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islet Reveals Segmentation in the Amphioxus Hindbrain Homolog

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The vertebrate embryonic hindbrain is segmented into rhombomeres. Gene expression studies suggest that amphioxus, the closest invertebrate relative of vertebrates, has a hindbrain homolog. However, this region is not overtly segmented in amphioxus, raising the question of how hindbrain segmentation arose in chordate evolution. Vertebrate hindbrain segmentation includes the patterning of cranial motor neurons, which can be identified by their expression of the LIM-homeodomain transcription factor *islet1*. To learn if the amphioxus hindbrain homolog is cryptically segmented, we cloned an amphioxus gene closely related to *islet1*, which we named simply *islet*. We report that amphioxus *islet* expression includes a domain of segmentally arranged cells in the ventral hindbrain homolog. We hypothesize that these cells are developing motor neurons and reveal a form of hindbrain segmentation in amphioxus. Hence, vertebrate rhombomeres may derive from a cryptically segmented brain present in the amphioxus/vertebrate ancestor. Other *islet* expression domains provide evidence for amphioxus homologs of the pineal gland, adenohypophysis, and endocrine pancreas. Surprisingly, homologs of vertebrate *islet1*-expressing spinal motor neurons and Rohon-Beard sensory neurons appear to be absent.

Key Words: **amphioxus; hindbrain; *islet;* motor neuron; rhombomere; segmentation.**

INTRODUCTION

Since the dawn of evolutionary theory, comparative anatomists have hypothesized that vertebrates evolved from an ancestor with a segmented body plan, including a segmented head (Balfour, 1878; Neal, 1914; Goodrich, 1930b; Gilland and Baker, 1993). This theory was formulated not only because diverse vertebrates have segmented heads as embryos, but also because amphioxus (cephalochordata), the closest living relative of the vertebrates (Wada and Satoh, 1994; Schaeffer, 1997), also has a segmented head (Willey, 1894; Goodrich, 1930b). However, as the molecular patterning mechanisms of head segmentation are elucidated, it will be important to make comparisons between amphioxus and vertebrates to be more certain head segmentation did not arise convergently.

Hindbrain rhombomeres are a prominent example of segmentation in the vertebrate head. Rhombomeres consist of segmentally repeating units that include overt morphological boundaries, iterated cell types, and iterated gene expression domains (reviewed in Lumsden and Krumlauf, 1996). Recent molecular studies have provided excellent evidence that amphioxus has a homolog of the vertebrate hindbrain (reviewed in Holland and Holland, 1998). However, the embryonic amphioxus hindbrain region lacks overt boundaries, and the segmentation of gene expression domains has not been demonstrated (Nieuwenhuys, 1998; Williams and Holland, 1998). Serially iterated cell types are present in the hindbrain region of late amphioxus larvae and adults (Bone, 1959, 1960; Lacalli and Kelly, 1999), but it is unclear whether these arise from a fundamentally segmented embryonic hindbrain region.

In vertebrates, the early segmental arrangement of hindbrain neurons has been disclosed by examining genes such as *islet1,* which are expressed during neuronal development. *islet* genes encode LIM-homeodomain transcription factors involved in many aspects of embryogenesis (Tsuchida *et al.*, 1994; Tokumoto *et al.*, 1995; Ahlgren *et al.*, 1997). *islet1* is expressed in all developing vertebrate motor neurons and has been shown to be required for their specification in the mouse (Ericson *et al.*, 1992). *islet1* reveals the segmental patterning of cranial motor neurons

Amphioxus *islet* sequence data from this article have been deposited with the GenBank Data Library under Accession No. AF226616.

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in the hindbrain and posterior midbrain at a time when rhombomere boundaries are present and rhombomere-specific genes such as *krox-20* and *hoxb1* are expressed (Chandrasekhar *et al.*, 1997; Prince *et al.*, 1998). It is the coincident repetition of these serially iterated hindbrain features that defines rhombomere segmentation (Bateson, 1894; Lumsden and Krumlauf, 1996).

To investigate possible cell-type segmentation in the hindbrain homolog, we cloned a homolog of *islet1* in the amphioxus *Branchiostoma floridae*. We found a single *islet* gene homolog which we refer to as amphioxus *islet*. *islet* is expressed in several restricted domains of cells during embryonic development, including segmental clusters of ventral cells in the amphioxus hindbrain region. We hypothesize that this *islet* expression reveals a form of segmentation in the amphioxus hindbrain region homologous to that in vertebrates. Furthermore, we suggest that the more prominent, overt segmentation of vertebrate hindbrain rhombomeres may have derived from serially repeating neurons present in the amphioxus/vertebrate ancestor. Additionally, we consider what the other domains of *islet* expression suggest about the homology of these expressing tissues with several vertebrate organs including the pineal gland, adenohypophysis, and endocrine pancreas.

MATERIALS AND METHODS

Collection

Amphioxus adults (*B. floridae*) were collected from Old Tampa Bay, Florida, separated by sex, and induced to spawn in the lab by electrical stimulation (Holland and Holland, 1993). Eggs were fertilized *in vitro* and allowed to develop at 23°C.

cDNA Library Screening

For screening, we used a library constructed from mRNA isolated from 6- to 20-h postfertilization (h) amphioxus embryos and packaged in the Lambda ZAP II vector (Stratagene; Langeland *et al.*, 1998). Approximately 5 × 105 pfu were screened at low stringency with ^{32}P-labeled DNA probes made from two *Pst*I restriction fragments of zebrafish *islet1* cDNA (Inoue *et al.*, 1994). The first fragment included the region encoding the LIM1 and LIM2 domains, the second the homeodomain and *islet*-specific domain. Five cDNAs were identified, cloned, and found to be of identical sequence. Hybridization and wash conditions were identical to those described in Langeland *et al.* (1998).

