Developmental and evolutionary implications of labial, Deformed and engrailed expression in the Drosophila head

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Summary

Prior developmental genetic analyses have shown that labial (lab) and Deformed (Dfd) are homeotic genes that function in the development of the embryonic (larval) and adult head. Using antibody probes to reveal the spatial distribution of the lab and Dfd proteins in embryonic and imaginal tissues, we have assessed the respective roles of these genes through an analysis of the correspondence of their expression patterns with their mutant phenotypes. With regard to imaginal development, lab and Dfd occupy adjacent non-overlapping expression domains in the peripodial cell layer of the eye–antennal disc, in patterns that are consistent with their adult mutant phenotypes and published fate maps. During embryogenesis, lab and Dfd exhibit limited overlapping expression in areas that are of no obvious significance to the development of larval head structures, but also in areas that may have consequences for imaginal development. The head of Drosophila and other cyclorrhaphous Dipterans is characterized by an extreme morphological difference between the larval and adult stages. Given this unique ontogenetic and phylogenetic history and the observation that homeotic transformations produced by the lab, Dfd, and proboscipedia (pb) loci are manifested only in the adult, we suggest that distinct regulatory paradigms evolved for homeotic gene function in the development of the larval versus adult head. Finally, a detailed examination of the engrailed (en) expression pattern in the embryonic head strengthens the view of insect morphologists that the clypeolabrum evolved from the fusion of paired labral appendages.

Key words: Deformed, Drosophila, engrailed, head segmentation, labial, protein localization.

Introduction

The maggot represents an extreme degree to which juvenile development among the insects has diverged from the evolutionary course that produced the adult, until the young insect has become an independent creature in no way structurally related to its parents... The structural disparity between the larva and the adult in the Cyclorrhapha is due to the specialized form that the larva has acquired, rather than to that of the adult fly. The larval head of these flies in particular has become so highly modified in a specific way that it is difficult to understand how it has been evolved from a head of more usual structure.

R. E. Snodgrass, 1953

Drosophila belongs to the suborder Cyclorrhaphora, the members of which are considered the most evolutionarily advanced of all insects. As reflected in the above comments of Snodgrass (1953), the Drosophila head is characterized by an extreme morphological difference between the larval and adult stages. Specifically, the larva head is not well-developed and is withdrawn deep into the thoracic cavity. Thus, despite the ease with which Drosophila is manipulated both genetically and molecularly, its ontogeny reflects a specialized or advanced condition that is bound to complicate our understanding of the common molecular genetic mechanisms underlying developmental processes within insects and other arthropods. This is especially true of the head. Not surprisingly, little scientific attention has been devoted to such an analysis of head development.

Despite overall appearances, the insect head is thought to be a segmented structure, arising during the course of evolution by the coalescence of a number of trunk segments with a non-segmental acron. The acron, the anterior-most division, is considered homologous to the annelid prostomium, reflecting the view that the ancestors of the insects were annelid-like. Although there is a large body of comparative morphological and embryological evidence to support this view, the actual
segmental organization of the head has been the subject of much-heated debate. [Reference to the problem of head segmentation in insects may be found in Matsuda (1965), Anderson (1972, 1973), Jura (1972), Rempel (1975), Richards and Davies (1977) and Jürgens et al. (1986).] Inspection of a Drosophila embryo at the segmented-germ-band stage (c.f. Turner and Mahowald, 1979) provides a nice illustration of the problem: whereas bilateral appendages or 'lobes' constitute the gnathocephalon (mandibular, maxillary, and labial), no such indications of metamersism are evident on the procephalic and clypeolabral lobes of the pre-oral head. Nonetheless, this latter region is thought to include vestiges of intercalary, antennal, and labral segments.

The homeotic genes of the Antennapedia complex (ANT-C) direct the development of head and thoracic segments (for review, see Mahaffey and Kaufman, 1988). These include Antennapedia (Antp), Sex comb reduced (Scr), Deformed (Dfd), proboscipedia (pb), and labial (lab), of which only Dfd, pb, and lab function almost exclusively in the head. In this report, we focus on the expression of lab and Dfd for three reasons: first, lack of function of either gene results in the failure of head involution and associated lethality; no homeotic transformation is apparent in mutant embryos (Merrill et al., 1987, 1989; see also, Regulski et al. 1987 and Jürgens, 1988). Second, the pattern of lab expression is uniquely localized to the intercalary 'segment' of the procephalon (Diederich et al. 1989). Because this enters into controversial morphological territory, we also present a detailed account of the engrailed (en) expression pattern in the embryonic head for insights into its segmental constitution. Finally, it was of interest to determine whether the developmental program initiated by lab and Dfd involved overlapping expression, since developmental genetic analyses had indicated similar mutant phenotypes for both of these genes, particularly in adult tissues, where a homeotic transformation of head to thorax is observed (Merrill et al. 1987, 1989).

