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The Hox Paradox: More Complex(es) Than Imagined

Victoria E. Prince[1]

Department of Organismal Biology and Anatomy, Committees on Developmental Biology, Neurobiology, Genetics and Evolutionary Biology, The University of Chicago, 1027 E. 57th Street, Chicago, Illinois 60637

An understanding of the origin of different body plans requires knowledge of how the genes and genetic pathways that control embryonic development have evolved. The Hox genes provide an appealing starting point for such studies because they play a well-understood causal role in the regionalization of the body plan of all bilaterally symmetric animals. Vertebrate evolution has been characterized by gene, and possibly genome, duplication events, which are believed to have provided raw genetic material for selection to act upon. It has recently been established that the Hox gene organization of ray-finned fishes, such as the zebrafish, differs dramatically from that of their lobe-finned relatives, a group that includes humans and all the other widely used vertebrate model systems. This unusual Hox gene organization of zebrafish is the result of a duplication event within the ray-finned fish lineage. Thus, teleosts, such as zebrafish, have more Hox genes arrayed over more clusters (or "complexes") than do tetrapod vertebrates. Here, I review our understanding of Hox cluster architecture in different vertebrates and consider the implications of gene duplication for Hox gene regulation and function and the evolution of different body plans.

Key Words: **Hox genes; Hox clusters; gene duplication; vertebrate evolution; teleosts; zebrafish; hindbrain.**

CLUSTERED ORGANIZATION OF HOX GENES

The Hox genes were first characterized in the fruitfly, *Drosophila melanogaster*, where eight linked *Antennapedia* class homeobox genes make up the Homeotic complex (Lewis, 1978). These eight genes encode homeodomain transcription factors that are characterized by their role in conferral of segmental identity along the primary body axis, from anterior to posterior (AP; reviewed by McGinnis and Krumlauf, 1992). Thus, mutations in the fly Hox genes lead to dramatic homeotic phenotypes, where one body segment takes on the identity of another. Homologous Hox genes have been found in every bilaterian animal investigated (de Rosa *et al.*, 1999), and in all cases analyzed, the genes show a clustered organization, although gene and cluster number vary. Most importantly, wherever tests have been applied, the Hox genes have proven to play critical roles in determining AP identity.

Comparative analyses of Hox cluster organization have revealed that variations in Hox gene number between species reflect an evolutionary history characterized by two types of duplication events: tandem duplication and whole cluster duplication. Current models suggest that single cluster organizations, like that of *D. melanogaster*, arose via the tandem duplication of ancestral Hox genes (Kappen *et al.*, 1989; Kmita-Cunisse *et al.*, 1998). A single cluster organization appears to be common to all protostomes, and a single cluster with seven genes was in place in the ancestor of all bilaterians (Fig. 1) (de Rosa *et al.*, 1999). A single Hox cluster is also assumed to be characteristic of primitive deuterostomes, with the cephalochordate amphioxus having the cluster with the largest number of genes (Figs. 1 and 2) (Ferrier *et al.*, 2000).

It has long been supposed that gene duplication events could have played a vital role in allowing vertebrates to achieve their complexity of form through evolution of new gene functions (Ohno, 1970). The origin of vertebrates was associated with major expansions in gene number, possibly as a result of two rounds of whole genome duplication via polyploidization (often referred to as the "2R" hypothesis, for "two rounds" of duplication; Friedman and Hughes, 2001; Sidow, 1996). Such duplications would result in

[1] Fax: (773) 702-0037. E-mail: vprince@midway.uchicago.edu.

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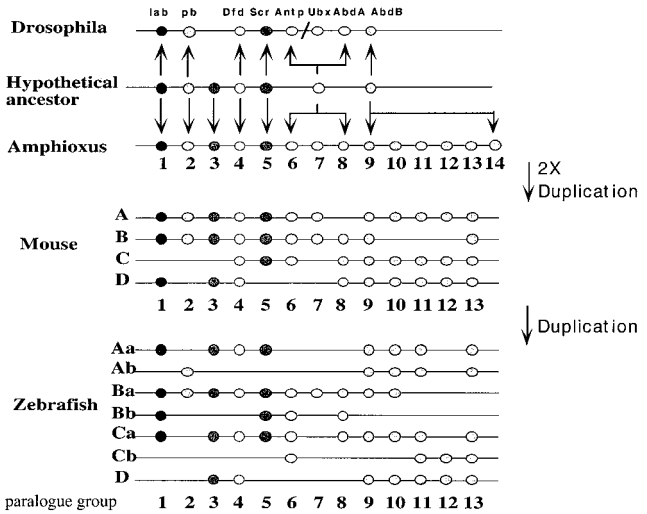

FIG. 1. Hox gene organization in *Drosophila melanogaster*, amphioxus, mouse, and zebrafish. A hypothetical ancestral condition is also shown. Shades of gray indicate the most closely related genes.

duplication of entire Hox clusters, and consistent with the 2R hypothesis, tetrapod vertebrates have four clusters of Hox genes (reviewed by Holland *et al.*, 1994; Sidow, 1996). Mouse and human have had their Hox cluster organizations fully described, and they share an identical 39-gene organization over 4 clusters, A–D (reviewed by McGinnis and Krumlauf, 1992; see also Zeltser *et al.*, 1996). The genes fall into 13 paralogue groups, with most paralogue groups having less than a full complement of 4 genes as a result of secondary gene losses (Fig. 1). A large number of Hox genes have also been isolated from frog (*Xenopus laevis*) and chick (*Gallus gallus*), and in each case, there is no evidence to suggest differences from the 39-gene mammalian organization (e.g., Godsave *et al.*, 1994; Ladjali-Mohammedi *et al.*, 2001). Thus, available data strongly suggest that a 4-Hox cluster organization is the primitive condition of crown-group tetrapods. Similarly, a PCR survey of Hox genes in the more basal lungfish (Longhurst and Joss, 1999) is also consistent with a 4-cluster condition. Nevertheless, we need more complete data from lungfish, as well as from the coelacanth, before we can conclude that there has been complete conservation of the 4-Hox cluster organization throughout the sarcopterygians (lobe-finned fishes).

If 2R happened, can we estimate when each genome duplication event would have occurred? The cephalochordate amphioxus may provide the best approximation of the preduplication vertebrate ancestor. The first 10 Hox genes in the single amphioxus Hox cluster show clear homology to the first 10 mammalian paralogue groups (Fig. 1) (Garcia-Fernandez and Holland, 1994). The most basal group of the vertebrates is the cyclostomes, comprising hagfish and lampreys (Fig. 2), which probably form a monophylectic group (Mallatt and Sullivan, 1998). These jawless vertebrates (Agnatha) might be expected to fall into an intermediate state between an ancestral, single Hox cluster organization and a derived, 4-cluster organization. Recently, 2 groups have published extensive analyses of the Hox clusters of the sea lamprey (*Petromyzon marinus*) (Force *et al.*, 2002; Irvine *et al.*, 2002). Both groups have isolated and mapped genomic Hox clones, to extend previous analyses based on PCR surveys (Pendelton *et al.*, 1993; Sharman and Holland, 1998). In both of the new studies, the data point to a minimum of 3 Hox clusters, with a 4th cluster considered likely. Irvine and colleagues (2002) built neighbor-joining trees based on sequences of individual homeodomains and were unable to distinguish between models where only one

