Among the transcription factor gene families, Pax genes play important and unique roles in morphological patterning of animal body plans. Of these, Group I Pax genes (Pax1 and Pax9) are expressed in the endodermal pharyngeal pouches in many groups of deuterostomes, and vertebrates seem to have acquired more extensive expression domains in embryos. To understand the evolution of Pax1/Pax9-related genes in basal groups of vertebrates, their cognates were isolated from the Japanese marine lamprey, Lampetra japonica. RT-PCR of larval lamprey cDNA yielded two different fragments containing vertebrate Pax1- and Pax9-like paired domains. The Pax9 orthologue was isolated and named LjPax9. Whole-mount in situ hybridization revealed that this gene was expressed in endodermal pharyngeal pouches, mesenchyme of the velum (the oral pumping apparatus) and the hyoid arch, and the nasohypophysial plate, but not in the somitic mesoderm of the lamprey embryo. These expression patterns could be regarded as a link between the basal chordates and the gnathostomes and are consistent with the phylogenetic position of the lamprey. Especially, the appearance of neural crest seemed to be the basis of velar expression. Homology of the velum and the jaw is also discussed based on the LjPax9 expression in the first pharyngeal pouch and in the velar mesenchyme. We conclude that Pax9 genes have sequentially expanded into new expression domains through evolution as more complicated body plans emerged. © 2000 Academic Press

Key Words: Pax genes; lamprey; embryo; pharynx; endoderm; evolution.

INTRODUCTION

The genes of the Pax family encode transcription factors containing a paired domain and are involved in various aspects of animal morphogenesis (reviewed by Goulding, 1992; Gruss and Walther, 1992; Strachan and Read, 1994; St-Onge et al., 1995; Dahl et al., 1997). Nine members of this gene family are recognized in mammals, and these can be further classified into four subgroups (Groups I to IV) based on structural features and amino acid sequences. This suggests hierarchical duplications in the evolutionary history of this family, providing an ideal topic for studies of evolutionary developmental biology.

In vertebrates, Pax genes occupy a central position in developmental cascades to pattern the body plan. For example, Pax3, Pax7 (Group III), and Pax6 (Group IV) genes are involved in regional specification of the neural tube (Walther and Gruss, 1991; Jostes et al., 1991; Goulding et al., 1993; Conway et al., 1997; Ericson et al., 1997; reviewed by Chalepakis et al., 1993), Pax2, -5, and -8 (Group II) genes are expressed in the mid-hindbrain boundary as isthmic organizer components in anteroposterior patterning of the neuraxis (Asano and Gruss, 1992), and many of the Pax family members also function in establishment of a number...
of specific cell lineages and in organogenesis (Koseki et al., 1993; Barnes et al., 1996; Wallin et al., 1994).

Comparative analyses of Pax gene family expression patterns have suggested that the developmental functions of each orthologue are rather well conserved within vertebrates (Krauss et al., 1991; Püschel et al., 1992; Glardon et al., 1997, 1998). In larger scale evolution including changes in the body plan, however, regulatory genes usually have acquired additional expression domains in crown groups that exhibit more complex body architecture (Wada et al., 1998). Of the Pax genes, Pax1 and -9 (Group I) are expressed in pharyngeal slits of vertebrate embryos (Wallin et al., 1996; Müller et al., 1996), a pattern that appears to be conserved in distantly related animal embryos belonging to the deuterostomes (Holland and Holland, 1995; Ogasawara et al., 1999). Similar expression patterns are retained in the urochordates (Ciona intestinalis; Ogasawara et al., 1996) and cephalochordates (Holland and Holland, 1995, 1996), in which branchial arches show diverse patterns of development. A Pax1/Pax9 orthologue was also found in a hemichordate, Ptychodera flava, and its expression in pharyngeal slits supports the homology of gill slits of this animal with those in chordates (see Ogasawara et al., 1999). In vertebrates, however, the gene expression domains include more structures than in other animal groups, such as the somite-derived skeletogenic mesoderm, limb bud, mandibular arch, and pharyngeal pouch and its derivatives, for which these genes are involved in organogenesis (Hofmann et al., 1998; Peters et al., 1998; Henderson et al., 1999). In this line of evolutionary studies, the expression of the Pax1/Pax9 group in the embryonic lamprey is now a missing link. Of particular interest is the number of genes or history of duplication of the genes, since only gnathostomes are known to possess two members in Group I (Dahl et al., 1997).

