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Differential Expression of Three *labial* Genes during Earthworm Head Regeneration

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The earthworm provides an excellent model for investigating regeneration. Here we report the full-length cloning of three *labial* genes (*Pex-lab01*, *Pex-lab02*, and *Pex-lab03*) in the earthworm *Perionyx excavatus*. To analyze their expression pattern during head and tail regeneration, we used the reverse transcription-polymerase chain reaction. Our results indicate that the three *labial* genes were expressed only in the head-regenerating tissues. Also, we found that the expression of *Pex-lab01* and *Pex-lab02* is up-regulated, and this indicates their involvement in wound healing and the blastema formation processes during early head regeneration.

Key words: *labial* genes; *Perionyx excavatus*; head regeneration

It is well known that hydra and planarians can completely regenerate a new organism from a small body fragment.[1,2] Compared to hydras and planarians, which have been widely used in invertebrate regeneration research, the earthworm (Annelida: Oligochaeta) is morphologically more complex, in that it has a well-developed central nervous system, a closed blood-vascular system, a coelom, and segmentation.[3]

Furthermore, there are several reasons the earthworm provides a unique and valuable model to investigate the mechanism of regeneration. First, earthworm regeneration is relatively rapid. *Perionyx excavatus* (used in this study) is able to regenerate an amputated head and tail completely within 25 d post-amputation. Secondly, in contrast to regeneration in other animals, earthworm regeneration of a complete head and tail requires the reformation of various tissues and organs, including a central nerve system, heart, clitellum, blood-vascular system, testis, ovary, intestine, nephridia, and setae. Thirdly, earthworm regeneration is bidirectional. It is thought that the earthworm is the highest evolutionary form capable of regenerating an anterior portion containing a central nerve system, heart, and clitellum.[4,5] These properties, together with its metameric morphology and ease of culture and handling, make *P. excavatus* an excellent material for studies of regeneration mechanisms.

The *Hox* genes consist of a large family of genes that encode homeodomain transcription factors.[6,7] *Hox* genes in vertebrates have been found to be involved in several other processes, including patterning of the limb bud axis, hematopoeisis, and organogenesis.[8,9] In addition, *Hox* genes have been to be expressed in tissues that have the ability to regenerate. These tissues include the planarian,[10,11] *Xenopus* tail,[12] urodele appendages,[13] and zebrafish fins.[14] The expression of *Hox* genes during a regeneration event indicate positional identity and represent re-patterning of the tissues in a manner similar to that occurring during their initial development.[15] *Hox* genes have also been found to play a role in wound repair and regenerative wound healing.[12,16,17]

In the present study, we cloned the full-length cDNAs of three *labial Hox* genes, *Pex-lab01* (GQ223405), *Pex-lab02* (GQ223406), and *Pex-lab03* (GQ223407), from the earthworm, *Perionyx excavaus*. We analyzed temporal changes in the expression of these three *labial* genes during head and tail regeneration. Semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR) analysis revealed that all three *labial* genes were expressed only during head regeneration. In addition, we report the results of RT-PCR analysis of the transcript distribution of these genes during early head regeneration. Our results indicate that the three *labial* genes have different expression patterns during the early head regeneration processes. This is first report on *labial* gene expression during head and tail regeneration in annelids.

Materials and Methods

Experimental animals and amputation for regeneration. Sexually mature *P. excavatus* worms were reared by the method described in Cho *et al.* 2003.[18] Except where noted otherwise, all the worms were cut into three body regions (anterior, trunk, and posterior) (Fig. 1).

