The segmental pattern of otx, gbx, and Hox genes in the annelid
Platynereis dumerilii

Patrick R. H. Steinmetza,a,1 Roman P. Kostyuchenkob, Antje Fischer,a and Detlev Arendta,

aDevelopmental Biology Unit, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69012 Heidelberg, Germany
bDepartment of Embryology, State University of St. Petersburg, Universitetskaya nab. 7/9, 199034 St. Petersburg, Russia

Author for correspondence (email: detlev.arendt@embl.de)

1Present address: Department for Molecular Evolution and Development, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.

SUMMARY Annelids and arthropods, despite their distinct classification as Lophotrochozoa and Ecdysozoa, present a morphologically similar, segmented body plan. To elucidate the evolution of segmentation and, ultimately, to align segments across remote phyla, we undertook a refined expression analysis to precisely register the expression of conserved regionalization genes with morphological boundaries and segmental units in the marine annelid Platynereis dumerilii. We find that Pdu-otx defines a brain region anterior to the first discernable segmental entity that is delineated by a stripe of engrailed-expressing cells. The first segment is a “cryptic” segment that lacks chaetae and parapodia. This and the subsequent three chaetigerous larval segments harbor the anterior expression boundary of gbx, hox1, hox4, and lox5 genes, respectively. This molecular segmental topography matches the segmental pattern of otx, gbx, and Hox gene expression in arthropods. Our data thus support the view that an ancestral ground pattern of segmental identities existed in the trunk of the last common protostome ancestor that was lost or modified in protostomes lacking overt segmentation.

INTRODUCTION

Arthropod and annelid brains are morphologically similar with anterior supra-esophageal ganglia connected to a segmental chain of ventral ganglia via circum-esophageal connectives (Bullock and Horridge 1965). This shared morphology had traditionally been interpreted as obvious homology and was used as strong argument in favor of common descent of annelids and arthropods from segmented common ancestors (Articulata) (Cuvier 1817; Siewing 1985). However, the homology of annelid and arthropod segments has been called into question by the “New Animal Phylogeny” that places arthropods and annelids into distinct protostome super-phyla (Ecdysozoa and Lophotrochozoa), together with a majority of nonsegmented groups (Adoutte et al. 2000). Convergent evolution of a segmented nervous system in annelids and arthropods has accordingly been considered a more parsimonious scenario than the multiple secondary loss of head segmentation (Davis and Patel 1999; Seaver 2003).

Recent molecular studies have elucidated different modes of segment formation in anterior and posterior segments in various animal groups. The Delta/Notch/Hairy oscillating system plays a role in vertebrate somite formation and in segmentation from the posterior growth zone in spiders and cockroaches; yet it is not involved in the formation of anterior-most segments in the same species, which are presumably defined by a gap gene-driven mechanism (Stollewerk et al. 2003; Tautz 2004; Dequéant and Pourquié 2008; Pueyo et al. 2008). In insects, myriapods, crustaceans, pycnogonids (sea spiders), chelicerates, and onychophorans, the three anterior most segments are homologized based on conserved anterior Hox expression boundaries (hox1/labial in the second and hox4/deformed in the third segment) (Hughes and Kaufman 2002b; Jager et al. 2006; Eriksson et al. 2010) (Fig. 1A). Also, it is reasonable to assume that the anterior expression boundary of fushi-tarazu/lox5 in the fourth segment, as found at least during some stages of development in the chelicerates Archegozetes and Cupiennius, the centipede Lithobius, the millipede Glomeris and the crustacean Daphnia, represents the ancestral arthropod state (Telford 2000; Hughes and Kaufman 2002a). However, the complete change of function to a pair-rule gene in insects and the dynamic anterior boundary in some of the investigated arthropods (CNS expression in the third segment of Cupiennius; retraction to the fifth segment during later development in Daphnia) indicate that ftz is less conserved as a regional marker gene and thus less useful for interphyletic comparison than the highly conserved hox1 and hox4. Beyond that, the anterior expression boundary of gbx/unplugged appears to posteriorly abut otx in chordates,
hemichordates, polychaetes, and Drosophila (Rhinn and Brand 2001; Hirth et al. 2003; Lowe et al. 2003; Steinmetz et al. 2007; Urbach 2007). However, the limited data set available for other arthropods (especially for gbx) does not yet allow firm conclusions to what extent this pattern can be generalized across arthropods. Thus, taken with caution in the cases of gbx/unplugged and fz, the set of conserved anterior and posterior expression boundaries of orthologous homeobox genes constitutes a conserved molecular topography, an “arthropod ground plan” of segment identities (Fig. 1A) that can be used as a basis for comparing segment identities between arthropods and annelids.

