# The Drosophila Genes *disconnected* and *disco-related* Are Redundant With Respect to Larval Head Development and Accumulation of mRNAs From Deformed Target Genes

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## ABSTRACT

HOM-C/hox genes specify body pattern by encoding regionally expressed transcription factors that activate the appropriate target genes necessary for differentiation of each body region. The current model of target gene activation suggests that interactions with cofactors influence DNA-binding ability and target gene activation by the HOM-C/hox proteins. Currently, little is known about the specifics of this process because few target genes and fewer cofactors have been identified. We undertook a deficiency screen in *Drosophila melanogaster* in an attempt to identify loci potentially encoding cofactors for the protein encoded by the HOM-C gene *Deformed* (*Dfd*). We identified a region of the X chromosome that, when absent, leads to loss of specific larval mouthpart structures producing a phenotype similar to that observed in *Dfd* mutants. The phenotype is correlated with reduced accumulation of mRNAs from Dfd target genes, though there appears to be no effect on Dfd protein accumulation. We show that these defects are due to the loss of two functionally redundant, neighboring genes encoding zinc finger transcription factors, *disconnected* and a gene we call *disco-related*. We discuss the role of these genes during differentiation of the gnathal segments and, in light of other recent findings, propose that regionally expressed zinc finger proteins may play a central role with the HOM-C proteins in establishing body pattern.

HOX genes encode homeodomain-containing transcription factors that specify body pattern during embryogenesis in all metazoans (McGinnis *et al.* 1984; Scott and Weiner 1984; Scott *et al.* 1989; McGinnis and Krumlauf 1992; Krumlauf 1994; Manak and Scott 1994). Each individual hox gene is expressed in a specific anterior/posterior domain wherein the encoded protein will specify regional identity through activation of a specific set of target genes (Garcia-Bellido 1977; Andrew and Scott 1992). Loss of a specific hox gene disrupts pattern formation because appropriate target genes are not activated in the region controlled by that gene. However, the mechanisms underlying target gene selection and activation by hox proteins are unclear because the DNA-binding properties of the proteins encoded by different HOM-C/hox genes are quite similar (Hoey and Levine 1988; Affolter *et al.* 1990; Florence *et al.* 1991; Dessain *et al.* 1992; Ekker *et al.* 1994; Walter *et al.* 1994; Biggin and McGinnis 1997). In general, hox proteins bind to a consensus sequence with the nucleotides TAAT at the core. Surrounding bases can influence binding strength, but there is significant overlap in the binding abilities of the various hox proteins. However, understanding how hox proteins selectively activate the appropriate target genes is critical to understanding the pattern formation process. In the fruit fly *Drosophila melanogaster* these genes are located in the Antennapedia and Bithorax complexes and are referred to collectively as the HOM-C genes (Lewis 1978; McGinnis and Krumlauf 1992).

Recent data indicate that interactions with cofactors play an important role in target gene selection. For example, the Extradenticle/Pbx proteins (Exd) participate in cooperative binding with hox proteins, and the heterodimer has a more specific DNA recognition site than the HOM-C/hox protein alone (Chan *et al.* 1994; Chan and Mann 1996; Mann and Chan 1996). This increases the specificity of DNA binding and thereby could lead to differential activation of specific target genes. Support for this model comes from studies like that of Chan *et al.* (1997), where they show that a small change in the sequence of the heterodimer binding site in a labial response element converts the element into a Dfd response element. Cooperative binding with Exd also increases the binding strength of the protein/DNA interaction as well as influencing hox protein/DNA-binding specificity (Chan and Mann 1996; Chan *et al.* 1997; Pinsonneault *et al.* 1997). Though Exd is a critical component of HOM-C/hox protein function, it seems unlikely that a single cofactor will account for the diversity and specificity needed for target gene selection in all tissues. Yet, to date only the Exd cofactor has been well characterized, though there is evidence that other cofactors exist (Li *et al.* 1999; Ryoo *et al.* 1999).

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In an attempt to identify potential cofactors functioning with the protein encoded by the HOM-C gene *Deformed* (*Dfd*), we carried out a genetic screen using deficiencies available from the Bloomington Drosophila Stock Center. We identified one region of the X chromosome that appears likely to encode such a factor. We show that embryos lacking this region have disruptions of the larval cephalopharyngeal skeleton similar to those seen in *Dfd* mutants. In addition, Dfd target gene expression is altered in these embryos, though there appears to be no effect on *Dfd* expression itself. We show that these defects are due to the loss of two functionally redundant, neighboring genes encoding zinc finger transcription factors. One gene, *disconnected* (*disco*), has been previously described as a gene necessary for neural connectivity (Steller *et al.* 1987; Heilig *et al.* 1991). The second gene, *disco-related*, encodes a related protein. We discuss the role of these genes in target gene selection during gnathal development and, in a broader sense, as a possible universal mechanism of HOM-C/hox protein function.

## MATERIALS AND METHODS

**Drosophila stocks and culture:** *Dfd^16^*, *Df(1)4b18*, *Df(1)19*, *Dp(1Y)shi^+ 1*, *Df(1)sd72b*, and the flies in the deficiency kit were obtained from the Bloomington Indiana Drosophila Stock Center. *Df(1)XR14* was a gift from G. Haddad (Yale University). *Dp(1;4)81j6e* was provided by S. Faulhaber and M. Tanouye (University of California, Berkeley). *disco^1^* flies were provided by J. Hall (Brandeis University). Flies were reared on standard cornmeal-agar-molasses medium.

**Cuticle analysis:** Embryos were collected and prepared for cuticle examination following procedures described in Pederson *et al.* (1996). Females were allowed to lay eggs for several hours, and a known number of embryos (between 200 and 300) were placed onto a grid in groups of 10 on a new collection plate. The embryos were aged for at least 24 hr and the number and phenotype of the hatched and unhatched larvae were determined. In many experiments the collection plate with the hatched larvae was placed into a bottle containing standard Drosophila medium and the larvae were allowed to develop into adults. The phenotypes of the surviving adults were then determined. The *yellow* (*y*) mutation was often used to distinguish between the different classes of larvae. *y* larvae have lighter colored mouthparts than those carrying the y+ gene.

***disco* clones:** The disco subclones used in RNAi and whole embryo *in situ* were derived from a λ clone of the 14B region (Surdej *et al.* 1990; a gift of R. Miassod, Lab. Genet. Biol. Cell., CNRS, Marseille, France). A 4.3-kb *Eco*RI fragment containing disco was used to generate dsRNA for RNAi, and a 1.8-kb *Not*I-*Xho*I fragment from this was used in RNAi and whole embryo *in situ* experiments. *In situ* localization of mRNAs followed a modification of the procedure of Tautz and Pfeifle (1989). *In situ* hybridization analysis used ribonucleotide probes generated with an RNA transcription kit (Stratagene, La Jolla, CA) and DIG-11-UTP (Boehringer Mannheim, Indianapolis). Anti-DIG-AP (Boehringer Mannheim) was used to detect hybridization.

**PCR amplification and cloning of *disco-r*:** The 5′ and 3′ coding domains of disco-r were amplified from genomic DNA using Taq DNA polymerase (QIAGEN, Valencia, CA). The 5′ primers used were ATCAGCACCACCCACATTTGC and TCTCTGCAGATAATCCTGTCC. The 3′ primers used were ATGAGATTGATAGCATGCCGC and ATCAGTGGATAAC CAGCGTGG. Primers were obtained from Operon Technologies (Alameda, CA). Amplification was carried out using the following parameters: 95° for 40 sec, 55° for 1 min, 72° for 2 min for 40 cycles. The two reactions each yielded a product of 1.5 kb. PCR products were cloned into pCRII using a TA cloning kit (Invitrogen, San Diego, CA).

