Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: https://www.tandfonline.com/loi/tbbb20

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To cite this article: Sung-Jin CHO, Myung Sik LEE, Eun Sik TAK, Eun LEE, Ki Seok KOH, Chi Hyun AHN & Soon Cheol PARK (2009) Gene Expression Profile in the Anterior Regeneration of the Earthworm Using Expressed Sequence Tags, Bioscience, Biotechnology, and Biochemistry, 73:1, 29-34, DOI: 10.1271/bbb.80391

To link to this article: https://doi.org/10.1271/bbb.80391

Biosci. Biotechnol. Biochem., **73** (1), 29–34, 2009

Gene Expression Profile in the Anterior Regeneration of the Earthworm Using Expressed Sequence Tags

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Received June 11, 2008; Accepted September 30, 2008; Online Publication, January 7, 2009
[doi:10.1271/bbb.80391]

In order to gain insight into the gene expression profiles associated with anterior regeneration of the earthworm, *Perionyx excavatus*, we analyzed 1,159 expressed sequence tags (ESTs) derived from cDNA library early anterior regenerated tissue. Among the 1,159 ESTs analyzed, 622 (53.7%) ESTs showed significant similarity to known genes and represented 338 genes, of which 233 ESTs were singletons and 105 ESTs manifested as two or more ESTs. While 663 ESTs (57.2%) were sequenced only once, 308 ESTs (26.6%) appeared 2 to 5 times, and 188 ESTs (16.2%) were sequenced more than 5 times. A total of 803 genes were categorized into 15 groups according to their biological functions. Among 1,159 ESTs sequenced, we found several gene encoding signaling molecules, such as *Notch* and *Distal-less*. The ESTs used in this study should provide a resource for future research in earthworm regeneration.

Key words: earthworm; anterior regeneration; expressed sequence tags; signaling molecules

A few animal species have the remarkable capacity of being able to regenerate a missing part of their body after amputation. The process of epimorphic regeneration, involving de-differentiation, cell proliferation, and re-differentiation, provides a useful model for investigating both normal development and differentiation.[1)] It is thought that the earthworm is the highest evolutionary species capable of regenerating an anterior portion containing the central nerve system, heart, and clitellum.[2)] Annelids constitute a large and diverse taxon of marine, freshwater, and terrestrial segmented worms. Traditionally regarded as comprising three distinct classes, polychaetes, oligochaetes, and leeches,[3)] they are now thought to be paraphyletic with respect to Echiura, Pogonophora, and Sipuncula.[4)] Clitellata (uniting oligochaetes and leeches) is a major annelid grouping, accepted as monophyletic on the basis of both molecular and morphological criteria.[5)] Although the potential for anterior regeneration depends on earthworm species, *Perionyx excavatus*, which was used in the present study, is a species highly capable of anterior regeneration. This species can complete anterior regeneration with restructuring of reproductive organs (*i.e.*, testis, ovary, seminal vesicle, and clitellum) within 2 weeks of amputation.[6)] Additionally, cell-cell communication and biosynthesis actively take place during the early stages of regeneration to induce de-differentiation, to regulate the proliferation of pluripotent cells, and to respecify the fates of such cells to reconstruct the missing organs.[7)] Therefore, analysis of transcripts in the early stage of anterior regeneration of the earthworm might provide a unique and valuable data set for understanding the molecular mechanism of the loss and gaining of cell identity that occurs during anterior regeneration, and to identify the candidate molecules responsible for controlling the de-differentiation and re-differentiation of the pivotal system, which includes the central nervous system.

Transcriptome analysis, using expressed sequence tags (ESTs), provides a rapid and simple data set for the genes expressed in a given tissue. Among the biological techniques for transcriptome analysis, the generation of ESTs, which are single-pass sequences generated from the partial sequencing of randomly selected cDNA clones,[8)] is the simplest method of profiling the transcriptome, and is also particularly useful in the development of cDNA microarrays for the systematic identification of differential gene expression.[9,10)] Large-scale analysis of ESTs might constitute an effective method of rapidly analyzing gene expression, characterizing gene functions, and discovering new genes that are important to specific developmental and physiological events.[11)]

This study provides the gene expression profiles of earthworm regeneration, establishing 622 ESTs representing 338 genes and 537 unknown ESTs from the early anterior regeneration of the earthworm *P. excavatus*. Some novel genes discovered in this study appear to be involved in early anterior regeneration. Furthermore, we identified several genes of signaling molecules that might be involved early in the regeneration processes. Semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) analyses confirmed that these candidate genes were significantly upregulated during anterior regeneration.

