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

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

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

Introduction

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

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

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

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

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

Results and Discussion

Genomic location of the disco gene

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

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

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

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

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

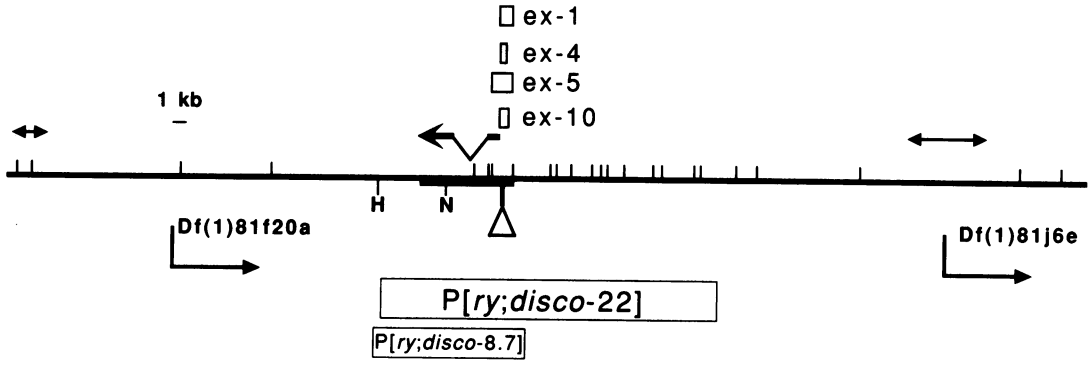

Fig. 1. Schematic diagram of the X chromosome around polytene region 14B3-4. The centromere is to the right. Arrows below the line mark the approximate break points of the deficiencies relevant to locating the *disco* gene; the arrows point in the direction of the deleted material which extends into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

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

Rescue of the disco mutant phenotype

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

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

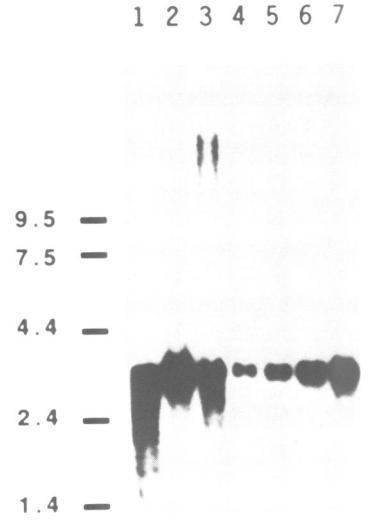

Fig. 2. Transcription of the *disco* gene throughout development. PolyA^{+} RNA isolated from different stages of development was probed with a fragment of genomic DNA from within the coding region of the gene. Positions of size markers (Promega) are indicated. 1, 0–6 h embryos; 2, 0–24 h embryos; 3,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

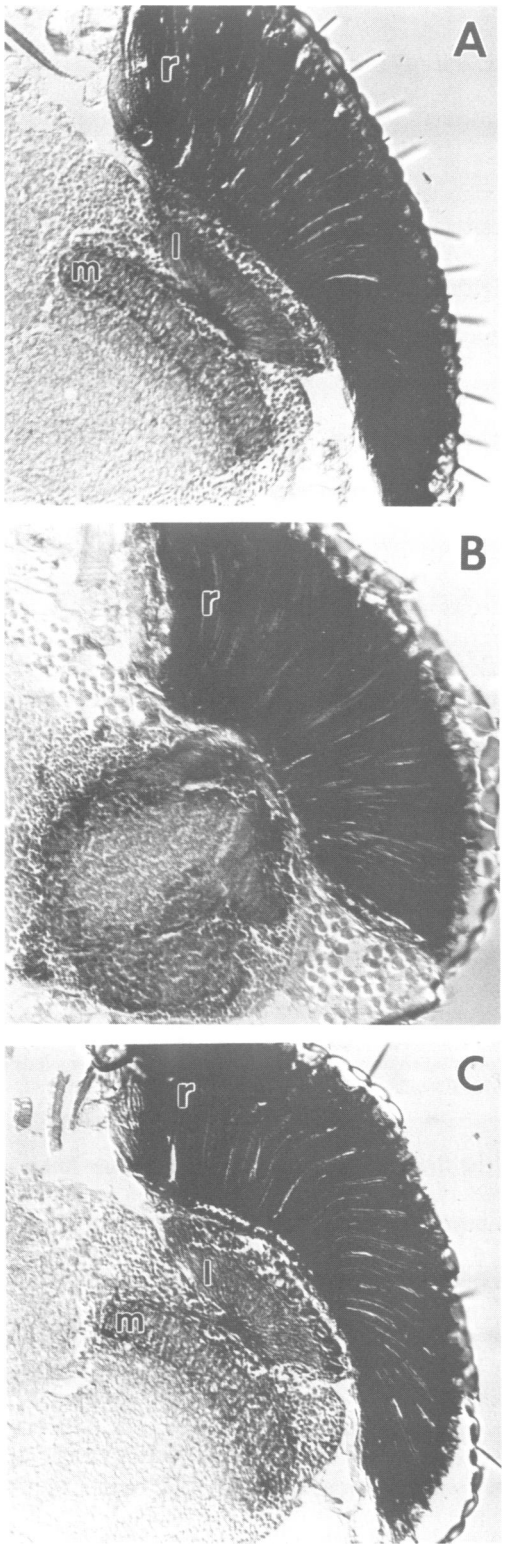

Fig. 3. Horizontal sections through heads of $disco^{1}$ mutant flies and their brothers carrying the P[*ry*;*disco*-22] construct stained with Mab 24B10. Panel **A** is a section through the optic lobes of a wild type fly. Panel **B** is a section through the head of a male $disco^{1};ry^{506}$ fly;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

