Expression of estrogen-receptor related receptors in amphioxus and zebrafish: implications for the evolution of posterior brain segmentation at the invertebrate-to-vertebrate transition

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SUMMARY The evolutionary origin of vertebrate hindbrain segmentation is unclear since the amphioxus, the closest living invertebrate relative to the vertebrates, possesses a hindbrain homolog that displays no gross morphological segmentation. Three of the estrogen-receptor related (ERR) receptors are segmentally expressed in the zebrafish hindbrain, suggesting that their common ancestor was expressed in a similar, reiterated manner. We have also cloned and determined the developmental expression of the single homolog of the vertebrate ERR genes in the amphioxus (AmphiERR). This gene is also expressed in a segmented manner in a region considered homologous to the vertebrate hindbrain. In contrast to the expression of amphioxus islet (a LIM-homeobox gene that also labels motoneurons), AmphiERR expression persists longer in the hindbrain homolog and does not later extend to additional posterior cells. In addition, AmphiERR and one of its vertebrate homologs (ERRα) are expressed in the developing somitic musculature of amphioxus and zebrafish, respectively. Altogether, our results are consistent with fine structural evidence suggesting that the amphioxus hindbrain is segmented, and indicate that chordate ERR gene expression is a marker for both hindbrain and muscle segmentation. Furthermore, our data support an evolution model of chordate brain segmentation: originally, the program for anterior segmentation in the protochordate ancestors of the vertebrates resided in the developing axial mesoderm which imposed reiterated patterning on the adjacent neural tube; during early vertebrate evolution, this segmentation program was transferred to and controlled by the neural tube.

INTRODUCTION

Segmentation is the process that subdivides tissues into repeated, identical subunits that will later acquire particular characteristics according to their position. In adult vertebrates, this phenomenon is particularly obvious in the spinal cord where vertebrae are iterated elements sharing identical features, such as the presence of dorsal sensory- and ventral motor roots although the territories they serve vary along the axis. This importance of segmentation is also reflected by the complexity of the vertebrate head, the formation of which depends on brain segmentation. In addition to cryptic segmentation in the diencephalon, the hindbrain displays an overt segmentation, which is required for proper craniofacial morphogenesis (Trainor and Krumlauf 2000). Hindbrain is transiently subdivided into repeated elements called rhombomeres. Neural crest cells emerging from the hindbrain are specified according to their antero-posterior position and migrate through the whole cephalic region to help pattern definite territories. In addition to this morphological segmentation, each rhombomere displays a specific gene expression code, as exemplified by Hox genes (Prince et al. 1998).

The evolution of the vertebrate complex head, as a distinctive trait of their phylum, has been extensively discussed (Guthrie 1995; Holland 2000; Holland and Chen 2001). Regarding this issue, the cephalochordate amphioxus (Branchiostoma floridae), being the closest living invertebrate relative to the vertebrates, represents a powerful tool. The general vertebrate brain organization is indeed conserved in the amphioxus since structures homologous to fore- and hindbrain have been identified (Holland and Holland 1999), but simpler than in the vertebrates, as the amphioxus hindbrain homolog...
is not segmented at the gross anatomical level and does not give rise to neural crest cells. However, amphioxus genes have been cloned that display an iterated expression pattern in the hindbrain. For instance, the LIM homeobox gene islet is expressed in a repeated manner throughout the posterior part of the amphioxus nervous system (Jackman et al. 2000; Jackman and Kimmel 2002). This suggests that even though morphological segmentation is not obvious, molecular segmentation already exists in the amphioxus. However, expression domains for amphioxus genes previously proposed as hindbrain markers (Jackman and Kimmel 2002), although suggestive of a hindbrain homology with vertebrates, are transiently expressed and their expression pattern is not exactly homologous to those of their vertebrate orthologs. For instance, whereas amphioxus islet has been reported to be only expressed in the hindbrain homolog, its vertebrate islet 1 counterpart not only labels the hindbrain but is also expressed in the whole spinal cord (Appel et al. 1995). Furthermore, the developmental mechanisms directing amphioxus and vertebrate hindbrain segmentation could be different. Indeed, it has been proposed that in amphioxus signals originating from the segmented head mesoderm trigger neural segmentation (Mazet and Shimeld 2002). In contrast, the vertebrate hindbrain segmentation program is initiated entirely within the neural tube.