Sequence Analysis

An alignment and phylogenetic tree of the translated amino acid sequences from representative *islet* cDNAs was constructed using the Clustal X computer program (Thompson *et al.*, 1997). This program employs the neighbor-joining method (Saitou and Nei, 1987) and was adjusted to exclude positions containing gaps from the analysis and correct for multiple substitution events. Confidence values at branch nodes were determined by the program from 1000 replicate bootstrap resamplings of the alignment data. GenBank accession numbers of genes used in constructing the alignment and tree (Fig. 2) were *Rattus rattus Islet-1,* 57613; *Gallus gallus Islet-1,* 1708560; *Danio rerio islet1,* 1708559; *R. norvegicus Islet-2,* 1708563; *G. gallus Islet-2,* 1708562; *D. rerio islet2,* 1708561; *D. rerio islet3,* 1708564; *Ciona intestinalis Ci-isl,* 3150146; and *Drosophila melanogaster islet,* 1895062.

Southern Analysis

Genomic DNA was isolated from a single *B. floridae* adult and cut separately with five restriction enzymes (*Bam*HI, *Sac*I, *Xho*I, *Pst*I, and *Xba*I), and a Southern blot of these digests was probed with a ^{32}P-labeled 826-bp *Hin*dIII amphioxus *islet* cDNA fragment (Sambrook *et al.*, 1989). The probe comprised the 5′ end of the gene including the LIM1, LIM2, and homeodomain, but excluding the *islet*-specific domain. The blot was first washed at high stringency (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS, 64°C) and exposed to film. For lower stringency tests, the blot was reincubated with probe, washed at lower temperatures (55 and 45°C), and reexposed to film.

mRNA in Situ Hybridization

Embryos were fixed as described in Langeland *et al.* (1998), except that they were stored in 100% methanol instead of ethanol after fixation. A ribonucleotide probe was synthesized from the 826-bp *Hin*dIII 5′ fragment of the cDNA and *in situ* hybridization was performed as described (Langeland *et al.*, 1998). To facilitate probe penetration, embryos neurula stage and older (>11 h) were digested in a 10 μg/ml solution of proteinase K in PBS + 0.1% Tween 20 for 10 min (12- to 15-h embryos) or for 30 min (>15 h). The signal from the probe was somewhat weak, thus we allowed the *in situ* reactions to develop for several days and examined several hundred embryos from each stage to be sure we were seeing the entire expression pattern. A sense probe was made from the same restriction fragment as a control and found to have no detectable expression. For sections, fully developed embryos were embedded in Epon resin and cut in 3.5- (transverse sections) or 7.5-μm (horizontal section) slices (Langeland *et al.*, 1998). Names of developmental stages follow the convention established in Holland *et al.* (1996).

RESULTS

Isolation and Characterization of Amphioxus islet

We screened an amphioxus embryonic cDNA library with probes made from the zebrafish *islet1* cDNA, independently isolating five clones of a single amphioxus cDNA. The cDNA identified contains the two LIM domains, the homeodomain, and the *islet*-specific domain characteristic of *islet* genes (Fig. 1). Figure 2 shows a phylogenetic tree constructed from an alignment of the conserved regions of the translated amino acid sequences of our cDNA and representative *islet* cDNAs. The branching pattern of the tree strongly suggests (98% bootstrap value) that the amphioxus cDNA is no more closely related to one or the other of the two groups of vertebrate *islet* genes, thus we named it simply *islet*. Amphioxus *islet* is more similar to vertebrate *islet* genes than are either the *Ciona* (urochordate) or *Drosophila* (arthropod) *islet* homologs, but the bootstrap confidence value at this level of the tree is less robust.

