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Duplication events and the evolution of segmental identity

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SUMMARY Duplication of genes, genomes, or morphological structures (or some combination of these) has long been thought to facilitate evolutionary change. Here we focus on studies of the teleost fishes to consider the conceptual similarities in the evolutionary potential of these three different kinds of duplication events. We review recent data that have confirmed the occurrence of a whole-genome duplication event in the ray-finned fish lineage, and discuss whether this event may have fuelled the radiation of teleost fishes. We then consider the fates of individual duplicated genes, from both a theoretical and an experimental viewpoint, focusing on our studies of teleost Hox genes and their functions in patterning the segmented hindbrain. Finally, we consider the duplication of morphological structures, once again drawing on our experimental studies of the hindbrain, which have revealed that experimentally induced duplicated neurons can produce functionally redundant neural circuits. We posit that the availability of duplicated material, independent of its nature, can lead to functional redundancy, which in turn enables evolutionary change.

INTRODUCTION

The teleost fishes have radiated broadly and are a remarkably speciose group; more than 23,000 different species have been described (Nelson 1994), a number that rivals the sum of all other vertebrate species (Fig. 1). For several teleost species, whole-genome sequencing has been completed (*Fugu rubripes*, Aparicio et al. 2002; *Tetraodon nigroviridis*; Jaillon et al. 2004) or is well underway (*Danio rerio, Oryzias latipes*). Recently Jaillon et al. (2004) unequivocally demonstrated that a whole-genome duplication event occurred in the ray-finned fish lineage leading to the teleosts. This finding has led to the tempting, but currently untested, hypothesis that there is a causal relationship between whole-genome duplication and the teleost radiation. Whether or not such a link exists, teleost fishes have been through a whole-genome duplication, and this coupled with the genetic and embryological tractability of many teleosts, is providing a convenient system to explore the consequences of gene duplication from evolutionary, developmental, and functional perspectives. In complement to these genetic data, the morphology of teleost fishes has long been studied. In particular, many instances have been reported of duplications of structural elements. Here we consider whether duplication of genes and of morphological structures can be considered within a common framework; in both instances duplication provides a general means to facilitate diversification and the formation of novelties.

WHOLE-GENOME DUPLICATION EVENTS IN THE VERTEBRATE LINEAGE

Many authors have theorized that the duplication and subsequent modification of an existing gene was a more probable way to create genetic novelty than creating genes de novo (reviewed by Taylor and Raes 2004). In addition Ohno (1970) proposed that several rounds of whole-genome duplication might have fuelled early vertebrate evolution, one phase of duplication facilitating the invertebrate-to-vertebrate transition, and a second enabling vertebrate diversification. Ohno did not specify the timing or number of duplication events that had occurred and only later did this theory become known as the "2R hypothesis," for two rounds of duplication close to vertebrate origins (Hughes 1999). Although it is now widely accepted that duplications did indeed occur during early vertebrate evolution, it is still under debate whether these duplicates arose simultaneously or are derived from lineage-specific duplication events (Furlong and Holland 2004).

Teleost fishes, a major subgroup of the ray-finned bony fish (Actinopterygii; Fig. 1), show a huge variation in morphology, behavior, ecology, and physiology (Nelson 1994). It has been proposed that teleost fish are so successful and diverse because their common ancestor underwent a whole-genome duplication before their explosive radiation (Holland et al. 1994; Amores et al. 1998; Postlethwait et al. 1998; Wittbrodt et al. 1998; Meyer and Schartl 1999). Like the "2R hypothesis," this theory has been controversial since it was

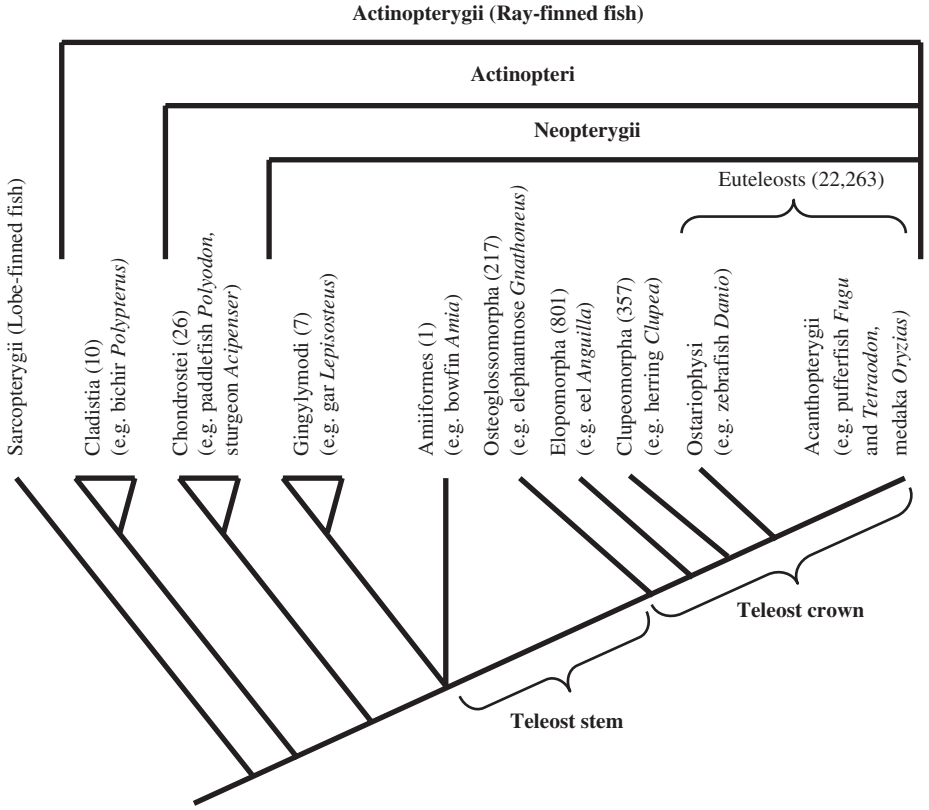

Fig. 1. Phylogeny of Osteichthyes—bony fishes. Phylogeny and species numbers (indicated in brackets) adapted from Nelson (1994).

first proposed (Robinson-Rechavi et al. 2001). Although most ray-finned fishes are diploid, recent polyploidization has occurred multiple times in independent lineages and several extant species remain polyploid (Le Comber and Smith 2004). The occurrence of a whole-genome duplication event in the ray-finned fish lineage implies that all post-duplication species are ancient polyploids or "paleopolyploids," although they may have returned to a diploid state.