Nuclear receptors form a family of ligand-dependent transcription factors implicated in a variety of developmental and physiological processes (Laude and Gronemeyer 2002). These receptors can be recognized through their typical domain organization, with a highly conserved DNA-binding domain localized in the middle of the molecule and a ligand binding, transactivation domain in the C-terminus. However, for some members of the family, referred to as "orphan receptors", no ligand has been identified to date (Giguère 1999). This is the case for estrogen-receptor-related (ERR) α, β, and γ receptors (NR3B1, 2 and 3, respectively) which may activate transcription in a constitutive manner (Xie et al. 1999), although the existence of an unidentified ligand has also been suggested (Vanacker et al. 1999a; reviewed in Horard and Vanacker 2003). While ERR receptors have been suggested to positively or negatively interfere with the signaling driven by estrogens (Vanacker et al. 1999b; Lu et al. 2001; Kraus et al. 2002; reviewed in Giguère 2002), little is known of their developmental roles. During mouse embryonic development ERRα is expressed in a variety of tissues (Bonnelye et al. 1997) whereas ERRβ expression is restricted to the developing placenta (Pettersson et al. 1996) to the formation of which it contributes (Luo et al. 1997). Besides its complex pattern of expression in the brain (Hermans-Borgmeyer et al. 2000), the territories in which ERRγ is expressed during mouse development have not been thoroughly characterized.

To gain insight into the developmental roles of the ERR genes, we have cloned their zebrafish orthologs and determined their expression pattern during development (Bardet et al. 2004). Among other expression sites, we found that three zebrafish ERR receptors are expressed in the hindbrain in an iterated manner. Furthermore zebrafish ERRα is also expressed in a segmented manner in the somites. We next cloned the single ERR ortholog in the amphioxus (AmphiERR) and determined its expression pattern. AmphiERR is expressed in the hindbrain homolog where it most likely labels dorsal compartment (DC) motoneurons, and also in somitic mesoderm. Strikingly AmphiERR is expressed in a segmented manner in both tissues. Our results show that a structure similar to the segmented hindbrain was already present before the separation between invertebrates and vertebrates. Our data also support a model in which neural segmentation in invertebrate chordates requires signaling originating from the segmented mesoderm. During early vertebrate evolution this signaling was evidently transferred to the hindbrain and the neural tube.

**MATERIALS AND METHODS**

**Molecular cloning**

One microgram of total RNA extracted from pooled amphioxus larvae (24-h post fertilization) was retrotranscribed using MMLV reverse transcriptase (Gibco BRL, Cergy, France) and submitted to nested polymerase chain reaction (PCR) using degenerated primers located in the conserved DNA binding and ligand-binding domains. PCR products were cloned into pCR2.1 vector (Invitrogen, Cergy, France) and individual clones were sequenced. *B. floridae* (2 to 4 days of development) cDNA library was screened using standard methods. RACE experiments were performed using the 5′/3′ RACE kit (Roche, Meylan, France) following the manufacturer’s instructions. Cloning of zebrafish ERRs is described elsewhere (Bardet et al. 2004).

Sequences of the degenerated oligonucleotides used:

5′: GA(T/C)TA(T/C)GCITCIGGITA(T/C)CA
3′: GIGTIG(T/C)CA(G/A)CA(G/A)(T/C)ATICAAGG
3′ nested: CAITTCIC(T/C)T(G/C)TA(G/A)(T/C)ATICAAGG

GenBank accession number for ERRs:

AmphiERR: zebrafish ERRα: AY556395; zebrafish ERRβ: AY556396; zebrafish ERRγ: AY556397.

