RESEARCH ARTICLE

# Identification of Four *Engrailed* Genes in the Japanese Lamprey, *Lethenteron japonicum*

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**We have isolated four homologs of *Engrailed* genes from the Japanese lamprey, *Lethenteron japonicum*, an agnathan that occupies a critical phylogenic position between cephalochordates and gnathostomes. We named these four genes *LjEngrailedA, LjEngrailedB, LjEngrailedC*, and *LjEngrailedD*. *LjEngrailedA, LjEngrailedB*, and *LjEngrailedD* share a major expression domain in the presumptive midbrain–hindbrain boundary region of the central nervous system, although their levels and timing of expression differed. On the other hand, *LjEngrailedC* transcripts were in the pharyngeal ectoderm and the ventral ectoderm of the body wall. In addition, *LjEngrailedA* was expressed in the ventral side of the epibranchial muscle precursors. *LjEngrailedD* transcripts were seen in the mesodermal cells of the mandibular arch and later in a group of cells responsible for the formation of the upper lip, lower lip, and velum. Our results provide clues to the evolution of these structures as well as a possible scenario for duplication events of *Engrailed* genes. *Developmental Dynamics 237:1581–1589, 2008.* © 2008 Wiley-Liss, Inc.**

**Key words:** *Engrailed*; lamprey; homeodomains; midbrain; hindbrain; epibranchial muscle; mandibular arch

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## INTRODUCTION

The *Engrailed* (*En*) genes encode a highly conserved homeodomain-containing transcription factor and play pivotal roles in morphogenesis in invertebrate and vertebrate development (Gibert, 2002). In *Drosophila, engrailed* is known as a selector gene, as it is involved in the development of appendages and in the establishment of segments, whereby it specifies the posterior identity of each compartment (Garcia-Bellido and Santamaria, 1972; Lawrence and Morata, 1976). *En* homologs have been cloned from several protochordates (Holland et al., 1997; Imai et al., 2002) and many vertebrate species (Joyner et al., 1985). The cephalochordate *Branchiostoma floridae* and urochordate *Ciona intestinalis* are reported to have only a single *En* gene, whereas chick, mouse, and humans contain two members of the *En* family, *En-1* and *En-2* (Joyner et al., 1985; Joyner and Martin, 1987; Poole et al., 1989; Holland et al., 1997; Imai et al., 2002). Whole genome duplication prior to the origin of teleosts produced four *En* homologs in zebrafish: *eng1a* (renamed *eng1*), *eng1b, eng2a* (renamed *eng2*), and *eng2b* (renamed *eng3*) (Ekker et al., 1992; Force et al., 1999). Four *En* paralogues, *En-1A, En-1B, En-2A,* and *En-2B* have also been identified in the tetraploid *Xenopus laevis* (Holland and Williams, 1990; Hemmati-Brivanlou et al., 1991). In cephalochordates and vertebrates, segmentally reiterated *En* expression has been observed in the paraxial mesoderm (Davidson et al., 1988; Ekker et al., 1992; Hol-

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land et al., 1997). In amphioxus, metameric expression of *AmphiEn* in the forming somites was observed (Holland et al., 1997). Somitic *En-1* expression was also observed in the muscle pioneer-like cells in the primitive cartilaginous dogfish *Scyliorhinus canicula* (Tanaka et al., 2002). In zebrafish, *eng1a* and *eng2a* expression was also observed in a subset of muscle precursor cells in the myotomes, such as muscle pioneers, during somitogenesis (Ekker et al., 1992). A small cluster of mesodermal cells in the mandibular arch also express *eng2a* and *eng2b* during zebrafish embryogenesis (Ekker et al., 1992). These cells seem to be the jaw muscle precursor cells derived from the paraxial mesoderm (Hatta et al., 1990) and similar mandibular *En* expression is observed in *Xenopus laevis* (Hemmati-Brivanlou et al., 1991), chick (Gardner and Barald, 1992), and mouse (Logan et al., 1993). *En*-expression has been observed in the somites of chick and mice specifically in the dermis precursor cells (Davis et al., 1991; Gardner and Barald, 1992).

*En* expression is also observed in neurons of hemichordates and various chordates (Davis et al., 1991; Holland et al., 1997; Imai et al., 2002; Lowe et al., 2003). In the hemichordate *Saccoglossus kowalevski*, which lacks a centralized nervous system, *En* is strongly expressed in the anterior mesosome of the neurogenic ectoderm as a narrow single band (Lowe et al., 2003). In chordates, the ectoderm becomes partitioned into the neuroectoderm and the non-neural ectoderm (also named general ectoderm; Holland, 2005), both of which appear to be patterned along the anterior-posterior axis by a common mechanism (Holland, 2005). In amphioxus, *AmphiEn* is expressed in a small cluster of cells in the dorsal nerve cord and the cerebral vesicle, and it is transiently expressed in a band of cells of the general ectoderm (Holland et al., 1997; Holland, 2005). In *Ciona intestinalis*, *Ci-En* is restricted to two domains of the central nervous system during embryogenesis: the midbrain and the midbrain-hindbrain boundary (Imai et al., 2002). In zebrafish, *Xenopus*, chick, and mouse, *En* genes are expressed in a single broad stripe in the boundary between the presumptive midbrain and hindbrain of the neural epithelium (Davis et al., 1991; Ekker et al., 1992). Recent studies in mice showed that the development of the tectum (midbrain) and cerebellum (hindbrain) depends upon the dose of En proteins expressed in the midbrain-hindbrain boundary (Sgaier et al., 2007). In chick and mouse embryos, ectodermal expression of *En-1* is seen in the ventral compartment of the body epidermis at the pre-fin/limb bud stage and then in the ventral ectoderm and ventral apical ridge of limb buds where it positions limbs at the dorso-ventral boundary (Loomis et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Tanaka et al., 1998). This limb-positioning mechanism of En-1 appears to be also conserved in the primitive cartilaginous dogfish (Tanaka et al., 2002).

