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# Number, identity, and sequence of the *Drosophila* head segments as revealed by neural elements and their deletion patterns in mutants

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ABSTRACT **The development of the insect head tagma involves massive rearrangements and secondary fusions of segment anlagen during embryogenesis. Due to the lack of reliable morphological markers, the number, identity, and sequence of the head segments, particularly in the pregnathal region, are still a matter of ongoing debates. We examined the complex array of internal structures of the embryonic *Drosophila melanogaster* head such as the sensory structures and nerves of the peripheral and stomatogastric nervous systems, and we used embryonic head mutations causing a lack of overlapping segment anlagen to unravel the segmental identity and the sequence of the neural elements. Our results provide evidence for seven distinct segments in the *Drosophila* head, each characterized by a specific set of sensory neurons, consistent with the proposal that insects, myriapods, and crustaceans share a monophyletic evolutionary tree from a common annelid-like ancestor.**

The insect body is composed of metamerically repeated units. While segmentation is obvious in the "trunk" region, the segmental organization of the head is still obscure (1–7). By distinct cuticular features, three gnathal segments (mandibula, maxilla, and labium) can be distinguished in the posterior head region. The anterior pregnathal region, however, is poor in diagnostic morphological structures (8). Morphological and evolutionary studies based on epidermal head structures (5, 8), coelomic cavities, or brain regions (1–4) have so far failed to unambiguously determine the number and identity of pregnathal segments in the insect head. Due to the evolutionary changes that resulted in the "acephalic" appearance of higher dipteran larvae, the segmental composition of the *Drosophila* head was analyzed only recently. The detailed fate map analysis of a few cuticular specializations of the larval *Drosophila* head by laser ablation studies suggested six segments—i.e., three pregnathal and three gnathal segments (5). This interpretation was questioned through a detailed analysis of the expression patterns of the segment polarity genes engrailed (*en*) and wingless (*wg*), which serve as molecular markers for metamers in the prospective trunk and gnathal regions of the *Drosophila* embryo. This analysis suggested the possibility of seven instead of only six head segments (7). Here we present additional and more substantial evidence for seven head segments which is based on detailed analysis of internal head structures such as sensory organs. Their axons fasciculate to give rise to seven distinct sensory projections to the brain. The analysis of the patterns of sense organs deleted in various head segmentation mutants allows us to make an assignment of the sense organs to their segments of origin.

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## MATERIALS AND METHODS

*Drosophila melanogaster* strains were kept under standard conditions; wild-type and mutant embryos were obtained as described (9). The *en* expression patterns of embryos lacking *tor* activity (collected from homozygous $tor^{PM}$ females) (10, 11) and of embryos homozygous for the mutations $hkb^{2}$ (12), $croc^{5F59}$ (gift of G. Jürgens), Df(1)KA14 uncovering *otd* (13), $ems^{9Q64}$ (14, 15), $btd^{XG}$ (16), or Df(1)62g18 uncovering *gt* (17, 18) were analyzed (6, 9, 13–21) by antibody staining of *en*-encoded protein (22); the sensory organs, sensory nerves, and the stomatogastric nervous system (SNS) were examined by 22C10 antibody staining (23). Examination of whole mount preparations of wild-type and mutant embryos was done as described (7, 24). Stages of embryos refer to the staging by Campos-Ortega and Hartenstein (25).

## RESULTS

The expression pattern of the segment polarity gene *en* serves as a molecular marker for metamers in the *Drosophila* trunk region. Its pattern in the head region (Fig. 1) suggested a total of seven metamers (7). To establish the segmental composition of the head by morphological markers, we examined specific internal head structures in wild-type embryos, such as the sensory organs and nerves of the PNS and SNS (summarized in Fig. 1). Using 22C10 monoclonal antibody stainings (23), we confirmed the known set of PNS/SNS elements—i.e., 15 sensory organs and 5 sensory nerves (24–26). In addition, we discovered 5 additional PNS sensory organs and 2 sensory nerves (Fig. 2). The *Drosophila* head therefore contains a total of 20 distinct sensory organs and 7 sensory nerves which were found to project into seven different portions of the embryonic brain (for the summary, nomenclature, and description see Fig. 1). The sensory nerves SNI, -II, and -III project into the supraesophageal ganglion and SNIV, -V, -VI, and -VII project into the subesophageal ganglion. SNI (labral nerve) collects axons from the PNS elements ep, pch1, dpo, hpo, and lho; SNII (also termed Bolwig nerve) collects them from the Bolwig light sensory organ; SNIII, from the do, ch1, and the dmp; SNIV (papilla nerve; pan), from the dlp and pao; SNV (laterophargyngeal nerve; lpn), from the apo and lpo; SNVI (maxillary nerve; mxn) from the ao, to, vo, ch2, and hmo; and SNVII (labial nerve; lan), from the hy and lbo (details in Figs. 2 and 3).