Materials and methods

Immunological staining

The immunological staining procedures used here were essentially the same as those described in Mahaffey and Kaufman (1987) and Mahaffey et al. (1989). A mouse anti-injected (inv) monoclonal antibody was used to reveal the en expression pattern (Coleman et al. 1987). Double labeling of embryos was accomplished by simultaneously incubating embryos with rabbit anti-lab and rat anti-Dfd polyclonal antibodies (Diederich, 1989; Diederich et al. 1989), or in combination with the anti-inv antibody. This was followed by an incubation with the appropriate species-specific secondary antibodies (BioRad) conjugated directly to alkaline phosphatase (AP) or horseradish peroxidase (HRP).

For embryos that were to be sectioned or dissected, it was necessary to stain them darkly compared to the level of staining that was acceptable for observations of whole-mounts. Sections were obtained using a cryostat (Slee): after staining, embryos were gently blotted of excess water, frozen in O.C.T. compound (Miles Scientific), serially sectioned at 10 micron thickness, and mounted under a coverslip in Gurr's aqueous mounting media. The best retention of tissue and morphological detail was obtained by not attempting to remove the O.C.T. compound prior to mounting with a coverslip. Overlapping expression was discernable because of the black color that is produced when the brown (HRP) and blue (AP) precipitates mix in the same cell (Boorsma, 1984). Scanning multiple focal planes of many specimens allowed us to determine whether a single cell was expressing both gene products of interest or whether a 'blue' cell overlaid a 'brown' cell.

Two methods were employed for an examination of lab and Dfd expression in imaginal tissue. For singly stained tissue, late-staged third instar larvae were first torn in half under a dissecting microscope in 0.9 % NaCl and 0.02 % Triton X-100 using watchmakers forceps. Anterior and posterior halves were then separated, turned inside out, and immediately transferred to the same (fresh) solution on ice until approximately 30 larvae had been dissected. The tissue was fixed for 10 min in ice-cold acetone, followed by 10 min in ice-cold methanol. This fixation procedure generally resulted in lower background staining compared to fixation by paraformaldehyde. Endogenous peroxidase activity was inactivated by treating the tissue with 0.3 % H2O2 in methanol at room temperature for 45 min, followed by two methanol washes. Antibody incubations and staining reactions using HRP-coupled secondary antibodies and diaminobenzidine (DAB) were the same as those used for embryos, with the exception that the staining of the eye-antennal discs of Figs 4A and 5B was intensified by adding NiCl2 at 0.04 % to the reaction mixture (NiCl2 causes the normally reddish-brown precipitate to turn purple). After staining and rinsing in water, imaginal discs were dissected from the larval body halves and mounted in Gurr's aqueous mounting media.

For doubly stained imaginal tissue, the protocol of Pattatucci and Kaufman (1991) was followed. Briefly, dissected larvae were fixed for 1 min in a 1:1 mixture of heptane: 3 % paraformaldehyde in PBS (PBS is 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4), transferred to fresh 3 % paraformaldehyde/PBS without heptane for 10 min and then rinsed in PBS. Tissues were stained sequentially, first for lab using the rabbit anti-lab antibody and an HRP-coupled goat anti-rabbit Fab' secondary antibody (Protos Immunoresearch) and DAB. This was followed by incubations with the rabbit anti-Dfd antibody and an AP-coupled goat anti-rabbit secondary antibody (Boehringer-Mannheim). All antibody incubations were done in PBS, 0.1 % BSA, 8 % normal goat serum, 0.2 % Triton X-100. The AP staining was performed in a reaction mixture adapted from Van Rooijen et al. (1984) and composed of three parts: Solution 1 consisted of 20 mg ml−1 naphthol AS phosphate (Sigma) in N,N-dimethyl formamide. Solution 2 consisted of 60 mg ml−1 Fast Blue BB Base (Sigma) in 2 % HCl, to which an equal volume of 4 % NaNO2 was added. The final reaction mixture comprised 3.66 ml 85 mM Tris (pH9.8), 21 mM MgCl2, 11 % dimethyl sulfoxide, and 5 mm levamisole, to which 25 μl solution 1 and 50 μl of filtered solution 2 were added. The staining reaction occurred in the dark for ~30 min and was terminated by washing in PBS. Tissues were dissected and mounted in Aqua Polymount (Polysciences) and photographed on a Zeiss AXiophot microscope using Kodak VRG 100 print film.