**Isolation of *disco-r* cDNAs:** The *disco-r* cDNA clone CK00522 (Research Genetics, Huntsville, AL) was used to make a fluorescein-labeled probe for the screening of an embryonic cDNA library (Zinn *et al.* 1988). Detection of the hybridized probe was carried out using anti-fluorescein-AP and CDP-Star (New England Nuclear, Boston). Approximately $1.5 \times 10^5$ clones were screened and four disco-r cDNAs were recovered. Currently, only the largest (3.1 kb) has been characterized.

**Mapping of Df breakpoints:** DNAs from single mutant and wild-type embryos or larvae were prepared using the method of Gloor *et al.* (1993). Mutant larvae were recognized by the altered mouthpart structures at the terminal developmental stage. Primer pairs capable of amplifying small stretches of genomic DNA flanking the deficiency breakpoints were obtained from Operon Technologies. The following amplification parameters were used: 95° for 40 sec, 55° for 1 min, 72° for 2 min for 40 cycles.

**RNAi:** Preparation of dsRNA and injection of embryos followed the procedure of Brown *et al.* (1999), except that embryos were collected for only 20 min prior to dechorionation and injection. We have found that injection at this earlier stage yields a higher percentage of phenocopy larvae (J. W. Mahaffey, unpublished observation). For *disco-r*, a 2.5-kb *Eco*RI fragment from the 3′ portion of the 3.1-kb cDNA was used to prepare the dsRNA. For *disco* we used both genomic clones described above. Preparation and examination of larval cuticles followed the procedure of Pederson *et al.* (1996).

## RESULTS

**Screen for potential cofactors of Dfd:** To identify genomic regions potentially encoding cofactors, we screened chromosome deficiencies available from the Bloomington Drosophila Stock Center using three criteria: (1) disruption of maxillary and/or mandibular cephalopharyngeal components of the first instar larva, (2) normal expression of *Dfd*, and (3) alteration of Dfd target gene expression. We identified two deficiencies of the X chromosome that met these criteria. One, *Df(1)sd72b*, is known to remove the gene *exd* (Peifer and Wieschaus 1990; Rauskolb *et al.* 1993). As mentioned above, *exd* is a cofactor required for hox protein function, so it was not unexpected that loss of this chromosome interval would affect head development. In contrast, the phenotype of *Df(1)4b18* (reported to remove 14B8-14C1, Stanewsky *et al.* 1993) could not be attributed to loss of a known gene.

The segmental origin of larval cephalopharyngeal structures has been well defined (Jürgens *et al.* 1986; Campos-Ortega and Hartenstein 1997). (Here we will use the terminology found in FlyBase for descriptions of the components of the cephalopharyngeal skeleton, see http://firefly.bio.indiana.edu.) Formation of many of these structures requires the action of three
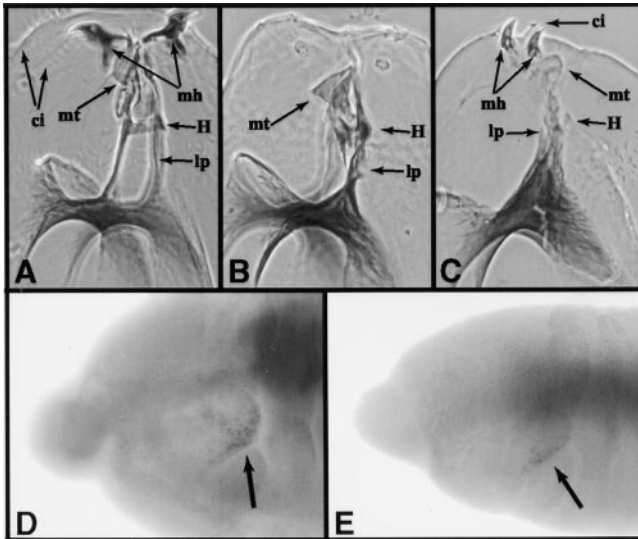
FIGURE 1.—*Df(1)4b18* potentially removes a Dfd cofactor. (A) Mouthpart structures from a wild-type first instar larva. (B) The terminal phenotype of an embryo homozygous for *Dfd¹⁶*. Absence of the cirri and mouth hooks are most notable, but the lateral process is truncated and the lateral bar of the H-piece is missing. (C) The terminal phenotype of an embryo hemizygous for *Df(1)4b18*. Note that the bases of the mouth hooks are missing and the H-piece and median tooth are faint. The number of cirri is reduced, and the remaining cirri are disorganized and misshapen. (D) Accumulation of mRNA from the Dfd target gene *1.28* in a wild-type, stage 14 embryo. *1.28* mRNA accumulates along the posterior edge of the maxillary lobe. (E) Similarly staged embryo hemizygous for *Df(1)4b18*. Note the reduction of *1.28* mRNA accumulation along the posterior edge of the maxillary lobe. In this embryo, slight staining is observed in a few cells along the edge of the lobe. Expression of *1.28* is not altered in other areas of the embryo. In D and E, anterior is to the left and dorsal upward. ci, cirri; H, H-piece; lp, lateral process; mh, mouth hooks; mt, median tooth.

HOM-C genes, *Sex combs reduced* (*Scr*) for labial-derived structures (PATTATUCCI *et al.* 1991; PEDERSON *et al.* 1996), *Dfd* for structures originating in the maxillary and mandibular segments (MERRILL *et al.* 1987; REGULSKI *et al.* 1987), and *labial* for structures derived from the intercalary segment (MERRILL *et al.* 1989). Embryos lacking any one of these HOM-C genes have characteristic defects in the cephalopharyngeal skeletal and sensory structures that arise from the affected segments. For example, embryos lacking *Dfd* (Figure 1B) are missing the maxillary cirri, the ventral organ, the dental sclerite, and the lateral bar of the H-piece from the maxillary segment (MERRILL *et al.* 1987; REGULSKI *et al.* 1987). The mouth hooks, composite structures derived from the maxillary and mandibular segments, are also absent, and the lateral process is truncated anteriorly. Though not entirely removed, a portion of the maxillary sense organ is missing and the remaining structure does not align properly with the antennal sense organ.

Half the male progeny from a cross of *Df(1)4b18/ FM7c* to *FM7c/Y* lack a portion of the 14B region of the

X chromosome. (In our descriptions below we refer to such embryos and larvae as hemizygous, for, though they lack the 14B region, they are hemizygous for the X chromosome.) Many of the structures missing or disrupted in unhatched larvae hemizygous for *Df(1)4b18* are the same as those altered in embryos homozygous for mutations in *Dfd* (Figure 1). The terminal larvae lack the base of the mouth hooks and dental sclerites. The lateral process is truncated near the H-piece, which is also disrupted. The few remaining cirri are misshapen and disorganized. The maxillary portion of the maxillary sense organ does not fuse with the antennal portion. Overall, this phenotype is similar to that of embryos homozygous for strong hypomorphic mutations of *Dfd* (MERRILL *et al.* 1987).

Loss or disruption of many *Dfd*-specific structures in *Df(1)4b18* embryos coincides with reduced mRNA accumulation from at least some Dfd target genes. Though many genes are likely to be regulated by Dfd, currently only four genes have been identified as strong candidate target genes: *Dfd* (through autoactivation), *Distal-less* (*Dll*), *1.28*, and perhaps *Serrate* (*Ser*; KUZIORA and MCGINNIS 1988; O'HARA *et al.* 1993; MAHAFFEY *et al.* 1993 and PEDERSON *et al.* 2000; WIELLETTE and MCGINNIS 1999, respectively). Expression of *Dll* and *Dfd* are not altered in embryos hemizygous for *Df(1)4b18*; however, maxillary expression of *1.28* is reduced. Normally, *1.28* mRNA accumulates in cells along the posterior edge of the maxillary lobe (Figure 1D). In embryos hemizygous for *Df(1)4b18*, *1.28* mRNA is reduced below the level of detection in most maxillary cells, though we occasionally observe some accumulation in a few cells (Figure 1E). *1.28* accumulation in other tissues is unaffected.