EVIDENCE FOR A FISH-SPECIFIC WHOLE-GENOME DUPLICATION EVENT

The number of gene orthologs in teleosts relative to those of tetrapods provided the first indication of a whole-genome duplication event specific to the ray-finned fishes. Studies of Hox gene cluster numbers (reviewed by Prohaska and Stadler 2004), as well as of non-Hox genes (e.g., Taylor et al. 2003), provided initial support for the ray-finned fish specific duplication hypothesis, but it remained necessary to show that the extra copies of genes present in fish were the result of a large-scale duplication and not merely the result of independent smaller-scale duplications. Mapping experiments of zebrafish and *F. rubripes* paralogs (duplicate genes) (e.g. Postlethwait et al. 1998; Smith et al. 2002) revealed that whole genomic regions had been duplicated, whereas phylogenetic analyses of duplicate teleost genes generally suggested that paralogs did indeed arise from a single duplication event (Taylor et al. 2003).

The release of genome sequence data enabled analysis of homology across entire genomes for the first time, providing more conclusive evidence of a ray-finned fish specific whole-genome duplication (e.g., using *F. rubripes,* Christoffels et al. 2004; Vandepoele et al. 2004 and *O. latipes*, Naruse et al. 2004). The recent sequencing of the *T. nigroviridis* genome has provided definitive proof of a ray-finned fish-specific genome duplication (Jaillon et al. 2004); this study differed from previous work by virtue of its high level of sequence coverage, which when combined with mapping data enabled the sequence to be anchored to chromosomes. The genome-wide distribution of duplicates was identified and shown to lie on paralogous chromosomes. As expected following a whole-genome duplication, all chromosomes were involved. The *T. nigroviridis* genome was also compared with mouse and human, genomes that have not undergone the fish-specific duplication. This analysis identified extensive regions of double synteny, where two *T. nigroviridis* domains mapped to a single tetrapod location. These results were exactly what

would be expected following whole-genome duplication and when added to the body of work reviewed above provide overwhelming evidence in favor of a whole-genome duplication event in the ray-finned fish lineage.

Now that a whole-genome duplication has been established, two questions remain: where in the ray-finned fish lineage did the duplication event occur, and when did duplication occur? It is essential to answer these questions to begin to test the hypothesis that the duplication facilitated teleost radiation. The duplication would need to have occurred within the teleost stem group (Fig. 1) to support the theory that a whole-genome duplication fuelled adaptive radiation.

POSITIONING THE RAY-FINNED FISH SPECIFIC WHOLE-GENOME DUPLICATION

The position of a duplication event relative to a speciation event can be calculated by phylogenetic analysis of duplicated genes. The goal is to identify the species that pre- and post-date the whole-genome duplication. It should be noted that the accuracy of such a phylogenetic approach is reduced if the rate of evolution varies between paralogs or between taxa (Kondrashov et al. 2002). Sequence saturation may also have been reached in ancient duplicates, distorting tree topologies, as the sequence changes observed do not equal the actual level of divergence. However, methods are available that can identify and remove such sites (Van de Peer et al. 2002).

Hox cluster analysis in teleosts implies that the most recent point at which the whole-genome duplication event could have occurred was in the euteleost lineage (Fig. 1). The duplication event is assumed to have occurred after divergence of the lobe- and ray-finned fishes because all tetrapods analyzed to date have only four Hox clusters whereas the euteleosts have seven or eight (Koh et al. 2003). Furthermore, it seems likely that the duplication post-dates the origin of the bichir *Polypterus*, as only a single bichir HoxA cluster has been discovered in contrast to the euteleosts that have two (see Fig. 1) (Chiu et al. 2004). The search for gene duplicates in non-teleost species has received only limited attention and it is therefore not yet possible to determine precisely where the duplication occurred relative to the teleost radiation. However, analysis of several non-Hox duplicate genes supports the conclusion that the duplication pre-dates euteleost divergence (Taylor et al. 2003). In addition, duplication of ion and water transporter genes in eels (Elopomorpha, a basal teleost lineage) (Cutler and Cramb 2001) also suggests the duplication event occurred before the euteleost radiation, although these data remain contentious as Robinson-Rechavi et al. (2004) did not find support for eel genome duplication. Phylogenetic analysis of *sox11*, *frizzled8*, and *tyrosinase* in a range of ray-finned fishes suggests that the duplication occurred after the separation of Chondrosteans (e.g., sturgeon) and Gingylymodi (e.g., gar), but before the divergence of Osteoglossomorphs (a basal teleost lineage, e.g., elephantnose) (see Fig. 1) (Hoegg et al. 2004). An examination of these genes in Amiiformes (e.g., bowfin, see Fig. 1) is currently missing, and it will also be important to expand the repertoire of genes investigated and include a wider range of basal teleost species to allow the genome duplication to be definitively placed with respect to phylogeny. Finally, as evolutionary analysis depends on the strength of the existing phylogeny it should be noted that there remains significant disagreement regarding relationships among the ray-finned fishes. Inconsistencies exist between relationships derived from morphological and molecular data (Inoue et al. 2003), and critically, the nonteleost relationships remain controversial. The conflicts between trees derived from morphological and molecular data need to be resolved in order to place the fish-specific duplication and explore its significance.