**Phylogenetic analysis**

Protein sequences were aligned automatically by Clustalw (Thompson et al. 1994) with manual correction in SeaView (Galtier et al. 1996). Phylogenetic reconstruction was done using amino-acid alignments. Only complete sites (no gap) were used. Tree was constructed by Neighbor-Joining (Saitou and Nei 1987) with distances corrected for rate heterogeneity between sites, using a gamma law of parameter alpha estimated in Tree-Puzzle (Schmidt et al. 2002), with eight categories. Reliability of nodes was estimated by 1000 bootstrap replicates (Felsenstein 1985).
**Expression analysis**

In situ hybridizations on amphioxus embryos were performed according to Holland et al. (1996). Anti-sense probes were synthesized from complete cDNA cloned in pcCS2+ plasmid using T7 polymerase after linearization (DIG RNA labeling kit, Roche). In situ hybridizations of zebrafish embryos were performed according to Thisse et al. (1993). All zebrafish probes were synthesized from partial cDNA (around 1 kb from the DNA- up to the ligand-binding domains thus including the highly divergent hinge domain) cloned in pBluescript, using the same DIG RNA labeling kit (T3 polymerase for ERRα, and ERRγ, T7 polymerase for ERRβ). After photography of the whole mounts, specimens were counterstained in Ponceau S, embedded in Spurr’s resin, and sectioned (Holland et al. 1996).

**RESULTS**

**ERR genes are expressed in a segmented manner in zebrafish hindbrain**

We have previously reported the cloning and expression of five ERR species in the zebrafish (Bardet et al. 2004). The present report will mainly extend the data concerning the segmented expression in the hindbrain of the three zebrafish ERRs (α, β, and γ) genes that are expressed in the hindbrain. There were differences as well as striking similarities among the hindbrain expression domains of the three related genes (Fig. 1). At 36 hpf, ERRα was detected in the hindbrain region in two patches of cells in r5 and 6 (Fig. 1A; and not in r4 as mistakenly stated in Bardet et al. 2004). Transverse section revealed that ERRα was expressed in a ventro-lateral position (Fig. 1B). ERRβ also displayed a segmented expression in the hindbrain at 36 hpf (Fig. 1C). As for ERRα, ventrolateral positions of the labeling was also revealed by cross-sections (Fig. 1, D and E). More laterally, ERRβ was also detected in structures whose position corresponds to the trigeminal (tg), the anterior and posterior lateral line ganglia (allg and pllg, respectively; Fig. 1, C and D; Andermann et al. 2002). At 36 hpf ERRγ was detected in all three posterior cranial ganglia (tg, allg and pllg; Fig. 1, F and G) and displayed a segmented expression in the rhombomeres in ventro-lateral position (Fig. 1, F–H). The rhabdicular expression of ERR genes appeared in a sequential order (data not shown) as summarized in Fig. 1I. In addition, cohybridization indicated that at least a subset of rhabdicular cells co-expressed ERRβ and ERRγ at 24 hpf and 36 hpf (data not shown). To confirm the segmented nature of ERR genes expression, we used valentino mutant, where the r5 and r6 fail to individualize (giving rise to an undetermined rX rhombomere; Moens et al. 1996). In this context, ERRγ expression is lacking (or at least deeply attenuated) at the level of the affected rhombomere at 24 hpf (compare Fig 1, J and K). Thus ERRs are robust markers of the hindbrain segmentation in the zebrafish.

**Identification of a unique ERR receptor in amphioxus**

To analyze the evolutionary conservation of the segmented expression of ERRs, we used the amphioxus (Branchiostoma floridae), a cephalochordate that displays no overt hindbrain segmentation at the gross anatomical level. Furthermore, a unique ERR gene may be predicted in this species given that (i) its genome is not duplicated (Panopoulou et al. 2003) and (ii) unique copies of ERR were found in other invertebrates such as C. intestinalis (Dehal et al. 2002) and D. melanogaster (Ostberg et al. 2003). We first isolated an ERR cDNA fragment by RT-PCR using total RNA extracted from amphioxus larvae (24 h post fertilization) and degenerate primers located in the two conserved regions of nuclear receptors: the DNA binding domain (DBD) and the ligand binding domain (LBD). This experiment gave rise to a fragment that was used to screen an amphioxus cDNA library. Partial cDNAs were obtained and 5’ RACE was then performed to produce complete cDNAs that were subsequently sequenced. A single ORF in the cDNA was predicted to produce a putative protein of 455 amino acids, hereafter referred to as AmphiERR (NR3B4). This sequence was aligned with zebrafish ERRs (Fig. 2A). AmphiERR bears the typical DNA binding domain of nuclear receptors organized in two zinc finger modules with cysteines in fixed positions and a putative ligand-binding domain located in the C-terminal end of the protein with 12 conserved z-helices (Laudet and Gronemeyer 2002).