Results

The pattern of engrailed expression in the embryonic head

The en protein has been shown to be localized within
The periodic pattern of expression that is so striking in the epidermis and central nervous system (CNS) of metameric regions also extends anteriorly into regions of the head that are not obviously segmented. This includes that portion of the head anterior to the 'mouth' (the preoral head), which is considered to have evolved from the fusion of three ancestral segments (Rempel, 1975; Struhl, 1981; Jürgens et al. 1986). DiNardo et al. (1985) suggest that the pattern of en-expressing cells in this region marks the position of vestiges of these segments.

The initial observations of DiNardo et al. (1985) were used as a framework for a more detailed examination of the en expression pattern in the embryonic head. This analysis was prompted by the observation that en-expressing cells of the procephalon provide visually striking boundaries for the domains of lab and Dfd expression (Mahaffey et al. 1989) and thus might provide insights into the controversial segmental makeup of the head. High magnification views of the en expression pattern in the embryonic head are presented in Fig. 1.

In contrast to the gnathocephalon, thorax, and abdomen, most of the en-expressing cells of the procephalon appear relatively late, towards the end of germ-band extension but prior to segmentation, and are evident more as clusters of cells rather than as stripes of cells (Fig. 1A,B). We detect five discrete clusters on either side of the procephalon, though DiNardo et al. (1985) report seeing only four clusters. This discrepancy may be attributed to the close juxtaposition of two of these cell clusters (labeled an? and Ir? in Fig. 1B).

Because the en expression pattern does not encircle the head, as it does in the trunk, DiNardo et al. (1985) proposed boundaries for the ancestral segmental primordia by drawing imaginary lines. We have modified the position of these putative boundaries (dotted lines, Fig. 1B) to account for the fifth cell cluster, and have tentatively labeled the vestigial segments [(in) intercalary; (an) antennal; (Ir) labral] based on the work of comparative insect morphologists (e.g. Anderson 1972, 1973; Rempel 1975; see also Jürgens et al. 1986).

During segmentation of the germ band (stage 11; embryonic stages are of Campos-Ortega and Hartenstein, 1985) all of the en-expressing cell clusters of the preoral head, with the exception of those associated with the clypeolabral lobe, appear to delaminate from the ectodermal surface (Fig. 1E,F) to incorporate into the supraesophageal ganglia of the CNS (Fig. 1G). The apparently non-neural cell clusters of the clypeolabral lobe, on the other hand, organize as two broad bands as this structure retracts during the process of head involution (Fig. 1G). Thus, the final number of cell clusters observed in the head of a near-mature embryo is consistent with our observation of earlier-staged embryos (Fig. 1B) that five areas of en-expressing cells (including those of the clypeolabrum) originate in the preoral head. Furthermore, a simple counting of 'segments', as suggested by the periodic pattern of these cells, indicates that one of the cell clusters may be associated with the archicerebrum or acron, the anterior-most division (Fig. 1G). Finally, en expression is apparent in cells that make up the dorsal ridges (Fig. 1E); these develop later into the dorsal fold (Fig. 1H).

The striking combination of lab and en expression patterns, particularly during early stages of embryogenesis (see Mahaffey et al. 1989), lends support not only to the suggestion that en-expressing cells mark vestiges of ancestral segments, but also to the extension of segmental boundaries as proposed in Fig. 1B, at least for those cells associated with the intercalary and antennal 'segments'. In Fig. 2A,B this relationship is shown for a later-staged embryo (stage 13) after the germ band has retracted. By this stage both en and lab are coexpressed within a small number of cells of the posterior intercalary 'segment'; these cell clusters, along with most lab-expressing cells, delaminate from the ectoderm to reside at the base of each 'brain' lobe (see Fig. 3E). Interestingly, the anterior domain of Dfd expression extends to these same en-expressing cell clusters of the intercalary 'segment' (Fig. 2C,D and see below).

A direct comparison of labial and Deformed expression patterns during embryogenesis

With the availability of different species-derived antisera, we were able to compare directly the lab and Dfd expression patterns. We were particularly interested in addressing the extent to which their respective patterns overlapped. Such overlapping expression not only would provide an explanation for the apparent overlap and similarity of mutant defects produced by lab and Dfd, but also would provide requisite knowledge towards elucidating the biochemical contexts within which they functioned.