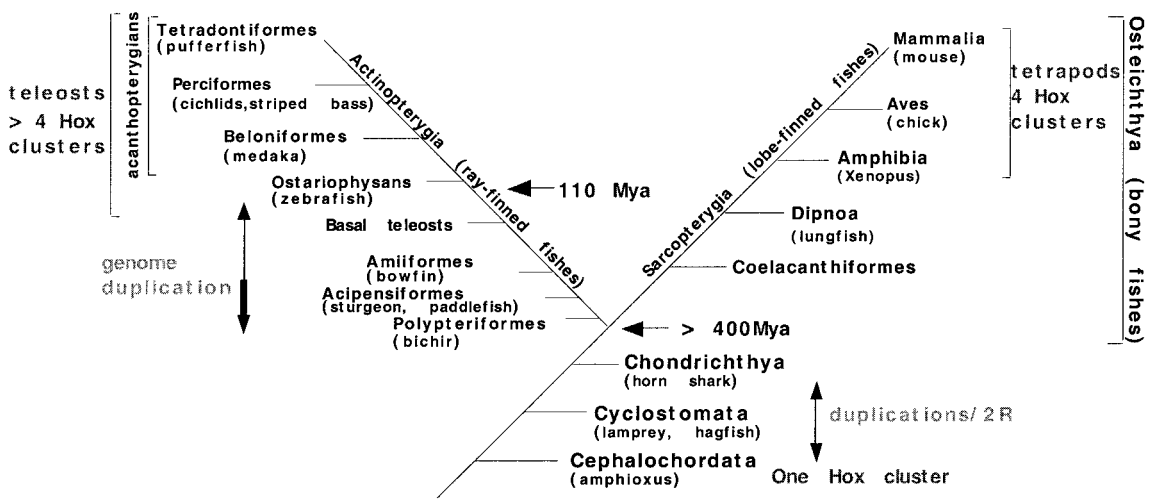

FIG. 2. Vertebrate phylogeny showing Hox cluster number and putative duplication events. (Based on Carroll, 1988).

round of duplication occurred before divergence of the jawed (gnathostome) and jawless vertebrates, versus all duplications predating the split. By contrast, Force and colleagues (2002) concatanated the lamprey homeodomain sequences that lay in contigs, to provide additional informative characters. Using this strategy, their extensive tree analysis of Hox genes across the vertebrates suggests that only one duplication event occurred prior to the divergence of the agnathans and gnathostomes, with a second duplication event occurring within the lineage leading to lampreys. Consistent with this model, both groups found pairs of lamprey Hox genes that are more closely related to one another than to any Hox gene from a gnathostome. Taken together, these data support the idea that 1 round of Hox duplication occurred before the divergence of the agnathans and gnathostomes, and a 2nd occurred in the gnathostome lineage. Ultimately, a complete linkage map of all the lamprey Hox genes will help to confirm this model.

An alternative model to the 2R hypothesis has been put forward by Ruddle and colleagues (Bailey *et al.*, 1997). According to this model, there were not two rounds of duplication in the lineage leading to tetrapods, but three. The existence of only four Hox clusters in the tetrapods is explained either by incomplete duplications (of a single cluster) or by losses of clusters following whole genome duplication events. Bailey and colleagues (1997) used sequence from *collagen* genes linked to Hox clusters to reconstruct a likely duplication scenario whereby the ancestral Hox cluster was D-like, which duplicated to create an A-like cluster from which the B and C clusters arose in turn (D(A(B,C))).

The Ruddle group has recently expanded its studies to include the horn shark (*Heterodontus francisci*). This species is cartilaginous, a member of Chondrichthyes, another basal group of vertebrates and a sister group to the bony fishes (Osteicthyes; Fig. 2). Investigations of the horn shark have so far revealed the presence of only two Hox clusters, M and N. However the sequences and organization of the Hox genes within these clusters suggest that M is homologous to the A cluster, while N is homologous to the C or D cluster, as described in mammals (Kim *et al.*, 2000). If the three-duplication event model is correct, the horn shark may ultimately prove to have only three Hox clusters in total, representing a stage before duplication of the common ancestor of the B and C clusters. However, there has been little additional data supporting the three-duplication event model, and the recent extensive tree building of Force and colleagues (2002) strongly supports two sequential duplication events [(AB)(CD)] leading to a four-cluster Hox organization in Sarcopterygia. It therefore seems likely that chondrichthyans (cartilaginous fish) and sarcopterygians (lobe-finned osteichthyans) will eventually prove to share a very similar four-cluster Hox organization, in which case the putative two rounds of duplication both occurred before the origin of chondrichthyans.

Despite the prevalence of the 2R hypothesis, the phylogenetic analyses of Hughes and colleagues (e.g., Friedman and Hughes, 2001; Hughes *et al.*, 2001) do not provide strong support for two rounds of genome duplication in the vertebrate stem lineage. The Hox genes themselves are notoriously uninformative for detailed phylogenetic analysis because of their remarkable sequence conservation. Thus, Hughes *et al.* (2001) constructed phylogenetic trees for other sets of duplicated genes lying on the Hox-bearing chromosomes of human. The trees for different gene families have different topologies, which the authors interpret as revealing that the duplicated genes did not arise simultaneously and are rather the result of numerous independent small-scale duplication events. However, these different topologies could also reflect recombination/conversion events between closely related genes shortly after duplication or may be an artefact of rapid evolution subsequent to duplication. Furthermore, the newly available human genome sequence reveals extensive synteny between the four Hox-bearing clusters (although less evidence for larger-scale duplication events; International Human Genome Sequencing Consortium, 2001). Whether or not the four Hox clusters of the sarcopterygian vertebrates arose as a result of two rounds of genome duplication within the vertebrate stem lineage, the appearance of more than one Hox cluster correlates well with the origin of vertebrate specific characters, such as neural crest, epibranchial placodes, and an elaborated brain. If the role of Hox genes in regionalization of the body plan is considered in the light of the idea that gene duplications can provide new genetic material for selection to act upon (Ohno, 1970), it can be hypothesized that some of the specialized characters of the vertebrates, and the variation within the vertebrate body plan, are essentially a result of the availability of additional Hox genes (Holland *et al.*, 1994). A more simplistic notion is that there is a direct relationship between number of Hox genes and complexity of morphology.

IMPLICATIONS OF HOX CLUSTER ORGANIZATION FOR GENE REGULATION AND FUNCTION

The clustered organization of Hox genes shows an obvious relationship to their mode of expression. Thus, expression domains along the primary axis of developing embryos reflect the locations of individual genes within the clusters, such that more 3′-located genes have more anterior expression domains. This orderly relationship is termed spatial colinearity, and in vertebrates, there is also a temporal colinearity, such that the most 3′ genes have the earliest onsets of expression, with a sequential activation of adjacent more 5′ genes. The stage of this initial sequential activation of Hox genes is the most conserved developmental stage among the vertebrates, the “phylotypic stage.”

The clustered organization of the Hox genes is assumed to play a vital role in the establishment of colinear expression, although the mechanisms of this process remain somewhat obscure (reviewed by Duboule, 1998). Control of

Hox gene expression has been well studied by using transgenic approaches in the mouse (reviewed by Capecchi, 1997), and as with other eukaryotic enhancers, the Hox regulatory elements include series of independent modules. For example, retinoic acid response elements (RAREs) are frequently important for proper Hox gene regulation in mouse, and in many cases, gene-specific regulatory elements have been shown to mediate positive autoregulation or cross-regulation by other Hox genes. However, the transgenic approach has also revealed that adjacent Hox genes can share enhancer elements or compete for them (Gould *et al.*, 1997; Sharpe *et al.*, 1998; Zakany *et al.*, 1997). As pointed out by Duboule (1998), this kind of complex intertwining between regulatory elements of adjacent Hox genes may be a consequence of the tight clustering of the genes rather than its cause: It may explain the maintenance of clustered organization, yet not help us to understand how clustered organization arose in the first place.

