Structural Evolution of *Otx* Genes in Craniates

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Using a degenerate PCR approach, we performed an exhaustive search of *Otx* genes in the reedfish *Erpetoichthys calabaricus,* the dogfish *Scyliorhinus canicula,* and the hagfish *Myxine glutinosa.* Three novel *Otx* genes were identified in each of these species, and their deduced protein sequences were determined over a large C-terminal fragment located immediately downstream of the homeodomain. Like their lamprey and osteichthyan counterparts, these nine genes display a tandem duplication of a 20–25-residue C-terminal domain, which appears to be a hallmark of all craniate *Otx* genes identified thus far, including the highly divergent *Crx* gene. Phylogenetic analyses show that, together with their osteichthyan counterparts, the dogfish and reedfish genes can be classified into three gnathostome orthology classes. Two of the three genes identified in each of these species belong to the *Otx1* and *Otx2* orthology classes previously characterized in osteichthyans. The third one unambiguously clusters with the *Otx5*/*Otx5b* genes recently characterized in *Xenopus laevis,* thus defining a novel orthology class. Our results also strongly suggest that the highly divergent *Crx* genes identified in humans, rodents, and oxen are the mammalian representatives of this third class. The hagfish genes display no clear relationships to the three gnathostome orthology classes, but one of them appears to be closely related to the *LjOtxA* gene, previously identified in *Lampetra japonica.* Taken together, these data support the hypothesis that the *Otx* multigene families characterized in craniates all derive from duplications of a single ancestral gene which occurred after the splitting of cephalochordates but prior to the gnathostome radiation. Using site-by-site sequence comparisons of the gnathostome Otx proteins, we also identified structural constraints selectively acting on each of the three gnathostome orthology classes. This suggests that specialized functions for each of these orthology classes were fixed in the gnathostome lineage prior to the splitting between osteichthyans and chondrichthyans.

Introduction

Comparative analyses of an increasing number of chordate gene families have suggested that massive gene duplications have taken place during early vertebrate evolution, after the emergence of cephalochordates but before the splitting between chondrichthyans and osteichthyans (defined as a group containing both actinopterygians and sarcopterygians, including tetrapods). Several authors have proposed that by releasing the constraints acting on duplicated genes, these genetic events may have facilitated the gain of new functions and substantially contributed to the morphological complexity displayed by vertebrates among chordates (Ohno 1970; reviewed in Holland et al. 1994). *Orthodenticle*-related homeobox genes, which have been identified with a high level of similarity over the homeodomain in representatives of all three major metazoan taxa, diploblasts (Müller, Yanze, and Schmid 1999; Smith et al. 1999), protostomes (Finkelstein et al. 1990; Bruce and Shankland 1998; Umesono, Watanabe, and Agata 1999), and deuterostomes, provide an example of such gene duplications. In deuterostomes, these genes, termed *Otx* genes, have been characterized in a wide range of species, including echinoderms (Gan et al. 1995), enteropneusts (Harada et al. 2000), ascidians (Wada et al. 1996), cephalochordates (Williams and Holland 1998), and a number of vertebrates. While they are present as single-copy genes in echinoderms, urochordates, and cephalochordates, small multigene families have been described in most vertebrates studied (references below). These gene duplications have been followed by functional diversifications of the different paralogs, as best illustrated in mice using targeted gene inactivation techniques. In this species, three paralogous *Otx* genes have been identified, *Otx1*, *Otx2*, and *Crx*. *Otx1* and *Otx2* display a dynamic expression pattern in the developing head and sense organs, with a characteristic expression domain at 10.5 days postcoitum (dpc) over the whole prosencephalon and mesencephalon (Simeone et al. 1992), while *Crx* is specifically transcribed in the retinal photoreceptors and the pinealocytes of the epiphysis (Furukawa, Morrow, and Cepko 1997). Each of these three genes is involved in some aspects of head development, but the associated mutant phenotypes substantially differ. Mouse *Otx2,* which, unlike *Otx1* and *Crx,* is transcribed as early as 5.5 dpc in mouse embryos, is required during gastrulation for the initial induction of anterior neuroectoderm, a process which relies on cellular and molecular interactions with the adjacent visceral endoderm layer (Acampora et al. 1995; Rhinn et al. 1998). The resulting $Otx2^{-/-}$ phenotype, which is characterized in the neural tube by the specific deletion of anteriormost neuromeres, shows some similarity to the deletion of the protocerebrum induced in *Drosophila* by an *orthodenticle* null mutation, thus suggesting that an ancestral role in brain specification may have been conserved between these phylogenetically distant species (Hirth and Reichert 1999). On a shorter evolutionary scale, among chordates, expression patterns of amphioxus and ascidian

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Key words: *Otx,* craniate phylogeny, gene duplication, dogfish, reedfish, hagfish.

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Mol. Biol. Evol. 18(9):1668–1678. 2001

Otx genes do support their involvement in anterior neuroectoderm specification through inductive interactions with mesendoderm (Wada et al. 1996; Williams and Holland 1996). *Otx1*$^{-/-}$ mice display a complex phenotype, characterized by a variety of defects, including pituitary hormone deficiencies and behavioral and morphological abnormalities (Acampora et al. 1996). Among the latter, the deletion of several gnathostome-specific characteristics, such as the lateral semicircular canal of the inner ear or the ciliary process, is of particular interest, since it suggests that the diversification of the *Otx1* form may have been linked to their emergence during evolution (Mazan et al. 2000). Finally, in line with its expression pattern, *Crx* is essential for the differentiation of retinal photoreceptors and is also involved in the genetic control of circadian entrainment (Furukawa et al. 1999).