Agnathans are regarded as a basal group of the vertebrate lineage and show a typical bilateral pattern of pharyngeal pouch development specific to vertebrates. In the present study, we isolated members of the Pax1/Pax9 gene family in a Japanese marine lamprey, Lampetra japonica, and examined expression patterns of the putative orthologue of the Pax9 gene in a series of developing embryos.

**MATERIALS AND METHODS**

**Embryos.** Adult male and female lampreys (L. japonica) were collected in a tributary of the Miiomote River, Niigata, Japan, during the breeding season (late May through June). The eggs were artificially fertilized and kept in fresh water at 18°C or in 10% Steinberg’s solution (Steinberg, 1957) below 23°C. Embryos were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS). Since embryological development is easily affected by temperature, they were staged morphologically according to the table of Tahara (1988) for L. reissneri, a brook species of L. japonica.

**Construction of cDNA libraries and isolation of cDNA clones for lamprey Pax1/Pax9-related gene.** Total RNA was extracted from the head region of larval L. japonica by the AGPC method (Chomczynski and Sacchi, 1987). Poly(A)^+ RNA was purified using Oligotex dT 30 beads (Roche, Tokyo, Japan). Complementary DNA was synthesized and constructed as described previously (Ogasawara et al., 1996).

A L. japonica larval head cDNA library was constructed using the Uni-ZAP II vector (Stratagene). The sense-strand oligonucleotide primer Pax-unif, 5'-GTNAACTCAAGCCTTN-GNGGGNTNTT-3', which corresponds to the amino acid sequence VNQLGGVF, and the antisense oligonucleotide primer Pax-R3, 5'-AAGCGTGAGTTCGTCTTCCCAAGCG-3', which corresponds to the amino acid sequence FAWEYRDKL, were synthesized based on conserved paired domains from Caenorhabditis elegans, Drosophila, tunicates, amphioxus, and vertebrates. Using the poly(A)^+ RNA extracted from the head region of larval lamprey, target fragments were amplified by RT-PCR and two types of PCR products were obtained. These PCR fragments were randomly labeled with [32P]dCTP (Amersham, Buckingham, UK), and 5.5 x 10^6 phages of a cDNA library were screened by hybridization in 6x SSPE, 0.1% SDS, 1x Denhardt's solution, 50% formamide at 42°C for 16 h and washing in 2x SSC - 0.1% SDS at 50°C for 30 min, 1x SSC - 0.1% SDS at 50°C for 30 min, 0.1x SSC - 0.1% SDS at 50°C for 30 min. Isolated clones were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Roche, NJ).

**Sequences comparisons and molecular phylogenetic analyses.** Sequences were aligned using the Seqxppl 1.9 manual aligner for Macintosh (Gilbert, 1993). Phylogenetic analyses were performed on the amino acid sequences of the paired domain. Estimation of molecular phylogeny was carried out by the neighbor-joining method (Saitou and Nei, 1987) using the Clustal V (Higgins et al., 1992) program. Confidence in the phylogeny was assessed by bootstrap resampling of the data (x>100) (Felsenstein, 1985).

**Northern blotting analyses.** Total RNA was isolated by the AGPC method (Chomczynski and Sacchi, 1987), and poly(A)^+ RNA was purified with Oligotex dT 30 beads. Northern blot hybridization was carried out using standard procedures (Sambrook et al., 1989), and filters were washed under high-stringency conditions (hybridization, 6x SSPE, 0.1% SDS, 1x Denhardt's solution, 50% formamide at 42°C for 16 h; washing, 2x SSC - 0.1% SDS at 60°C for 30 min, 1x SSC - 0.1% SDS at 60°C for 30 min, 0.1x SSC - 0.1% SDS at 60°C for 30 min). Entire regions of CDNA s were labeled with [32P]dCTP using a random prime labeling kit (Boehringer Mannheim, Indianapolis, IN) for hybridization probes.