Previous studies revealed only few molecular similarities between annelid and arthropod segment formation. In the phyllodocid annelid Platynereis dumerilii (Prud’homme et al. 2003), but not in other annelids investigated (Patel et al. 1989; Wedeen and Weisblat 1991; Seaver et al. 2001; Seaver and Kaneshige 2006), orthologs of the arthropod segment polarity genes wingless (wg) and engrailed (en) demarcate early forming segment boundaries. In addition, recent observations show that in Platynereis and insects, but so far not observed in other annelids (Kang et al. 2003; Seaver and Kaneshige 2006), the Hedgehog signaling system is required for the establishment and maintenance of segmental morphology supporting homology of segmentation at least within protostomes (Dray et al. 2010). Remarkably, however, the annelid pattern of en and wg is out of register by half a segment with the arthropod pattern, in that adjacent wg and en stripes demarcate segment boundaries in the annelid as opposed to parasegmental boundaries in the arthropods (located between the later-appearing segment boundaries; Fig. 1A) (Damen 2002b; Prud’homme et al. 2003). As in arthropods, an anterior-to-posterior sequence of otx + (Bruce and Shankland 1998; Arendt et al. 2001), gbx + (Steinmetz et al. 2007) and Hox + regions (Kourakis et al. 1997; Irvine and Martindale 2000; Kulakova et al. 2007; Fröbius et al. 2008) appears to convey regional identities to the series of anterior segments in the annelid embryo and larva, as inferred from various expression studies. However, while the expression of Pdu-otx appears to be presegmental and that of the Hox genes staggered in the segmental region, a precise alignment of these molecular regions with early developing segmental boundaries has not been undertaken. The matter is further complicated by the unclear affiliation of the “tentacular” cirri which are head appendages present directly anterior of the first chaetigerous segment in Platynereis (Hempelmann 1911) and many other phyllodocids (Pleijel and Dahlgren 1998; Rouse and Pleijel 2001). It is unclear if they represent a first, incomplete or “cryptic” segment (Hempelmann 1911) similar to the reduced head segments in other polychaetes (Rouse and Pleijel 2001), or a nonsegmental part of the peristomium (Schroeder and Hermans 1975). Does this “cryptic” segment indeed exist and how do the anterior and posterior expression boundaries of otx, gbx, and Hox-genes relate to the first molecular and morphological manifestations of segmentation in an annelid? How does this pattern compare with the arthropod ground pattern?

Here, by reassessing engrailed expression as marker for anterior segment boundaries (Prud’homme et al. 2003) and by analysis of early segmental blocks of musculature, we corroborate the existence of a first, most anterior, “cryptic” larval segment in the Platynereis larva. By double whole-mount in situ hybridization (Tessmar-Raible et al. 2005) we spatially correlate the stripes of en expression (and thus, the prospective boundaries of all larval segments) with the anterior expression boundaries of Hox and gbx genes. We show that the first larval segment expresses gbx, directly abutting the otx + peristomium, and spatially correlate the anterior expression...
boundaries of *hox1*, *hox4*, and *lox5* with the second, third, and fourth larval segment. Overall, this arrangement matches the arthropod ground pattern. Furthermore, the first, *gbx* + *Platynereis* segment and its putative homologous arthropod parasegment share the absence of a commissure and a ganglion located on the circum-esophageal connectives while all subsequent (para-) segments have segmental ganglia with commissures. Our results indicate homology of the anterior most four (para-) segments in annelids and arthropods, which would imply the frequent loss of this pattern concomitant with segmentation in many protostome groups.