On this point, it should also be noted that the vertebrate Hox genes are clustered far more tightly than those of the fruitfly. The intergenic distances in the mouse are an order of magnitude smaller than those of *Drosophila*, implying that the mechanisms of gene regulation must differ significantly between these species. For example, individual enhancers within the *D. melanogaster* Bithorax complex, which comprises a 300-kb region including the *Ubx*, *abd-A*, and *Abd-B* genes, are functionally separated from one another by "boundary elements," thus preventing the kind of enhancer sharing and competition that appears prevalent in vertebrate Hox clusters (e.g., Hagstrom *et al.*, 1996; Zhou *et al.*, 1996). Furthermore, the Hox genes of *D. melanogaster* are separated into two individual clusters, with a split between the *Antp* and *Ubx* genes. In a related species *D. virulis*, there are similarly two individual clusters, yet the split is between *Ubx* and *abd-A* (Von Allmen *et al.*, 1996). Such cluster breakdown is not found in more primitive insects such as *Tribolium* (Beeman *et al.*, 1993), emphasizing that the drosophilids are highly derived insects. Nevertheless, the differences in general cluster organization between insects and vertebrates suggest that the evolutionary forces underlying retention of clustered organization may also vary between phyla.

The temporal aspect of colinearity is also likely to play an important role in maintaining the organization of the vertebrate Hox clusters. Temporal colinearity may depend on chromatin accessibility. Gene transposition experiments have suggested that there is a progressive release of a repressive configuration that allows Hox genes to be sequentially activated in turn, from 3′ to 5′, as their chromatin becomes accessible (Kondo *et al.*, 1998; van der Hoeven *et al.*, 1996). In accord with this proposal, a long-range repressive element 5′ to the mouse HoxD cluster has been identified (Kondo and Duboule, 1999). These experiments emphasize the importance of a gene's position within the cluster for establishment of colinear expression; thus, deletion or transposition of individual genes would have a negative impact on the entire cluster and be selected against.

Extensive functional studies in both flies and mice have established that the basic functions of Hox genes are well conserved: Hox genes act as selectors of regional identity along the primary body axis. Mutational analysis in *Drosophila* has established that gain-of-function mutations tend to cause posteriorizing homeotic transformations, where the identity of a segment anterior to the normal expression domain of the gene is altered to resemble the more posterior segment; conversely, loss-of-function mutations cause anteriorizing transformations. In the tetrapod vertebrates, misexpression and null mutant analyses have revealed that similar rules apply, although the situation is rendered significantly more complex by the existence of not one but four Hox clusters. Also, unlike *Drosophila*, the vertebrate Hox gene expression domains often overlap in the posterior; however, the genes tend to act at or close to their anterior expression limits. The vertebrate Hox genes are expressed primarily in the CNS and the paraxial mesoderm. Consistent with these expression patterns, alterations to Hox expression lead to changes in morphology of the mesoderm-derived vertebrae (reviewed by Burke, 2000), the segmentally organized neurons of the hindbrain (reviewed by Lumsden and Krumlauf, 1996), and the derivatives of the cranial neural crest (reviewed by Trainor and Krumlauf, 2001).

More than one member of a vertebrate Hox paralogue group is often expressed in a given location, and these paralogous genes tend to have partially redundant functions. For example, null mutants of the mouse *Hoxa3* gene have defects in neural crest-derived structures (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). By contrast, null mutants of the *Hoxd3* gene show transformations in the first two cervical vertebrae (Condie and Capecchi, 1993). Although these phenotypes are nonoverlapping, double mutants of both *Hoxa3* and *Hoxd3* reveal redundancy between the genes (Condie and Capecchi, 1994). In this particular case, the differences in the phenotypes of the individual mutations must be a consequence of differences in the *cis*-regulatory control of the two genes, rather than of differences in their coding sequences, as *Hoxa3* and *Hoxd3* are functionally interchangeable in "gene-swap" experiments, where one coding sequence is replaced with the other in the normal genomic context (Greer *et al.*, 2000). Although the overall expression patterns of the two genes appear similar, the details of their *cis*-regulation, including variations in level of expression, have profound consequences. Disparate functions of individual paralogues may often depend largely on their *cis*-regulation; this in turn suggests that novel Hox regulation mechanisms must have arisen during evolution of the vertebrates as Hox genes came to pattern new features of the vertebrate body plan.

A recent study (Manzanares *et al.*, 2000) has begun to address the degree of conservation between Hox gene regulation in vertebrates (mouse or chick) and in the cephalochordate amphioxus, which as described above approximates a preduplication ancestral condition. As amphioxus does not have vertebrate-specific structures, such as neural

crest cell derivatives and neurogenic placodes, one might expect that amphioxus Hox regulatory elements would be incapable of driving expression within these regions of a vertebrate. However, contrary to this expectation, regulatory elements from the most 5′ amphioxus Hox genes were shown to drive restricted expression of reporter genes in neural crest and placode derivatives, as well as in the neural tube of both mouse and chick. This finding implies that the basic machinery for Hox expression in vertebrate-specific tissues was already in place in the common ancestor of cephalochordates and vertebrates, more than 520 million years ago (Mya) (based on the fossil record; reviewed by Holland and Chen, 2001). This can be explained by assuming that the vertebrates continue to use the same basic set of upstream regulators of Hox expression as did their ancestors, suggesting that the control of expression of these regulators has been modified during vertebrate evolution, such that they are present in vertebrate-specific structures. Consistent with this model, expression of the amphioxus reporter constructs is retinoic acid-dependent, and RAREs can be recognized in the amphioxus Hox regulatory sequences, suggesting that retinoid signaling is important for Hox activation throughout the chordate lineage.

Manzanares and colleagues (2000) further showed that, although the regulatory elements for particular amphioxus Hox genes can target reporter gene expression to those vertebrate tissues in which the orthologous vertebrate Hox genes are expressed, these amphioxus sequences cannot target the reporter genes to precisely the correct locations; the anterior limits of reporter gene expression lie posterior to the expression limits of the vertebrate Hox orthologues. Hence, the details of vertebrate Hox expression patterns, for example, in specific segments of the developing hindbrain, may well depend on enhancer elements that are unique to the vertebrate lineage. This latter finding is consistent with the hypothesis that vertebrate Hox genes have evolved novel forms of regulation, and is also in accord with the general paradigm that gene duplication can facilitate the arisal of novel gene regulation.

HOX CLUSTER DUPLICATION IN THE RAY-FINNED FISH LINEAGE

The 4-Hox cluster organization of the mammals was initially assumed to be a general characteristic of all the jawed vertebrates (Gnathostomata). Early PCR screens for teleost Hox genes (Misof and Wagner, 1996; Misof *et al.*, 1996) offered hints that this might not be the case, with 5 Hox genes identified in paralogue group 9 of the killifish. Ultimately, detailed studies of the zebrafish Hox genes, including complete linkage analysis, revealed at least 48 Hox genes arrayed over 7 clusters in this ostariophysan teleost (Amores *et al.*, 1998). Based on both sequence and comparative linkage analysis to adjacent non-Hox genes, the 7 clusters (Aa, Ab, Ba, Bb, CA, Cb and D) have been assigned as duplicates of the 4 mammalian Hox clusters (Fig. 1); a duplicate D cluster was either lost during evolution or missed in the initial analysis. Taken together with other synteny relationship analyses between zebrafish and mammals, these data further suggest an additional whole genome duplication event in the lineage leading to zebrafish (Gates *et al.*, 1999; Postlethwait *et al.*, 2000).

