Conservation of Pax gene expression in ectodermal placodes of the lamprey

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Abstract

Ectodermal placodes contribute to the cranial ganglia and sense organs of the head and, together with neural crest cells, represent defining features of the vertebrate embryo. The identity of different placodes appears to be specified in part by the expression of different Pax genes, with Pax-3/7 class genes being expressed in the trigeminal placode of mice, chick, frogs and fish, and Pax-2/5/8 class genes expressed in the otic placode. Here, we present the cloning and expression pattern of lamprey Pax-7 and Pax-2, which mark the trigeminal and otic placodes, respectively, as well as other structures characteristic of vertebrate Pax genes. These results suggest conservation of Pax genes and placodal structures in basal and derived vertebrates. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Placodes and neural crest cells share many similarities. Both are unique to vertebrates and originate between the neural plate and the surrounding non-neural epidermis (Baker and Bronner-Fraser, 1997; Webb and Noden, 1993). Both migrate and contribute to neurons in cranial sensory ganglia. These similarities suggest embryonic and/or evolutionary relationships between these two populations.

In the head of vertebrate embryos, ectodermal placodes are detectable as discrete regions of thickened ectoderm (reviewed by Webb and Noden, 1993; Baker and Bronner-Fraser, 2001). Placodal cells subsequently ingress or invaginate to form cranial sensory ganglia as well as the paired sense organs (eyes, ears, nose, lateral line; see Baker and Bronner-Fraser, 2001). The trigeminal and epibranchial placodes form only sensory neurons, while the lens placode forms only lens cells; the otic placode forms both the acoustic ganglion as well as sensory and support structures of the inner ear.

Recent studies suggest that the identity of different placodes may in part be specified by the expression of different Pax genes. Pax-6 is expressed in the lens and nasal placodes. Pax-3 is expressed in the ophthalmic trigeminal placode and the olfactory placode but in no other placodes. Pax-2 is expressed in the otic and epibranchial placodes. Although Pax-2 is the earliest molecular marker described in presumptive otic ectoderm in the chick (Groves and Bronner-Fraser, 2000), evidence from fish, amphibians and mice suggests that other genes (such as Pax-8, Dlx-5 and Dlx-3) may be expressed before Pax-2 in the developing otic placode (Acampora and Simeone, 1999; Depew et al., 1999; Ekker et al., 1992; Heller and Brändli, 1999; Pfeiffer et al., 1998).

Interestingly, many of the Pax null mice have defects in corresponding placode development. For example, Pax-6 mutants (Small eyes) have an eye and nose phenotype (Hogan et al., 1986; Grindley et al., 1995). The Splotch mutant mouse, which carries a Pax-3 deletion, has major defects in the ophthalmic lobe of the trigeminal ganglion, suggesting that Pax-3 is also important in its development in the mouse (Tremblay et al., 1998). Furthermore, Pax-2 mutants have inner ear defects, lacking the cochlea and most of the vestibulo-acoustic ganglion (Torres et al., 1996). Thus, Pax genes appear to be required for proper development of appropriate placode derivatives. This suggests that a ‘Pax code’ of expression and function plays an important role in specifying placodal identity.
To understand the events that led to the evolutionary origin of vertebrate features such as the placodes, it is essential to understand the early development and patterns of gene expression in placodes and their derivatives in numerous vertebrates, including basal vertebrates. To this end, we have characterized molecular markers for placodes in the lamprey, Petromyzon marinus. We have concentrated on the Pax genes expressed in the otic and trigeminal placodes, that form the ear and trigeminal ganglia, respectively.

2. Methods

2.1. Lamprey embryos

Spawning adults were collected from streams near the Hammond Bay Biological Station, Millersburg, MI. Males and females were stripped of gametes and embryos were reared at 18 °C according to Piavis (1961). Embryos were fixed for 1 h at room temperature in 4% formaldehyde, 0.1 M MOPS (pH 7.4), 1 mM MgSO₄, 2 mM EGTA, then dehydrated in methanol, and stored at −20 °C in 100% methanol and staged according to Tahara (1988).