As expected, there is a high level of sequence identity within these domains between species (over 90% for the DBD, close to 60% for the LBD when comparing amphioxus to zebrafish sequences).

Using the Neighbor-Joining method, we then analyzed the phylogenetic relationships of AmphiERR to others ERRs (Fig. 2B). Low bootstrap values were obtained for the position of zebrafish ERRα, probably because of a high mutation rate. AmphiERR sequence branched at the root of vertebrate (human and zebrafish) ERRs and is most likely the unique amphioxus homologue of the ancestral ERR.

**Expression of ERR during amphioxus development**

We determined the expression pattern of AmphiERR using in situ hybridization of amphioxus embryos (Fig. 3). Expression was first detected at 14 h of development (neurula stage) in somites 2 to 6 (Fig. 3, A and B) and then extended to newly formed posterior somites (Fig. 3, E–G). Transverse sections show that AmphiERR is expressed in the dorsal part of the muscular masses of the somites (Fig. 3, C and D). These will later give rise to superficially-located slow muscle types (reviewed in Lacalli 2001), a population that is labelled by AmphiERR until at least 3 days of development. In contrast, AmphiERR expression was always excluded from somite 1,
Fig. 1. Expression of estrogen-receptor related (ERR) genes in the zebrafish hindbrain. (A and B): ERRα (A) Dorsal view of the head (anterior to the left) of 36 hpf embryos, showing ERRα expression in the diencephalon (arrow) and in cell patches in rhombomeres (r) 5 and 6 (arrowheads). Double arrowhead: muscle expression in the trunk. Position of the otic vesicle (ot.) is indicated. (B) Cross section through level x in (A), showing ventro-lateral ERRα expression corresponding to motoneurons (arrowhead). (C-E): ERRβ (C) Dorsal view of the head of 36 hpf embryos (anterior to the left) showing ERRβ expression in the diencephalon (arrow), in cell patches in all rhombomeres. ERRβ is also expressed in trigeminal ganglia (double open arrowhead), anterior and posterior lateral line ganglia (open arrowhead and open arrow, respectively). (D) Cross section through level x in (C) showing ERRβ expression in motoneurons (arrowhead) and anterior lateral line ganglia (open arrowhead). (E) Cross section through level y in (C) showing ERRβ expression in motoneurons (arrowhead). (F-H): ERRγ (F) Dorsal view of the head of 36 hpf embryos (anterior to the left) showing ERRγ expression in the epiphysis (asterisk), the diencephalon (arrow), in cell patches in the rhombomeres. ERRγ is also expressed in the trigeminal ganglia (open double arrowhead), in the anterior and posterior lateral line ganglia (open arrowhead and open arrow, respectively). Position of the ot. is indicated. (G) Cross section through level x in (F) showing ERRγ expression in motoneurons (black arrowhead) and in anterior lateral line ganglia (open arrowhead). (H) Cross section through level y in (F) showing ERRγ expression in motoneurons (arrowhead). (I) Summary of the spatio-temporal expression of ERR genes. Hatched bars indicate weaker gene expression. Position and numbering of the rhombomeres, as well as of the ot., are schematized on the left. (J and K) Loss of ERRγ expression in valentino mutants. Dorsal views of 24 hpf wild type (J) or val mutant (K) embryos (anterior to the left) hybridized with an ERRγ probe. Rhombomeres are numbered, rX corresponds to the undetermined r5–r6 equivalent in val. ERRγ expression is absent in rX in val mutant as compared with r5 and r6 in wild type (wt) animal.
which only contains deep, fast fibres (Fig. 3, A and B). In addition to somites, individual mesodermal and epidermal cells also began expressing AmphiERR, starting at 24 h of development (Fig. 3, K–M). By three days of development, expression in these cells tended to disappear or at least to be strongly reduced (Fig. 3Q). At these later stages, AmphiERR was also weakly transcribed in pharyngeal endoderm cells (Fig. 3Q).