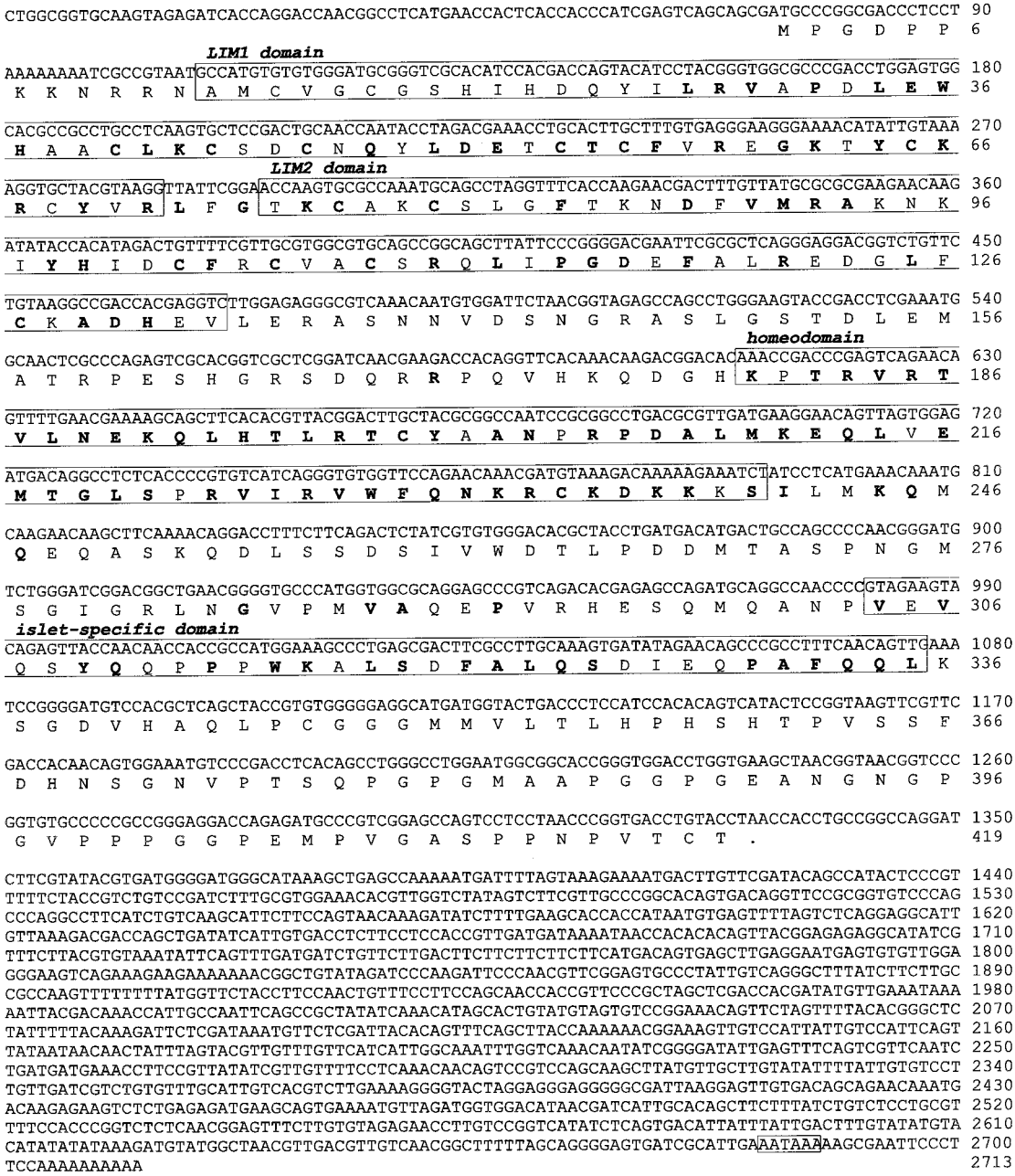

FIG. 1. The sequence of amphioxus *islet* contains characteristics of the *islet* gene family. Nucleotide sequence is shown above the putative amino acid translation. Boxes mark the LIM1 domain (108–285), LIM2 domain (294–471), homeodomain (609–792), *islet*-specific domain (983–1077), and polyadenylation signal (2681–2687). Domains are from Thor and Thomas (1997), except the *islet*-specific domain, which we have expanded based on our alignment information. Amino acid positions found to be identical between all of the *islet* genes included in our phylogenetic analysis (Fig. 2) are highlighted in bold.

Finding multiple *islet*-positive clones with the same sequence led us to suspect that amphioxus may have only one *islet* gene in its genome. To examine this idea more closely, we probed a Southern blot of genomic DNA digested separately with five restriction enzymes with a probe made from the amphioxus *islet* cDNA (Fig. 3). The single bands seen in lanes 1 and 2 suggest that *islet* is present at only one genomic locus and there are no other genes closely related to *islet* in the amphioxus genome. The multiple bands in the other lanes may be the result of cutting within

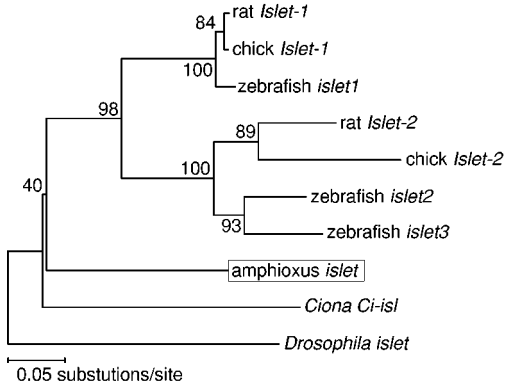

FIG. 2. Amphioxus *islet* is an outgroup of vertebrate *islet* genes. Pictured is a neighbor-joining phylogenetic tree of translated amino acid sequences from representative *islet* family genes. Numbers at branch nodes indicate percentage confidence values based on 1000 replicate bootstrap resamplings of the alignment data. Vertebrate *islet* genes fall into two categories with amphioxus *islet* no more closely related to either group. This arrangement agrees with theories of vertebrate-specific genome duplication (see Discussion). See Materials and Methods for sequence references.

introns contained at this locus; however, a Southern blot cannot rule out certain other possibilities (see Discussion).

islet Is Expressed Segmentally in the Neural Plate

We first detect *islet* mRNA expression in late gastrulae at 10 h in the anterior neurectoderm (arrow, Fig. 4A) and in the underlying endoderm (arrow, Fig. 4B; see below for further description of the endodermal expression pattern). By 11 h, the neurectodermal expression has localized to two columns of cells in the neural plate adjacent to the midline, with the strongest expression in the posterior of each column (arrow, Fig. 4C). After another hour (12 h), the columns have resolved into serially iterated clusters (numbered in blue; Fig. 4D). The anterior four clusters contain a small number of cells, perhaps two or three on each side of the midline. Transverse sections reveal that these cells are located in the ventral neural plate (arrow, Fig. 4E), except at the level of cluster 5 (Fig. 4F; see below). By 14 h, six somites have fully developed, and all but the anteriormost segmental *islet*-expressing cells are positioned adjacent to somite borders (arrowheads, Fig. 4G).

The anteriormost cluster (cluster 1) appears to be different from the rest of the ventral *islet*-expressing clusters. Unlike the others, cluster 1 is not located at a somite border, being instead positioned at the level of the myocoele of somite 1 (arrow, Fig. 4D). Also, cluster 1 is not bilaterally symmetrical like the others, having more cells on the right side than the left, and is more spread out along the anterior/posterior axis (Fig. 4G).

