in the *disco* mutant background, since the *disco* mutation is over an attached X. Preliminary double-labeling experiments showed expression of both *fasII* and β -gal in 29 of 52 embryos (55.7%) (data not shown). Of these 29 embryos, 19 (65.5%) co-expressed in the optic lobe primordium (data not shown).

D. Larval Expression of the Promoter-*lacZ* Fusion

The C50.1S1 enhancer trap pattern of β -galactosidase expression in the larval central nervous system shows a band that wraps around each brain lobe, sparse dispersal over the developing lamina, a regular array in the ventral nerve cord and in the antennal discs (Figure 17A). Heat-shock treatment of these enhancer trap larvae had no effect on the pattern of expression (data not shown). Similarly, progeny larvae from a cross of the C50.1S1 line and the heat-shock *disco* line had a similar expression pattern whether they were heat-shock treated (data not shown), or not treated (Figure 17B).

Figure 17. Promoter-*lacZ* Fusion Expression in Larval Brains.

Whole mounts of third instar larval CNSs and the attached eye-antenna imaginal discs. X-gal reactions reveal β -galactosidase expression patterns. Anterior is to the left. Scale bar, 200 μ m.

A. The C50.1S1 enhancer trap shows the expression pattern of *disco* in the larval CNS and attached eye-antennal imaginal discs. A band of expression wraps around each brain lobe; there is a sparse dispersal of stained cells across the lamina and in a regular array along the ventral nerve cord. Heat shocked enhancer trap larvae showed the same pattern of expression (data not shown).

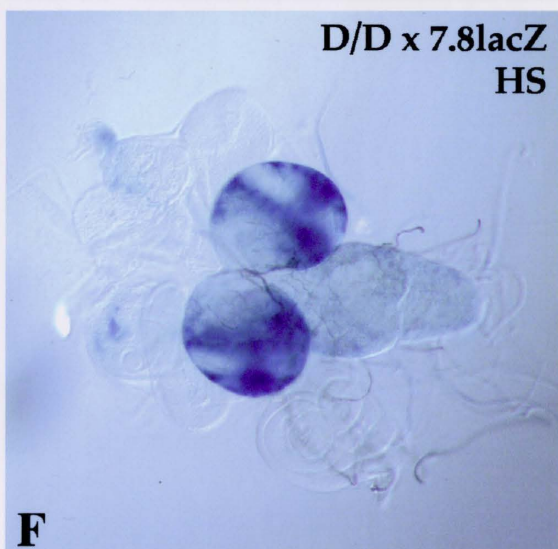
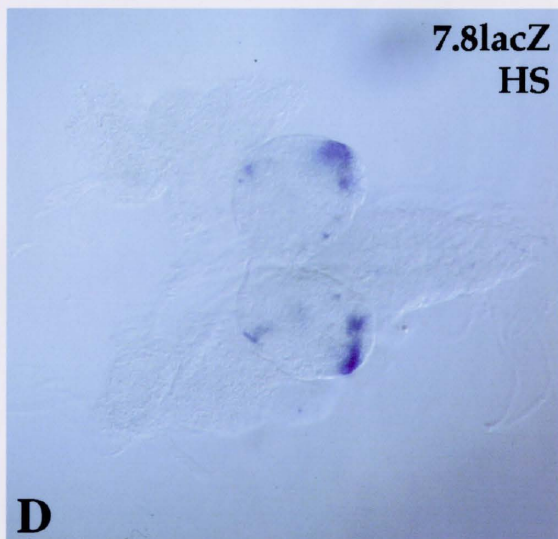
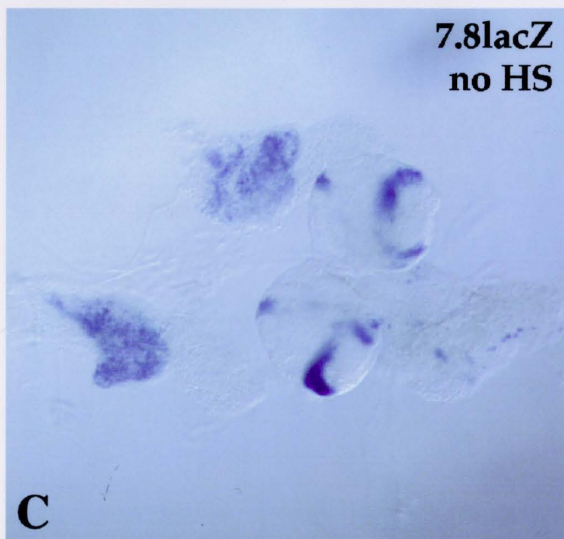
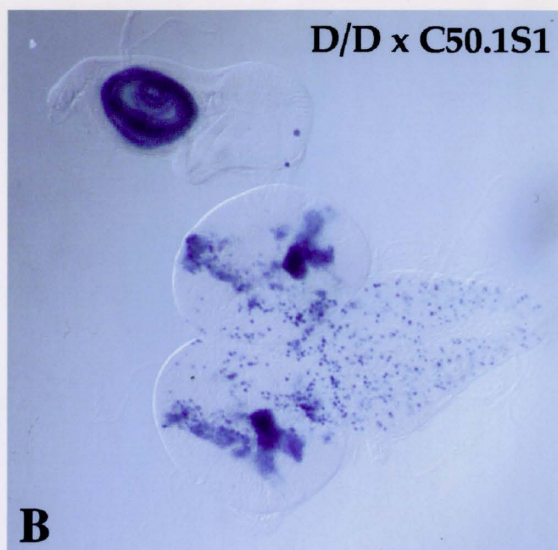
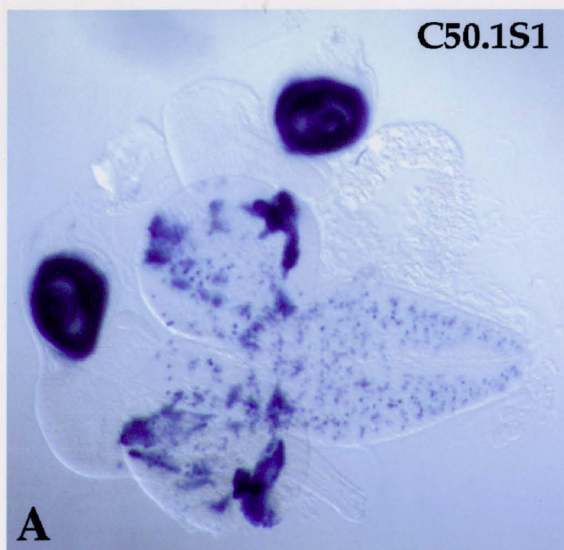
B. Larval brains dissected from C50.1S1 crossed to heat shock *disco* (D/D), show a pattern of expression like wildtype *disco*. Progeny from the cross were also heat shocked, and no changes were seen in the patterns of expression in these specimens (data not shown).

C. The expression pattern of β -galactosidase in non-heat shocked (no HS) larval brains, ventral nerve cord and attached imaginal discs from promoter-*lacZ* fusion third instar larvae. There seemed to be a reduction in overall expression, but the basic pattern of a band enwrapping the brain lobes, in individual cells along the ventral nerve cord, and in the antennal disc remained.

D. Heat shocked (HS) larvae carrying the promoter-*lacZ* fusion show an expression pattern similar to wildtype and to non-heat shocked promoter-*lacZ* larvae, although not as strong. This particular specimen is a weak example, as there is no visible expression in the antennal discs.

E. Non-heat shocked progeny larvae from D/D crossed to the promoter-*lacZ* fusion show expression in the brain lobes, along the ventral nerve cord and in the antennal discs.

F. Heat shocked progeny from heat shock *disco* crossed to the promoter-*lacZ* fusion. Although this particular specimen shows no expression in the antennal discs, observations of other specimens indicated that the overall pattern of expression did not change from wildtype. Expression was seen in the brain lobes, ventral nerve cord and the antennal discs.



Optic lobes, ventral nerve cords and their attached eye-antennal imaginal discs were dissected from third instar larvae homozygous for the promoter-*lacZ* fusion insert and showed a precise reporter gene expression pattern. X-gal staining revealed a band of β -galactosidase wrapping each brain lobe, some expression in the ventral nerve cord and weak staining of the antennal imaginal discs (Figure 17C). Heat-shock treatment of homozygous *7.8lacZ* larvae did not substantially change this pattern of expression dramatically (Figure 17D). All heat-shocked and non-heat-shocked specimens showed optic lobe expression and antennal disc expression, but with some variability in the strength.

Larval brains from progeny of *7.8lacZ* transformants crossed to *hsdisco* were also dissected. Figure 17E shows a typical pattern of expression of β -gal observed in non-heat-shocked larvae: expression in the antennal discs, bands enwrapping the brain lobes and expression in individual cells along the ventral nerve cord. Heat-shock treatment of progeny larvae from *7.8lacZ* crossed to *hsdisco* did not change the pattern of expression, as seen in Figure 17F. Similarly, the amount of staining in the brain lobe varied from very weak and barely visible, to quite widespread. However, the basic pattern of bands enwrapping the lobes was observed in all specimens (n=28).

Thus, both the larval and embryonic expression patterns conferred by the pCaSpeRAUG β -gal7.8 promoter fusion are similar to the wildtype *disco* expression pattern and are not affected by ubiquitous expression of Disco protein.

Identification of P1 Clones Containing the *disco* Locus

Two putative Disco binding sites have already been identified within the *disco* locus (Lee et al., 1999). There is also the possibility of other *cis*-acting regulatory sequences situated upstream of the *disco* gene. Thus, sequencing of the intron and the 5' upstream region from genomic DNA was attempted. Bacteriophage P1 clones containing genomic *Drosophila* sequence from chromosome region 14 were screened with a radiolabeled *disco* probe. Of the 13 P1s probed, only two P1 clones were found to contain the *disco* locus: DS00447 and DS06529 (Table 8). The bacteriophage P1 DS00447 contains the 14B1-14B4 region and DS06529, contains the region 14B1-8. Several attempts to sequence the genomic DNA of the *disco* locus from these two bacteriophage P1s failed, thus the use of these clones was abandoned.

Table 8. Bacteriophage P1s Screened with *disco* Probe.

Bacteriophage P1 Name	Map Position	Disco?
DS05435	14A6-7	-
DS00252	14B1-4	-
DS00447	14B1-4	+
DS02166	14B1-8	-
DS06529	14B1-8	+
DS00335	14B3-4	-
DS05497	14B3-4	-
DS01678	14B14-C2	-
DS03852	14B14-C5	-
DS04627	14B14-C5	-
DS06547	14B14-C5	-
DS06579	14B14-C5	-
DS06440	14C3-8	-

- Indicates the clone did not contain the *disco* locus
+ Indicates the clone contained the *disco* region

Sequencing and Molecular Analysis of *disco* Locus

Sequencing of the region upstream of the *disco* locus and the *disco* intron was accomplished by obtaining templates from the two plasmids carrying promoter-*lacZ* fusions, pCaSpeRAUG β -gal7.8 and pCaSpeR β -gal6.5 (Figure 3). Both constructs contain genomic DNA from the *disco* region.