**Genomic Southern analyses.** High-molecular weight genomic DNA of L. japonica was extracted from a single adult by the standard procedure (Sambrook et al, 1989). After exhaustive digestion with EcoRI, HincII, SalI, and BglII, and 1% agarose gel electrophoresis, the DNA fragments were blotted onto Hybond-N + nylon membranes (Amersham). The blots were hybridized with random-primed [32P]dCTP using a random prime labeling kit (Boehringer Mannheim, Indianapolis, IN) for hybridization probes.
8.0, 1 mM EDTA, 150 mM NaCl). The samples were then postfixed and incubated in hybridization buffer (50% formamide, 5× SSC, 1% SDS, 50 μg/ml yeast tRNA, 50 μg/ml heparin sulfate, 0.1% Chaps, 5 mM EDTA, 1% blocking solution (Boehringer Mannheim)). Hybridization was performed in freshly prepared hybridization buffer containing 2.0 μg/ml probe for overnight (O/N) at 70°C.

After hybridization, the specimens were washed in solution 1 (50% formamide, 5× SSC, 1% SDS) for 30 min at 70°C and the solution was substituted gradually by solution 2 (0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, 0.1% Tween 20). RNase A was added at a final concentration of 50 μg/ml in solution 2 and incubated for 30 min at room temperature. The embryos were then washed twice for 30 min each in 50% formamide, 2× SSC at 70°C, and once in 0.2× SSC/0.3% Chaps at 70°C. For immunological detection of digoxigenin epitopes, the embryos were blocked with blocking solution for 60 min before incubation with 1:4000-diluted anti-digoxigenin–APFab fragments (Boehringer Mannheim) at 4°C for O/N. The specimens were then washed five times for 1–2 h each time and O/N in washing buffer (Boehringer Mannheim) at room temperature. Alkaline phosphatase activity was detected with NBT/BCIP in detection buffer (Boehringer Mannheim). The stained specimens were postfixed in PFA/PBS and photographed. Stage 27 embryos were embedded in Technovit 8100 (Heraeus Kulzer, Friedrichsdorf, Germany) and sectioned at a thickness of 7 μm.

Some of the stained embryos were dissected under the microscope for observation of the mesodermal expressions.

RESULTS

Pax1/Pax9-Related Genes in L. japonica

RT-PCR using larval lamprey cDNA and degenerate oligonucleotide primers corresponding to the conserved paired domain sequences yielded two types of clones containing a 296-bp PCR fragment encoding the Pax1/Pax9-related paired domain. Comparison of the nucleotide sequences of these PCR fragments revealed that these sequences were 94.7% identical (Fig. 1). The most remarkable difference was an in-frame triple-nucleotide displacement of GAC to ACG (Fig. 1) causing an amino acid change from asparagine, characteristic of Pax1, to threonine, characteristic of Pax9 (cf. Fig. 4A). Molecular phylogenetic analyses by the neighbor-joining method based on the amino acid sequences encoded by these fragments revealed that they were Pax1-like and Pax9-like paired domains (not shown): the clone containing the Pax1-like paired domain was named pLjP1-PCRF and that containing the Pax9-like paired domain was named pLjP9-PCRF. One of the most distinctive differences was the presence of a HinClI site in pLjP9-PCRF, which was absent in pLjP1-PCRF (Fig. 1). The HinClI site in pLjP9-PCRF was confirmed by sequencing of the Pax9 cDNA clone and also by Southern blotting analyses (see below).

Genomic Southern blotting analyses using the 32P-labeled pLjP1-PCRF fragment encoding Pax1-like paired domain as a probe detected two to three bands in the lanes of the EcoRI (about 5.8 and 4.3 kb), HinClI (about 7.2, 2.2, and 1.5 kb), SalI (about 14.0, 8.6, and 1.8 kb), and BglII (about 13.0 and 4.9 kb) digests (Fig. 2A). When we used the pLjP9-PCRF fragment as a probe, the results of genomic Southern blotting were the same as those obtained with the Pax1-like paired domain (Fig. 2B). When we used the pLjP9-PCRF fragment as a probe, the results of genomic Southern blotting were the same as those obtained with the Pax1-like probe (not shown). These results suggested that the nucleotide sequences of paired domains were very similar, and the probes cross-reacted and both genes were detected. Northern blotting analyses using a 32P-labeled PCR fragment containing the paired domain detected a slightly broad signal of about 3.4 kb (Fig. 2B).