Application of rapid amplification of cDNA ends (RACE)-PCR for the three labial genes. We performed RACE-PCR to obtain additional 5′ and 3′ sequences for the three *Pex-lab* genes. For 5′ and 3′-RACE, 1 μg of poly $(A)^+$ mRNA isolated from embryos at various stages was reverse transcribed using the SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA) according to manufacturer's instructions. Each *lab*-specific primer was designed from the homeobox sequence: 5′-CGGACGATTCGTTCCGTTCCTCTTCGG-3′ (5′-

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Abbreviations: *Hox*, homeobox; lab, *labial*; RACE-PCR, rapid amplification of cDNA ends-polymerase chain reaction; sqRT-PCR, semi-quantitative reverse transcriptase-PCR

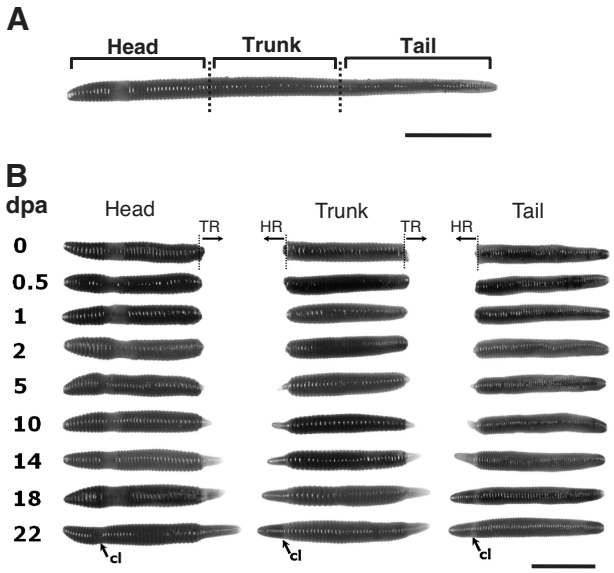

Fig. 1. Regeneration of Head, Trunk, and Tail.

A, Dorsal view of adult *P. excavatus* and body fragments (head, trunk, and tail) where amputations were performed. B, Regeneration from the head, trunk, and tail fragments. The broken line indicates the amputation level. TR, tail regeneration; HR, head regeneration; dpa, days post-amputation; cl, clitellum. Scale bars, 10 mm.

RACE) and 5′-CACTTCAGCCGTTACCTGACTCGCTCCAGG-3′ (3′-RACE) for *Pex-lab01*; 5′-ATTGTTGTTGTTGTTGCTTGGTG-ACG-3′ (5′-RACE) and 5′-ACTAACAAGCAGCTGACGGAACTGG-AAAAG-3′ (3′-RACE) for *Pex-lab02*; and 5′-GATGATTCAGTTGT-CGTCTGTCTGCG-3′ (5′-RACE) and 5′-GAACTGGAAAAAGAG-TTCCACTTCAACAAG-3′ (3′-RACE) for *Pex-lab03*. Touch-down PCR cycling conditions were as follows: 1 cycle of 94 °C for 1 min, 5 cycles of 94 °C for 30 s and 72 °C for 3 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, and 25 cycles of 94 °C for 30 s, 68 °C for 1 min, and 72 °C for 2 min. The PCR product of the expected size was excised from an agarose gel, and purified cDNA fragments were cloned into pCR 2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed to competent *Escherichia coli* TOP10 cells, which were plated onto selective medium. Recombinant clones were selected by blue/white screening. DNA minipreparation of single white clones was performed with Plasmid Spin kits (Genemed, Seoul, South Korea) according to the manufacturer's instructions. Digestion with restriction endonuclease *Eco*RI was performed to ensure the presence of inserted cDNA fragments. The purified clones were sequenced with an ABI 310 DNA Analyzer (Applied Biosystems, Foster City, CA) using the M13 reverse primer.