Due to the complex pattern of the PNS/SNS elements in the wild-type embryonic head (Fig. 1), their segmental organization and sequential order cannot be established directly. We therefore made use of mutations causing specific deletions of different but overlapping portions of the larval head. We examined the PNS/SNS structures in embryos which lack the activity of torso (*tor*), a key component of the maternal terminal organizer system, or the activity of the zygotic segmentation genes huckebein (*hkb*), crocodile

Abbreviations: SNS, stomatogastric nervous system; PNS, peripheral nervous system; CNS, central nervous system.

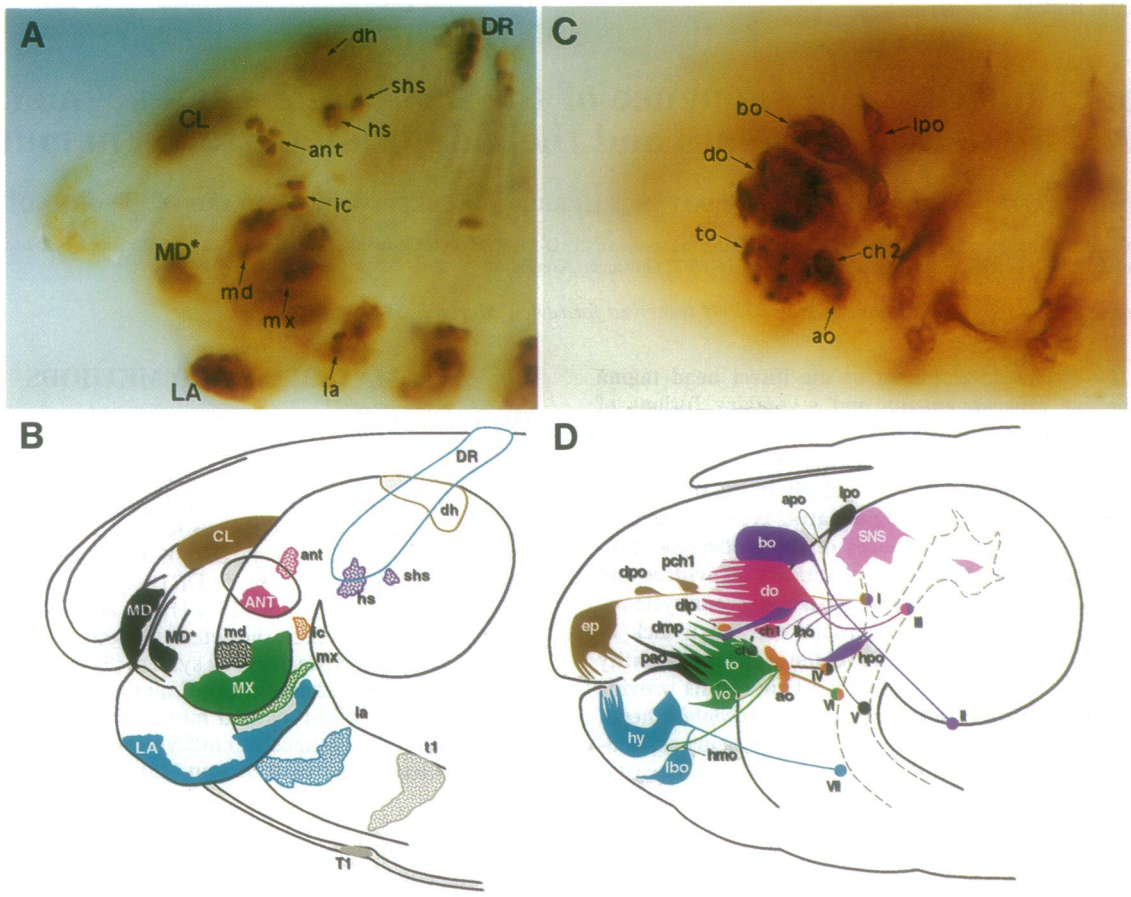


FIG. 1. Expression pattern of the segment polarity gene *en* (*A* and *B*) and the peripheral nervous system (PNS) (*C* and *D*) in the head region of the *Drosophila* wild-type embryo. Orientation of embryos: anterior left, dorsal up. (×460.) For abbreviations see *B*. (*A*) *en* antibody (22) staining pattern at embryonic stage 14; due to the optical section, only part of the *en* expression pattern is seen. (*B*) Schematic representation of the corresponding *en* expression pattern. Epidermal and central nervous system (CNS) *en* pattern elements, distinguished by solid (uppercase letters) and stippled drawing (lowercase letters), can be attributed to individual segments which are obfuscated due to morphogenetic movements during head formation (7). Different colors refer to segmental identity as established by the mutant analysis (see text, Figs. 3 and 4, and summary in Fig. 4*G*): blue, labial segment; green, maxillary segment; black, mandibular segment; orange, intercalary segment; red, antennal segment; purple, ocular segment; brown, clypeolabrum of the labral segment [note that due to the weak *en* staining in the dorsal hemispheres (7), we used only the more reliable clypeolabral *en* expression to examine labral identity in mutants]; and gray, prothoracic segment. Abbreviations: T1, t1, prothoracic segment; LA, la, labial segment; DR, dorsal ridge (part of the labial segment); MX, mx, maxillary segment; MD, md, mandibular segment; MD*, mandibular *en* expression in the floor of the pharynx; ic, intercalary segment (which lacks an epidermal counterpart in the stage-14 embryo); ANT, ant, antennal segment; hs, head spot; shs, secondary head spot (hs and shs in the CNS are part of the preantennal ocular segment; note that they have no epidermal