

Evolution of regeneration and fission in annelids: insights from *engrailed*- and *orthodenticle*-class gene expression

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SUMMARY

The recent explosion of information on the role of regulatory genes in embryogenesis provides an excellent opportunity to study how these genes participate in post-embryonic developmental processes. We present a detailed comparison of regulatory gene expression during regeneration and asexual reproduction (by fission) in the segmented worm *Pristina leidyi* (Annelida: Oligochaeta). We isolated three genes from *Pristina*, one homolog of *engrailed* and two homologs of *orthodenticle*, and characterized their expression in different developmental contexts. In situ hybridization studies on worms undergoing normal growth, regeneration and fission demonstrate that in all three processes, *Pl-en* is expressed primarily in the developing nervous system, and *Pl-Otx1*

and *Pl-Otx2* are expressed primarily in the anterior body wall, foregut and developing nervous system. Our data reveal extensive similarities between expression during regeneration and fission, consistent with the idea that similar developmental processes underlie these two types of development. Thus, we argue that in these annelids fission may have evolved by recruitment of regenerative processes. Furthermore, by comparing our data to existing data from leech embryos, we find evidence that embryonic processes are re-deployed during regeneration and fission.

Key words: Regeneration, Fission, Post-embryonic development, Annelid, Gene expression, Evolution, Homeobox gene, *orthodenticle*, *engrailed*, *Pristina leidyi*

INTRODUCTION

How can embryogenesis, regeneration and fission converge on a common endpoint (the adult morphology) from drastically different starting points (a one-celled egg versus part of a multicellular adult body)? Post-embryonic developmental phenomena such as regeneration and agametic reproduction (i.e. asexual reproduction not involving germ cells, such as budding and fission) add a fascinating dimension to the challenge of understanding animal development. Both embryogenesis and regeneration/agametic reproduction involve the establishment of body axes, regional localization and tissue differentiation. However, while embryogenesis is initiated by a single large cell, regeneration and agametic reproduction are initiated by numerous small cells acting in concert. In addition, while the egg cell is originally undifferentiated, cells that participate in regeneration/agametic reproduction can arise from differentiated tissues (Berrill, 1952; Goss, 1969; Ferretti and Géraudie, 1998; Sánchez Alvarado, 2000). Furthermore, while body axes and organ systems are established de novo in a developing embryo, body patterning during regeneration/agametic reproduction must respect the body axes already established in the mature tissues, and new parts of an organ system must become connected to the pre-existing parts in such a way as to form an integrated and functional whole.

Although virtually all animals are capable of embryonic development, the ability to regenerate or reproduce agametically varies widely (Vorontsova and Liosner, 1960; Brusca and Brusca, 1990), sometimes even between closely related species (Scadding, 1977; Bely, 1999). This highlights the fact that there has been extensive evolution of post-embryonic capabilities. Furthermore, many animals can regenerate but cannot reproduce agametically, indicating that these two phenomena are in some way distinct. Nevertheless, regeneration and agametic reproduction appear to be related: agametic reproduction has evolved primarily within groups capable of extensive regeneration (Vorontsova and Liosner, 1960), and all animals capable of agametic reproduction whose regenerative capabilities have been investigated can regenerate.

Although the established animal model systems of developmental biology have been extremely useful for investigating embryogenesis, most of these animals undergo determinate growth, have poor regenerative capabilities, and are not capable of agametic reproduction. This has left a striking gap between our understanding of embryonic and post-embryonic developmental processes. We have chosen to work on the segmented worm *Pristina leidyi* (Annelida: Oligochaeta: Naididae), which is capable of several different forms of post-embryonic development (Van Cleave, 1937). This tiny freshwater worm grows continuously as an adult, is capable of

regenerating a new head and tail, and routinely undergoes agametic reproduction by paratomic fission (Fig. 1), in which a new head and tail are intercalated in the middle of a worm's body. *Pristina* is not known to reproduce sexually under laboratory conditions. However, its embryos are very similar to those of its close relatives, the leeches, which have been the subject of numerous embryological studies (Irvine and Martindale, 1996; Shankland and Savage, 1997), but which cannot add segments as adults, regenerate or reproduce agametically. *Pristina* therefore provides an exceptional opportunity to investigate relationships between embryogenesis, regeneration and agametic reproduction.

Developmental regulatory genes known to play important roles in establishing global and regional domains in metazoan embryos should be particularly informative in uncovering links between different forms of development. A small but growing number of studies have already demonstrated that such genes are often expressed during regeneration (e.g. amphibians – Gardiner et al., 1999; fish – Akimenko et al., 1995; Laforest et al., 1998; hydra – Bosch, 1998; planarians – Bagnù, 1998).

We now report the isolation of oligochaete homologs of two homeobox genes, *engrailed* (*en*) and *orthodenticle* (*otd/Otx*), and describe their expression patterns during adult growth, regeneration, and paratomic fission in *Pristina*. These genes were chosen because they play crucial and putatively conserved roles in neurogenesis (*en*, *Otx*), segmentation (*en*), and head patterning (*Otx*) in several phyla (Finkelstein et al., 1990; Simeone et al., 1993; Patel, 1994; Wada et al., 1996; Holland et al., 1997; Bruce and Shankland, 1998; Duman-Scheel and Patel, 1999). We compare gene expression patterns between fission and regeneration to test the hypothesis that the evolution of fission in *Pristina* involved recruitment of regenerative processes. Furthermore, to investigate the possibility that embryonic developmental pathways are redeployed during post-embryonic development, we compare our gene expression data for regeneration and fission with published data for leech embryos.

MATERIALS AND METHODS

Culturing *Pristina leidyi*

Pristina worms were obtained from Carolina Biological Supply (misidentified as the naidid *Stylaria*) and identified using the key of Kathman and Brinkhurst (Kathman and Brinkhurst, 1998). Adult worms (typically <8 mm in length) were cultured at room temperature in spring water with brown paper towel as substrate, and fed a mixture of green algae and diatoms. Under these conditions, worms grow continuously and reproduce by paratomic fission every few days (Fig. 1).

Regeneration studies

Worms were anesthetized in 5% ethanol in spring water and cut with a scalpel under a dissecting microscope. To permit the most direct comparisons between fission and regeneration, for anterior regeneration studies worms were decapitated at segment boundary VII/VIII (see Brinkhurst and Jamieson, 1971 for segment nomenclature), removing exactly those structures normally produced anteriorly during fission. As the presence of fission zones in regenerating worms could interfere with the posterior regeneration process (Galloway, 1899), worms were decidualized for posterior regeneration studies by cutting two segments in front of the anterior-most fission zone (corresponding to a cut ranging from segment boundary XI/XII to XVI/XVII), or at segment boundary XIV/XV if