The molecular basis for these functional diversifications has been addressed by experiments consisting of the replacement of either Otx1 or Otx2 coding regions by the paralogous form in knock-in mice. These experiments have shown that differential transcriptional controls could largely, but not exclusively, account for the differences observed between *Otx1*$^{-/-}$ and *Otx2*$^{-/-}$ mutant phenotypes. When expressed under *Otx2* endogenous regulatory sequences, *Otx1* can completely restore the gastrulation defects observed in *Otx2*$^{-/-}$ mice. However, at later stages, the specification of the prosencephalon and the mesencephalon remains defective (Acampora et al. 1998; Suda et al. 1999). On the other hand, *Otx2* can compensate for most *Otx1*$^{-/-}$ defects in knock-in mice except for the deletion of the lateral semicircular canal in all animals studied, and with lower penetrance for several eye defects, including the absence of ciliary process (Morsli et al. 1999; Acampora et al. 1999).

A comparative analysis of vertebrate *Otx* genes in a wide range of species provides an alternative approach to address the molecular mechanisms underlying their functional diversification and possible links to gene duplication events. Most studies thus far have focused on osteichthyan species. *Otx* genes have been characterized in zebrafish (Li et al. 1994; Mori et al. 1994; Mercier et al. 1995), two anurans, *Xenopus laevis* (Pannese et al. 1995; Kablar et al. 1996) and *Eleutherodactylus coqui* (Fang and Elinson 1999), chick (Bally-Cuif et al. 1995), and several mammals (Simeone et al. 1992; 1993). In all of these species, genes clearly related to the mouse *Otx1* and *Otx2* paralogous forms have been unambiguously recognized, thus demonstrating the presence of two orthology classes among osteichthyans. Comparative analyses with amphioxus *Otx* have provided strong evidence that the corresponding duplication event took place prior to the splitting between actinopterygians and sarcopterygians but after the emergence of cephalochordates (Williams and Holland 1998). In contrast, *Crx* genes, which are divergent members of the gene family, have been found only in mammals (mice, rats, humans, and oxen). Although these genes have aroused much interest in the past few years due to their involvement in human hereditary retinal degeneration, their origin is thus far completely unknown (Furukawa, Morrow, and Cepko 1997; Chen et al. 1999). In addition, other *Otx* genes have been isolated in *Danio rerio* (*DrOtx3*; Mori et al. 1994; Mercier et al. 1995) and, recently, in *X. laevis* (*XlOtx5* and *Otx5b*; Kuroda et al. 2000; Vignali et al. 2000). No orthological relationships have been described between these genes and any of their vertebrate counterparts, and the chronology of the corresponding duplication events is unclear. Finally, three *Otx* genes have also been identified in *Lampetra japonica* (*LjOtxA* and *LjOtxB*; Ueki et al. 1998) and *Petromyzon marinus* (*PmOtx*; Tomsa and Langeland 1999). Their relationship with their osteichthyan counterparts remains to be clarified.

To further characterize orthological relationships among vertebrate *Otx* genes and gain new insights into the structural evolution of craniate Otx proteins, we have started a characterization of these genes in two species which belong to phylogenetic groups of early emergence among craniates, the hagfish *Myxine glutinosa* and a chondrichthyan, the dogfish *Scyliorhinus canicula*. The reedfish *Erpetoichthys calabaricus*, which belongs to the Cladistia, a group of basal emergence among actinopterygians, has also been included in this analysis. We report here partial sequences of three *Otx* genes in each of these species. Their comparison with other vertebrate sequences provides new insights into orthological relationships among gnathostome *Otx* genes and into the structural constraints acting on their protein products.

Materials and Methods

Characterization of *Otx* Genes in *E. calabaricus, S. canicula,* and *M. glutinosa*

To identify *Otx* genes, a seminested degenerate PCR approach was taken, starting from genomic DNA and using the 5′ primers 5′-GTNTGGTTYAA-RAAYMG-3′ and 5′-GCNAARTGYMGNCARCA-3′, which, respectively, encode the VWFKNR and AKCRQQ protein motifs, and the 3′ primer 5′-ARNA-CYTGRAAYTTCCA-3′, which encodes the WKFQVL protein motif. The cycling conditions were (1) denaturation, 95°C for 1 min; (2) annealing, 50°C for 1 min; and (3) elongation, 72°C for 2 min in the standard *Taq* DNA polymerase buffer. For each species, a heterogeneous population of amplified fragments of 550–700 bp in length were subcloned in linearized pTZ19R, and 24 independent recombinants were picked and sequenced. Sequences showing less than five nucleotide differences were inferred to derive from the same gene, with differences corresponding either to polymorphisms or to *Taq* DNA polymerase errors. The sequences corresponded to the consensus of a minimum of three independent clones.

Molecular Phylogenetic Analysis

Otx protein sequences were retrieved from the GenBank database. Their accession numbers are as follows: *Branchiostoma floridae* Otx, AF043740; *P. marinus* Otx, AF099746; *L. japonica* OtxA, AB012299; *L. japonica* OtxB, AB012300; *D. rerio* Otx1, D26172; *D. rerio* Otx2, D26173; *D. rerio* Otx3, D26174; *X. laevis*

Otx2, U19813; *X. laevis* Otx2b, Z46972; *X. laevis* Otx5, AB034702; *X. laevis* Otx5b, AJ251846; *Homo sapiens* Otx1, P32242; *H. sapiens* Otx2, P32243; *H. sapiens* Crx, AF024711; *Rattus norvegicus* Otx1, L32602; *R. norvegicus* Crx, AB021129; *Mus musculus* Otx1, P80205; *M. musculus* Otx2, P80206; *M. musculus* Crx, U77615; *Bos taurus* Crx, AF154123. Two sequences were obtained from Kablar et al. (1996), *X. laevis* Otx1 and *X. laevis* Otx4. All these sequences were manually aligned using the ED program of the MUST package (Philippe 1993). The nine new sequences determined in this study were added to this alignment of chordate Otx proteins, allowing for the comparison of 31 sequences.