We also detected AmphiERR in neural derivatives, such as the presumptive frontal eye and the presumptive photoreceptor
Fig. 3. AmphiERR expression demonstrated by in situ hybridization of developing Branchiostoma floridae. All side and dorsal views with anterior toward left; all cross sections viewed from tail end of animal. Scale lines for whole mounts = 50 μm; scale lines for cross and frontal sections (counterstained pink) = 25 μm. (A) Side view of 14-h neurula showing strong expression in the second through sixth somite on either side and weak expression in some cells of the presumptive frontal eye (arrow). (B) Dorsal view of (A) showing expression in frontal eye cells (single arrow), presumptive photoreceptor cells of Hess (arrowhead) and muscular somites, excluding the first (tandem arrow). (C) Cross section through level x in (B) showing strong expression in the muscle cells of the somites, but no detectable transcripts in the neural plate (arrow). (D) Cross section through level y in (B) showing strong expression in the photoreceptor cells of Hess in the neural plate and weaker expression in somitic muscles. (E) Side view of 19-h neurula showing conspicuous expression in the muscular somites. (F) Dorsal view of (E) showing expression in the somites and in the photoreceptor cells of Hess (arrowhead) as well as in some neurons (one of which is indicated by the arrow). (G) Frontal section through (F) showing expression in somites and, more medially, in neurons. (H) Cross section through level x in (G) showing expression in neurons (arrow). (I) Cross section through level y in (G) showing expression in neurons (arrow) and somitic muscle cells (arrowhead). (J) Cross section through level z in (G) showing expression in somitic muscle cells (arrowhead). (K) Twenty-four-hour embryo with focus on upper surface showing transcripts in individual epidermal/mesodermal cells. (L) Twenty-four-hour embryo with focus on lower surface showing transcripts in individual epidermal/mesodermal cells. (M) Cross section through level x in (K) and (L) showing expression in individual mesodermal cell (arrow) and individual epidermal cell (arrowhead). (N) Dorsal view of 24-h embryo showing transcripts in somitic muscle cells and neurons. (O) Frontal section through (N) showing transcripts in muscle cells and neurons; pigment cup cells have appeared medial to the photoreceptor cells of Hess (arrow and enlarged in inset). (P) Cross section through level x in (O) showing expression in somitic muscle cells and in photoreceptor cells of Hess; the arrow indicates the pigment cup cells. (Q) Side view of a 3-day larva with weak expression in somitic muscle cells and endodermal cells of the pharynx and strong expression in neurons. The inset is an enlarged dorsal view of the expressing neurons; the most anterior (arrowhead) are at the posterior end of the cerebral vesicle, and the most posterior are the photoreceptor cells of Hess (arrow). (R) Cross section through the level of the arrowhead in (Q) showing expression in several neurons located ventrally in the neural tube.
of Hesse (Fig. 3B) and other neuronal derivatives starting at 19 h of development. Indeed, AmphiERR was expressed in paired cells from the border between somites 1 and 2 and up to somite 4 (Fig. 3, F–H). At 24 hpf, this expression was expanded to six pairs of cells from the level of somites 2 to 5 (Fig. 3, N and O). This clearly segmented expression persisted until at least 3 days of development with an additional expression domain in the posterior part of the cerebral vesicle (Fig. 3Q). Based on their wedge-shaped morphology and on their ventro-lateral position within the nerve cord (Fig. 3I), the paired cells expressing AmphiERR are likely to represent DC motoneurons that innervate superficial muscular fibers (Lacalli and Kelly 1999).