**Phenotype and mapping of deficiencies removing 14B:** We used other chromosomal aberrations with breaks near 14B to further map the position of the gene or genes responsible for the head defects. Figure 2 shows the larval head phenotype and the mapping data for these deficiencies. Note, since *Df(1)sd72b* and *Df(1)19* delete *exd* (PEIFER and WIESCHAUS 1990), we crossed these deficiencies to *Dp(1Y)shi⁺1*. This duplication covers *exd* but extends only as far as 14A (PEIFER and WIESCHAUS 1990; RAUSKOLB *et al.* 1993) and does not rescue the head defects of *Df(1)4b18*.

Larval mouthpart structures appear normal in hemizygous *Df(1)4b18* embryos when *Dp(1;4)81j6e* also is present (data not shown). This indicates that the locus responsible for the head defect lies distal to the break of *Dp(1;4)81j6e* but within the region removed by *Df(1)4b18*. A slight larval head defect is observed in *Df(1)sd72b/Dp(1Y)shi⁺1* embryos (Figure 2B). The bases of the mouth hooks are slightly reduced as are the H-piece and dental sclerites, but the cirri appear to be complete, and the lateral process is not truncated.

The mouthparts of *Df(1)XR14* and *Df(1)19/Dp(1Y) shi⁺1* embryos (Figure 2, D and E) are more severely disrupted than are those of embryos hemizygous for
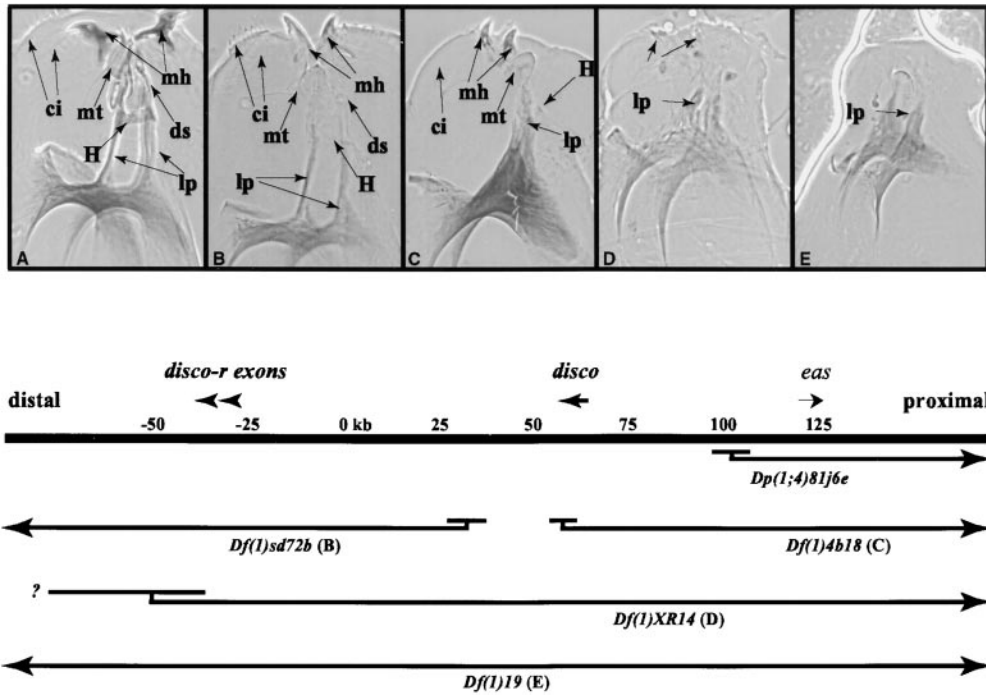
FIGURE 2.—Deficiency mapping of the 14B region. (A–E) Terminal stage cuticle preparations showing the phenotypes of the larval heads produced by embryos hemizygous for the various deficiencies. (A) Wild type. (B) *Df(1)sd72b*. Note the reduced mouth hook bases. The median tooth and the dental sclerite are faint. (C) *Df(1)4b18*. The phenotype of this deficiency is described in Figure 1. Note that this phenotype is more severe than that of *Df(1)sd72b* in B. (D) *Df(1)XR14*. The mouth hooks, dental sclerite, and H-piece are absent, the median tooth is faint, and the lateral process is shortened. We occasionally see small, partially sclerotized structures that may be remnants of the mouth hooks in a few embryos (arrows) and one or two cirri-like structures as well. (E) *Df(1)19*. The phenotype is very similar to that seen in embryos hemizygous for *Df(1)XR14*, but slightly more severe. All that remains of the affected structures is the truncated lateral process. Labels are as in Figure 1 with the addition of the dental sclerite (ds). The map shows the positions of the deficiency breakpoints in 14B. Proximal and distal are in reference to the centromere. Lines below the chromosomal map indicate the deleted region with the arrow pointing in the direction of the deletion. The small bars at the breakpoints indicate the region of uncertainty of the break position. The positions of *disco* and the two exons of *disco-r* are indicated; *eas* is provided for reference. The 0-kb map position is that of SURDEJ *et al.* (1990). The letter in parentheses following the deficiency name corresponds to the cuticle image above. *Dp(1;4)81j6e* was created by a subsequent deletion of the 14B5 to 15A interval from a fourth chromosome duplication of 13F to 16A (FALK *et al.* 1984). *Df(1)19* removes 13F through 14E (STELLER *et al.* 1987; PEIFER and WIESCHAUS 1990), while *Df(1)sd72b* is smaller, extending from 13F1 to 14B1. *Df(1)XR14* removes the interval between 14B1-2 and 14D1-2 (STANEWSKY *et al.* 1993).

*Df(1)4b18.* In these terminal larvae, the mouth hooks and cirri are absent, and the lateral process is reduced further than that in *Df(1)4b18* larvae. In addition, the H-piece and the hypostomal sclerites (structures are derived from the labial segment) also are absent. In addition, the antennal sense organ is usually not observed. The phenotype of *Df(1)19/Dp(1;Y)shi⁺1* embryos usually appears more severe than that of *Df(1)XR14*. For instance, though the cirri are usually absent in *Df(1)XR14* hemizygous larvae, we occasionally observe what might be remnants of the cirri and small bits of partially sclerotized material that may be remnants of the mouth hooks.

We mapped the positions of the 14B breakpoints of these deficiencies using genomic Southern blotting and PCR analyses (see map in Figure 2). Having a molecular map of the region (SURDEJ *et al.* 1990) as well as information from the Berkeley Drosophila Genome Project (ADAMS *et al.* 2000; RUBIN *et al.* 2000) facilitated this mapping. The distal break of *Df(1)4b18* lies within a 10-kb fragment including the *disco* coding region to about 8 kb distal of *disco*. Therefore, this deficiency removes *disco* and extends proximally to the gene *no on or off transient A* (STANEWSKY *et al.* 1993). The 14B breakpoint

of *Dp(1;4)81j6e* had been mapped previously to a fragment about 20 kb distal of the gene *easily shocked* (*eas*) (HEILIG *et al.* 1991; PAVLIDIS *et al.* 1994), and we confirmed this location. We mapped the 14B break of *Df(1)sd72b* to a 15-kb fragment 20–35 kb distal of *disco*. Therefore, the *disco* gene is intact, and only genes distal to *disco* are removed. We were unable to find the distal endpoint of *Df(1)XR14* within the interval examined; however, genetic tests indicate that it does not extend as far as *exd*. Taking the mapping and phenotypic data together we conclude that the gene or genes whose loss leads to the larval head defects reside between the proximal break of *Df(1)sd72b* and the distal break of *Dp(1;4)81j6e*. Thus, the region of interest is within a 75-kb interval centered on the gene *disco*.