Isolation and Characterization of L. japonica Pax1/Pax9-Related Gene of cDNA Clones

Using pLjP1-PCRF and pLjP9-PCRF as probes, we screened a larval lamprey cDNA library and found eight
cDNA clones. Sequencing of these clones revealed that all
contained the same Pax9-like paired domain that was
included in pLjP9-PCRF. The longest clone included 1977
nucleotides excluding the poly(A) tail and contained a
single open reading frame that encoded a polypeptide of 393
amino acids (Fig. 3). In addition to the paired domain, this
clone also included octapeptides, but not the homeodo-
main, showing the structural features characteristic of
Pax1/Pax9-related genes. Analyses of the amino
acid sequence with other group I, II, and Pox meso-
genes as well as Group
I, and
Drosophila Pax9-related (Group I) genes. Analyses of the amino
acid sequences of Group I,
Pax9
Pax4/6
together with those of chick
Pax9
mouse
Pax9
ascidian
Pax9
C. elegans
K07C11.1
Pax9
C. elegans
K07C11.1
Pax9
C. elegans
K07C11.1
Fig. 2. (A) Genomic Southern blotting analysis of the L. japonica Pax1/Pax9-related genes. Aliquots of genomic DNA isolated from
a single adult were digested separately with EcoRI, HindIII, SalI, and
BglII. 10 µg of digested genomic DNA was loaded per lane, and
blots were hybridized with the 32P-labeled DNA probes of the
paired domain. The numbers indicate sizes (in kb) of the signals. (B)
Northern blots of poly(A)− RNA prepared from larval lamprey were
hybridized with the 32P-labeled paired domain DNA probes. 10 µg
of poly(A)− RNA was loaded in each lane. The numbers indicate
sizes (in kb) of signals.

Expression Pattern of LjPax9 in Developing L. japonica Embryos

The earliest expression of LjPax9 was observed around
stage 23.5 when the cheek process had already clearly
formed on the surface of the embryonic head (Fig. 5A). The
transcripts were found in the rostral endoderm surrounding
the first and future second pharyngeal pouches. The expres-
sion of LjPax9, therefore, seemed to take place after
endodermal protrusion. As embryos developed, the order of
pouch expression followed that of the appearance of the
pouches, which proceeded from anterior to posterior (Figs.
5A–5G). The last pouch to express LjPax9 was pouch 8, the
caudalmost permanent pharyngeal pouch of the lamprey
(Fig. 5G, stage 26). The morphology of the first pouch differs
from that of the others in that it never perforates and lies in
an oblique plane; the posterior pouches protrude laterally
and expand along dorsoventral axis (Figs. 5E and 5F). No
other overt expression domains were found in the endoder-
mal region, including the endodermatyle, indicating that LjPax9
serves as a specific marker of laterally protruding pharyn-
geal pouches.

From early stages, the lateral wall of the pouches ceased
expressing LjPax9, possibly corresponding to the perforat-
ing part of the endoderm; this loss of expression did not
occur in the first pouch that does not open externally. In
later stages, LjPax9 expression was restricted to the medial
lining of the branchial arch and not in the epithelium
covering the lateral lamella of the arch (Figs. 5E–5J).

From stage 25.5 onward, the pouch expression began to be
restricted to the medial portion of the posterior wall (or the
rostromedial wall) of the third and more caudal pharyngeal
(or real branchial) arches (Figs. 6A and 6B). This part of the
endoderm covers a part of the branchial arch called the
ciliary band (Figs. 6A and 6B; Kuratani et al., 1997; "Wim-
perschnur" of von Kupffer, 1896). In the first and second arches, no such changes occurred in this pouch (Fig. 5G, also see Fig. 7). Around this stage, a new expression domain appeared in the ectodermal nasohypophysial plate (Fig. 5E). At stage 28, \( LjPax9 \) expression was found in the velum (Fig. 5I), the pumping apparatus derived from the mandibular arch of the lamprey, where the expression was mainly seen in the mesenchyme. On histological sections, this expression was seen to be heavily accumulated in the cells surrounding the muscle (Figs. 6C and 6D), implying that the positive cells were of neural crest origin (see below). Mesenchymal expression was also seen in the second pharyngeal arch (Figs. 6C and 6D). In the trunk region of the same histological specimen, \( LjPax9 \) expression was not observed in the somitic mesoderm, nor was it found in any part of the mesoderm when whole-mount stained embryos ranging from stage 23 to 29 were dissected under the microscope and carefully observed.