2.2. Isolation of cDNA sequences

2.2.1. Lamprey Pax-7

Degenerate oligonucleotides in the forward (HKIVEMA) and reverse (FERTHY) direction were synthesized and used to amplify by polymerase chain reaction (PCR) a 567 bp fragment from an embryonic staged Lambda Zap II cDNA library (Stratagene, La Jolla, CA; kindly provided by Dr. Jim Langeland, Kalamazoo, MI). PCR denaturation was carried out for 6 min at 94 °C. After denaturation, 30 cycles of PCR were carried out; individual cycles included 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 45 s. A final extension step of 6 min at 72 °C was included. The amplified PCR fragment was cloned into the pGEM-T Easy vector (Promega) and used to screen approximately 5 × 10⁵ pfu of the embryonic cDNA library. Hybridization of the 3²P-labelled PCR fragment probe was in 50% formamide, 5X SSC, 0.1% Wattman 3M, 100 µg/ml heparin, 1X Denhardt’s, 0.1% CHAPS. Washes were performed in 0.2X SSC at 65 °C and embryos developed in NBT/BCIP (Gibco BRL) according to the manufacturer’s instructions.

2.2.2. Lamprey Pax-2/5/8

Nested degenerate oligonucleotides were synthesized in forward (outside VNBRPL; inside VELAHQG) and reverse (outside AWEIRD; inside PTMFAW) directions and used with PCR to amplify a 138 bp fragment. PCR conditions were as described above. The fragment was used to screen a staged embryonic cDNA library as described above and two plaques were isolated and sequenced. These were combined to form a single contiguous sequence.

2.3. In situ hybridization

Embryos were collected as described above. In situ hybridization was essentially as described by Tomsa and Langeland (1999). Briefly, digoxigenin-labeled antisense riboprobes were synthesized from full-length clones to Lamprey Pax-7 (LampPax-7) and Lamprey Pax-2 (Lamp-Pax-2) using the Promega Riboprobe in vitro Transcription System. Hybridization of probes was performed overnight at 65 °C in hybridization solution containing: 50% formamide, 5X SSC, 0.1% Tween 20, 5 mM EDTA, 1 mg/ml tRNA, 100 µg/ml heparin, 1X Denhardt’s, 0.1% CHAPS. Washes were performed in 0.2X SSC at 65 °C and embryos developed in NBT/BCIP (Gibco BRL) according to the manufacturer’s instructions.

2.4. Neurofilament staining

Fixed embryos, as described above, were rinsed in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin, 0.1% Triton X-100, and 5% heat-inactivated goat serum. Neurofilament antibody (NF-M; kindly provided by Dr. Virginia Lee) was diluted 1:300 and embryos incubated 1 h at room temperature followed by 4 × 30-min washes in PBS. Embryos were incubated overnight at 4 °C in secondary antibody, goat anti-mouse IgG conjugated to alkaline phosphatase, diluted 1:2000, rinsed in PBS and developed in NBT/BCIP as above.

2.5. Sectioning

Stained embryos were dehydrated through a graded series of ethanol, cleared through two changes of propylene oxide (PO) and infiltrated for 1 h with a 1:1 mixture of PO and Epon Araldite, followed by overnight infiltration in 100% Epon Araldite. Embryos were embedded in fresh Epon Araldite and polymerized 72 h at 60 °C. Ten-micrometer sections were cut with a glass knife and mounted onto albumin-coated slides. Slides were coverslipped in fresh Epon Araldite and polymerized overnight at 60 °C.

2.6. Phylogenetic analysis

Accession numbers used for the phylogenetic analysis of Pax-3/7 group genes were: Drosophila Paired, P06601; ascidian Pax-37, BAA12289; amphioxus Pax-37, AAF89581; zebrafish Pax-3, AAC41253; zebrafish Pax-7c, AAC41255; quail Pax-3, AF000673; chick Pax-7, BAA23005; mouse Pax-3, S15031; mouse Pax-7, AAG16663; human Pax-3, P23760; and human Pax-7, P23759. For phylogenetic analysis of Pax-2/5/8 group genes, accession numbers were as follows: ascidian Pax-258, AB006675; amphioxus Pax-258a, AF53762; zebrafish Pax-2.1, AAD19296; zebrafish Pax-2.2, AAC31811; zebrafish Pax-5, AAC31812; zebrafish Pax-8, AAC31813; Xenopus Pax-2, AAD52680; Xenopus Pax-5, AJ010503; mouse Pax-2, P32114; mouse Pax-5, NP032808; mouse...
Pax-8, A56925; human Pax-2, Q02962; human Pax-5, A44063; and human Pax-8, Q06710. Full-length protein sequences and conserved regions were aligned and compared for each Pax gene group. Alignments were made using CLUSTALW (Thompson et al., 1994) and neighboring joining trees using the method of Saitou and Nei (1987) were constructed with complete deletion of missing alignment sites using MEGA2 (Kumar et al., 2001).

