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REVIEW ARTICLE

Genetic and developmental bases of serial homology in vertebrate limb evolution

Ilya Ruvinsky[1,*] and Jeremy J. Gibson-Brown[2,‡]

[1]Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
[2]Department of Biology, Washington University, St Louis, MO 63130, USA
*Present address: Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114, USA
‡Author for correspondence (e-mail: gibbro@biology.wustl.edu)

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SUMMARY

Two sets of paired appendages are a characteristic feature of the body plan of jawed vertebrates. While the fossil record provides a good morphological description of limb evolution, the molecular mechanisms involved in this process are only now beginning to be understood. It is likely that the genes essential for limb development in modern vertebrates were also important players during limb evolution. In recent years, genes from a number of gene families have been described that play important roles both in limb induction and in later patterning processes. These advances facilitate inquiries into several important aspects of limb evolution such as their origin, position along the body axis, number and identity. Integrating paleontological, developmental and genetic data, we propose models to explain the evolution of paired appendages in vertebrates. Whereas previous syntheses have tended to focus on the roles of genes from a single gene family, most notably *Hox* genes, we emphasize the importance of considering the interactions among multiple genes from different gene families for understanding the evolution of complex developmental systems. Our models, which underscore the roles of gene duplication and regulatory 'tinkering', provide a conceptual framework for elucidating the evolution of serially homologous structures in general, and thus contribute to the burgeoning field seeking to uncover the genetic and developmental bases of evolution.

Key words: Vertebrate, Limb, Evolution, Development, T-box gene, *Hox* gene, *Pitx1*, Selector gene, Serial homology

INTRODUCTION

The last decade has witnessed a dramatic revival of interest in understanding the connection between developmental process and morphological change during evolution (Raff, 1996; Gerhart and Kirschner, 1997). Much insight has been gained by comparing the expression patterns and functions of homologous developmental genes among different taxa (Akam, 1995; Carroll, 1995). These surveys emphasize the redeployment of pre-existing genes in the evolution of novel developmental pathways, and build upon the classical idea that regulatory changes, rather than biochemical changes in proteins, are a major driving force in morphological evolution (Wilson et al., 1974; King and Wilson, 1975). According to this paradigm, pre-existing genetic modules are reshuffled and 'tinkered with' over time to generate the diversity of body plans (Jacob, 1977; Von Dassow and Munro, 1999; Raff and Sly, 2000). Clearly the duplication of pre-existing genes will therefore have profound evolutionary implications. This process appears to have been of particular importance for the evolution of developmental complexity in vertebrates (Ohno, 1970; Holland et al., 1994; Ruvinsky et al., 2000b).

The vertebrate limb has long been the subject of considerable interest to both evolutionary and developmental biologists. In his classic book, '*On the Nature of Limbs*', Richard Owen (1849) outlined the basic problems that still define the field today. One of the characteristic features of jawed vertebrates (gnathostomes) is the presence of two, and no more than two, sets of paired appendages; limblessness, evident in several independent lineages such as caecilians, snakes and whales, is a derived feature resulting from the secondary loss of these structures. The forelimbs and hindlimbs of tetrapods are homologous to the pectoral and pelvic fins of fish, respectively. Moreover, similarities in their bone patterns reveal that forelimbs are homologous to hindlimbs, a phenomenon referred to as serial homology.

After 150 years of inquiry we are now in a position to provide explanations for these observations at the molecular level. Of particular interest are the questions relating to the origins of limbs. How did the characteristic number of limbs evolve? How did their distinctive morphologies become specified in the course of vertebrate evolution? Additionally, vertebrate limbs provide an excellent model system with which to study the molecular bases of serial homology.

Recently, two models have been presented to account for the evolution of vertebrate limbs at the molecular level. Tabin and Laufer (1993) suggested that pectoral fins evolved as a consequence of the rostral homeotic transposition of a pre-existing set of pelvic fins resulting from the novel redeployment of *Hox* genes. Coates and Cohn (1998, 1999) proposed a model which explained the evolution of limb positioning as a result of the co-option of a '*Hox* code' that had originally evolved in splanchnic (gut) mesoderm to regulate rostrocaudal patterning of the digestive tract. Since important discoveries have recently been made in understanding the molecular bases of vertebrate limb specification and development, this review provides a more comprehensive account of the molecular steps likely to have been involved in the evolution of paired appendages in vertebrates.

Any molecular model seeking to explain a morphological transformation in the deep evolutionary past has to satisfy three basic criteria. First, the molecular components proposed to be responsible for the evolution of a trait are likely to be involved in the specification of that trait in extant organisms. Second, the transitional forms through which the trait is proposed to have evolved must be consistent with evidence derived from the fossil record. Finally, the timescale inferred for the evolution of the molecular events proposed has to be consistent with the paleontological evidence.

We first review evidence from the fossil record revealing the timing and sequence of events involved in the evolution of vertebrate appendages. We then provide an overview of the molecular developmental biology of the vertebrate limb, as it pertains to the specification of the limb fields and the initiation of bud outgrowth. Next, we present molecular models to account for the evolution of serial homology and distinct morphologies in vertebrate limbs. Finally, we propose several experiments designed to evaluate the validity of these models, providing a framework for future research in the field.