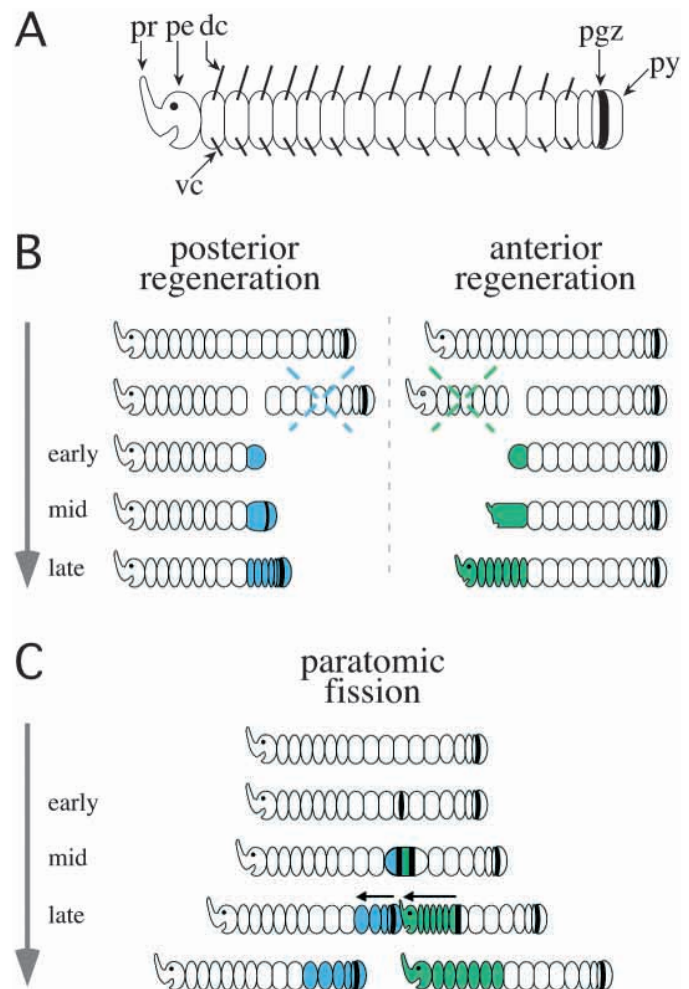


Fig. 1. Regeneration and paratomic fission in *Pristina leidyi*. Anterior is towards the left in this and all other figures. (A) Adult worms grow continuously by adding new segments from a subterminal posterior growth zone (pgz). Trunk segments bear bristle-like projections called chaetae (dc, dorsal chaetae; vc, ventral chaetae), which occur in paired dorsolateral and ventrolateral bundles roughly in the middle of each segment. The prostomium (pr) is the asegmental tissue in front of the mouth (greatly elongated in *Pristina*), the peristomium (pe) is the asegmental tissue around the mouth and the pygidium (py) is the posterior asegmental cap of tissue. (B) After posterior or anterior amputation, worms wound-heal, form a blastema (an undifferentiated mass of cells), and replace the missing structures through regeneration. (C) During paratomic fission, a worm forms a zone of cell proliferation (a fission zone) in the middle of its body. This zone splits into two proliferative zones, each forming new tissues anteriorly. A new head and tail are thus intercalated in the middle of the original worm's body, forming a transiently linked chain of worms. Multiple fission zones may be present in a worm: younger fission zones form in progressively more anterior segments (see Fig. 9). In growth, regeneration and paratomic fission, new segments are added in an anterior to posterior direction. Blue, new posterior tissue; green, new anterior tissue; black, regions of cell proliferation (fission zone or posterior growth zone).

no fission zone was visible. After amputation, worms were cultured individually at room temperature in spring water. After 2 days, posteriorly amputated worms were fed, as this was found to accelerate the posterior regeneration process.

Worms were fixed (see below) at multiple time points during regeneration, beginning 12 hours after amputation and then daily over the course of 5 days, by which time both anterior and posterior regeneration were normally complete. Because regeneration rate varied considerably between individuals, we refer to the stage of regeneration (early, mid, late, see Fig. 1) rather than the exact time point.

Isolation and sequencing of *engrailed* and *orthodenticle* homologs

To isolate *en*- or *Otx*-class genes, small fragments (including part of the homeobox) were amplified from genomic DNA through two rounds of PCR using degenerate primers. Primers were EN-A⁺ and EN-D⁻ for the first *en* PCR, EN-B⁺ and EN-C⁻ for the second *en* PCR, OTX-A⁺ and OTX-B⁻ for the first *Otx* PCR, and OTX-C⁺ and OTX-B⁻ for the second *Otx* PCR. Primer sequences (written 5' to 3', followed by corresponding amino acids) were:

EN-A⁺: GATGARAARMGICCMGIAC (DEKRPR)
 EN-D⁻: TGRTRTAIARICCYTGIGC (AQGLYNH)
 EN-B⁺: GARAARMGTCCIMGIACIGC (EKPRPTA)
 EN-C⁻: TGTGCCATIARYTGIARIGC (ALQLMAQ)
 OTX-A⁺: MGTAARCAARMGTMGIGARMGIAC (RKQRRERT)
 OTX-B⁻: TGAACCTCKGAYTCTGGIARATT (NLPESTVQ)
 OTX-C⁺: ACGACTTTYACKMGIRCICA (TTFTR[T/A/Q])

For the first PCR, 30 cycles were performed at 60°C to 45°C (temperature decreased 0.5°C/cycle) annealing, followed by 20 cycles at 50°C annealing. For the second PCR, 35 cycles were performed at 50°C annealing.

To obtain additional sequences, we performed 3'RACE for all three genes isolated by degenerate PCR (*Pl-en*, *Pl-Otx1*, *Pl-Otx2*, see Results), as well as 5'RACE for *Pl-en*. For 3'RACE, total RNA (isolated from ~50 mg of actively fissioning worms, using RNazol [Tel-Test]) was reverse transcribed according to Frohman (Frohman, 1990). For 3'RACE, gene-specific primers for the first and second PCR, respectively, were EN-F⁺ and EN-H⁺ for *Pl-en*, OTX-D⁺ and OTX-E⁺ for *Pl-Otx1*, and OTX-M⁺ and OTX-N⁺ for *Pl-Otx2*:

EN-F⁺: ACAGCCGGGCAACTCGAACG (TAGQLER)
 EN-H⁺: CGACAGCAAATACCTCACCG (DYSKYLTE)
 OTX-D⁺: TACTCGAGACGCTGTTCC (VLETLFH)
 OTX-E⁺: AAGACTCGTACCAGAC (KTRYPD)
 OTX-M⁺: AAAACGCGGTATCCGGAC (KTRYPD)
 OTX-N⁺: ACATATTCACCCGAGAGG (DIFTREE)

For 5'RACE of *Pl-en*, total RNA was reverse transcribed (5'RACE System, GIBCO BRL) using EN-L⁻, and the gene-specific primers for the first and second PCRs were, respectively, EN-M⁻ and EN-N⁻:

EN-L⁻: AACCCGACGAATGATTG (NHSSG)
 EN-M⁻: AGCCCTGAGCCATCAACTG (QLMAQG)
 EN-N⁻: AAGGGCTAGTTGGTTTC (RNQLAL)

Conditions and anchor primers for all RACE amplifications were those of Frohman (Frohman, 1990).

PCR products were cloned into pGEM-T (Promega) and sequenced using standard methods. We used NCBI BLAST searches (Altschul et al., 1990) to identify related sequences. Amino acid sequences of conserved gene regions (specifically, homeodomains and *en* domain EH5) were used to generate neighbor-joining trees using PAUP* (version 4.0b2, Swofford, 1999).

Whole-mount in situ hybridization

The cloned 3'RACE fragments of the *en* homolog (*Pl-en*) and the two *Otx* homologs (*Pl-Otx1* and *Pl-Otx2*) served as templates to synthesize sense and antisense digoxigenin-labeled riboprobes (Boehringer Mannheim). Probe lengths were *Pl-en*, 567 bp; *Pl-Otx1*, 2.2 kb; and *Pl-Otx2*, 2.1 kb.

We used an in situ hybridization protocol based on that of Nardelli-Haeffliger and Shankland (Nardelli-Haeffliger and Shankland, 1992) for leech embryos. Relevant specifications or deviations from the published protocol are as follows. Worms were relaxed 15 minutes in 10 mM MgCl₂/5 mM NaCl/1 mM KCl/8% ethanol prior to fixation; Pronase E digestion lasted 10 minutes; hybridization was carried out using ~0.6-1 ng/μl unhydrolyzed probe at 58°C (*Pl-en*) or 67°C (*Pl-Otx1/Otx2*); the post-hybridization RNase treatment was omitted; preabsorbed antibody was used at 1:2000 dilution; color reactions proceeded for 1-3 hours. Some worms were incubated in Hoechst 33258 (1 μg/ml) to stain nuclei. Specimens were mounted in 70-90% glycerol and photographed (with or without Nomarski optics) on a Zeiss Axioskop microscope using a Spot digital camera (Diagnostic Instruments) or on a Zeiss Axiophot microscope using 35 mm slide film.