3. Results

An important first step in understanding evolution of the vertebrate placodes is to identify molecular markers of placode development in diverse species, including basal vertebrates. Here, we concentrate on identifying the sequence and expression pattern of lamprey genes homologous to Pax-3/7 (for the trigeminal placode) and Pax-2/5/8 (for the otic placode). Basal chordates contain a single copy of Pax-3/7. Here, we concentrate on identifying the lamprey Pax-3/7 homologue. PCR with degenerate primers was used to amplify a 567 bp fragment of a lamprey Pax-3/7 group gene spanning portions of the paired domain, an octapeptide, and a homeodomain characteristic of Pax-3/7 group proteins (Fig. 1). Fig. 2A compares the lamprey Pax-3/7 putative amino acid sequence to mouse and amphioxus Pax-3 and Pax-7 orthologues.

The lamprey Pax-3/7 sequence contains a paired domain between amino acids (aa) 24 and 152, a conserved octapeptide from aa 178 to aa 185, and a homeodomain spanning aa 210 to aa 269. Comparison of the lamprey sequence with the respective paired domain, octapeptide, and homeodomains shows 92, 100, and 97% identity with amphioxus, 95, 88, and 98% identity with mouse Pax-3, and 96, 100, and 100% identical to mouse Pax-7 (Fig. 2A).

CLUSTALW (Thompson et al., 1994) was used to construct a full-length sequence alignment of Pax-3/7 genes and MEGA2 (Kumar et al., 2001) was then used to build a neighbor joining tree (Fig. 2B; Saitou and Nei, 1987). Based upon this sequence comparison to other Pax-3/7 group genes, we infer the lamprey gene we have isolated is orthologous to vertebrate Pax-7 genes (LampPax-7; Fig. 2B). LampPax-7 is available from the GenBank database (accession number AF411465). Partial sequencing in the 5’ region of alternate library clones and sequencing of the isolated PCR fragment revealed no differences from the clone used for analysis, suggesting all clones are from a single Pax-3/7 orthologue. Although we were able to isolate only a single Pax-3/7 group gene, this does not preclude the possibility of independent duplication of Pax-3/7 in Petromyzon.

3.2. Developmental expression of lamprey Pax-7

LampPax-7 RNA transcripts are first seen after neural tube closure at stage 21 (Fig. 3A). A stripe of expression is seen dorsally in the rostral neural tube. Expression within the somites can also be seen by stage 21 (Fig. 3A) and is seen in later stages (Fig. 3C,F). By stage 22, RNA expression extends along the entire length of the dorsal neural tube and is found in the trigeminal placode (Fig. 3B,C); however, LampPax-7 expression never extends into the forebrain (Fig. 3C,D,F). Sectioning through a stage 23 embryo (Fig. 3D,E) revealed restriction of transcript to the dorsal region of the neural tube.

In addition to the neural tube, LampPax-7 is observed in the trigeminal placode and developing ganglion (Fig. 3C,F). Neurofilament antibody staining was used to verify that the LampPax-7 expression domain overlaps with that of neurons and axonal processes in the trigeminal ganglion. By stage 23, neurofilament immunoreactivity reveals that differentiation of nerve fibers in the trigeminal root has begun (Fig. 3G; see also Kuratani et al., 1998). In the stage 27 larva (Fig. 3H), neurofilament staining is prominent in the differentiated cranial nerves, including the ophthalmicus profundus (V1) and the maxillomandibular nerves (V2), that originate from the trigeminal ganglia (see Kuratani et al., 1998).
al., 1997). These overlap with regions of LampPax-7 expression.