Relative quantification of gene expression by RT-PCR. Head and tail regenerated tissues were sampled at 0, 0.5, 1, 3, 6, 12, 18, and 24 h, and at 2, 4, and 7 d from the trunk body region (Fig. 1B). Total RNA samples were prepared with TRIzol reagent (Sigma, St. Louis, MO) according to manufacturer's instructions for each time point using approximately 20 worms. The total RNA (3 µg) obtained was reverse transcribed with a first-strand cDNA synthesis kit (BD Biosciences, Palo Alto, CA). The PCR mixture (50 µl) contained 10 × Taq buffer, Taq DNA Polymerase (Qiagen, Valencia, CA), 2.5 µM of dNTPs, an appropriate set of primers, and the cDNA (50 ng) synthesized from the each time point as template (Fig. 1B). The β-actin gene was used as an internal standard. The following primers were used: 5′-GGGAA-GGATGAGACATGGT-3′ (forward) and 5′-AAGTTCGTCCGATGT-CACC-3′ (reverse) for *Pex-lab01*; 5′-ACGGTCAGAAGAAGTCAC-3′ (forward) and 5′-TTGAAGTGGAACTCCTTT-3′ (reverse) for *Pex-lab02*; 5′-GAACTGTCTGACCAGCTCAT-3′ (forward) and 5′-GACCCATGTACCCGTAGTC-3′ (reverse) for *Pex-lab03*; and 5′-CATTGTCACCAACTGGGATG-3′ (forward) and 5′-CTCGAACAT-GATTTGGGTCA-3′ (reverse) for β-actin. The PCR reactions were performed under the following cycling conditions: initial denaturation at 94 °C for 5 min, followed by 25–35 cycles (for β-actin and *Pex-lab* orthologs, respectively) at 94 °C for 30 s, 50–55 °C (depending on the primers) for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The primer sets yielded PCR products of 312, 213, 126, and 198 bp in length for *Pex-lab01*, *Pex-lab02*, *Pex-lab03*, and β-actin respectively (Fig. 3A). The extent of amplification was chosen empirically so as to avoid saturation of the amplified bands. To quantify the PCR products, each sample was electrophoresed in 1.5% agarose gel and stained with ethidium bromide. The band intensities were measured with a BIORAD Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA).

Orthology and phylogenetic analysis. The sequences obtained were compared with GenBank Database (NR, non-redundant) through BLASTX. Alignments of all three *labial* genes were performed using the Clustal X 1.81 program.[19] To assign earthworm *labial* genes, phylogenetic analysis was carried out by the neighbor-joining (NJ) method using the MEGA 2 program.[20] Bootstrap analysis was performed with 1,000 replications. The sequences listed in Fig. 2 were employed in our alignment and phylogenetic analyses (GenBank accession numbers as indicated).

Results and Discussion

Regeneration from head, trunk, and tail fragments

Earthworm regeneration is thought to occur mainly *via* de-differentiation and subsequent re-differentiation of cells, without any contribution from totipotent stem cells.[21] In earthworm head regeneration, amputation of segments up to the clitellar region of the body induces wound healing and blastema formation in the severed segments.[22] Wound healing is closed by superficial epiblasts (originating from the body wall epithelium) coming from undifferentiated epidermal cells.[3] It is generally accepted that blastemal cells are heterogeneous in origin.[23] The process of epimorphic regeneration involving de-differentiation, cell proliferation, and re-differentiation provides a useful model for investigating the mechanisms of normal development as well as differentiation.[24]

P. excavatus has high regenerative ability.[4] When it is cut transversely into three parts, the anterior part can completely regenerate a tail from the posterior plane within 22 d, the posterior part with a tail regenerates a head (the head part, consisting of the clitellum: segments

12 to 17) from the anterior plane also within 22 d. A new head and tail are consistently regenerated from the anterior and posterior cut ends respectively of the trunk piece (Fig. 1).

As shown in Fig. 1, at the 12-h and 1-d stages, extrusion of the intestine was observed, and wound healing was in progress. Outgrowth of the regenerating part was observable from 2 to 5 d stage. The dark red color of the normal segments was in marked contrast to the much lighter colored new regenerating tissue during the early weeks of regeneration. The regenerated head and tail became distinct 10 d after amputation. At 14 d, segmentation of the regenerated head and tail was observed the followed by rapid growth and pigmentation (Fig. 1B).