MORPHOLOGICAL EVIDENCE REGARDING THE EVOLUTION OF PAIRED APPENDAGES IN VERTEBRATES

The commonly accepted scheme of chordate evolution (Fig. 1) shows that modern vertebrates evolved from a basal invertebrate chordate, morphologically similar to the extant cephalochordate, amphioxus (Carroll, 1988). Possible candidates for such an organism include the Middle Cambrian chordates *Pikaia* (Conway Morris, 1982) and *Haikouella* (Chen et al., 1999), and the Early Cambrian chordates *Cathaymyrus* (Shu et al., 1996a) and *Yunnanozoon* (Chen et al., 1995; for an alternative interpretation of this fossil see Shu et al., 1996b). The body plan of these animals primitively lacks paired appendages.

The first vertebrates to emerge in the course of evolution were jawless fish (agnathans), which appeared in the fossil record at the Cambrian/Ordovician boundary and underwent an extensive radiation in the Silurian (Carroll, 1988; Janvier, 1996). Whereas there is much debate regarding the details of the phylogenetic relationships of modern and extinct lineages within this, most likely, paraphyletic assemblage, one thing is clear: the modern radiation of jawed vertebrates with two sets of paired appendages derives from a jawless, finless ancestor (Forey and Janvier, 1993; Coates, 1994). It is therefore of particular interest to survey the status of paired fin evolution in agnathan fish. Unfortunately, only two divergent and possibly paraphyletic representatives survive to the present day, the hagfish and lampreys. Neither shows any evidence of paired appendages, although, at least in the case of lampreys, this may not represent a primitive condition (Forey and Janvier, 1993). The lack of extant agnathans with paired fins confines the analysis to extinct forms. These animals, commonly referred to as ostracoderms, are extinct agnathans characterized by the extensive development of bony plates covering much of the body (hence the name 'shell-skinned'). Even though the fossil record must be considered incomplete, available evidence suggests that several distinct lineages of ostracoderms experimented with lateral protrusions that resemble pectoral fins by their location along the anteroposterior body axis. These include pectoral flaps in thelodontids, pectoral spines and lateral folds in anaspids, and pectoral extensions of the head shield in heterostracans (Carroll, 1988; Coates, 1994; Janvier, 1996). However, true gnathostome-type muscular appendages are first evident only in a subgroup of ostracoderms known as osteostracans, which phylogenetic analyses place closest to the gnathostome ancestor (Forey and Janvier, 1993). Even if modern jawed vertebrates were not derived from within this group, a shared ancestry with such organisms suggests that the presence of pectoral appendages may be a shared-derived character (synapomorphy) of a group containing both osteostracans and gnathostomes. Importantly, nowhere in the fossil record of pre-gnathostome evolution can any evidence be found of a fish lacking pectoral fins but with a single set of paired fins at the pelvic level.

The emergence of jawed vertebrates near the Ordovician/Silurian boundary was followed by the largest radiation of extant vertebrate species. These modern forms can be divided into two major groups (Metscher and Ahlberg, 1999). The first is chondrichthyans (cartilaginous fish), which includes sharks, skates, rays and chimaeras. The second is osteichthyans (bony fish), which is further divided into actinopterygians (ray-finned fish) and sarcopterygians (lobe-finned fish and tetrapods). Teleosts are prominent members of the former group, whereas tetrapods constitute the majority of the latter. While it is uncertain which specific lineage of agnathans gave rise to the gnathostomes, it is quite clear that all gnathostomes primitively possessed two sets of paired appendages, and no chordates other than gnathostomes possess this trait (Carroll, 1988; for a possible exception to this rule see Märss and Ritchie, 1998). Furthermore, it is thought that the pectoral and pelvic fins of a chondrichthyan are homologous to the pectoral and pelvic fins of a teleost, which in turn are homologous to the forelimbs and hindlimbs of a tetrapod (Owen, 1849; Goodrich, 1958; Coates, 1994; Shubin et al., 1997). This fact has profound implications for the study of vertebrate limb evolution in at least two respects. First, it implies a commonality of developmental mechanisms involved in the outgrowth and patterning of these appendages. Second, it suggests that all evolutionary transitions between the limbless body plan of a primitive agnathan, and that of a fish with two sets of paired fins, occurred between the origin of an osteostracan-like gnathostome ancestor and the origin of jawed vertebrates, a relatively short period of time (Fig. 1).