DATING THE RAY-FINNED FISH SPECIFIC DUPLICATION

The age of a duplication event (expressed in millions of years ago; Ma) can be calculated from rates of synonymous substitutions (nucleotide changes that do not alter amino-acid usage); if the rate of substitution has clock-like properties then the time since two sequences diverged can be calculated. By applying this approach to several zebrafish genes Taylor et al. (2001) estimated the fish-specific genome duplication to have occurred around 350 Ma. As resolution is decreased by the likelihood that the sequences have reached saturation, they proposed that the duplication occurred earlier than 300 Ma but after the divergence of the ray- and lobe-finned fishes 450 Ma. The age of duplications can also be calculated from linearized phylogenetic trees (Takezaki et al. 1995). Vandepoele et al. (2004) and Christoffels et al. (2004) have applied this approach to *F. rubripes* data, and their conclusions concur reasonably well with one another and with the results of Taylor and colleagues: they estimate that the ray-finned fish duplication occurred 320 and 350 Ma, respectively.

The age of the duplication event can also be determined if the timing of a critical divergence event is known. However, there are current disagreements about the age of speciation events in the ray-finned fish lineage. Fossil, morphological, molecular, and biogeographical data provide incongruent timings. For example, fossil data suggest that living teleosts radiated as recently as 161 Ma (Teleost crown node, Fig. 1; Patterson 1993) and that the split of teleosts from gar and *Amia* is minimally 270 Ma (Teleost stem node, Fig. 1). By contrast, molecular studies have proposed that the crown teleosts originated 256–312 Ma (Kumazawa and Nishida 2000).

It is necessary to have accurate speciation dates in order to interpret the results provided by clock-based methods. A ray-finned fish specific genome duplication occurring before 320–350 Ma (Christoffels et al. 2004; Vandepoele et al. 2004) would predate the origin of the teleost stem lineage by so long that it is inconsistent with the hypothesis that genome duplication fuelled the radiation. However, ongoing work using complete mitochondrial genomic data in combination with newly identified fossil calibration points, is reinvestigating the timescale for key events in ray-finned fish evolution (M. Coates, personal communication). The key question is whether support exists for a range-extension of the teleost stem group deep into the Palaeozoic, some 50 Ma earlier than the current earliest fossil marker. If so, then a major obstacle to the hypothesized causal link between duplication and teleost radiation will be removed.

In summary, there remains a paucity of data relating to the timing of the whole-genome duplication event that occurred in the ray-finned fish lineage. Although the available data place the duplication event minimally in a euteleost ancestor, we need to more accurately determine where and when this event occurred. This will require analysis of a wide range of genes in multiple noneuteleost species and interpretation will hinge on the establishment of an accurate ray-finned fish phylogeny. Molecular clock analysis has been limited to *F. rubripes* and zebrafish so far, and this work also needs to be extended to other species using the correct date for the teleost radiation. In this way, it may be possible to derive a correlation between whole-genome duplication and speciation; accurate information is essential if we are to test the hypothesis that genome duplication fuelled the teleost radiation.

THE FATES OF DUPLICATED GENES

How can duplicated genes diverge to produce genetic novelty? Immediately after a large-scale duplication event duplicated pairs of genes will function identically to play entirely redundant roles. Classical models suggest that the most likely outcome of this redundancy is the ultimate loss of one of the pair, as deleterious mutations in one of the two genes will not have any negative impact on fitness and will therefore not be selected against (Haldane 1933). Only in those rare cases where one gene mutates to have a new and beneficial function will both be positively selected and retained in the genome. This kind of "neofunctionalization" was suggested to provide a critical facilitating force in vertebrate evolution by Susumu Ohno in his influential 1970 book. However, genomic evidence has revealed that duplicate genes are actually retained far more frequently than this classical model would suggest, and this has led to development of additional models to explain preservation of duplicate gene pairs in the genome (reviewed by Prince and Pickett 2002).

One of the most widely applicable models to explain the preservation of duplicate genes is "subfunctionalization," which was recently considered in a population genetics context by Force and colleagues in their Duplication–Degeneration–Complementation (DDC) model (Force et al. 1999; Lynch and Force 2000; Lynch et al. 2001). The basic concept underlying the DDC model is that each of the two duplicate genes may undergo degenerative losses, and that these losses will affect complementary components of the function of the unduplicated ancestral gene. As a consequence, both gene duplicates must be preserved in order for all the functions of the original unduplicated gene to continue to be performed. These components of gene function are termed "subfunctions" and in order for DDC processes to act they must be independently mutable (reviewed by Prince and Pickett 2002). Independently mutable subfunctions will often parallel genetic modules; for example, they are likely often to correlate with *cis*-regulatory elements, as these can function in a modular fashion. Consider an example where the ancestral gene is expressed in the "head" and the "tail" under control of two separate *cis*-regulatory elements (schematized in Fig. 2A). Following duplication of the ancestral gene, one of the two duplicate genes may acquire degenerative mutations in the "head" element and the other in the "tail" element; now both genes are necessary to ensure both head and tail expression, and thus both genes are preserved in the genome.

Mutation of an independently mutable subfunction is neutral as long as that subfunction is retained in the other duplicate. Like other neutral processes, subfunctionalization is highly dependent upon effective population size: only in small populations (less than 10,000) are duplicate genes likely to be preserved through DDC processes (Lynch and Katju 2004). Preservation of duplicate genes in populations via DDC processes can result in each duplicate having fewer selective constraints in relation to the ancestral gene because of a reduction in pleiotropy. This in turn can allow each preserved duplicate to begin to explore mutational space closed to the ancestral locus, and to develop novel functions that might have imposed too high a selection cost on the ancestral gene. Thus, one could consider DDC processes as acting to extend the time that duplicates are preserved for long enough to allow novel gene functions to arise.