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Abbreviations: EST, expressed sequence tag; NCBI, National Center for Biotechnology Information; nr, nonredundant; EGF, epidermal growth factor

Materials and Methods

Experimental animals and RNA isolation. Sexually mature *P. excavatus* worms were reared following the method described by Cho *et al.* 2003.[12] Except where noted otherwise, all amputations were performed at the level of the 2nd segment anterior to the clitellum. Total RNA of the anterior regenerating tissues (mixture of 0.5, 1, 3, 6, 12, 18, and 24 h after amputation) was isolated using the TRI Reagent (Sigma, St. Louis, MO). Poly $(A)^+$ RNA was purified from total RNA using Dynabeads mRNA DIRECT™ Kit (Invitrogen Dynal AS, Oslo, Norway) according to the manufacturer's instructions.

Construction of cDNA library and sequencing. A directional cDNA library was constructed using the pCMV-script XR cDNA Library Construction Kit (Stratagene, La Jolla, CA). About 1,500 bacterial clones were randomly chosen from the plates and grown overnight in 1.5 ml of LB medium. The plasmid DNA was prepared using Plasmid Spin kits (Genemed, Seoul, South Korea) and stored at $-20\,^{\circ}C$ Sequencing reactions were performed in a MJ Research Gradient Cycler using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template with T3 primer using an ABI 3700 sequencer (Applied Biosystems).

Bioinformatic analysis. The vector sequences were trimmed from the raw sequence data using VecScreen software of the National Center for Biotechnology Information (NCBI). The sequence of each EST was also edited manually, to remove ambiguous bases, poly (A) tracts, and poor-quality sequences (nucleotide sequences shorter than 150 bp). All edited sequences were assembled into groups using SeqMan II software (DNASTAR, Madison, WI). Each sequence was compared with the non-redundant (nr) NCBI protein sequence database using BLASTX algorithms. Sequences that did not match any known sequences in the protein database were further analyzed at the nucleotide level using the nr BLASTN program. Sequence similarities were considered to be statistically significant only when the *E*-value was less than 1×10^{-5}. The sequences determined were deposited in the DNA Database in Japan (DDBJ accession nos. BP998465-BP999623). DNA sequences showing similarity to known genes were classified into different functional groups based on the categories described by Lee *et al.* (1999).[13]

Semi-quantitative RT-PCR and multiple alignments. The nucleotide sequences of the primers and the PCR conditions used in semi-quantitative RT-PCR are listed in Supplemental Table 2 (Supplemental Table 2; see *Biosci. Biotechnol. Biochem.* Web site). Anterior regenerate tissues obtained at 0, 0.5, 1, 3, 6, 12, 18, and 24 h (with approximately 20 worms used per time point) were sampled and total RNA was isolated as described above. Each total RNA (2 μg) was reverse transcribed using a first-strand cDNA synthesis kit (BD Biosciences, Palo Alto, CA). We determined the appropriate conditions for amplification cycles (25–35 cycles) to occur in the linear range. The β-actin gene was used as a positive control. The products were analyzed by electrophoresis on an 8% polyacryamide gel, visualized by ethidium bromide staining, and photographed using a BIORAD Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA).

Multiple alignments of the ESTs were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw) and the GeneDoc program (Corpet, 1988).[14] The following sequences were employed in our analysis of multiple alignments (GenBank accession numbers): PER10262 (BP998711): sea urchin (AAA29996), ascidian (NP_001037825), and beetle (XP_972305); PER11262 (BP999002): fruit fly (AAF47279), mouse (CAB37647), and human (P56177); PER10719 (BP999026): cattle (AF487464), fruit fly (CAA27641), and human (CAC34820).