Because the above-mentioned regions of *En* expression, including muscle of the oral apparatus, tectum, and cerebellum, and function are major morphological innovations along the chordate lineage, examination of *En* expression in agnathan lampreys should provide further understanding of vertebrate evolution. As Vertebrates acquired the jaw after the origin of agnathan vertebrates, expression pattern of lamprey *En* genes in the pharyngeal regions should provide significant insights into the acquisition of the jaw in vertebrate evolution. The optic tectum and cerebellum are the novel morphological innovations in vertebrate evolution; thus delineating the expression patterns of the lamprey *En* genes should provide further understanding for the evolution of the subdivisions of the brain. Although the lamprey lacks paired fins, some agnathan fossils seem to have one set of paired fins, and these ancestral agnathan fish might have already had dorso-ventral compartmentalization. Thus, examination of *En* gene expression patterns in the body ectoderm of lamprey embryos could provide insight into the evolutionary process of these appendages (Tanaka et al., 2002).

In lampreys, only a single *En* sequence has been identified in *Lampetra fluviatilis* and *Petromyzon marinus* (Holland and Williams, 1990; Force et al., 1999). Indeed, the hagfish also has two *En* homologs, although they seem to have duplicated within the lineage (Holland and Williams, 1990). En protein distribution has been studied in the lamprey using the polyclonal antiserum *aEnhb-1* raised against mouse En protein (Holland et al., 1993). However, no *En* mRNA expression patterns have been described in lamprey during embryogenesis.

In this study, we obtained 3 novel *En* sequences *LjEnB, LjEnC*, and *LjEnD*, in a Japanese lamprey, *Lethenteron japonicum*. Moreover, the nucleotide sequence of the previously reported partial *LjEnA* gene (Takio et al., 2007) was extended and its expression was analayzed throughout embryogenesis. Phylogenetic and expression analyses provided valuable insights into the evolution of vertebrate novel morphologies, as well as a possible model of duplication events whereby *En* genes have undergone subfunctionalization of their roles.

## RESULTS

### Identification of *En* Sequences in the Lamprey *L. japonicum*

To identify *En* genes in the lamprey *L. japonicum*, we used an RT-PCR approach with stage-21 to -30 embryos using degenerate primers. This strategy led to amplification of several fragments of *En* genes. Three of them, which we named *LjEngrailedA* (*LjEnA*; Takio et al., 2007), *LjEngrailedB* (*LjEnB*), *LjEngrailedC* (*LjEnC*), were closely related to gnathostome *En* genes and were further characterized. We extended their sequences using a combination of 5′ and 3′ RACE to yield 873-, 784-, and 167-bp fragments, corresponding, respectively, to each gene. The ortholog of *LjEnC* was then extended 264 bp using primers against the *EnC* sequences from sea lamprey (*Petromyzon marinus*) retrieved from the NCBI Trace Archive database (http://www.ncbi.nlm.nih.gov/Traces/). The sequence of *LjEnC* was further extended using 3′ RACE to yield 661-bp fragments. An additional *En* gene, *P. marinus EnD* (*PmEnD*), was also found in this search. The ortholog of *PmEnD* in *L. japonicum, LjEnD* (402 bp), was isolated by genomic PCR using *PmEnD*-specific primers. Se-