**Identification of *disco-related*:** Two groups have searched for transcribed regions in the 14B interval (SURDEJ *et al.* 1990; HEILIG *et al.* 1991), and the only gene they identified that is expressed during embryogenesis is *disco*. Though null alleles of *disco* are semi-viable and do not cause defects in larval head development (STELLER *et al.* 1987; HEILIG *et al.* 1991), *disco* is expressed in the gnathal lobes during embryogenesis (LEE *et al.* 1991). Therefore, it seemed possible that the

**A**

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MNIGHEKHPPHFHPHPQPHPHPRAAAASEVHRLAAGGSVSPPPPPSTSSSLLYHLPANTN     60
AATAATVATVSASYRHPLQGHERQQNQHHHPYQHQHHHYHQHHPHLPTGGGNVHLSRES    120
SPASMVMTPSRRSSPPLPPLPLTTHPIHPHSHSHSHPHPHPHPLPLALPLRHPLALNANA   180
GGHQSPSRSSGSVQQTSSHAQSSAQSMSVHFSQLAHAQLHLQNQLQQKLSAAASRNNNNN   240
NNNNNNNSSSTGSGTGTGNSTPTHDPNPMNPLSDLQNMQPFDFRKISSAAALGAFGGPLP   300
LPQSPTEFGQHHHHSQQQQHQHHLHRNQFFNAMAMAYHLPPPPPPPPPDSRSPPSPPPGV   360
GQYQHQAGSGSGSGSGSGSGSSSGAGSVDDSAGKQQRNSDKVSASGSCSSSQRMTRLALS   420
SNMRSSRKTPHSPGGGGAGGNGGSVGGGGGKRQWGSMPANLGTQFINPVTGKKRVQCNVC   480
LKTFCDKGALKIHFSAVHLREMHKCTVDGCSMMFSSRRSRNRHSANPNPKLHSPHLRRKI   540
SPHDGRSAQPHPLLLQAPNGLMAGLAPFGSFPLLTPPPDLRHHAMGGSGAGSGAGVGALE   600
LKHGQDYLQRSYLDAGRFEGQRRKLAMENEHTEDEDDDEILEVGIHMAGDDDDDEADGDE   660
DEDDDDPDGIVVVGDEIDSMPLDHENDNDNENENDESDERSTSAVSSLSQTKEQSSPGKV   720
VQSGLEECHTYAHAHAHALTHGHAHSLGHAHALAQMAANKRKRKSQNPVRCPVQSDENSG   780
ENSIDYDVAADLSIKKVRLPAQAAASAKESEVGESTKSIPSPPLTPYGDLRPVGLCIKTE   840
QDHEQELDQEQGQERKREQEQEPEPDPVATASKSEMKLEEQETRPADKEDDNEEDEQVSP   900
PVVTTLDLSRAAAAIKLEPLEEINYEADENRYVRIKQELMGGDESLAEESSDKTANHNNN   960
NNNNNNININNSVRLESDNGLEASELEEREPEPETEIEPEHEHEHEPEPETEVEVPEVPI  1020
DKENPLKCTACGEIFQNHFHLKTHHQSVHLKLHHKCNIDGCNAAFPSKRSRDRHSSNLNL  1080
HRKLLSTSDDHGLLHAPVMPATDPLLELMSLNLNNNKSGFHHSAMVGSSAGGAGGVNPAA  1140
VGSIQAEILARICAGAHAHGLNVPLCFEALQHRFAVGHGHAGYPLIAGDGSPPSPRLFLN  1200
HGGGASPLLFAGLPRMPRFPQLTPHMLAASAGNTAAAAAAAAAAAAGMGGLSPFCRRTSS  1260
DSNSQHSITPPPKRSRSQSRSPDHCVHPAHAGDTTGITEDSGQRQSPDRIS*          1311
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**B**

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disco       WGSPPINLAG QFINPATGKK RVQCSICFKT FCDKGALKIH FSAVHLREMH
dr-5   454  ...M.A..GT .....V.... ....NV.L... .......... .....H....
dr-3  1005  HEPE.ETEVE VPEV.IDKEN PLK.TA.GEI .QNHFH..T. HQS...KLH.


disco       KCTVEGCNMV FSSRRSRNRH SANPNPKLHS PHIRRKISP- --HDGRTAQ
dr-5        ....D..S.M .......... .......... ..L......- --....S..  549
dr-3        ..NID...AA .P.K...D.. .S.L.--..R KLLSTSDDHG LL.APVMPA 1101
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FIGURE 3.—Putative sequence of the protein encoded by *disco-r* and alignment of the zinc finger motifs. (A) Conceptual translation of the Disco-r protein from the genomic ORFs and partial sequence of the 3.1-kb cDNA. The translation begins at the first Met-initiated open reading frame in the 5′ exon and extends to the stop codon in the 3′ exon. The splice between the two ORFs was determined from the cDNA sequence. Each boldface region contains one pair of zinc fingers. The predicted $M_r$ is about 140 kD. (B) Alignment of the Disco zinc finger motifs with those from Disco-r. Dots indicate identity with the Disco sequence and dashes indicate gaps. The expected Zn-binding residues are in boldface type. The putative DNA-binding domain is in italics. Note the identity between the DNA-binding domains of Disco and the first pair of zinc fingers of Disco-r. The DNA-binding domain of the second pair from Disco-r is less similar. The numbers refer to the amino acid position in the putative protein shown in A.

lack of *disco* somehow was involved in causing the larval head defects. The discrepancy between the phenotype of *disco* mutations and the embryonic expression pattern was resolved by finding two previously unidentified open reading frames (ORF) encoding peptides related to Disco within the 14AB region. Using information obtained from the Berkeley Drosophila Genome Project (ADAMS *et al.* 2000) and generating and sequencing fragments to span existing gaps in the contigs, we established that these ORFs are located 90–95 kb distal of *disco* (see map in Figure 2). We isolated a cDNA that spans the intervening sequence between these two ORFs, demonstrating that they are two exons of a single gene. We refer to this gene as *disco-related* (*disco-r*). Both *disco* and *disco-r* are transcribed from the same strand of DNA, proximal to distal along the chromosome. The putative Disco-r protein contains two pairs of zinc fingers, each related to the single pair in Disco, but there is little or no similarity outside these domains (Figure 3A). Of particular note is the sequence of the first pair of zinc fingers in Disco-r, which is nearly identical to the sequence of the zinc finger pair in Disco (Figure 3B). The amino acids forming the DNA recognition domains are identical, indicating that these proteins could bind to the same DNA sequence. The second pair

of zinc fingers in Disco-r, though related to Disco, is somewhat more divergent.

***disco* and *disco-r* are redundant genes together responsible for the larval head defect:** That *disco* and *disco-r* encode related proteins and knowing the phenotypes and positions of the deficiency breakpoints in the 14B region indicate that one or both of these genes could be involved in morphogenesis of the larval head. However, since mutations in the *disco* gene are viable, we suspected that the genes might have redundant functions. Recently we and others have used double-stranded (ds) RNA interference (RNAi) to generate null phenocopies of specific genes in Drosophila (KENNERDELL and CARTHEW 1998; BROWN *et al.* 1999; MISQUITTA and PATERSON 1999). We prepared dsRNA from our PCR clone of *disco* and from the 2.5-kb *disco-r* cDNA fragment (see MATERIALS AND METHODS). Injecting either of these dsRNAs into wild-type embryos had little or no effect on development. Most injected embryos hatched and were found wiggling in the halocarbon oil. For example, using the *disco-r* cDNA as the template, of the 56 embryos that developed, 50 embryos were wild type, and 6 had general head defects not resembling the defects observed in the deficiency embryos (data not shown).