In phylogenetic reconstructions, the Otx2 sequence from *H. sapiens* was excluded because of its identity to mouse Otx2 over the amplified segment. Phylogenetic trees were constructed using neighbor-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP), and maximum-likelihood (ML) algorithms using the MUST, version 2000, package (http://bos.snv.jussieu.fr/must2000.html); PAUP, version 3.1.1 (Swofford 1993); and PROTML, version 2.3 (Adachi and Hasagawa 1996), respectively. In the NJ analysis, distances were computed with the method of Kimura (1983). The MP tree was obtained by 100 random-addition heuristic search replicates and the tree bisection-reconnection (TBR) branch-swapping option. ML trees were constructed by the quick-add OTUs search with the JTT-f model of amino acid substitution, retaining the 2,000 top-ranking trees. Bootstrap proportions (BPs) were calculated by analysis of 1,000 replicates for NJ and MP and by the RELL method (Kishino, Miyata, and Hasegawa 1990) on the 2,000 top-ranking trees for ML.

Results

Identification of Three *Otx* Genes in *E. calabaricus, S. canicula,* and *M. glutinosa*

To identify *Otx* genes in *E. calabaricus, S. canicula,* and *M. glutinosa,* we used a PCR-based strategy, taking advantage of the high phylogenetic conservation of protein motifs located between residues 47 and 59 of the homeodomain (VWFKNR and AKCRQQ) and at the C-terminal ends of all vertebrate Otx proteins (W(K/R)FQVL). Degenerate amplification primers were designed to span these motifs, which delimit a large protein domain, ranging in mice from 186 to 252 amino acids for Otx1 and Otx2, respectively. The corresponding coding region is contained within a single exon in mouse, as well as in amphioxus, suggesting that this genomic organization may be conserved among all vertebrates. All PCR amplifications were performed starting from genomic DNA.

In each studied species, this PCR strategy led to the amplification of a heterogeneous population of fragments, approximately 550–700 bp in length, which were subcloned and sequenced. Sequence comparisons of 24 subclones revealed the presence of three different fragments in the amplification products obtained from each of the three species under study, *E. calabaricus, S. canicula,* and *M. glutinosa.* These nine fragments unambiguously contain Otx coding sequences, as shown by their deduced amino acid sequences, which display a number of conserved motifs or residues previously identified in vertebrate Otx proteins. All share with their vertebrate counterparts a glutamine-rich stretch of variable length ($Q_{1–5}$, positions 1–5 in the alignment; see *Supplementary Material*) immediately following the AKCRQQ motif, and the $KXRX_{1–2}KXK$ motif (positions 16–23), which is also found in Amphi Otx. Except for one sequence of *M. glutinosa* (termed MgOtxD) which displays a divergent version (ILWNPA), they also contain the highly conserved (S/A)(I/L)WSPA motif (positions 111–116). This motif has been identified in a wide range of metazoan Otx proteins, including those encoded by the flour beetle *Tc-otd2* and the jellyfish *Pc-Otx* genes. Finally, sequences showing a similarity to the (D/E)CLDYK(D/E)(Q/P) motif (positions 365–376), called the Otx tail and present immediately upstream of the C-terminal W(K/R)FQVL motif in all deuterostome Otx proteins, can be unambiguously identified at the expected position in each case. The nine *Otx* genes thus identified were termed *EcOtx1, EcOtx2,* and *EcOtx5* in *E. calabaricus*; *ScOtx1, ScOtx2,* and *ScOtx5* in *S. canicula*; and *MgOtxA, MgOtxC,* and *MgOtxD* in *M. glutinosa,* according to their orthology relationships with their vertebrate counterparts (described below).

Phylogenetic Analysis of *Otx* Genes in Craniates

The partial Otx protein sequences determined in *E. calabaricus, S. canicula,* and *M. glutinosa* were included in an exhaustive alignment containing their cephalochordate and vertebrate counterparts (see *Supplementary Material*). All of the craniate sequences could be confidently aligned over extended regions of similarity (bold characters in the alignment), which were used for phylogenetic analyses. Outside of these regions, more variable intervening segments were excluded from the analysis. From the alignment of 380 amino acids, 148 positions were kept, among which 101 corresponded to informative sites for parsimony. Phylogenetic reconstructions were carried out using the NJ, MP, and ML methods (fig. 1). A single most-parsimonious tree (length = 479; retention index [RI] = 0.595; consistency index [CI] = 0.698) was obtained, and the length (L) of the ML tree was $\ln L = -2{,}615.9$. Although the analyses were conducted unrooted, the resulting trees are shown rooted on *MgOtxC* and *MgOtxD* for convenience of presentation (fig. 1). All three analyses confirm the presence of the *Otx1* and *Otx2* orthology classes already characterized in osteichthyans. In addition to the *Otx1*- and *Otx2*-related genes identified in zebrafish, Xenopus, and mammals, each of these classes contains one of the genes newly characterized in *E. calabaricus* (*EcOtx1* and *EcOtx2*) and *S. canicula* (*ScOtx1* and *ScOtx2*). These two classes are always found to be monophyletic whatever the reconstruction method used. However, the clustering of the gnathostome *Otx2* sequences is more highly supported (with BP values ranging from 75% to 97%) than the clustering of the *Otx1*-related paralogous genes (with BP reaching a maximum