Studies of other teleost fishes have shown that additional Hox clusters are very unlikely to be a zebrafish-specific character; all the different teleosts that have been analyzed appear to have passed through a cluster duplication event. Thus, a linkage map for the acanthopterygian teleost medaka (*Oryzias latipes*) again reveals seven clusters of Hox genes (Naruse *et al.*, 2000). The zebrafish data also allowed reinterpretation of the description of a four cluster organization for the tetraodontiform pufferfish (*Fugu rubripes)*: Its four clusters appear most likely to comprise two A clusters, one B and one C, with the likelihood of other clusters yet to be found (Amores *et al.*, 1998; Aparicio, 2000). Preliminary analysis of the perciform African cichlid (*Oreochromis niloticus)* has also revealed at least six Hox clusters (Malaga-Trillo and Meyer, 2001). Finally, although only four Hox clusters have been recognized to date in the perciform striped bass (*Morone saxatilis),* these represent one A, two B, and one C cluster (Ed Stellwag, personal communication). Taken together, the presence of duplicate Hox clusters in these divergent teleost groups (Fig. 2) suggests that an entire Hox complement duplication event, relative to the four-cluster state seen in mammals, is a shared feature of the teleosts that must have occurred in their common ancestor, perhaps in a primitive ray-finned fish species (actinopterygian). As teleost morphology shows no obvious greater complexity than that of the sarcopterygian vertebrates, which have only four Hox clusters, these findings essentially refute the idea of a direct relationship between number of Hox genes and complexity of morphology. Nevertheless, teleosts demonstrate a fascinating degree of variation, and the availability of additional Hox genes may have played a major role in allowing this variation to arise, thus facilitating the teleost radiation.

These data may further suggest that not just the Hox clusters, but also the entire genome, was duplicated in a teleost ancestor. Although there is significant support for this hypothesis (e.g., Amores *et al.*, 1998; Meyer and Schartl, 1999; Postlethwait *et al.*, 1998; Taylor *et al.*, 2001a), it has been challenged by Robinson-Recharvi and colleagues, who propose that teleost duplicate genes are the result of several local duplication events (Robinson-Rechavi and Laudet, 2001; Robinson-Rechavi *et al.*, 2001b,c). Differences over data interpretation have since led to an animated debate in the literature (Robinson-Rechavi *et al.*, 2001c; Taylor *et al.*, 2001c). Rather than add to this debate, which will eventually be resolved by analysis of new data, I will merely point out that whether this duplication affected the entire genome of a teleost ancestor, or merely some subcomponent of the genome that included the Hox clusters, almost all of the implications for teleost evolution that I discuss below remain valid.

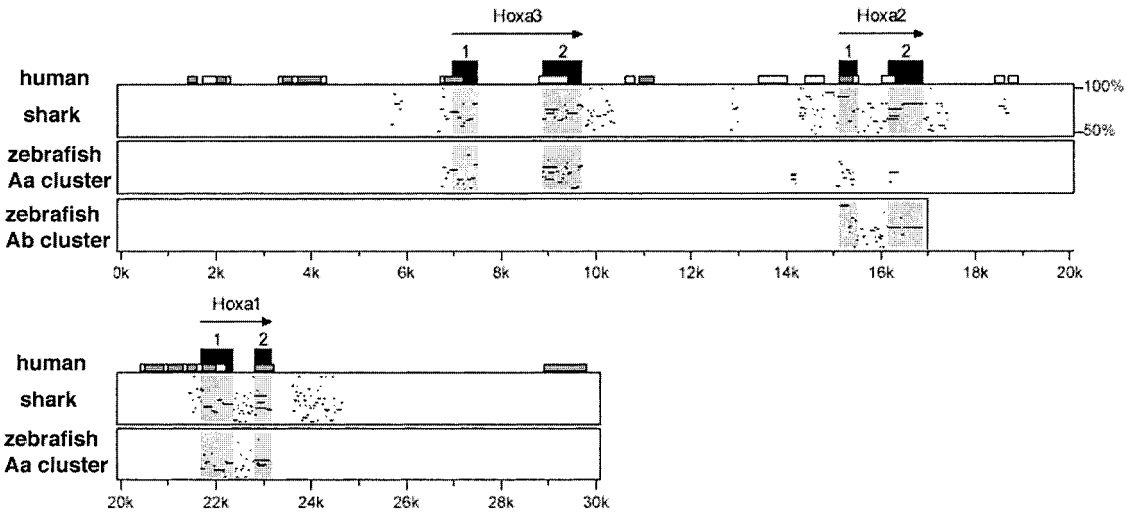

FIG. 3. Pipmaker plot (Schwartz *et al.*, 2000) comparing human and horn shark HoxA clusters with the hoxAa and hoxAb clusters of zebrafish. This strategy allows a *hoxa2a* pseudogene to be recognized. Blast analyses of this sequence show homology to previously isolated *Hoxa2* genes. However, there are STOPS in all three frames. The zebrafish hoxAa sequence was primarily derived from the zebrafish sequencing project at the Sanger Institute (hoxAa genomic sequence at Accession No. AL645756; hoxAb genomic sequence at http://www.sanger.ac.uk/cgi-bin/nph-getblast?humpub/zebrafish all+dZ31B14.00422), with gaps filled by our own sequencing of the HoxA1a–A3a intergenic sequence (Kheirbek and V.E.P., unpublished data).

These findings also leave open the question of when the duplication event that produced more than 4 Hox clusters in the teleosts occurred. The major vertebrate groups, the lobe-fined and ray-finned fishes, diverged more than 400 Mya (Fig. 2) (Carroll, 1988); thus, the duplication must have occurred subsequent to this time. The teleost species for which Hox cluster organization is known represent a radiation that started more than 100 Mya (Nelson, 1994); this represents the most recent date at which the duplication could have occurred. However, Taylor *et al.* (2001a) have recently estimated that the genome duplication may have occurred more than 300 Mya, based on analysis of nucleotide substitution rates in the 3rd codon position of 15 duplicated zebrafish genes. More accurate pinpointing of the time of the duplication will require analysis of basal actinopterygian fishes, such as Polypteriformes (bichir), Acipensiformes (sturgeon, paddlefish), or Amiiformes (bowfin); several groups have already begun to explore Hox genes in these basal species.