Two competing theories have been advanced to explain the

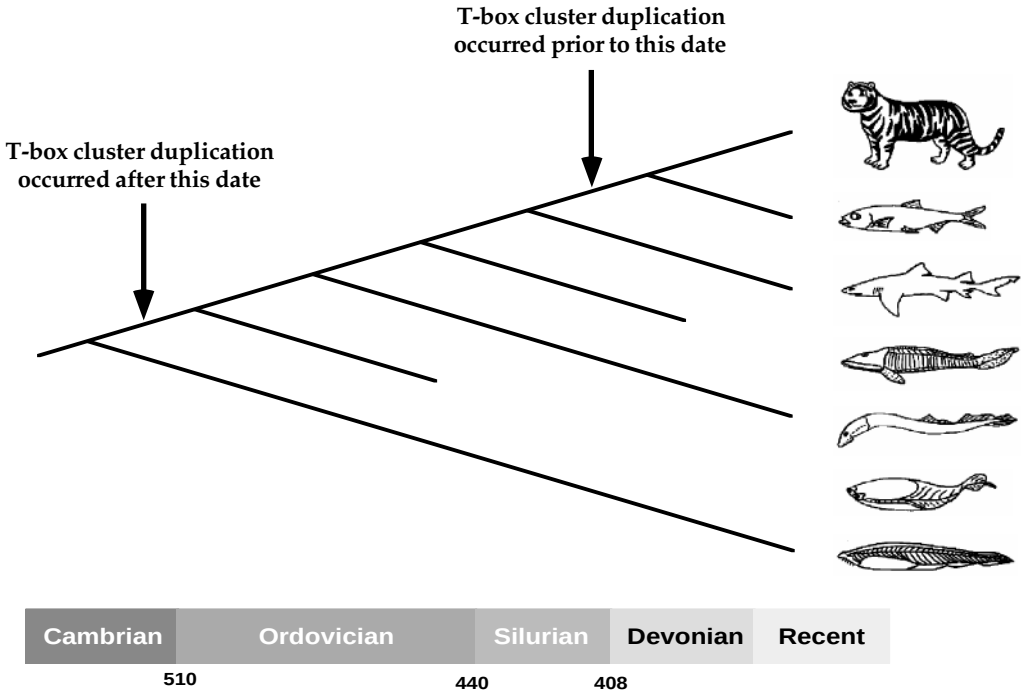

Fig. 1. Schematic representation of commonly accepted phylogenetic relationships and dates of divergence among major chordate lineages as inferred from paleontological evidence. Truncated terminal branches indicate extinct taxa. Starting at the bottom, the following lineages are shown. The cephalochordates, represented by amphioxus. One of the earliest known vertebrates, *Sacambambaspis*, a jawless fish lacking paired appendages. An extant agnathan, the lamprey. An osteostracan, *Hemicyclaspis*, a jawless fish with a single set of paired appendages at the pectoral level. The radiation of jawed vertebrates is represented by three lineages: cartilaginous fish (represented by a shark), bony fish (represented by a teleost), and tetrapods (represented by a tiger). The geological time scale is indicated in millions of years before present.

origin of paired vertebrate appendages from the structures already present in the body plan of an ancestral vertebrate (reviewed by Goodrich, 1958; Coates, 1994; Bowler, 1996). The first hypothesis, known as the 'gill-arch' theory, posits that pectoral fins represent a modified rendition of a posterior branchial arch, with the origin of pelvic fins being explained by a subsequent posterior transposition of this structure. The alternative, the 'lateral-fold' theory, proposes that paired fins were derived from paired lateral fin folds postulated to have run along the length of the body of a hypothetical vertebrate ancestor (see Coates, 1994; Shu et al., 1999). Both theories are based on evidence from comparative anatomical studies and the fossil record. Both contain points regarded to be fatal flaws by their opponents, and even their validity has been questioned (Bemis and Grande, 1999). For these reasons we do not currently favor either of these hypotheses over the other. Determining which, if either, of the two theories reflects the actual path of vertebrate limb evolution remains beyond the scope of this work.

In summary, current interpretation of the fossil record suggests that the origin of two sets of paired appendages in modern gnathostomes proceeded via two discrete steps. First, an anterior set of paired fins evolved at the pectoral level in a previously limbless agnathan. Later, a second set evolved at a more posterior axial level to produce a pair of pelvic fins. The ages of key fossils imply that these two steps must have happened within a relatively short span of geological time.

MOLECULAR DEVELOPMENTAL BIOLOGY OF THE VERTEBRATE LIMB

The vertebrate limb has long been a favorite model for developmental biologists studying pattern formation during embryogenesis. Molecular mechanisms underlying limb outgrowth and patterning have therefore been relatively well characterized (reviewed by Johnson and Tabin, 1997; Schwabe et al., 1998). In brief, the limb bud is initiated as a distal projection from the body wall by the proliferation of cells in lateral plate (flank) mesoderm. The axial level at which this outgrowth commences is currently believed to be determined by *Hox* genes. Several lines of evidence point in this direction. First, their nested patterns of expression along the anteroposterior body axis, known as the '*Hox* code', determine the unique morphologies of reiterated axial structures (Kessel and Gruss, 1990; Krumlauf, 1994; Favier and Dollé, 1997). Second, in species with different axial morphologies, changes in expression patterns of homologous *Hox* genes tend to correlate with morphological changes in the body plan (Burke et al., 1995; Cohn and Tickle, 1999). Third, application of Fibroblast Growth Factor (FGF)-soaked beads to the flank of chick embryos can induce ectopic limbs that develop as either wings or legs, depending on the axial level of bead application (Cohn et al., 1995), and the patterns of *Hox* gene expression in the flank of embryos in which ectopic limbs are being induced mimic the expression patterns observed in the limb fields of endogenous limbs of the same identity (Cohn et al., 1997). Finally, and perhaps most convincingly, a loss-of-function mutation in mouse *Hoxb5* can shift the axial position of the limb bud (Rancourt et al., 1995).