In agreement with the indirect evidence provided by the en double-labeling study (Fig. 2), lab and Dfd are coexpressed within prospective neural cells of the intercalary 'segment'. Prior to the onset of germ-band retraction this coexpression is very weak, if it occurs at all (Fig. 3A,B), but is convincing by the time of germ-band retraction (stage 12; Fig. 3C,D). Ultimately these cell clusters reside at the bases of the supraesophageal ganglia as shown in the dissected CNS from a late-staged embryo displayed in Fig. 3E (arrow). Thus, it appears that lab, Dfd, and en are coexpressed within a small number of prospective neural cells in a region of the head that marks the boundary between gnathocephalon and procephalon. In other regions of the embryo, which are likely to contain progenitor cells of imaginal tissue (see also below), lab and Dfd coexpression is evident in a few cells located in an area ventral to the dorsal ridges (arrows, Fig. 3F) but which does not include the dorsal ridges.
Expression of labial in tissues of the third instar larva

In addition to its essential role for embryonic head development (and viability), lab is also necessary for the proper development of the adult head capsule (Merrill et al. 1989). Thus, it is likely that lab function is localized to nascent imaginal tissue, specifically the eye-antennal discs, from which most of the adult head will develop. Dissected tissues from third instar larvae, in which lab protein accumulation was detectable, are shown in Fig. 4. All aspects of the larval expression pattern are consistent with embryonic expression patterns and/or with the known genetic function of lab in imaginal development.

Expression was observed within cells located at the bases of the supraesophageal ganglia (Fig. 4B) and within cells that compose a short section of the midgut (Fig. 4C); these patterns of expression are consistent with earlier patterns witnessed in embryonic stages (Diederich et al. 1989). In addition to these tissues, lab expression was also detectable in the eye-antennal discs (Fig. 4A); however, the origin of the eye-antennal discs and the lab expression pattern within them are not so obvious. The physical arrangement of the paired eye-antennal discs, as they occur in a third instar larva, is displayed in Fig. 5A. During embryogenesis, precursor cells of these discs are likely to be located on the procephalic lobe (pl), ventral dorsal ridge (dr), and posterior maxillary lobe (see also Diederich et al. 1989). In the dorsal ridge proper, Dfd-expressing cells appear anterior and medial to, and do not overlap with, lab-expressing cells. Arrows on thorax point to presumptive sensory anlagen expressing lab.
Fig. 4. Expression of lab in tissues of the third instar larva. (A) Dissected left eye-antennal disc (composite focal planes); anterior end faces top of page. lab protein accumulation is apparent only in nuclei of the peripodial cell layer. Over the antennal (a) portion of the disc, this expression is restricted to the lateral edge (a tear interrupts the continuum of expression); above the eye (e) portion faint expression encompasses a broader domain. (B) Dissected CNS (composite photo). lab-expressing cells are located at the base of each supraesophageal ganglion (sp), within the apparent tritocerebrum [vnc ventral nerve cord]. (C) Dissected section of the midgut. lab expression is detectable within large polytene nuclei that compose a short section of the midgut.

Fig. 5. Dfd expression in tissues of the third instar larva. (A) A composite photo showing the approximate in situ relationship of the eye-antennal discs (a,e), in dorsal view. Note their point of attachment to the cephalopharyngeal skeleton (cps), anteriorly, and to the supraesophageal ganglia (sp), posteriorly [vnc ventral nerve cord]. (B) Dissected left eye-antennal disc. Dfd expression is evident only in nuclei of the peripodial cell layer, including most cells over the antennal (a) region. The positions of the 'a' and 'e' are roughly the same in panels A and B. (C) Dissected labial disc, highly magnified, showing Dfd expression at the base of the disc epithelium. Because dissections of this small disc are difficult, not all Dfd-expressing cells may be exposed here; loose cells appear adjacent to the disc. (D) Dissected CNS, ventral view. Dfd expression is localized to nuclei within the subesophageal-derived portion of the ventral nerve cord. The 'sp' in panels A,D identify the left 'brain' lobe.