RESULTS

Pl-en: an *engrailed* homolog from the oligochaete *Pristina*

The degenerate PCR fragment (236 bp), the 3'RACE fragment (567 bp) and the 5'RACE fragment (242 bp) that we amplified from *Pl-en* together comprise 667 bp (GenBank Accession Number AF336055), including 410 bp of presumed coding sequence and 257 bp of presumed 3'UTR.

Pl-en is clearly a member of the *en*-class gene family: just downstream of the homeodomain, it possesses an amino acid motif (domain EH5) unique to and conserved across *en*-class genes from both protostomes and deuterostomes (Fig. 2) (Bürglin, 1994). In addition, phylogenetic analyses based on the homeodomain and domain EH5 place *Pl-en* within a poorly resolved but strongly supported (by bootstrap analysis) *en* clade (data not shown), and a BLASTP search identifies *ht-en*,

	homeodomain (EH4)	domain EH5
<i>Pl-en</i> (oligochaete)	EKRPRTSFTAGQLERLKRFFDSSKYLTEERRQSLARELSLNESQIKIWFQNKRAKMKKAS (100%)	GVRNQLALQLMAQGLYNHS
<i>ht-en</i> (leech)A..GD..A.....SEN.....Q..TC..K..N..... (80%)	..K.....
<i>CT-en</i> (polychaete)ND..Q...E..ECNR.....D...T..N.....I..S.. (75%)T.....
<i>Ilyanassa en-a</i> (snail)SE..S.....ECR.....T..RH..A..G..T.....I..S.. (73%)	..K.E..M.....
<i>smox-2</i> (trematode)	L.....VP..K..SQ...EKNR...DEL..KK..T..D.R...V.....T..... (68%)	AQ.C...H...E.....
<i>en</i> (fruitfly)A.SSE..A.....NENR.....R...Q..SS..G..A.....I..ST (70%)	..SK.P.....T
<i>ARTen</i> (artemia)A...E..S...H..NENR.....R...D.....G.H.N.....N...L..S.. (73%)	..QK.P.....
<i>AmphiEn</i> (amphioxus)A..SE..Q...K..QENR.....Q..D.....K.....I..A (77%)G...H.....
<i>En-1</i> (mouse)	D.....A...E..Q...A..QANR..I..Q...T..Q.....I..T (75%)	..IK.G...H.....

Fig. 2. Homeodomain and domain EH5 of *en*-class genes. Percent amino acid identities to *Pl-en* are shown for the homeodomain in parentheses. Dots indicate residues identical to *Pl-en*. Sequences are from Wedeen et al. (Wedeen et al., 1991), Dick and Buss (Dick and Buss, 1994), Wray et al. (Wray et al., 1995), Webster and Mansour (Webster and Mansour, 1992), Poole et al. (Poole et al., 1985), Manzanares et al. (Manzanares et al., 1993), Holland et al. (Holland et al., 1997) and Logan et al. (Logan et al., 1992).

A

	homeodomain		
<i>Pl-Otx1</i> (oligochaete)	LDVLETLFHKTRYPDIFMREEVAMKINLPESRVQVWFKNRRAKCRQQQ	(100%)	(85%)
<i>Pl-Otx2</i> (oligochaete)	..C..S..Q.....T.....Q.....S.....	(85%)	(100%)
<i>Lox22-Otx</i> (leech)	QRRERTTFTRTQ.....Q.....	(98%)	(88%)
<i>Pd-Otx</i> (polychaete)A.....S.....Q.....L.....	(94%)	(88%)
<i>otd</i> (fruitfly)A.....A.....G.....L.....L.....	(92%)	(83%)
<i>DjotxA</i> (planarian)	T..D.....Q..EI..LH.E.N.....L.D.ISS.....E..KN	(71%)	(69%)
<i>Otx1</i> (mouse)S.....A.....A.....L.....	(94%)	(85%)
<i>CnOtx</i> (hydra)	R.....KA.....DM.G..M...V.....K...A.A.....F.RSR	(75%)	(71%)
<i>Crx</i> (mouse)S...EE..A..A..Q...VYA.....R	(81%)	(75%)
<i>paired</i> (fruitfly)	...C...SAS...E..RA.ER.Q...YT...L.QRT..T.A.I...S...RL.K.H	(58%)	(63%)
<i>goosecoid</i> (fruitfly)	K..H..I..EE..EQ..AT.D..H...VVL..QL.L.VD.K.E..E.....W.K.K	(58%)	(58%)
<i>otp</i> (mouse)	.K.H..R..PA..NE..RS.A..H.....L.LR.G.T.....Q...WKKRK	(65%)	(63%)

B

	carboxy-terminal region
<i>Otx1</i> (mouse)	SAWKLNFN SPDCLDYKDQ ---ASW---RFQVL*
<i>LjotxB</i> (lamprey)	SAWKLN FTHADCLDYKDQ ---NAW---KFQAL*
<i>Hroth</i> (ascidian)	GLNSYSNAG VDCLDYKDQ ---TPSW---KFQVL*
<i>SpOtx</i> (sea urchin)	HMPMGAM SSAECIDGKEQ ---PQW---KFQSL*
<i>Crx</i> (mouse)	GTWKF TYNMDFLDYKDQ ---SAW---KFQIL*
<i>Pl-Otx1</i> (oligochaete)	SAGSSLS SPVDCIDYKDAMI ANPW---SKFQNL*
<i>Pl-Otx2</i> (oligochaete)	GPRVNGLS PELVEDRKE -----W---YKFQAL*
<i>Lox22-Otx</i> (leech)	HPFN NETYNEFVGDNKE -----WVTT KFQAL *
<i>DjOtxA</i> (planarian)	SQSN DDNQKLYGDYK -----IETIT*
<i>DjOtxB</i> (planarian)	SGALS SNYS DPHTATWPL TMPT INKDFGQWTQ*
<i>otd</i> (fruitfly)	ITRI ITR INTRITTAII ISSNT IMMNSDR I *
<i>Tc Otd1</i> (beetle)	TGYSAM GMAAPH HQNF GPRHPP DCSMEFAN MA *
<i>Tc Otd2</i> (beetle)	MSHSQ FGN LE TSW GKSR DESS WFYNS GW ERK*

Fig. 3. Homeodomain and C-terminal region of *Otx*-class and related genes. (A) Upper sequences are *Otx*-class genes; lower sequences are closely related, non-*Otx*-class genes. Percent amino acid identities to *Pl-Otx1* (left column) and *Pl-Otx2* (right column) are given in parentheses. Dots indicate residues identical to the top reference sequence; the arrowhead points to the lysine characteristic of *Otx*-class genes. (B) Amino acid motifs shared between deuterostomes (top) and protostomes (bottom) are in bold; the conserved domain is boxed; broken lines represent alignment gaps; asterisks represent termination codons. Sequences are from Bruce and Shankland (Bruce and Shankland, 1998), Arendt et al. (Arendt et al., 2001), Finkelstein et al. (Finkelstein et al., 1990), Umesono et al. (Umesono et al., 1999), Simeone et al. (Simeone et al., 1993), Smith et al. (Smith et al., 1999), Furukawa et al. (Furukawa et al., 1997), Frigerio et al. (Frigerio et al., 1986), Goriely et al. (Goriely et al., 1996), Simeone et al. (Simeone et al., 1994), Ueki et al. (Ueki et al., 1998), Wada et al. (Wada et al., 1996) and Li et al. (Li et al., 1996).

the *en*-class gene from the leech *Helobdella triserialis* (Wedeen et al., 1991), as the most similar sequence to *Pl-en*.