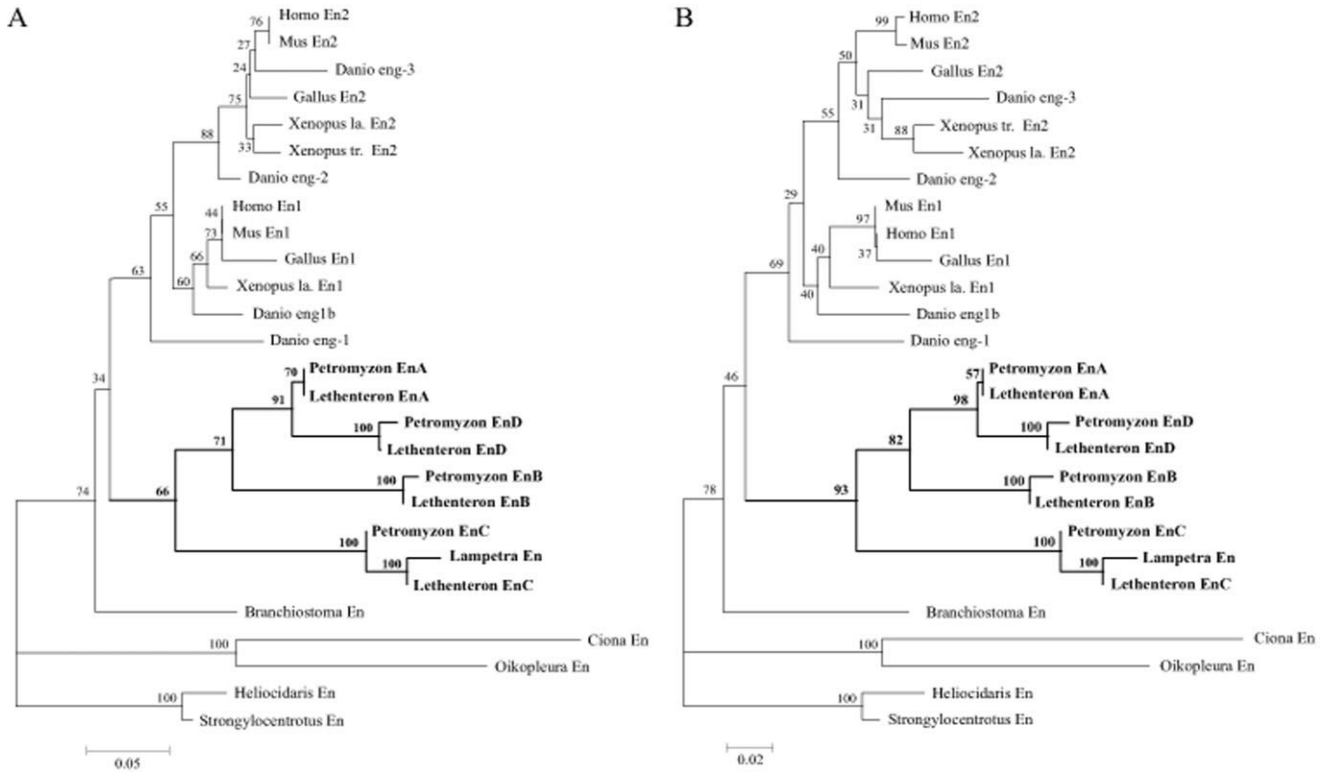


**Fig. 1.** Phylogenetic analysis of *L. japonicum* and *P. marinus En* genes. The NJ phylogenetic trees of the *En* genes constructed with the (**a**) amino acid (JTT model) and (**b**) nucleotide (first and second codon positions, Tamura-Nei model) sequences (see Experimental Procedures section). Only the conserved EH1–5 regions were used for both analyses. Numbers above branches represent bootstrap values with 10,000 replicates.

quence analysis of these fragments and comparison with the *En* gene from a variety of species showed that these four clones are orthologs of *engrailed* (Fig. 1a). Phylogenetic analysis was performed using four *En* gene sequences of *L. japonicum* with those of various chordates and sea urchins. The *En* sequences of sea lamprey and *P. marinus* retrieved from the NCBI Trace Archive database were also used for the analysis. NJ (neighbor-joining) trees were constructed with both amino acid and nucleotide (first and second codon positions) datasets. Interestingly, all the lamprey *En* genes are grouped together in both phylogenetic trees (Fig. 1).

In addition, to confirm the orthology among the eight *En* sequences in the lampreys, we performed a maximum-likelihood estimation of *Ks* (synonymous substitution rate) for the EH1-5 regions of the four *En* genes using the PAML 3.14 program. The *Ks* values calculated between *P. marinus* and *L. japonicum* are as follows: *Ks* (*EnA*) = 0.08, *Ks* (*EnB*) = 0.45, *Ks* (*EnC*) = 0.35, and *Ks* (*EnD*) = 0.18. In the previous study, *Ks* values have been calculated for several other orthologous genes between the two genera, and they ranges from 0.01 to 0.36 (Kuraku and Kuratani, 2006). Because the *Ks* values for *En* genes are similar to those of other genes, *PmEnA, PmEnB, PmEnC*, and *PmEnD* are probably orthologs of *LjEnA, LjEnB, LjEnC*, and *LjEnD*, respectively.

The tree topology within the lamprey clade is the same for the amino acid (Fig. 1a) and nucleotide (Fig. 1b) datasets. Among them, *PmEnA, PmEnB, PmEnC*, and *PmEnD* in *P. marinus* form sister relationships of *LjEnA, LjEnB, LjEnC*, and *LjEnD* in *L. japonica*, respectively. These results suggest that the four *En* genes were duplicated in the lamprey lineage before the divergence of *L. japonicum* and *P. marinus*.

## Expression of Lamprey *LjEn* Genes

Expression of *LjEnA* was examined in lamprey developmental stages 21–28. Staining was absent until stage 22, when faint expression first appeared in the neural tube at the presumptive border between the future midbrain and hindbrain (Takio et al., 2007; arrowheads in Fig. 2a,b). By stage 25, expression in the neural tube was more intensified (arrowheads in Fig. 2c,d). *LjEnA* was expressed in this region until at least stage 28 (Fig. 2e–n, q), the last embryonic stage examined. Additional expression was first observed in a group of rostral myotomes at stage 25 (arrows in Fig. 2c,d). By stage 25.5, the number of labelled myotomes increased and extended rostrally (arrows in Fig. 2e–g, o). By stage 27, *LjEnA* was expressed in the rostral end of the extended infraoptic muscle precursor cells (arrows in Fig. 2h–j, r) and remained expressed at least until stage 28 (arrows in Fig. 2k–m).

Expression of *LjEnB* first appeared at stage 22 in the neural tube at the future midbrain-hindbrain boundary (arrowheads in Fig. 3a,b). Expression in the neural tube persisted during subsequent stages, and became more intensified (arrowheads in Fig. 3c,d).