Cluster 5 also appears different from those in the rest of the neural tube ("5", Fig. 4D). *islet* is expressed more strongly at this level from 11 h (arrow, Fig. 4C) and continues to be expressed more strongly until about 20 h. Transverse sections through 12-h embryos at this level reveal strong expression in the dorsolateral edges of the neural plate (arrow, Fig. 4F) in addition to expression more ventrally (arrowhead, Fig. 4F). The dorsal cells at this level eventually develop a wedge-shaped morphology different from the round morphology of the ventral *islet*-expressing cells (arrow, Fig. 4I). Wedge-shaped, dorsal *islet*-expressing cells also appear a few hours later one somite's length back, near the somite 5/6 boundary. These cells are not visible at early neurula stages and begin to appear only at about 24 h (data not shown).

By the late neurula/early larval stage of 21 h, *islet* is expressed at its highest level in the neural tube, with seven clusters visible (numbered in Fig. 4H). Soon after this stage the organization of these clusters becomes more complex, as some *islet*-expressing cells are now located away from somite boundaries, and not all are in bilateral arrangements (Fig. 4I). By 30 h, *islet* expression is more difficult to detect in the neural tube (Fig. 4K; note switch to right-side view). By 47 h, *islet* expression appears to have mostly faded from

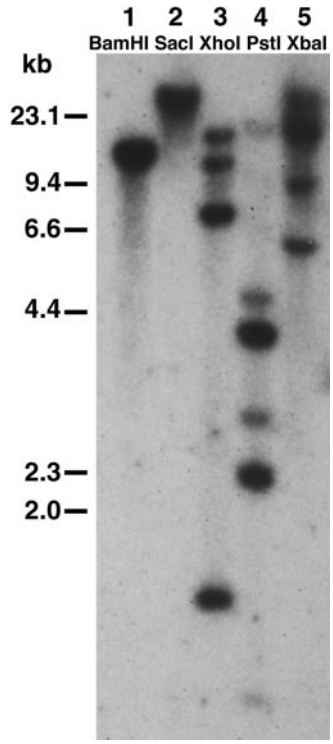

FIG. 3. Southern analysis suggests amphioxus has a single *islet* gene. Blot of genomic DNA digested with five restriction enzymes was probed with an 826-bp 5′ fragment of the amphioxus *islet* cDNA. The single bands in lanes 1 and 2 suggest the presence of no other genes closely related to *islet* (see Discussion). Shown for clarity is a high-stringency blot, but blots of lower stringency showed no additional bands (see Materials and Methods).