**DISCUSSION**

**Group I Pax Genes of L. japonica**

Pax1/Pax9-related genes encode polypeptides with the paired domain and octapeptide but without a homeodomain. In higher vertebrates, two \( Pax1/Pax9 \)-related genes named \( Pax1 \) and \( Pax9 \) have been reported in human, mouse, chick, and fish. On the other hand, only one \( Pax1/Pax9 \)-related gene has been isolated from invertebrates: amphioxus, ascidians, acorn worms, sea urchins, flies, and nematodes. Therefore, one ancestral \( Pax1/Pax9 \)-related gene may have duplicated and evolved to \( Pax1 \) and \( Pax9 \) during the evolution of vertebrates.

In the present study, we isolated two different clones containing lamprey \( Pax1/Pax9 \)-related PCR fragments. The nucleotide sequences of these fragments closely resembled each other. One of the clones (pLjP1-PCRF), however,
encoded an aspartate residue at position 86 in the paired domain, which is typical for Pax1 (Figs. 1 and 4A). In contrast, the other clone pLjP9-PCRF encoded a threonine residue characteristic of Pax9 at this position (Figs. 1 and 4A). Since this amino acid change arose from triplet nucleotide displacement, it was not due to PCR-induced mutation. Furthermore, genomic Southern blotting analyses using these paired domains as probes alluded to the possibility that there are two closely related Pax1/Pax9-related genes in the genome (Fig. 2A). The two signals in the EcoRI and BglII digests might have been derived from the loci of both genes. In the HincII digest, two of the three signals may have been derived from the Pax9-like gene and one from the Pax1-like gene, because only the Pax9-like paired domain has the HincII site. These observations favor the hypothesis that L. japonica possesses two Pax1/Pax9-related genes, Pax1 and Pax9, in single copies per haploid genome, though the definite conclusion waits for the isolation of the hypothetical LjPax1, which has not been successful so far.

On Northern blotting analyses using the paired domains as probes, we detected only a single slightly broad band in the larval lamprey (Fig. 2B). On screening of a larval cDNA library, we isolated only the same type of cDNA clone containing the paired domain and octapeptides without homeodomain. These structural profiles support strongly the idea that the isolated cDNA clone encodes a Group I, Pax1/Pax9-related protein (all the other group members possess the homeodomain). Amino acid residues in this paired domain (phenylalanine 2, threonine 86, and arginine 90) and octapeptides (asparagine 5) are characteristic of Pax9 (Figs. 4A and 4B). Finally, molecular phylogenetic analyses indicated that the isolated cDNA clone encoded Pax9 of lamprey. These results suggested that the signal observed on Northern blotting was the Pax9 transcript, and the expression level of the gene which contained the Pax1-like paired domain appeared to be very low in the larval lamprey. Alternatively, the lengths of the putative Pax1 and Pax9 transcripts may be similar, resulting in apparently single
FIG. 5. Expression pattern LjPax9 in L. japonica embryos. Expression patterns of LjPax9 are shown successively in staged embryos (stages 23.5 to stage 29). Embryos are shown as lateral views except for F, which is a ventral view. In some of the embryos, the latest pharyngeal pouches to express LjPax9 are indicated by arrows. Note that expression of the gene followed the sequence of pharyngeal pouch development, which proceeds in a rostral-to-caudal direction. Asterisks in A and C indicate nonspecific staining of the embryo. The morphology of the first pharyngeal pouch (p1) differs from that of the others in that it develops in an oblique plane (E and F). This pouch is never perforated during development. From stage 27 onward, a de novo and temporary expression domain appears in the velum (double arrows in I). Abbreviations: cp, cheek process; llp, lower lip; nhp, nasohypophysial plate; p1–8, pharyngeal pouches (numbered); ulp, upper lip. Bars, 100 μm.
Further studies are required for isolation and characterization of the clone for Pax1-like paired domain-containing gene.