FATES OF DUPLICATED GENES

It has long been thought that gene duplication could play a vital role in providing new genetic material for natural selection to act upon, and multiple models have been put forward to predict the fates of duplicated genes. The "classical" model of gene duplication holds that because duplicated genes are initially identical they can be considered functionally redundant, and stresses the role of the acquisition of novel function in the retention of gene duplicates (Ohno, 1970). This model suggests that, following duplication, only one of the two copies needs to be maintained for ancestral function to be retained. Thus, once one of the two genes acquires a strongly deleterious mutation, further mutations can accumulate in that gene unchecked. As deleterious mutations are far more likely than beneficial ones (Lynch and Conery, 2000), the retention of both duplicated genes as a result of acquisition of some key novel function (neo-functionalization) is thought to be an extremely rare event. According to this model, loss of duplicated genes is a common and relatively rapid evolutionary event. In accordance with the model, the majority of duplicated Hox genes have been lost from the zebrafish clusters. Furthermore, there remains evidence of pseudogenes in some of the locations where a duplicate would be expected to lie. For example, Amores *et al.* (1998) described a pseudogene in the location of *hoxA10a*. Not all the Hox cluster sequence has yet been analyzed, and so it is likely that other pseudogenes remain to be discovered. Indeed, recently available zebrafish genomic sequences (Sanger Centre zebrafish genome sequencing project; Kheirbek and V.E.P., unpublished data), have allowed me to compare the duplicate HoxA clusters of zebrafish with the HoxA clusters of both human and horn shark, to recognize a previously undescribed pseudogene at the location of *hoxA2a* (Fig. 3).

Despite the predictions of the classical model, that many more duplicates will be lost than retained, vertebrate genomes appear to be rife with ancient gene duplicates

(Nadeau and Sankoff, 1997). To explain this conundrum, new theories have been put forward. Gibson and Spring (1998) have suggested that changes in multidomain proteins are likely to have dominant negative effects, and thus duplicate genes may be retained indefinitely despite their functional redundancy, because altered forms have negative impact. Force and colleagues (Force *et al.*, 1999; Lynch and Force, 2000) have proposed a model of "subfunctionalization" that may be more generally applicable. The Force model suggests that the modular nature of eukaryotic gene enhancers may lead to a partitioning of gene functions following duplication, such that complementary expression domains (spatial or temporal) are lost through degeneration of individual regulatory elements for each duplicate. Enhancers could also change with respect to the levels of gene expression, so that duplicates produce some lower amount of protein than did the ancestral, preduplicate gene. Such changes could lead to the duplicates retaining complementary functions—both duplicates will then be required to recapitulate the original gene function (referred to as the duplication–degeneration–complementation, or "DDC" model; Force *et al.*, 1999). These complementary mutations ensure that both gene copies are retained in the genome. An important extension of this model is that once gene functions are divided between the duplicates, each gene may be freed to evolve along a novel trajectory once the constraint of functioning in multiple contexts is removed.

The limited data available suggest that different teleosts do not all share a common Hox cluster architecture. Rather, there appear to have been different patterns of Hox gene losses subsequent to the genome duplication event. For example, the zebrafish has a *hoxC1a* and a *hoxC3a* gene (Amores *et al.*, 1998), but in the pufferfish, these are pseudogenes (Aparicio *et al.*, 1997). Similarly, Malaga-Trillo and Meyer (2001) have described several differences in the architecture of the HoxA clusters of zebrafish, striped bass, pufferfish, and an African cichlid. This variability in cluster organization contrasts markedly with our understanding of a stable tetrapod Hox cluster organization. Nevertheless, rather than being an exception within the vertebrates, this variable architecture should perhaps be considered the rule, as teleosts make up the majority of vertebrate species (about 25,000 fish species have been described; Nelson, 1994). Indeed, it has been suggested that the variable Hox organizations may have a direct relationship with the diversity of morphologies among the teleosts (Meyer and Schartl, 1999; Wittbrodt *et al.*, 1998).

RESOLUTION OF ZEBRAFISH HOX GENE DUPLICATES

The zebrafish provides a tractable model system to examine the functional significance of Hox gene duplications. Using comparative sequence, expression, and functional studies, we can begin to investigate what events have allowed retention of select pairs of zebrafish Hox gene duplicates (although many duplicate genes have been lost, at least 10 duplicates have been maintained). If novel functions could be uncovered for zebrafish Hox genes, this would be consistent with the hypothesis that the availability of duplicate Hox genes was important in facilitating the teleost radiation. Ideally, we would compare zebrafish Hox genes to those of a species that approximates the ancestral, preduplication condition. Unfortunately, information on the Hox genes of the basal actinopterygians, which are most likely to provide such a comparison group, has not yet reached the literature.

However, Hox genes are unusually conserved in their sequence, clustered organization, and regulation, which permits (even requires) comparisons to be made over wide evolutionary distances. Indeed, Hox genes are so conserved at the level of protein function that, in some cases, they can be functionally substituted for one another between different phyla (e.g., Lutz *et al.*, 1996). Thus, informative comparisons can be made between zebrafish and such phylogenetically distant osteichthyans as mice, allowing us to take advantage of the wealth of data concerning mouse Hox gene expression and function. This approach relies on the assumption that the four-cluster organization, seemingly widespread in sarcopterygians, reflects the ancestral osteichthyan condition. While this assumption already seems reasonable, it would be even more strongly supported were two additional Hox clusters to be found in the chondrichthyan horn shark, bringing the total number of Hox clusters to four in the sister group to Osteicthyes.

There are a total of 48 Hox genes described for the zebrafish (compared with 39 for mouse and human), yet despite this difference in gene number, the majority of zebrafish Hox genes show expression patterns that are essentially similar to those of their murine orthologues (Prince *et al.*, 1998a,b). One interesting exception to this rule is the zebrafish *hoxA1a* gene (McClintock *et al.*, 2001), which is discussed in more detail below. Although the zebrafish is well known for its tractability as a genetic model system, no homeotic mutants have been uncovered in large-scale forward genetic screens. Zebrafish Hox mutants would be expected, based on our knowledge of mouse, to cause alterations in vertebral morphology and hindbrain segmental identity. The large-scale zebrafish mutagenesis screens were not designed to identify such phenotypes, and thus it is unsurprising that homeotic mutants have not yet been found.

Nevertheless, other types of mutant have provided useful information about zebrafish Hox gene function. In particular, the phenotype of the *lazarus (lzr)* mutant has suggested that zebrafish Hox genes must play very similar functional roles to mammalian Hox genes (Pöpperl *et al.*, 2000). The *lzr* mutant affects a Pbx gene, zebrafish *pbx4*; Pbx proteins are Hox cofactors, binding together with Hox proteins on their target sequences to provide proper specificity to regulation of the downstream targets (reviewed by Mann and Affolter, 1998). The zebrafish *pbx4* gene provides the major Pbx cofactor acting during early development, and in its

absence there are multiple defects within the developing hindbrain region. All of the phenotypes have been interpreted as corresponding to losses of Hox gene function, by analogy to known mouse Hox mutants (Pöpperl *et al.*, 2000). This interpretation has been further supported by our analysis of Hox gene "knock-down" phenotypes, where Hox protein translation is blocked by using antisense reagents (Hunter and Prince, 2002; McClintock *et al.*, 2002, see below).

Comparison of the *lzr* mutant phenotype with the phenotypes of mouse Hox mutants does not reveal any major zebrafish-specific Hox gene functions during early development: The *lzr* phenotype largely phenocopies null mutants of mouse Hox genes. It should be remembered, however, that any novel late functions of these Hox genes would not be recognized due to the lethal nature of the *lzr* mutant. Furthermore, zebrafish Hox genes may have evolved Pbx-independent functions that would be unaffected by the *lzr* mutant. Whether or not the zebrafish Hox genes have taken on novel functions (a question that remains wide open at present), we do know that some duplicate genes were retained. A comparative approach can be used to try to establish the mechanisms underlying these retentions.