As pointed out above and in contrast to vertebrates, the amphioxus hindbrain homolog is not segmented at the gross anatomical level (Holland and Holland 1998). However, the fine structural data (Lacalli and Kelly 1999) and the iterated expression of AmphiERR suggests that the hindbrain homolog is fundamentally segmented. It has been previously suggested that the amphioxus islet gene could be a similar marker for hindbrain segmentation since it is expressed as a series of seven paired spots spread throughout the nerve cord (Jackman et al. 2000; Jackman and Kimmel 2002). Given that AmphiERR also presented this type of pattern, we compared the expression domains of both genes (Fig. 4). As expected, islet was expressed at 20 hpf in the nerve cord in a segmented manner. Sections of embryos at this stage of development revealed a heterogeneous expression of this gene within the nerve cord. Indeed, in some of the sections, ventro-lateral expression was observed (Fig. 4B) which most likely corresponded to DC motoneurons, while on others islet was expressed in laterally positioned cells (Fig. 4C). These cells could correspond to sensory neurons by analogy with zebrafish islet 1 and islet 2 that label Rohon-Beard (sensory) neurons in the dorsal neural tube (Appel et al. 1995). Notably, islet expression was also observed in the floor plate. Double in situ hybridizations (Fig. 4, D–F) revealed that islet and AmphiERR share common expression regions in the anterior half of the embryo which corresponds to the amphioxus hindbrain homolog (Holland and Holland 1998). However, the sequentially repeated expression of islet corresponded only partially to that of AmphiERR and does not always occur in DC motoneurons. Furthermore, islet was also detected more posteriorly suggesting that this gene may be involved in segmentation of the whole nerve cord. In contrast, AmphiERR expression was limited to motoneurons of the hindbrain homolog. Altogether our results allow us to propose ERR genes as conserved markers of posterior brain segmentation.
DISCUSSION

Hindbrain segmentation is conserved between cephalochordates and vertebrates

The hindbrain is a typical vertebrate structure transiently characterized during development by a repetition of subunits called rhombomeres. Individual rhombomeres can be identified morphologically through their clear boundaries and also through expression of a given combination of molecular markers. For instance vertebrate Krox20 is only expressed in rhombomere 3 (r3) and r5 (Wilkinson et al. 1989). Within rhombomeres, individual cranial nerves will differentiate and innervate distinct muscular territories. The evolutionary origin of the segmented hindbrain of vertebrates is unclear since no gross morphological segmentation can be observed in cephalochordates, the sister group of vertebrates.

In the amphioxus we have found ERR labeling in neuroectodermal structures, the frontal eye, the two initial photoreceptor cells of Hesse, and six pairs of additional cells at the level of somite 2-6. These cells have the typical wedge-shape of DC somatic motoneurons with axons extending posteriorly (Lacalli and Kelly 1999). This view is further supported by the fact that no such labeling was observed at the level of somite 1, whose anterior neural territory does not contain DC motoneurons. Furthermore, the distance between the cells expressing AmphiERR is compatible with the intervals predicted to exist between DC motoneurons (Lacalli 2002). Nevertheless only five pairs of DC motoneurons have been reported (Lacalli and Kelly 1999). However, these cells were identified by tracing back axons on serial sections of the anterior nerve cord. The sampling of the sections used, together with the asymmetry that can be observed within a pair of neurons renders it possible that an additional DC motoneuron pair might have been missed (T. Lacalli, personal communication). It is thus very likely that AmphiERR is an exclusive marker of all DC motoneurons.

A number of genes, such as AmphiNk2-1 (Venkatesh et al. 1999), AmphiFoxB (Mazet and Shimeld 2002), AmphiKrox, and AmphiShox (Jackman and Kimmel 2002), have been identified whose expression demonstrated segmentation of the amphioxus neural tube, including the hindbrain homolog. Vertebrate hindbrain segmentation can also be observed through the LIM-homeodomain transcription factor Islet1 that patterns cranial nerves (reviewed in Hobert and Westphal 2000). An amphioxus islet gene has been cloned that is expressed as an iterated series of cells identified as motoneurons (Jackman et al. 2000; Jackman and Kimmel 2002).