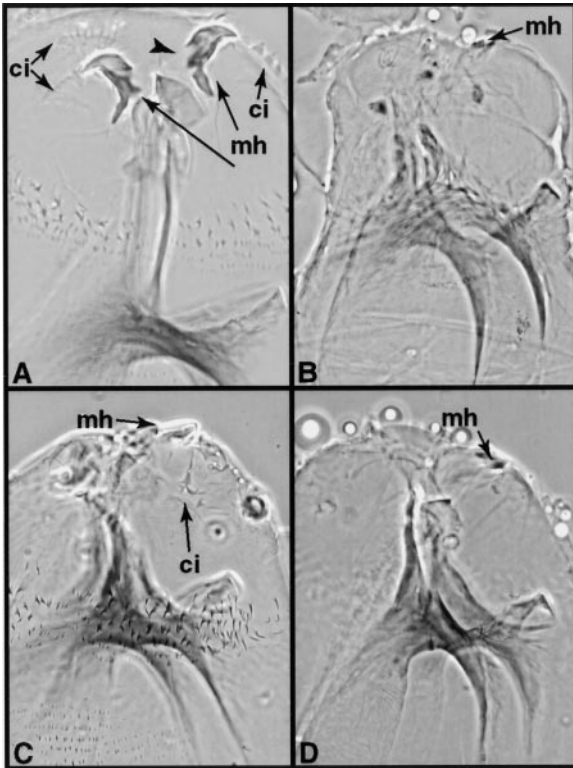To remove the functions of both genes, we injected

FIGURE 4.—RNAi phenocopy of the deficiency phenotypes. (A) Homozygous *disco¹* first instar larval cuticle. Although the mouthparts are usually complete, we occasionally note slight reductions in the base of the mouth hooks (arrowhead). (B) Terminal phenotype of the hemizygous *Df(1)XR14* embryo as shown in Figure 2D. (C and D) Homozygous *disco¹* embryos injected with dsRNA synthesized from the 2.5-kb *disco-r* cDNA fragment. Note the nearly complete absence of mouth hooks, shortened lateral process, and absence of cirri as seen in hemizygous *Df(1)XR14* embryos. Abbreviations are as in Figure 1.

dsRNA synthesized from the *disco-r* cDNA into embryos homozygous for the mutation *disco¹*. Homozygous *disco¹* embryos develop into normal larvae with an occasional, slight reduction in the mouth hook base (Figure 4A). However, injecting *disco-r* dsRNA into *disco¹* homozygous embryos caused the majority of these embryos to fail to hatch, and the mouthparts of the unhatched larvae were disrupted in a manner similar to those observed in the deficiencies described above (Figure 4, C and D). Of the 44 developed larvae from one experiment, 3 were similar to *disco¹*, 34 resembled embryos hemizygous for the deficiencies, and 7 had general head defects not resembling the deficiency embryos. Of the 34 larvae appearing similar to the deficiencies, a few resembled larvae hemizygous for *Df(1)4b18* (Figure 4C), while most had more severe head defects (Figure 4D). This indicates that the head defects associated with deficiencies of 14B are due to the loss of these two genes that have redundant functions during gnathal lobe development.

**Comparison of *disco* and *disco-r* expression during embryogenesis:** The distributions of *disco* mRNA and protein have been described (LEE *et al.* 1991). Here we compare *disco-r* mRNA accumulation with that of *disco* using *in situ* localization (Figure 5). *disco* mRNA is first detected during the late syncytial blastoderm stage in a cap of cells at the posterior end of the embryo, excluding the pole cells (late stage 4; stages according to CAMPOS-ORTEGA and HARTENSTEIN 1997). As gastrulation begins the posterior *disco*-expressing cells invaginate, and new accumulation of *disco* mRNA is detected in two bands of cells anterior and posterior to the dorsal portion of the cephalic furrow (Figure 5, A and B). The invaginating cells will form the posterior midgut rudiment and the amneoproctodeal invagination. By stage 10, when the germ band reaches full extension, *disco* mRNA is no longer observed in the posterior midgut, but accumulates in the proctodeum, along the cephalic furrow and on the dorsal side of the clypeolabrum. Formation of the gnathal lobes (mandibular, maxillary, and labial) is preceded by expression of *disco* in the three lobe primordia (Figure 5C) but not in the ventral region of these head segments. We note that there is a gap of one or two nonstaining cells between each lobe primordium, so that, at least during this stage, not all cells of the lobe accumulate *disco* mRNA. *disco* mRNA also is present in cells along the lateral edge of the acron and in the proctodeum, the optic lobe, and the clypeolabrum.

In contrast to *disco*, *disco-r* mRNA is not detected until late stage 10 to early stage 11 when it accumulates in the proctodeum and the dorsal clypeolabrum (Figure 5F). *disco-r* mRNA is not detected in the gnathal region until after segmentation is apparent (stage 11, Figure 5J). From this point onward the distribution of *disco-r* is nearly indistinguishable from that of *disco*, though we have not examined expression in the nervous system in detail. By the end of stage 11 both genes are expressed in the mandibular, maxillary, and labial lobes and in primordia of the leg discs. Low levels of mRNA also accumulate in each of the abdominal segments, in a position analogous to the leg disc primordia, but this staining soon disappears (visible in Figure 5, G and J, but absent in H and K; see COHEN *et al.* 1991). As the germ band continues to contract, *disco* and *disco-r* mRNAs accumulate in the visceral mesoderm and slightly later in the dorsal vessel (Figure 5, H and K). During stage 14 and later, *disco* mRNA is detected in the peripheral nervous system (PNS). We have not detected *disco-r* in the PNS, though transcripts from both genes are detected later in a few cells of each neuromere along the ventral nerve cord. Finally, we can detect mRNA from both genes in cells of the gnathal lobes during head involution until accessibility of the mRNA is blocked by cuticle synthesis (Figure 5, I and L).