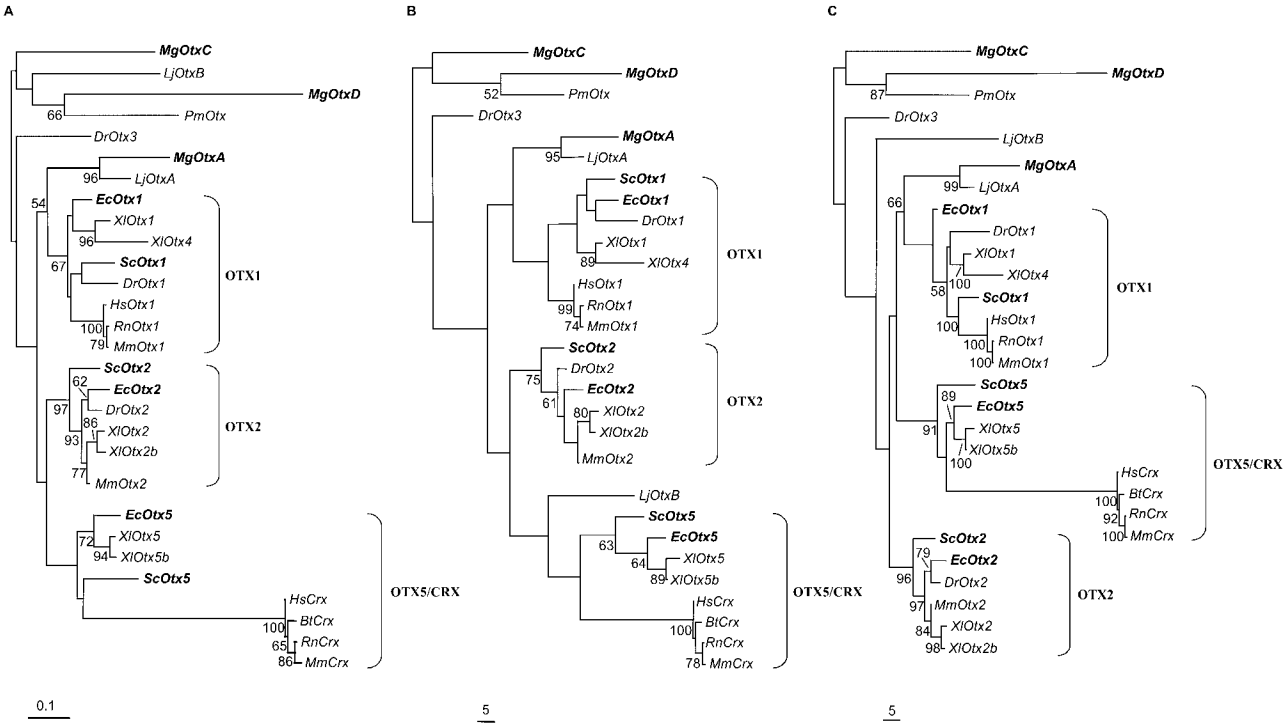

FIG. 1.—Phylogenetic relationships of *Otx* genes within craniates. *A–C,* Phylogenetic trees calculated using distance, maximum-parsimony and maximum-likelihood methods, respectively. Although no root has been assumed in this analysis, the trees are shown rooted with MgOtxC and MgOtxD sequences for convenience of presentation (see *Results*). Bold names correspond to the sequences determined in this study, and species nomenclatures are homogenized using the following abbreviations: *Mm, Mus musculus*; *Rn, Rattus norvegicus*; *Hs, Homo sapiens*; *Xl, Xenopus laevis*; *Dr, Danio rerio*; *Ec, Erpetoichthys calabaricus*; *Sc, Scyliorhinus canicula*; *Lj, Lampetra japonica*; *Pm, Petromyzon marinus*; *Mg, Myxine glutinosa*; *Bf, Branchiostoma floridae.* Numbers indicate bootstrap proportions supporting the corresponding nodes. Only bootstrap values >50% are shown. The three orthology classes identified in gnathostomes are indicated by vertical brackets. The scale bar represents (*A*) the number of estimated differences for a unit branch length, (*B*) the number of steps, and (*C*) the number of substitutions per position for a unit branch length.

of 67% for NJ). Inside the *Otx2* class, the classical phylogeny of gnathostomes is recovered by the three reconstruction methods, except for the position of actinopterygians, which form a paraphyletic group in the MP tree (BP < 50%). In particular, the early emergence of *S. canicula* is always supported by high bootstrap values (93%, 61%, and 97% in NJ, MP, and ML analyses, respectively). The splitting of actinopterygians and sarcopterygians into two well-defined groups also receives strong support in NJ and ML analyses (BP = 62% and 79% for actinopterygians, 77% and 84% for sarcopterygians). Inside the *Otx1* class, the branching orders differ substantially depending on the reconstruction method used. If the monophyly of mammals, as well as the close relationship of *Otx1* and *Otx4* sequences in *X. laevis,* are always recovered and supported by high bootstrap values, the relative position of the two actinopterygians (*E. calabaricus* and *D. rerio*) and the chondrichthyan (*S. canicula*) is not resolved.

In addition to the *Otx1* and *Otx2* classes, a third group, containing representatives of the major gnathostome taxa (chondrichthyans, actinopterygians, sarcopterygians including amphibians, and mammals) was found in all three tree reconstructions. This group contains the two closely related genes recently characterized in Xenopus (*XlOtx5* and *XlOtx5b*) and the third gene identified in *E. calabaricus* and in *S. canicula* (thereafter named *EcOtx5* and *ScOtx5*, respectively), together with the mammalian *Crx* genes. While the corresponding bootstrap values remain low in NJ and MP analyses, the existence of this third class receives good support in the ML analysis (BP = 91%). Inside this class, *EcOtx5*, *XlOtx5*, and *XlOtx5b* always form a monophyletic group, as do the mammalian *Crx* genes, but the relative positions of *ScOtx5* and these two groups differ between the three reconstruction methods. This variability may be a consequence of the high divergence displayed by Crx sequences. However, *Crx* genes are always found in the same clade as *Otx5* genes. The most parsimonious interpretation of these data is that *Crx* genes are orthologous to *Otx5* genes but underwent an acceleration in their rate of evolution following the amphibian/amniote separation. In line with this hypothesis, we have been unable to find an *Otx5* gene in the mouse or a *Crx* gene in *Xenopus* using the PCR strategy described here, whereas mouse *Crx* and *Xenopus Otx5* gene fragments could be readily amplified by this method (data not shown).

As for the third gene identified in zebrafish (*DrOtx3*), it appears unrelated to the *Otx1*, *Otx2*, or *Otx5*/*Crx* classes in our tree reconstructions, always emerging before their separation. This result is incongruent with previously published studies which showed a clustering of *DrOtx3* and *DrOtx1* (Williams and Holland 1998).

However, the early emergence observed in our analyses for *DrOtx3* is poorly supported. Furthermore, when only gnathostome sequences were included in the phylogenetic analyses, the zebrafish *DrOtx1* and *DrOtx3* sequences clustered together (data not shown). Together with the site-by-site analysis of the alignment in the more variable regions (see below), these results suggest that *DrOtx3* may be a divergent member of the *Otx1* class.