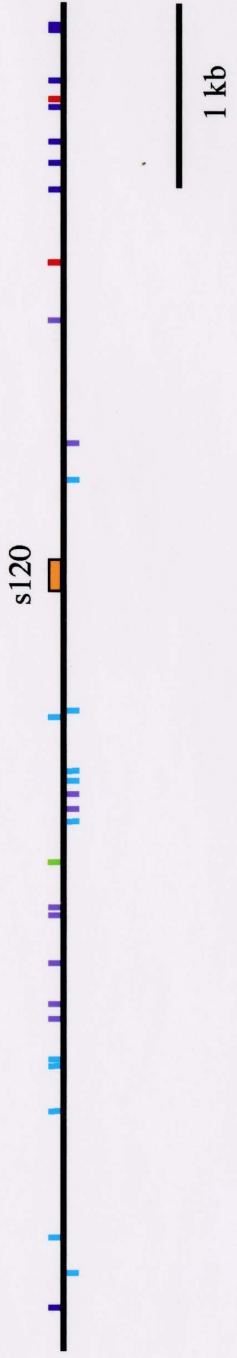
Sequences were aligned and the consensus sequence of 12.8 kb was analysed using BLAST sequence comparison algorithm revealing no significant similarities with previously reported nucleotide or amino acid sequences, other than *disco* (accession number X56232). The 12.8 kb of sequence included 7 kb of upstream sequence, the first *disco* exon, the intron and part of the second exon.

The intron was found to be 2904 bp long, starting at + 413 and ending at + 3314 (relative to the transcriptional start site). It contained a 5' splice site donor consensus sequence (AAG G|TA TGT A), as well as a 3' splice site acceptor consensus sequence (TTT TCC AG|G). The sequence of the intron is shown in Appendix B.

Approximately 7 kb (6956 bp) of *disco* 5' upstream region was sequenced and analysed (Appendix B). The core promoter region of 50 bp flanking the transcriptional start site lacks both the traditional TATA box normally found in the -25 to -30 region and the Initiator element (TCA G/T TC) normally found in the -25 to +25 region. Within 200 bp upstream of the transcription start site, the GC content is 26 and 32% and the AT content about 16 and 26%. The upstream sequence was also searched for consensus sequences of known regulatory elements. Within 400 bp of the transcription start site, a putative Sp1 binding site (consensus, GGGCGG) was found at -407 (Figure 18). Throughout the 7 kb of upstream sequence are scattered eight putative *caudal*-binding sites (consensus, TTTATG) (Dearolf et al., 1989), 11 putative *myb*-binding sites (consensus, C/T AAC G/T G) (Biedenkapp et al., 1988), eight putative *zeste* recognition sequences (consensus, T/C GAG T/C G)

Figure 18. Diagram Showing Putative Transcription Factor Binding Sites.

The horizontal line represents the genomic region, upstream of the *disco* locus. Several sequences identical to the binding sites of known *Drosophila* transcription factors are marked: 8 *caudal*-binding sites (consensus, TTTATG), 11 *myb*-binding sites (consensus, C/T AAC G/T G), one SP1 binding sites (consensus, GGGCGG), 8 *zeste* recognition sequences (consensus, C/T AAC G/T G) and a single *gcm*-binding site (consensus, AT G/A CGGGT). Neither the TATA element nor the Initiator sequence were found in the proximal promoter region.



(Benson and Pirrotta, 1988) and a single putative *gcm*-binding site (consensus, AT G/A CGGGT) (Schreiber et al., 1997) was found at -4345 (Figure 18).

Two putative Disco binding sites have been previously reported (Lee et al., 1999) and were both found within the 12.8 kb sequence. The 270 bp s280 site (Figure 19B) was found within the intron between +1099 and +1368 and the other putative binding site, s120, was found to be 134 bp long (-3025 to -2893). The binding site sequences differed by one or two base pairs from those previously reported (Figure 19A, B) (Lee, 1994).

Summary of Promoter Region Studies

Studies of the promoter-*lacZ* fusion construct indicate that the 7 kb of *disco* upstream sequence, containing the s120 putative Disco binding site, is sufficient to drive proper *disco* gene expression. In the presence of excess Disco protein the pattern of expression does not change. Sequencing and analysis of the upstream region revealed a number of putative transcription factor binding sites.

Figure 19. Sequences of the Two Putative Disco Protein Binding Sites.

The two putative Disco protein binding sites were found with binding assays and the regions sequenced (Lee et al., 1999). Both are sense strand sequences. Bolded are the one base pair differences found between this and the previously reported sequences (Lee, 1994).

A. The sequence of the s120 binding site found about 2.9 kb upstream of the *disco* transcription start site.

B. The s280 binding site sequence. The site is located in the *disco* intron, approximately 1 kb downstream of the *disco* transcription start site.

A

1 GATCTGGATG GGTATGGGTA TGGGTATGGG AATGAGGATA GAGGATAGAG GACGGAGAAT
61 GGTAATGGTA ATCGTAATGG CAATGGGATG GAGAATGAGA ACCTTGGACG ACAAAGTGTG
121 AAAAAATGATT GATC

B

1 GATCTACCAT CTACCATCTA AAGCGTCTCC CAACTCTCGG AATTCCTCAA TCTTGTCGTT
61 TCTTGTCCTG GGTACTTFC AATTTTTGCT GCCTTTGCCA CTGCCGATAA TCAAAAAACG
121 TTTTCGTCCGA GAGCCATCAA AAGTTGTGAT TGGTTTTTCC CCTACCCCCC AGCTGCCCAA
181 CATCACCCCC CCTTTCACCA TCGGTGGCTT AAGACATTTT AAGCGTTTGA CAAAAATAAT
241 TACCCATGTA CATGCATACT CACCTCGATC

CHAPTER 4

Discussion

4.1 TISSUE-SPECIFIC MISEXPRESSION OF DISCO

The *disconnected* gene encodes a transcription factor containing two transactivating regions and two zinc-finger motifs. Its loss of function phenotypes are nervous system defects which are most prominent in the visual system. However, the function of *disco* in development is yet unknown. Probing the function of a gene and its contribution to gene-regulatory pathways can be accomplished by gene overexpression. Such gain of function mutations allow for screening of mutations that modify the phenotypes caused by overexpression with subsequent identification of proteins which interact with the gene of interest and their function in a developmental pathway.

To create gain of function mutants the *disco* gene was ectopically expressed using the GAL4-UAS system developed by Brand and Perrimon (1993). Disco protein was overexpressed in specific tissues at various developmental stages and the resulting progeny were scored for dominant phenotypes. In embryos, the integrities of the embryonic peripheral and central nervous systems were assessed; third instar larval eye-imaginal discs and optic lobes were examined for developmental defects; and in adults, phenotypes of the compound eyes were assessed. Evidence suggests that expression of Disco very early in embryogenesis leads to disruptions in development, as does expression of Disco in undifferentiated cells. Also, preliminary experiments using the anti-apoptotic baculovirus P35 protein

assessing Disco involvement in the cell death pathway showed partial phenotype rescue by P35.

CNS Phenotypes Resemble Previously Described Double Mutants

The CNS phenotypes resulting from overexpression of Disco were all similar to one another. The commissures and connectives were either weak or missing and there was some lateral displacement from the midline. Misexpression of Disco with T80-GAL4 mildly disrupted axon pathfinding. In these gain of function mutants, commissures and connectives formed, but there was evidence of axonal tracts entering the intercommissural space, as well as weakening of the longitudinals. *sca*-GAL4 directed Disco expression to neuroblasts and neurons of the CNS resulting in ventral nerve cords with weak longitudinals, missing commissures and lateral displacement of several neuromeres from the midline. Most axon tracts were absent in embryos where Disco expression was driven by *sev*-GAL4. In a minority of progeny embryos with *en*-GAL4 directed Disco expression, the CNS phenotype resembled a combination of both *sca*-GAL4 and *sev*-GAL4 phenotypes: minimal commissure and connective formation, with lateral displacement from the midline. *ey*-GAL4 driving Disco expression to groups of cells neighbouring the ventral nerve cord resulted in the formation of aberrant axon tracts, with some semblance of commissures and connectives. Embryos where *elav*-GAL4 directed Disco expression had difficulty developing,

resulting in CNS phenotypes which were not a direct consequence of the ectopic expression, but a consequence of the morphology of the embryo.

Disco protein misexpression led to CNS phenotypes with severe reductions in axonal growth and lateral displacement from the midline. From phenotypes described in the literature, Disco gain-of-function CNS phenotypes most closely resemble double mutants of the pointed group. These mutations include: *pointed (ptd) rhomboid*, *ptd Star*, *ptd kette*, and *orthodenticle ptd* double mutants (Klamt, 1993; Hummel et al., 1999). In *rhom* and *S* mutants there is either a loss of midline glial cells or these cells have lost their functions (Klamt, 1993). Double mutants, *ptd rhom* and *ptd S*, show a reduction in the number of commissural fibers crossing the midline (Klamt, 1993). Genetic analyses have shown that *pointed* also acts in the midline glia. The genes *kette* and *orthodenticle* function in midline neurons (Finkelstein et al., 1990; Hummel et al., 1999) and in *ptd kette*, *otd ptd* double mutants, there is a disruption in the development of both midline neurons and glia leading to weak, missing and aberrant axon tracts (Hummel et al., 1999). The gain of function of Disco in neurons of the midline (*sca*), and in groups of cells neighbouring the ventral nerve cord (*ey*), had an effect on axon growth and pathfinding. Thus, it may be speculated, that like *ptd* and *kette*, Disco may have a function in the development of neurons or glia or may function as part of a repulsive or attractive cue pathway for growth cones.

PNS Phenotypes

Every GAL4 line that was used to drive tissue specific misexpression of Disco caused a perturbation in the development of the PNS. In each case the ISN was found misrouted between clusters within a segment or between segments. Also, neuronal clusters were affected by misexpression of Disco – either individual neurons developed in the wrong place, or entire clusters were mispositioned. However, in embryos where *en*-GAL4 and *ey*-GAL4 directed Disco misexpression, PNS phenotypes appeared to be an indirect consequence of the morphology of the embryo. Misexpression of Disco, led to embryos having difficulty developing, rather than leading to a direct effect on PNS development. In these embryos, the basic segmental pattern of the PNS was barely recognizable, with a concomitant reduction in the number of neurons.

The PNS phenotypes of the gain of Disco function embryos are difficult to compare to previously identified mutations that affect only a single aspect of PNS development because each PNS defect caused by Disco misexpression with different GAL4 drivers, falls into several mutant categories. However, from the encountered mutant phenotypes some conclusions can be drawn. Since there were no obvious increases or decreases in neuronal cell numbers, Disco does not function as a neurogenic gene. The overall morphologies of neurons were like wildtype, thus Disco does not function in the initial steps of neurogenesis. However, the mislocation of neurons and neuronal clusters, and the misrouting of ISNs suggest that overexpression of Disco may

disrupt genetic interactions of final differentiation, axonogenesis and pathfinding.