***disco-r* expression in *Df(1)4b18* embryos:** The results from our RNAi analyses indicate that both *disco* and *disco-r* functions must be removed to disrupt larval head development. Therefore, it is surprising that embryos hemizygous for *Df(1)4b18* develop with mouthpart de-
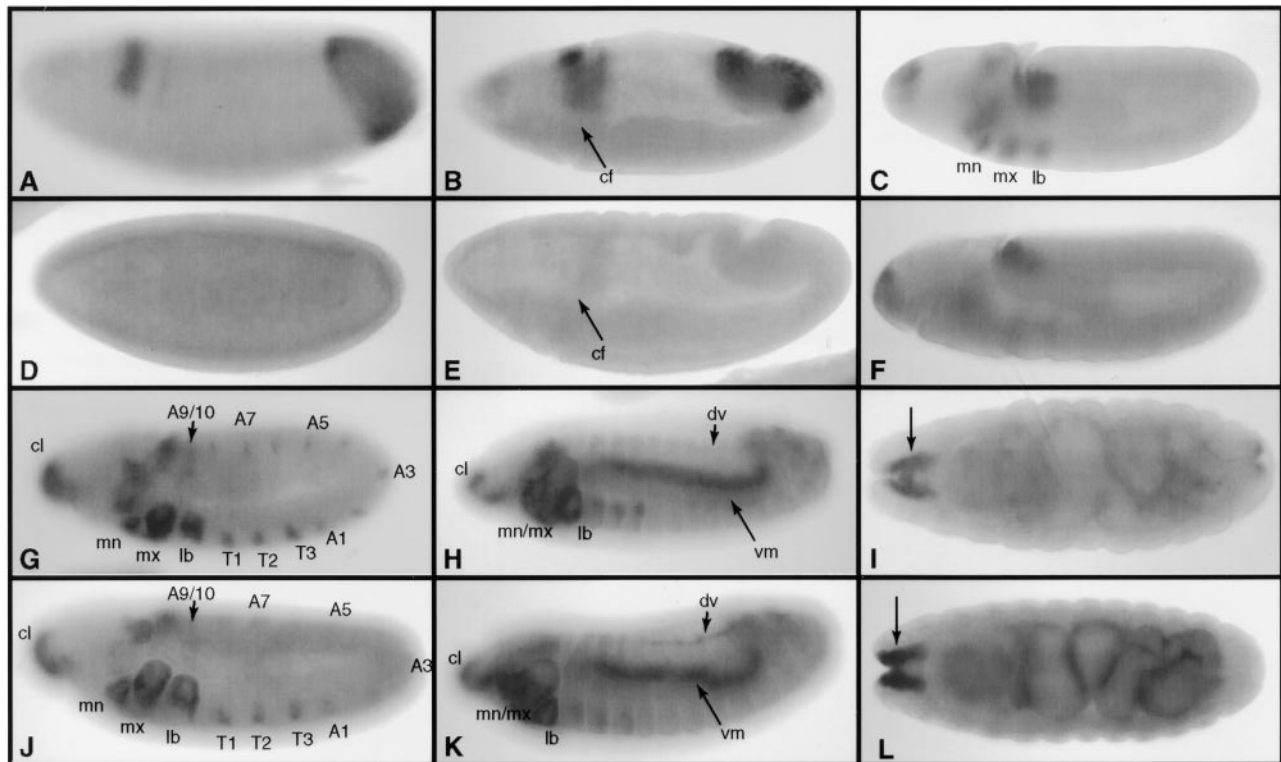
FIGURE 5.—*In situ* localization of mRNA from *disco* and *disco-r*. A–C and G–I are *disco*; D–F and J–L are *disco-r*. All embryos except in I and L are oriented anterior to the right, dorsal up. (A) *disco* mRNA in an early embryo after cellularization of the blastoderm. Note the cap at the posterior pole and the two stripes flanking the dorsal cephalic furrow. (B) As the germ band begins to extend, *disco* mRNA is detected in the clypeolabrum at the anterior-dorsal tip of the embryo. (D and E) *disco-r* is not detected in similarly aged embryos. (C) As the germ band reaches full extension (late stage 10) *disco* mRNA accumulates in the gnathal lobe primordia. Note the few nonstained cells between the stained regions. Staining is also detected in the optic lobe and in the posterior-lateral acron, near the remnant of the cephalic furrow. (F) *disco-r* is first detected at early stage 11 in the proctodeum and clypeolabrum. (G and J) As the germ band begins to contract and the gnathal lobes form, the distributions of *disco* (G) and *disco-r* (J) appear to be identical. Both genes are expressed in the gnathal lobes and in the leg disc primordia. Staining resembling that in the disc primordia extends through the abdominal segments, but as shown in H and K, the abdominal expression does not persist through germ band contraction (stage 12). The cells of the visceral mesoderm and dorsal vessel also express both genes. (I and L) Ventral views of embryos undergoing head involution stained to detect *disco* and *disco-r* mRNA, respectively. mRNA continues to accumulate in the gnathal region during head involution (arrow). The location of the stained cells anticipates the mouthpart defects of the deficiencies. mRNA from both genes is still present in the visceral mesoderm. cf, cephalic furrow; cl, clypeolabrum; dv, dorsal vessel; vm, visceral mesoderm; mn, mandibular lobe; mx, maxillary lobe; lb, labial lobe; T# and A#, thoracic and abdominal segments, respectively.

fects, because this deficiency removes only *disco* (see above). One possible explanation is that the deficiency alters expression of *disco-r* without removing the gene. To determine whether or not this was the case, we examined the accumulation of *disco-r* transcripts in embryos hemizygous for *Df(1)4b18* (Figure 6). *disco-r* mRNA is first detected about stage 11 as in wild-type embryos; however, in the mutant embryos *disco-r* transcripts are not distributed throughout the gnathal lobes. Instead, mRNA accumulates in small clusters of cells in the ventral-posterior region of each lobe (Figure 6, B and D). In addition, where there is normally only a low level of transient *disco-r* mRNA in the abdominal segments, these regions stain more intensely, and staining persists for a longer period in *Df(1)4b18* hemizygous embryos (Figure 6B). As germ band contraction continues, the abdominal staining subsides, but staining remains re-

stricted to the small clusters of cells in the gnathal lobes. During later development, when labial and maxillary lobes have migrated to the edges of the stomodeum, the mRNA is no longer detectable in the gnathal lobes (Figure 6F). Accumulation in other areas appears to be unaffected by the deficiency. This altered distribution of *disco-r* mRNA is caused by the deletion and not by the loss of *disco* alone, as embryos homozygous for *disco[1]* appear to have normal *disco-r* mRNA distribution (data not shown).