counterparts); CL, clypeolabrum; dh, *en* expression in the dorsal brain hemispheres. DR and dh are outlined to indicate that the segmental assignment is not based on the mutant analysis (summarized in Fig. 4). Instead, DR could be assigned to the labial segment by tracing it to the early *en* expression in the blastoderm. (*C*) Optical section through a stage-15 wild-type embryo stained with 22C10 antibodies. The focus shows a fraction of head sensory organs relevant to the mutant analysis (Figs. 3 and 4). (*D*) Schematic representation of the PNS and SNS patterns (stage-15 embryo). Twenty identified sensory organs and the projections of the seven sensory nerves (SNI–VII) are shown. Color code refers to segmental identity of the elements as revealed by mutant analysis (see text, Figs. 3 and 4, and summary in Fig. 4*G*). Colored circles at the end of the seven sensory nerves indicate the segmental origin of the sensory organs projecting into a distinct portion of the brain. Previously identified head sensory organs are described in refs. 24–26; newly identified elements are shown in Fig. 2. Cuticular structures of the dlp, dmp, and hpo (defined below) were described (5, 6); we identified their neuronal counterparts. The dlp lies between the do and the to; its axon contributes to SNIV. The dmp axon fasciculates with SNIII; its cell body lies within the do but its distal sensory projection is next to the dlp. The hpo shows two sensory projections; it fasciculates with the lho and projects into the root of SNI (Fig. 2). Abbreviations: in the labial segment, hy, hypophysis; lbo, labial organ; in the maxillary segment, to, terminal organ; vo, ventral organ; ch2, chordotonal organ with two scolopidia; hmo, hypomaxillary organ; in the mandibular segment, pao, papilla organ; lpo, lateropharyngeal organ; apo, anterior pharyngeal organ; in the intercalary segment, dlp, dorsolateral papilla; ao, associated organ; in the antennal segment, do, dorsal organ; ch1, chordotonal organ with one scolopidia; in the ocular segment, bo, Bolwig organ; dmp, dorsomedial papilla; hpo, hypopharyngeal organ; lho, laterohypopharyngeal organ; in the labral segment, ep, epiphysis; dpo, dorsopharyngeal organ; pch1, pharyngeal chordotonal organ. The apo, hmo, and lho could not always be scored in the different mutant backgrounds; colored outlines indicate a segmental assignment for these organs. The SNS (in pink) derives from the stomodeum, which also gives rise to the esophagus.