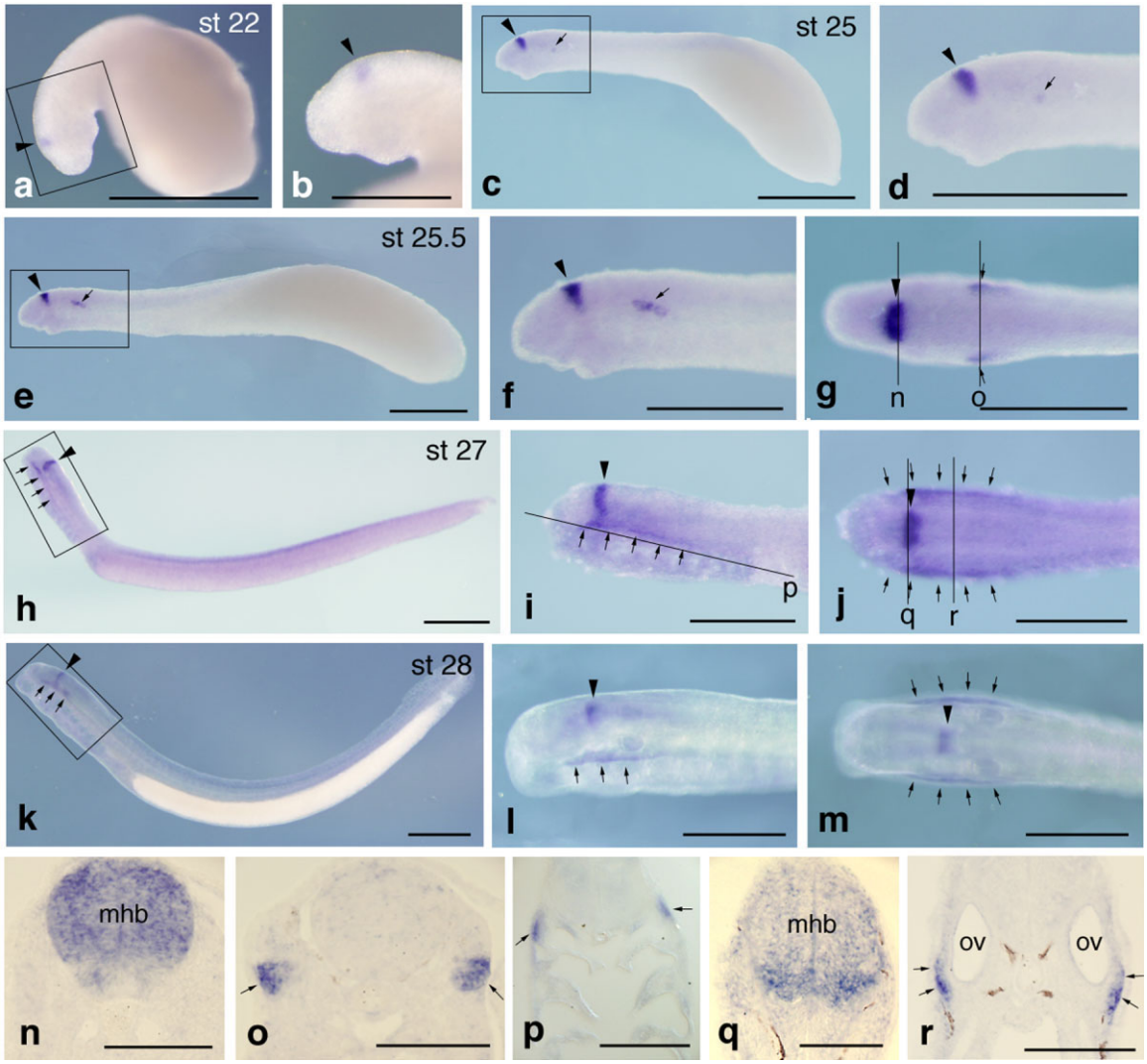


Fig. 2.

At stage 25, cells expressing *LjEnB* were detectable at the midbrain-hindbrain boundary (arrowheads in Fig. 3e–g). Weak *LjEnB* expression remained at the border between the midbrain and hindbrain at least until stage 28 (arrowheads in Fig. 3h–m).

Faint expression of *LjEnC* first appeared at stage 25 on the ventral side of the pharyngeal ectoderm (arrows in Fig. 4a–c). Expression of *LjEnC* was detected in the ectoderm over the pharyngeal arches at stage 26 (arrows in Fig. 4d–f). By stage 27, *LjEnC* expression intensified in the pharyngeal ectoderm (arrows in Fig. 4g–i, k) and was maintained until stage 28.5 (data not shown). By stage 29, *LjEnC* expression in the pharyngeal ectoderm was much lower (data not shown). On the ventral side of the body ectoderm,

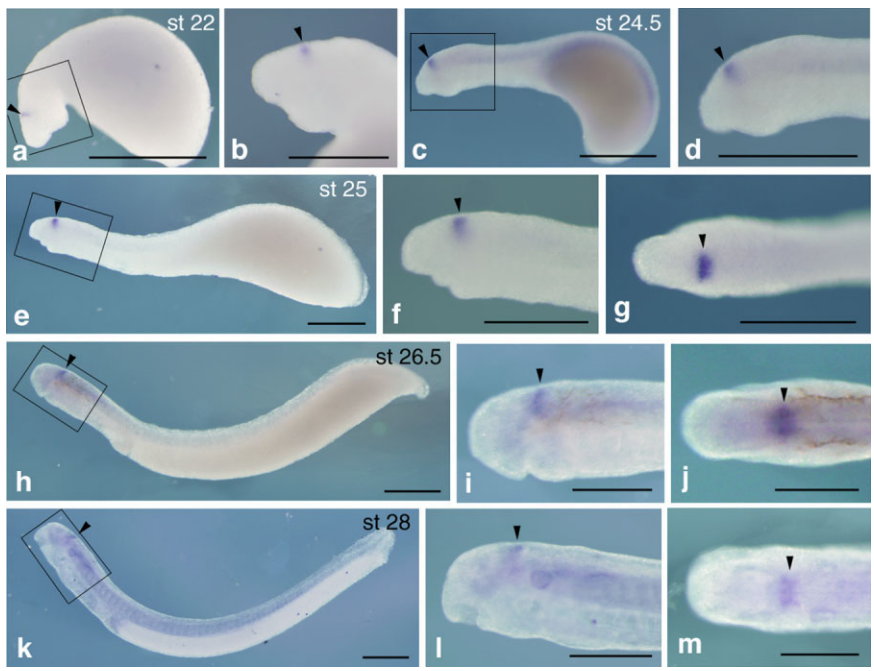


Fig. 3.

very faint *LjEnC* expression was first seen at stage 26 (Fig. 4d). These signals were seen from the heart level to the cloaca level (arrows in Fig. 4j, l,m), but no signals were detected in the ectoderm caudal to the cloaca (Fig. 4n). The *LjEnC* expression boundary is almost at the dorsal margin of somatopleure in the body ectoderm (arrows in Fig. 4j, l,m) and its expression persisted through stage 28 (data not shown).

*LjEnD* transcripts were first detected at stage 23 in a few cells of the mandibular arch and in the border of the presumptive midbrain-hindbrain region (Fig. 5a,b). At stage 25, the mesoderm of the mandibular arch organizes a band of cells on both sides of embryos (arrows in Fig. 5c,d) that persists through stage 25.5 (arrows in Fig. 5f–i). An increase in *LjEnD* expression was also observed in the wall of the prospective midbrain-hindbrain boundary at stage 25 (arrowheads in Fig. 5c–e) and was seen in the same domain until stage 25.5 (arrowheads in Fig. 5f,g). By stage 28, *LjEnD* expression was seen in the upper and lower lip mesoderm and in the velum, but expression in the neural tube was down-regulated (Fig. 5j,k). The weak staining observed in the caudal part of the body at stages 25 and 28 (Fig. 5c, j) appeared to be artefacts, as the sense probes were also trapped in the same region (data not shown).