Pl-Otx1 and *Pl-Otx2*: two orthodenticle paralogs from *Pristina*

Although only single *Otx* homologs have been identified from the leech *Helobdella triserialis* (Bruce and Shankland, 1998) and the polychaete *Platynereis dumerilii* (Arendt et al., 2001), we isolated two homologs from the oligochaete *Pristina leidy*: *Pl-Otx1* and *Pl-Otx2*. Using degenerate primers, we amplified two distinct 123 bp *Otx*-like homeodomain fragments (differing at 25 bp and 7 amino acids), and then isolated 3'RACE fragments (each ~2 kb) for both genes using primers specific to each gene copy. In the homeodomain, the two genes are only 85% identical at the amino acid level (72% at the nucleotide level; Fig. 3), and downstream of the homeodomain the two genes are so divergent that over most of this region they cannot be aligned reliably. Thus, it appears likely that these sequences represent two distinct *Otx* paralogs, rather than alleles of a single locus. Based on inferred amino acid translations, for each gene we have isolated 145 bp (of an expected 180 bp) of the homeodomain and the entire coding region downstream of the homeodomain (1220 bp for *Pl-Otx1*; 937 bp for *Pl-Otx2*), as well as some 3'UTR (~800 bp for *Pl-Otx1*, ~1000 bp for *Pl-Otx2*). These sequences have been deposited in GenBank (Accession Numbers AF336056 and AF336057).

Both *Pristina* genes are clearly members of the *Otx*-class gene family: their homeodomains possess a lysine at position 50 (Fig. 3), a residue that is unusual at this position but characteristic of *Otx*-class genes (Galliot et al., 1999), and phylogenetic analyses using the homeodomain place both genes within a poorly resolved but strongly supported (by

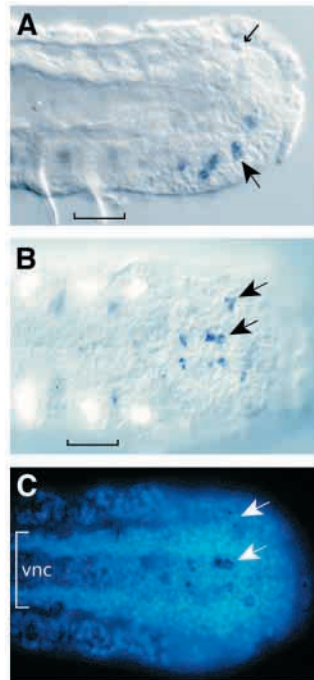
bootstrap analysis) *Otx* clade (data not shown). In addition, although most of the *Pl-Otx1* and *Pl-Otx2* post-homeodomain regions are highly divergent and cannot be confidently aligned to other *Otx*-homologs (or to each other), the C-terminal ends of both *Pristina* genes display a short conserved motif that is found in *Otx*-class genes from leech and diverse deuterostomes (Fig. 3); a subset of the motif is present in one of the two planarian homologs. This motif includes both the deuterostome 'tail motif' of Furukawa (Furukawa, 1997) and the terminal motif identified by Bruce and Shankland (Bruce and Shankland, 1998). Based on available sequence, neither *Pristina* paralog shows an obviously closer resemblance to the leech gene *Lox22-Otx*. Although *Lox22-Otx* is most similar to *Pl-Otx1* in the homeodomain, in the C-terminal region it is more similar to *Pl-Otx2*. Gene expression sheds no light on this matter, as the two genes display largely similar (though not identical) expression patterns (see below). Thus, orthology assignments among the annelid homologs must be postponed until additional data become available.

Expression of *Pl-en* during adult growth, regeneration and fission

In situ hybridization controls using the sense probe gave no staining, or at most a faint and diffuse haze in dense tissues, suggestive of nonspecific probe trapping. Using the antisense probe, *Pl-en* expression was detected primarily in newly formed tissues; in older segments, all segmental expression fades below detection levels. *Pl-en* expression patterns are very similar during adult growth, regeneration and fission.

In growing adults, *Pl-en* is expressed in several cells at the anterior limit of the pygidium (Fig. 4A). In the posterior growth zone and in young, recently formed, segments, *Pl-en* is expressed

Fig. 4. *Pl-en* expression during posterior growth. (A) lateral view; (B,C) ventral views. (A,B) In growing adults, *Pl-en* is expressed in dorsolateral cells (A, small arrow, positive cell slightly out of focus) near the anterior limit of the pygidium, as well as in bilaterally symmetrical ventral and ventrolateral cells (A,B, large arrows) of the posterior growth zone and young segments. (C) Visualization of nuclei of the specimen in B, as revealed by Hoechst 33258 staining, demonstrates that ventral *Pl-en* staining in young segments localizes primarily to the ventral nerve cord (C, white bracket). Nuclei of the ventral nerve cord form a 'U' in cross section; thus, in this ventral view, the outer margins of the nerve cord appear more densely nucleated. The black bracket in A,B marks a fully formed segment. Vertical scale bar: 50 μ m.



in scattered ventral and ventrolateral cells (Fig. 4A-C). These cells are unidentifiable in the growth zone, but in young segments, most localize to the ventral nerve cord (Fig. 4C).

During regeneration, *Pl-en* is first expressed in mid-stage blastemas in dorsolateral cells near the anterior margin of the new pygidium, and in scattered ventral cells (Fig. 5). These ventral cells form a salt-and-pepper pattern that is not obviously bilaterally symmetrical or iterated along the AP axis. This ventral expression is more extensive than that seen during adult growth, perhaps reflecting the fact that during regeneration, multiple segments can form nearly simultaneously from a blastema (whereas during adult growth, segments clearly form sequentially). During late stages of regeneration, once the new tissues are becoming visibly segmented, expression resembles that during adult growth (data not shown).

During fission, *Pl-en* is once again expressed in the same body regions as in regeneration and adult growth. Beginning in the early stages, the anterior pygidium expression is detectable just anterior to the constriction marking the plane of fission (i.e. in the new tail of the anterior worm; Fig. 6A). At mid and late stages of fission, as the tissues of the new head and tail become visibly segmented, *Pl-en* is transiently expressed ventrally and ventrolaterally (Fig. 6B,C), including in cells of the developing ventral nerve cord.

Expression of *Pl-Otx1* and *Pl-Otx2* during adult growth, regeneration and fission

The expression patterns of *Pl-Otx1* and *Pl-Otx2* are largely similar, and are therefore described together with qualitative differences mentioned only where pertinent. However, it should be noted that *Pl-Otx2* expression tended to be more intense and/or more extensive than *Pl-Otx1* expression, even though the two probes were of nearly identical size, and expression studies for the two

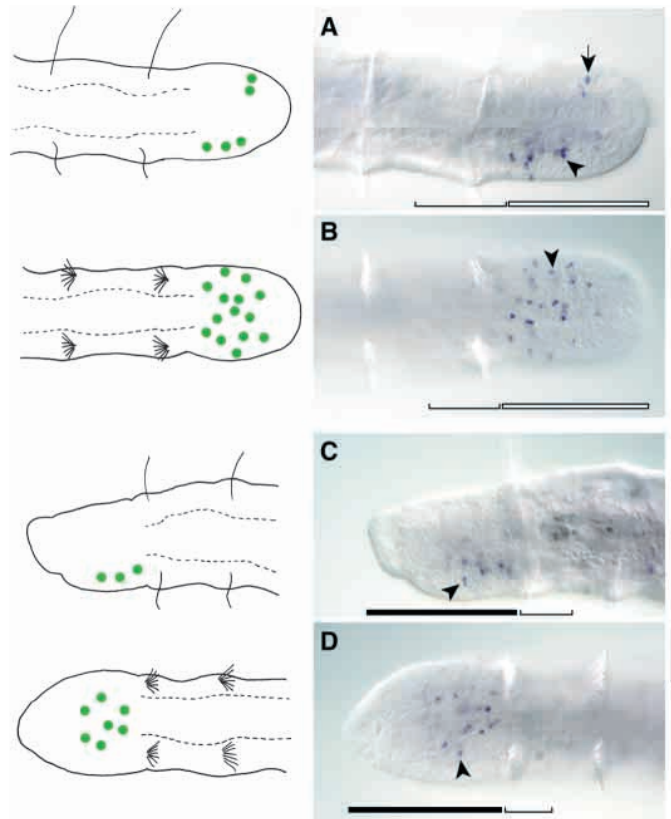


Fig. 5. *Pl-en* expression during posterior and anterior regeneration. (A,C) Lateral views; (B,D) ventral views. In diagrams of expression (left), two original (non-amputated) segments are included, broken line represents gut outline and color highlights the major expression domains. (A,B) Mid-stage posterior regeneration: *Pl-en* is expressed in a few cells (A, arrow) near the anterior limit of the regenerated pygidium, and in scattered ventral cells of the blastema (A,B, arrowheads) in regions where new segments are being formed. A is a composite of two images of the same specimen taken at slightly different focal planes. (C,D) Mid-stage anterior regeneration: *Pl-en* is similarly expressed in scattered ventral cells of the blastema. In this and all other figures, a white bar marks new posterior tissue and a black bar marks new anterior tissue. A bracket identifies the location of the original (non-regenerated) segment closest to the site of amputation. Vertical scale bar: 50 μ m.