The lamprey and hagfish *Otx* sequences cannot be confidently assigned to any of the three classes identified in gnathostomes. Their relative branching orders appear variable depending on the reconstruction method used and most of the time are poorly resolved. A major exception lies in the clustering of *LjOtxA* with one of the hagfish genes (thereafter termed *MgOtxA*), which is observed in NJ, MP, and ML analyses. A close relationship between these two sequences is supported by high bootstrap values (96%, 95%, and 99% in NJ, ML, and MP analyses, respectively). In addition, a clustering of *MgOtxD* and *PmOtx* was obtained in the three phylogenetic reconstructions with bootstrap values above 50%. However, because of the high degree of divergence of these two sequences, we cannot exclude that this result may be due to a long-branch attraction artifact.

Phylogenetic Analysis of Amphioxus and Craniate Otx Sequences

In an attempt to root the craniate Otx phylogenetic tree, amphioxus Otx sequence was added for a new phylogenetic analysis (fig. 2). The portions of the alignment which were taken into account were the same as those previously used. Among the 150 sites used in this analysis, 136 corresponded to variable sites and 102 were informative for parsimony. As in the previous analysis, phylogenetic reconstructions were carried out using the NJ, MP, and ML methods. Ten equiparsimonius trees were found (length = 517; RI = 0.684; CI = 0.594), and the length of the ML tree was $\ln L = -2{,}790.86$.

The three gnathostome *Otx* classes and the cyclostome *OtxA* class were recovered, with slightly less support than in the previous analyses. The ML analysis provided the strongest support for the monophyly of the *Otx2* and *Otx5*/*Crx* classes (96% and 92%, respectively), but weak support concerning *Otx1*-related genes (41%). In the MP analysis, the three classes were recovered in the 10 MP trees found, albeit with relatively low bootstrap values (38%, 63%, and 42%, respectively, for the *Otx1*, *Otx2*, and *Otx5*/*Crx* classes). The NJ analysis also confirmed the presence of the three classes, with relatively low bootstrap values for the *Otx1* and *Otx5*/*Crx* classes (59% and 33%) and a higher one for *Otx2* (92%).

The close relationship observed between LjOtxA and MgOtxA in the previous analysis was recovered with high bootstrap values in all three reconstruction methods. No clear conclusion could be reached concerning the relative order of emergence of the three gnathostome orthology classes, the cyclostome *OtxA* class, and the other lamprey and hagfish genes (*PmOtx*, *LjOtxB*, *MgOtxC*, and *MgOtxD*). It appeared variable depending on the reconstruction method used (data not shown), and none of the groupings appeared to be supported by significant bootstrap values (fig. 2).

Structural Evolution of the *Otx1*, *Otx2*, and *Otx5*/*Crx* Orthology Classes in Gnathostomes

To independently assess the existence of the three orthology classes identified in the phylogenetic analysis and gain deeper insight into their structural evolution, we performed a systematic search of motifs or residues specifically maintained in each of them. This analysis was carried out for the portions of the alignment which were included in the phylogenetic analysis and extended to the more variable intervening regions (see alignment). In the latter, no strongly supported alignment can be proposed between all gnathostome Otx proteins, but significant similarities can be identified within each of the four classes, respectively, containing Otx1/Otx4, Otx2/Otx2b, Otx5/Otx5b, and Crx sequences. In particular, the highly variable regions (positions 59–105 and 121–193) located immediately upstream and downstream of the WSP motif show an extensive divergence between Otx1, Otx2, Otx5, and Crx proteins but can be unambiguously aligned within each of these four classes. These close relationships strongly support the assignment of *Sc*/*EcOtx1*, *Sc*/*EcOtx2*, and *Sc*/*EcOtx5* to the *Otx1*, *Otx2*, and *Otx5* orthology classes, respectively. Most of the repetitive stretches of serine and histidine (positions 62–75, 257–263, 290–299, and 307–317), previously identified as hallmarks of osteichthyan *Otx1*-related genes, are also conserved in *EcOtx1* and *ScOtx1* but absent from all other craniate sequences, suggesting that they correspond to synapomorphies of the *Otx1* orthology class. The only exception is the histidine stretch located at positions 290–299, which appears to be restricted to a single additional H in *ScOtx1*. From position 257 to position 299, *ScOtx1* sequence also provides new clues for understanding the structural evolution of *Otx1*-related genes, since it displays a 7-amino-acid motif (positions 270–276) which has been deleted in all osteichthyan Otx1 proteins but is present in all other craniate sequences. While the relationship between osteichthyan Otx1 sequences and other vertebrate sequences remained unclear over this domain, the identification of this ancestral characteristic makes it possible to confidently align them with their craniate counterparts.

In gnathostomes, the characterization of *Otx1*-, *Otx2*-, and *Otx5*-related coding sequences in a wide range of species, including in all cases a chondrichthyan, at least one actinopterygian, and one osteichthyan, makes it possible to identify residues which have been selectively conserved within each of these three classes. A total of 15, 10, and 14 such amino acids, respectively, selectively maintained among Otx1, Otx2, and Otx5 protein sequences, could be identified. These residues are shaded in the alignment (*Supplementary Material*). They are present not only in the most variable regions of the proteins, which were excluded from the phylogenetic analysis, but also within the protein domains