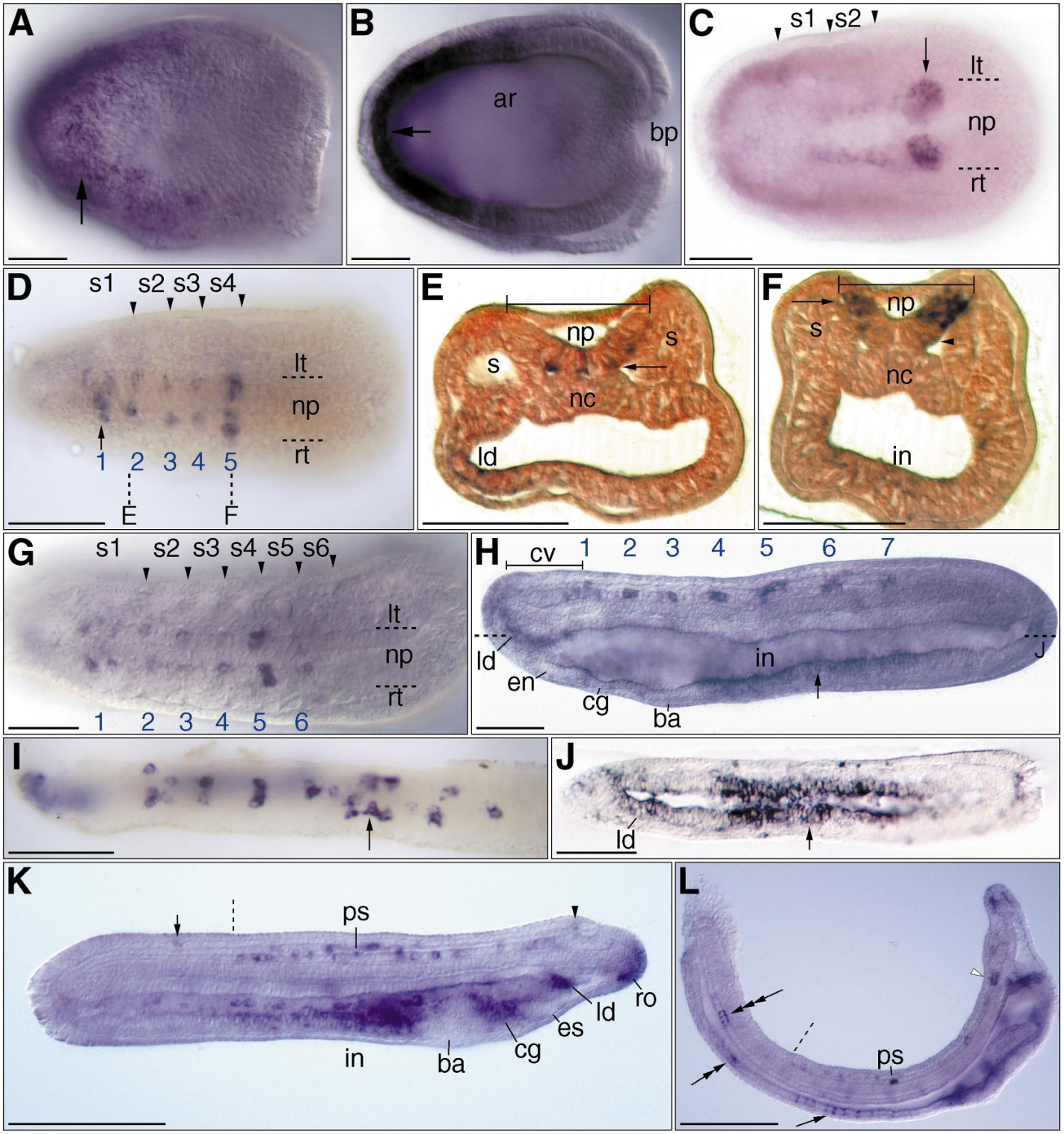

FIG. 4. *islet* mRNA expression reveals segmentation in the amphioxus neural plate. (A–D) Dorsal views, anterior to left. Expression is initiated in the late gastrula. (A) 10-h gastrula showing *islet* expression in the anterior neurectoderm (arrow). (B) Deep focus of embryo from (A) revealing expression in the anterior endoderm (arrow). (C) 11-h neurula. Expression in the neurectoderm is limited to two longitudinal stripes in the neural plate (np). Boundaries between numbered somites (s) are shown with arrowheads as in (D) and (G). (D) At 12 h *islet* is expressed in five bilateral pairs of clusters in the neural plate (numbered in blue). Vertical dashed lines indicate positions of sections in (E) and (F). (E) Transverse section from a 12-h embryo near the somite 1/2 boundary revealing expression in the ventral neural plate (arrow) and the left gut diverticulum (ld). Bracketed bar indicates width of neural plate. (F) 12-h transverse section through *islet* cluster 5. Expression is in the dorsal (arrow) as well as the ventral (arrowhead) neural plate and in the cells lining the intestine (in). (G–J) Anterior to left. (G) 14-h neurula, dorsal view. Neural expression is now localized adjacent to the borders of the first six somites. (H) 21-h late neurula, left-side view.

the neural tube except in the posterior cerebral vesicle (open arrowhead, Fig. 4L), and by 63 h, neural tube expression was barely detectable (data not shown).

We were sometimes able to visualize a few small, weakly expressing cells in the dorsal cerebral vesicle just posterior to the neuropore (arrowhead, Fig. 4K). This domain was present only in the later larvae we examined (>24 h) and probably due to its low level of expression we were unable to locate it in sectioned specimens.

We never saw *islet* expression in the neural plate posterior to the somite 7/8 boundary. At 30 h, when larvae have developed more than 15 somites and have begun moving via their myotomal muscle (Stokes and Holland, 1994), we were able to detect neural *islet* expression only anterior to somite 8 (dashed line, Fig. 4K). This remained the case even at 47 h (dashed line, Fig. 4L) up to the latest stages we investigated (63 h; data not shown).

islet Is Also Expressed in Certain Nonneural Tissues

The endodermal expression of *islet* seen in the late gastrula is maintained in the intestine and parts of the pharynx throughout the later stages which we examined. At 10 h, *islet* is expressed strongly in the anterior endoderm lining the archenteron (arrow, Fig. 4A). A uniform level of expression is maintained in the anterior endoderm from this stage until later neurula stages (data not shown).

By 21 h, a more complex pattern of endodermal expression has developed (Fig. 4H). The most anterior endodermal expression is in the forming left anterior gut diverticulum ("ld", Figs. 4E, 4H, 4J, and 4K). *islet* is also expressed in much of the pharyngeal endoderm including the club-shaped gland ("cg", Figs. 4H and 4K). However, expression seems to be excluded from the endostyle ("es", Figs. 4H and 4K), the branchial anlage ("ba", Figs. 4H and 4K), the mouth (data not shown), and the gill slits after they have formed in later larvae (data not shown). This pharyngeal expression pattern is maintained in the oldest larvae we examined (63 h, data not shown).

More caudally, *islet* is expressed in the developing intestine ("in", Fig. 4F). It is initially expressed along much of the length of the intestine (arrows, Figs. 4H and 4J). By 47 h, intestinal expression is restricted to three domains: several ventral cells in the anterior intestine (arrow, Fig. 4L), a ventral patch of cells more posterior (double arrow, Fig. 4L), and most caudally in three or four dorsal cells (triple arrow, Fig. 4L).

Additionally, *islet* is expressed in cells in and just underlying the rostral epithelium ("ro", Fig. 4K) and in isolated cells in the epidermis located mostly on the lateral flanks of later larvae (data not shown).

DISCUSSION

islet Gene Evolution

Our screening efforts have identified a single amphioxus *islet* gene. In contrast, more than one *islet* gene has been found in several vertebrate species (Gong *et al.*, 1995). To investigate the possible presence of other amphioxus *islet* gene homologs, we screened a genomic Southern blot with a probe made from an 826-bp, 5′ fragment of the *islet* cDNA (Fig. 3). The single bands in lanes 1 and 2 of the Southern suggest the presence of a single amphioxus *islet* gene, with the multiple bands in lanes 3–5 the result of the cutting of this single gene within intron sequences. However, a Southern blot cannot distinguish between the presence of a single gene copy and the following two scenarios: that there are multiple *islet* genes within approximately 15 kb on the same chromosome (the size of the band in lane 1) or that both lanes 1 and 2 contain more than one band of the same size cut from multiple *islet* genes. We feel that the Southern results can best be explained by the presence of a single amphioxus *islet* gene, but acknowledge that questions of gene copy cannot be truly resolved until the entire genome of the organism in question has been sequenced (e.g., *Caenorhabditis elegans;* Chervitz *et al.*, 1998).