Disco Expression During Embryogenesis Is Lethal

Lethality amongst the GAL4 line crosses to UAS-*disco* was prevalent. It was found that embryonic Disco overexpression disrupted development and led to lethality, as shown by expression from the two enhancer traps, A62 and A90. These GAL4 lines, by maternal contribution, directed Disco overexpression at very early stages of development and the consequence of this was no survivors. Further supporting evidence was provided by the *en*-GAL4 enhancer trap, which regulated the onset of Disco misexpression to stage 4/5 of embryogenesis. Such early and ubiquitous expression led to developmental defects believed to be responsible for the lethality. Expression of Disco to neurogenic regions by *sca*-GAL4, beginning at embryonic stage 5, caused severe defects in the nervous system, and led to lethality, although there were some adult escapers. These survivors developed with a mutant eye phenotype. *elav*-GAL4 also directed Disco expression during embryogenesis, starting at stage 12, to all post-mitotic neurons, including the R cells of the eye. Progeny had difficulty developing resulting in lethality. However, one cross using a different UAS-*disco* transformant line allowed several escapers that all had a wildtype phenotype. This suggests that the level of Disco protein expressed from this particular line was not sufficient to cause complete lethality, nor did it induce an eye phenotype. Lethality was

also the end result when embryonic PNS and CNS defects were quite penetrant and severe, namely those of *ey*, *sev* and T80-GAL4s, all of which drive expression during embryogenesis. After embryogenesis is complete, Disco misexpression to specific tissues, such as the eye imaginal disc by *gmr*, *elav* and *Rh1*-GAL4s, has no effect on viability. Thus, overexpression of Disco during embryogenesis and the consequence of nervous system defects leads to lethality.

Determining Stage of Lethality

The stages of lethality were deduced based on observations of survivorship to a particular stage of development. Survival of progeny to early larval stages was seen in crosses of the enhancer traps A90 and A62 with UAS-*disco*, suggesting larval lethality. *en* and *elav*-GAL4-directed Disco expression did not allow development past the embryonic stages, as inferred by the observation of absence of larvae. Stage of lethality of progeny with Disco expression directed by *sca*, *sev* and T80-GAL4s could not be deduced. There were adult escapers from *sca*-GAL4 crossed to UAS-*disco*; both the *sev* and T80-GAL4 lines were balanced with *CyO*, making it impossible to distinguish UAS-*disco* progeny carrying the GAL4 from the *CyO* carrying progeny. Progeny from any of these three GAL4 lines could have survived to any developmental stage.

To confirm the precise stage of lethality, observations of survivorship should be done at each stage of development. Embryonic lethality could be

inferred from comparisons of the number of hatched eggs versus the number of unhatched eggs. If eggs hatch, larval versus pupal lethality could be determined by the number of emergent adults from a specific number of larvae.

Larval Optic Nerve Phenotypes

Eye imaginal discs were dissected from larvae in which the promoter-GAL4 driving *Disco* expression had an effect on the development of the adult compound eye. *Disco* misexpression to cells anterior to the morphogenetic furrow using *ey*-GAL4 resulted in a marked reduction in the number of photoreceptor cells developing, as visualized by MAb 24B10. Also, in some specimens, the LON was found to be longer than in wildtype. In *gmr*-GAL4;UAS-*disco* eye imaginal discs photoreceptors developed like that in wildtype, the retinal projections developed with a few tangles in the optic lobe, and some of the LONs could not find their target in the central brain. However, these LONs were not as extensive like those of *ey*-GAL4;UAS-*disco*; they simply had difficulty reaching their target. Defects in the LON, in the photoreceptors and in the retinal projections were not observed in the eye-imaginal disc and brain complexes of the *sca*-GAL4;UAS-*disco* genotype. The increased length of the LON in *ey*-GAL4;UAS-*disco* progeny, as well as the misrouted LON in the central brain of the *gmr*-GAL4;UAS-*disco* progeny suggest that overexpression of *Disco* in the larval brain and eye discs may have an effect on LON target recognition.

Ectopic Expression of Disco in the Eye Imaginal Disc

The compound eye phenotypes generated by ectopically expressing Disco at different times in the development of the eye-imaginal disc can be interpreted with regards to the stage of cell differentiation. The more extreme phenotypes of missing or smaller eyes were generated by driving Disco expression to the undifferentiated cells anterior to the morphogenetic furrow by *ey*-GAL4. Posterior to the furrow, some cells have differentiated into photoreceptor neurons and ectopic expression of Disco to these cells using *sca*-GAL4 and *gmr*-GAL4 seemed to have no effect on their development. These compound eyes are wildtype in size and shape, but the interommatidial bristles are misplaced and lenses are fused. Thus, this phenotype suggests that when Disco is expressed in all cells posterior to the furrow, it only has an effect on those cells which have not yet differentiated. These would be the cone cells and sensory bristle cells which do not differentiate until the late pupal stages. Furthermore, Disco expression directed to the eye imaginal disc with *Rh1*-GAL4 at the late pupal stage, after all cells have differentiated, had no effect on the development of the compound eye. All these results suggest that expression of Disco in undifferentiated cells may disrupt their differentiation.

Which Eye Cells Are Specifically Affected By Disco Misexpression?

The mutant eye phenotypes produced by Disco misexpression may be due to the inability of cells to complete differentiation or due to cell death. Several methods may be used to determine which cells are affected, and whether or not the mutant phenotypes are due to misdifferentiation. One method involves staining the mutant retinas with phalloidin, which would show the overall organization of the compound eye. Sectioning the retina would show greater detail of the tissue architecture and would show if cells were absent, misdifferentiated or simply misdeveloped.

Is Disco Involved in the Cell Death Pathway?

When Disco was expressed in undifferentiated cells anterior to the morphogenetic furrow, the resulting compound eye was smaller than wildtype or it was completely absent. Thus, directing Disco expression to undifferentiated cells either directly induces cell death, or does not allow cells to develop according to their fate, which then default to cell death. It has previously been shown that the baculovirus protein P35 blocks apoptosis in *Drosophila* (Hay et al., 1994; Grether et al., 1995; Warrick et al., 1998). By expressing P35 in the eye disc, an attempt was made to rescue the mutant eye phenotypes caused by misexpressing Disco with *ey* and *gmr*-GAL4s. P35 expression was directed to GLASS-expressing cells posterior to the morphogenetic furrow by *gmr*-P35. The effort to rescue apoptosis, that may be

occurring anterior to the furrow due to *ey*-GAL4 directed Disco expression, with P35 expression posterior to the furrow, did not seem to have an effect. However, all emergent flies did possess both compound eyes of wildtype structure, although smaller in size than wildtype. In flies with Disco expression driven by *gmr*-GAL4, the expression of P35 in the same cells did not rescue the mutant phenotype. Only in three compound eyes was a very small patch of normal ommatidia observed. Perhaps, this inability to rescue the *gmr*-GAL4 mutant phenotype was due to insufficient availability of Glass protein, since both the GAL4 and the P35 constructs require Glass protein for expression.

The question of whether Disco expression in specific cells directly activates programmed cell death or whether it causes problems in cell differentiation remains unanswered. Results from expressing the baculovirus P35 protein from a UAS construct may provide an answer. This method will allow the expression of P35 in the same cells as UAS-*disco*, at the same time in development. The only drawback may be insufficient production of GAL4 to drive expression from two UAS constructs.

Future Studies

The purpose of overexpressing Disco in tissue and time-specific manners was to generate dominant phenotypes for further study. The results obtained suggest that Disco misexpression in neurons leads to axon pathfinding difficulties and/or cell differentiation difficulties. The question

of Disco involvement in neuronal and glial cell development and differentiation can be addressed using GAL4 lines that drive expression to specific subsets of cells in the midline in combination with enhancer traps that show expression in midline cells. The line *sim*-GAL4.AA142 drives expression solely in the midline glia (Crews et al., 1988). AA142 is an enhancer trap that shows *lacZ* expression specifically in the midline glia (Klamt et al., 1991). After crossing to UAS-*disco*, the CNS phenotype could be revealed by MAb BP102 labeling and the presence or absence of the midline glial cells by anti- β -gal staining. Misexpression of Disco to the midline glia could also be accomplished using *slit*-GAL4.AA142. Other GAL4 lines, such as *omb*-GAL4, which drives expression to glial cells of the visual system (Poeck et al., 1993), and *ftz*-GAL4, which drives expression to axons in the pCC longitudinal connective pathway, could also be used to misexpress Disco. Both *omb* and *ftz*-GAL4 lines may result in progeny with axon pathfinding difficulties in the visual system and CNS, respectively. Also, to help identify which cells in the midline may be affected by Disco misexpression, recombinants of the GAL4 lines already used to misexpress Disco and one of two enhancer traps, AA142 or AE60, can be created. The AA142 enhancer trap shows expression in the midline glia and the AE60 enhancer trap shows expression in six VUM neurons and two MP1 neurons (Klamt et al., 1991).

The dominant phenotypes that were established in this work by Disco overexpression using the GAL4-UAS system can be used for further studies. It should be possible to perform a second site mutagenesis screen for enhancers or suppressors of these phenotypes, eventually leading to the identification of possible *disco* interacting proteins.

4.2 AUTOREGULATION OF DISCO

The expression pattern of Disco during embryogenesis and in the larval brains is quite widespread. It is known that *disco* expression is regulated by *tinman* in developing heart cells (Bodmer, 1993) and by *Distal-less* in the leg imaginal discs (Cohen et al., 1991). It has also been shown that in *disco* mutant embryos, Disco mRNA expression is not found in the embryonic optic lobe primordium and that this expression is restored by upregulated expression of Disco protein from a heat-shock promoter (Lee et al., 1999). Thus, it is believed that Disco autoregulates its expression in the optic lobe primordium. Supporting evidence was provided by the identification of two putative Disco binding sites, s120 and s280, within the *disco* locus (Lee et al., 1999). To determine whether the binding sites were necessary or sufficient for *disco*-dependent expression, constructs were made which incorporated different parts of the *disco* locus fused to a *lacZ* reporter gene. Expression from one of the constructs, pCaSpeR β -gal6.5 containing the s280 binding site, has been shown to bear little resemblance to the endogenous *disco* expression pattern, including no β -gal expression in the developing optic lobe primordium (Lee, 1994). From this it was concluded that the s280 site is not sufficient to drive proper expression of the *disco* gene (Lee, 1994). In order to determine whether the s120 binding site is alone necessary or sufficient for Disco autoregulation in the optic lobe primordium, we germ-line transformed embryos with a promoter-*lacZ* fusion construct (pCaSpeRAUG β -gal7.8) containing 7 kb of upstream sequence, including the s120 binding site, and examined the *lacZ* expression pattern.

Expression Pattern of the Promoter-*lacZ* Fusion Recapitulates *Disco*

Both embryonic and larval CNS expression patterns of the pCaSpeRAUG β -gal7.8 construct resembled those of endogenous *disco*, at least qualitatively. In embryos, β -gal expression was detected in gnathal segments, the antennomaxillary complex, the optic lobe primordia and weak expression was detected in the visceral mesoderm. In the larval CNS, a band of β -gal expression was seen enwrapping each optic lobe, in scattered cells along the ventral nerve cord and in the antennal discs. There was no change in the patterns or strength of expression in neither embryos nor larvae carrying a single copy of the promoter-*lacZ* fusion and the heat-shock *disco* construct. Progeny carrying both these constructs were heat-shock treated, and the pattern of expression remained like wildtype. β -galactosidase expression was not upregulated as one may have expected by the excess *Disco* protein binding to the s120 site thereby increasing β -galactosidase expression.