**Dfd target gene expression in mutants lacking both *disco* and *disco-r*:** Since *Df(1)XR14* removes both *disco* and *disco-r*, we looked at Dfd target gene expression in embryos hemizygous for this deficiency (Figure 7). As mentioned above, there are four potential target genes of Dfd: *1.28, Dll, Dfd*, and perhaps *Ser* (MAHAFFEY *et al.* 1993 and PEDERSON *et al.* 2000; O'HARA *et al.* 1993;
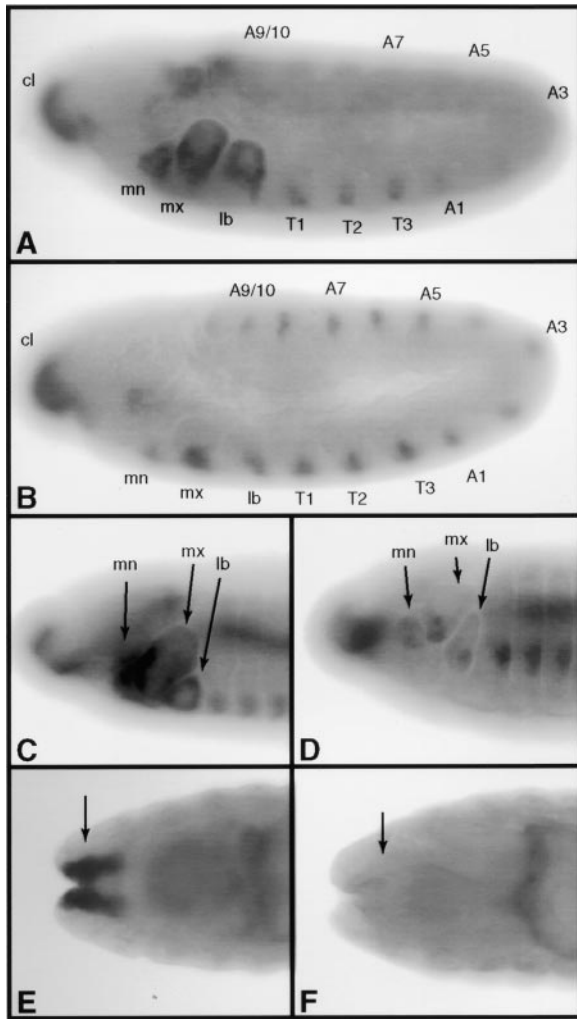
FIGURE 6.—Distribution of *disco-r* mRNA in embryos hemizygous for *Df(1)4b18*. (A) Stage 11 wild-type embryo. (B) Similarly staged *Df(1)4b18* hemizygous embryo. Note the reduced staining in the mandibular, maxillary, and labial lobes where staining is present in ventral-posterior clusters of cells instead of throughout the lobes. (C) Wild-type stage 14 embryo. (D) Similarly staged *Df(1)4b18* hemizygous embryo. (E) Wild-type embryo during head involution, ventral view. (F) Head involution in *Df(1)4b18* hemizygous embryo. At this stage, *disco-r* mRNA is no longer detectable in the gnathal region of the hemizygous *Df(1)4b18* embryo. Abbreviations are as in Figure 5.
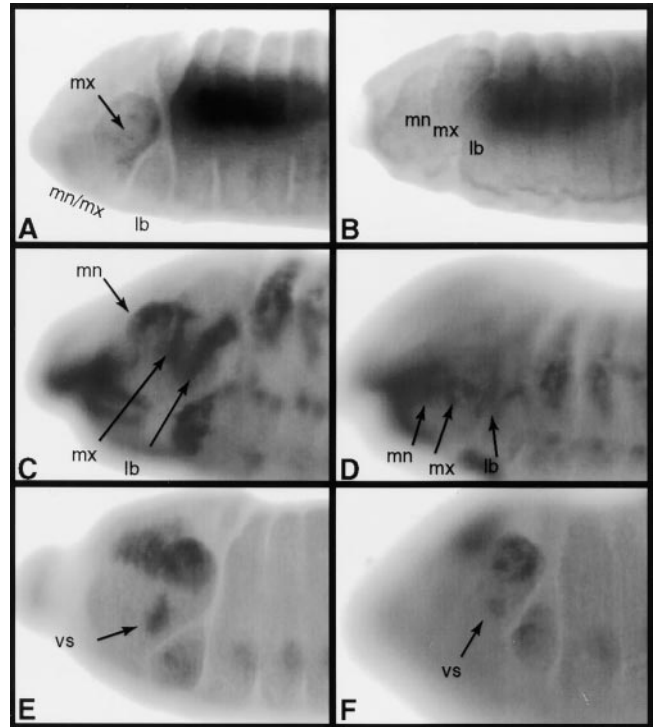


FIGURE 7.—Target gene expression in embryos lacking *disco* and *disco-r*. (A, C, and E) Wild-type embryos. (B, D, and F) Hemizygous *Df(1)XR14* embryos. (A and B) *1.28* mRNA accumulation. We did not detect any *1.28* mRNA in the maxillary lobe of this mutant embryo, though in a few others we noted a low level of staining along the posterior edge of the lobe, particularly in the midlateral cells. In A, the arrow points to the posterior maxillary cells that accumulate *1.28* mRNA in the wild-type embryo. (C and D). *Serrate* mRNA accumulation. In wild-type embryos (C) *Ser* mRNA accumulates throughout the mandibular lobe and in the anterior and posterior of the maxillary and labial lobes. In hemizygous *Df(1)XR14* embryos (D), *Ser* mRNA was not detected in the maxillary and labial lobes, but was present in a few cells of the mandibular lobe. The slight darkening of the gnathal lobes in D is due to staining below the lobes that is not altered in the mutants. (E and F) *Distal-less* mRNA accumulation in the ventral spot of the maxillary lobes. Note the reduced staining of the ventral spot in the *Df(1)XR14* embryo (F). Abbreviations are as in Figure 5 with the addition of the *Dll* ventral spot (vs).

Kuziora and McGinnis 1988; Wiellette and McGinnis 1999, respectively). We can identify *Df(1)XR14* hemizygous embryos after germ band contraction because the labial lobes fail to migrate ventrally, the mandibular lobes do not fuse with the maxillary lobes, and the maxillary lobes do not fully rotate. Note, these are also characteristics of mutant embryos lacking the HOM-C genes *Dfd* and *Scr* (Merrill *et al.* 1987, 1989).

*1.28* mRNA is not detected in the maxillary lobes of most *Df(1)XR14* hemizygous embryos (Figure 7, A and B). Occasionally we note slight staining in a few cells, mainly along the posterior edge of the lobes, but this often approaches background levels (data not shown).

In wild-type embryos, *Ser* mRNA accumulates throughout the mandibular lobes and along the lateral anterior and posterior edges of the maxillary and labial lobes (Figure 7C). The proteins encoded by the HOM-C genes *Dfd* and *Scr* are necessary for this expression (Wiellette and McGinnis 1999). In the absence of *Dfd*, *Ser* mRNA does not accumulate in the mandibular lobes or in the anterior portion of the maxillary lobes; absence of *Scr* causes loss of *Ser* mRNA in the posterior maxillary-anterior labial and posterior labial domains. Staining in embryos lacking both *disco* and *disco-r* appears similar to a combination of the HOM-C mutant patterns; *Ser* mRNA is not detected in the maxillary and labial regions, though a few cells stain in the mandibular lobe (Figure 7D).

We also examined *Dll* mRNA distribution in embryos

hemizygous for *Df(1)XR14*. In wild-type embryos *Dll* mRNA accumulates in a large number of cells in the anterior-lateral portion of the maxillary lobe and in a smaller group of cells more ventrally located (Figure 7E). *Dll* mRNA accumulation in the ventral cells requires Dfd while expression in the anterior-lateral region does not (O'Hara *et al.* 1993). In embryos lacking *disco* and *disco-r* the ventral *Dll* spot forms but is smaller than that in wild-type embryos (Figure 7, E and F). We conclude from these observations that the loss of the *disco* and *disco-r* can have varying effects on accumulation of mRNAs from Dfd target genes. *Ser* and *1.28* are absent or occasionally detected at low levels, and *Dll*, although reduced, is present.

*Dfd* is also a target through autoactivation (Kuziora and McGinnis 1988), and an explanation for the phenotype we observe in larvae lacking *disco* and *disco-r* might be that the encoded proteins are required for normal *Dfd* expression. Failure of the autoactivation process results in loss of Dfd protein from the maxillary cells after stage 10 (Pinsonneault *et al.* 1997). (It is unlikely that *disco* and *disco-r* are involved in activating *Dfd*, as *Dfd* mRNA and protein accumulate prior to *disco-r* expression, and our results indicate that *disco-r* is sufficient for normal cephalopharyngeal development.) We collected *Df(1)XR14* embryos and stained these embryos with antibodies recognizing the Dfd protein (Mahaffey *et al.* 1989). Dfd protein accumulates in the maxillary cells throughout development of the mutant embryos (data not shown) indicating that Disco and Disco-r are not likely required for autoactivation of *Dfd*.

**disco** and **disco-r** are not targets of Dfd: Another possible cause of the larval head defect might be that *disco* and *disco-r* are themselves Dfd target genes that, once activated by Dfd, are necessary for further development of the gnathal lobes. If this is the case we would not expect to see *disco* and *disco-r* mRNAs accumulate in embryos lacking *Dfd*. We, therefore, collected embryos from a cross of *Dfd^16* heterozygous parents and detected *disco* and *disco-r* mRNA accumulation by *in situ* hybridization. *Dfd^16* has a nonsense mutation before the homeobox (Zeng *et al.* 1994), and the phenotype of homozygous *Dfd^16* embryos is identical to that of embryos carrying deficiencies of *Dfd*, indicating that this is a null allele (Merrill *et al.* 1987). We observe no difference in *disco* or *disco-r* mRNA accumulation between embryos homozygous for *Dfd^16* and wild-type embryos, indicating that Dfd is not required to activate *disco* or *disco-r* (data not shown).