Classical embryological experiments have demonstrated that limb outgrowth is initiated by signals from lateral plate mesoderm to the overlaying ectoderm, resulting in the induction of an apical ectodermal ridge (AER), a thick cord of cells at the interface of the dorsal and ventral aspects of the limb bud (Saunders, 1948). Once established, the AER provides signals to maintain high proliferation rates in the distal part of the bud mesenchyme, the progress zone (Summerbell et al., 1973). Another function of the AER is to induce formation of the zone of polarizing activity, an

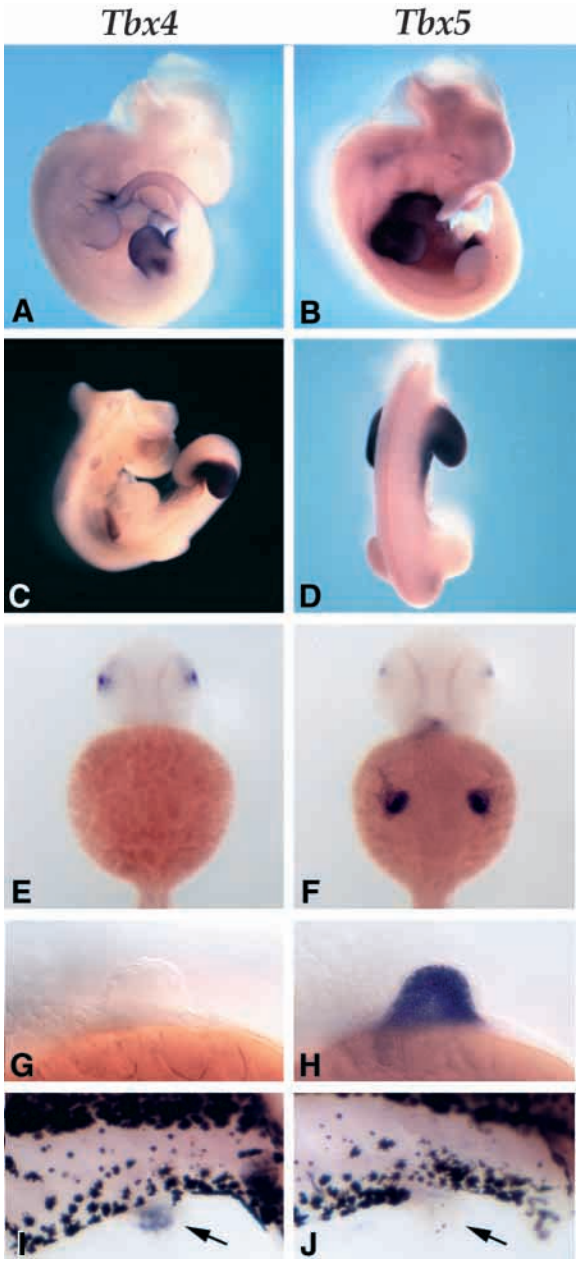

Fig. 2. Conserved expression of *Tbx4* (A,C,E,G,I) and *Tbx5* (B,D,F,H,J) in vertebrate appendages. (A,B) Mouse. (C,D) Chick. (E-J) Zebrafish. (E,F) Dorsal views showing pectoral fin buds. Lateral views of pectoral (G,H) and pelvic (I,J) fin buds (arrowed), anterior to the left. Modified from Gibson-Brown et al. (1996, 1998) and Ruvinsky et al. (2000a).

organizer responsible for generating the anteroposterior polarity of the limb bud (Saunders and Gasseling, 1968; Tickle et al., 1975). Throughout the entire process of limb development there exists extensive cross talk between the different signaling centers. Thus the final morphology of the adult limb is the product of a complex network of interacting molecular determinants acting during embryogenesis (reviewed by Tabin, 1995; Johnson and Tabin, 1997; Schwabe et al., 1998).

It is currently thought that the role of the initial inducing signal emanating from the mesoderm is played by FGF10 (Ohuchi et al., 1997). Induction is achieved by activating FGF8 in the ectoderm (Crossley et al., 1996; Vogel et al., 1996), which initiates tissue cross-talk, mediated by FGF receptor 2 (FGFR2; Xu et al., 1998). This model was tested by analyzing mouse mutants lacking FGF10 (Min et al., 1998; Sekine et al., 1999) and FGFR2 (Xu et al., 1998). Remarkably, mice mutant for either gene were limbless, suggesting essential roles played by these two molecules in the initial induction of limb outgrowth.

Cells of the lateral plate mesoderm differentiate to produce the skeletal elements of the limb. Other tissues, such as the musculature, nervous and vascular systems, arise from cells that invade the limb bud from the adjacent somites and neural crest (Gilbert 2000). Importantly, leg-to-wing and wing-to-leg transplantations in chick embryos show that the identity of the limb resides in the mesodermal, rather than the ectodermal, component of the bud (Saunders et al., 1959; Isaac et al., 1998). Furthermore, similar experiments demonstrate that limb identity is determined prior to the commencement of outgrowth. Mesodermal cells from the limb field, dissected at the pre-bud stage, are competent to direct the development of an appendage, the identity of which is consistent with their axial level of origin (Zwilling, 1955). In search of the bases of limb identity, therefore, one must concentrate on elucidating specific molecular differences distinguishing the populations of lateral plate mesoderm cells at the prospective pectoral and pelvic levels. This search points in the direction of a problem that remains elusive despite the substantial progress made in understanding the basic biology of vertebrate limb development. Why are pectoral and pelvic appendages so similar in their general design, yet so different in their specific morphologies?