Embryos double-labeled with *fasII* and β -galactosidase provide further evidence of the promoter-*lacZ* fusion recapitulating the *disco* expression pattern in the proper tissues, namely in the optic lobe primordium and gnathal segments. Expression of β -galactosidase was also seen along the LON. The shape of the pattern of expression along the LON indicates that the expression is probably within the glial cells that ensheath the LON. Thus, if *disco* is indeed expressed in the glial cells of the LON, its role in the development of the LON may be via these glial cells.

***disco* Promoter Region**

Eukaryotic gene promoters are often very complex because they must often function to regulate transcription in specific tissues at specific times in development. Promoter regions of various genes have been extensively studied and have been divided into two regulatory regions: the proximal region, which contains the core promoter adjacent to the transcription start site, and the distal region, which functions as an enhancer or suppressor (reviewed by Novina and Roy, 1996). The core promoter may contain elements that independently direct basal transcription, namely the TATA box, the Initiator element (reviewed by Lo and Smale, 1996) and the Downstream elements (Arkhipova, 1995).

Approximately 7 kb of sequence upstream of the *disco* locus was sequenced and analysed. The proximal promoter region lacks both the TATA box (at -25/-35) and the Initiator sequence (at -25/+25bp). The novel class of Downstream Elements (+20/+30), identified by Arkhipova (1995) are common in *Drosophila* promoters, but do not have a single consensus and their location is not strictly fixed. These elements could not be identified in the *Disco* promoter region because of their diversity and ambiguity. Transcriptional control of the *disco* gene must be accomplished by other regulatory sequences. The sequence of the 5' flanking region was found to contain many putative transcription factor binding sites, several of which are for the *myb* transcription factor. *myb* transcripts are found early in development, at preblastoderm stages (Katzen et al., 1998). Expression of *myb* continues during cellular blastoderm and germ band extension stages, but at

reduced levels (Katzen et al., 1998). At later stages, *myb* transcripts are present at high levels only in the CNS (Katzen et al., 1998). It is possible that maternal *myb* at the preblastoderm stages interacts with the Disco promoter. Thus, in wildtype embryos, *myb* may regulate *disco* transcription, allowing normal development. When Disco protein was overexpressed via GAL4 drivers early in embryogenesis, regulation of transcription by *myb* was impossible, and this may have disrupted development and led to lethality.

Another identified binding site in the *disco* promoter region was for the *glial cells missing* transcription factor. *gcm* functions in the specification of lateral glia (Hosoya et al., 1995; Jones et al., 1995) by way of the *pointed* regulatory pathway (Giesen et al., 1997). Perhaps, in a wildtype background, *gsm* binds to the regulatory sequence near the *disco* locus, regulating *disco* transcription, thereby allowing normal development of the CNS. However, when Disco is overexpressed in differentiating cells of the CNS using GAL4 drivers, *gcm* cannot bind the promoter to regulate the amount of Disco protein. The increased amounts of Disco protein may perturb the *pointed* pathway, which may lead to the dominant CNS phenotypes that resemble *pointed* double mutants.

The *disco* core promoter varies substantially from a typical *Drosophila* promoter, in that it contains neither a TATA box nor an Initiator element. However, the upstream region seems to be composed of multiple sequence elements which may act together to achieve proper levels of transcription in the proper tissues.

Future Studies of the Promoter Region

The pCaSpeRAUG β -gal7.8 promoter-*lacZ* fusion confers the wildtype *disco* expression pattern, including β -galactosidase expression in the optic lobe primordium. Further studies, involving expression in a *disco* mutant background could indicate if the entire 7 kb of 5' flanking sequence to the *disco* locus is necessary for regulation of Disco expression in the optic lobe primordium.

Further analysis of the *disco* promoter region could reveal other regulatory elements within the sequence. To define the minimum sequences necessary to confer wildtype expression various deletion constructs can be created and germ-line transformed. Perhaps, if the *disco* gene exists in another *Drosophila* species and functions in the same manner, the sequences can be compared to reveal conserved motifs. These motifs can either be deleted, or used in promoter-*lacZ* fusion constructs and expression studied both *in vivo* and *in vitro*.

APPENDIX A
Chromosomal Location of UAS-*disco* Insertions

Table A-1. Chromosomal Location of UAS-*disco* Insertions.

Chromosomal location of the P-element insertions was determined by crossing homozygous progeny to balancer lines in a *yw* background. Progeny from this cross carrying the balancer and coloured eyes, was then backcrossed to the host strain, *yw*. Separation of the eye colour from the balancer marker, indicated that the insertion was located on that chromosome.

Parent Line (G ₁)	UAS- <i>disco</i> Transformant Line	Location of Chromosomal Insertion
unknown	1	III
	2	II
	4	ND
	44	III
14	17	X
	20	II
	22	II
17	6	ND
	36	III
24	16	III
	32	III
33	24	ND
	29	II
34	19	ND
40	38	ND
	43	III
	46	III
47	11	ND
	37	ND
	41	ND
85	48	III

ND not determined

APPENDIX B**Sequences**

Figure B-1. Intron Sequence.

The sequence of the intron was determined using the bi-directional primer walking method. The primers used are listed in Table 4. The putative Disco binding site, s280 (Lee et al., 1999), was found roughly 1 kb downstream of the transcription start site. The s280 Disco binding site is highlighted.

TATGTATAGTTCCGAGTAAAGTTGATTTGCCGTCGGCACTTCAAGCAAAGBAATTCAAAATCAAGTGAAGTCATAGTGAATATTTTTCGAGA
90
ATACATATCAAGCTCATTTCAACTAAACGGCAGCCGTGAAGTTCGTTTCTTAAAGTTAAGTTCACCTCAGTATCACTTATAAAAAGCTCT

TCAGAGGAGTATTTTCTAAAAAGAAATCCTGGTGCCATAAAAATATTACCCATTCAAGAATTGTGTATGAAATAGGAACGCCAACAAATAG
180
AGTCTCCTCATAAAAGATTTTCTTTAGGACCACGGTATTTTATAATAGGGTAGTTCCTTACACAAATACCTTATCCTTGGCTGTGTATC

GATTCCTTTTCGGTAAATAATGATCTTGTAGGATCACATAGGCTGCTAGTGGCATTAAATATGGTATTACCATTTTCATCGGATCTCTG
270
CTAAGAAAAGCACCATTATTAAGTACAGCAATCCTAGTGTATCCGACGATCACCGTAAATATACCATAATGGTAAAAGTACCTTAGAGAC

GTGATGTGCTGCCATCTTTTACCTCTAGTCTATGGAATTTCTTATAATATCCTGACTTTGCTAATGAAAACAGCTTGTAAATCCAAATG
360
CACTACAGCAGCGTAGAAAATGGAGATCAGATACCTAAAAGAAATATTAAGGACTGAAAACGATTTAACTTTTGTGCAACAATTAGGTTAC

TTGTAACCCATTTTGTCTATTTGCTGCGTGAGCTTCGACGACAAAAGTGAATGCTTTCGGGGAAACGCCAAATGTCAAAGTTGTA
450
AAGCATTTGGGTA AAAACAGATAAACGACGCACTCGAAGCTGCTGTTTTTCACTACAGAAGCCCCCTTTCGGGTTTACAGTTTCAACAT

ATTAAGATTTTGTCTCGGCTTTTCGAGCTCTGTTTTCTCGTTGATGCTCGGACCATCGGCGTTGTCTATCTTTGGACCATCGCATGCC
540
TAATTCATAAACAGACGCCAAAAGCTCGAGACAAAAGAGCAACTACAGGCTGTAGCCGCAACAGTAGAAAACCTGTAGCGTACGGG

CTTGCAATGCCCTTGTGCCATCGACTCTGCTTATTAATGTACTACTATTAACAGCAACTACAAACAGCAATATAGCCGCGGATTC
630
GAACGTACGGGGACACGGGTAGCTGAGGACGAATATATACATGATGATRAATGTCGTTGATGTTGTCTTAAATACGGCGGCCTAAC

CTCACAATCGGGCATCTTCCATCCCAACTGCCACACACCGTACTCCACAGATCTTGGATCTACCATCTACCATCTAAAGCGTCTCCCAA
720
GAGTGTAGACCCGTAGAAGGTAGGGTTGACGGTGTGTGCGATGAGGTGCTAGAACCTAGATGGTAGATGGTAGATTTCCGACAGAGGTT

CTCTCGGAATCTTCAATCTTGTCTGTTCTTCTTTGGTACTTTCAACATTTTGTCTGCTTTGCCACTGCCGATAATCAAAAACGTTT
810
GAGAGCCTTAAAGGTAGAACAGCAAGAACAGAACCCATGAAAGTGTAAAACGACGGAAACGGTACGGCTATTAGTTTTTTGCAAA

CGTCCGAGAGCCATCAAAAGTTGTGATGGTTTTTCCCTACCCCCAGCTGCCCAACATCACCCCCCTTTCACCATCGGTGGCTTAAG
900
GCAGGCTCTCGGTAGTTTTCAACACTAACCAAAAAGGGGATGGGGGTCGACGGTTGTAGTGGGGGGAAAGTGGTAGCCACCGGAATTC

ACATTTTAAGCGTTTGACAAAATAATTACCCATGTACATGCATACTCACCTCGATCTGTCGACCCGGTGCACCTGAGAGAAAATAGAGAG
990
TGTAATAATCGCAAACTGTTTTTAATAAGGGTACATGTACGTATGAGTGGAGCTAGACAGCTGGCGCACGTGACTCTCTTTTATCTCTC

GTGTATTGTTTTCCGTTGCGCCTTATTGTGCTATGCAAGATGTTATGATGCTTAGATCTGAAATGTGTAAAAGATCTATATTTTT
1080
CAACATACAAAAGGCACCGGATACACGATACGTTCTTACAAATACTACGAATCTAGAACTTACACAATTTCTAGATATAAAA

TTTGAGTTGAATCTCTTTTCAAAAGATCTTGGAAAGATAAAAAGGTATAGGCACCTTCAACTCATAATGATAAGTAACTTATAATGAT
1170
AAACTCAACTTAGAGAAAAGTTTTCTAAGAACCCTTCTATTTTTCCATATCCGTGGAAGTTGAGTATACTATATCAATGAAATATTACTA

CTAGAAAATTTGTCAACATCTTTGGGTCATCTCTATTTCTGAAATGGTAAAGAAATATGGTAAACATATTTTCGGCCACTAGTCAGAAA
1260
GATCTTTTACACCGTTGTAGAAACCCAGTAAAGACTTACCATTGCTTATACCATTTGTTATAAAAAGCCGTGGATCAGCTCTT

CTGATTACTTCCAGTACCTGACARTTAGTTTTTTTCCGCCCTTTTTGCTCTTCTTTTTTTCTTGCGCTTTCGCATTTTCGCCGT
GACTAATGAAGGTTTCATGGAGTTAATTCAAAAAAGGGGGGAAAACGAGAAGAAAAAGAACCGGAAGCGTAAAAGCGGCACA 1350