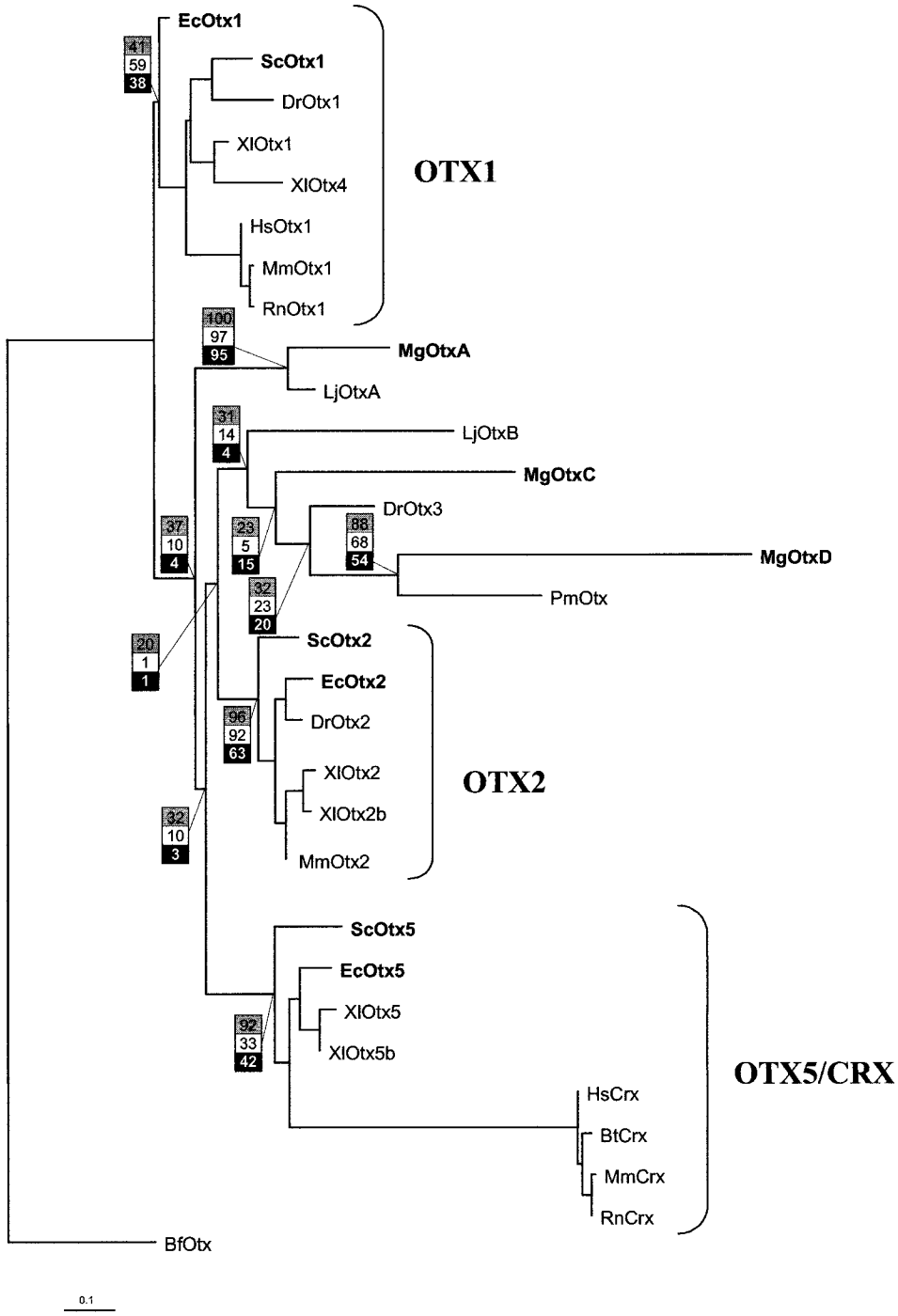

FIG. 2.—Phylogenetic relationships between craniate and amphioxus *Otx* genes. The tree was reconstructed using the maximum-likelihood method. Squared numbers indicate bootstrap proportions supporting the corresponding node in each reconstruction method: maximum-likelihood, neighbor-joining, and maximum-parsimony bootstrap values are shown in black characters on a gray background, black characters on a white background, and white characters on a black background, respectively. For sake of clarity, bootstrap values obtained inside each of the *Otx1*, *Otx2*, and *Otx5*/*Crx* classes were omitted. The tree is rooted using amphioxus Otx (BfOtx). Species abbreviations are defined in figure 1. The scale bar represents the number of substitutions per position for a unit branch length.

which can be unambiguously aligned between paralogous forms.

Using these distinctive features as a criterion, we studied the assignment of the zebrafish *Otx3* and the mammalian *Crx* genes to the *Otx1*, *Otx2*, and *Otx5* classes identified in gnathostomes. Over the protein domain studied, *DrOtx3* displays 10/15 (positions 41, 43, 61, 62, 123, 219, 257, 262, 263, and 308) and 1/10 residues (position 284) specific for *Otx1*- and *Otx2*-related genes, respectively, but no residue specific for the *Otx5* class. This suggests that it may be a divergent member of the *Otx1* orthology class. In line with this conclusion, it ex-

hibits the serine and histidine stretches which are also characteristic for this class (positions 62–74, 257–266, 290–294, and 307–313). As for the four mammalian *Crx* genes, their deduced amino acid sequences share 9 selectively conserved residues out of 14 with *Otx5* sequences but appear unrelated to *Otx1* or *Otx2* forms over the residues specific for these classes. This result thus supports their orthological relationship with *Otx5* genes, as already pointed out by the phylogenetic analysis.

The Otx sequences characterized in lamprey and in hagfish show no clear relationship with either the gnathostome Otx1, Otx2, or Otx5 form over the residues specific for each class. In contrast, *L. japonica* and *M. glutinosa* OtxA proteins share a total of eight residues which are not found in any other craniate Otx form. While the ancestral state cannot be determined in most cases, the G (position 358) which corresponds in both LjOtxA and MgOtxA to the N shared by the cephalochordate and all other craniate Otx sequences (except Crx forms, which display a T) is likely to correspond to a derived character.

Otx Tail Duplication in Craniate Otx Proteins

Phylogenetic reconstructions taking the whole protein sequence into account have provided strong evidence that the duplication events which have given rise to the lamprey *Otx* genes and the gnathostome *Otx1* and *Otx2* orthology classes took place after the splitting between the cephalochordate and vertebrate lineages. In line with this conclusion, it has been shown that these genes share sequence features which are not observed in echinoderms, ascidians, or cephalochordates. In particular, all of them display an imperfect tandem duplication of an 18–25-amino-acid motif containing the Otx tail (domains A and B in the alignment; see *Supplementary Material*). In contrast, a single copy is present in echinoderms, ascidians, and amphioxi (Williams and Holland 1998).