Expression Pattern of LjPax9 in the Lamprey and Evolution of the Pharynx: Pharyngeal Pouches—Plesiomorphic Expression

In the present study, expression of a Pax9 gene cognate was first observed in the developing agnathan species. In addition to the paired domain, the probe for in situ hybridization included the 3' UTR of the cDNA that would not be highly conserved with the putative paralogue of LjPax9, and furthermore, our preliminary in situ hybridization using only the paired domain did not give specific signals in the whole-mount embryos (not shown). The expression data presented in this study, therefore, are most likely to reflect the LjPax9 expression specifically. It was expressed in the endodermal pharyngeal pouch, nasohypophysial plate, and...
velar epithelium and mesenchyme. Similar expression patterns have been detected not only in vertebrates, but also in other chordates and even a hemichordate, *P. flava* (Ogasawara et al., 1999). This latter group expresses an ancestral form of *Pax1/9* in the endodermal pharyngeal pouches. In vertebrates, *Pax1* and *Pax9* genes have been suggested to play roles in the development of the pharyngeal endoderm and its derivatives; *Pax9* gene disruption leads to the loss of pharyngeal pouch derivatives such as the thymus, parathyroid, and ultimobranchial body (Peters et al., 1998). The presence of *Pax1/Pax9* genes in the lamprey, which does not possess a thymus, implies roles of these genes upstream of pharyngeal endodermal differentiation. In *Pax9*-deficient murine embryos, the defect is already seen in the early stage of pouch development (Peters et al., 1998). The *Pax9* gene seems, therefore, to function in the primary stage of pharyngeal segmentation, both in ontogeny and in phylogeny. Moreover, these genes are associated with paired pharyngeal pouches and not the medial derivatives such as the thyroid, development of which is regulated by another Pax group gene (*Pax8*) (Plachov et al., 1990). It would be of interest to determine whether *Pax2/5/8* homologues are associated with the endostyle in ammocoetes or in other chordate species.

**Vertebrate-Specific Expression**

Some *Pax9* expression domains are found only in vertebrates; the expression of *LiPax9* in the velar mesenchyme (Figs. 6C and 6D) resembles *Pax9* expression in the mandibular arch ectomesenchyme of gnathostome embryos (Ten Berge et al., 1998), which is homologous to the lamprey velum. Similar conservation was again encountered in the nasal–hypophysial complex (Fig. 5G). These expression domains are missing in invertebrates and are probably synapomorphic to vertebrates.

In the mouse, *Pax9* expression in the mandibular mesenchyme is associated with tooth development (Peters et al.,

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**FIG. 7.** Summary of *LiPax9* expression in the developing pharynx of the lamprey. Horizontal sections of early (left) and late (right) *L. japonica* embryos are shown schematically. *LiPax9* was expressed in a series of endodermal pouches, of which the indentation lateral to the oropharyngeal membrane (opm) corresponds to pharyngeal pouch 1 (p1). The velum develops rostral to this pouch. In later development, *LiPax9* becomes mesenchymally expressed also in the first and second pharyngeal arches. The endodermal expression of *LiPax9* was restricted to the rostral surface of ciliary bands (medial portions) in caudal branchial arches. No such expression was seen in arch 1 or 2, which do not differentiate into ciliary bands. This mesenchymal expression was absent from velar muscle and is probably associated with neural crest-derived ectomesenchyme.
From the expression profile of this gene, the domain apparently corresponds to the crest-derived ectomesenchyme (Ten Berge et al., 1998; Peters et al., 1998; Lu et al., 1999). In amniotes, the neural crest-derived ectomesenchyme has been shown to play fundamental roles in patterning of head muscles and for differentiation of major groups of connective tissues in the craniofacial region (reviewed by Noden, 1988). In the lamprey, neural crest-derived cells have also been observed to migrate into the pharynx, including the mandibular arch, and populate the outer layer of the mesenchyme similar to that in gnathostome embryos (Horigome et al., 1999). These cells are also thought to become skeletal as in gnathostomes (Langill and Hall, 1988). Although more detailed observations are necessary, it seems likely that in the lamprey LjPax9 is also expressed in the mandibular arch ectomesenchyme, although its expression in the mandibular mesoderm (Kuratani et al., 1999) could not be excluded (Fig. 6D). Moreover, the developmental role of LjPax9 in the mandibular mesenchyme of lamprey larvae is unclear, since mammalian Pax9 has been shown to be prerequisite for tooth development and the lamprey does not possess mineralized teeth (Peters et al., 1998). Further experiments are necessary to address this question.