GTACATACACTCATTTAATGCTATTGTTGTCATCGTTACTAATGAGCCCCATAAAAGCGACCATCTCCTGGCTTCTGCAACTGTGTG
CATGTATGTGAGTAAAAATACGATAACAACAGTAGCAATGATTTACTTCGGGTATTTTTGCTGGTAGAGGACCGAAGGACGTGACACAC 1440

CGTGTACCTGTATGCTGATGTTGTTGAGCCATAGTCTATGTTGTTTTTCCGGCTTAACTTTTATAGCTGGCATTTGGGCAAGCGAATGCGAA
GCACAAATGGACATACGGACATACACTCGGTATCAGCATAACCAAAAGCCGAATTGAAAATTCGACCGTAACCCGTTCCTTACGCTT 1530

TGTTGTTTCGACTTTTGAGATCGAATCCAGATAGCCAGCCAGCATCGCACACAGATCATCACCATCGTATCCGCATCTCACCGCAA
ACACACAAGCTGAAAACTCTAGCTTAGGCTATCGGTCGGTGTGTAGCGTGTGTCTAGTAGGGTAGCAGTAGGGCTAGAGTGGCGTT 1620

TGTCCCAAGTTTGTGAGCGCCCTGGGTTCCGTTCACTTCTTTTTTTTTTTTTACCCTGATTTGTTTCGTTTTTAAAGTCAAGTC
AACAGGGTCAAAAACACTCGGGGACCCAGCCAAGTGAAGAAAAAATGGGGACTAAACAAGCAAAAATTGCAGTTTACG 1710

GACTCTCACBATTACGGTAGCGAACCAGTCCACBATAAAAAAGAGCTAGCTTCGGCTTACGATTTGCTTTTGTGTTTTGGCTTTGGC
CTGACGAGTGTTAATGCCATCGCTTCCTCAGGTGTTATTTTTTTCGATCGAGACCGAAATCTAAACGAAAACCAAAACCAAAACCG 1800

TTTAACTTCGACCGATCGGATCCAAGTCAAGTCCGACACTGATCGCCGCATGTTGTCGTCGCCGTCGACGTTGTCAGAGCGGATTTTA
AAATGAAAGCTGGCTAGCTTAGGTTTACGCTTCAGGCTGTGACTAGCGGCTACACAGCAGCGGCAGTGCACAGCTCTCCGCTAAAAT 1890

ATGGCCAGAAGAACCAGAAAGCTGTAAGATTTCCGTCGAGTAAATGAGGCAATGTCACCTTACACAAGTAATCGCCAGTGTAGTG
TACCCTTCTTTGGCTTTTTCGACATTTCTAAGCGAGCTCATTTACTCCGTTACACGTAATGTGTTCATTACCGGTCACAAATCAC 1980

TACAGTGGCCCTTGCTACGACAGAAATGGGTGCATAGGAACCAAAATGATAAAGATTAAACAATGATGTAGCCTCAGTCACTGGGAT
ATGTCACCCGGAACCGATCTGTCTTTAACCCAGTATCTTGGTTTTACTATTTCBATTGTTTTACTACATCGCAGTCAAGTCAAGCTTA 2070

ACCCCTTTGATTATTTCAAAATACAAAACATGGCAACTCTAGTGTTCACTGTACTATCGGCTCGACATGAAAGATATGCCGCCATCG
TGGGAAAACATAAAGTTTTATGTTTGTACCCTGAGATCAACAAGATGACATGATAGCCGAGCTGTACTTTCTATACGGCCGTTAGC 2160

GTGTCGCGCATATAAATGTTGGCTTGTCTCGAGTTATGTTBAGCAGTGTGTTGTTTACCAATAAATCTAGCTGGATTTGCACAAATTTGCC
CACAGCGGCTATATTTACAAAGCGAAGACTCAATAACATTCGTCAACACAGAATGGTATTTAGATCGACCTAAAAGTGTTCACCCG 2250

TAAGCTCCTTGGAAGCTTTCACTTGGGAATTTCCGTAATACACTTGAGATATGACTTTAGAGCGCTTTCCAAATGCTATAGATTAGTGCTT
ATTCGAGGAACCTTCGAAAGTGAACCTTAAAGCCATTATGTGAACTCTATACTGAAATCTCGGAAAAGTTACGATATCTAATCAGGAA 2340

TGCGGTTGGCAATGCAGTCATATGTACATATACATTCGCCTCAGATATTAATCTATACTTTAAGGATATCGAAATTAATTAATGAAAAA
AACCAGCACCCTTACGTCAGTATACATGATATGTAGCGGAGTCTATAATTAGATATAGAATTCCTATAGCTTATAATTAATTAATTTT 2430

GCCATTTGAACGCAATGGGATTTTTCTCGAAGCACACAGCTGGCAGCCTTAAAGACTTTCCTTTAAATCTGGATTTCAAGATGTATC
CGTBAACTTGTGTTACCTTAAAAGGAGTCTCGTGTGTGCAGCCGTCGGAATTTCTGAAAAGAAAATTTAGACCTBAAGTCTTACATAG 2520

TTACAGCAATGACCAACTAATCGCTGAATCCACTGATTAATGAGACCCCCCCCCCCAAATAAAGTTTTCTCCCTTACTTATTTT
AATGTCTTACTGTTGATTAGCGACATTAGGTGACTAATTACTCTGGGGGGGGGGGGTTTTTTTCAAAAGAGGGGAACGAATAAG 2610

GACCCTCTGACTTTTCGCACCTCTAATTGCGCATCAATGACCACTGCCAATGTCAGGTCAGGAGTCAATGTCCATGTCCCAGGAC
CTGGCAGAACTGAAAAGCGTGAGATTAACGGCTAGTTACTGGTGACGGGTACAGGGTCCAGGTCTCAGTTACAGGTACAGGGCCGTG

CACAGACCATTCACCAATTTGCCCTGCTGACTCTCGGCGATAAGGTTGATTCCAGTTCCCAAGTGTAGTTGCGCAATCGGATTTGCCCC
GTCTCTGGTAAGTTGTTAABCAGGGACGACTGAGAGCGCTATTCCAACCTAAGGTC AAAGGTC AACATCAACGGGTTAGCCTAACAGGGG

GCAACCCCTGGCAATAGTTTTGATTAGGACTGACCGATTCAGTCTCCTGATTGACACTCCCTGATTGACACTCTAACGATTCGTTT
CGTTGGGACCGTTATCAAAGCTAATCCTGACTGGCTAAGTGACTGAAGGACTAACTGTGAGGGACTAAGTGTGAGGATTGCTAAGCAAA

CTGTTTCTTGTGTTTTCAG
GACAAAGAACAAACAAAAGGTC

Figure B-2. The Sequence of the 5' Flanking Region to the *disco* Gene.

The upstream region from the *disco* gene was bi-directionally sequenced by primer walking. Primers used are listed in Table 3. Approximately 7 kb was sequenced and analysed. The putative Disco binding site, s120 (Lee et al., 1999), was found roughly 2.8 kb upstream of the *disco* transcription start site and is indicated within the sequence by a shaded box.

GATCCGTGGCCCTTTAATACCCCTGCATCCGCATGTCGCGGTGCATAAATAAAACTGTAAACCACACAAATAATCCCCAGATAGTCGAGTC
90
CTAGGCACCGGAAATATATGGGACGTAGGCGTACAGCGCCACGTATTAATTTTGTACATTTGGTGTGTTTATTAGGGTCTATCAGCTCAGC

GAGTGGACGAGTGCCTCCAGATTCATATACCATATATTCGGCATCTGGGGTGGCTGTCCCAATTCGATGGTAGAAATGCTAGATGGG
180
CTCACCTGCTCACGACGGGGTCTAAGGTATATGGTATATAAGCGCTAGACCCACCGACAAGGGTAAAGCTACCATCTTACGATCTACCC

AATGGTATCTCATCTGGGTCCATCATTAGGGCATGGTGAACCTTGTTCGCCAATCAGTGAATCACTTTGTGTGTTTCGTATACATACAT
270
TTACCATAGAGTAGACCAAGGTAGTAATCCCGTACCACCTTTGAACAAGGGCTTAGTCACTTAGTGAAACACACAAGCATATGTATGTA

AATCAATGATGTGCCCGGAGGGTGCACCTCTAATGTCTTTAATAGTTTGTACCGACCTACGAGCAATTACTAAACATATCCTCAA
360
TTATGTAACACACGGGCGCTCCACAGTTGGAGATTACAGGAAATATCAAAACATGGCTGGATGCTCGTTAATGATTTGTATAGGAGTTT

CTCTCGCACACACACACACACAGAAACACACACACAGTTACACAGTAGCAGGGAAATAGAAAATCTCGGAAAACCTACCCCAA
450
GAGAGCGT

AAATGCATTAATTAATGCTCGATGTAGGCTCTCTCCCTGGATATTTACTGAGTATGGGTGTGGCTGTCCGCACTGTGTGTAGTGAAATG
540
TTTACGTATTAATAACGAGCTACACTCCAGAGAGGACCTATAAATGACTCATACCCACACCGAACAGCGGTAGACACACTCACCTTTTAC

TATTAAGCCCATGCCTCTAAATGAATCCAAATATAAAATATACCCGAAAGGATTCAGCTGTTGAGATTTTTTCGAAAGGCTCGATTT
630
ATAATTCGGGTACGGAGATTTACTTARGGTTTATATTTTATATGGGGCTTTCTTAAGTTCGACAACTCTAAAAAAGCTTTCCGAGCTAAK

CAATAAGGAACAACAATTTTATTCAAAATGGCACTGGATAGCTTTCTGTTGATTTCTGCTATTTTAGGGTTTGTAAATAAAAATTTTCG
720
GTTAATCCCTTCTTTGTTTTAAATAAGTTTTACCGTTGACCTATCGAAAGCAACTAAAGACGATAAAATCCAAACATTTATTTTATAAAGC

AATATTAATAAACTATATACAAATAGGAAATGCTTTTTCAGACTATTTGCTTTGTTTTTATTTGAATTTTTTTTGTATTTGATTTGGTATTA
810
TTATAATTTTTTGTATATATGTTATCTCCTTTACGAAAAGTCTGATAACAGAAACAAATTTACTTTAAAAAATCAITTAACATATCCATAT

CACAAGCTTTCTTACTACATCTATTCATTTCTTATTTGCGCGTTTCTTCTTTTAAAATTAATTTGAAAATTTGAAACTCAATATACTAT
900
GTGTTCGAAAGAATGATGATAGATAGTAAAGATAACCGCCAAAAGAAGAAATTTTAAATTTACTTTTAAACTTTGAGTPTATGATA

GTACTTCATCAGATCTTTGCTCAGGATAGTTGTAAATTTCACTTTTCACATCATTTTGCCTGTAATAATATTTAATAATACGAAAAGA
990
CATGAGTAGCTTAGAAACGAGTCTAATCAACATTAAGTGAAGTGTAGTAAACGAACTTTTATATAAATTAATTAATGTCCTTTCTE