T-BOX GENES IN DEVELOPMENT…

During the last 15 years many gene families have been discovered that play a variety of roles in vertebrate embryogenesis. Important among these are the T-box genes, which encode a family of transcription factors sharing a conserved domain with the classical mouse *Brachyury (T)* gene (Bollag et al., 1994). A feature universally conserved among all T-box gene products is a domain of about 160-180 amino acids (Papaioannou and Silver, 1998). This conserved region, the T-domain, binds DNA in a sequence-specific manner (Kispert and Herrmann, 1993; Muller and Herrmann, 1997), allowing the gene products to function either as activators or repressors of transcription of downstream target genes (Kispert et al., 1995; He et al., 1999; Smith, 1999; Papaioannou, 2000).

The first detailed study to examine the expression patterns of T-box genes in developing mouse embryos suggested that these genes are likely to play important roles in development (Chapman et al., 1996). Five genes, *Tbx1-Tbx5*, were found to be expressed in dynamic spatiotemporal patterns suggestive of a possible role in inductive tissue interactions. Interestingly, closely related paralogs were found to have strikingly similar, yet distinct, expression patterns. Subsequent studies in the mouse and other organisms have extended these observations to other family members (Papaioannou and Silver, 1998; Papaioannou, 2000).

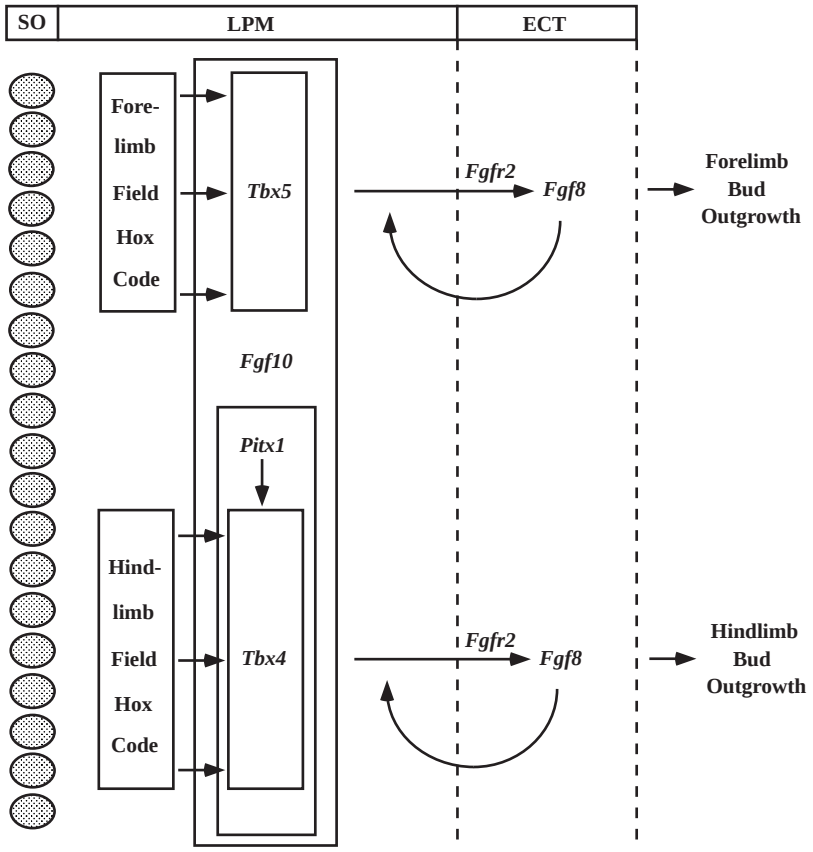

Fig. 3. A genetic model for the specification of limb position and initiation of bud outgrowth. *Hox* genes expressed within the lateral plate mesoderm specify the positions at which forelimbs and hindlimbs will develop. This positional (axial) information leads to limb-specific T-box gene expression within the prospective limb fields. Initially, *Fgf10* is expressed by all cells of the lateral plate mesoderm. Subsequently, *Tbx4* and *Tbx5* are activated in the prospective limb fields, where their interaction with the *Fgf10*/*Fgf8* positive-feedback loop initiates bud outgrowth. *Fgf10* is later required to maintain T-box gene expression in the outgrowing buds. *Pitx1* expression in posterior flank mesoderm is independently induced and extends in a broader rostocaudal domain than that of *Tbx4*. *Pitx1* positively interacts with *Tbx4* to maintain its expression. SO, somites; LPM, lateral plate mesoderm; ECT, ectoderm.