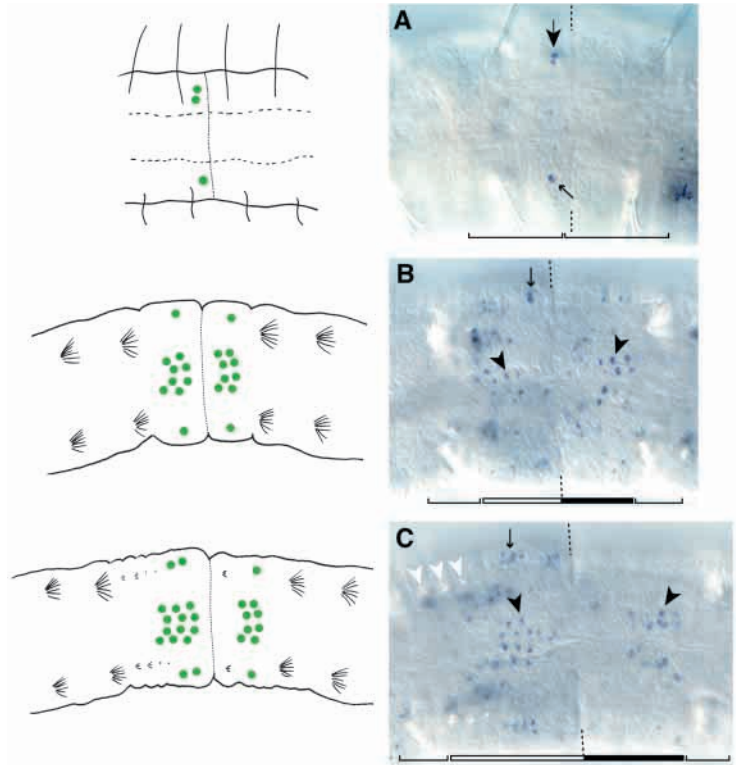
genes were always performed in parallel, on the same batches of fixed worms, and using comparable probe concentrations.

Sense controls of both genes produced no staining or, at most, a faint haze in dense tissues, as described for *Pl-en*. *Pl-Otx1/Otx2* are primarily expressed transiently in developing tissues, with expression fading below detection levels once tissues are fully formed. However, *Pl-Otx1* (but not *Pl-Otx2*) is also expressed in a single medial cell of the ventral ganglia of certain fully formed midbody segments (Fig. 7C).

During adult growth, *Pl-Otx1/Otx2* are expressed in scattered ventral and ventrolateral cells of the posterior growth zone and newly formed segments (Fig. 7A,B), including in cells of the developing ventral nerve cord.

During mid to late stages of both anterior and posterior regeneration, when the new tissues are becoming visibly segmented, *Pl-Otx1/Otx2* expression resembles that seen during adult growth (Fig. 8A,B,F). This is the only phase of expression

Fig. 6. *Pl-en* expression during fission. (A) lateral view; (B,C) ventral views. In diagrams of expression (left), two original segments are shown on either side of the fission zone. (A) *Pl-en* expression is first detected in young fission zones in dorsolateral (large arrow) and ventrolateral (small arrow) cells near the anterior margin of the developing pygidium. The positive dorsolateral cells persist through late stages of fission, becoming the anterior pygidium expression seen during posterior growth and regeneration. A is a composite of two images of the same specimen taken at slightly different focal planes. (B,C) During mid (B) and late (C) stages of fission, *Pl-en* is expressed in scattered ventral cells (black arrowheads) and ventrolateral cells (small arrow) of developing anterior and posterior segments, often in a recognizably segmentally iterated pattern. The ventral expression is localized primarily to the ventral nerve cord. Expression fades below detectable levels in older (more anterior) segments of the fission zone, once these have produced chaetae (white arrowheads). Brackets mark one original (parental) segment on either side of the fission zone. A broken line marks the fission plane, along which the anterior and posterior worms will eventually separate. Vertical scale bar: 50 μ m.



seen during posterior regeneration. In contrast, *Pl-Otx1/Otx2* are expressed extensively during early and mid stages of anterior regeneration, primarily in the body wall and foregut. When a small anterior blastema is formed, intense expression is detected near its anterior limit, in bilaterally symmetrical lateral crescents following the contour of the body wall (Fig. 8C,D). As this expression fades, a lateral cluster of unidentified cells transiently express *Pl-Otx1/Otx2* on either side of the blastema (Fig. 8E). At mid and late stages of regeneration, *Pl-Otx1/Otx2* are strongly expressed deep within the body in the regenerating foregut (Fig. 8E,F). Once the different regions of the foregut are distinguishable, expression of *Pl-Otx1/Otx2* localizes specifically to the pharynx. When regeneration is nearly complete (and sometimes in normal, fully formed heads), dorsolateral patches of staining cells occur just below the body wall surface (Fig. 8G), lateral to each lobe of the cerebral ganglion. Some naeidid species (though not *P. leidyi*) have pigmented eyespots in this location (Dehorne, 1916). As *Otx*-class genes are expressed in the eyes and/or associated nerve cells in a number of animals (Finkelstein et al., 1990; Simeone et al., 1993; Vandendries et al., 1996; Umesono et al., 1999), it is possible that *Pl-Otx1/Otx2*-expressing cells are associated with light-sensing organs, although studies of naeidids with eyespots will be needed to confirm this.

The spatial and temporal expression patterns of *Pl-Otx1/Otx2* during fission strongly resemble those during regeneration. During late stages of fission, *Pl-Otx1/Otx2* are expressed in the new head and tail, in ventral/ventrolateral cells of developing segments (Fig. 9D), primarily in the ventral nerve cord. In addition, during early and mid stages of fission, *Pl-Otx1/Otx2* are expressed extensively and exclusively in the developing head of the fission zone, primarily in the body wall and foregut. When a trunk segment first begins to form a fission

zone, *Pl-Otx1/Otx2* are intensely expressed in left and right lateral crescents which follow the contour of the body wall (Fig. 9A,B) in a manner strikingly similar to expression seen during early anterior regeneration (Fig. 8C,D). Once the fission zone develops a constriction that marks the fission plane, this expression is clearly limited to the anterior-most region of the

Fig. 7. *Pl-Otx2* expression during posterior growth and *Pl-Otx1* expression in fully formed mid-body segments.

(A) Lateral view; (B,C) ventral views. See text for explicit comparisons of *Pl-Otx1* and *Pl-Otx2* expression. (A,B) During growth, *Pl-Otx2* is expressed ventrally in young segments, primarily in the developing ventral nerve cord (arrows). (C) *Pl-Otx1* is expressed in a single, unpaired cell of the ventral ganglion of several contiguous midbody segments, typically beginning in segment VI (left arrow). A bracket marks a fully formed segment in each panel. Vertical scale bar: 50 μ m.