ACRCCATTAATCCATAAATAAGCGGGTTCGAACAGGGATATCGATGACCTAAACATATTTTACTAGACGGGGTCACTTGGGCATCTAA
1080
TGTGGTCAATTAAGGTATTTATTTCCGCCAAGCTTGGTCCCTATAGCTACTGGATTTGTATAAATGATCTGCCCCAGTAGACCCCTAGATT

AATGCTTGGATCAGCCAGCAGGATTCAGGATTCAGTCTCTTCAGATGTTGGTGGCCAGGATCGATTTGCTAATCCATTTGCCATGCCAT
1170
TTGACGAACCTTAGTGGTCTCTAAGTCTTAAGCTCAGGAAGTCTACACCCACCCGGTCTAGCTAACGATTAGGGTAAACGTTACGGTA

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6938
GCGTAGAA

APPENDIX C***in Vitro* Analysis of the *disco* Promoter**

INTRODUCTION

Assaying Gene Regulation

Insect cell lines are often used in gene expression studies due to their capability in accurate translation and processing of proteins, their ease of use, ease of maintenance and the ability to control experimental parameters. Cultured *Drosophila* embryonic Schneider line 2 (S2) cells (Schneider, 1972), have been used to study gene expression by the transient activity of a recombinant protein. In one study, 5' deleted actin 5c distal promoter regions were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and transfected into S2 cells (Chung and Keller, 1990). The transformed cells were assayed for transient expression of CAT (Chung and Keller, 1990). Regulation of CAT activity from deletion constructs allowed identification of ten important regulatory regions of the distal actin 5c promoter (Chung and Keller, 1990). Other bacterial reporter genes (eg. *lacZ*) may also be fused with a promoter of interest and then assayed for expression. This technique of assaying for regulatory elements which mediate expression of a promoter, can be modified to study autoregulatory activity of a promoter. By expressing the regulatory protein of interest in the same cells as the promoter-reporter gene fusion, one can assay for reporter expression and thus, assay autoregulatory activity.

The expression vector pMT/V5-HisB (Invitrogen, Catalog No. 4120-20, Table 2) is used to conduct gene expression studies in cultured insect cells.

The sequence of a regulatory protein can be inserted downstream of the inducible metallothionein (MT) promoter such that when MT is induced, the protein is expressed. Since cloning into pMT/V5-HisB generates a fusion protein with a V5 epitope tag, protein expression can be monitored with the anti-V5 antibody. DNA mediated co-transfection of a regulatory protein within this construct with a promoter-reporter gene fusion allows autoregulation to be assayed. This system is useful for assaying transient expression to define *cis*-acting regions required for proper gene expression and regulation.

This Work

Within the *disco* locus itself, two binding sites for the Disco protein were found by immunoprecipitation (Lee et al., 1999). One of the binding sites, which is estimated to be 280 bp long (s280), is located within the intron, while the other 120 bp site (s120) is 5' to the *disco* transcription start site (Lee et al., 1999). Several plasmids were created to test whether one or both of the binding sites are sufficient for regulation of *disco* mRNA expression. Two of these constructs, a promoter-*lacZ* fusion consisting of 7.8 kb of 5' flanking DNA of the *disconnected* gene (pCaSpeRAUG β galBK7.8, Table 2) and one consisting of 6.5 kb of the *disco* locus, including the intron, first exon and several kb of upstream sequence (pCaSpeR β galCN6.5, Table 2), differ in the *disco* binding site they each encompass (Figure 3) (Lee, 1994).

In order to assay for autoregulation of *disco* mRNA expression, the *disco* coding region was cloned into the multiple cloning site of the pMT/V5-HisB plasmid to create the pMT/V5-HisB-*disco* vector. Upon induction with copper sulfate, this fusion construct produces Disco protein with a V5 epitope tag. It is believed that the Disco protein may bind to the putative binding site (s120 or s280) and activate transcription of the *lacZ* gene in cells co-transfected with both this fusion construct and with one of the two promoter-reporter fusions. Expression of β -galactosidase was used as an indication whether the particular binding site is sufficient for autoregulation of Disco protein expression *in vitro*.

METHODS AND MATERIALS

Drosophila Cells - Designation and Karyotype

An embryonic cell line established by Schneider (1972) was used for DNA transfections. Schneider line 2 (S2) cells are epithelial-like and the line consists of only XX cells which are 60-80% tetraploid (Schneider and Blumenthal, 1978). The cells were cultivated in complete DES Expression Medium and 10% heat-inactivated Fetal Bovine Serum at 24°C.

Transfection of Schneider Line 2 Cells

Recombinant proteins were expressed in *Drosophila* S2 cells using the DROSOPHILA EXPRESSION SYSTEM (DES-Inducible Kit, Invitrogen, Catalog No. K4120-01). S2 cells were grown (under sterile conditions) in Complete DES Expression Medium with L-glutamine containing 10% heat-inactivated Fetal Bovine Serum (GIBCO BRL, Catalog No. 10438-026) and penicillin-streptomycin (GIBCO BRL, Catalog No. 15140-122) to a final concentration of 50 units penicillin G and 50 µg streptomycin per milliliter of medium, at 24°C. Every four to five days, cells were subcultured into 10 ml of fresh Complete DES Expression Medium in 75cm² tissue culture flasks (Corning, Catalog No. 430720). Cultured cells were prepared for transfection by seeding approximately 3 × 10⁶ S2 cells in a 35 mm plate in 3 ml of Complete DES Expression Medium. Cells were grown for 16 hours, or until they reached a density of 2 to 4 × 10⁶ cells/ml.

Each DNA transfection required the following transfection mixes: Solution A: 36 µl of 2 M CaCl₂, 19 µg of each recombinant DNA and double-processed tissue culture water (Sigma W-3500) to bring the final volume to 300 µl; Solution B consisted solely of 300 µl of 2X HEPES-Buffered Saline (50 mM HEPES, 1.5 mM NaH₂PO₄, 280 mM NaCl, pH 7.1). Solution A, containing recombinant DNA, was added dropwise to Solution B, which was continuously mixed by mild vortexing, and the combined solution was incubated at room temperature for 40 minutes to generate a fine precipitate. This solution was mixed and added dropwise to cells for transfection. After addition of each drop the cells were swirled. Transfected cells were incubated

for a further 16 to 24 hours at 24°C. Using a pipette, cells were dislodged from the plate and transferred to a 15 ml conical tube (Sarstedt, Catalog No. 62-554-002). The cells were spun down for 5 minutes at 1,000 rpm and resuspended in 1 ml of complete medium. After the cells were washed this way twice, they were resuspended in 3 ml of fresh Complete DES Expression Medium and replated into the same vessels. To induce expression from the vector pMT/V5-HisB, 17 µl of 100 mM CuSO₄ was added to the cells in medium for 24 hours. On the fourth day, cells were harvested by dislodging them from the plate and were washed twice in 1 ml of 1X PBS. Cells were then assayed for expression of *disco* and *lacZ*.

Immunolabeling of S2 Cells

To assay S2 cells for protein expression, they were adhered to dry microscope slides that had been briefly dipped in subbing solution (1% gelatin and 0.1% chromium K sulfate, (Ashburner, 1989)). Cells dislodged from petri plates were washed and resuspended in 1 ml of 1X PBS. 200 µl of harvested cells were allowed to adhere for 30 minutes to the centre of a subbed slide. Non-adhered cells were washed off by briefly dipping the slides in 1X PBS (in coupling jars). Adhered cells were fixed for 10 minutes at room temperature with 2% paraformaldehyde (in PBS) and the fixative was washed off in 1X PBS with three ten minute washes. After pre-incubation of cells in blocking solution (Normal Goat Serum and PBS, 1:15) for 30 minutes, primary antibody (anti-V5 or anti-β-galactosidase) was added to the adhered cells and

adsorbed overnight at 4°C. Three ten minute washes in 1X PBS were used to remove both the NGS and antibody. The cells were again blocked with NGS and PBS for 30 minutes at room temperature before secondary HRP-conjugated antibody was added and allowed to adsorb for 3 hours. After three 10 minute washes in 1X PBS, 100 µl of staining solution (250 µg/ml of DAB and CoCl₂ in PBS) was added to each slide for two minutes. 3% hydrogen peroxide was then used to initiate the enzymatic reaction between HRP and DAB, which was stopped by dipping the slides into 1X PBS. Cells were then dehydrated in a series of ethanol washes (50%, 70%, 90%) and twice with absolute ethanol (3 minutes each). After allowing excess ethanol to evaporate, cells were immediately overlaid with D.P.X. mounting medium (Aldrich Chemical Company, Inc., Catalog No. 31,761-6) and covered with a microslip. Preparations were allowed to set overnight before examining under a microscope.

Bradford Protein Assay

A Bradford protein assay (Bradford, 1976) was conducted to evaluate the total amount of protein within the transfected cells. Two days after transfection, S2 cells were harvested, pelleted at 3,000 rpm for 5 minutes and resuspended in 1 ml of 1X PBS. Each sample was split into two equal 500 µl aliquots. One set of aliquots was pelleted and stored on ice for use in the β-galactosidase assay. The other set of aliquots was also pelleted but the cells were resuspended in 50 µl of lysis buffer (50 mM Tris.HCl, 150 mM NaCl, 1%

Nonidet P-40, pH 7.8). The cell suspension was incubated on ice for 10 minutes and the nuclei and debris pelleted at 15,000 rpm for 15 minutes to create a cell-free extract. The supernatant (lysate) was transferred to a fresh tube. 2 μ l of each lysate sample was added to a glass culture tube (Fisher Scientific, Catalog No. 14-961-29) containing 800 μ l sterile water and 200 μ l of Bio-Rad Dye Reagent Concentrate (Catalog No. 500-0006) and allowed to incubate at room temperature for 15 minutes. A Cary IE UV-Visible Spectrophotometer was used to measure absorbance at 595 nm. Testing of all cell-free protein samples was done in triplicate. Following the assay, the protein amount for each sample was calculated by averaging the three readings.

β -galactosidase Activity Assay

To assess β -galactosidase activity, cells were incubated with o-nitrophenyl- β -D-galactopyranoside (ONPG). β -galactosidase catalyses a colourimetric reaction, where colourless ONPG is hydrolysed to yellow o-nitrophenol. This assay was performed both with whole cell extracts (including nuclei) and with total protein extracts (cell-free extracts). Pelleted whole cells (which were stored on ice) were resuspended in 201 μ l of 0.1 M sodium phosphate pH 7.5 and each sample was mixed with 3 μ l of 100X Mg buffer and 66 μ l 1X ONPG (Sambrook, 1989). From the cell-free extracts, 100 μ g of protein was added to 201 μ l of 0.1 M sodium phosphate, 3 μ l of 100X Mg buffer and 66 μ l ONPG. Triplicate ONPG hydrolysis reactions were prepared

in glass culture tubes (Fisher Scientific, Catalog No. 14-961-29). Each sample was incubated in a 37°C water bath until either a faint yellow colour appeared or until 30 minutes had passed. All reactions were terminated by addition of 500 µl of 1 M Na₂CO₃ and to obtain a clear supernatant the samples were centrifuged for 5 minutes at 15,000 rpm. The optical density was measured at 420 nm (OD₄₂₀) for each sample using a Cary IE UV-Visible Spectrophotometer. β-galactosidase activity per mg of protein for the cell-free protein extracts, in Miller units, was calculated using the formula:

$$(\text{OD}_{420} \times V) / \Delta T \times 0.0045 \times M$$

where V = ml of sample, ΔT = incubation time in minutes, M = mg of protein in sample.