These observations indicate that regeneration of a complete individual from each of the three body fragments is accomplished by a combination of epimorphic recovery of the head, clitellum, and tail, and morphallactic transformation of old segments into the appropriate segments so as to produce the correct body proportions. Moreover, our experiments indicate that this bidirectional regeneration can be artificially induced under laboratory conditions.

Identification of the three labial *genes from* P. excavatus

Previous studies have identified 11 *Hox* genes, including the three *labial* genes in the earthworm *P. excavatus*.[18] PCR amplifications were performed on genomic DNA extracted from *P. excavatus*, using the *labial*-specific (encoding the TNFTNKQ peptide) and the Helix3 degenerate primer set. These fragments (residues 6–53) encode portions of the homeodomain of the predicted *labial*-class genes, designated *Pex-lab01*, *Pex-lab02*, and *Pex-lab03*.

In this study, for *Pex-lab01*, *Pex-lab02*, and *Pex-lab03*, we used 5′- and 3′-RACE-PCR on first-strand cDNAs for an additional sequence, and obtained a *Pex-lab01* open reading frame (ORF) encoding 273 amino acids plus 5′-UTR (73 bp) and 3′-UTR (243 bp); a *Pex-lab02* ORF encoding 343 amino acids plus 5′-UTR (132 bp) and 3′-UTR (94 bp); and a *Pex-lab03* ORF encoding 145 amino acids plus 5′-UTR (57 bp) and 3′-UTR (293 bp). Three in-frame stop codons lay upstream of the presumed start codon, suggesting that we obtained the complete ORFs of the three *labial* genes (Fig. 2A, B, and C). Our data, which represent 5′- and 3′-UTR sequences of the three PG1 ortholog homeodomains, strongly confirm that *P. excavatus* has at least three PG1 orthologs, and hence that two independent duplications of PG1 must have occurred in this species. Like other *labial*-class genes, our results also showed diverse hexapeptide sequences of *Pex-lab* duplicates: WMTMRS, WLTVRR, and WMTVKR for *Pex-lab01*, *Pex-lab02*, and *Pex-lab03* respectively. In addition, the linker region connecting the hexapeptide to the N-terminal arm of the homeodomain was observed to be somewhat variable in length among the three Pex-lab genes: 21, 41, and 14 amino acids for *Pex-lab01*, *Pex-lab02*, and *Pex-lab03* respectively (Fig. 2E). It has been found that both the hexapeptide and its position relative to the homeodomain influence the developmental and DNA-binding specificity of a *Hox* gene.[25,26] The homeodomain sequences of the *P. excavatus labial* orthologs, *Pex-lab01*, *Pex-lab02*, and *Pex-lab03*, were most similar to the PG1 homeodomains of the polychaetes (*Nvi-lab* and *Pdu-Hox1*), cephalopod (*Esc-lab*), brachiopod (*Lan-lab*), flatworm (*Pnox3*), and leech (*Htr-Lox7*). In particular, *Pex-lab03* has high sequence similarity to the *labial* orthologs from polychaete (*Nvi-Hox1*, 96%) and cephalopod (*Esc-lab*, 95%) (Fig. 2E). They display PG1-specific A29, N41, and T43 residues,[27] and cluster as a clade on phylogenetic analysis (Fig. 2D and E). This information might be useful in understanding the molecular mechanisms that figure in head regeneration. In addition, exploring the regulatory regions of the duplicated genes should provide valuable information on the evolution of *Pex-lab* function and its implications in earthworm regeneration.

Temporal expression patterns of the Pex-lab01*,* Pex-lab02*, and* Pex-lab03 *genes as revealed by RT-PCR during head and tail regeneration*

It has been observed in several model organisms that *Hox* genes function in pattern formation along the anterior-posterior axis during regeneration in hydra[28] and Planarians,[10,11,29] but the function of the *Hox* genes during annelid regeneration is still uncertain.