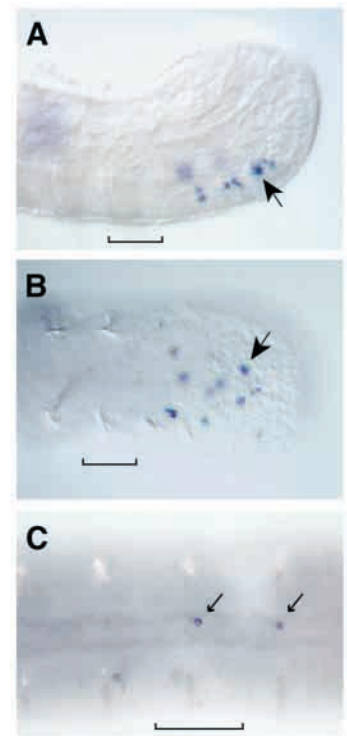
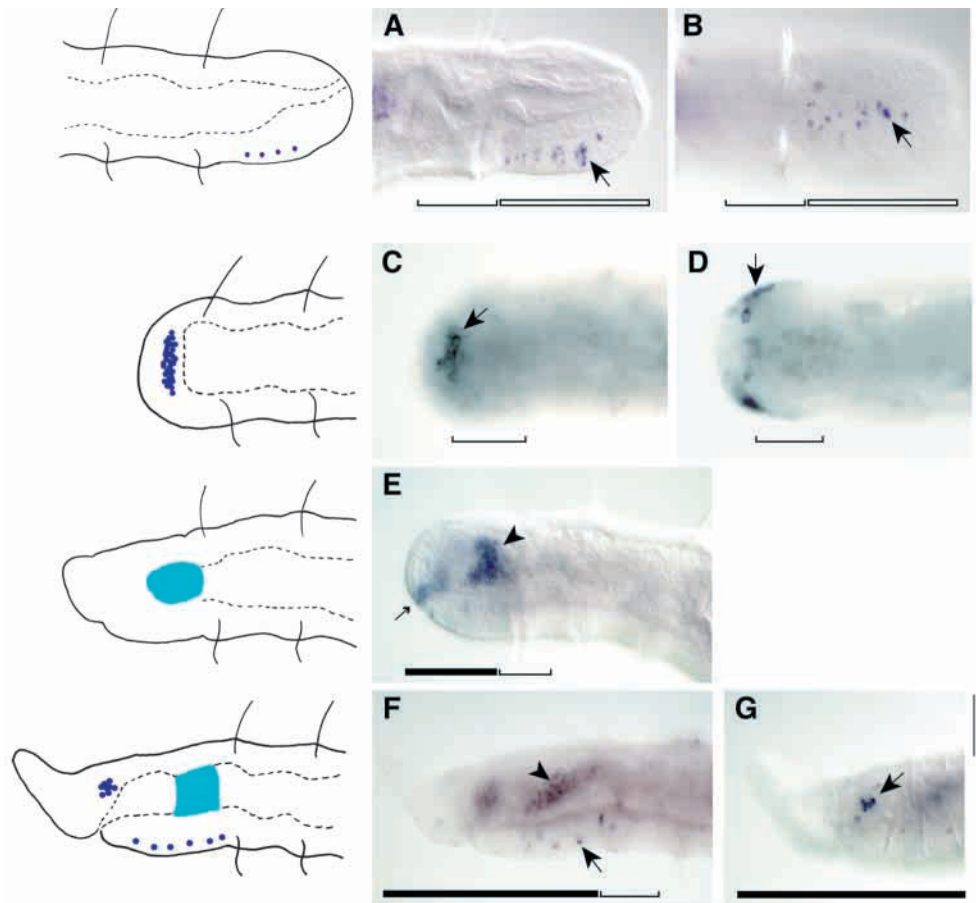


Fig. 8. *Pl-Otx2* expression during posterior and anterior regeneration. (A,C,E-G) Lateral views; (B) ventral view; (D) dorsal view. See Fig. 5 for explanation of expression diagrams (left). (A,B) Mid-stage posterior regeneration: *Pl-Otx2* is expressed in ventral cells of the blastema (arrows), primarily in regions where the ventral nerve cord of new segments is forming. (C,D) Early anterior regeneration: *Pl-Otx2* is expressed intensely in lateral crescents on each side of the developing blastema (arrows). (E) Mid-stage anterior regeneration: deep cells of the developing foregut strongly express *Pl-Otx2* (arrowhead), and a lateral cluster of unidentified cells near the anterior margin of the blastema transiently expresses *Pl-Otx2* (small arrow). (F,G) Late anterior regeneration: foregut expression (F, arrowhead) persists, localizing specifically to the pharynx. In addition, *Pl-Otx2* becomes expressed in scattered ventral cells of the blastema (F, arrow), mostly in the developing ventral nerve cord, and in a bilaterally symmetrical cluster of dorsolateral cells (G, arrow), possibly associated with an eyespot (see text). Vertical scale bar: 50 μ m.



new developing head (Fig. 9B). Rather than the expression fading at this point, however, as it does during anterior regeneration (Fig. 8E,F), the lateral crescents of *Pl-Otx2* (though not *Pl-Otx1*) expression join dorsally and split into an anterior and a posterior field, thus defining the outer edges of a dorsal wedge of tissue. The developing prostomium emerges dorsally from this wedge. *Pl-Otx2* expression encircles the prostomium's base throughout its development (Fig. 9C,D,E), and marks the boundary between the prostomium and the peristomium. During comparable stages, *Pl-Otx1* is expressed only in a few cells near the base of the prostomium (data not shown). At mid stages of fission, bilaterally symmetrical, lateral clusters of unidentified cells transiently express *Pl-Otx1/Otx2* just behind the plane of fission, below the surface of the body wall (data not shown), similar to expression seen during anterior regeneration. During mid stages of fission, *Pl-Otx1/Otx2* also become expressed in bilaterally symmetric groups of cells surrounding the gut, just behind the plane of fission (Fig. 9C). As development proceeds, this expression localizes to the developing pharynx (Fig. 9D), as in anterior regeneration blastemas. At late stages of fission, positive cells forming the putative 'eyespot' expression (also seen during anterior regeneration) become detectable (Fig. 9F).