However, we have no reason to suspect the presence of more than a single amphioxus *islet* gene. Only one *islet* homolog has been found in the urochordate *C. intestinalis* (Giuliano *et al.*, 1998) and in the arthropod *D. melanogaster*

The seven neural *islet* clusters are numbered. Cluster 1 is located in the posterior cerebral vesicle (cv). In the gut, the left diverticulum, club-shaped gland (cg), and intestine also express. Dashed lines indicate position of horizontal section in (J). (I) 21 h, dorsal view. Dorsal cells in cluster 5 adopt a wedge-shaped morphology (arrow). Ventral structures were cut away to allow light through to the neural plate. (J) Horizontal section of the dorsal gut cavity, 21 h. Expression is strongest in the intestine (arrow) and left diverticulum. (K) 30 h. Larva is shown in right-side view to correctly display the asymmetrical, right-side pharyngeal structures. Neural expression is only seen anterior to somite 8 (dashed line, see line in (L) also). The posterior somites are smaller than the anterior ones at this stage, thus the somite 7/8 boundary is located in the posterior part of the animal. Dorsal posterior spot is in the epidermis (arrow). Gut expression is similar to (H): noticeably absent in the endostyle (en) and branchial anlage (ba). Expression is also visible in the rostrum (ro) and faintly in the dorsal cerebral vesicle (arrowhead). (L) 47-h larva, right-side view. Neural tube expression is largely faded except in the posterior cerebral vesicle (open arrowhead). Intestinal expression has become localized to anterior ventral (arrow), more posterior ventral (double arrow), and posterior dorsal (triple arrow) clusters of cells. Other labels: ar, archenteron; bp, blastopore; lt, left; nc, notochord; ps, pigment spot; rt, right. Scale bars, 50 μm.

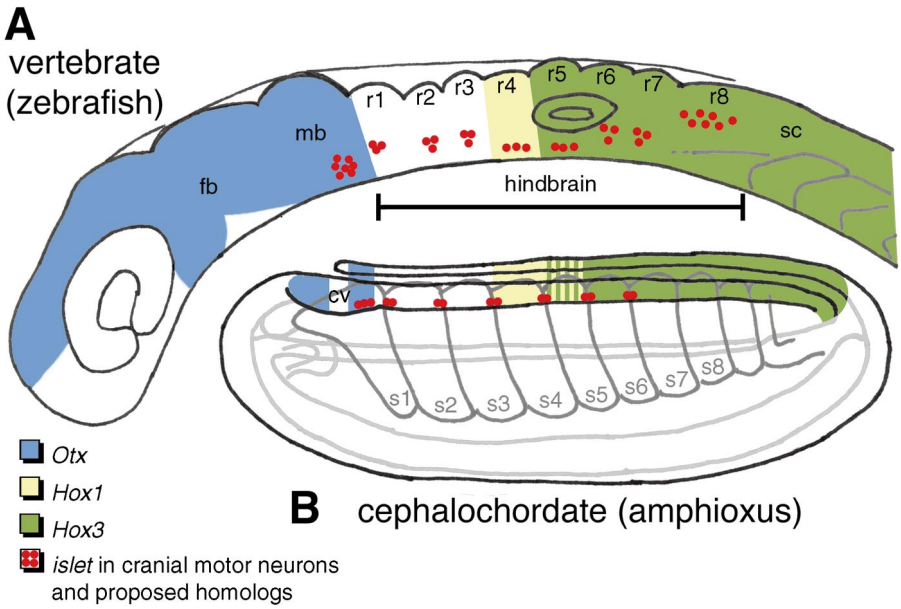

FIG. 5. Vertebrate (A) hindbrain segmentation may have derived from cell-type segmentation, like that revealed by amphioxus *islet* (B). We hypothesize that amphioxus *islet* (red, B) marks amphioxus homologs of *islet1*-expressing vertebrate cranial motor neurons (red, A) and reveals segmentation in the amphioxus hindbrain homolog. A left-side view of a 21-h zebrafish head (A) is sketched above an 18-h amphioxus embryo (B). Only neural tube gene expression is shown. The vertebrate hindbrain is located posterior to the expression of *Otx* genes (Li *et al.*, 1994), with *Hox1* and *Hox3* genes expressed in its caudal half (Prince *et al.*, 1998). The expression of homologs of these genes suggests that the amphioxus neural tube posterior to the cerebral vesicle back to about the level of somite 8 is homologous to the hindbrain (Holland *et al.*, 1992; Holland and Garcia-Fernàndez, 1996; Williams and Holland, 1996). Zebrafish cranial motor neurons are drawn as they are revealed by *islet1* expression (Chandrasekar *et al.*, 1997 and our own observations (data not shown)). Scale of zebrafish is approximately twice that of the amphioxus. Amphioxus sketch is modified from Hatschek (1892), the zebrafish after Kimmel *et al.* (1995). Labels: cv, cerebral vesicle; fb, forebrain; md, midbrain; r, rhombomere; s, somite; sc, spinal cord. Region of *AmphiHox-1* and *AmphiHox-3* overlap is indicated by vertical stripes.

(Thor and Thomas, 1997), suggesting that ancestrally *islet* was present only in a single copy. Additionally, the analysis of many gene sequences and conserved syntenic arrangements between amphioxus and vertebrates has led several researchers to hypothesize that the genome of early vertebrates duplicated after the vertebrate and amphioxus lineages diverged (Holland and Garcia-Fernàndez, 1996; Amores *et al.*, 1998; Patton *et al.*, 1998).