The presence of three PG1 genes in *P. excavatus* suggests that two duplication events of PG1 have occurred in the evolutionary lineage leading to this species. In the present study, RT-PCR analysis was performed on cDNA derived from various regenerating tissues (Fig. 3). *P. excavatus* can regenerate along the body axis anteriorly (head regeneration from tail), bidirectionally (head and tail regeneration from trunk), and posteriorly (tail regeneration from head) (Fig. 1). Preliminary data suggested that the expression patterns of the three *lab* genes during bidirectional regeneration are be the same as those of anterior and posterior regeneration (data not shown), which entails their expression only in head regeneration and not in tail regeneration. Hence in for our experiments and analysis of the expression patterns of the three labial genes, we used the trunk region (bidirectional regeneration) during various stages of head and tail regeneration using sqRT-PCR (Fig. 3).

As a first step, we used sqRT-PCR assays to evaluate the expression levels of the three *labial* genes in the trunk head and tail regenerating tissues (0, 12, and 24 h, and 2 and 4 d after amputation). We found that all three *labial* genes were expressed in head regenerating tissues, whereas none of these genes was detected in tail regenerating tissues (Fig. 3B). These results suggest that all three *labial* genes can be expressed only in head regenerating tissues.

To further characterize the expression of the three *labial* genes during head regeneration, we performed sqRT-PCR on the three genes at 0, 12, and 24 h, and 4 and 7 d after amputation. It appears that *Pex-lab01* and *Pex-lab02* are absent in intact tissues (0 h) and are expressed only from 12 to 24 h during anterior regeneration. In contrast, *Pex-lab03* is expressed in intact tissues (0 h) and until 24 h into regeneration (Fig. 3C). We observed that the *Pex-lab01* and *Pex-lab02* genes were not expressed in intact worms but were expressed in the head regenerating tissues (Fig. 3C). This apparent turning on of expression of the *Pex-lab01* and *Pex-lab02*