Results and Discussion

Overview of ESTs from the anterior regenerates

The cDNA library established with anterior regenerates contained 9.2×10^4 primary clones. The average length of the cDNA fragments was 935 bp, and more than 91% of the clones contained cDNA fragments that were longer than 0.5 kb. Table 1 summarizes the results of EST analysis of anterior regenerate gene expression. The 1,159 ESTs analyzed consisted of 663 singletons and 140 clusters, indicating the presence of 803 genes in our data set. Of the total ESTs, 53.7% (622 ESTs) showed similarity to known genes in the GenBank nonredundant database with *E*-values lower than 10^{-5}. Of the 622 ESTs, 170 ESTs (27.3%) matched genes identified from annelids, including oligochaetes, hirudineans, and polychaetes. The remaining 46.3% (537 ESTs) matched unknown sequences in GenBank or matched known genes with insignificance (*E*-value $> 10^{-5}$).

Table 1. Composition of ESTs Analyzed by BLAST Search

ESTs categories	Number of ESTs (%)	Number of clusters (Number of comprised ESTs)	Number of singletons
A. Sequences matched significantly[a]			
BLASTX (nr) matches	622 (53.7)	105 (389)	233
Annelids	170 (14.7)	19 (153)	17
Oligochaeta	150 (12.9)	16 (141)	9
Hirudinida	18 (1.6)	3 (12)	6
Polychaeta	2 (0.2)	—	2
Others	452 (39.0)	86 (236)	216
B. Sequences not matched or matched insignificantly[b]	537 (46.3)	35 (107)	430
Total	1159 (100.0)	140 (496)	663
Total of ESTs		803	

[a] *E*-value $< 10^{-5}$
[b] *E*-value $> 10^{-5}$

Expression profiles in the anterior regenerates

Figure 1 shows the expression profiles of the genes identified in the anterior regenerates of the earthworm. Among 803 genes, while 663 ESTs (57.2%) were sequenced only once, 308 ESTs (26.6%) were sequenced 2 to 5 times, and 188 ESTs (16.2%) were sequenced more than 5 times. Considering that more than 57% of the ESTs exhibited a single appearance (Fig. 1) and that the 12 most highly expressed genes accounted for no more than 13% of all the transcripts analyzed (Table 2), the gene expression profile in the anterior regenerate was much less polarized than that in the midgut, at which the expression of the top 12 genes accounted for 21.2% of overall transcripts analyzed.[15] Nine of the 12 genes with the highest expression accounted for 9.9% of overall expression, and appeared to be related to energy production (Table 2). The classification system employed in Table 3 essentially divides the identified ESTs into three major categories, as previously established by Lee *et al.* 1999,[13] with the 803 genes belonging to these categories (Table 3, Supplemental Table 1 (Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site)): category A (AI-AIX), which consists of structural and enzymatic housekeeping proteins, contained 252 genes comprising 504 ESTs with a redundancy factor of 2.0 and accounted for 43.5% of all the transcripts analyzed; category B (BI-BIII), which consists of cell-cell communication molecules, contained 68 genes comprising 97 ESTs with a redundancy factor of 1.43 and accounted for 8.4% of all