(*croc*), orthodenticle (*otd*), empty spiracles (*ems*), buttonhead (*btd*), or giant (*gt*). In the head anlage of a wild-type blastoderm, these genes are functionally expressed in distinct regions (10–14, 16–18, 27) which correlate with the lack of *en* expression in the respective regions of the mutant embryos (6, 13–15, 18–21). An example of this analysis, which shows overlapping deletion patterns of sensory elements in *ems* and *btd* mutant embryos, is shown in Fig. 3. In *btd*, the mandibular, the intercalary, and the antennal segments are deleted (6). Likewise, the intercalary segment and the antennal and ocular segments are absent from *ems* mutant embryos. Comparison of *ems* and *btd* mutants reveals that hpo, bo, and dmp are missing from *ems* embryos but present in *btd* embryos. Thus, these sensory elements must derive from the segment deleted in *ems* but not in *btd* mutants—i.e., the ocular segment. The pao, lpo, and apo are deleted in *btd* but not in

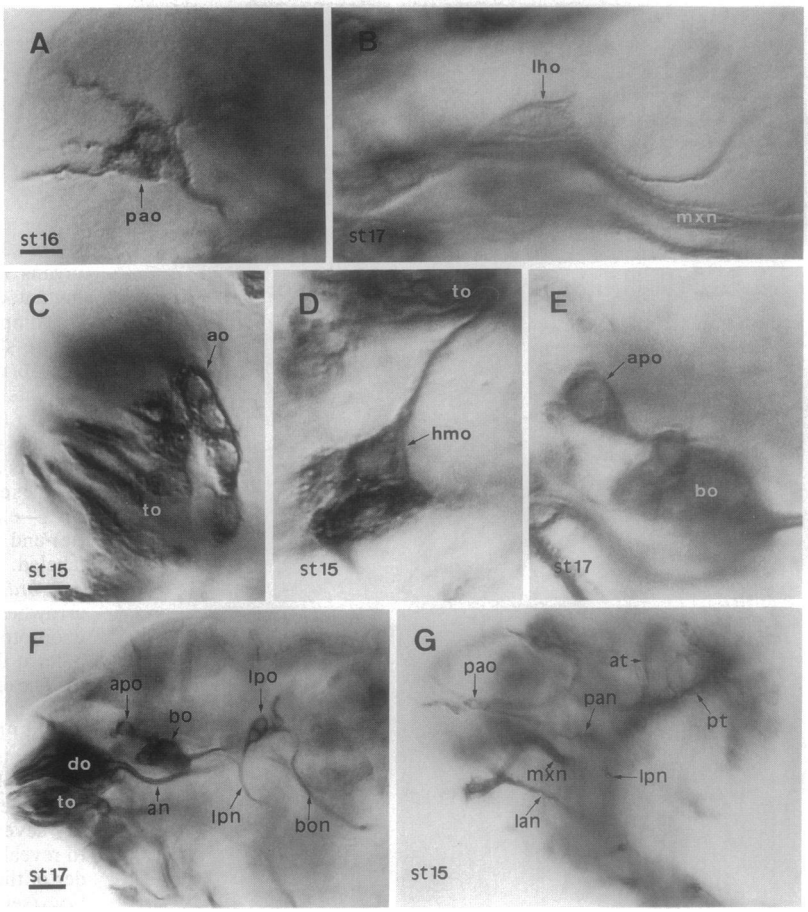


FIG. 2. Newly identified sensory organs and their fasciculation patterns in the *Drosophila* head region as revealed by 22C10 antibody staining. Stages (st) are indicated at the lower left of each panel. (*A*) The papilla organ (pao). It consists of two cells at stage 14. Three (or more) cells and three sensory projections close to the terminal organ (to) are seen at stage 16. The pao, but not the to, is deleted in *btd* mutant embryos (see Figs. 3 *A–C* and 4 *B* and *G*). Thus, they represent different organs. (*B*) Laterohypopharyngeal organ (lho). It appears as a single neuron at stage 14, which fasciculates with the axons of the hypopharyngeal organ (hpo) and then with the root of the labral nerve (SNI) (see Fig. 1 and Fig. 4*G*). (*C*) Associated organ (ao). It consists of about four cells (first seen at stage 13) which are tightly associated with the to. It is not part of the to, since it is absent from both *ems* and *btd* mutant embryos while the to is present (see Figs. 3 *A–C*, *G*, and *H*; 4 *B*, *C*, and *G*). Its axons project to the SNVI (Figs. 1 and 4*G*). (*D*) Hypomaxillary organ (hmo). It consists of a few cells (stage 14; close to the labial organ) which project to SNVI (Fig. 1). (*E*) Anterior pharyngeal organ (apo). It consists of a single neuron (first seen at stage 15) located dorsal to the Bolwig organ (bo) at stage 16–17 and fasciculates with SNV. (*F* and *G*) Among the seven sensory nerves, newly identified were the lateropharyngeal nerve (*F*; lpn, SNV) and the "papilla