Of central importance for the subject of this review is the observation that four particular T-box genes, *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5*, are expressed during limb development (Gibson-Brown et al., 1996). Transcripts of *Tbx2* and *Tbx3* are expressed in similar patterns in the anterior and posterior margins of the outgrowing forelimb and hindlimb buds. In contrast, *Tbx4* and *Tbx5* reveal complementary expression. Whereas *Tbx5* transcripts are detected only in the forelimb bud, *Tbx4* transcripts are found almost exclusively in the hindlimb (Fig. 2A,B). Furthermore, the onset of expression of both genes in their respective limb fields, prior to the formation of a morphologically discernable bud, precedes that of any other known limb-field-specific marker. Subsequent studies in the chick (see below), *Xenopus* (Takabatake et al., 2000) and zebrafish (Tamura et al., 1999; Yonei-Tamura et al., 1999; Begemann and Ingham, 2000; Ruvinsky et al., 2000a) demonstrate that the characteristic expression of these four T-box genes in the limbs is a feature conserved among jawed vertebrates (Fig. 2C-J). The expression patterns of *Tbx4* and *Tbx5* were interpreted as an indication of their involvement in specifying limb identity during embryogenesis (Gibson-Brown et al., 1996).

Later functional analyses of T-box genes in the chick reinforced this idea (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). *Tbx5* and *Tbx4* are expressed in lateral plate mesoderm throughout the forelimb and hindlimb fields, respectively, prior to the initiation of bud outgrowth. This expression is retained in leg-to-wing and wing-to-leg mesenchymal tissue grafts, consistent with the previously reported retention of graft identity following such transplantations (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998). Also, in ectopic limbs induced by application of FGF-soaked beads, expression of T-box genes correlates with axial level and future identity: more rostral limbs mainly express *Tbx5* and develop as wing-like mosaic limbs, while more caudal limbs mainly express *Tbx4* and develop as leg-like mosaic limbs (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). It has also been shown that the newt *Tbx5* gene (Nv*Tbox1*) is expressed during forelimb, but not hindlimb, regeneration (Simon et al., 1997).

To establish whether *Tbx4* and *Tbx5* expression is sufficient to confer specific limb-type identity, constructs containing these genes were ectopically expressed during embryogenesis in the chick (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). These experiments resulted in the partial transformation of limb identities as seen by both morphological and molecular markers. Wing-like characteristics were induced in the leg upon misexpression of *Tbx5* constructs and leg-like features were seen in the wing after misexpression of *Tbx4* constructs. These results showed that both genes are capable of inducing alternative fates upon ectopic expression, and confirmed the idea that these genes are involved in the establishment of limb identity.

Additional inferences regarding the roles of T-box genes during limb development can be made from analyses of mutations in the human *TBX3* and *TBX5* genes. The former cause ulnar-mammary syndrome in patients heterozygous for apparent loss-of-function alleles (Bamshad et al., 1997). A wide variety of forelimb malformations, which are characteristic of this condition, indicate the critical role

played by *TBX3* in anteroposterior patterning of the forelimb (Bamshad et al., 1995). Mutations in human *TBX5* cause Holt-Oram syndrome (Basson et al., 1997; Li et al., 1997). Limb defects in heterozygotes range from subtle hand abnormalities to phocomelia (severe limb shortening), revealing an important function of this gene in the process of forelimb growth and patterning.

Taken together, expression patterns, embryological manipulations and mutant phenotypes highlight distinct and essential roles played by *Tbx2-Tbx5* genes during limb development in gnathostomes. Specifically, *Tbx5* and *Tbx4* appear to be involved in determining forelimb and hindlimb identity, respectively, and *Tbx2* and *Tbx3* are likely to be involved in anteroposterior limb patterning (Gibson-Brown et al., 1996, 1998; Bamshad et al., 1997; Yonei-Tamura et al., 1999; Ruvinsky et al., 2000a).

At what level do these genes fit into the cascade of molecular components specifying limb position and outgrowth? Because *Fgf10* is transiently expressed by all cells of the lateral plate mesoderm prior to the expression of *Tbx4* and *Tbx5* in the flank (Ohuchi et al., 1997; Isaac et al., 2000), its expression must be initiated in a *Tbx4/Tbx5*-independent manner. Likewise, because initial expression of *Tbx4* and *Tbx5* is induced in the prospective limb mesoderm in limbless *Fgf10* mouse mutants (Sekine et al., 1999), these genes must be induced independently of *Fgf10* expression and bud outgrowth. Because T-box gene expression is subsequently lost in the mutant embryos, *Fgf10* does appear to be required later to maintain their expression (Fig. 3). The axial position of the limb bud is likely to be determined by the action of *Hox* genes. Thus *Tbx5* is activated as a result of a 'read out' of the '*Hox* code' for the pectoral appendage, whereas *Tbx4* is expressed as an 'interpretation' of the '*Hox* code' characteristic of the pelvic appendage (Gibson-Brown et al., 1998; Fig. 3). Since they delineate the territories from which bud outgrowth will initiate, and specify the identity of the limbs that subsequently develop, *Tbx4* and *Tbx5* can be viewed as 'selector genes' of limb position and identity (Weatherbee and Carroll, 1999).