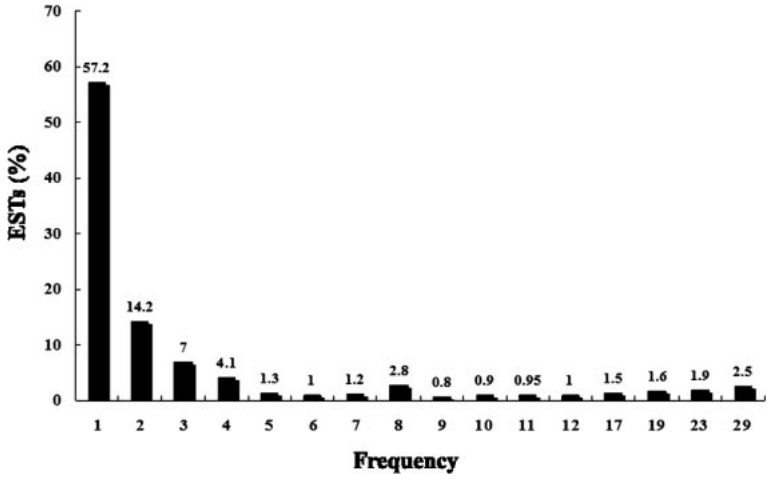

Fig. 1. Expression Profiles and Sequencing Redundancy in Analysis of ESTs from the Regenerating Tissue of the Earthworm *P. excavatus*. Of 1,159 ESTs analyzed, while 663 ESTs (57.2%) were sequenced only once, 308 ESTs (26.6%) appeared 2 to 5 times, and 188 ESTs (16.2%) were sequenced more than 5 times.

the transcripts analyzed; and category C, which consists of transcription factors or other genes, contained 18 genes comprising 21 ESTs and accounted for 1.8% of all the transcripts analyzed with a redundancy factor of 1.16. The remaining 465 genes comprising 537 ESTs (46.3%) did not significantly match any known genes.

Among the recognized protein-coding genes, gene category AVI, containing many typical mitochondrial genes, such as cytochrome c oxidase and NADH dehydrogenase subunits, appeared to be the largest, accounting for 11.6% of expression in the early regenerate. The gene categories related to protein synthesis (category AV, 11.3%), intracellular signal transduction molecules (category BII, 5.8%), transportation and binding proteins (category AI, 4.9%), and cytoskeleton and membrane proteins (category AIV, 4.9%) were surveyed. The redundancy factor indicates the frequency of repeated sequencing. Other categories had redundancy factors exceeding 2.0, including transportation and binding proteins (category AI, 2.59) and protein synthesis (category AV, 2.1) (Table 3). The top three genes with the high redundancy factor in category AI were ferritin, ATP synthase subunit 6, and hemerythrin, in that order. Ferritin, an iron-storing protein, appeared to account for about 1% all the transcripts analyzed. The gene of this protein is highly expressed in the midgut of the earthworm *Eisenia andrei* (1.5% of total transcripts analyzed),[15] and was considered to relate to globin formation. Hemerythrin is an invertebrate-specific non-heme-iron oxygen transport protein found in sipunculids, priapulids, brachiopods, and annelids.[16] Previous studies have shown that this protein is a cadmium scavenger[17] and an innate immune molecule defending against bacterial invasion due to its ability to bind oxygen and iron.[18] The high redundancy factor of category AV was attributable mainly to the high expression of genes encoding ribosomal proteins, accounting for 11.3% of overall expression (Table 3). We identified 34 60S ribosomal proteins and 19 40S ribosomal proteins, with redundancies of 2.1 and 2.6 respectively. Of the ribosomal proteins, L23 which is regarded as a cell growth regulator that activates p53 appeared at the highest frequency of 8,[19] and 21 ribosomal proteins were sequenced more than 3 times.

Table 2. The 12 Most Highly Expressed Genes in the Anterior Regenerate of the Earthworm *P. excavatus*

ESTs no. (Accession no.)	Number of clones (% frequency)	Putative identification
PER11247 (BP998571)	23 (1.98%)	Cytochrome c oxidase subunit II
PER10573 (BP998493)	19 (1.64%)	Cytochrome c oxidase subunit III
PER11114 (BP998556)	17 (1.47%)	Cytochrome c oxidase subunit I
PER10052 (BP998609)	12 (1.04%)	Ferritin
PER10555 (BP998621)	11 (0.95%)	ATP synthase F0 subunit 6
PER11038 (BP998650)	10 (0.86%)	Cytochrome b
PER11029 (BP998670)	9 (0.78%)	NADH dehydrogenase subunit 3
PER11141 (BP998679)	8 (0.69%)	Hemerythrin
PER10142 (BP998471)		Ribosomal protein L23
PER10233 (BP998687)		NADH dehydrogenase subunit 1
PER10049 (BP998632)		NADH dehydrogenase subunit 2
PER10168 (BP998640)	7 (0.6%)	Myosin regulatory light chain LC25