nerve" (*G*; pan, SNIV); SNV is established at stage 14 and collects axons from the apo and the lpo (see Fig. 1). SNV collects the axons of the pao and the dlp and projects independently into the supraesophageal ganglion at stage 15 (see Fig. 1). At later stages, SNV is in close contact to (or fasciculated with) SNVI. However, these nerves are separated in the vicinity of the CNS and they enter the subesophageal ganglion at independent positions. Note that only one focal plane is shown; for overview see Figs. 1*D* and 4*G*. Abbreviations refer to antennal nerve (an, SNIII), anterior trunk of supraesophageal neuropile (at), Bolwig nerve (bon, SNII), labial nerve (lan, SNVII), maxillary nerve (mxn, SNVI), and posterior trunk of supraesophageal neuropile (pt). [Bars represent 7 μm (*A* and *B*), 5 μm (*C–E*), and 13 μm (*F* and *G*).]

*ems* mutant embryos. Thus, they represent structural elements of the segment deleted in *btd* but not in *ems* mutants—i.e., the mandibular segment. Both mutant embryos have in common the lack of the do, the dlp, and the ao. This means that these organs derive from segments that are deleted in both *ems* and *btd* mutant embryos—i.e., the antennal and intercalary segments. Although the SNS is present in both mutants, those elements which are associated with the esophagus are altered, probably as a secondary effect owing to disruptions in the posterior wall of the pharynx (a derivative of the intercalary segment).

We also examined the PNS/SNS elements in embryos lacking *tor*, *hkb*, *croc*, *otd*, or *gt* activity. As summarized in Fig. 4 *A–F*, analysis of the deletion patterns of sensory elements and the lack of *en* expression in mutant embryos allowed us, in a manner exemplified for *btd* and *ems* (Fig. 3), to assign each sensory element to a particular *en* spot and, hence, to a specific head segment anlage. In this way, the complex arrangement of the PNS/SNS elements could be resolved. Each pattern element is shown to originate from one out of seven different units. According to this analysis, each of these units is characterized by a defined set of sensory organs and one nerve. On the basis of their correlation with distinct *en* expression domains, they can also be aligned in sequential order on the blastoderm fate map (Fig. 4*G*). These data imply that the pregnathal region is composed of four metameric units: the labral, ocular, antennal, and the intercalary segment, followed by the gnathal region containing the mandibular, maxillary, and labial segments (summarized in Fig. 4*G*).