Evolution of the jaw has been an intriguing topic in comparative embryology. Traditionally, the cheek process of the lamprey embryo represents the mandibular arch and the first pharyngeal pouch. The velum, which develops from the cheek process, has been compared to the mandibular arch of gnathostomes (reviewed by Mallat, 1996; Janvier, 1996). However, the endodermal pocket found caudal to the velum (named the first pouch in the present study) differs markedly from the corresponding pouch in gnathostomes; the shape of the pocket is also different and it never perforates during development, while the first pouch of gnathostomes always opens as spiracles. In the embryonic pharynx also, this pocket appears different from ordinary pouches (Figs. 5E, 5F, and 7). The present study revealed that this pharyngeal pouch marker was also expressed in this structure, confirming the serial homology of this endodermal structure as a pharyngeal pouch. This was also consistent with the close relationship between the velum and the mandibular arch (reviewed by Mallat, 1984; Janvier, 1996).

Gnathostomes and Lampreys

The lamprey embryos did not seem to express LjPax9 in some other tissues in which gnathostome Pax9 genes are reported to be expressed, the synapomorphic domains associated with gnathostome or more restricted groups within gnathostomes. In mammalian and avian development, Pax1/Pax9 genes are expressed in somites or their derivatives and are involved in somitic differentiation in response to sonic hedgehog signal from the notochord (Spörle and Schughart, 1997; Borycki et al., 1998; Hacker and Guthrie, 1998; Watanabe et al., 1998; Peters, 1999; Aruga et al., 1999; Furumoto et al., 1999). Similar mesodermal expression has also been reported in zebrafish embryos (Nornes et al., 1996). In the present study, no such expression was seen in the lamprey somites as observed by dissecting stage 23 to 29 whole-mount embryos under the microscope. Given the assumption that the number of sclerotomal cells is very small in the lamprey embryo (Maurer, 1906), however, it is not ruled out that there were a few LjPax9-expressing cells lateral to the notochord, which were not possible to detect with the available technique.

In summary, the lamprey Pax9 gene is expressed in the pharyngeal pouch endoderm and mandibular ectomesenchyme as well as in the nasohypophysial plate ectoderm, while the homologues of hemichordates, tunicates, and amphioxus are expressed only in the endodermal derivatives. Furthermore, Pax1/Pax9 genes of gnathostomes have acquired more expression domains (limb bud and probably somites) than in lampreys. Along the phylogenetic tree, the position of the lamprey lineage exactly fits the transition of Pax9 gene expression domains and this animal may repre-
sent the basal state of vertebrate Pax9 gene expression (Fig. 8). In such an evolutionary context, it is important that gene duplication itself does not appear to be the basis of evolution of the body plan; Pax9 and Pax1 genes have not differentiated into discrete expression domains. Rather, Group I Pax genes as a whole appear to have expanded their expression domains into multiple organ systems during evolution toward gnathostenomes (Fig. 8). The evolution of the neural crest cells is associated with the mandibular mesenchyme expression of these genes. These cells constitute the essential component of the vertebrate-specific developmental plan as the “fourth” germ layer (reviewed in Hall, 1998). With the evolution of the neural crest, the Pax9 gene seems to have acquired new expression domains simultaneously. Lampreys are now thought to represent a secondarily acquired limbless condition (Janvier, 1996). It is not known, therefore, whether limb-bud expression of the Pax9 gene is synapomorphic to gnathostenomes or to vertebrates.

As is often emphasized with Hox genes, the evolution of the animal body plan may in part rest upon duplication of regulatory genes and subsequent diversification of their functions. The apparent evolutionary trend in Pax9 genes highlights a contrasting pattern of evolutionary strategy, in which functions of regulatory genes expand into new domains within embryos. In this context, expression patterns of the putative paralogue, LjPax1, which have not yet been identified, would be of great interest in evaluation of Pax gene evolution.

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