3.3. Cloning and characterization of a lamprey Pax-2/5/8 group gene

To study ear development in the lamprey, we first isolated a Petromyzon homologue of Pax-2/5/8. PCR with degenerate oligonucleotides was used to amplify a 138 bp fragment of the paired domain of a Pax-2/5/8 orthologue from a staged embryonic cDNA library. The fragment was used as a probe to screen the cDNA library from which two clones were isolated. The clones were sequenced and a single contiguous sequence constructed and found to contain a 1173 bp ORF that encoded 390 amino acids of a putative Pax-2/5/8 orthologue and 1.8 kb of noncoding sequence 3’ to the coding region (Fig. 4A). The putative protein sequence contains a paired domain (aa 18–142), an octapeptide sequence (aa 189–196) and a truncated homeodomain (aa 228–257) characteristic of Pax-2/5/8 group genes. Amino acid sequences for mouse and amphioxus Pax-2/5/8 group genes are shown in Fig. 5A for comparison. The paired domain shows 96% similarity to AmphiPax-2/5/8 and mouse Pax-2, 98% similarity to mouse Pax-5, and 93% similarity to mouse Pax-8. The octapeptide shows 100% similarity to amphioxus and mouse Pax-2 orthologues and 88% similarity to mouse Pax-5 and mouse Pax-8 genes while the partial homeodomain shows more variation, having only 38% similarity to the partial homeodomain of AmphiPax-2/5/8 and 65, 65, and 61% similarity to mouse Pax-2, Pax-5, and Pax-8 partial homeodomains, respectively (Fig. 5A).

We were able only to isolate two cDNA clones from the staged embryonic cDNA library. These are of the same gene. However, differences in sequence length and base composition were seen between the amplified PCR frag-

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**Fig. 1.** Nucleotide and putative amino acid sequence of LampPax-7. The paired domain, octapeptide, and homeodomain are shaded green, red, and blue, respectively.
ment and the corresponding region of the isolated cDNA clones (Fig. 4B). An apparent 75 bp deletion in the PCR fragment (138 bp) is not seen in the library sequence (213 bp), and 31 basepair substitutions resulted in ten differences between the putative amino acid sequences of the PCR fragment and the library clone (Fig. 4B). While these differences suggest the presence of other Pax-2/5/8 genes in the lamprey, we have not yet been able to isolate these members. Thus, we have performed a phylogenetic analysis on the single gene we have isolated.

Zebrafish, the most basal vertebrate studied to date, contains each of the three members of the Pax-2/5/8 subfamily (Pfeffer et al., 1998) while invertebrates appear to contain a single Pax-2/5/8 member (Kozmik et al., 1999; Wada et al., 1998). Thus, we were interested in determining the phylogenetic position of the Petromyzon Pax-2/5/8 orthologue. A full-length alignment of the lamprey Pax-2/5/8 contiguous sequence to other vertebrate Pax-2/5/8 subfamily genes was made using CLUSTALW (Thompson et al., 1994). Sequences with gaps were deleted from the alignment and a neighbor-joining tree constructed using MEGA2 (Kumar et al., 2001). From this analysis, we provi-

Fig. 2. (A) Comparison of LampPax-7 putative amino acid sequence to amphioxus Pax-3/7, mouse Pax-3 and mouse Pax-7. Residues that match the consensus sequence are shaded black. (B) Neighbor-joining tree (Saitou and Nei, 1987) of Pax-3/7 subfamily genes constructed from full-length CLUSTALW alignment of amino acid sequence with complete deletion of missing sites in the alignment. Numbers at nodes indicate bootstrap values from 500 iterations.
sionally infer that the clone we have isolated is the lamprey orthologue to the vertebrate Pax-2 gene (LampPax-2; Fig. 5B). LampPax-2 is available on the GenBank database (accession number AF411466).

3.4. Expression of LampPax-2

LampPax-2 transcripts are expressed at the mid-hindbrain boundary by stage 21 (Fig. 6B), as defined by otx expression in the lamprey midbrain and lamprey Pax-6 expression in the forebrain and hindbrain (Tomsa and Langeland, 1999; Murakami et al., 2001). This is similar to the Pax-2 expression pattern in the isthmus region of higher vertebrates.