Expression patterns of some anterior regeneration-related genes

In order to determine the expression levels of regeneration-related genes during early anterior regeneration, we used sqRT-PCR with total RNA isolates of both intact tissue and regenerating fragments obtained at 0.5, 1, 3, 6, 12, 18, and 24 h after amputation (Fig. 2). The results of sqRT-PCR revealed that the expression levels of three genes (*fibropellin*, *Distal-less*, and *neuronal nicotinic acetylcholine receptor*) were highest in the regenerating fragments at about 3–18 h after amputation.

Notch is an essential gene encoding a signaling receptor that is required throughout development to regulate the spatial patterning, timing, and fate of cells in both vertebrates and invertebrates.[20,21] Recent studies have shown that during the eye development of *Drosophila*, *Notch* does not specify eye field identity by promoting homeotic genes, but instead influences eye

Table 3. Distribution of ESTs Analyzed Genes in the Anterior Regenerate of the Earthworm *P. excavatus*

	Classes of gene function and gene annotation		No. of ESTs	No. of genes	Redundancy factor	Expression (%)
	A. Functions that many kinds of cells use					
AI	Transportation and binding proteins for ions and other small molecules		57	22	2.59	4.9
AII	RNA processing, polymerizing, splicing, and binding proteins, and enzymes		25	21	1.19	2.1
AIII	Cell replication, histones, cyclins and kinase, DNA polymerase, DNA modification		9	7	1.29	0.8
AIV	Cytoskeleton and membrane proteins		57	33	1.73	4.9
AV	Protein synthesis co-factors and ribosomal proteins		130	62	2.1	11.3
AVI	Intermediary synthesis and catabolism enzymes		135	46	2.93	11.6
AVII	Stress response, detoxification, and cell defense proteins		45	26	1.73	3.9
AVIII	Protein degradation and processing, proteases		28	20	1.4	2.4
AIX	Transportation and binding proteins for proteins and other macromolecules		18	15	1.2	1.6
		Total	504	252	2	43.5
	B. Cell-cell communication					
BI	Signaling receptors and ligands		5	4	1.25	0.4
BII	Intercellular signal transduction pathway molecules, including kinase and signal intermediates		67	45	1.49	5.8
BIII	Extracellular matrix proteins and cell adhesion		25	19	1.32	2.2
		Total	97	68	1.43	8.4
	C. Transcription factors and other gene regulatory proteins					
CI	Sequence-specific DNA-binding proteins		16	14	1.07	1.4
CII	Non-DNA-binding proteins that had positive or negative roles		4	3	1.3	0.3
CIII	Chromatin proteins other than AIII with regulatory functions		1	1	1	0.1
		Total	21	18	1.16	1.8
	D. Not enough information to classify					
DI	Sequence not matched or matched insignificantly		537	465	1.15	46.3
		Total	537	465		46.3
	Total		1159	803		100

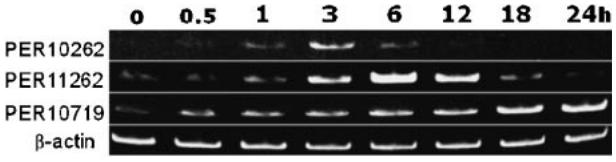

Fig. 2. Semi-Quantitative RT-PCR Analyses of *fibropellin* (PER10262), *distal-less* (PER11262), and *neuronal nicotinic acetylcholine receptor* (PER10719) mRNAs in Early Anterior Regeneration.