SDS-PAGE and Western Blotting

Mini-PROTEAN II gel assemblies (BIO RAD, Catalog no. 170-3930) were used for casting and running of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (see Table C-1 for stock solutions). 12% SDS-polyacrylamide gels were used for charge separation of proteins. Protein separating gels consisted of 3.3 ml H₂O, 2.5 ml Tris.HCl pH 8.8, 4.0 ml 30% acrylamide mix, 100 µl 10% SDS, 100 µl 10% ammonium persulfate (APS) and 5 µl N, N, N', N', - tetramethylethylenediamine (TEMED, Fluka, Catalog No. 87689) and were allowed to polymerize at room temperature before polymerization of the stacking gel. The stacking gel solution (3.0 ml H₂O, 1.25 ml Tris.HCl pH 6.8, 0.66 ml 30% acrylamide mix, 50 µl 10% SDS, 50 µl 10%

APS and 5 μ l TEMED) was allowed to polymerize with combs in place, to make wells for loading of protein samples. After polymerization of the stacking gel, the combs were removed, the cast gels were placed in the Mini-PROTEAN II chamber and submerged in Running Buffer (Table C-1). 50 μ g of total protein from each sample was combined with 4X SDS gel loading buffer (Sambrook, 1989) and the volume was brought up to 30 μ l with lysis buffer. The samples were boiled for 3 minutes before loading onto the SDS-polyacrylamide gels. Gels were placed in the Mini-PROTEAN II chamber filled with Running Buffer (Table C-1) and run for approximately 40 minutes at 200 V until the bromophenol blue dye had run off the gel. Gels were removed from the assemblies and transferred to Whatman paper. Immobilon-N transfer membrane (PVDF by Millipore, Catalog No. INHV00010) was presoaked in methanol before being used in the protein transfer sandwich. To transfer proteins from the gels, PVDF, Whatman paper and gel sandwiches were made of a sponge, two pieces of Whatman paper, SDS-PAGE gel, PVDF, two pieces of Whatman paper and another sponge. This sandwich was soaked in cold Transfer Buffer (Table C-1) before it was placed in the Mini-PROTEAN II chamber. The chamber was filled with cold Transfer Buffer, and the proteins were transferred at 100 V for one hour at 4°C.

The transfer membranes were removed from the sandwiches, placed in small trays and rinsed twice with 30 ml of Post-Blot Buffer (Table C-1) for 10 minutes. This and all subsequent reactions were carried out at room temperature with gentle agitation on a shaker. Non-specific binding of antibody to the protein samples on the transfer membrane was blocked using

skim milk powder (2.5 g milk powder) and Nonidet P-40 (NP-40) (30 μ l) dissolved in 50 ml Post-Blot Buffer. After one hour of blocking, 5 μ l of primary antibody (anti-V5 or anti- β -galactosidase) was added to a fresh 50 ml sample of skim milk and NP-40 dissolved in Post-Blot Buffer, for a 1:10,000 dilution. After the primary antibodies were allowed to bind for one hour, the membrane was washed with three successive changes of Post-Blot Buffer with dissolved NP-40. HRP-conjugated secondary antibodies (1:10,000) were added to 50 ml of fresh Post-Blot Buffer/skim milk/NP-40 and were allowed to bind for 30 minutes. The secondary antibodies were rinsed away with two 10 minute washes in Post-Blot Buffer and NP-40, and two 10 minute washes in 10 mM Tris pH 7.4. The HRP was detected using Enhanced Chemiluminescence (ECL, Amersham LIFE SCIENCES, RPN2106) and Kodak Scientific Imaging Film (X-OMAT AR, Catalog No. 165 1454). Autoradiographs were exposed for 10 seconds, 30 seconds and 2 minutes at room temperature and developed using a Kodak M35A X-OMAT Processor.

Table C-1. Stock Solutions for SDS-PAGE and Western Blotting.

Solution	Composition
30% Acrylamide mix	58.4 g acrylamide (Fluka, Catalog No. 01699) 1.6 g bisacrylamide (Aldrich, Catalog No. 148326) at 200 ml H ₂ O
0.5 M Tris.HCl pH 8.8	36.34 g Tris Base (Fisher Scientific, Catalog No. BP152) add 150 ml H ₂ O, adjust pH to 8.8 at 200 ml H ₂ O
1.5 M Tris.HCl pH 6.8	6.06 g Tris Base add 60 ml H ₂ O, adjust pH to 6.8 at 100 ml H ₂ O
1 M Tris.HCl pH 7.4	60.57 g Tris Base add 400 ml H ₂ O, adjust pH to 7.4 at 500 ml H ₂ O
10% SDS	5 g sodium dodecyl sulfate (Fluka, Catalog No. 71736) at 50 ml H ₂ O
10% APS	1 g ammonium persulfate (Sigma, Catalog No. A9164) at 10 ml H ₂ O
Running Buffer	14.4 g glycine 3.0 g Tris Base 1 g SDS at 1000 ml H ₂ O
Transfer Buffer	2.84 g Tris Base 13.51 g glycine 300 ml methanol 0.30 g SDS at 1500 ml H ₂ O
Post-Blot Buffer	9.0 g NaCl 10.0 ml 1 M Tris.HCl pH7.4 at 1000 ml H ₂ O

RESULTS

Successful Transfection Shown by Immunolabeling with Anti-V5 Antibody

Schneider line 2 (S2) cells were transfected with the following DNA constructs: a) no DNA (control), b) pMT/V5-HisB-*disco*, c) 6.5 kb promoter-*lacZ* fusion (6.5*lacZ*), d) 7.8 kb promoter-*lacZ* fusion (7.8*lacZ*), e) pMT/V5-HisB-*disco* and 6.5*lacZ* and f) pMT/V5-HisB-*disco* and 7.8*lacZ*. This transfection set was done in duplication, such that one set of cells was induced with copper sulfate, and the other set remained uninduced. Cells from both sets were then washed, adhered to microscope slides and fixed. The adhered cells from each sample were exposed to anti-V5 primary antibody, anti-mouse HRP secondary antibody and reacted with DAB. In all uninduced samples, cells remained colourless, indicating an absence of the Disco protein with a V5 epitope tag (Table C-2). Induced cells only transfected with pMT/V5-HisB-*disco* and induced cells co-transfected with pMT/V5-HisB-*disco* and 6.5*lacZ* had approximately 1 cell in 200 immunolabel, indicating the presence of Disco-V5 protein (Table C-2). The transfection rate of pMT/V5-HisB-*disco* with 7.8*lacZ* was 20 fold higher than that of pMT/V5-HisB-*disco* with 6.5*lacZ*, as approximately 1 in 10 cells was immunolabeled (Table C-2).

Table C-2. S2 Cell Immunoreactivity to Anti-V5 and Anti- β -gal.

	Not Induced		Induced	
	anti-V5*	anti- β -gal**	anti-V5*	anti- β -gal**
S2 untransfected	–	1/4 to 1/2	–	1/4 to 1/2
pMT/V5-His- <i>disco</i>	–	1/8	1 in 100	1/2 to 3/4
6.5lacZ	–	1/2	–	1/2
pMT/V5-His- <i>disco</i> and 6.5lacZ	–	1/2	1 in 200	1/4
7.8lacZ	–	1/2	–	1/2
pMT/V5-His- <i>disco</i> and 7.8lacZ	–	1/2 to 3/4	1 in 10	1/4 to 1/2

* Approximate number of cells immunolabeled with anti-V5 antibody

** Approximate number of cells immunolabeled with anti- β -galactosidase antibody

Immunolabeling of S2 cells with Anti- β -gal Antibody

Results of antibody labeling with rabbit anti- β -galactosidase antibody were unclear. Both uninduced and induced samples showed β -galactosidase expression in about 1/4 to 1/2 of the cells (Table C-2). Cells co-transfected with pMT/V5-HisB-*disco* and the promoter-*lacZ* fusions, showed no dramatic increase in β -gal expression – the same proportion of cells immunolabeled in both induced and uninduced sets (Table C-2).

Bradford Protein Assay

We were particularly interested in expression from the *7.8lacZ* fusion gene and due to time constraints had to scale down the experimental samples. The second sample set consisted of: a) untransfected S2 cells, b) pMT/V5-HisB-*disco*, c) *7.8lacZ* and d) pMT/V5-HisB-*disco* and *7.8lacZ*. Each transfected cell sample was mixed with copper sulfate to induce transcription from the metallothionein promoter. Transfected cells were harvested, washed and divided into two aliquots of equal volume. Both aliquots of each sample were centrifuged, one of which was resuspended in S2 lysis buffer for use in the Bradford Protein Assay.

The total amount of protein in each extract was quantitated in triplicate and averaged. The amount of protein extracted from lysed cells that were not transfected with DNA was determined to be 3.4 $\mu\text{g}/\mu\text{l}$. Cells transfected with pMT/V5-HisB-*disco* alone and cells co-transfected with pMT/V5-HisB-*disco* and *7.8lacZ*, each contained 2.6 $\mu\text{g}/\mu\text{l}$ of total protein. Cells transfected with only *7.8lacZ*, contained a total of 4.0 $\mu\text{g}/\mu\text{l}$ of protein.

Western Blots

50 μg of total protein from each cell-free sample: lane 1) no DNA (untransfected S2 cells), lane 2) pMT/V5-HisB-*disco*, lane 3) pMT/V5-HisB-*disco* and *7.8lacZ* and lane 4) *7.8lacZ* was run on a SDS-PAGE gel, transferred to a PVDF membrane and probed with antibodies. One transfer membrane

was probed with anti-V5 antibody to visualize the presence of the Disco protein with its V5 epitope. A band at approximately 71.5 kDa (69 kDa Disco and 2.5 kDa V5 tag) was seen in protein samples from cells transfected with only pMT/V5-HisB-*disco* and in cells co-transfected with pMT/V5-HisB-*disco* with 7.8*lacZ*, lanes 2 and 3 respectively (Figure C-1A). These results indicate that the cells were efficiently transfected, and that the MT promoter was induced to transcribe of the Disco-V5 protein. The second transfer membrane was probed with mouse anti- β -galactosidase antibody to detect any β -galactosidase which may have been produced. Many weak bands were seen in all samples, showing that this anti- β -galactosidase antibody seemed to bind to unspecific proteins, including those of the negative control (non-transfected cells) (Figure C-1B).