Fig. 6. Direct comparison of lab and Dfd expression patterns in larval tissues. Dfd-expressing cells are blue and lab-expressing cells are brown. (A) Dissected right eye-antennal disc; the lab expression domain is lateral to that of Dfd (A tear in the peripodial membrane interrupts the Dfd domain). (B) High magnification (different specimen) of anterior disc attachment site (see white-boxed region in A); despite mounting artifacts resulting in a convoluted peripodial cell layer, there is apt to be no overlap of lab and Dfd expression. (C) High magnification (different specimen) of lateral eye-antennal boundary (see black-boxed region in A). Note precise juxtaposition of Dfd-expressing cells medial to lab-expressing cells. (D) Dissected larval CNS (third instar), dorsal view. (E) High magnification of boxed region in D. Dfd-expressing cells are ventral to the plane of focus; there is no overlap of expression.
All imaginal discs can be thought of as flattened sacs of cells, consisting of a disc epithelium and an overlying cell layer known as the peripodial ‘membrane’. Within the eye–antennal discs, expression of the *lab* protein is limited to nuclei of the peripodial cell layer (Fig. 4A). However, even within this cell layer, *lab* expression is spatially restricted, and judging from staining intensity, appears at varying levels of abundance. Specifically, *lab*-expressing cells begin anteriorly at the apices of the discs, continue posteriorly over the lateral aspects of the antennal portion, and finally, spread out over a large area of the eye-portion of the disc. Within this latter region, expression is very faintly detected.

Expression of Deformed in tissues of the third instar larva

Dissected tissues from third instar larvae, in which Dfd protein accumulation was evident, are shown in Fig. 5. Consistent with its earlier embryonic pattern, Dfd expression within the CNS constitutes a stripe of cells at the anteroventral-most portion of the ventral nerve cord (Fig. 5D). Laterally, this domain of expression abuts the *lab*-expressing domains in each of the ‘brain’ lobes but does not overlap with *lab*-expressing cells (Fig. 6D,E), in contrast to the embryonic CNS expression pattern (Fig. 3E).

Dfd expression is also observed within a small number of cells located at the base of each labial disc (Fig. 5C), near their point of attachment to the cephalopharyngeal skeleton. These cells compose a portion of the disc epithelium, as opposed to belonging to the thinner peripodial cell layer. The labial discs give rise to adult mouthpart structures known as the labial palps. Dfd expression in these discs is consistent with the observations of Merrill et al. (1987) that Dfd mutant effects include a region of the labial palps. Interestingly, this expression may represent a later, secondary activation of the Dfd gene, i.e. expression that is discontinuous with earlier embryonic patterns: the labial discs reportedly derive from the embryonic labial segment (Poulson, 1950); however, there is no detectable accumulation of Dfd protein in this segment, at least prior to head involution when it is still discernable (Jack et al. 1988; Diederich, 1989).

In accordance with developmental genetic predictions (Merrill et al. 1987), Dfd is expressed in the eye–antennal discs (Fig. 5A,B). As observed for *lab*, Dfd-expressing cells are present only in the peripodial membrane and are spatially restricted within this cell layer, in agreement with the RNA localization data of Chadwick and McGinnis (1987). From the point of attachment of the discs to the cephalopharyngeal skeleton, anteriorly, Dfd-expressing cells occupy a broad domain into central and medial aspects of each disc, posteriorly. The posterior boundary of this domain ends rather abruptly near the eye portion of the disc. This contrasts with *lab*-expressing cells, which not only extend further posteriorly, but also show a more gradual decrease in staining intensity (Fig. 4A). Overall, the levels of protein accumulation, judging from staining intensity, appear to be more uniform for cells expressing Dfd than those expressing *lab*.

Direct comparison of *lab* and Dfd expression in the eye–antennal disc

By itself, the peripodial ‘membrane’ is a nondescript cell layer; it is essentially the underlying disc epithelium that constitutes what is recognizable as the eye–antennal disc. Moreover, preparation of this disc for microscopic examination inevitably results in the shifting of the peripodial cell layer relative to the underlying disc epithelium, and thus distorts the in situ relationship of the two cell layers. Therefore, the disc epithelium can be used only in a limited sense as a reference for comparing expression patterns between different whole-mount specimens. By applying double-labeling techniques to imaginal tissue, we were able to circumvent this problem and to address the extent to which *lab* and Dfd overlap in their expression to influence determinative events in the adult head.

Fig. 6 presents representative imaginal tissue doubly-stained to reveal both the *lab* and Dfd proteins. As noted earlier, we detect no overlapping expression in the CNS of the third instar larva (Fig. 6D,E), in contrast to the pattern of coexpression evident in the embryonic CNS (Fig. 3C,E). Additionally, examination of many preparations of eye–antennal discs (Fig. 6A–C) has led us to conclude that the domains of *lab* and Dfd expression are apt to be non-overlapping in this tissue as well, although it is possible that a few co-expressing cells escaped our detection given the difficulties inherent in observations of this cell layer. Overall, the *lab*-expressing domain of the eye–antennal disc occupies a region of the peripodial cell layer that is adjacent and lateral to that of Dfd, resulting in a sharp demarcation between the two staining patterns (Fig. 6A,C).