**MATERIAL AND METHODS**

**Animal culture**

*Platynereis* embryos and larvae were obtained from an established breeding culture at the EMBL Heidelberg.

**Labeling of cellular outlines**

Trochophore larvae were incubated for 15 min in 5 μM BODIPY564/570 coupled to propionic acid (Invitrogen, San Diego, CA, USA) in natural sea water, then rinsed twice in natural sea water and mounted in a 1:1 mixture of natural sea water and 7.5% MgCl2 to prevent muscular contractions.

**Wholemount in situ hybridization and immunohistochemistry**

Larvae were fixed in 4% paraformaldehyde/1.75% PBS Tween-20 for 2 h. Single color and double fluorescent wholemount in situ hybridizations followed established protocols (Tessmar-Raible et al. 2005). For immunohistochemistry, a monoclonal antibody raised against acetylated α-tubulin (1:250; SigmaT6793, Sigma, St. Louis, MO, USA) was used.

**Phalloidin labeling**

Embryos were fixed in 4% PFA in PBS+0.1% Tween-20 (PTW), for 50 min at room temperature, rinsed in PTW 2 × 20 min and stored in PTW at 4°C up to maximum 7 days. The larvae were Proteinase K-digested and postfixed as described previously (Tessmar-Raible et al. 2005). The larvae were incubated 1–3 nights shaking at 4°C in rhodamine–phalloidin (Molecular Probes) 1:100. Afterwards the larvae were washed 3 × 10 min and 3 to 5 × 30 min in PTW and stored in 87% glycerol containing 2.5 mg/ml of anti-photobleaching reagent DABCO (Sigma) at 4°C. Larvae were mounted between a slide and a cover slip, separated by three layers of adhesive tape.

**Confocal microscopy and image analysis**

Single confocal images were taken on a Leica TCS SPE (Leica Microsystems CMS, Mannheim, Germany) with a × 40 oil immersion objective at 0.5–1 μm intervals. Surface rendering and 3D modeling of muscle structures were performed using Imaris (Bitplane) and colorized in Photoshop (Adobe). All other 3D reconstructions were done using ImageJ.

**RESULTS AND DISCUSSION**

The molecular comparison of arthropods and annelid segments had so far been mainly hampered by the difficulty to unequivocally assign regional marker gene expression to early developing segments and regions in annelids. Here, we reassess the previously described *engrailed* gene (Prud’homm et al. 2003) as an anterior segment boundary marker to precisely correlate homeobox gene expression borders before the occurrence of morphological segment boundaries in *Platynereis*. Anterior to the previously described three ectodermal rows of *engrailed* + cells that extend from the ventral midline to the dorso-lateral appendage-forming regions (Prud’homm et al. 2003), we identify an additional, shorter row that persists through larval development, visible from 24 hpf onwards (Fig. 2, A–F, and H). Two-color double in situ hybridization shows that this anteriormost *engrailed* + cell row posteriorly abuts the *otx* + peristomium at the anterior border of the trunk ventral nerve cord anlage (Fig. 3, A–D). Between 24 and 32 hpf, a fifth row of *en* + cells appears in the prospective growth zone of the pygidial region (Fig. 2, C–F, arrow). Between 36 and 48 hpf (when mediolateral patterning occurs and neural differentiation starts), the *engrailed* + rows disintegrate in the medial neuroectoderm (Fig. 2, C–F), similar to the medial portion of cell rows positive for some NK homeobox genes that likewise show segmental expression at earlier stages (Saudemont et al. 2008). During the same period, segmental structures such as chaetal sacs (Fig. 2E) or ciliary bands (Fig. 2H, marked by α-tubulin in situ hybridization) become morphologically apparent on the lateral side (Steinmetz et al. 2007), critically depending on Hedgehog signaling (Dray et al. 2010). Consistent with previous reports, we show by double in situ hybridization that the persisting lateral parts of the *engrailed* + rows are positioned at the anterior segment boundary abutting the ciliated band-marker α-tubulin at posterior segment boundaries (Fig. 2H).