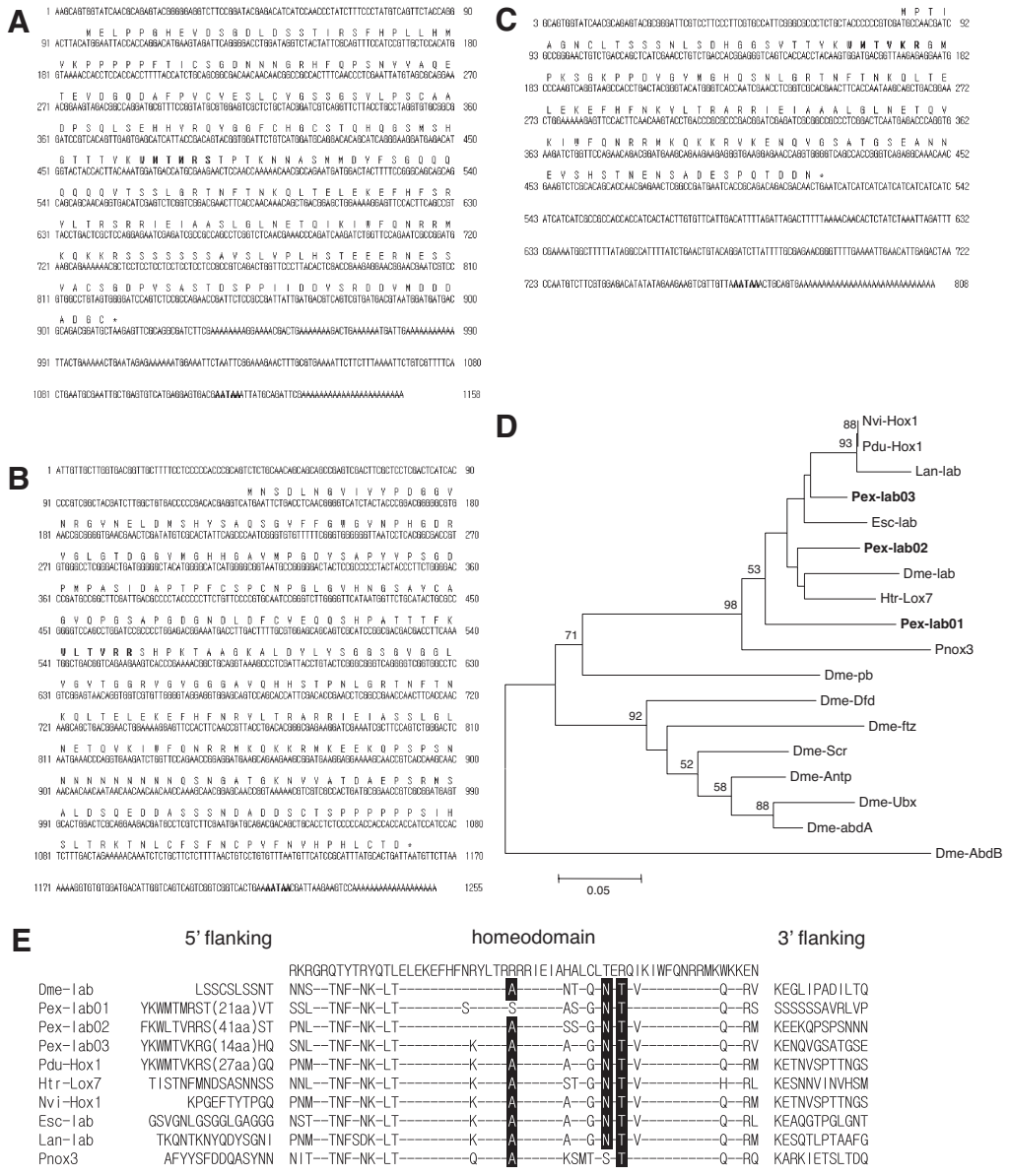

Fig. 2. Nucleotide Sequence of *Pex-lab01*, *Pex-lab02*, and *Pex-lab03* ORFs.

A–C, Nucleotide sequences of *Pex-lab01* (A), *Pex-lab02* (B), and *Pex-lab03* (C) cDNA; conceptual translation is indicated by a single-letter code. The hexapeptide specific to PG 1 is indicated in bold. Asterisks indicate the stop codon and the polyadenylation signal. D, Phylogenetic relationships among *labial* sequences based on neighbor-joining analysis of deduced amino acid sequences. The numbers at the nodes are scores from 1,000 bootstrap re-samplings of the data. Unlabeled nodes are supported by a bootstrap value lower than 50%. E, Alignment of amino acid sequences of the *labial* gene homeodomain and flanking regions. The sequences of *Pex-lab01*, *Pex-lab02*, and *Pex-lab03* were aligned in comparison with those from other lophotrochozoans and *Drosophila* homeodomains. All sequences are aligned to the *Drosophila Antennapedia* homeodomain. Dashes indicate identity to *Antp*. The dataset includes sequences from polychaete (*Nvi-* and *Pdu-*), oligochaete (*Pex-*), leeches (*Htr-*), cephalopoda (*Esc-*), brachiopoda (*Lan-*), flatworm (*Pnox-*) and Drosophila (*Dme-*). The following sequences were employed in our alignment and phylogenetic analyses (GenBank accession numbers): *Drosophila lab* (NM_057265), *pb* (NM_057322), *Dfd* (NM_057853), *Scr* (X14475), *Antp* (NM_079525), *ftz* (NM_058159), *Ubx* (NM_080500), *abdA* (NM_057345), *AbdB* (NM_080157); oligochaete (*P. excavatus*). *Pex-lab01* (GQ223405), *Pex-lab02* (GQ223406), *Pex-lab03* (GQ223407); polychaete (*Nereis virens*) *Nvi-Hox1* (AF151663), (*Platynereis dumerilii*) *Pdu-Hox1* (CAD43607); leech (*Helobdella triserialis*). *Htr-Lox7* (Y10888); flatworm (*Polycelis nigra*) *Pnox3* (L41848); Cephalopoda (*Euprymna scolopes*) *Esc-lab* (AH011177); Brachiopoda (*Lingula anatina*) *Lan-lab* (AF144672).

genes during head regeneration might be associated with blastema formation, and indicates that these genes are candidates for early head regeneration processes.