LampPax-2 is also expressed in the otic placode (Fig. 6B) and developing otic vesicle by stage 23 (Fig. 6C,D), continuing through stage 25 (Fig. 6E,F). This suggests remarkable conservation of Pax-2 staining in both jawless and jawed vertebrates. Expression in the developing pronephros is detected beginning at stage 21 (Fig. 6A) and remains later in embryogenesis (Fig. 6D,F). Additionally, expression is seen in spinal interneurons (Fig. 6C,F) by stage 23. At stage 25, LampPax-2 transcripts are also seen in the endostyle, a larval organ that at metamorphosis is transformed into the thyroid (Fig. 6F).

4. Discussion

The cranial ectodermal placodes are unique to the vertebrates and form much of the peripheral nervous system of the head. In order to study events leading to both early development and evolution of the vertebrate placodes, it is...
Fig. 4. (A) Nucleotide and putative amino acid sequence of LampPax-2. The paired domain, octapeptide, and truncated homeodomain characteristic of Pax-2/5/8 group genes are shown boxed in green, red, and blue, respectively. (B) Alignment of 138 bp fragment amplified by PCR with the 213 bp sequence of the LampPax-2 library clone DNA.
necessary to have appropriate markers. In higher vertebrates, a number of molecular markers characteristic of particular placodes have been identified. For example, Pax-3 is expressed in the ophthalmic trigeminal placode and the olfactory placode but in no other placodes. Interestingly, the *Splotch* mutant mouse, which carries a Pax-3
deletion, has major defects in the ophthalmic lobe of the trigeminal ganglion, suggesting that Pax-3 is also important in its development in the mouse (Tremblay et al., 1998). Pax-2 is expressed in the otic and epibranchial placodes and Pax-6 is expressed in the lens and nasal placodes. The differential expression of Pax genes in different placodes suggests an intriguing correlation such that there may be a ‘Pax code’ for specification of various placodes. Interestingly, many of the Pax null mice have defects in corresponding placode development; for example Pax-2 mutants have inner ear defects (Torres et al., 1996) and Pax-6 mutants have an eye and nose phenotype (Hogan et al., 1986; Grindley et al., 1995), suggesting that this code is both morphological and functional.

We have explored whether the Pax code was already present in basal agnathans by isolating Pax genes from the lamprey and examining their early distribution pattern relative to developing cranial ganglia and sensory structures. We focused on two Pax gene families: Pax-3/7 and Pax-2/5/8. Our results show that lampreys have similar types of Pax genes to those observed in higher vertebrates with patterns of expression that are highly reminiscent of those observed in gnathostomes. Interestingly, when we isolated a Pax-3/7 homologue, the full-length cDNA corresponded most closely to Pax-7. Though this is still a member of the Pax-3/7 family, its distribution pattern closely resembled that of Pax-3 in higher vertebrates, being expressed in the trigeminal placode and ganglion, and neural tube. In the dorsal neural tube, Pax-3 and Pax-7 have been shown to have overlapping expression patterns. However, Pax-7 distribution has not been described in the trigeminal placode. Thus, it is possible that lampreys use Pax-7 in place of the Pax-3 used by higher vertebrates. There is precedence for this type of paralogue switching since Slug and Snail are used differently in chick versus mouse (Sefton et al., 1998). Alternatively, it is possible that higher vertebrates have overlapping expression of Pax-3 and Pax-7 in some placodes and this pattern has yet to be described. Yet another possibility is that there is but a single Pax-3/7 group gene in the lamprey though this has not yet been determined. Resolution of this issue awaits cloning of the lamprey Pax-3 homologue as well as examination of the Pax-7 distribution pattern in the placodes of higher vertebrates.

The lamprey Pax-2/5/8 homologue that we isolated groups most closely with gnathostome Pax-2 and has a distribution pattern highly reminiscent of Pax-2 in other species. It is expressed in the otic placode and later in the otic vesicle, as well as in the midbrain/hindbrain junction. Southern analysis (not shown) suggests the existence of other Pax-2/5/8 family members in lamprey. Additionally, Wada et al. (1998) suggest that an initial vertebrate Pax-2/5/8 duplication led to Pax-2/5 and Pax-8. Taken together with our placement of LampPax-2 (Fig. 5B), this suggests the possibility of at least one additional member in this subfamily in the lamprey.