Bruce *et al.* (2001) performed the first study to investigate why a pair of zebrafish Hox genes have been retained rather than one gene being lost from the genome. In this study, the expression patterns of zebrafish *hoxB5a* and *hoxB5b* were compared to that of the single mouse *Hoxb5* gene, and found to recapitulate its overall expression. The zebrafish *hoxb5* duplicates have different, but overlapping, expression patterns, yet appear to share identical biochemical functions as assessed by a gain-of-function approach. Thus, it seems that in this case, zebrafish *hoxb5a* and *hoxb5b* represent a partitioning of expression domains with respect to the murine *Hoxb5* gene. Assuming that the murine *Hoxb5* gene reflects the ancestral osteichthyan state, the Hox duplication in the teleost lineage appears to have lead to a subfunctionalization for these zebrafish *hoxB5* duplicates in accordance with the DDC model. Further tests of the model would include demonstrating that these two zebrafish genes are able to functionally substitute for one another, although it should be remembered that, even when the DDC model is invoked to explain the fixation of gene duplications, this does not rule out subsequent alterations that might obscure initial functional equivalence. It would also be of interest to explore the regulatory sequences of the zebrafish *hoxB5a* and *hoxB5b* genes, to attempt to identify degenerative changes in the zebrafish sequences that underlie the presumed partitioning of the ancestral expression domain.

Chiu *et al.* (2002) have recently investigated the molecular evolution of HoxA clusters across the major gnathostome lineages: They compared complete HoxA cluster sequences of zebrafish, human, and horn shark. Duplicate genes have been retained for three of the more 5′-located zebrafish HoxA genes, yet the duplicated zebrafish clusters did not show evidence for the kind of complementary degenerative changes in *cis*-regulatory elements that the DDC model predicts. Instead, the two zebrafish HoxA clusters, as well as the one reported striped bass HoxA cluster, showed a conspicuous loss of putative *cis*-regulatory elements that are conserved between human and horn shark. The authors conclude that the changes they have found in the zebrafish sequences are consistent with adaptive modification rather than the more passive mechanisms associated with subfunctionalization. By contrast, comparative sequence analysis of the intergenic region between *Hoxb2* and *Hoxb3* of human, mouse, zebrafish, fugu, and striped bass has revealed extensive conservation of transcription factor binding sites (Scemama *et al.*, in press). The conserved sites have been shown to be important for proper expression of mouse *Hoxb2*, and consistent with conserved function of these elements, the expression patterns of the vertebrate *Hoxb2* orthologues are largely conserved. Interestingly, in several cases, the binding sites occur in different orders in different species, and such reorganization of small *cis*-regulatory elements may make it difficult for large-scale alignment techniques to pick up functional homology.

Recent studies in my own lab have also focused on the question of why some Hox duplicates have been retained in the zebrafish genome. We have concentrated on the four zebrafish Hox genes comprising paralogue group (PG) 1, which include a pair of duplicates with respect to the four-cluster state, *hoxB1a* and *hoxB1b*. In this case, we have found good evidence for an ancient subfunctionalization between the duplicates. However, we additionally find evidence for a subsequent more complex situation of "function shuffling" among the members of the paralogue group.

FUNCTION SHUFFLING AMONG PG1 GENES

The PG1 genes are a particularly good system in which to investigate potential subfunctionalization because two of the three mouse genes have had both gene function and regulation studied in great detail. These experiments have shown that mouse *Hoxa1* and *Hoxb1* are necessary for proper development of the hindbrain. In zebrafish, as in mouse and chick, hindbrain morphology is conceptually simple, with overt segmentation dividing the hindbrain into seven lineage-restricted compartments termed rhombomeres (r1–r7 from A to P). This basic organization is conserved across the vertebrates, and there are a wealth of molecular and neuroanatomical markers that allow the identity of individual rhombomeres to be unambiguously recognized (reviewed by Moens and Prince, 2002).

Hoxa1 and *Hoxb1* are coexpressed in the mouse hindbrain from the early stages of gastrulation, with an identical anterior expression limit at the presumptive boundary between r3 and r4 (Barrow *et al.*, 2000; Frohman *et al.*, 1990; Murphy and Hill, 1991; Wilkinson *et al.*, 1989). This

expression is dependent on retinoic acid response elements (RAREs) that lie 3′ of each gene. *Hoxa1* expression is very transient in r4, retracting posteriorly out of the hindbrain during early somite stages. In contrast, *Hoxb1* expression is stably maintained in r4, while expression is gradually lost from r5 and r6 to leave an r4 "stripe" of *Hoxb1* expression. This r4 *Hoxb1* domain is maintained by an autoregulatory positive feedback mechanism, which is dependent on three defined Hox/Pbx binding sites upstream of *Hoxb1* (Pöpperl *et al.*, 1995).

Mutant analysis of mouse *Hoxa1* and *Hoxb1* has revealed that these two paralogues play divergent, but partially redundant, roles in patterning the hindbrain. The prime function of the *Hoxb1* gene is to confer proper r4 identity, as loss of *Hoxb1* function results in major alterations to the r4-derived facial motor neurons, which no longer undergo their normal migration behavior (Gaufo *et al.*, 2000; Goddard *et al.*, 1996; Studer *et al.*, 1996). By contrast, loss of *Hoxa1* function causes a radical reduction in the AP extent of r4 and r5, with an accompanying reduction in the size of the adjacent otic vesicle (Carpenter *et al.*, 1993; Chisaka *et al.*, 1992; Lufkin *et al.*, 1991; Mark *et al.*, 1993); thus, *Hoxa1* is rather unusual as it is important for setting up proper segmental organization of the hindbrain, not just for conferral of segmental identity. Double knockouts of both *Hoxb1* and *Hoxa1* show synergistic phenotypes (Barrow *et al.*, 2000; Gavalas *et al.*, 1998; Rossel and Capecchi, 1999; Studer *et al.*, 1998) revealing redundancy of function between the paralogues.

In the zebrafish, the *hoxB1* duplicates, *hoxB1a* and *hoxB1b*, have expression profiles that are intriguingly similar to those of mouse *Hoxb1* and *Hoxa1*, respectively (McClintock *et al.*, 2001), although zebrafish *hoxB1a* lacks the early gastrula-stage expression shown by mouse *Hoxb1*. By contrast, the zebrafish orthologue of mouse *Hoxa1*, zebrafish *hoxA1a*, is not expressed in presumptive r4, and thus cannot play a role in early patterning of this hindbrain territory (McClintock *et al.*, 2001; Shih *et al.*, 2001). Hence, expression data suggest the hypothesis that zebrafish *hoxB1a* and *hoxB1b* are the functional equivalents of mouse *Hoxb1* and *Hoxa1*, respectively.

A new "knock-down" technology, using stabilized antisense morpholinos, has allowed us to test directly the functions of the zebrafish *hoxB1* duplicates (McClintock *et al.*, 2002). We have demonstrated that zebrafish *hoxB1a* and *hoxB1b* do indeed play similar roles to mouse *Hoxb1* and *Hoxa1*. Thus, the zebrafish *hoxB1a* gene, like mouse *Hoxb1*, is required for proper migration of facial nerve neurons from r4 and for its own positive regulation. The *hoxB1b* gene, like mouse *Hoxa1*, is required for proper segmental organization of the hindbrain, and for development of a normally sized otic vesicle. How can our finding that a zebrafish *HoxB* duplicate gene and a mouse *HoxA* gene are functionally equivalent be reconciled with the DDC subfunctionalization model? Data emerging from the zebrafish sequencing project have helped us to develop a model to explain our findings.