## DISCUSSION

The present study is the first to demonstrate that the lamprey genome contains at least four *En* genes. Based on the phylogenetic results, it is likely that *LjEnC* diverged first, and subsequently another duplication produced *LjEnB*. The sister relationship of *LjEnA* and *LjEnD* is strongly supported. Therefore, at least three duplication events of *LjEn* genes seem to have occurred after divergence of the lamprey lineage from other vertebrates. It remains unclear whether the *LjEn* genes originated from chromosomal-scale duplications only from our results. However, given that multiple copies of *Hox* and related genes such as *Dlx* were found in the lamprey genome (Force et al., 1999; Neidert et al., 2001; Irvine et al., 2002; Takio et al., 2007), it is possible that the *LjEn* genes diverged by duplications involving those *Hox* cluster. Similar to previous analyses of other lamprey genes (Ueki et al., 1998; Ogasawara et al., 2000; Myojin et al., 2001), the phylogenetic tree of lamprey *En* genes does not provide enough resolution, especially when, and in which animal lineages, the duplication events of the analyzed genes took place. In order to resolve this, further genomic analyses by comparison of various gene cognates as well as synteny analyses are required in the future.

Neural expression of an *En* homolog has been observed as two distinct stripes in the central nervous system of *Ciona intestinalis*, in a few cells of the cerebral vesicle of amphioxus, and at the midbrain-hindbrain boundary of zebrafish, *Xenopus*, chick, and mouse (Davis et al., 1991; Ekker et al., 1992; Holland et al., 1997; Imai et al., 2002). The major neural expression domain of *LjEnA* (Takio et al., 2007), *LjEnB*, and *LjEnD* is the midbrain-hindbrain boundary, as previously shown by using a polyclonal antiserum (α*Enhb-1*) raised against mouse En protein in *L. japonicum* (Holland et al., 1993). Thus, *En* expression in the central nervous system appears to be one of shared features of all the vertebrates (Takio et at., 2007; see Fig. 6). Redundancy in the function of *En* genes in the midbrain-hindbrain boundary has been demonstrated in mice by the complete absence of the tectum and cerebellum in *En-1;En-2* double knockout mice (Liu and Joyner, 2001). Although *En-1* and *En-2* have overlapping expression patterns, their levels and timing of expression differ in various organisms (Ekker et al., 1992; Wurst et al., 1994). For example, in mice, *En-1* is first expressed in the midbrain-hindbrain boundary 12 hr earlier than *En-2* and this earlier expression of *En-1* is required for the initial establishment of the midbrain-hindbrain region (Wurst et al., 1994). Furthermore, recent studies in *En* conditional knock-in and knock-out mice showed that En proteins subdivide the tectum and the cerebellum into distinct domains in a dose-dependent manner (Sgaier et al., 2007). Lamprey *En* genes *LjEnA* and *LjEnB* became detectable in the midbrain-hindbrain boundary at stage 22 and continued to be expressed in the same region until at least stage 28, whereas *LjEnD* transcripts were first detected in a few cells of neuroepithelium at stage 23 and disappeared by stage 26. Our results suggest that agnathan lampreys, which have multiple subdivided brains, have already acquired the same fundamental mechanisms of partitioning the midbrain and the hindbrain as gnathostomes.

Despite conserved expression of *En* in the midbrain-hindbrain boundary among chordates (Holland, 2005), *LjEnC*, a homolog of an *En g*ene identified in *Lampetra planeri* (Holland and Williams, 1990), was not expressed in the central nervous system, but rather was expressed in the ectoderm of pharyngeal arches and the body wall (Fig. 4). In chordates, the ectoderm is partitioned into the neuroectoderm and the general ectoderm during gastrulation (Holland, 2005). Although the number of neurons seems to have decreased gradually in the general ectoderm during chordate evolution, several genes known to be involved in the patterning of the cen-

**Fig. 2.** Expression pattern of *LjEnA* during embryonic development. **a, c, e, h, k:** Lateral view of stage 22, 25, 25.5, 27, and 28 embryos, respectively. **b, d, f, i, l:** Higher magnification of the head region of the photographs shown in a, c, e, h, and k, respectively. **g, j, m:** Dorsal view of the head region of the photographs shown in e, h, and k. **n, o:** Transverse sections of a stage-25.5 embryo at the levels indicated by the lines in g. **p:** A horizontal section of a stage-27 embryo at the level indicated by the line in i. **q, r:** Transverse sections of a stage-27 embryo at the levels indicated by the lines in j. The hybridization signals are seen in the midbrain-hindbrain boundary (arrowheads) and epibranchial muscles (arrows). mhb, midbrain-hindbrain boundary; ov, otic vesicle. Scale bars = 1 mm (a), 500 μm (b–m), 100 μm (n–r).

**Fig. 3.** Expression pattern of *LjEnB* during embryonic development. **a, c, e, h, k:** Lateral view of stage 22, 24.5, 25, 26.5, and 28 embryos, respectively. **b, d, f, i, l:** Higher magnification of the head region of the photographs shown in a, c, e, h, and k, respectively. **g, j, m:** Dorsal view of the head region of the photographs shown in e, h, and k. The hybridization signals are seen in the midbrain-hindbrain boundary (arrowheads). Scale bars = 1 mm (a), 500 μm (b–m).