However, several differences can be noted between the expression of AmphiERR and islet. From a temporal point of view, islet expression appears much earlier (at 12 h when somite 4 is formed) than that of ERR (whose first neuronal labeling appears at 19 h). The latter distinctly persists at least up to 3 days of development whereas islet expression has faded at 30 h. The difference in regional expression between the two genes is even more striking. The caudal-most site of AmphiERR expression (the photoreceptor of Hesse) is at the level of the boundary between somites 4 and 5, whereas islet expression extends back to the boundary between somites 6 and 7. Islet has been suggested to label the whole amphioxus hindbrain homolog. However, it could be argued that the posterior boundary of the amphioxus hindbrain is not clearly defined because of the lack of markers. For instance, whereas the anterior expression limit of vertebrate Hox5 paralogous genes abuts on the boundary between the hindbrain and the spinal cord, AmphiHox5 expression pattern has not been determined to date. In vertebrates islet genes are expressed not only in the hindbrain, but also more caudally in the neural tube (Appel et al. 1995). By analogy, the possibility exists that, in amphioxus as well, islet labels posterior neural structures and not only the hindbrain homolog. In contrast, expression of ERR genes in the zebrafish is posteriorly limited to the hindbrain in which each rhombomere expresses a combination of the ERR genes. Alternatively it is possible that islet labels the whole amphioxus hindbrain homolog and that the posterior domain of expression is a vertebrate innovation. In this case, AmphiERR would mark a subdomain of the hindbrain, displaying a more refined segmentation pattern, in which DC motoneurons are found.

Whatever the case, it is worth noting that all markers found to date are transiently expressed and define embryonic fields in which functional cells will differentiate. In contrast, AmphiERR is expressed during a longer period of time and defines functional structures (likely DC motoneurons). The fact that islet is a marker of DC motoneurons (Jackman et al. 2000) is challenged by our experiments. Indeed, these cells have only been described in the anterior part of the nerve cord and project caudally to innervate the whole set of somites (Lacalli 2002). Coexpression of AmphiERR and islet has been detected in the anterior part of the nerve cord, but evidently neural cells expressing islet extend much more posteriorly. It is therefore possible that islet labeling was detected in some, but not all, DC motoneurons. This is supported by the ventrolateral islet expression observed on cross sections. In contrast the medio-lateral expression found on certain cross sections suggests that islet is also expressed in ventral compartment (VC) motoneurons or in sensory neurons. In agreement with the latter hypothesis, it should be remembered that zebrafish LIM homeobox genes including islet1 also labels the sensory Rohon–Beard neurons (Appel et al. 1995).

In summary, our results lead us to propose ERR genes as markers of hindbrain segmentation. Since this is true for amphioxus and zebrafish, it is likely that segmented ERR expression was present in the hindbrain equivalent of the last common ancestor of cephalochordates and vertebrates.
**ERR in motoneuron-muscle complex**

Slow and fast muscles can be clearly identified during zebrafish development. Fast fibers originate from the lateral presomitic cells present in the segmental plate, whereas slow fibers originate from adaxial cells, adjacent to the notochord. The latter cells migrate radially across the somitic mass and differentiate in muscle pioneer cells and in superficial muscle layer (Devoto et al. 1996), two structures that express zebrafish ERRz (Bardet et al. 2004 and data not shown). ERRz is expressed in muscular masses during mouse embryonic development (Bonnelye et al. 1997). Furthermore, preliminary results indicate that this receptor is preferentially expressed in slow fibers versus fast ones in rat muscles (P. L. Bardet and J. M. Vanacker, unpublished). The slow muscle tropism is therefore a conserved feature of ERRz expression.

Trunk musculature in amphioxus is composed of (i) deep, fast fibers used in escape, emergency swimming; (ii) intermediate fibers which are thought to constitute a reserve for further differentiation; and (iii) superficial slow fibers which are dedicated to slow regular swimming (Lacalli and Kelly 1999). In the amphioxus, these structures can be identified during somitogenesis. The different types of muscle fibers are innervated by specific motoneurons: fast fibers are innervated by VC motoneurons whereas slow fibers receive influx from DC motoneurons. Axons from the latter neurons lie entirely in the nerve cord, and muscle cells send processes dorsally where they contact neurons at the level of the DC (reviewed in Lacalli 2001). We found that AmphiERR is expressed in each newly formed somite, at a superficial location, suggesting that this receptor labels slow fibers but not fast ones. This view is further supported by the fact that AmphiERR is not expressed in somite 1 which only comprises deep, fast fibers. The transcription of AmphiERR in the mesoderm as well as in DC motoneurons (but not in other types of muscle-innervating nerves), is consistent with the expression of this receptor in both the nervous and the muscular compartments of a given nerve-muscle complex.