Total RNA was extracted at the indicated times. β-actin was used as a positive control. Primers and RT-PCR were carried out under conditions indicated in Supplemental Table 2 (Supplemental Table 2; see *Biosci. Biotechnol. Biochem.* Web site).

primordium formation by controlling cell proliferation.[22] We identified six contigs related to the EGF-like protein family, including *fibropellin*, *Notch* receptors, and their ligands, such as *DeltaD* and *Serrate* (category BII in Supplemental Table 1 (Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site)). A contig annotated as fibropellin (ESTs no. PER10262, accession no. BP998711), which is an EGF-repeat containing protein, exhibited an amino acid similarity higher than 45% to other EGF-related genes, including the *Notch* receptor previously established in other animal species, with an *E*-value of 6e-47 (Supplemental Table 1, Supplemental Fig. 1a (Supplemental Table 1, Fig. 1a; see *Biosci. Biotechnol. Biochem.* Web site)). It appeared that this EGF-repeat protein, which was not expressed in intact tissues (0 h), began to be expressed as soon as 1 h (Fig. 2) postamputation. This increased expression sharply peaked at 3 h, and then suddenly returned to control level at about 12 h after amputation (Fig. 2). Furthermore, there is evidence that *Notch* signaling can induce regeneration of the spinal cord in a frog tadpole,[23] and that in zebrafish, *Notch* expression is in these cases up-regulated in regenerating hearts and fins very early after amputation, suggesting that activation of the *Notch* signaling pathway precedes regenerations.[24] In the anterior regeneration of the earthworm, very early activation of a *Notch*-like receptor might be a regeneration-inducing pathway that precedes the regeneration process.

DNA-binding proteins are important transcription regulators that can induce a developmental process in which undifferentiated cells become specific cell types. In the regeneration of the hydra, a *Distal-less*-like homeobox gene, *cnox3*, is differentially expressed at maximal level at 2 d after cutting, suggesting that this gene plays a specific role in the regenerative processes.[25] In amphibian tail regeneration, expression of this gene appears to be localized to the muscle masses and the cells of the ependymal tube, which gives rise to part of the central nervous system, whereas in the adult these cells do not have such a function.[26] In addition, the expression pattern of *Dlx-3*, an amphibian homolog of *Distal-less*, in limb regeneration indicates a correlation between *Dlx-3* expression and the establishment of the outgrowth-permitting epidermis at a nerve-dependent stage.[27] The *Distal-less* gene is necessary for limb development, especially in determining its proximodistal patterning. Although the function of *Distal-less* in limbless animals has not been clearly established, the regeneration process carries the promise of elucidating the molecular significance of this gene during evolution and development. The present study identified the homeobox-containing DNA binding proteins *Distal-less* (ESTs no. PER11262, accession no. BP999002) (category CI in Supplemental Table 1 (Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site)). The EST annotated as *Distal-less* exhibited an amino

acid similarity higher than 75% to other *Distal-less* genes reported in other animal species (Supplemental Fig. 1b (Supplemental Fig. 1b; see *Biosci. Biotechnol. Biochem.* Web site)). During anterior regeneration of the earthworm, the expression of *Distal-less* appeared to be up-regulated within the first few hours, and reached a distinct peak at 6 h after amputation, followed by gradual decrease to the intact level (Fig. 2). Up-regulation of *Distal-less* expression occurred within 6 h of amputation, and it was maintained over the 24 h, which suggests involvement in the regenerative processes during early anterior regeneration.

Communication between the nervous system and the surrounding tissues is essential for regeneration in the earthworm[2] and amphibia.[28] There is accumulating evidence that neurotransmitters and their corresponding ligand-gated ion channel receptors can regulate the growth, differentiation, and plasticity of developing central nerve system neurons in vertebrates.[29] In earthworm anterior regeneration, a nicotinic receptor for acetylcholine (ESTs no. PER10719, accession no. BP999026) was identified (Supplemental Table 1, Supplemental Fig. 1c (Supplemental Table 1, Supplemental Fig. 1c; see *Biosci. Biotechnol. Biochem.* Web site)), and it appeared to be up-regulated within 30 min after amputation, and then gradually increment until the time examined (Fig. 2). This suggests that the chemical and electrical signaling mediated by this receptor play an important role in the early phase of earthworm anterior regeneration, including brain reformation. This is consistent with several lines of evidence that heterologous expression of this receptor is responsible for neurite outgrowth, synaptic plasticity, and neuronal development in mammals.[30,31]

Acknowledgments

This work was supported by grant no. R21-2005-000-10006-0 from the National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology.

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