## DISCUSSION

Comparative morphological studies on a variety of insect species led to numerous theories concerning the segmental organization of the insect head, placing the number of segments as low as three and as high as seven (1–4). In the head of a Saltatoria, the camel cricket, seven "morphogenetic units" have been defined by ablation experiments (30). In

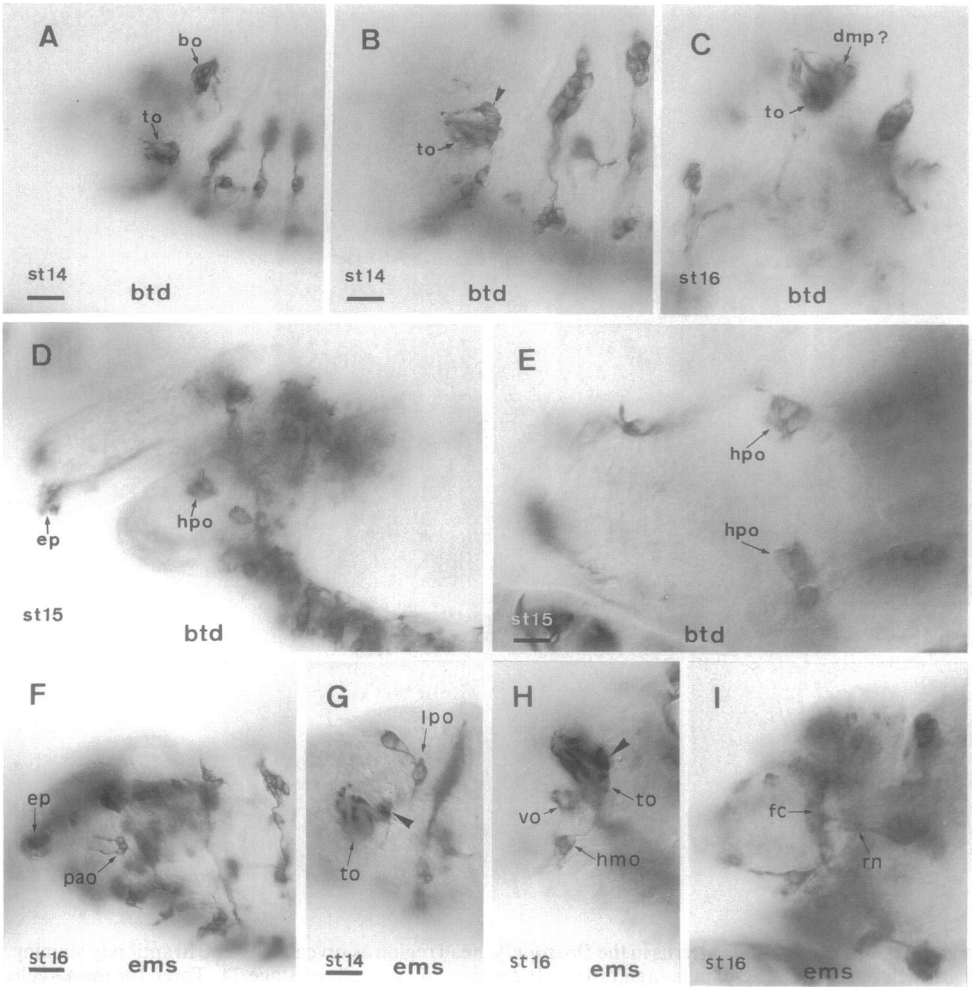


FIG. 3. Diagnostic head sensory organs in *btd* (*A–E*) and *ems* (*F–I*) mutant embryos. The to (*A–C*), bo (*A*), dmp (*C*), ep (*D*), and hpo (*D* and *E*) are shown between stages (st) 14 and 16 of head development in *btd* mutants. The ep and pao (*F*), the lpo (*G*), to (*G* and *H*), and vo and hmo (*H*) are shown to be present in *ems* mutants at the corresponding stages. Representative elements of the SNS—e.g., the frontal commissure (fc) and the recurrent nerve (rn) are labeled. They are present in *ems* (*I*) and *btd* mutants (not shown). The arrowheads in *B*, *G*, and *H* label the chordotonal organ with two scolopidia at the dorsoposterior side of the to. The examples shown here indicate the lack of distinct and overlapping sets of PNS/SNS elements in *btd* and *ems* mutants (see also text). Similar results obtained with the other head mutant embryos allowed us to identify seven metameric head units and to reveal the anterior-to-posterior order of the seven segments in the embryo (see text and summary in Fig. 4). For abbreviations see Figs. 1 and 2. [Bars represent 20 μm (*A* and *F*) and 13 μm (*B–E* and *G–I*).]