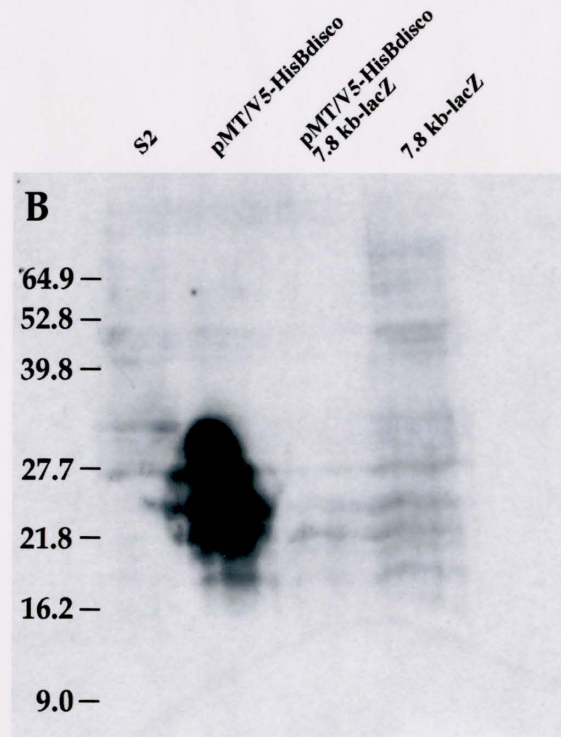
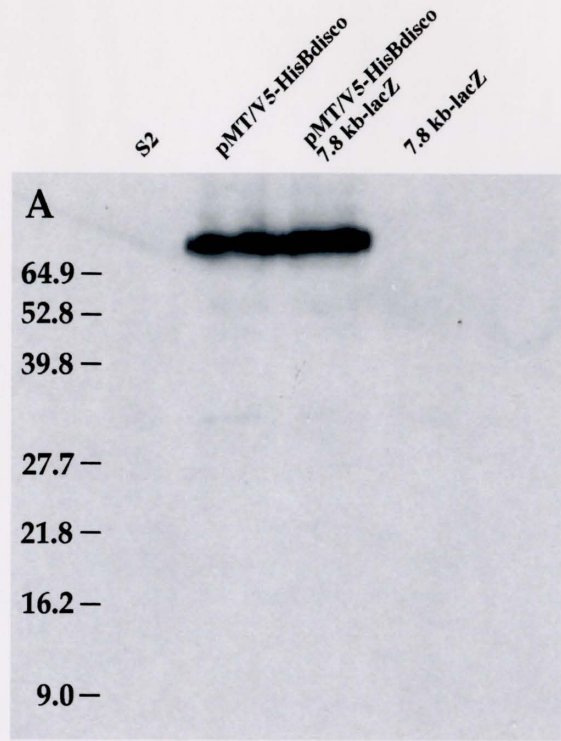
When the same samples were run on a SDS-PAGE gel for a second time and the proteins probed with rabbit anti- β -galactosidase antibody, a positive control of purified β -gal protein (Boehringer Mannheim, Catalog No. 567 779) was run alongside. Again, the antibody bound proteins other than the 108 kDa *E.coli* β -gal protein – several other bands were visible in the positive control (data not shown). In the experimental samples, bands above 125 kDa indicate that β -gal may have been present in the cells. However, due to the unspecific binding of the antibody conclusions about the presence of β -galactosidase cannot be drawn. Results from this Western blot are inconclusive.

Figure C-1. Western Blots of S2 Cell Extracts.

SDS-PAGE of proteins extracted from DNA transfected S2 cells. Molecular weight markers: 64.9 kDa, 52.8 kDa, 39.8 kDa, 27.7 kDa, 21.8 kDa, 16.2 kDa and 9.0 kDa (Gibco BRL Benchmark Prestained Protein Ladder, Catalog No. 10748-010). Protein extract samples (all from induced cells) were loaded onto gels in the following order: untransfected S2 cells; pMT/V5-HisB*disco*; pMT/V5-HisB*disco* with 7.8*lacZ*; 7.8*lacZ* alone.

Blot A was incubated with a 1/17,000 dilution of mouse anti-V5 antibody followed by a horseradish peroxidase-conjugated secondary antibody and developed with enhanced chemiluminescence. Bands are seen above 64.9 kDa. We assume this is the 71.5 kDa Disco-V5 fusion protein. It was present in cells that were transfected with the pMT/V5-HisB-*disco* construct and induced by copper sulfate.

Blot B was incubated with a 1/10,000 dilution of mouse anti- β -galactosidase antibody followed by a horseradish peroxidase-conjugated secondary antibody and developed with enhanced chemiluminescence. Faint bands are seen in all lanes, including the negative control of untransfected cells, indicating that the antibody is not specific to *E.coli* β -galactosidase protein. The dark spot in Blot B is an artifact.



β -galactosidase Activity Assay

The β -galactosidase Activity Assay was used as an indirect method of measuring reporter protein enzyme activity. This assay, which measures the hydrolysis of o-nitrophenyl- β -D-galactopyranoside (ONPG) by β -galactosidase, was performed because it is the most sensitive assay for determination of presence of β -galactosidase. A positive control of pure β -galactosidase protein (0.5 μ g) hydrolysed colourless ONPG to yellow o-nitrophenol within 5 minutes of incubation at 37°C. In this sample, β -gal activity was calculated from the absorbance readings, to be very high, 6319.6 \pm 144.4 (SEM) Miller units (Table C-3). The protein sample from untransfected S2 cells was found to have a very low enzymatic activity, 29.7 \pm 4.2 (SEM) Miller units (Table C-3). Protein extracts from cells transfected with pMT/V5-HisB-*disco*, the promoter-reporter construct or both, also had very low enzymatic activities (Table C-3). These activities are comparable to that of untransfected cell sample.

Absorbance at 420 nm (OD_{420}) readings were also taken for the whole cell samples. Readings of samples from DNA transfected cells were virtually the same as the negative control (untransfected S2 cells). Readings for the positive control of pure β -gal protein were between 18 and 65 times higher than those of the whole cell samples, both untransfected and transfected (data not shown). Although, enzymatic activity was not calculated for these samples, the absorbance readings provide supporting evidence for the β -galactosidase activities determined for the cell-free protein extracts.

Table C-3. β -galactosidase Activity of Cell-free Protein Samples Extracted from S2 cells.

Protein Sample		β -galactosidase Activity (Miller units)* \pm SEM
no S2 cells	β -gal	6319.6 \pm 144.4
	no DNA	29.7 \pm 4.2
S2 cells transformed with	pMT/V5-HisB-disco	24.3 \pm 0.3
	7.8lacZ	25.2 \pm 0.2
	pMT/V5-HisB-disco and 7.8lacZ	24.3 \pm 0.1

* mean of triplicate readings

DISCUSSION

S2 Cells Expressed V5 and β -galactosidase

S2 cells were co-transfected with pMT/V5-HisB-*disco* and 7.8lacZ. Expression of Disco-V5 was induced with copper sulfate and the cells were immunolabeled with the anti-V5 antibody. The transfection efficiency with pMT/V5-HisB-*disco* was found to be approximately 1 in 10 cells. When immunolabeled with anti- β -gal antibody these cells showed β -galactosidase expression in about 1/4 to 1/2 of all cells. However, expression of β -galactosidase, as seen by immunolabeling, was detected in similarly high

amounts in untransfected cells and in cells transfected with either pMT/V5-HisB-*disco* or the promoter-*lacZ* fusion. These results indicate that S2 cells may contain endogenous β -galactosidase activity that is indistinguishable from the bacterial fusion gene.

Further evidence of efficient transfection of S2 cells with the pMT/V5-HisB-*disco* vector, was obtained from the Western blot probed with anti-V5 antibody. Bands above 64.9 kDa suggest the presence of Disco protein with the V5 epitope tag and these were seen in samples transfected with pMT/V5-HisB-*disco* alone, and co-transfected with pMT/V5-HisB-*disco* and 7.8*lacZ*. Blots assayed with anti- β -galactosidase antibody, showed numerous faint bands in each of the lanes, including the negative control. These protein samples were used in a second blot that included a positive control of pure *E.coli* β -galactosidase protein. Here several bands were seen with the control, indicating that the antibody recognizes several proteins. In all other samples, including the negative control, a band which may be the 108 kDa *E.coli* β -galactosidase band, was seen (results not shown). However, due to both unspecific antibody binding and the possibility of endogenous *Drosophila* β -galactosidase protein presence in the cultured cells, no conclusions can be drawn from these results, as to whether the 7.8 kb of *disco* promoter sequence containing the s120 binding site is sufficient for *disco* autoregulation.

A gene coding for β -galactosidase has been found in *Drosophila melanogaster* (Knipple and MacIntyre, 1984; Knipple et al., 1991), purified and characterized (Fuerst et al., 1987). The *Drosophila* β -galactosidase-1 enzyme is a glycoprotein and a homodimer with a molecular weight of 160 kDa (Fuerst et al., 1987). A study has shown it to be endogenously expressed in larval,

pupal and adult tissues (Schnetzer and Tyler, 1996). Studies involving cultured insect cells have shown *Drosophila* β -galactosidase to be induced by ecdysone (Best-Belpomme et al., 1978), however the exact nature of the induction is unknown. Other studies using cultured S2 cells have also found background β -galactosidase activity (Cherbas, L., pers. communication). In *Drosophila* transformed with the *E.coli* β -galactosidase gene, X-gal staining did not distinguish between true fusion gene activity and endogenous β -galactosidase activity (Schnetzer and Tyler, 1996). Thus, the *E.coli lacZ* gene should be used with caution in fusion studies or an alternative reporter gene that is not endogenously expressed in *Drosophila* should be employed. Successful reporter function has been achieved using the green fluorescent protein (GFP) isolated from jelly fish (Yeh et al., 1995).

No β -galactosidase Activity

Neither S2 cell staining nor the Western blots provided results as to the sufficiency of the *disco* upstream region containing the s120 binding site in Disco autoregulation. A more sensitive β -galactosidase activity assay using hydrolysis of ONPG as a measure, was used to study expression from the promoter. The positive control of *E.coli* β -galactosidase protein was found to have an enzymatic activity approximately 213 times higher than any of the other total protein samples. The level of β -gal activity in samples from transfected cells was similar to the negative control of untransfected cells, indicating that *lacZ* expression was not induced by the presence of Disco

protein. Low OD_{420} readings for the whole cell samples add support to the idea that there was no β -galactosidase activity in any of the samples.

β -galactosidase-1 enzyme has been found to constitute less than 0.02% of total soluble protein or 2 to 3 ng per adult fly (Fuerst et al., 1987). This is a relatively small amount, and could account for the low basal amount of enzymatic activity seen in each protein sample. The β -galactosidase activity assay was the most sensitive of the three assays performed to monitor reporter gene expression. Thus, based on the fact that no increase β -galactosidase activity was seen in the protein samples from cells co-transfected with the promoter-*lacZ* fusion and the pMT/V5-HisB-*disco* constructs, we suggest that the 7 kb of upstream sequence and the s120 binding site are not sufficient for Disco autoregulation.

There may be several reasons why the single *cis*-acting Disco binding site was insufficient to regulate Disco expression. In these experiments, a low co-transfection rate of the two plasmids pMT/V5-HisB-*disco* and 7.8*lacZ* may hinder Disco binding, either because not enough Disco protein was produced, or because not enough s120 binding sites were available for binding. Disco binding to the s120 site alone may not be sufficient to stimulate expression of the reporter gene. Disco autoregulation is thought to be tissue-specific, thus the cultured cells may not have been able to provide the tissue-specific factors required to regulate transcription from this binding site in the promoter.

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