These findings depict an apparent discrepancy between morphological and molecular segmental markers; we observe four rows of *engrailed* + cells as opposed to only three pairs of ciliated bands and parapodia. Likewise, four *nk4/tinman*-expressing rows of cells were reported previously (Saudemont et al. 2008). To solve this mismatch, we have inspected larval morphology more closely and observe a distinct, chaetal-sac-like anlage anterior to that of the first chaetigerous, “real” larval segment at 52 hpf (Fig. 2G, “I”). At about 5 days of development, an anterior pair of tentacular cirri develops in the corresponding region that is innervated by axons projecting onto the axonal bundles left and right of the mouth (circum-esophageal connectives) without forming a commissure (Fig. 4, A–F) (Gilpin-Brown 1958). These cirri resemble the second, posterior pair of tentacular cirri that form from the first chaetigerous segment during metamor-
phosis (Fig. 4B), when this segment loses its chaetae in the process of cephalization (Hempelmann 1911). Also, muscle stainings with rhodamine-phalloidin reveal that after 5 days of development, the anterior tentacular cirri region exhibits a muscle organization similar to subsequent chaetigerous segments (Fig. 4, G–J). Although fewer in number, the anterior...

Fig. 2. Single (A–F) and two-color (H) in situ hybridization of the Platynereis anterior segment boundary marker Pdu-engrailed (A–F, H) and the ciliary marker Pdu-α-tubulin (H) indicates the presence of four larval segments, also visualized by the fluorescent, cellular outline marker propionic acid-BODIPY564/570 (G). Lateral view tilted anteriorly (A–E) or ventral view (F–H). (G) Single confocal section. (H) 3D reconstruction of confocal sections. I–IV, larval segments; Arrowheads, *engrailed* rows at anterior larval segment boundaries; Arrows, *engrailed* row in the prospective posterior growth zone; Continuous line, position of the prototroch ciliated band; Dotted lines, segment boundaries; Dashed line in (G), outlines of the invaginated parapodial and tentacular cirri “sacs”; Crosses, the position of the parapodial sacs; Dashed line in (F), approximate border of the ventral plate neuroectoderm.
tentacular cirri muscles are arranged—as in chaetigerous segments—into pairs of ventral oblique muscles (Fig. 4, H–J, yellow and blue) and more medial appendage muscles (Fig. 4, H–J, orange). While the ventral oblique muscles are morphologically similar and thus directly comparable between first and subsequent larval segments (Fig. 4, H–J, yellow and blue), the v-shaped appendage muscles of the first larval segment differ from more complex parapodial appendage muscles of the subsequent segments, hampering the direct correlation of the v-shaped muscles reaching toward the cirri to a particular set of parapodial muscles (Fig. 4, H–J, orange).

These morphological observations together with the existence of a first engrailed stripe anterior to the first chaetigerous segment reveal the existence of a first, “cryptic” segment in the anterior tentacular cirri region, as proposed already a century ago (Hempelmann 1911). This notion is further supported by recent fate-mapping studies that show that the anterior pair of cirri does not originate from the otx1 peristomium (developing from the 2a, 2b, and 2c micromeres), but like all following metameric segments from the 2d micromere (Ackermann et al. 2005).