Gene expression in nerve and muscle is tightly regulated with information originating from one compartment acting on the other. Examples of such cross-talk involve neuron-secreted ligands and receptors expressed on the post-synaptic membrane (reviewed in Schaeffer et al. 2001). On the other hand, transcription factors belonging to the same gene family have been reported to be expressed in both compartments, but in this instance, different members of the family are found in nerve and muscle. This is the case for the ETS gene family, where combinations of ER81 and/or PEA3 are expressed in motor and sensory neurons (Lin et al. 1998), whereas GABP is the predominant ETS member in muscle cells (Schaeffer et al. 1998; Khurana et al. 1999). To our knowledge, expression of an identical gene (AmphiERR) in both the nerve and muscle cells that establish functional contacts with each other is a unique feature of ERR genes.

The identity of the ERR-expressing cells in the zebrafish hindbrain remains to be determined. Indeed, we performed double in situ hybridizations with ERRγ and either islet 1 or evx 1 (data not shown). As in the amphioxus, expression of islet1 does not overlap that of ERRγ, thus excluding the latter as labeling cranial nerves. In the zebrafish hindbrain Evx1 is expressed in a segmented manner in the primary and secondary interneurons (Thaëron et al. 2000). Although ERR-positive cells have striking similarities with the evx1+ secondary interneurons, technical limits prevented us from demonstrating a coexpression of both genes.

**Evolution of the mechanisms of hindbrain segmentation**

Several lines of evidence suggest that retinoic acid (RA) is a key determinant in the regulation of vertebrate hindbrain segmentation and rhombomere identity (reviewed in Gavalas and Krumlauf 2000; see also Dupe and Lumsden 2001). RA is produced by the somitic mesoderm adjacent to the posterior brain and establishes an antero-posterior (AP) gradient of concentration with the help of Cyp26 gene (an RA-degrading enzyme) expressed in the midbrain (Swindell et al. 1999). In...
the amphioxus RA has also been shown to exhibit AP patterning activities (Holland and Holland 1996; Escriva et al. 2002). However this is not concomitant with the establishment of morphologically conspicuous rhombomeres. Based on these and other observations, including gene expression pattern, Mazet and Shimeld (2002) have recently proposed that posterior brain segmentation in ancestral chordates was regulated by vertical signals, originating from the adjacent mesoderm, which is also segmented in the head, in contrast to the situation in vertebrates.

The data presented here support this hypothesis. Indeed, we suggest that a segmentation control program, first active in the mesoderm, is revealed by AmphiERR segmented expression in somites. In some way, over evolutionary time, mesoderm then instructs the neuroectoderm to undergo the segmentation program. This might also be recapitulated during development by AmphiERR expression in motoneurons, which appears later as compared with that in the somites. It should also be stressed that the compartments that express AmphiERR (DC motoneurons and slow fiber muscles) are intimately connected. To our knowledge, AmphiERR is the only marker to date to display segmented expression both in the mesoderm and in the neuroectoderm.

This hierarchy of control (summarized in Fig. 5A) could have been present in primitive protochordates, a remnant of which can be observed in the amphioxus. As a first step towards the vertebrate situation, the instructive program triggered by the segmented mesoderm might have been lost (Fig. 5B). This could have been accompanied by a reinforcement of the influence of the posterior signaling (Fig. 5C). Ultimately the instructive program has been transferred to the neural tube leading to morphologically segmented hindbrain in which RA plays a prominent role (Fig. 5D). At some point head mesoderm segmentation, now dispensable for neural segmentation, was lost (Guthrie 1995; Kuratani 1997). As a final step, the segmented hindbrain controls the patterning of the head mesoderm through the migration and differentiation of neural crest cells (a vertebrate innovation), to give rise to the complex vertebrate head (Fig. 5D). Whether an already present segmentation program (as measurable at the level of gene expression) triggered the iterated appearance of given cell type, or whether the ancestral existence of segmented neurons led to repeated gene expression is not clear. Whatever the case, it has been suggested (apropos of islet expression; Jackman et al. 2000) that this phenomenon eventually led to morphological segmentation. Expression of ERR in segmented neurons in the amphioxus posterior brain is in favor of the latter hypothesis.

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