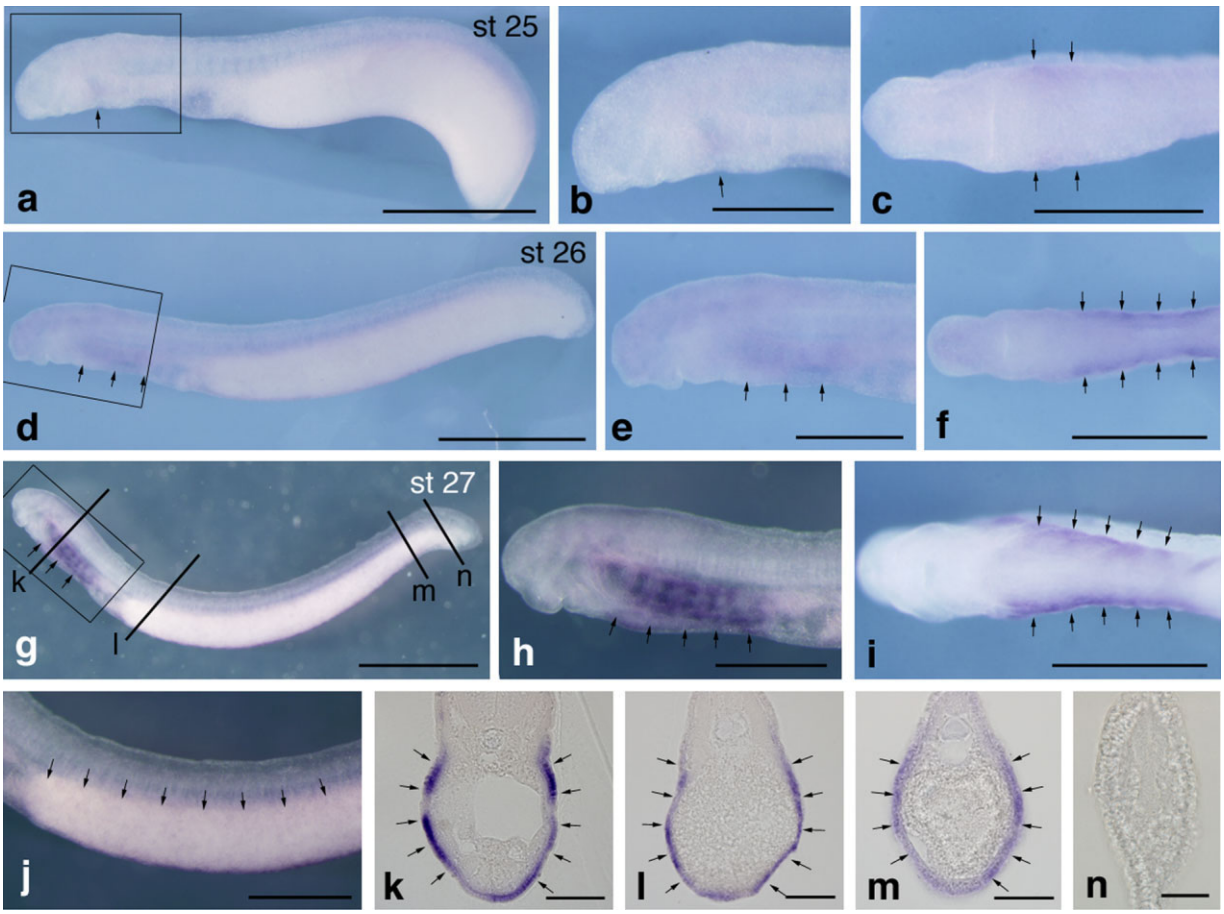


**Fig. 4.** Expression pattern of *LjEnC* during embryonic development. **a, d, g:** Lateral view of stage 25, 26, and 27 embryos, respectively. **b, e, h:** Higher magnification of the head region of the photographs shown in a, d, g. **c, f, i:** Ventral view of the head region of the photographs shown in a, d, and g. **j:** Lateral view of the trunk region of the photograph shown in g. **k–n:** Transverse sections of a stage-27 embryo at the levels indicated by the lines in g. The hybridization signals are seen in the pharyngeal ectoderm and the ectoderm of the ventral side of body (arrows). Scale bars = 500 μm.

tral nervous system are regionally expressed in the general ectoderm (Holland, 2005). In amphioxus, *En* as well as *Distal-less* and *Pax6* are expressed in both the neuroectoderm and the general ectoderm (Holland et al., 1996, 1997; Glardon et al., 1998). Interestingly, *LjOtxA* is expressed in the pharyngeal ectoderm of lamprey *Lethenteron japonicum*, as is *LjEnC* (Murakami et al., 2001). *LjEnC* expression was also observed in the ectoderm of the ventral side of the body wall (Fig. 4). In chick, mouse and dogfish embryos, *En-1* is expressed in the ectoderm of the ventral side of the body from the forelimb/pectoral fin level to the hindlimb/pelvic fin/cloaca level (Loomis et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Tanaka et al., 1998, 2002). The ectodermal expression of *En-1* in the body wall is critical for the positioning of the apical ectodermal ridge of the