Discussion

Dfd and lab: A comparison of expression patterns with mutant defects in the adult head capsule

In the larva, both *lab* and Dfd display little overlap in mutant phenotype (e.g. disrupted H-piece) that cannot be explained as a secondary consequence of a failure of head involution (Merrill et al. 1989). In agreement with this observation, these genes exhibit no apparently significant overlap in their embryonic expression patterns: Dfd expression is limited primarily to the gnathocephalon, whereas *lab* is restricted chiefly to the procephalon. This contrasts with the apparent overlap and similarity of mutant defects that are produced in the adult head capsule when the function of either gene is eliminated by somatic recombination (Merrill et al. 1987, 1989). We were somewhat surprised to find that there also appears to be no overlap in their respective expression patterns in the eye–antennal disc of the third instar larva, even though both patterns are limited to the peripodial cell layer of this disc.
The most detailed fate map that exists for the eye–antennal disc was produced by Haynie and Bryant (1986). One of the approaches utilized by these authors was the transplantation of disc fragments into larval hosts followed by a scoring of differentiated structures after the host had undergone metamorphosis. Using these results in combination with gynandromorph-derived fate maps and a working knowledge of the morphology of the adult head, these authors were able to deduce that the peripodial 'membrane' actually gives rise to a significant portion of the posterior head (see also Sprey and Oldenhave, 1974). Thus, the expression patterns of \textit{lab} and \textit{Dfd} in this cell layer provide a satisfying confirmation of their analysis. Similarly, the peripodial cells of the imaginal antennae of the butterfly, \textit{Pieris}, were shown by Eassa (1953) to contribute to a large portion of the adult head capsule.

In general, the mutant effects of \textit{Dfd} extend throughout most regions of the posterior head (Merrill et al. 1987), whereas those of \textit{lab} are limited primarily to its dorsal half (Merrill et al. 1989). Consistent with this pattern, \textit{Dfd} has a correspondingly broad domain of expression, whereas \textit{lab} expression is more narrowly defined, at least in those areas where it is strongest. Although there is very good general correspondence between expression pattern and mutant defects for \textit{lab} and \textit{Dfd}, it is difficult to present confidently a structure-by-structure analysis. This can be attributed to (1) insufficient detail and/or inaccuracies in the eye–antennal disc fate map; (2) difficulties in discerning the precise boundaries between disc epithelium and peripodial membrane; and (3) uncertainties about the total extent of the mutant phenotype as revealed in somatic clones (see Merrill et al. 1989). One example is provided by the maxillary palps, which are absent in both \textit{lab} and \textit{Dfd} clonal tissue: They have been mapped to the anterolateral edge of the disc epithelium but apparently not to the overlying peripodial membrane, which is, however, the site of both \textit{lab} and \textit{Dfd} expression. Nonetheless, the expression patterns of \textit{lab} and \textit{Dfd}, in conjunction with those of other loci involved in head development, may provide useful markers for the future refinement of the eye–antennal disc fate map, particularly in the largely ignored peripodial cell layer.

In the embryo, limited coexpression of \textit{lab} and \textit{Dfd} is observed in prospective neural cell clusters (along with \textit{en}) of the intercalary segment (Fig. 3C,E) and in the vicinity of the dorsal ridges (Fig. 3F) in cells that are likely to compose a portion of the presumptive eye–antennal anlage. Interestingly, this overlap of expression apparently does not extend to corresponding imaginal tissues (Fig. 6). If true, this observation raises the possibility that the manner by which \textit{lab} and \textit{Dfd} serve to influence the developmental hierarchy that produces adult head structures may involve early coexpression followed by a 'refinement' to mutually exclusive expression domains. The developmental significance, if any, of coexpression in the neural cell clusters remains to be determined. However, in the presumed eye–antennal anlage, the behavior of a temperature sensitive \textit{lab} allele (Merrill et al. 1989) in temperature shift studies indicates that the adult function of \textit{lab} is likely to reside during embryogenesis, in which case the coexpression with \textit{Dfd} may play an important role for imaginal development. Moreover, this early coexpression may hold the key to resolving the apparent paradox of overlapping mutant defects in the face of (later) non-overlapping expression in the eye–antennal discs.