According to the DDC model, the duplicates would be expected to divide out the ancestral expression domain. In accord with the model, the *hoxB1b* gene has an expression pattern resembling the gastrulation phase of murine *Hoxb1* expression, while the *hoxB1a* gene has an expression pattern resembling the later "r4 stripe" phase of mouse *Hoxb1* expression. The DDC model also predicts the degeneration of discrete complementary *cis*-regulatory elements in the two duplicates. We find that, although *hoxB1b* possesses a 3′ RARE with a two-nucleotide spacer between the half sites, similar to the one which in mouse *Hoxb1* confers gastrulation stage expression, we are unable to detect such an element 3′ of *hoxB1a*, consistent with its lack of an early expression phase. Similarly, zebrafish *hoxB1a* retains perfect copies of all three Hox/Pbx binding sites, which in mouse *Hoxb1* confer autoregulation in r4, yet *hoxB1b* has point changes in each of the individual sites, consistent with the absence of a late r4 expression domain for this gene. This degeneration of different regulatory modules in each of the two duplicates is likely to have been sufficient to allow preservation of the two genes as postulated by the DDC model (Fig. 4), but leaves open the question of how the function of a *HoxA* gene could have shifted to a *HoxB* gene.

Our model for how *hoxB1b* came to take on the role that in mouse is played by *Hoxa1* has been influenced by our expression analyses of vertebrate *Hoxa1* orthologues. We have shown that the zebrafish *hoxA1a* gene is expressed at late neurulation stages in a small, bilaterally located group of neurons in the ventral midbrain (McClintock *et al.*, 2001; Shih *et al.*, 2001). As midbrain expression has not generally been described for Hox genes, this domain seems at first observation to reflect a potential neofunctionalization event. However, our comparative analyses have demonstrated that midbrain expression is more likely a primitive characteristic of the vertebrate PG1 genes. Thus, we find expression of *Hoxa1* orthologues in a similar group of cells not only in another teleost, medaka, but also in the sarcopterygian chick (C. Jozefowicz. and V.E.P., unpublished observations). Furthermore, we have confirmed a previous description of midbrain expression for *Xenopus Hoxa1* (Kolm and Sive, 1995). As *Xenopus* and chick combine both hindbrain and midbrain expression domains of *Hoxa1*, we hypothesize that these two separate expression domains represent the ancestral condition. In the zebrafish, *hoxB1b* has taken on the hindbrain patterning role of tetrapod *Hoxa1*, which may have freed *hoxA1a* to lose its hindbrain expression domain while retaining the ancestral midbrain patterning role (Fig. 4). We have termed this phenomenon function shuffling (McClintock *et al.*, 2001, 2002), and it relies upon a phase of partial functional redundancy between nonorthologous genes, in this case the paralogous *hoxA1a* and *hoxB1b*. These data reveal that it may be essential to study an entire group of related genes to fully understand the consequences of a particular duplication event.

We have also been able to combine the morpholino knock-downs with mRNA misexpression to test the degree

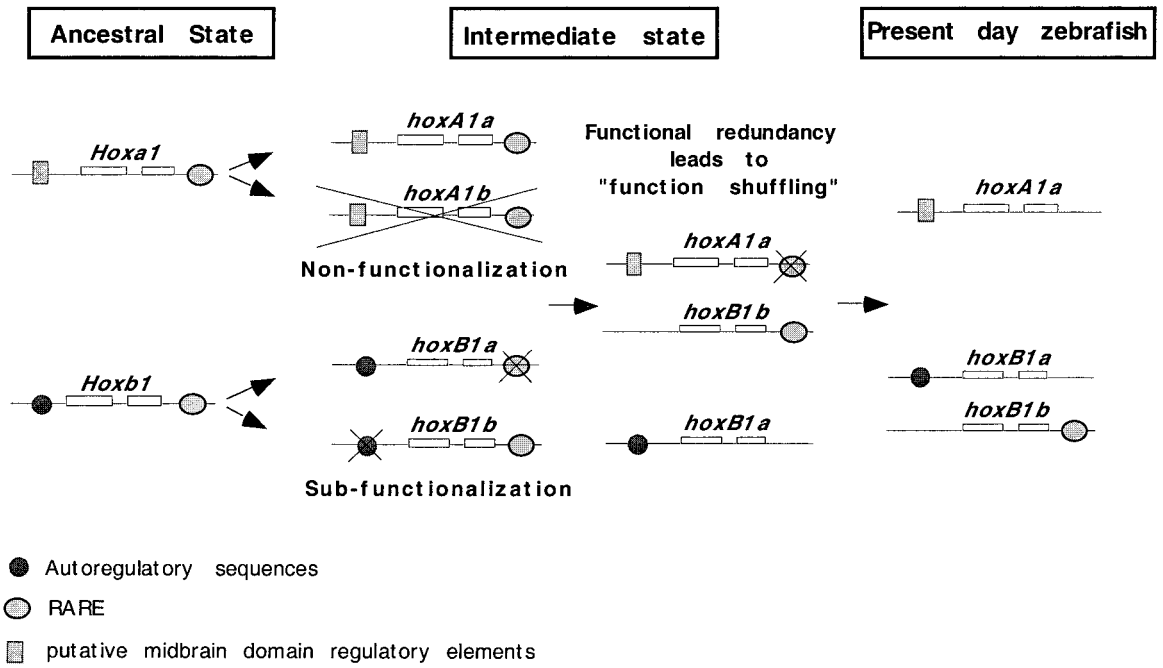

FIG. 4. Model outlining the evolutionary mechanism of Hox PG1 gene "function shuffling." The *cis*-regulatory elements characterized for the mouse and human *Hoxa1* and *Hoxb1* genes [3′ RAREs (blue), Hox/Pbx binding sites (red)] are assumed to be present in the ancestral, pre-"third"-duplication, condition. We also postulate the presence of a regulatory domain directing midbrain expression of *Hoxa1* (mauve), although no such domain has yet been characterized. The duplication event in the lineage leading to teleosts produced redundant copies of both *Hoxa1* and *Hoxb1* in an ancestor of the zebrafish. The *hoxA1b* duplicate was eventually lost by accumulation of deleterious mutations ("nonfunctionalization") as predicted by classical models. In contrast, the *hoxB1a* and *hoxB1b* genes accumulated complementary degenerative changes in their *cis*-regulatory elements such that *hoxB1a* lost early, RARE-mediated expression, and *hoxB1b* lost autoregulation. This led to retention of the duplicate genes, as both were required to maintain the expression pattern and function of the single *Hoxb1* ancestral gene (subfunctionalization), as predicted by the DDC model. As *hoxA1a* and *hoxB1b* shared similar coding sequences and expression patterns, these two genes were now functionally redundant with respect to a role during gastrulation in setting up segmental organization of the hindbrain. These nonorthologous genes were thus able to go through another "subfunctionalization" event, such that *hoxA1a* lost its early RARE-mediated expression, which was retained by *hoxB1b*. Thus, *hoxB1b* became essential for proper hindbrain segmentation, the role played in the ancestral state by *Hoxa1*. Retention of the *hoxA1a* gene in the lineage leading to zebrafish was presumably dependent on a function that was not redundant with *hoxB1b*, possibly a role in midbrain patterning. We term this rearrangement of PG1 gene roles "function shuffling."

of interchangeability of Hox PG1 coding sequences (McClintock *et al.*, 2002). In these experiments, we attempted "rescue" of knock-down phenotypes with different mRNAs. We found that mouse *Hoxb1* can functionally substitute for either zebrafish *hoxB1a* or *hoxB1b*, consistent with the model that the two zebrafish duplicates have subfunctionalized the ancestral roles that in mouse continue to be played by the single *Hoxb1* gene. However, we also found that, although *hoxB1a* can functionally substitute for *hoxB1b*, the reciprocal is not true. Thus, *hoxB1b* has lost the capacity to allow proper migration of facial nerve neurons. Once again, this is consistent with the model of Force and colleagues (Force *et al.*, 1999; Lynch and Force, 2000): Their DDC model states that, although complementary degeneration of *cis*-regulatory elements is what initially allows maintenance of a pair of duplicates, it does not prevent the individual genes from then becoming "fine-tuned" to their separate functions or eventually taking on novel functions.