If the evolution of the placodes was a vertebrate-specific event, it is likely to have been associated with genome-wide duplication events that occurred at the origin of the vertebrates (see Holland et al., 1994). Such gene duplications are permissive for the evolution of new gene functions, since at
least one of the duplicated genes can diverge and become co-opted for new functions. Our results suggest that lamprey may have multiple genes in the Pax-2/5/8 gene subfamily and perhaps in the Pax-3/7 subfamily as well, consistent with the possibility that expansion of these gene families facilitated evolution of the vertebrate placodes. In contrast, amphioxus has a single Pax-2/5/8 homologue and there is no comparable expression pattern of this transcript in midbrain/hindbrain junction or otic vesicle (Kozmik et al., 1999). This suggests a novel function for Pax-2/5/8 in the most basal extant vertebrates in establishing the ear and mid/hindbrain junction. Alternatively, Wada et al. (1998) have suggested that placodes are not a new feature of vertebrates. Based upon ascidian Pax-258 expression in the atrial primordia, and the origination of these cells from epidermal thickenings, Wada et al. suggest placodes originated in ascidians as the precursors to the atrial primordia which develop sensory cells similar to those of the vertebrate inner ear (Bone and Ryan, 1978).

Placodes give rise to characteristic derivatives including ciliated sensory receptors which are either neurons, as in the olfactory placode, or dedicated receptors, as in the otic placode and lateral line system. There is some evidence for similar types of cells in invertebrate chordates. For example, ascidians have paired epidermal primary sensory neurons along the tails of the tadpole (Torrence and Cloney, 1982; Crowther and Whittaker, 1994). These cells are putative mechanoreceptors, derived from dorsal epidermal cells. Interestingly, they express homologues of vertebrate Pax-3 and Pax-7 at the neurula stage (Wada et al., 1996). Thus, there is precedent for expression of Pax genes in the differentiated sensory cells of ascidians that resemble those derived from vertebrate ectodermal placodes. Analogously, adult ascidians have ciliary mechanoreceptors with gelatinous cupulae, similar to vertebrate otic and lateral line receptors, whose cilia are also embedded in gelatinous cupulae (Bone and Ryan, 1978).

An important emerging theme in evolution of novel functions for existing genes is that the type of gene networks used for differentiation are preserved and co-opted for new and developmentally earlier functions. Thus, the whole genetic toolkit is present in all animals but used for new functions in different evolutionary contexts. A good example of use of ‘old’ genes for new functions is illustrated by Pax-6. There is good evidence that Pax-6 controls eye development in flies and mice (Halder et al., 1995). However, the eyes of these two animals are quite different in morphology and developmental program. This suggests that the protostome/deuterostome ancestor utilized Pax-6 for a more basal function such as photoreceptor differentiation. Later, Pax-6 was co-opted to take on more upstream functions in addition to roles in cell differentiation. Similar co-options are likely to have occurred for other Pax genes. For example, the basal function of Pax-3/7 may have been for differentiation of mechanoreceptor-type cells. Acquiring such new functions for old genes may ultimately have facilitated the emergence of a novel cell type in vertebrates – the ectodermal placodes.

Placodes share many interesting properties with neural crest cells and the two cell types together represent a defining feature of vertebrates. Both placodes and neural crest cells form at the border between the neural plate and ectoderm, both give rise to migratory cells and both contribute to sensory neurons. The Pax genes may well represent the molecular link between invertebrates and vertebrates with respect to evolution of the placodes based on their basal use in photoreceptors of the eye and mechanoreceptors of the ear followed by more upstream use in development of vertebrate placodes. No similar molecular/evolutionary link has been found in genes involved in neural crest formation. One intriguing hypothesis is that the placodes may have evolved as the first vertebrate feature and may represent the evolutionary precursors to neural crest cells that later became incorporated into the neural tube. Future work will examine the molecular, embryological and evolutionary relationships between these two fascinating cell types that form defining traits of the vertebrate body plan.

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