To establish the molecular identities of the now four Platynereis larval segments (one cryptic plus three chaetigerous segments), we spatially correlated the anterior boundaries of otx, gbx, hox1, hox4, and lox5 expression with the engrailed + anterior segment boundaries and the otx + peristomium by two-color double WMISH (Fig. 3). We found that the previously identified boundary between peristomial otx and gbx expression (Steinmetz et al. 2007) coincides with the anterior border of the “cryptic” first segment demarcated...
by the anterior most *engrailed* cell row (Fig. 3, A–F). In the segmented trunk, the anterior expression borders of *hox1* (Fig. 3, G and H), *hox4* (Fig. 3, I and J), and *lox5* (Fig. 3, K and L) register with the *Pdu-en* stripes of the second, third, and fourth larval segment, respectively. However, only the dorsal part of the *hox4* and the ventral part of the *lox5* anterior expression boundaries overlap *engrailed* and thus coincide with the annelid segment boundaries (Fig. 3, I–L). The remaining *hox4* and *lox5*, and the entire *gbx* and *hox1* anterior boundaries lie posteriorly adjacent of *engrailed* in the middle of the annelid segment (corresponding to the arthropod segment boundary). A comparison between *Platynereis* segment and arthropod parasegment identities thus reveals striking molecular and morphological similarities (summarized in Fig. 1): in both cases, an anterior most *gbx/unplugged* + and *hox1/labial*-negative (para-) segment lacking commissural axons is followed by a second *hox1/labial* +, a third *hox4/deformed* + and a fourth *lox5/fushi-tarazu* + (para-) segment. This prospective ancestral pattern is found highly conserved in chelicerates (Rogers and Kaufman 1996; Damen et al. 1998; Telford and Thomas 1998; Telford 2000; Damen 2002b) (where, however, *unplugged* expression is so far unknown) and is conserved in insects with the exception of *fushi-tarazu* that adopted a novel function as a pair-rule gene (Hughes and Kaufman 2002b). This comparison indicates evolutionary correspondence of the first antennal/cheliceral segment in arthropods with the first larval (cryptic) segment in the annelid (Fig. 1) bearing the first pair of tentacular cirri. This would suggest that the first insect antennae are homologous to the anterior pair of tentacular cirri in *Platynereis*, but not to annelid or onychophoran antennae, which both form from the most anterior *six3* + territory (Steinmetz et al. 2010). The *Platynereis otx/gbx* boundary lies, as in all bilaterians so far investigated, anterior of the Hox-expressing region

*Fig. 4.* Relative position (A, B), innervation (C–F) and musculature (G–J) of head appendages and anterior segments before (A, C–J) and after metamorphosis (B). (A) Seven days old; (B) 5 weeks old; (C–J) 5 days old *Platynereis* worms. (C, E) Reconstructions of confocal stacks of acetylated α-tubulin-immunostained animals visualizing the axonal scaffold and ciliary bands. (D, F) Schematics of animals in (C, E) highlighting the main axonal fibers. Frame in (E) marks approximately the region magnified in (C). Z-projection (G), 3D-reconstruction of confocal sections (H, I) and schematics (J) of rhodamine–phalloidin stained animals. Color code in (D, F): Red, cerebral ganglion axons; Pink, Circum-esophageal connective axons innervating the anterior tentacular cirri and the esophageal nervous system; Brown, Axons of the first chaetigerous and following segments. Color code in (H–J): Yellow, anterior oblique muscles; Blue, posterior oblique muscles; orange, “cirral” and “parapodial” muscles; background colors depicting segmental identities as in Fig. 1. (A, B) Dorsal views. (C, D, H) Ventral–lateral views. (E–G, I, J) ventral views. Scale bar: 40 μm. a, antennae; plp, palpae; peris, peristomium; prost, prostomium; am, antennal muscle; 1.pp, first pair of parapodia; 2.pp, second pair of parapodia; atc, anterior pair of tentacular cirri; ptc, posterior pair of tentacular cirri; I–IV, segments 1–4.
(Damen 2002a; Steinmetz et al. 2007). In Platynereis, it demarcates a fundamental morphological border between the head that originates from all four embryonic quadrants in a quadriradial-symmetrical manner, and the segmented trunk ectoderm (deriving entirely from the bilateral-symmetrically dividing 2d micromere) (Ackermann et al. 2005). So far, the ancestral position of the otx/gbx boundary in the frame of the developing arthropod segments is not clear due to the lack of comparative gbx expression data and missing otd/engrailed co-localization data for all arthropod groups except Drosophila (Hirth et al. 2003) and Tribolium (Li et al. 1996). However, the restriction of otd expression to the protocerebral regions of Tribolium (Li et al. 1996) and chelicerates (Telford and Thomas 1998; Simonnet et al. 2006) suggests an ancestral otx/gbx boundary at the anterior border of the first parasegment—comparable to the situation in Platynereis (Fig. 1). The position in Drosophila, where otd expression stretches into deutocerebral regions (Hirth et al. 2003; Urbach 2007), is so far not described for other arthropod species and appears secondarily modified. Further comparative co-expression studies will more clearly position the ancestral otx/gbx expression boundary in the developing arthropod brain.