Subfunctionalization models rely on the existence of independently mutable subfunctions. The modular nature of enhancers (Force et al. 1999, 2004) suggests that subfunctions often lie within *cis*-regulatory elements, but one interesting question is how do such subfunctions originally arise? Force et al. (2004) have proposed that the evolution of genes with multiple subfunctions may often be a consequence of "subfunction fission." Subfunction fission is proposed to occur when multiple functions under shared genetic control evolve to be under independent control. During the fission process, the expression domains of the gene in question do not change,

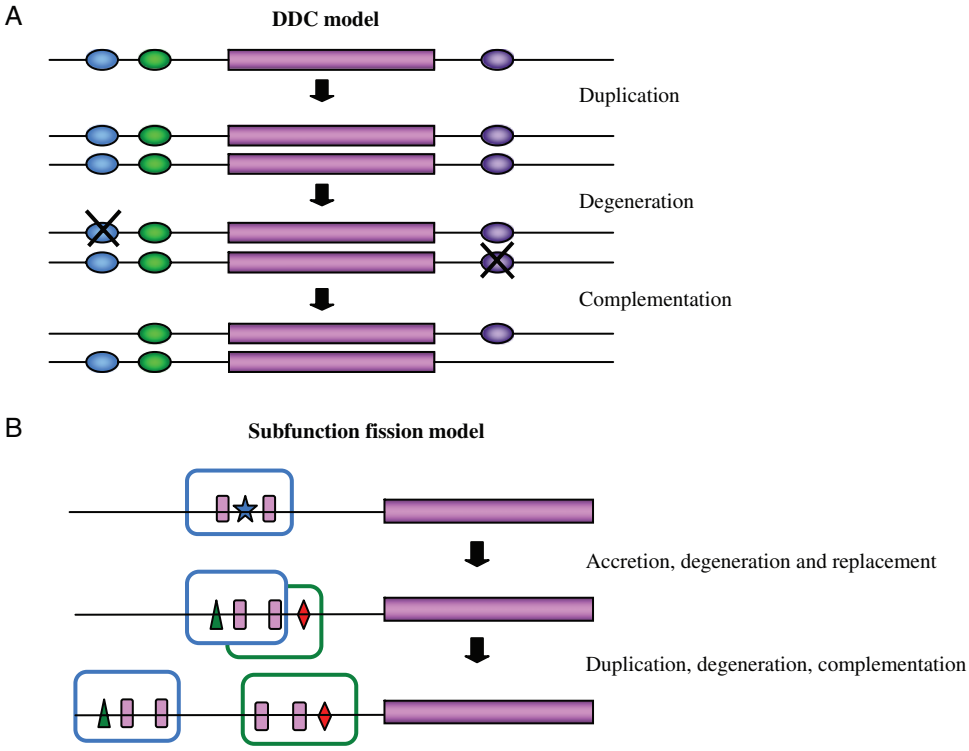

Fig. 2. (A) Duplication–Degeneration–Complementation (DDC) model. (B) Subfunction fission model.

only the mechanisms that control expression. The process relies on the acquisition of *cis*-regulatory elements that interact with tissue specific transcription factors; this allows replacement of completely shared regulatory sites with independent binding sites to produce a semi-independent enhancer. Localized duplication of the semi-independent enhancer, followed by degenerative mutations analogous to those occurring during DDC processes, can ultimately produce entirely independent *cis*-regulatory regions, each critical to an independent subfunction of the gene (Fig. 2B). The accrual of such genetic modularity, like subfunctionalization itself, is more likely to occur in small populations (Force et al. 1999), and its existence is a prerequisite for preservation of duplicate genes via DDC processes.

EXPERIMENTAL APPROACHES TO GENE DUPLICATION: FUNCTIONAL COMPLEMENTATION BETWEEN TELEOST HOX GENES

Although models of gene evolution depending on DDC-like processes have been with us for some years, relatively few convincing examples of subfunction degeneration and functional complementation between duplicates have been described (Postlethwait et al. 2004). Although it is very difficult to date complementary degenerative events to specific periods after duplication, such events should nevertheless leave evidence of their occurrence in the sequence and functions of extant duplicate gene pairs. Unsurprisingly, examples of this have come primarily from work on teleost fishes, where not only has a genome duplication event been recognized, but in addition, genomic sequence information goes hand-in-hand with tractable developmental model systems. Our own studies have focused on teleost Hox patterning genes, in particular those that are expressed in the developing hindbrain region.

The Hox paralog group (PG) 1 genes have been well studied in a variety of vertebrates (Prince 2002), both with respect to their regulation and their functions in hindbrain patterning. In the zebrafish, degenerative mutations in *cis*-regulatory sequences appear to underlie the preservation of a pair of duplicated PG1 genes (*hoxb1a* and *hoxb1b*) in accord with the DDC model. The combined expression patterns of these two zebrafish genes together resemble the expression pattern of the single *Hoxb1* gene of tetrapods (e.g., mouse, chick, and *Xenopus*). Our sequence analysis showed that upstream of zebrafish *hoxb1a* lie a set of three autoregulatory sequences that are 100% conserved with those of the mouse *Hoxb1* gene (Fig. 3), consistent with the shared stable hindbrain expression domains of these genes. By contrast, *hoxb1b*, which is expressed only transiently in the developing hindbrain, has accrued point mutations in the autoregulatory sequences that are