…AND EVOLUTION

To address the question of whether or not specific T-box genes

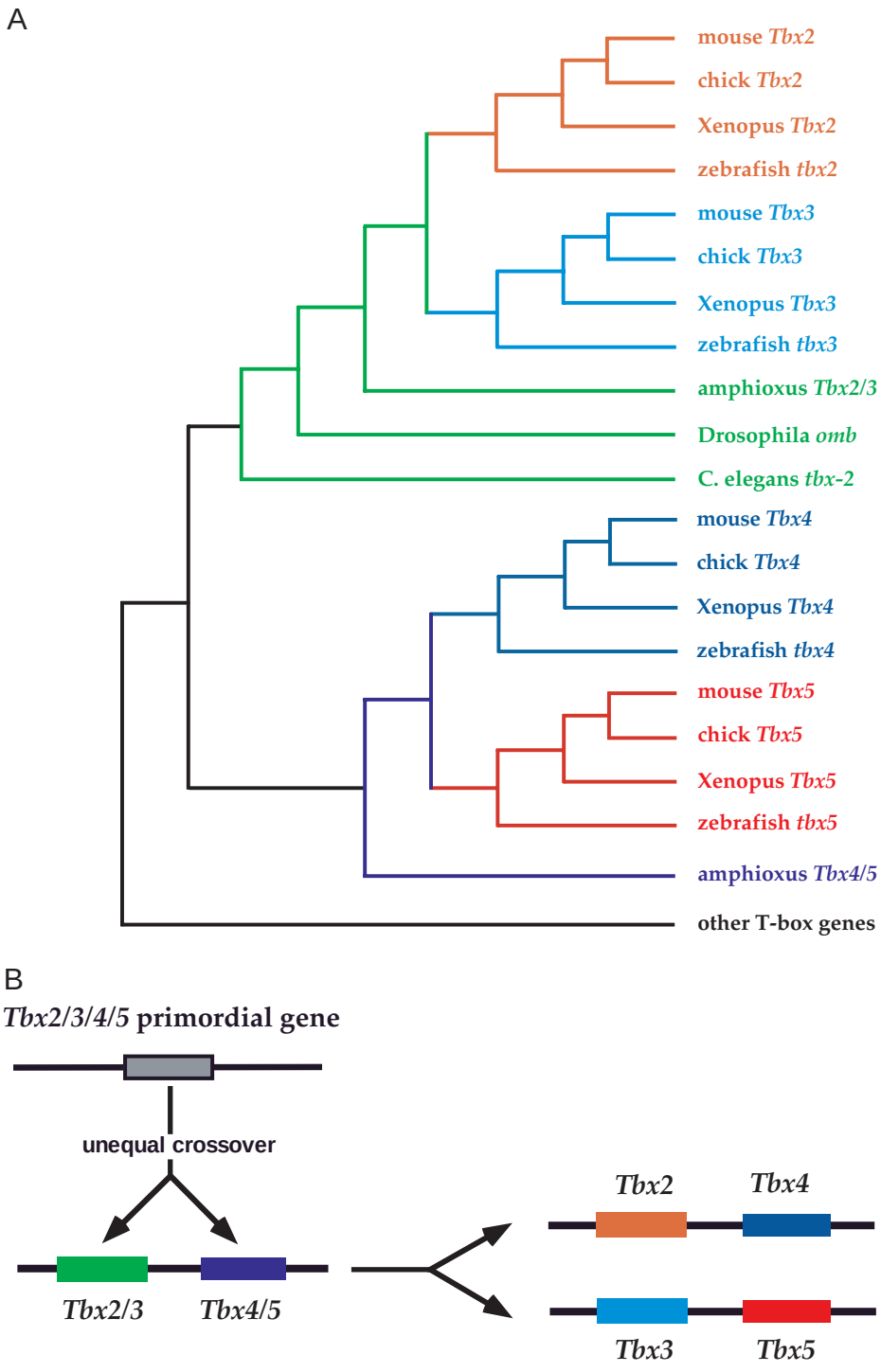

Fig. 4. Evolution of the *Tbx2-Tbx5* genes. (A) A schematic representation of the phylogenetic relationships of the *Tbx2-Tbx5* genes in a variety of species. Vertebrate orthologs are shown in the same color. Invertebrate genes are depicted in 'hybrid' colors (yellow + light blue=green, dark blue + red=purple), to indicate that two vertebrate genes are orthologous to each invertebrate gene. (B) A model for the evolution of two T-box gene clusters from a single primordial gene. Modified from Agulnik et al. (1996). The same color scheme is used in A and B, and in Figs 5 and 6.