**Molecular insights to morphological controversies**

Based primarily on embryological studies, it is generally regarded that the insect head comprises six segments and a non-segmental acron. The \textit{en} expression pattern, as first noted by DiNardo et al. (1985), is in excellent agreement with this classical theory of head segmentation. One aspect of this theory that had been particularly controversial concerned the segmental status of the clypeolabrum, which for many years was considered 'merely an outgrowth of the body wall in front of the stomodeum' (Rempel, 1975). Rejection of its segmental status was based, in part, on the belief that it was an unpaired structure. However, our observations of a bilateral \textit{en} expression pattern in the clypeolabrum (Fig. 1G) clearly suggests that this structure evolved from the fusion of paired labral appendages, in agreement with the conclusions of comparative embryological studies (cf. Rempel, 1975) and the \textit{Drosophila} fate-mapping study of Jürgens et al. (1986).

Similarly, we have noted here and elsewhere (Diederich et al. 1989) that the domain of \textit{lab} expression in the head identifies the intercalary segment. More traditional arguments in support of this claim come from observations of the ultimate fate of these cells. A majority of the \textit{lab}-expressing cells will delaminate from the ectodermal surface to incorporate at the base of each 'brain' lobe (Fig. 3E and 4B), a region that in other insects corresponds to the tritocerebrum, a derivative of the intercalary segment (Anderson, 1973; Rempel, 1975). Most of the remaining cells constitute the epidermis of the procephalic lobe and eventually incorporate into the dorsal pouch (frontal sac), the cells of which secrete the dorsal arms of the cephalopharyngeal skeleton (see Jürgens et al. 1986 for a description of larval head structures). In rare preparations where the \textit{lab} antisera has penetrated the cuticle of late-staged embryos (stage 17), \textit{lab}-expressing nuclei outline the dorsal arms even before pigmentation makes this structure discernible (data not shown). The dorsal arms appear to correspond to the anterior tentorial arms of other insects (cf. Fig. 94, Matsuda, 1965), which are themselves considered to be derivatives of the intercalary segment (Rempel, 1975). Finally, the expression of \textit{en} in the dorsal ridges and dorsal fold (see Fig. 1E,H) is puzzling with regard to the segmental derivation of these structures. Although it has been suggested that the dorsal ridges originate from the labial segment (Technau and Campos-Ortega, 1985), we would like to raise the possibility that the cells that make up these structures may derive instead from the intercalary segment, and that during the evolutionary reorganiz-
ation of the larval head, lost their overt continuity with the majority of lab-expressing cells on the anteroventral procephalic lobe. In support of this view, we note that (1) lab and en are coexpressed in the dorsal ridges/fold (Mahaffey et al. 1989), (2) an initial continuum of lab expression within the ventral procephalon becomes discontinuous by mid-embryogenesis (Diederich et al. 1989) and, (3) during the process of head involution, lab-expressing cells of the dorsal fold again become continuous with those of the procephalic lobe (Diederich et al. 1989).

The lack of association between en expression and the hypopharyngeal lobes suggests that these structures are merely an anterior extension of the sternal mandibular region destined to form the hypopharynx (cf. Turner and Mahowald, 1979). It appears unlikely that they represent transient paired appendages of the intercalary segment, in part because these are drawn as lateral not medial structures (cf. Anderson, 1972; Jura, 1972; Richards and Davies, 1977). Our estimation is that the hypopharyngeal lobes are homologous to the superlinguae of other insects (see Matsuda, 1965). It is noteworthy, perhaps, that these apparently same structures were considered by a few morphologists to represent a seventh head segment, a claim discredited by most other morphologists (Rempel, 1975; Richards and Davies, 1977).

The ontogenetic and phylogenetic relationship between embryonic and adult head segments: implications for homeotic gene function

Most holometabolous insects, including 'lower' Diptera such as the mosquito (Nematocera), have well developed larval heads. There is not a pronounced segregation of larval and adult tissue; imaginal cells appear during the larval stages, frequently just before pupation (Miall and Hammond, 1892). In 'higher' Diptera, on the other hand, the development of imaginal cells begins much earlier, during embryogenesis, continuing through the larval stages to form discrete structures known as imaginal discs.

Although the discs retain their connection to the larval epidermis, they are essentially independent of it. Anderson (1972) speculates that this condition evolved to permit 'greater larval specialization without destroying the capacity to develop into a normal adult insect.' Although the ontogenetic relationship between the larval and adult head among the 'higher' Diptera presents conceptual difficulties, clues to this relationship can be found in less advanced insects (e.g. Snodgrass, 1953). Indeed, in the head of 'lower' holometabolous insects, imaginal tissue develops in close association with larval structures; for example, imaginal antennae, labial and maxillary palps develop at the base of their respective larval counterparts (e.g. Eassa, 1953; Miall and Hammond, 1892). Because of this relationship, this tissue is more accurately referred to as imaginal 'folds' (Miall and Hammond, 1892) rather than as imaginal 'discs'.