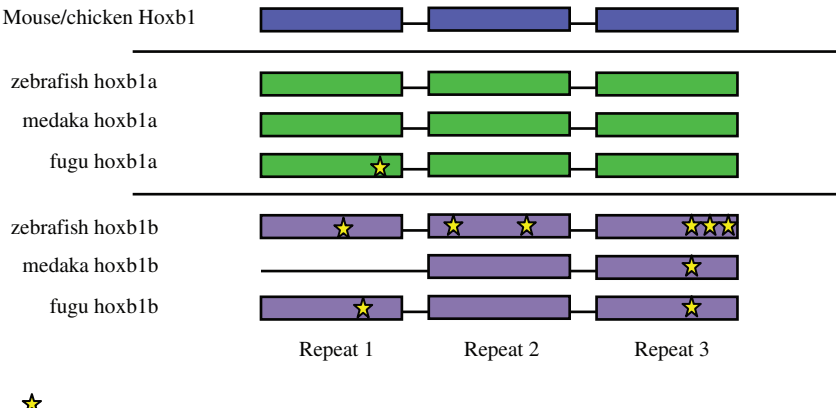

Fig. 3. Degeneration of teleost *hoxb1b* autoregulatory elements.

likely to be sufficient to abrogate its autoregulation (Fig. 3). The DDC model invokes complementary losses of subfunctions from duplicated genes, and again in accord with the model we are unable to find a neural specific retinoic acid response element downstream of zebrafish *hoxb1a*, although zebrafish *hoxb1b* and mouse *Hoxb1* share this element, which drives gastrulation-stage expression in the hindbrain. In summary, zebrafish *hoxb1a* has lost a downstream regulatory element that drives an early expression phase, while *hoxb1b* has deleterious mutations in the upstream autoregulatory element that drives a later expression phase.

One surprising aspect of our analysis of the zebrafish *hoxb1* duplicates is that the degenerate autoregulatory sequences that lie upstream of *hoxb1b* have accrued so few changes relative to functional autoregulatory sequences (Fig. 3). This apparent sequence conservation could be explained in one of two ways: either the mutations in the regulatory element of *hoxb1b* occurred relatively recently, and thus insufficient time has elapsed for additional degenerative changes to occur, or, these sequences play an unknown secondary role that is leading to their selection and maintenance. If the first explanation were correct, it would imply that the zebrafish *hoxb1* duplicates were preserved in the genome for a long period of their evolutionary history through some other mechanism than the one we have proposed, and have only recently fallen under new constraints that allowed mutations to occur in the autoregulatory sequences. If this explanation were accurate then it would predict that the *hoxb1* genes of other teleost species are unlikely to have been under similar selective pressures, and hence the fates of those duplicates might be quite different in different species.

To explore the fates of duplicated teleost genes we have begun a comparative study of *hoxb1* genes in three phylogenetically distant teleost species (zebrafish, medaka, and *F. rubripes*; Fig. 1). We find that the *hoxb1a* genes of all three species include highly conserved autoregulatory elements, whereas the *hoxb1b* genes have undergone a variety of degenerative changes (Fig. 3). These findings imply that the *hoxb1* genes of the last common ancestor of the euteleosts were indeed initially preserved through the DDC processes that we have explored in some detail for the zebrafish. We propose that in this ancestral species the *hoxb1b* autoregulatory sequences initially mutated to become nonfunctional; additional degenerative mutations were then acquired through genetic drift to ultimately produce the sequences we now find in zebrafish, medaka, and *F. rubripes*. In ongoing experiments, we are exploring the minimum degenerative changes that are sufficient to prevent function of the autoregulatory elements. Interestingly, our findings support the second of our initial hypotheses, that the "autoregulatory" sequences of the *hoxb1* genes in fact play an unknown secondary role that is subject to purifying selection.

The central tenet of Ohno's hypothesis that gene duplication facilitates evolution (Ohno 1970) is that the availability of duplicate genes should allow new gene functions to arise. By contrast, our functional analyses of zebrafish Hox genes from both paralog groups 1 and 2 have not revealed any new functions. Rather, we have found that when we compare zebrafish with mouse (our surrogate for a pre-duplication ancestral condition) the same Hox gene functions are in place, although these may be sub-divided among more or different genes within the same paralog group (Prince 2002). On reflection, our inability to determine novel functions for duplicated genes should perhaps not come as a surprise. The whole basis of subfunctionalization models is that genes are modular in nature and have pleiotropic functions. When we are considering developmental control genes, such as members of the Hox family, these multiple functions are just as likely to occur at different developmental stages as in different regions of the developing animal. As our current techniques to test gene function are targeted to the first few days of development, we may be missing new gene functions that act at later stages.

What we have been able to reveal is that the functions of a pair of duplicated genes, *hoxb1a* and *hoxb1b*, are not entirely interchangeable (see Prince 2002), and consistent with this, there is significant sequence divergence between the duplicates. These findings are consistent with the concept that once duplicates are preserved through DDC processes they will be freed to evolve along novel trajectories, and this in turn may lead to acquisition of novel functions. More sophisticated genetic approaches, for example selecting for conditional gene functions, may ultimately reveal these novel functions.

THE FATES OF DUPLICATED MORPHOLOGICAL STRUCTURES

Duplication of morphological structures, similar to duplication of genes, has long been considered a mechanism by which functional systems evolve. For example, in vertebrates, fundamental cranial structures such as the jaws and ears are usually interpreted as derivatives of the reiterated pharyngeal arch segments (Goodrich 1930; De Beer 1985), and the duplication of segments post-cranially has resulted in major variation among taxa in segment numbers and organization (Richardson et al. 1998). By generating new morphology, duplication events provide additional substrates for the evolution of structure and function (Lauder 1981, 1990; Friel and Wainwright 1997).