Our phylogenetic tree of *islet* genes places vertebrate *islet* genes into two groups, with amphioxus *islet* no more closely related to either group (Fig. 2). This tree is consistent with *islet* representing an unduplicated copy of a gene which in vertebrates has been duplicated. Sequence comparisons of many other amphioxus genes also support this model: several genes which have multiple copies in vertebrates appear to have only one copy in amphioxus (N. Holland *et al.*, 1996; L. Holland *et al.*, 1997; Patton *et al.*, 1998; Langeland *et al.*, 1998) and when amphioxus is found to have more than one copy, these duplicates appear to have stemmed from an independent, single-gene duplication event not shared with vertebrates (P. Holland *et al.*, 1995; Araki *et al.*, 1996; Shimeld, 1997).

islet Reveals Embryonic Hindbrain Segmentation

Recent gene expression studies suggest that amphioxus has a homolog of the vertebrate hindbrain. The amphioxus genes *AmphiOtx* (Williams and Holland, 1996), *AmphiHox-1* (N. Holland *et al.*, 1995), *AmphiHox-3* (Holland *et al.*, 1992), and *AmphiHox-4* (Wada *et al.*, 1999) are expressed in an anterior-to-posterior arrangement similar to their vertebrate homologs, suggesting regional homology between the vertebrate hindbrain and the amphioxus neural tube approximately adjacent to somites 2–8 (Fig. 5).

However, is this amphioxus hindbrain region segmented? We define segmentation using a widely known and long-standing definition put forth by Bateson (1894). He defined it as: "... a more or less coincident repetition of elements belonging to most of the chief systems of organs along an axis which corresponds to the long axis of the body." The vertebrate hindbrain includes at least three kinds of repeating elements: morphological boundaries between adjacent rhombomeres (Fraser *et al.*, 1990), segmentally arranged cell types such as motor neurons (Lumsden and Keynes, 1989), and segmental gene expression patterns (reviewed in Lums-

den and Krumlauf, 1996). Because the repetition of these elements are coincident, together they define the segmental units of the hindbrain: the rhombomeres.

Neither morphological boundaries nor segmental gene expression domains have been reported in the developing amphioxus hindbrain region (reviewed in Nieuwenhuys, 1998; Williams and Holland, 1998), but serially arranged motor neurons have been shown to be present in this region in late larvae (Lacalli and Kelly, 1999). However, perhaps due to the late stage examined, the arrangements of these cells are complex and it is unclear whether they are segmental with respect to the myotomes and the body plan in general. Earlier, at the peak of the neural expression of *islet,* there are six serially iterated, labeled clusters in the amphioxus hindbrain region (clusters 2–7; Figs. 4H and 5B). During neurulation, these clusters line up next to somite boundaries, demonstrating a coincidence of repetition that fits Bateson's definition of segmentation. We therefore hypothesize that *islet* expression reveals a form of segmentation in the amphioxus hindbrain homolog in the same way that *islet1* expression in cranial motor neurons reveals vertebrate hindbrain segmentation. The apparent absence of segmental morphological boundaries and regional gene expression patterns in the amphioxus hindbrain region raises the possibility that the overt segmentation of the vertebrate hindbrain may derive from the segmentally repeating neurons of an invertebrate ancestor. Segmental neurons may have laid the groundwork onto which gene expression and morphological boundaries were added later to make bona fide rhombomeres.

This hypothesis predicts that if there are other segmental parts to the amphioxus hindbrain region, they should consist of coincidentally repeating cell types and not morphological boundaries or gene expression domains. This idea can be tested by investigating amphioxus homologs of three types of vertebrate genes: other genes expressed in segmental cell types (i.e., *tag1,* Chandrasekar *et al.*, 1997), genes expressed at rhombomere boundaries (i.e., *FGF-3,* Mahmood *et al.*, 1995), and genes which are expressed in domains of one or a subset of rhombomeres (i.e., *krox-20,* Wilkinson *et al.*, 1989). There are many genes in each category which have yet to be investigated in amphioxus (for more examples, see Lumsden and Krumlauf, 1996).

islet May Reveal Motor Neuron Patterning

We do not know the identity of the segmentally arranged *islet*-expressing cells, but suspect they are motor neurons. In *Drosophila,* the single *islet* homolog is expressed in a subset of its motor neurons (Thor and Thomas, 1997). Closer to vertebrates, a *Ciona islet* homolog has been cloned, but its possible expression in motor neurons has yet to be investigated (Giuliano *et al.*, 1998). However, in vertebrates, *islet1* is expressed in all developing motor neurons and has been shown to be required for motor neuron development in mouse knockout studies (Ericson *et al.*, 1992). Amphioxus *islet,* as a likely unduplicated, close homolog of vertebrate *islet* genes (see above), may perform many of the same developmental functions which have been parceled out in vertebrate *islet* duplicates (Force *et al.*, 1999), including expression in developing motor neurons. Thus phylogenetic comparisons currently support the idea that *islet* may be marking some or all developing motor neurons in amphioxus.

In the amphioxus posterior cerebral vesicle and hindbrain region, two classes of somatic motor neurons and a few visceral motor neurons have been identified in 8- to 12-day larvae (Lacalli and Kelly, 1999). Similar to the segmental *islet*-expressing cells, these motor neurons are located ventrally in the neural tube. The late arrangements of these motor neurons seem similar enough to the early pattern of *islet*-expressing cells for the former to have possibly arisen from the latter. However, we were barely able to detect neural *islet* expression in 63-h larvae and not at all past this stage (data not shown), making it impossible to follow the early *islet*-expressing cells to their later larval phenotype. Establishing the connection between early amphioxus gene expression and late phenotype will have to wait until techniques to culture amphioxus embryos in the laboratory and trace the lineage of cells during development are developed (Stokes and Holland, 1994; Zhang *et al.*, 1997).