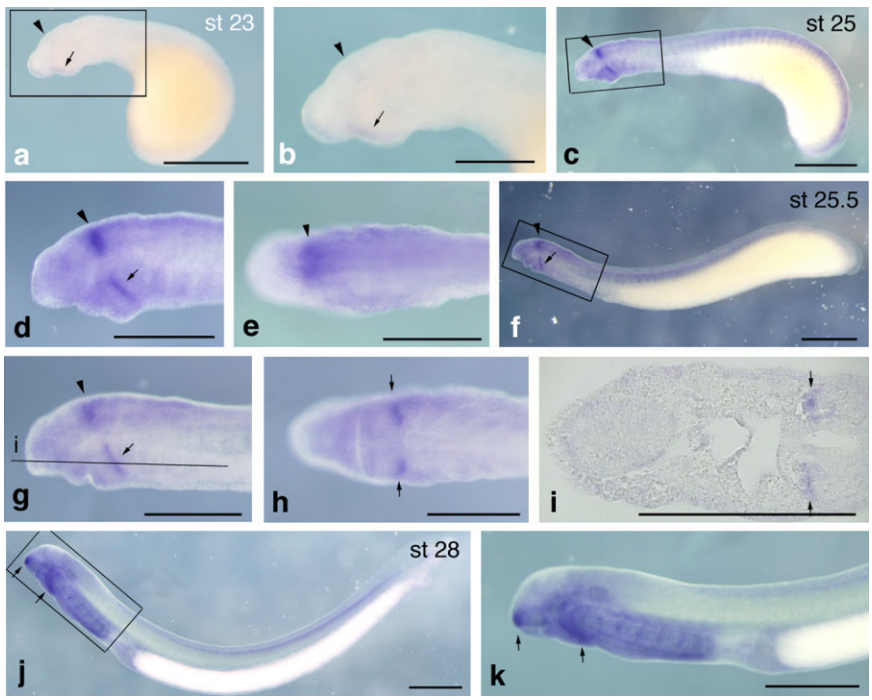


**Fig. 5.**

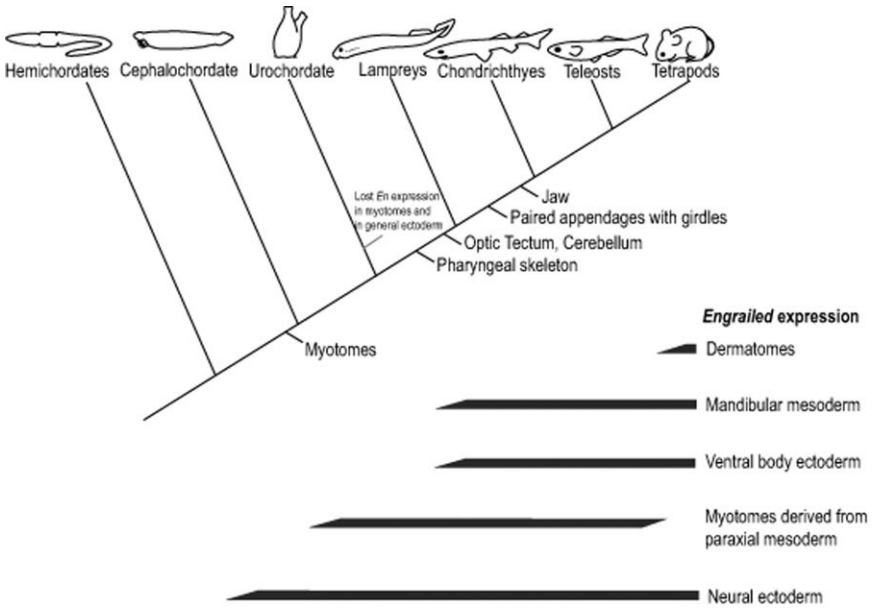


**Fig. 6.** Hypothetical scenario for the evolution of *En* expression. Phylogenetic tree indicating the probable timing of acquisition of the morphological characteristics in relation to different expression patterns for *En* during the developmental process (bold line). Hemichordates lack a central nervous system (CNS), but the entire ectoderm is neural. Expression of *En* is seen in the general ectoderm in hemichordate (Holland, 2005), which contains sensory cells. In chordates, *En* expression is seen both in the CNS and in the general ectoderm. *En* transcripts are seen in certain varieties of myotomes derived from the paraxial mesoderm. Expression of *En* is observed in the developing somites of the cephalochordates, amphioxus. In lampreys, *En* transcripts are observed in myoblasts derived from the first somites. In jawed fishes, *En*-positive myoblasts are observed in the muscle pioneer-like cells, whereas, in tetrapods, *En*-positive cells are seen in the dermatomes. Expression of *En* on the ventral side of the ectoderm of the body and limbs is conserved among jawed vertebrates, and similar ecotodermal *En* expression is observed in the ventral side of the body of lampreys. *En*-positive mesodermal cells are also observed within the mandibular arch of lampreys, as in gnathostomes; these cells differentiate into the muscles of the oral apparatus.

limb buds (Loomis et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Tanaka et al., 1998) and has only been observed in limbed vertebrates. The ectodermal expression of *LjEnC* may not correspond to that of *En-1* in limbed vertebrates because the *LjEnC* expression boundary is almost at the margin of somatopleure (Fig. 4l); however, the fact that the body ectoderm of the limbless agnathan has already acquired the *En* expression domain supports the view that the body ectoderm of ancestral agnathans may have been compartmentalized dorso-ventrally with *En* expression ventrally (Tanaka et al., 2002).

Transcripts of *LjEnA* were seen in a small cluster of myotomes in the first somite and later in the elongated infraoptic muscle precursors (Fig. 2). In the lamprey head, there are two sets of preotically located myotomes, such as supraoptic and infraoptic muscles. Although these myotomes were first identified as derivatives of the cephalic mesoderm, they arise from postotic myotomes that are secondarily shifted rostrally into the preotic region (Kuratani et al., 1999; Kusakabe et al., 2004; Kusakabe and Kuratani, 2007). Expression of *LjEnA* supports these previous conclusions that the infraoptic myotomes arise from the postotic somites (Fig. 2d–k). No similar expression pattern of En cognates has been found in other vertebrate embryos, and its developmental and morphological significance remains to be clarified.