In order to characterize better the expression pattern of the three *labial* genes during early head regeneration, we did sqRT-PCR using total RNA isolates of both intact tissue (0 h) and regenerating fragments at 0.5, 1, 3, 6, 12, 18, and 24-h, and at 2 d after amputation. The three *labial* genes showed markedly different patterns of expression during early head regeneration (Fig. 3D).

It appeared that *Pex-lab01* and *Pex-lab02*, which were not expressed in intact tissues (0 h) began to be expressed as soon as 3 and 12 h postamputation respectively (Fig. 3D). Expression of *Pex-lab01* increased gradually from 3 h after amputation, and reached the initial level after 24 h of regeneration (Fig. 3D). The pattern of *Pex-lab02* expression was similar to that of *Pex-lab01*; it began to be expressed only in the head regenerating tissues (Fig. 3B), was not detected in the intact worms, and was expressed during early head regeneration

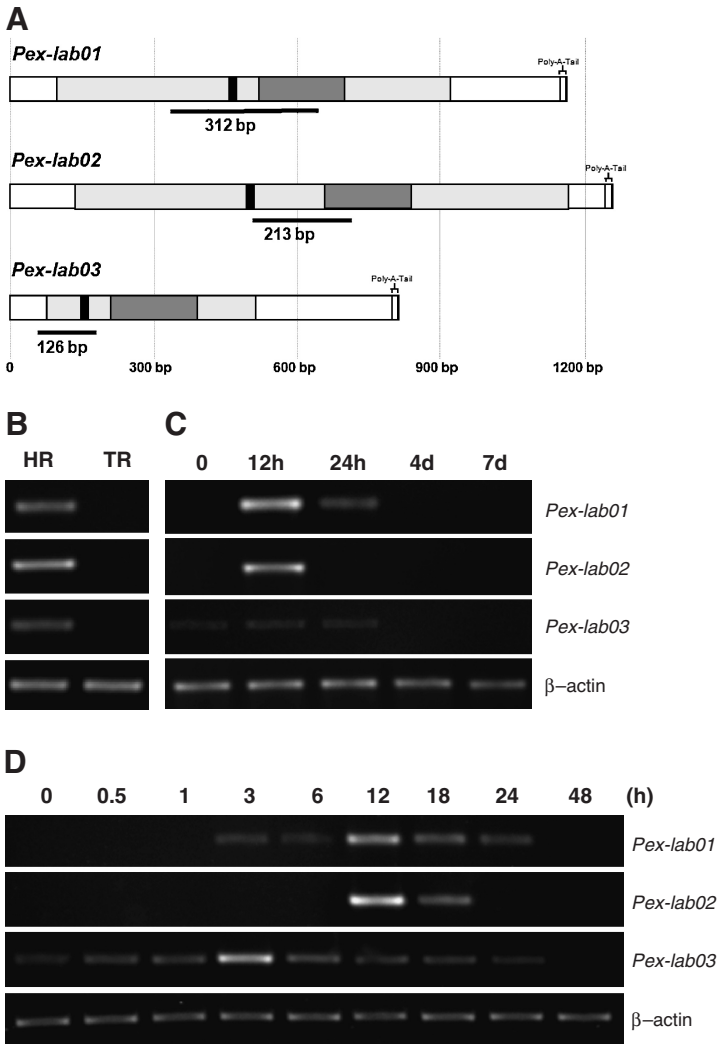

Fig. 3. Expression Patterns of the Three *labial* Genes as Revealed by RT-PCR.