Mechanisms proposed for morphological evolution through duplication parallel those proposed for gene evolution through duplication. For example, the concept of neofunctionalization, novel function arising through beneficial mutations in a redundant gene copy (Ohno 1970), has been proposed for morphology (Lauder 1981, 1982, 1990). One copy of a duplicated structure may evolve a new function whereas the other retains its initial role in the organism (Lauder 1981). Alternatively, increasing the number of structural elements by duplication may provide greater opportunity for diversification of the integrated functional system as a whole (Liem 1973). Rather than evolving independently, the duplicate structures together form a single more complex functional system that may nevertheless be able to evolve in directions constrained in the unduplicated state. This mechanism shares a conceptual framework with the genetic process of subfunctionalization: in both instances the duplicated "elements" are integrated to perform the original function of the unduplicated "element."

In biomechanical systems, structural duplication provides one mechanism for morphological decoupling (Lauder and Schaefer 1993; Schaefer and Lauder 1996). Decoupling of morphology (making structures functionally independent of another) provides increased complexity and opportunity for modification and diversification of the system (Schaefer and Lauder 1996). Whereas gene duplication occurs as a single event, it is thought that morphological duplication can occur either as a single evolutionary event (Liu et al. 2003; Hale et al. 2004), or alternatively as a gradual subdividing and remodeling of existing structures that results in independent units (Friel and Wainwright 1997); it is notable that this latter mechanism is conceptually similar to the hypothesis of subfunction fission.

PHYLOGENETIC APPROACHES TO MORPHOLOGICAL DUPLICATION: INCREASED FUNCTIONAL COMPLEXITY THROUGH THE SUBDIVISION OF TELEOST JAW MUSCLES

Morphological duplication and evolution have been primarily studied within a phylogenetic framework, as a well-resolved phylogeny makes it possible to test hypotheses for evolutionary transitions in the relationship of structure to function (Lauder 1990; Schaefer and Lauder 1996; Friel and Wainwright 1997). The jaws of teleost fishes have provided many examples of morphological diversification as part of the widespread decoupling of jaw elements that has occurred repeatedly through teleost evolution. Friel and Wainwright (1997, 1998, 1999) have explored changes in muscle function after the presumed evolutionary subdivision of the adductor mandibulae (jaw closer) muscles in a wide spectrum of tetraodontiform fishes. These muscles, represented by a single bilateral pair in basal members of the group, have independently subdivided several times during tetraodontiform evolution. These subdivisions have sometimes been incomplete (providing good evidence for subdivision rather than de novo duplication) and sometimes been complete, with the original pair giving rise to as many as eight separate muscles. Beyond duplication events, the adductor mandibulae muscles remain remarkably similar morphologically. Overall, muscle mass does not increase in the subdivisions relative to the original undivided muscle mass and, in all but two cases, the insertions of the duplicated muscles remain the same as in the unduplicated states. The variety of levels of muscle subdivision, the well resolved tetraodontiform phylogeny, and the diverse suite of tetraodontiform feeding behaviors has provided an ideal illustration of how such duplications may affect functional diversity.

To determine whether subdivision led to functional complexity of the jaws, Friel and Wainwright (1998, 1999) examined the electromyographic patterns of activity of paralogous muscles during feeding. In contrast to most teleosts, in which motor patterns of feeding are highly conserved when homologous muscles are compared across taxa (Friel and Wainwright 1997), tetraodontiform species show a diversity in the timing and amplitude of muscle activity when their muscle homologs are compared (Friel and Wainwright 1998, 1999). Thus, in the tetraodontiform jaw

muscles, increased morphological complexity is associated with increased functional complexity (Friel and Wainwright 1999). It has been suggested that this increase in functional complexity results in an increase in fine motor control for feeding (Friel and Wainwright 1998) and thus has important evolutionary consequences. An interesting question raised by Friel and Wainwright (1999) is whether physical or functional subdivision comes first. To begin to address this issue, two adductor mandibulae muscles that had not subdivided were examined by recording from regions that would correspond to subdivisions in other taxa (Friel and Wainwright 1999). In both cases, regional variation in muscle activity corresponded to differences in subdivisions of the same muscles in other taxa. These data suggest that functional subdivision can predate physical subdivision. However, subdivided muscles that do not differ in motor pattern have also been described, suggesting that the converse situation, where physical subdivision can occur before functional subdivision, also occurs (Friel and Wainwright 1998, 1999).

Exactly how similar are such instances of morphological duplications, and their evolutionary outcomes, to the gene duplications discussed earlier? Are the same models applicable? Both neofunctionalization and subfunctionalization models are dependent upon initial functional redundancy of a duplicate gene pair. In cases of morphological duplication are the duplicated structures functionally redundant? This can be very difficult to assess in extant forms where post-duplication diversification has already occurred. However, in cases where structural duplication occurred via subdivision, such as in the tetraodontiform adductor mandibulae muscles, it seems unlikely that the newly duplicated structures were capable of performing entirely equivalent functions; rather, they subdivided the ancestral functions of the single unduplicated structure. Nevertheless, even partial functional redundancy between duplicated structures might permit neofunctionalization or subfunctionalization. In any situation where duplicated morphological structures are equally capable of fulfilling a function played by the single ancestral structure, then either one of the duplicates is freed from the necessity to fulfill that function. This may merely lead to evolutionary loss through degenerative changes, but may also lead to the acquisition of a novel function via neofunctionalization processes. Similarly, if independently mutable subfunctions exist within duplicated structures, then DDC-like processes could theoretically act.

One difference between morphological and gene duplicates may lie in their relative degrees of modularity. Eucaryotic enhancers tend to act as independent modules, and can thus provide genes with a source of the independently mutable subfunctions that DDC processes require. By contrast, although morphological structures often fulfill multiple functions, the inherent integration of these different functions may preclude the existence of independently mutable subfunctions. As a consequence, we suggest that subfunctionalization, at least as defined via DDC, may be a less frequent outcome of morphological duplication than of gene duplication.