We were never able to detect *islet* expression in the posterior neural tube even after many more somites have formed caudal to somite 7 and expression is still detectable anterior to this level (dashed line, Figs. 4K and 4L). This is surprising because vertebrates have *islet1*-expressing motor neurons along much of the neural tube, from the midbrain back through to the posterior end of the spinal cord (Korzh *et al.*, 1993). It is also surprising because motor neurons were tentatively identified in the posterior neural tube of the larval and adult amphioxus (Bone, 1959, 1960). Unfortunately, the detailed work of Lacalli and Kelly does not extend caudal to somite 6, leaving the presence of more posterior larval amphioxus motor neurons in question. As stated above, the arthropod *D. melanogaster* has been shown to express its sole *islet* homolog in only a subset of its motor neurons (Thor and Thomas, 1997). This provides an example of an invertebrate in which *islet* marks only a subset of its motor neurons, raising the possibility that the situation may be similar in amphioxus. However, the amphioxus larval somatic motor neurons have large axons which descend caudally down the neural tube, allowing for the possibility that the posterior somites are innervated by anterior motor neurons which express *islet* in their development (Lacalli and Kelly, 1999). Thus it remains uncertain whether the lack of posterior *islet* expression is indicative of an absence of posterior motor neurons, the late development of such neurons, or examples of chordate motor neurons which do not express *islet* in their patterning.

The limited anterior/posterior extent of the segmental *islet*-expressing cells within the neural tube suggests that they may be homologs of a subset of vertebrate motor neurons: the cranial motor neurons. Cranial motor neurons are located in the hindbrain and posterior midbrain of

vertebrates (Gilland and Baker, 1993). As described above, gene expression has suggested that amphioxus has a hindbrain homolog, but has been inconclusive regarding the presence of an amphioxus midbrain homolog (L. Holland *et al.*, 1997; Williams and Holland, 1998). However, neuroanatomical work has suggested that amphioxus possess part of a midbrain, including a tectum and midbrain motor neurons (Lacalli, 1996). If *islet* indeed identifies the early development of amphioxus midbrain motor neurons, this suggests that the *islet*-expressing cells in the posterior cerebral vesicle may be homologs of vertebrate midbrain cranial motor neurons and that the more posterior *islet*-expressing cells may be homologs of vertebrate hindbrain cranial motor neurons. Future investigations of other vertebrate genes expressed in motor neurons in general (i.e., *Lim-3,* Tsuchida *et al.*, 1994) and cranial motor neurons more specifically (i.e., *phox2a,* Pattyn *et al.*, 1997) will test this hypothesis.

Other Homologies

islet has a complex expression pattern and is probably involved in several aspects of developmental patterning in addition to those described above. We outline below how these additional domains of *islet* expression contribute to arguments concerning the homology between several amphioxus and vertebrate structures.

Rohon-Beard cells are *islet1*-expressing dorsal sensory neurons in the posterior hindbrain and spinal cord of anamniote vertebrates (Korzh *et al.*, 1993; Kollros and Bovbjerg, 1997). Morphological studies have been inconclusive regarding the presence of Rohon-Beard homologs in amphioxus (Bone, 1960). The dorsal, *islet*-expressing cells at the level of somites 5 and 6 (Fig. 4I) may conceivably represent Rohon-Beard homologs. However, if this is the case, their limited anterior–posterior extent in the neural tube would indicate that they were homologous to posterior hindbrain and not spinal Rohon-Beard neurons.

The vertebrate epiphysis, which develops into the pineal gland, also expresses *islet1* (Inoue *et al.*, 1994). The faint spot of *islet* expression we detect in the dorsal cerebral vesicle of older larval stages (arrowhead, Fig. 4L) may be present in the developing lamellar body, lending strength to a morphological argument for homology of this structure with the vertebrate epiphysis (Lacalli, 1996).

islet is also expressed strongly in the left gut diverticulum and more weakly in the club-shaped gland of the pharynx (Fig. 4H). Whereas the homology of the club-shaped gland has remained difficult to assess (Goodrich, 1930a), the left diverticulum contributes to Hatschek's pit in the adult, a structure thought homologous to the vertebrate adenohypophysis (Whittaker, 1997; Gorbman *et al.*, 1999). *islet1* is expressed in the adenohypophysis of the rat (Thor *et al.*, 1991), strengthening the Hatschek's pit/adenohypophysis link.

islet1 has also been shown to be expressed in, and required for, the development of the endocrine islet cells of the mouse pancreas (Ahlgren *et al.*, 1997). Amphioxus does not have a discrete endocrine pancreas, but along with hagfish and lamprey, has several types of endocrine cells incorporated into the gut epithelium, some of which are possibly homologous to the pancreas-islet cells of mammals (Reinecke, 1981; P. Holland *et al.*, 1997). It is therefore possible that the intestinal expression of amphioxus *islet* is revealing the early development of these pancreas-islet homologs. The *islet*-expressing cells in the anterior intestine may be good candidates for such cells (arrow, Fig. 4L).

Finally, an area of *islet* expression is found in and perhaps just internal to the ectoderm at the tip of the head (rostrum, Fig. 4K). Vertebrate *islet1* is expressed in anterior, nonectodermal cells that appear to give rise to the hatching gland in the zebrafish embryo (Ericson *et al.*, 1992; Inoue *et al.*, 1994), but this expression quickly fades and no other vertebrate *islet* gene has been reported to be expressed rostrally outside of the neural tube during development.

CONCLUSION

The expression of a single gene cannot demonstrate homology, but it can be used as a character which, when considered along with others both molecular and morphological, can paint a picture of how animals are related and what their ancestors were like. The expression of *islet* strengthens links between the hindbrain region of vertebrates and amphioxus and suggests how part of rhombomere segmentation may have evolved, i.e., that segmental neurons likely appeared before boundaries. Further comparisons of developmental gene expression between amphioxus and vertebrates will no doubt greatly advance our understanding of the homology between the body plans of these animals and allow us to model more precisely what our ancestors were like before our lineages diverged so long ago.

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