DISCUSSION

Ancestral roles for *engrailed*

There has been considerable discussion regarding the ancestral

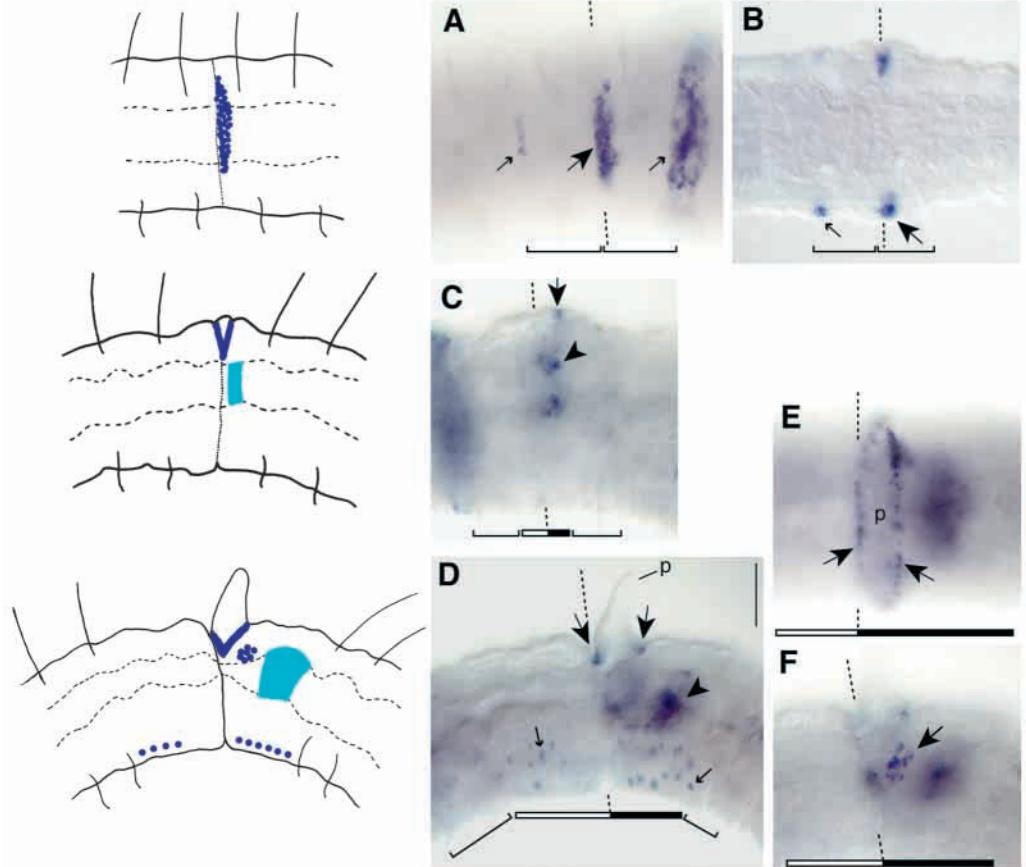
role(s) of *engrailed* in bilaterians. One of the most phylogenetically widespread, and thus presumably ancestral, roles of *en*-class genes is in embryonic neurogenesis (Holland, 1992; Lans et al., 1993; Holland et al., 1997; Duman-Scheel and Patel, 1999). In oligochaete annelids, we find that an *en* homolog is expressed in the developing nervous system during adult growth, regeneration and fission, suggesting that the involvement of *en*-class genes in neurogenesis is not limited to embryogenesis. Post-embryonic neuronal expression of an *en* homolog has similarly been reported for growing juvenile brittle stars (Lowe and Wray, 1997).

A more controversial hypothesis is that *en* may have played a role in segmentation in the common ancestor of annelids and arthropods (Wedeen and Weisblat, 1991), or perhaps even annelids, arthropods and chordates (Kimmel, 1996; Holland et al., 1997). The involvement of *en* in segmentation appears to be an ancestral feature for arthropods: in all arthropods investigated to date, *en* is expressed in a stripe of cells in the posterior region of nascent segments (e.g. Patel, 1994; Peterson et al., 1998), and functional studies in *Drosophila* demonstrate that *en* plays a direct role in delineating segments (Lawrence and Struhl, 1996). Among chordates, stripes of *en*-class gene expression precede signs of morphological segmentation in anterior somites of amphioxus embryos (Holland et al., 1997), but in other chordates, *en*-class genes are expressed only after segments are delineated, making the ancestral chordate pattern uncertain. Furthermore, arthropods and chordates are now believed to be more closely related to non-segmented phyla than they are to each other (Adoutte et al., 2000), making it far

Fig. 9. *Pl-Otx1* and *Pl-Otx2*

expression during fission. (B) *Pl-Otx1* expression; (A,C-D,F) *Pl-Otx2* expression. (A,C,D,F) Lateral views; (B,E) dorsal views. See Fig. 6 for explanation of expression diagrams (left). (A,B) Early fission: *Pl-Otx2* is expressed in the body wall in bilaterally symmetrical crescents (A,B, large arrows). Expression occurs just behind the plane of fission (evident in B by the constriction in the body wall), i.e. at the anterior limit of the new developing head. Worms in A,B possess multiple fission zones at different stages of development: the leftmost arrows in A,B indicate very recently initiated fission zones, in which expression is limited to a short strip of only a few cells, and the right-most arrow in A indicates the oldest fission zone in the panel, with more extensive staining. (C) Mid-stage fission: the lateral expression of *Pl-Otx2* extends dorsally to form a dorsal horseshoe of expression, which then splits dorsally into a posterior strip (arrow) and an anterior strip (not visible in this plane of focus). The prostomium emerges dorsally between these two strips of expression. At this stage of fission,

Pl-Otx2 is also expressed in the gut of the developing head (arrowhead). The staining in the left-most region of C is associated with a different, more anterior fission zone. (D-F) Late fission: the dorsally emerging prostomium (p) continues to be encircled by *Pl-Otx2* expression (D,E, large arrows). Foregut expression (D, arrowhead) persists, becoming localized to the pharynx. In addition, *Pl-Otx2* becomes expressed in scattered ventral/ventrolateral cells (including cells of the ventral nerve cord) in the developing anterior and posterior segments (D, small arrows), and in possible 'eyespot' (see text) in the developing head (F, arrow). Vertical scale bar: 50 μ m.



from certain that the *en* stripes in arthropods and amphioxus represent homologous phases of expression. (A recent investigation in several molluscs, including the metameric chitons, suggests a role for *en*-class genes in skeletogenesis, rather than metamere delineation (Jacobs et al., 2000).)

As one of the three major groups of segmented animals, annelids are of critical importance for understanding the evolution of segmentation. In leech embryos, *ht-en* expression is segmentally iterated (Wedeen and Weisblat, 1991; Lans et al., 1993). However, unlike the broad stripes of contiguous expression seen in arthropod and amphioxus embryos, expression in leech occurs in isolated non-contiguous cells (but see discussion of a possible asynchronous stripe of expression by Lans et al., 1993). Furthermore, recent experimental studies have failed to find evidence to suggest *en*-class genes are linked to the segmentation process in this annelid (Shain et al., 1998; Seaver and Shankland, 2000; Seaver and Shankland, 2001). We have extended investigations of *en* homolog expression to a second major group of annelids, the oligochaetes, and to new forms of development, namely adult growth, regeneration and fission. Although post-embryonic segmentation in oligochaetes involves large fields of small cells, much like arthropod or amphioxus embryonic segmentation, our studies in *Pristina* failed to uncover any obvious similarity to the segmental

expression patterns seen in arthropods and amphioxus. Rather, *Pl-en* expression occurs in scattered, isolated cells, primarily in the ventral body wall and nervous system (Figs 4-6). Functional investigations of annelid *en* homologs would be desirable, but currently no evidence suggests that *en* is involved in annelid segmentation, in striking contrast to what has been found in arthropods.

Otx-class genes and anterior specification during regeneration and fission

It seems clear that early in the evolution of bilaterians, the *Otx* homolog(s) acquired a role in defining anterior structures during embryogenesis. *Otx*-class genes are expressed almost exclusively in the extreme anterior structures in embryos of diverse bilaterians, including an annelid (Bruce and Shankland, 1998), several arthropods (e.g. Finkelstein et al., 1990; Li et al., 1996; Telford and Thomas, 1998) and a variety of chordates (e.g. Simeone et al., 1993; Wada et al., 1996; Williams and Holland, 1996; Tomsa and Langeland, 1999), as well as around the mouth of the radially symmetrical juveniles of sea urchins (Lowe and Wray, 1997). Our studies in *Pristina* demonstrate that *Otx*-class genes are also involved almost exclusively in head development during regeneration and fission. During both anterior regeneration and fission, the earliest phase of *Pl-*

Otx1/Otx2 expression occurs at the anterior limit of the new head, and occurs at very early stages of head development, before new morphological structures are apparent. Together, these findings suggest that *Pl-Otx1/Otx2* may be involved in the early processes of post-embryonic head specification. Interestingly, *Otx*-class genes in regenerating planarians also are associated primarily with the development of anterior structures (Stornaiuolo et al., 1998; Umesono et al., 1999).

New evidence that the evolution of paratomic fission involved recruitment of regenerative processes

Paratomic fission, in which a new head and tail are intercalated in the middle of a worm, has evolved multiple times in annelids (Giese and Pearse, 1975). Important similarities between the morphogenetic events of paratomic fission and regeneration exist for a number of asexual annelids (Dehorne, 1916; Berrill, 1952; Herlant-Meewis, 1953). These similarities, coupled with the observation that fission is far less common than regeneration among annelids, and tends to occur within larger taxonomic groups capable of regeneration, suggest that the evolution of fission may depend on recruiting regeneration processes for a role in reproduction. According to this hypothesis, then, paratomic fission in annelids is effected by initiating regeneration in the middle of an undamaged worm.