In 'higher' Diptera, on the other hand, three pairs of imaginal discs, labial, clypeolabral, and eye–antennal, contribute to the adult head. The eye–antennal disc, a single structure, bears the progenitors for the eye, antenna, maxillary palps, and a large part of the head capsule (Bryant, 1978; Haynie and Bryant, 1986). Thus, it would appear that the eye–antennal disc arose from the coalescence of formerly separate anlagen such as are evident in more primitive insects. Although this is implicit in the observations of Snodgrass (1953) and is a view held by Jürgens et al. (1986), others have argued on the basis of clonal analysis data that the eye–antennal discs originate from a single segment, namely the embryonic antennal segment (Morata and Lawrence, 1979; Struhl, 1981; see also Haynie and Bryant, 1986). The chief observation that prompted this conclusion was that because clonal restrictions are not observed until the third larval instar, the disc has a common cell lineage and thus originates from a single segment, as is observed for imaginal discs of the thorax and abdomen (Lawrence, 1981). However, this conclusion rests on the assumption that segments of the head are like those of the trunk.

That the eye–antennal disc might originate from a single segment seems counter to notions regarding the evolutionary process, that is, given the manner in which imaginal tissue develops in more primitive insects. Both comparative anatomical and developmental studies (e.g. Miall and Hammond, 1892; Eassa, 1953) on less advanced holometabolous insects have established homologies between larval and adult head structures. In those cases where anatomists have argued for the loss of a structure, for example of mandibles in the Lepidoptera and Diptera, no imaginal rudiments were observed to be associated with the larval mandibles. Thus, it is unlikely that the adult maxillary palps have been mislabeled, i.e. are actually of antennal origin.

As proponents of the view that the eye–antennal discs arose by the fusion of several segmental primordia, Jürgens et al. (1986) noted the juxtaposition of the larval (and presumably, imaginal) antennal and maxillary anlagen on the blastoderm fate map: With the introduction of head involution in the 'higher' Diptera, segmental anlagen that otherwise would remain separate, e.g. in 'lower' Diptera, now are positioned together within the frontal sacs. This fusion of segments, as witnessed in the preoral head region, appears to represent a trend in the evolution of the insect head (Rempel, 1975). Likewise, the ontogeny of the maggot head entails a fusion of the mandibular and maxillary lobes with the procephalic lobe, a coalescence that produces the 'anterior lobe' or 'pseudocephalon', complete with a multisegmentally-derived antennomaxillary sensory complex (Turner and Mahowald, 1979; Frederick and Denell, 1982). Thus, the loss of clonal restrictions may in fact be a necessary prerequisite for the morphogenetic processes that evolved in the fashioning of the larval head of 'higher' Dipterans.

Moreover, because the adult heads of 'higher' and 'lower' flies are not manifestly different, the evolution of these processes must have led to the generation of an eye–antennal disc without significantly affecting the development of the adult head.
In attempting to resolve the ontogenetic relationship between embryonic and adult head segments, it is tempting to view the embryonic expression of lab in the intercalary segment and that of Dfd in the maxillary segment as indicators of continuity between larva and adult. Although lab- and Dfd-expressing cells are in close juxtaposition in the vicinity of the dorsal ridges, this temptation must be tempered by the observation that Dfd expression in the labial disc appears to lack continuity with earlier embryonic expression.

It is worth stressing that during the evolution of the ‘higher’ Dipterans selective pressure was primarily on larval features, the head in particular (Snodgrass, 1953). We are intrigued by the implications this may have regarding the evolution of the ANT-C homeotic genes that function in the head: that concomitant with the evolution of the maggot body plan, lab, Dfd, and pb may have acquired divergent roles in the embryonic (larval) and adult stages of the life cycle compared to homeotic genes functioning in the thorax and abdomen. The differences observed in expression patterns among homeotic genes of the head and trunk, the absence of a larval homeotic transformation in lab and Dfd mutants, and the apparent lack of pb function in larval development per se (Pultz et al. 1988) suggest this might have been the case. Because the maggot head is an evolutionarily recent development, the implication is that additional regulatory mechanisms evolved for the development of this structure compared to those that were already in place for the more ancient adult head. Stated from an alternate viewpoint, the homeotic regulatory paradigm observed in the present day adult head is more reflective of the ancient or original condition than that seen in the more specialized embryo and larva. We think it likely that an analysis of the homologous genes in ‘lower’ Dipterans not only will elucidate the function of these genes in the highly evolved Drosophila, but also will provide a common link for understanding homeotic gene function in other insects and arthropods.

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References


Drosophila head development


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