Transcripts of *LjEnD* were observed in the mesodermal component of the mandibular arch (Fig. 5), which was almost identical to the distribution of the cells in *L. japonicum* labelled with a polyclonal antiserum, α*Enhb-1*, raised against mouse En protein (Holland et al., 1993). En proteins have been found in the mandibular arch myoblasts of zebrafish, *Xenopus*, chick, and mouse during embryogenesis (Hatta et al., 1990; Davis et al., 1991; Hemmati-Brivanlou et al., 1991). Morphological homologies of these muscles, however, remain unclear (Lubosch, 1938). In the lamprey, the developmental fate of the mandibular arch mesoderm has previously been described (Kuratani et al., 2004); vital dye-labeling into the lamprey mandibular mesoderm resulted in the labelling of upper lip, lower lip, and velar muscles, as well as the trabecular primordium (Kuratani et al., 2004) and the expression pattern of the muscle actin gene, *LjMA2*, suggested that myoblasts of the mandibular arch of lampreys migrated into the premandibular domain (Kuratani et al., 2004; Kusakabe and Kuratani, 2005). Although the *En*-expression in the mandibular arch muscles in gnathostomes and in lampreys functions in neuromuscular target recognition (Hatta et al., 1990; Holland et al., 1993), consistent with the innervation by the maxillomandibular portion of the trigeminal nerves (Kuratani et al., 2004), different subsets of the mandibular arch may express *En* genes; *En*-positive myoblasts differentiate into the masseter, temporalis, and pterygoid muscles in amniotes (Degenhardt and Sassoon, 2001), and into the levator arcus palatini and the dilator operculi muscles in zebrafish (Hatta et al., 1990). Taken together, *En* expression in the mandibular mesoderm appears to function in the specification of the mandibular arch muscle primordium among the branchiomerie, especially at early stages of development. Thus, consistent with the shared identity of the mandibular arch between gnathostomes and lampreys (Takio et al., 2004), expression of *En* in the mandibular mesoderm appears to have been

**Fig. 5.** Expression pattern of *LjEnD* during embryonic development. **a, c, f, j:** Lateral view of stage 23, 25, 25.5, and 28 embryos, respectively. **b, d, e, g, h, k:** Higher magnifications of the head region of the photographs shown in a, c, f, and j. b, d, g, k: Lateral view. e: Dorsal view. h: Ventral view. **i:** A horizontal section of a stage-25.5 embryo at the level indicated by the line in g. The hybridization signals are seen in the midbrain-hindbrain boundary (arrowheads) and mandibular regions (arrows). Scale bars = 500 μm (a, c–k), 200 μm (b).

Developmental Dynamics

established in the common ancestor of vertebrates (Fig. 6).

## EXPERIMENTAL PROCEDURES

### Embryo Collection

Adult male and female lampreys (*Lethenteron japonicum*) were purchased from Ebetsu fishery cooperative, Hokkaido, Japan, during the breeding season (early June). Spawning was induced, and embryos were reared to the desired developmental stages at 16°C in 10% Steinberg's solution (Steinberg, 1957). Lamprey embryos were staged according to Tahara's staging of *Lethenteron reissneri*, a species closely related to *L. japonicum* (Tahara, 1988). For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), then dehydrated in a graded methanol series and stored in 100% methanol at −20°C.

### Isolation of *En* cDNA Fragments From Lampreys

Total RNA extraction was performed on embryos from stages 21 to 30 using the RNAeasy kit (QIAGEN). First-strand cDNA was obtained by reverse transcription and used as a template for PCR reactions. The Japanese lamprey (*L. japonicum*) *LjEnA* partial sequence has previously been reported (Takio et al., 2007). *LjEnB* and *LjEnC* cDNA fragments were amplified using degenerate primers in forward (sequence encoding RYITEQRR or NFFIENIL) and reverse (WFQNKRAK, AQGLYNH, or WPAWVYCT) orientations. Isolated fragments were subcloned into the pGEMTeasy vector (Invitrogen). Three cDNAs closely related to the gnathostome En genes were further characterized. 5′ and 3′ RACE using the First Choice RLM-RACE kit (Ambion) were performed to obtain longer fragments. A large amount of genomic sequence data from sea lamprey (*Petromyzon marinus*) is available in the NCBI Trace Archive database (http://www.ncbi.nlm.nih.gov/Traces/). A local BLAST search (blastn and tblastn) was performed against *P. marinus* (~14 G bases) using the three *L. japonicum En* sequences as queries, and the orthologs of *LjEnA, LjEnB*, and *Lj EnC* were obtained. This research allowed us to extend the sequence of *LjEnC* using primers in forward (GGGGGD) and reverse (AQGLYNH) orientations. We then performed 3′ RACE to obtain longer fragments of *LjEnC*. An additional *En* gene (*PmEnD*) was also found in this search. The ortholog of *PmEnD* in *L. japonicum* (*LjEnD*) was isolated by genomic PCR using primers in forward (CRLRAEF) and reverse (AQGLYNH) orientations. The *LjEnA* (873 bp), *LjEnB* (784 bp), *LjEnC* (661 bp), and *LjEnD* (402 bp) partial coding sequences have been submitted to GenBank under accession numbers **EF546817, EF546814, EF546815**, and **EF546816**, respectively.

### Phylogenetic Analysis

In addition to lamprey, *En* genes from sea urchins (*Heliocidaris tuberculata* [U58775] and *Strongylocentrotus purpuratus* [XM_001189948]), tunicates (*Oikopleura dioica* [AY870647] and *Ciona savignyi* [AF518004]), amphioxus (*Branchiostoma floridae* [U82487]), and En-1 and En-2 in gnathostomes (*Homo sapiens, Mus musculus, Gallus gallus, Xenopus laevis, Xenopus tropicalis*, and *Danio rerio*) were used for phylogenetic reconstruction. Amino acid sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters. The En homology domains (EH1–5; Logan et al., 1992) were used for the phylogenetic analysis because other regions show high sequence heterogeneity. The Neighbor-Joining (NJ) phylogenetic trees of amino acid and nucleotide (first and second codon positions) datasets were constructed with JTT and Tamura-Nei models, respectively, by a MEGA3.1 program (Kumar et al., 2004). Bootstrap analysis was performed with 10,000 replicates for both trees.

### In Situ Hybridization

Whole-mount in situ hybridization was performed using protocols modified from Murakami et al. (2001). Embryos were dechorionated and then bleached by a series of 50, 100, 50% ethanol in PBT (PBS with 0.1 % Tween 20), and washed several times in PBT. Embryos were then cleared in 50% glycerol in PBT. Selected embryos were processed for frozen sections by dehydration in 30% sucrose in PBS followed by embedding in 7.5% gelatin in 15% sucrose for cryosections (15 μm).

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