This recruitment hypothesis is supported by our data, which demonstrate extensive similarities in the spatial and temporal expression of body patterning genes between fission and regeneration (compare Fig. 5 with Fig. 6, and Fig. 8 with Fig. 9). For example, at very early stages of head development during both fission and regeneration, *Pl-Otx1/Otx2* are expressed in lateral crescents at the new anterior limit of the developing heads. At later stages of both processes, *Pl-Otx1/Otx2* are expressed in the pharynx of the new foregut and *Pl-Otx1/Otx2* and *Pl-en* are expressed in a number of ventral cells, including cells of the ventral nerve cord of developing segments. Thus, the similarity between regeneration and fission is not only notable at the level of morphogenesis, but extends also to the underlying developmental regulatory genes.

Nevertheless, despite the cellular and molecular similarities between fission and regeneration, it is clear that paratomic fission is not simply regeneration. For example, the two processes are not perfectly correlated: not all annelids capable of regeneration can undergo fission (Berrill, 1952), and at least one annelid (a naudid) capable of fission cannot fully regenerate (Bely, 1999). Thus, at least some aspects of development presumably differ between the two processes. Interestingly, we found that *Pl-Otx2* expression associated with the development of the prostomium differs markedly between fission and regeneration. During fission, *Pl-Otx2* expression encircles the base of the emerging prostomium throughout its development (Fig. 9C-E), whereas during anterior regeneration, no such expression is detectable during any stage of prostomium regeneration (Fig. 8). These data suggest the intriguing possibility that to evolve fission, naudid worms had to evolve a novel way of generating the prostomium, perhaps because the structure is added terminally during regeneration, whereas it must be intercalated during paratomic fission. We might then expect to find additional differences between fission and regeneration by investigating the formation of the other asegmental terminal structure, the pygidium.

Relationship between embryonic and post-embryonic development

A major aim in undertaking this work was to determine whether, in annelids, genes involved in embryonic body patterning are also expressed during growth, regeneration and fission, and if so, whether these genes appear to play similar roles during different modes of development. It is clear from our studies in *Pristina* that homeobox genes such as *Pl-en* and *Pl-Otx1/Otx2* are indeed redeployed during post-embryonic development, and their transient and regional expression is consistent with a role in either body patterning or cell fate specification.

To investigate whether post-embryonic patterning resembles embryonic patterning, however, it is necessary to compare regeneration and fission to embryogenesis. Because *Pristina* is not known to reproduce sexually under laboratory conditions, we compare our findings with published data on embryonic expression of *en*- and *Otx*-class genes in embryos of the leech *Helobdella triserialis* (Wedeen and Weisblat, 1991; Lans et al., 1993; Bruce and Shankland, 1998). Leeches and oligochaetes are both clitellate annelids and share a form of direct development characteristic of this group (Anderson, 1973). For comparisons of *en*-class genes, it should be noted that we assayed mRNA distribution in *Pristina*, while protein distribution was investigated in *Helobdella*. With respect to *Otx*-class genes, our study and Bruce and Shankland's study were both performed using in situ hybridization, with nearly identical protocols and probe regions.

Despite the drastic differences between developing embryos, regenerating adults and fissioning adults, several striking parallels emerge from a comparison of leech embryonic expression and oligochaete post-embryonic expression. During anterior regeneration and fission, the earliest *Pl-Otx1/Otx2* expression is in the anterior body wall (Figs 8C,D, 9A,B). Similarly, in leech embryos, the earliest detected expression of *Lox22-Otx* is in the anterior-most ectoderm of the germinal plate. In both *Pristina* and *Helobdella*, early expression is broad and not localized to a particular morphological structure. Although it is far from certain that these represent functionally equivalent phases of expression, *Otx*-class genes do appear to play an early role in defining anterior tissue domains in annelids during both embryonic and post-embryonic head development.

Otx-class genes are also expressed in the developing foregut during both regeneration/fission (in *Pristina*) and embryogenesis (in leech). In *Pristina*, *Pl-Otx1/Otx2* are expressed in the developing pharynx (Fig. 8E,F, 9C,D), while in leech embryos, *Lox22-Otx* is expressed in the proboscis (thought to be homologous to the oligochaete pharynx; Anderson, 1973), as well as in the esophagus. Interestingly, during oligochaete embryogenesis, regeneration and fission, the pharynx arises from a deep mass of cells that becomes hollow, and is distinct from the ectodermal cells that will invaginate to form the lining of the buccal cavity (Dehorne, 1916; Anderson, 1973). Therefore, both tissue morphogenesis and *Otx*-class gene expression support the idea that pharynx formation occurs by similar processes in both embryonic and post-embryonic development.

Finally, during regeneration and fission, as well as adult growth, *Pl-en* and *Pl-Otx1/Otx2* are expressed in a few cells of developing ventral nerve cord ganglia in *Pristina* (Figs 4-9), just as their homologs are in *Helobdella*. Therefore, *en*- and

Otx-class genes appear to be involved in the development of particular neuronal phenotypes during embryonic as well as post-embryonic development.

Despite these extensive similarities, differences in body patterning between embryogenesis and regeneration/fission are also suggested by our data. For example, in leech embryos, *Lox22-Otx* is intensely expressed in a ring around the developing mouth throughout much of early development. In contrast, circum-oral expression was never observed in regenerating or fissioning *Pristina*. *Pl-Otx1/Otx2* occurs in the lateral (*Pl-Otx1/Otx2*) and dorsal (*Pl-Otx2*) body wall (Figs 8, 9), but the new mouth is described as invaginating from the ventral surface in naeid worms (Dehorne, 1916), far from the site of *Pl-Otx1/Otx2* expression. During later stages of fission (but not regeneration), circum-prostomial *Pl-Otx2* expression marks the boundary between the prostomium and peristomium of *Pristina*. No comparable expression has been described for leech embryogenesis. However, the comparison between leech and naeid heads is made difficult by the fact that leeches have a highly modified anterior end, and it is not clear where (or even if) the prostomium/peristomium boundary exists in leeches. Nevertheless, the expression patterns described for leech embryogenesis and *Pristina* regeneration and fission are difficult to reconcile, and suggest that some aspects of mouth and/or prostomium development may differ between them. To confirm that the above comparisons do indeed reflect differences between embryonic and post-embryonic development, rather than differences between leeches and oligochaetes, it will be important to investigate embryos of the closest possible relative to *Pristina*.

Investigations of amphibian limb patterning based on grafting experiments provided some of the first direct evidence suggesting that some patterning mechanisms are similar between embryonic and post-embryonic development (Muneoka and Bryant, 1982; Muneoka and Bryant, 1984). Since then, several studies have investigated expression of body patterning genes during regeneration, primarily in vertebrates, hydra and planarians (see Introduction), but only a handful of these have compared their findings specifically with embryonic patterning. The existing studies demonstrate that some genes expressed during embryogenesis are re-expressed, and in a similar way, during regeneration (Del Rio-Tsonis et al., 1995; Loosli et al., 1996; Stark et al., 1998; Cadinouche et al., 1999; Gardiner et al., 1999; Technau and Bode, 1999). However, some studies have also uncovered differences between embryogenesis and regeneration in the relative timing of expression of genes (Gardiner et al., 1995), as well as differences with respect to which tissues express a particular gene (Akimenko et al., 1995). Extending studies of post-embryonic development to include a broader range of taxa remains crucial if we are to achieve a general understanding of how post-embryonic development is effected in metazoans. Our study of *Pristina*, which represents the first analysis of developmental regulatory gene expression during annelid regeneration, and the first during fission, lends support to the idea that embryogenesis, regeneration, and fission employ largely similar, but clearly not identical, body patterning mechanisms.

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