Although the phylogenetic approach to morphological duplication has provided multiple examples of structural and functional diversity, such comparative studies remain limited in the information they can provide on the duplication event itself and on the initial mechanistic basis of the evolution of duplicated structures. However, techniques for genetic manipulation in model organisms have recently allowed experimental generation of morphological duplicates (del Toro et al. 2001; Gehring 2001; Liu et al. 2003; Hale et al. 2004), providing case studies in which to examine both the potential origins of duplicate structures and the functional consequences of such duplication events.

EXPERIMENTAL APPROACHES TO MORPHOLOGICAL DUPLICATION: DUPLICATE TELEOST MAUTHNER CELLS ARE FUNCTIONALLY REDUNDANT

The brain is thought to be a highly evolutionarily constrained system because of the overlapping and integrated organization of neurons into circuits. It is therefore perhaps surprising that the examples of genetically induced duplication, in which functionality has also been addressed, all involve the nervous system. For example, when ectopic eye structures were induced in *Drosophila,* electroretinograms demonstrated that these eyes respond to visual cues (Gehring 2001). del Toro et al. (2001) found that mice mutant for the *Hoxa1* gene have ectopic neurons of hindbrain rhombomere (r) 2 identity in the more posterior r3 and r4. In normal mice r2 cells are involved in breathing rhythm generation, in the mutants the ectopic cells of r2 identity integrated into appropriate circuits and were able to drive the breathing rhythm in the absence of their endogenous r2 counterparts, implying functional redundancy. Finally, several studies on the duplication of Mauthner cells (M-cells), large reticulospinal neurons that drive startle behavior in fishes, have found that these new cells are morphologically integrated into the startle circuit and can function in behavior (Liu et al. 2003; Hale et al. 2004).

The M-cell initiated startle behavior of teleosts is a simple system that has been used widely to examine neural circuit organization and functions (Zottoli and Faber 2000; Eaton et al. 2001). The M-cells are a pair of large neurons with their cell bodies located in r4, one on each side of the segment. They have commissural axons that cross the hindbrain and extend the full length of the spinal cord to excite spinal interneurons and motoneurons. The startle behavior they elicit is a rapid turn away from a threatening stimulus. If the stimulus is sensed on the left side of the fish, the left M-cell fires and an action potential travels down its axon on the right side

of the fish ultimately causing right side muscle to contract and the fish to bend and turn away from the threat. This initial M-cell-elicited movement is generally followed by burst swimming away from the stimulus. In addition to the M-cells, other reticulospinal cells have been shown to function in startle behavior (Liu and Fetcho 1999). In particular, MiD2cm and MiD3cm cells, often considered serial homologs to the M-cells, in r5 and r6, respectively, function in response to startle stimuli directed at the animal's head whereas only the M-cells are active in response to tail-directed stimuli (Liu and Fetcho 1999).

Liu et al. (2003) examined M-cell duplication in the zebrafish mutant *notch1a*/*deadly seven (des)*, which develops multiple M cells in r4. These authors found that all of the cells were active in response to startle stimuli indicating that when such a duplication event occurs the duplicate neurons are receiving the appropriate sensory input and that the cells can elicit a normal behavioral response. They also examined M-cell axon collaterals in the spinal cord to determine whether the duplicate cells were all providing motor output. They found that the M-cells appeared to be dividing their connections to downstream neurons in the spinal cord. Each M-cell in the mutant had fewer collaterals than the individual M-cell in a wild type fish, and those collaterals generally had non-overlapping distributions suggesting that they were functioning together to drive a normal response.

We have also examined M-cell duplication in the zebrafish, but using duplicates that were generated by Hox gene misexpression (Hale et al. 2004). Previous studies (Alexandre et al. 1996; McClintock et al. 2001) had demonstrated that misexpression of zebrafish *hoxb1b*, or other PG1 Hox genes, causes duplication of the r4 M-cells at the level of r2. As in the case of the *des* mutant (Liu et al. 2003), we found that *hoxb1b*-induced duplicate M-cells responded appropriately to startle stimuli and the additional M-cells did not increase performance of the startle behavior (Hale et al. 2004). We also assessed the functionality of the duplicate neurons using cell-specific lesions. To test how an ectopic r2 M-cell was integrated into the startle circuit either the ectopic r2 M-cell, the endogenous r4 M-cell, or both, were laser ablated, and the effects on behavior assayed. We found that when either the r2 or the r4 cell on one side of the brain was lesioned, the remaining M-cell could drive a normal response. Thus, the r2 and r4 neurons have redundant functions in the startle behavior. By contrast, removal of both the r2 and the r4 cell on one side of the brain resulted in a startle of decreased performance comparable with that of a wild type fish with a single M-cell lesioned (Liu and Fetcho 1999; Hale et al. 2004). In summary, experiments from our own (Hale et al. 2004) and other labs (del Toro et al. 2001; Liu et al. 2003) have shown that new neurons, at their inception, can be integrated into functioning neural circuits and can play functionally redundant roles in behavior.

What are the implications of these studies on M-cell function in startle circuits with respect to duplication and divergence mechanisms? As we have been able to demonstrate experimentally that duplicated r2 and r4 M-cells function redundantly in startle behavior, we have previously suggested that such duplication could provide a substrate for the evolution of reticulospinal circuits via neofunctionalization mechanisms (Hale et al. 2004). One of the duplicate cells could potentially take on a new function without decreasing the performance of the startle behavior. This is a particularly compelling system in which to make such an argument as the startle behavior is the primary behavior used by fishes to respond to predator attack and is therefore closely related to the animal's fitness: changes in M-cell function that decrease startle performance are likely to be heavily selected against. We further suggested that the entire suite of reticulospinal neurons, which also function in behavior, may have initially evolved by a similar mechanism (Hale et al. 2004). For example, the serial homologs to the M-cells (MiD2cm in r5 and MiD3cm in r6) may have evolved through duplication. Similar ideas were put forward by Brunet and Ghysen (1999), who postulated that developmental isolation of subsets of neurons, both from one another and from the larger system, could allow changes to arise in one or both neuronal sets and thus facilitate evolution of new behaviors. Interestingly, these authors specifically invoked Hox genes as likely candidates to set up modular identity of individual segments, thus allowing each module to evolve independently.