Function shuffling may prove to be common among zebrafish paralogues. For example, it has recently been shown using morpholino-based knock-down that the zebrafish *eng2* and *eng3* genes have early developmental roles equivalent to that of the nonorthologous mouse *EN1* gene (Scholpp and Brand, 2001). Furthermore, function shuffling may not be limited to transcription factor genes: The secreted signaling molecule bmp2a from zebrafish appears to play an equivalent functional role to the nonorthologous *Xenopus* Bmp4 during dorsoventral patterning of gastrula-stage embryos (Nguyen *et al.*, 1998). On a practical note, these findings suggest that, in cases where orthology relationships are unclear, it may not help to assume that common function can help with

assignments—synteny relationships are more likely to be a reliable tool.

CONCLUSION AND FUTURE DIRECTIONS

There is no doubt that Hox gene functions are intimately associated with axial patterning, and therefore changes in Hox genes are likely to play a key role in the evolution of new body plans. Many researchers have emphasized the importance of alterations in *cis*-regulation of Hox and other developmental control genes for allowing differing morphologies to arise during evolution (reviewed by Carroll, 2000). Studies in invertebrates have tended to support the notion that *cis*-regulation can be "tinkered" with more easily than protein coding sequences, presumably because detrimental effects are less likely to result from sequence changes. However, recent work has revealed that alterations to Hox proteins, as opposed to alterations in regulation of Hox expression, can underlie major morphological transitions. Two studies (Galant and Carroll, 2002; Ronshaugen *et al.*, 2002) have demonstrated that insects lost their abdominal limbs, such that they have only six legs, as a result of functional changes in the Hox protein Ubx. These reports underscore the importance of considering both gene regulation and protein function as we try to unravel how changes in Hox genes have influenced vertebrate evolution.

Consistent with the idea that changes to Hox genes can underlie new morphologies, the large-scale gene duplications in the vertebrate stem lineage provided many additional Hox genes, which correlate with the innovations that characterize the vertebrates (reviewed by Holland *et al.*, 1994). It has been suggested that the additional duplication event in the lineage leading to teleosts such as the zebrafish provided yet more raw genetic material for selection to act upon, and that this may have facilitated the broad radiation of teleosts (Meyer and Schartl, 1999). Although the radiation of the teleosts has been underway for about 200 My, this time frame is relatively short in comparison with the distant origin of vertebrates, more than 520 Mya. Thus, studies of teleost fishes hold significant promise for allowing us to test the importance of changes in Hox genes for the generation of new forms. In order to pursue these studies, it will be important for the duplication event that has led to additional Hox clusters in teleosts to be more accurately dated. This will allow us to recognize the last common ancestor of animals with and without the extra duplication, and provide an appropriate comparison point for all future studies. To this end, upcoming new data on basal teleosts and ray-finned fishes will be invaluable.

To further investigate the roles of Hox genes within the radiating teleosts, it will be vital to study species within a phylogenetic framework. In particular, it will be important to correlate known morphological variation with differences in Hox organization and function. Species suitable for such studies might include the members of the tetraodontifomes, which have a remarkably variant morphology (Santini and Stellwag, 2002; Tyler and Sorbini, 1996). This will entail much hard work in determining details of Hox cluster architecture for a range of species, and thus it will be important to choose species wisely. It will also be useful to have a reliable means of disrupting gene function, and conveniently, the new morpholino technology for gene knock-down should be equally applicable to any system where early embryos can be microinjected. As the majority of fish species have embryos that develop externally, this opens up the prospect of broad comparative functional analyses.

Another approach to understanding the genetic basis of morphological evolution is to use variation among closely related species to identify loci that contribute to the observed variation. Peichel *et al.* (2001) have used quantitative trait locus (QTL) mapping to investigate variation in skeletal armor and feeding morphologies of the threespined sticklebacks, well-studied teleosts that have undergone rapid divergence and speciation over the last 15,000 years. This work has identified a large number of QTL associated with the differing morphologies. It will be interesting to know whether these QTL correlate with known developmental control genes, including Hox genes, although targeted studies of the expression patterns of specific Hox genes in morphologically distinct populations of sticklebacks have not yet revealed any correlations with the different morphologies (Ahn and Gibson, 1999).

It is important to note that gene duplication events may be important for allowing speciation to occur via mechanisms that are separable from the generation of new morphologies. Lynch and colleagues have postulated that "divergent resolution" of duplicate genes could cause speciation within populations that are temporarily geographically isolated (Lynch and Conery, 2000; Lynch and Force, 2000; reviewed by Taylor *et al.*, 2001a,b). This would rely upon specific pairs of duplicated genes undergoing different fates in different populations, for example, loss of different duplicate genes, or subfunctionalization versus nonfunctionalization. Such events would reduce the fecundity of future hybrids once the separated populations become reunited. Consistent with this hypothesis, the salmonid fishes, which have gone through a recent genome duplication event, are significantly more speciose than a sister taxon that has not (reviewed by Taylor *et al.*, 2001b). The more divergently resolved loci present, the more effective such an isolation mechanism would be, thus in the case of the radiating teleosts this model is more relevant to a whole genome duplication event than to a more limited, Hox-specific, duplication event.

What then can we learn from the Hox genes of the zebrafish, the teleost that is currently best understood at both the molecular genetic level, and in terms of its early development? It has been established that zebrafish has retained at least 10 duplicated Hox genes, opening up the possibility that, in some cases, duplicates were fixed because one of them attained a novel function. In the two

cases that have been investigated in detail, this appears not to be the case. The *hoxB5* duplicates have subfunctionalized in accordance with the DDC model (Force *et al.*, 1999), whereas the PG1 genes have gone through an interesting function shuffling, while still not undergoing any obvious neofunctionalization. However, it should be noted that neofunctionalization may prove difficult to recognize, especially in the absence of a complete knowledge of the primitive condition. Important changes could be subtle—for example, minor but critical changes in timing of gene expression, concentration of gene product, or origin of a new late expression pattern that would not be detected within the usual time frame of developmental expression studies. Furthermore, the comparative sequence analysis of Chiu *et al.* (2002) has provided evidence for adaptive modification in teleost Hox regulatory elements, suggesting that new expression domains may well have arisen following duplication. Alternatively, the 10 retained duplicate Hox genes may all prove to have undergone some variation on the subfunctionalization theme. Nevertheless, this would not undermine Ohno's hypothesis that gene duplication facilitates evolution by providing new genetic material and allowing genes to take on new functions. Rather, there may be other developmental control genes that have gained important novel functions subsequent to duplication, and very good candidates for such genes would be the downstream effectors of Hox function.

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