*Drosophila*, laser ablation experiments (5) and mutant analysis (6, 31) suggested six head segments and a nonsegmental acron corresponding to the optic region. The number of six head segments and their order had been challenged by the head patterns of segment polarity gene expression, which suggested a sequence of seven segments (7). Our analysis of the neural elements and their deletion in various head mutants are consistent with seven morphological units, which can be assigned to the three prominent gnathal and four pregnathal segments, placing the optic region to the second metamer (7) rather than to the nonsegmental acron (5).

Our finding does not settle the issue of how many segments contribute to the insect head, but it leaves the case open with respect to a final assignment of segment number and identity (6). The use of sensory organs and nerves in the head, which provide a rich repertoire of structures that can be scored in the various head segmentation gene mutants, provides evidence for seven rather than six segments. In fact, the case is set firmly if one accepts the argument that the number and order of these neural elements reflect the segmental organization of the head. We favor this argument for the following reason. There is no doubt that mandibulata evolutionarily derived from annelid-like ancestors and that primitive head development originated from a specialization of anterior trunk segments at an early step of mandibulata phylogeny (4). In the trunk, the same metameric cues which establish the distinct epidermal segment pattern also generate the neuromers as well as the segmentally arranged elements and nerves of the PNS. Provided that the metameric cues act conventionally in both the trunk and the head region, the estimate of seven head segments by PNS analysis would close an almost "endless dispute" (2) concerning the number and sequence of head segments in insects.

The recent criteria used to establish six head segments were based on cuticular structures, including appendage primordia and cuticular sense organs of the *Drosophila* embryo (6). For the reasons outlined above, we give more weight to the criteria used in the present study. They confirmed the previously established six segments and their identity. In addition, they suggest the presence of a fourth pregnathal segment, the ocular segment, placing it between the labral and the antennal segments. This assignment is consistent with the observation of the remnants of two pairs of coelomic cavities anterior to the antennal segment in several insect species and a crustacean (reviewed in refs. 1 and 7), and the two pairs of preantennal (labral, ocular) ganglia found in crustaceans (reviewed in refs. 1 and 32). The common number of four pregnathal head segments in both insects and crustaceans, although obscured by secondary fusions and rearrangements that occurred during the course of evolution, suggests that initially four metamers became included in the pregnathal head region of a common annelid-like ancestor (4). Thus, the finding of seven head segments in insects is also consistent with the view of a monophyletic evolutionary tree for the Mandibulata, which include insects, myriapods, and crustaceans.

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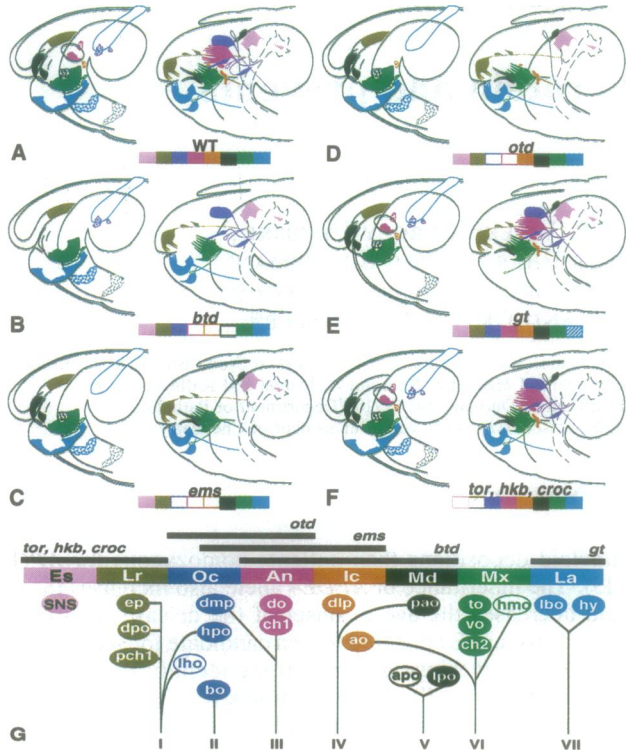