A, Diagram of the cDNA clone used in semi-quantitative RT-PCR experiments. Broken vertical lines indicate 300 bp. Light shading denotes the largest ORF, the small dark shaded box is the hexapeptide motif, and the larger dark shad box represents the homeobox. Dark lines indicate the positions and sizes of the RT-PCR products. B, Semi-quantitative RT-PCR analysis of *Pex-lab01*, *Pex-lab02*, and *Pex-lab03* expression during head (HR) and tail (TR) regeneration the trunk body region. C and D, RT-PCR was performed on the three *labial* genes (*Pex-lab01*, *Pex-lab02*, and *Pex-lab03*) at various time points during head regeneration from the trunk body region. The digital image of ethidium bromide-stained gels represents the expression patterns of *Pex-lab01*, *Pex-lab02*, and *Pex-lab03*. β-actin, used as a positive control, was amplified in parallel. To avoid saturation of the amplified PCR products, submaximal PCR amplification (30 amplification cycles for *Pex-lab* orthologs and for β-actin) was performed.

(Fig. 3C). During head regeneration, no expression of *Pex-lab02* was detectable at 0.5 to 6 h after amputation, but it was detected from 12 to 18 h after amputation (Fig. 3D). However, the expression of *Pex-lab03* appeared to be up-regulated within the first hours, and reached distinct peak 1 h after amputation, followed by a gradual decrease to the intact level (Fig. 3D). Expression of *Pex-lab03* was detected in both intact and regenerating tissues. It should be noted that *Pex-lab03* was expressed in intact worms, suggesting that pattern formation in earthworm occurs continuously.

In the flatworm *D. japonica*, two *Abdominal-B-like* genes, named *DjAbd-Ba* and *DjAbd-Bb*, were identified that are not involved in anterior-posterior axis development during regeneration,[30] but *Plox5* was expressed in the posterior region of the regenerating body pieces, suggesting its involvement in anterior-posterior patterning during regeneration.[11] In *D. trigrina*, a different flatworm species, two *Antp* orthologs, *Dthox-C* and *Dthox-E*, were reported, and their gene expression was not related to the anterior-posterior axis during body regeneration.[10] In the hydra, *cnox-1* and *cnox-2* showed spatially differential expression patterns, but appeared not to show the *Hox*-type expression pattern along the anterior–posterior axis.[28] In contrast, our results indicate that the three *lab* genes were expressed only in the head regenerating tissues, suggesting that the *lab* genes are involved in anterior-posterior patterning in earthworm.

Besides the these findings it is important to note that whereas planarians and the hydra regenerate *via* neoblasts that are widely distributed throughout the body,[2,28,31] earthworm regeneration is thought to occur mainly through the de-differentiation and re-differentiation of cells without any contribution from totipotent stem cells (neoblasts).[3,21] After wound healing, a regeneration blastema was formed at both ends of the fragment. At 24 h postamputation, de-differentiating blastemal cells appeared in the longitudinal muscle layer of the body wall facing the coelomic side (unpublished data). Thus blastema formation, by means

of de-differentiation of mature tissues and/or stem cells, is probably a key event in a the regeneration process.[3] The results suggest that the three *labial* genes play important roles in blastema formation during early head regeneration. It also appears that they play important roles in maintenance and functioning during early head regeneration.

In summary, we report on the characteristics of three duplicated *labial* genes from the earthworm *P. excavatus.* We found that earthworm regeneration can be considered both epimorphic and morphallactic regeneration. Thus earthworm regeneration is a useful model for elucidating the molecular basis of de-differentiation as well as body pattern formation in annelids. In addition, analyses of gene expression using the sqRT-PCR indicated that the *Pex-lab01* and *Pex-lab02* genes are the only ones that are specifically expressed during early head regeneration. To further elucidate the functions of this gene, additional studies including knockdown experiments are necessary.

Acknowledgments

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