The distribution of outputs of M-cells in the *des* mutant described by Liu et al. (2003) indicates that these cells are subdividing activity to the downstream cells. As each duplicate cell drives activity of only a subpopulation of spinal neurons, all the M-cells may need to function together to drive normal high performance behavior; unfortunately, as pointed out by Liu et al. (2003), testing this hypothesis using individual cell lesions is not feasible as the *des* mutant M-cell somata are located so close together. Nevertheless, if we assume that the multiple M-cells of the *des* mutant have subdivided function of the normal single M-cell, then could the *des* mutant phenotype be considered an instance of structural subfunctionalization? The mutant phenotype certainly shares some features with the final outcome of genetic subfunctionalization: the duplicate M-cells together play equivalent functions to the single, unduplicated "ancestral" M-cell. However, there is a limit to this analogy, because in this mutant animal none of the complementary degenerative mutations that underlie subfunctionalization have occurred. Nevertheless, the *des* mutant phenotype may be providing clues that evolution of reticulospinal circuitry could have proceeded via subfunctionalization mechanisms. This would of course hinge not only on duplication events leading to functional redundancy of duplicated cells, but also on the existence of independently mutable subfunctions. In future

studies it will be interesting to establish whether M-cells, and other reticulospinal neurons, are likely to have such independently mutable subfunctions. Such investigations would need to focus on whether these neurons possess intrinsic modularity, for example by determining whether their outputs to different downstream cells are independently mutable.

One major question that remains to be addressed with such experimental duplication studies is: what is the origin of the cellular material used to generate the duplicate cells? The duplicates may be new cells, or previously existing cells subdivided from the existing morphology or co-opted from another function. If the latter condition is the case, then another function may be sacrificed or diminished because of the loss of the original cell. The studies described above were all performed under lab conditions and, although behavior is maintained, fitness of the animal because of changes in other systems has not been addressed and it is not known whether these duplications have negative impacts on other aspects of an animal's biology. Despite this caveat, experimental studies on model systems nicely complement comparative studies in the context of a phylogeny to provide a more complete view of the role of duplication in morphological evolution. Experimental studies can assess the initial state and impact of a duplication, providing information on functionality, whereas the phylogenetic studies establish the outcome of evolution on those structures, providing information on generation of diversity.

Our own experimental focus has been on the segmented hindbrain, and one major question in this field is what form did the ancestral hindbrain take? Studies of the lamprey hindbrain have supported the model that reiterated hindbrain segments initially contained equivalent sets of reticular neurons that later diversified in function (Murakami et al. 2004). Although this model is appealing, it does not address why the number of hindbrain segments appears to be so invariant, especially when compared with the remarkable diversity in number of trunk mesodermal segments. Gilland and Baker (1993) reported conserved organization of the basic seven or eight rhombomere structure of the hindbrain among a wide range of gnathostomes, and the studies of Kuratani and colleagues on lampreys (Kuratani et al. 1998; Murakami et al. 2004) suggest that this organization may have already been present in an agnathan ancestor. One possibility is that the 7/8 rhombomere number has been well conserved because the hindbrain plays a central patterning role within the pharynx, coordinating migration of cranial neural crest into the adjacent pharyngeal arches (Lumsden et al. 1991; Schilling and Kimmel 1994; Horigome et al. 1999) as well as arch innervation by the cranial nerves (reviewed by Gilland and Baker 1993). However, a caveat to this argument is that stem group gnathostomes had highly variable numbers of pharyngeal arches (Janvier 1996), suggesting that the apparent conservation of cranial/pharyngeal organization among vertebrates may rather be an example of homoplasy. Nevertheless, once such a complex system has been established it is likely to be very resistant to major changes as a consequence of balancing selection or functional constraint. Although duplication of entire rhombomeres remains an unlikely evolutionary mechanism, significant variation nevertheless exists in the details of hindbrain organization across the vertebrates; experimental data from our labs and others have suggested that neuronal (and potentially gene) duplications could have been instrumental in generating this diversity (del Toro et al. 2001; Hale et al. 2004).

CONCLUSIONS

Now that it is clear that a whole-genome duplication occurred in the ray-finned fish lineage, teleost fishes are likely to become increasingly popular models in which to study the implications of genome duplication events. More accurate establishment of where in the phylogeny, as well as when in real time, this duplication event occurred will enable appropriate comparisons to be made of pre- and post-duplication species, and will allow the hypothesis that genome duplication fuelled teleost radiation to be tested. Of relevance to this issue, differential resolution of duplicate genes has been postulated to drive speciation even in the absence of any new gene functions (Lynch and Conery 2000). Teleosts will also continue to provide valuable models in which to study the implications of duplication of specific individual genes, and in the future such studies will need to extend to genetic networks; although, because of the very large gene families, the networks may prove more difficult to elucidate in teleosts than in other vertebrates. Finally, studies of morphological duplication have long exploited the advantages of the teleosts, and in the future this should allow possible links between gene duplication and structural duplication to be explored. Even if these two forms of duplication prove rarely to be causally related, they are clearly linked at an intellectual level. Consequently, theoretical approaches to gene evolution are likely to enrich our understanding of morphological evolution, and vice versa.

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