FIG. 4. Number, identity, and sequence of the head segments in *Drosophila*. *en* expression patterns (*Left*) and the PNS/SNS elements (*Right*) are shown in each panel. (*A*) Wild-type embryos. (*B–F*) Mutant embryos (exemplified in the case of the *btd* and *ems* mutants in Fig. 3). Colored bars represent head segments which are present (filled boxes) or absent (empty boxes) in the different mutant embryos (7, 19–26). (*B*) In $btd^{XG}$ the antennal, intercalary, and mandibular segments are deleted as revealed by the absence of the corresponding *en* spots in the head (left embryo) and sense organs (right embryo) (see also Fig. 3 *A–E*). (*C*) In *ems* the ocular, antennal, and intercalary segments are deleted (see mutant *en* pattern and bars below); the optic lobes are reduced as shown by crossing the enhancer trap line A6-2-45 (28) with the various mutants (not shown). Note that the ocular segment is absent from *ems*, the antennal and intercalary segments are absent from both *ems* and *btd*, and the mandibular segments are absent from *btd*. The sets of sensory organs deleted in *btd* and *ems* mutants show the corresponding overlap: bo, dmp, and hpo are deleted in *ems*; do, dlp, and ao, in both *ems* and *btd*; and pao, apo, and lpo, in *otd*. Thus, the bo, dmp, and hpo are derivatives of the ocular segment (purple); the pao, apo, and lpo are derivatives of the mandibular segment (black); and do, dlp, and ao are derivatives of the antennal–intercalary region (red and orange). (*D*) In *otd* mutant embryos, the ocular and antennal segments are deleted (left embryo) and the optic lobes are strongly reduced (data not shown; cf. *C*). The deletion of bo, dmp, and hpo confirms the allocation of these organs to the ocular segment. In addition, the do is absent, whereas the dlp and ao are present. Thus, the do is a derivative of the antennal segment (red) while the dlp and the ao arise from the intercalary segment (orange). (*E*) In *gt* mutant embryos, the labial segment is affected (stippled bar)—i.e., *en* labial expression is absent but the *en* stripe in the dorsal ridge (only outlined) is present (left embryo). Since the hy and lbo are deleted, they are derivatives of the labial segment (blue). By exclusion, the to and vo can be assigned to the maxillary segment (green). (*F*) Three mutants (*tor*, *hkb*, and *croc*) strongly affect or delete the clypeolabral expression of *en* and the SNS and organs ep, dpo, and pch1 fasciculating into SNI. The SNS derives from the stomodeum, which also gives rise to the esophagus (pink). The stomodeum is the most anterior ectodermal derivative, located just behind the anterior midgut in the blastoderm fate map (25). Since only the labral segment (brown) is affected in *tor*, *hkb*, and *croc* mutants, it must represent the most anterior segment. The deletion patterns of the other mutants indicate that the labrum is followed by the ocular, antennal, intercalary, mandibular, maxillary, and labial segments (see *G*). Note that in some cases structures or markers were strongly reduced but not completely deleted—e.g., the *en* antennal spot in the CNS persists sometimes in *ems* mutant embryos; remnants of the labral nerve and SNS can be observed in *hkb* and *croc* mutant embryos; the *en* expression in the CL of *hkb* mutant embryos is only partly deleted; and remnants can be found in *croc* mutants. The medially fused optic lobes in *tor* mutants (data not shown) are presumably due to deranged morphogenetic movements (29). (*G*) Summary of the mutant analysis. Black bars represent segments affected in the different mutant embryos, the anterior-to-posterior sequence (anterior is left) of the seven segments on the blastoderm fate map is shown below, and sensory organs (circles in color code) are assigned to particular segments. The segmental assignments of lho, hmo, and apo are only tentative as they could not be scored in mutants with high fidelity; lho are probably present in *btd* and *ems* but not in *otd* mutant embryos (data not shown). *otd* mutant embryos lack both *en* and *wg* ocular spots, whereas only the *en* ocular spot is affected in *ems* (6). Therefore, lho is tentatively located in the anterior portion of the ocular segment. For hmo and apo, the tentative segmental assignment is based on the fasciculation pattern of SNV and SNVI (roman numbers; see Fig. 2 for details). Abbreviations in addition to those described in Fig. 1: Es, esophagus; and the following segments: Lr, labral; Oc, ocular; An, antennal; Ic, intercalary; Md, mandibular; Mx, maxillary; and La, labial.


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