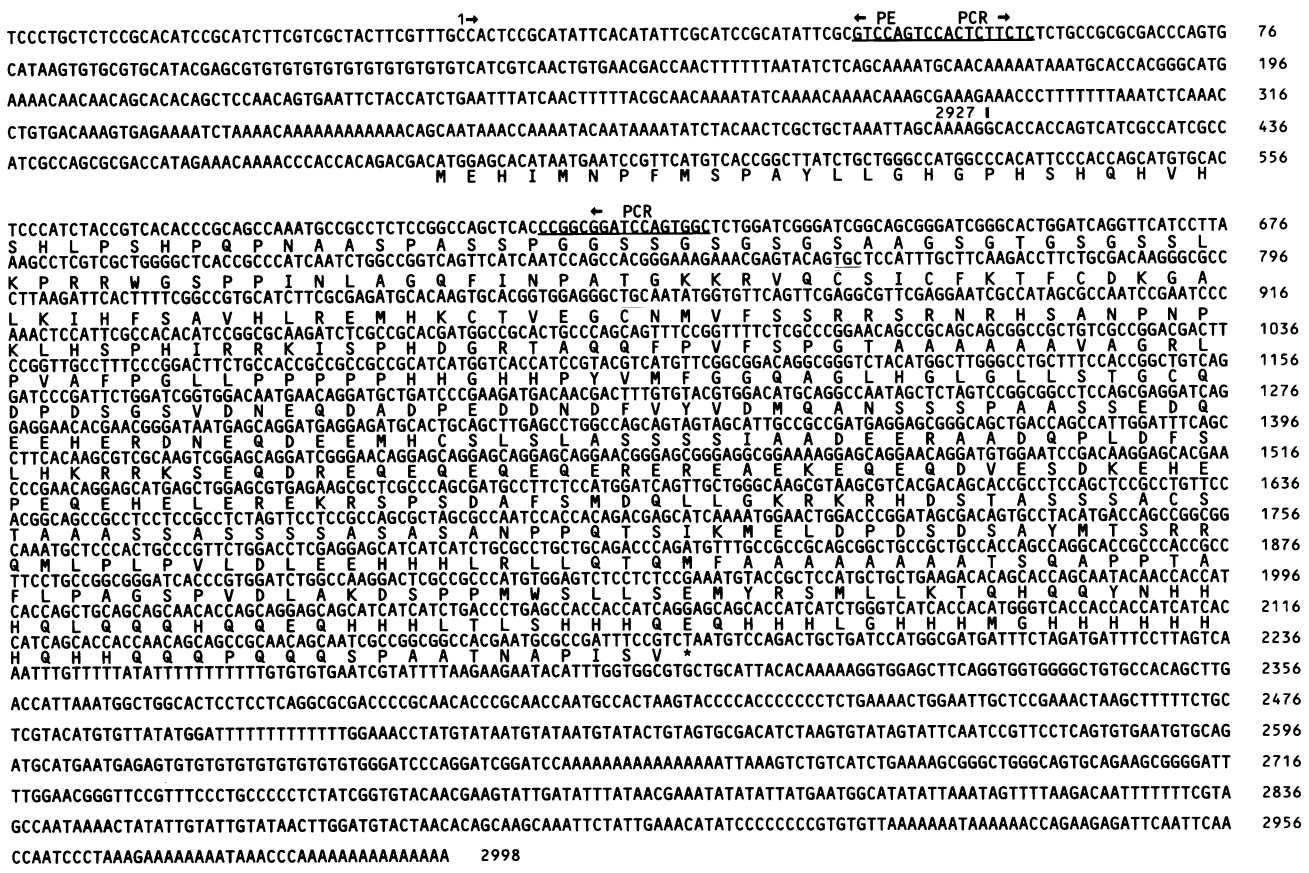

Fig. 4. Nucleotide sequence of the *disco* gene transcript. The sequence represents a composite of sequences determined by cDNA and genomic clones, PCR and primer extension. The primary transcript begins at the C residue below the numeral 1 on the figure and extends in the direction indicated by the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

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

Molecular characterization of the disco gene

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

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

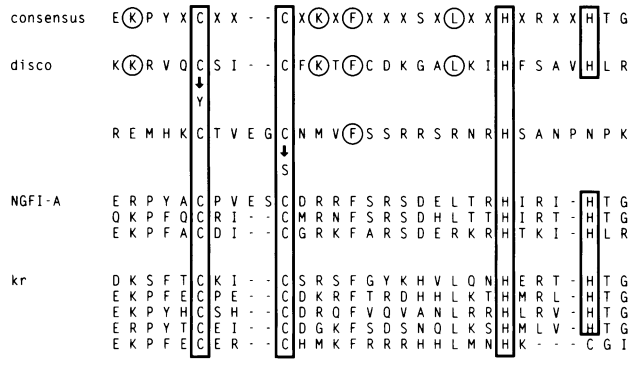

Fig. 5. The amino acid sequence surrounding the cysteine residues mutated in the EMS-induced *disco* mutant alleles compared with the zinc finger consensus and two zinc finger proteins. The consensus sequence was determined by the comparison of 148 zinc finger domains (Gibson *et al.*, 1988). Those amino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

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

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

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

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

Characterization of EMS-induced mutant alleles

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

Effects of multiple copies of the disco gene

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

Possible role of the disco gene

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

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

Materials and methods

Drosophila stocks

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

Molecular biology

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

Rescuing construct and transformation

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

Histology

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

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