We thus found high similarities between the Platynereis and the arthropod ground pattern of Hox expression. However, the Platynereis Hox expression data is less similar to other previously investigated annelids such as the polychaetes Capitella (Fröbius et al. 2008) and Chaetopterus (Irvine and Martindale 2000) or the leech Helobdella (Kourakis et al. 1997), possibly due to considerable tagmatization of the trunk (Chaetopterus, Capitella) (Irvine et al. 1999; Fröbius et al. 2008), or due to the secondary loss of a larval stage (Helobdella) in those species. An annelid ground pattern of Hox expression in the anterior segments is thus difficult to discern (Fröbius et al. 2008). Thus, the similarities between Platynereis and the arthropod ground pattern can be interpreted either as an extreme case of homoplaspy (i.e., ectodermal segmentation evolving twice independently in very similar spatial relationship to pre-existing regions), or as inheritance from a last common ancestor (and subsequent secondary modification in other groups). As the tagmatization of Chaetopterus, Capitella and of the leeches is derived within annelids, we tend to favor the latter scenario.

Yet, evolutionary conservation of the otx/gbx/Hox ground pattern in a specific segmental framework together with the conserved role in Hedgehog signaling in establishment of segmental morphology (Dray et al. 2010) would imply that the protostome ancestor already possessed segmented ectoderm that was subsequently lost in the lineages leading to today’s nonsegmented protostomes (e.g., nematodes, flatworms, and mollusks). The recent recognition of echiurans and sipunculans, nonsegmented at adult stages, as derived from segmented annelid ancestors shows that loss of segmentation might have occurred more often than assumed previously (Buchberger et al. 1996; McHugh 1997; Struck et al. 2007; Kristof et al. 2008). We propose that secondary loss of segmentation might be directly connected with the loss of the Hox ground pattern, as identified here. Notably, animals lacking all sign of segmentation, such as the nematode Capitella elegans (Aboubaker and Blaxter 2003), the abalone Haliotis asinina (Hinman et al. 2003), and the squid Euprymna scolopes (Lee et al. 2003), also lack all traces of these molecular regions (Graham et al. 1989; Holland et al. 1992; Simeone et al. 1992; Tallafuñ and Bally-Cuif 2002; Hughes and Kaufman 2002b; Hirth et al. 2003). Further comparative analysis of other segmented and nonsegmented ecdysozoans and lophotrochozoan groups, including the nonsegmented echiurans and sipunculans that likewise appear to be annelid-derived (Buchberger et al. 1996; McHugh 1997; Struck et al. 2007; Kristof et al. 2008), are needed to solve this issue.

Acknowledgment
This work was funded by a fellowship from the Luxembourg Ministry of Culture, Higher Education and Research to PRHS.

REFERENCES


Deterville, Paris.


Eriksson, B. J., Tait, N. N., Budd, G. E., Janssen, R., and Akam, M. 2010. Head patterning and Hox gene expression in an onychophoran and