Like their orthologs in sarcopterygians and in zebrafish, the *Otx1* and *Otx2* coding sequences identified in *E. calabaricus* and in *S. canicula* contain two copies of the C-terminal domain. To address the chronology of the duplication events that have generated the *Otx5*/*Crx* orthology class identified in gnathostomes and the multigene family characterized in hagfish, we examined each of the sequences reported in this study for the presence of this C-terminal repeat. Both domains A and B are unambiguously present at the C-terminal ends of the four Otx5 proteins identified in *X. laevis*, *E. calabaricus*, and *S. canicula* (positions 329–356 and 357–380). However, the presence of this tandem duplication has not been recognized in the amino acid sequence encoded by mammalian *Crx* genes (Furukawa, Morrow, and Cepko 1997). Since they cluster with *Otx5* genes in our phylogenetic reconstructions, we searched for the presence of a duplicated C-terminal domain in Crx sequences. Despite a 4-amino-acid deletion in the N-terminal part of domain A, all four mammalian Crx sequences contain the DSLEFKDPTGTWK motif, which can be unambiguously aligned with the C-terminal part of this domain (positions 337–356). Immediately downstream (positions 357–380), domain B can also be unambiguously recognized, although its N-terminal consensus in Otx1, Otx2, and Otx5 sequences (LNF, positions 357–359) is replaced by the motif FTY in Crx proteins. Although degenerate, the C-terminal repeat is thus present in Crx amino acid sequences. In hagfish, the three sequences identified also contain both domains A and B at their C-terminal ends (positions 329–356 and 357–380), with several single-amino-acid insertions or deletions.

Discussion

Orthological Relationships Among Craniate *Otx* Genes

We report here the identification and partial sequencing of three *Otx* genes in a polypterid, the reedfish *E. calabaricus*; a chondrichthyan, the dogfish *S. canicula*; and an agnathan, the hagfish *M. glutinosa*. Concerning the orthological relationships among gnathostome Otx genes, two strongly supported conclusions can be drawn from the phylogenetic analysis of these sequences. First, *Otx1*- and *Otx2*-related genes are present in all gnathostomes studied thus far, including the chondrichthyan *S. canicula*. Among extant vertebrates, a strict correlation is thus observed between the appearance of some gnathostome-specific characteristics which appear to be deleted in *Otx1*$^{-/-}$ mice (lateral semicircular canal of the inner ear, ciliary process) and the emergence of *Otx1*-related genes. This result supports the hypothesis that the diversification of gnathostome *Otx* genes is linked to the rise of these innovations during evolution (Mazan et al. 2000). Second, we show the presence of a novel orthology class, termed *Otx5*, in gnathostomes: this class unambiguously contains the *Otx5*/*Otx5b* genes recently identified in *Xenopus*, together with the third gene identified in *E. calabaricus* and *S. canicula*. In addition, our results also indicate that the highly divergent *Crx* genes, characterized in humans, rodents, and oxen, may be the mammalian representatives of this third group. In line with this hypothesis, *Xenopus Otx5*/*Otx5b* genes and rodent *Crx* genes share highly specific expression domains in the developing eye and epiphysis (Furukawa, Morrow, and Cepko 1997; Vignali et al. 2000). Such expression patterns substantially differ from the broad expression domains, spanning the whole prosencephalon and diencephalon displayed by all gnathostome *Otx1* and *Otx2* genes, thus supporting the relationship proposed between *Otx5* and *Crx* genes. Finally, in the hagfish *M. glutinosa*, as in gnathostomes, *Otx* genes form a small multigene family, comprising at least three paralogs. Like their lamprey counterparts, these genes show no clear relationship to the three orthology classes identified in gnathostomes. However, one of them is closely related to the *LjOtxA* gene previously isolated in *L. japonica*. This close relationship, which was highly supported by all our phylogenetic analyses, may simply reflect the clustering of both species in the same phylogenetic group. In line with this hypothesis, rRNA comparisons support the grouping of lampreys and hagfishes in the same phylogenetic group, the cyclostomes, which

is a sister group of the gnathostomes (Stock and Whitt 1992; Mallatt and Sullivan 1998). However, other analyses point to the basal emergence of hagfishes, considered a sister group of the vertebrates (lampreys and gnathostomes) (Janvier 1996; Rasmussen, Janke, Arnason 1998; Delarbre et al. 2000). Since no *OtxA*-related gene can be recognized among gnathostomes, this phylogeny would imply that *OtxA*-related genes either have been lost or have undergone a particularly high divergence rate in the gnathostome lineage after the splitting of lampreys.

Gene Duplications in the *Otx* Gene Family

The basal emergence of the chondrichthyans has recently been challenged on the basis of mitochondrial DNA analyses (Rasmussen and Arnason 1999). However, both the phylogenies obtained for *Otx2* and *Otx5* genes and the site-by-site analysis of gnathostome Otx1 sequences strongly support the basal position of the chondrichthyans as a sister group of osteichthyans. In this phylogeny, the identification of *S. canicula Otx* genes belonging to the three orthology classes characterized in osteichthyans indicates that the corresponding duplication events took place prior to the splitting between chondrichthyans and osteichthyans, more than 420 MYA (Benton 1990). The tandem repeat of the C-terminal motif observed in Otx1- and Otx2-related proteins, as well as in the three Otx proteins identified in *L. japonica* and *P. marinus*, has provided a strong argument that the corresponding genes all derive from duplications of a single ancestral gene which occurred after the splitting of cephalochordates (Ueki et al. 1998; Williams and Holland 1998; Tomsa and Langeland 1999). This conclusion can now be extended to the gnathostome *Otx5*/*Crx* genes and the three copies identified in hagfish, suggesting that the presence of this C-terminal repeat is a synapomorphy of all craniate Otx proteins. In contrast, the chronology of the duplication events that gave rise to the three orthology classes characterized in gnathostomes relative to the emergence of lampreys and hagfishes could not be resolved in our analyses. Independent duplication events provide the simplest hypothesis to account for the absence of sequence relationships between the lamprey or hagfish genes and the gnathostome *Otx1*, *Otx2*, and *Otx5*/*Crx* orthology classes. However, the possibility that gene duplications occurred prior to the splitting of lampreys and hagfishes cannot be formally ruled out. A relatively short time interval between such genetic events and the speciation events which generated these lineages could actually make orthological relationships with the gnathostome *Otx* genes difficult to detect.