## DISCUSSION

The two genes, *disco* and *disco-r*, appear to have redundant functions during development of the Drosophila larval head. Presence of either gene product is sufficient for normal development of the mandibular, maxillary, and labial lobes, but absence of both gene products disrupts development in these regions. The phenotype of terminal larvae lacking these two genes is strikingly similar to that of larvae lacking the HOM-C genes *Dfd* and *Scr*. *disco* was identified earlier as encoding a protein required for the formation of certain neural connections during embryonic and adult development of Drosophila (Steller *et al.* 1987). This does not appear to be a redundant function, because the phenotype was no more severe in *Df(1)19* hemizygous embryos that lack both *disco* and *disco-r* (Steller *et al.* 1987). At present, we do not know whether *disco-r* also has an independent role.

*disco* and *disco-r* encode proteins containing paired zinc finger domains, Disco with one pair while Disco-r has two pairs. The near identity of the Disco zinc finger pair and the first pair in Disco-r indicates that these proteins may bind to the same DNA sequence. This, along with overlapping distribution of mRNAs, likely explains the redundancy. However, the putative Disco-r protein contains a second pair of zinc fingers, and it is possible that these also influence DNA binding. If so, there may be some differences in the recognition site of these two proteins and, possibly, differences in their roles during development. It is worth noting that a mammalian gene, *basonuclin*, has been identified that encodes a protein with zinc finger domains similar to those in Disco (Tseng and Green 1992); Basonuclin contains three pairs of zinc fingers, so in this respect it is more similar to the Disco-r protein. We also identified an ORF in the *Caenorhabditis elegans* genome that encodes a peptide containing a single pair of zinc fingers quite similar to those in Disco; however, at this time little is known of the gene. Finding similar proteins in animals widely divergent from Drosophila indicates that at least some functions of Disco and/or Disco-r may be conserved during evolution.

*basonuclin* mRNA and protein accumulate in some cells that have the potential to divide, leading to the prediction that the protein is involved in regulation of cell proliferation (Tseng and Green 1994), though the protein is found in nonproliferating cells as well (Yang *et al.* 1997). Perhaps reduced cell proliferation in the gnathal lobes could cause the phenotype we observe, but we find no evidence that cell proliferation is altered in embryos lacking *disco* and *disco-r*. The maxillary lobes of embryos hemizygous for *Df(1)XR14* are nearly equal in size to those of wild-type embryos, and 4′,6-diamidino-2-phenylindole staining reveals that there are comparable numbers of nuclei in mutant and wild-type lobes (data not shown). Further, *Dfd* autoactivation and *1.28* expression occur at about the same time and in the same cells along the posterior-lateral edge of the maxillary lobes. In the absence of *disco* and *disco-r*, *Dfd* autoactivation occurs, but there is little or no accumulation of *1.28* mRNA. We conclude that the cells along the posterior edge of the maxillary lobes are viable and possess the correct homeotic identity, but there is a defect in mRNA accumulation from at least some Dfd target genes (*1.28* and *Ser*).

Other redundant gene pairs have been identified in Drosophila. Of particular note are the head gap gene *buttonhead* (*btd*) and the gene *D-Sp1* (Wimmer *et al.* 1993, 1996; Schöck *et al.* 1999). Like *disco* and *disco-r*, these two genes encode $C_2 H_2$ zinc finger proteins, but these proteins each have triplet zinc finger domains instead of paired domains as in Disco and Disco-r. *btd* and *D-Sp1* also are closely linked on the X chromosome, in division 9A. However, unlike *disco* and *disco-r*, the redundancy between *btd* and *D-Sp1* appears in their roles during neural development, not during segmentation (Schöck *et al.* 1999).

With this in mind, one may wonder whether the *disco* and *disco-r* are also head gap genes. The early distribution of *disco* mRNA may be suggestive, but we think that it is unlikely for the following reasons. Loss of *disco* and *disco-r* does not appear to cause a gap phenotype. We observe no loss of segments; the gnathal lobes form as expected. In addition, we do not observe a change in the distribution of the engrailed protein in the gnathal cells until head involution is underway, and then the changes appear to be due to improper migration of the gnathal lobes in the mutant embryos (data not shown). Further, *disco-r* function is sufficient for normal gnathal development, yet accumulation of *disco-r* mRNA in gnathal cells occurs well after segmentation. Finally, the process of segmentation in the gnathal region follows that of the trunk, relying on the gap, pair rule, and segment polarity functions (Lehmann and Nusslein-Volhard 1987; Mohler *et al.* 1989; Kraut and Levine 1991; Gallitano-Mendel and Finkelstein 1998; Schöck *et al.* 2000), though we note that *buttonhead* is required for development of the mandibular segment (Wimmer *et al.* 1993; Schöck *et al.* 2000). Taking this into consideration, it seems unlikely that *disco* and *disco-r* are head gap genes.

However, we suggest that *disco/disco-r* and *btd* may have similar roles. Recently, Schöck *et al.* (2000) presented evidence that the Btd protein is required along with the homeodomain-containing protein Empty spiracles (Ems) to specify intercalary identity. Ectopic Ems is capable of transforming regions only where Btd is present, indicating that Btd is necessary for Ems activity. Schöck *et al.* (2000) go on to demonstrate that Btd and Ems proteins can interact, and this can occur at the Btd zinc finger domain as well as elsewhere in the protein. From their studies and those of others, Schöck *et al.* (2000) conclude that Btd and Ems together specify intercalary identity, and that Btd represses phenotypic suppression of Ems. They go on to state that this supports the contention that Ems is an escaped HOM-C gene as proposed by Macias and Morata (1996).

Though repression of phenotypic suppression may occur, we propose that there is a more fundamental role for the proteins encoded by *btd* and *disco/disco-r*. We propose that these zinc finger-containing proteins are required along with the HOM-C proteins to activate the appropriate target genes necessary to establish segment identity. In the case of *disco* and *disco-r*, this is with Dfd and Scr during differentiation of the gnathal lobes. *disco* and *disco-r* have a lot in common with the HOM-C genes. They encode spatially restricted transcription factors. Absence of these genes causes a similar phenotype to loss of *Dfd* and *Scr*, suggesting a loss of segment identity. We suggest that, as with the HOM-C genes, *disco* and *disco-r* are needed to establish the appropriate transcriptional environment for gnathal segment identity. In an analogous manner, Btd and Ems are required for intercalary identity. Further, since Btd interacts directly with Ems, it seems possible that similar interactions may occur between other HOM-C proteins and zinc finger cofactors. It is tempting to speculate that this occurs with Disco/Disco-r and Dfd and Scr, but this may be a bit premature. Additional studies are necessary to determine if this model is correct, but the similarity of larvae lacking these genes to those lacking *Dfd* and *Scr* implies that the *disco* and *disco-r* function is crucial for normal pattern formation in the gnathal lobes.

With regard to general HOM-C/hox gene specification of body pattern, perhaps similar systems are in operation in other regions of the embryo that have gone undetected due to redundancy. There are numerous zinc finger encoding genes within the Drosophila genome (Rubin *et al.* 2000). Some of these are closely linked as are *disco* and *disco-r* and *btd* and *D-Sp1* (J. W. Mahaffey, unpublished observation). At present, evidence of such a mechanism involving zinc finger transcription factors has been detected in two regions of the embryonic head. Perhaps further studies will determine whether similar mechanisms are underway in other regions of the embryo. Finally, since genes encoding similar proteins to Disco and Disco-r are found in other animals, perhaps this is a conserved mechanism involved in establishing body pattern in all animals.

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