These results are very similar to those obtained in a number of other genetic systems in two respects. First, they show the presence of unambiguous orthology classes in all jawed vertebrates, including chondrichthyans. Similar conclusions have been reached in a number of other molecular studies also including chondrichthyan sequences (Sidow 1992; Duguay et al. 1995; Kandil et al. 1996; Li, Keith, and Evans 1996; Hahn et al. 1997; Flajnik et al. 1999). Second, in hagfish, as in lamprey, they provide an additional example of a multigene family resulting from duplications of a single ancestral copy in the craniate lineage. An absence of clear orthological relationships between the paralogous genes identified in these species and their gnathostome counterparts has also been a recurrent conclusion for the different studied systems (Sidow 1992; Li, Keith, and Evans 1996; Sharman and Holland 1998; Kuraku et al. 1999; Suga et al. 1999). Taken together, these results support the hypothesis that the duplication of *Otx* genes may have been part of massive amplification events which took place after the emergence of cephalochordates and prior to the splitting between chondrichthyans and osteichthyans (Williams and Holland 1998). However, a more precise chronology of such genetic events remains elusive.

Functional Evolution of Gnathostome *Otx* Genes

Several lines of evidence indicate that for some of the complex functions fulfilled by *Otx* genes in gnathostomes, paralogous Otx proteins may share largely equivalent biochemical properties. For instance, all the gastrulation defects observed in *Otx2*$^{-/-}$ mouse embryos, including the initial induction of anterior neuroectoderm, can be compensated when the mouse *Otx2* coding region is replaced by its *Otx1* counterpart in knock-in experiments (Acampora et al. 1998; Suda et al. 1999). Likewise, the replacement of Otx1 by Otx2 restores most of the brain and sense organ abnormalities observed in *Otx1*$^{-/-}$ mice (Acampora et al. 1999). In *Xenopus*, the phenotypes induced by overexpressions of *XlOtx2* and *XlOtx5*/*Otx5b* are also very similar, suggesting that both paralogs are involved in the early specification of anterior regions (Andreazzoli, Pannese, and Boncinelli 1997; Kuroda et al. 2000; Vignali et al. 2000). On the other hand, the identification of amino acid differences between Otx1, Otx2, and Otx5 proteins, which appear to be selectively maintained among a wide range of gnathostomes, provides strong evidence that different structural constraints act on each orthology class. Whatever the precise chronology of *Otx* genes duplication events in craniates, these structural constraints were fixed after the cyclostome/gnathostome splitting but prior to their radiation. This suggests that functions which are unique to each orthology class were also fixed in the gnathostome lineage, prior to the chondrichthyan/osteichthyan splitting. Results of knock-in experiments and comparisons of the expression patterns of gnathostome *Otx* genes are consistent with this hypothesis. The role played by mouse *Otx1* in the lateral semicircular canal formation could provide an example of such specializations, since it cannot be compensated for by the paralogous form *Otx2* in knock-in experiments. Concerning *Otx2*, it is not currently known whether the jaw defects observed in *Otx2*$^{+/-}$ mutant mice (Matsuo et al. 1995) can be compensated by paralogous genes. In contrast, the replacement of Otx2 coding sequence by the paralogous Otx1 form in mice results in a defective prosencephalon specification during neurulation, raising the possibility that this role may be specific to the *Otx2*

orthology class. As for the *Otx5*/*Crx* orthology class, it may have been recruited for specific roles in photoreceptor development, as suggested by the specific transcription pattern shared by amphibian *Otx5* genes in the developing eye and epiphysis and the analysis of $Crx^{-/-}$ mice. The analysis of *ScOtx5* expression should help to test this hypothesis.

Taken together, these results suggest that while some roles of *Otx* genes may be fulfilled by different combinations of paralogous genes depending on the species considered, each gnathostome orthology class has also been recruited for specific functions. Residues corresponding to selectively maintained differences between the three orthology classes may be important in determining the specificity of the molecular interactions which control these processes. The identification of such interactions, together with detailed analyses of the structure/function relationships of gnathostome *Otx* genes, will be required to elucidate the genetic and molecular mechanisms underlying their functional diversification.

Supplementary Material

Nucleotide sequences encoding the protein fragments reported in this study for the reedfish *EcOtx1*, *EcOtx2*, and *EcOtx5*, the dogfish *ScOtx1*, *ScOtx2*, and *ScOtx5*, and the hagfish *MgOtxA*, *MgOtxC*, and *MgOtxD* have been deposited in the GenBank database under accession numbers AF321406, AF321407, AF321408, AF321409, AF321410, AF321411, AF321412, AF321413, and AF321414, respectively. The alignment of the craniate and amphioxus Otx sequences are shown in supplementary material on the SMBE web site.

Acknowledgments

This work was supported by Fondation pour la Recherche Médicale and Société de Secours des Amis des Sciences postdoctoral fellowships to A.G. and funded by the Centre National de la Recherche Scientifique, the Université Paris-Sud, and the Fondation pour la Recherche Médicale. We thank Hervé Philippe for advice on phylogenetic reconstructions, Noëlle Narradon for her help in typing the manuscript, Lenny Rabinow for his careful reading of the manuscript, and Monique Pradels for her excellent technical assistance.

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WILLIAM JEFFERY, reviewing editor

Accepted May 11, 2001