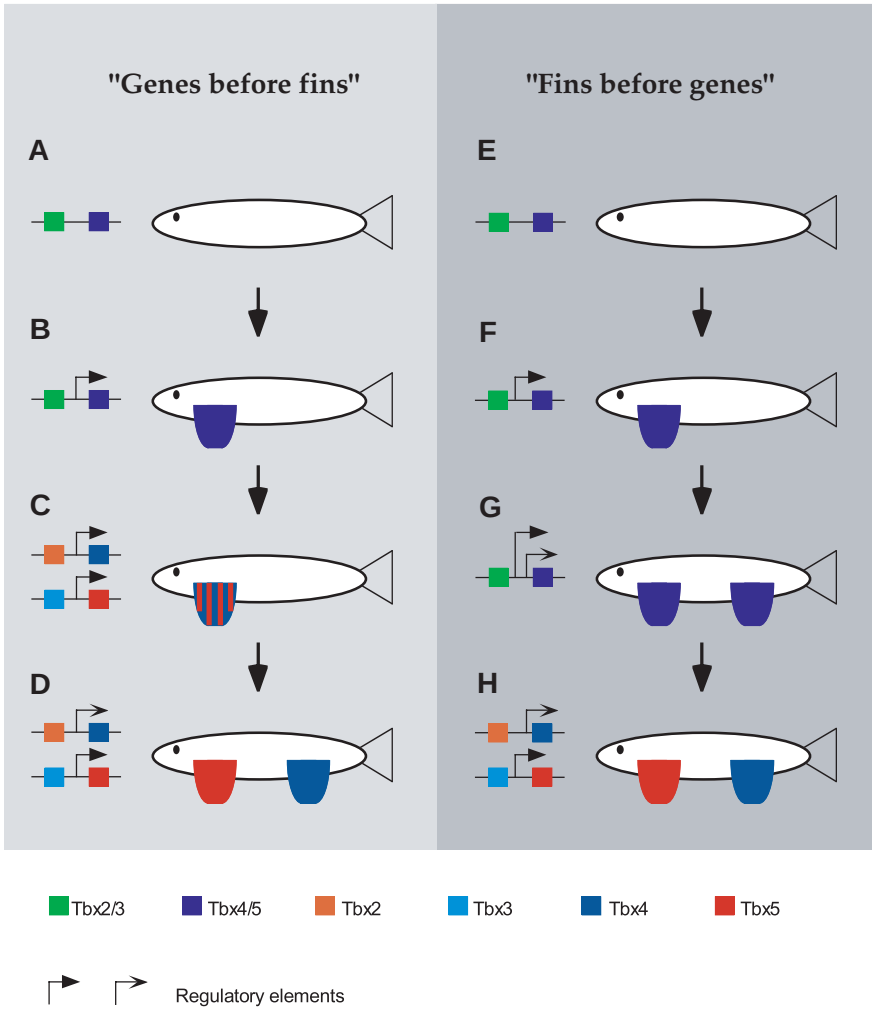

Fig. 5. Models for the evolution of serial homology of paired appendages in vertebrates. Two alternative scenarios are considered: 'genes before fins' (A-D) and 'fins before genes' (E-H). (A) A limbless ancestor of vertebrates possesses a single T-box cluster. (B) The *Tbx4/5* gene acquires a novel expression domain in lateral plate mesoderm, resulting in limb bud outgrowth at the pectoral level. (C) The T-box cluster undergoes duplication. Initially, the *Tbx4* and *Tbx5* genes are fully redundant and are coexpressed in the pectoral appendage. (D) Acquisition, by *Tbx4*, of a novel regulatory element (indicated by a different arrowhead) leads to the acquisition of a novel expression domain in posterior flank mesoderm, and to the reiteration of a limb outgrowth program at the pelvic level. Stages (E) and (F) are equivalent to stages (A) and (B), respectively. (G) *Tbx4/5* acquires a novel regulatory element (indicated by a different arrowhead), and leads to the acquisition of a novel expression domain, resulting in the outgrowth of pelvic appendages. (H) Following T-box cluster duplication, divergent evolution of *Tbx5* and *Tbx4* results in complementary expression patterns in pectoral and pelvic fins, respectively, each representing a subset of the original expression domain of the ancestral gene.

played a role during vertebrate limb evolution, it is essential to gain a clear understanding of the evolutionary history of the genes in question. A schematic representation of the phylogenetic relationships among *Tbx2-Tbx5* genes is depicted in Fig. 4A. This tree shows that there are two pairs of closely related vertebrate genes – *Tbx2* and *Tbx3*, and *Tbx4* and *Tbx5* – and that cognate genes within each pair diverged after the separation of the cephalochordate and vertebrate lineages (Ruvinsky et al., 2000b), but probably before the radiation of extant jawed vertebrates (Ruvinsky et al., 2000a; Fig. 1). In addition, the fact that branches of similar length lead, on the one hand to *Tbx2* and *Tbx3*, and on the other hand to *Tbx4* and *Tbx5*, prompted Agulnik et al. (1996) to suggest that these two duplications may have happened at about the same time in vertebrate evolution. Finally, the origin of the precursor genes, *Tbx2/3* and *Tbx4/5*, is ancient; they diverged from a single ancestral locus prior to the separation of the protostome and deuterostome lineages (Agulnik et al., 1996).

Interestingly, in the mouse genome *Tbx2* is tightly linked to *Tbx4* while *Tbx3* maps close to *Tbx5*. The most parsimonious interpretation of the phylogenetic and mapping results was proposed by Agulnik et al. (1996) and is depicted in Fig. 4B. According to this model, a gene ancestral to all four of the T-box genes under consideration, the *Tbx2/3/4/5* gene, underwent a tandem duplication, probably by unequal crossing-over, to produce a cluster of two tightly linked genes, *Tbx2/3* and *Tbx4/5*. Based on the phylogenetic analysis above, this event must have happened relatively early in metazoan evolution. Following separation of the vertebrate and invertebrate lineages, this original cluster then duplicated 'en masse' and, in the process, the two resulting copies were dispersed to two different chromosomal locations, giving rise to the arrangement seen in the mammalian genome today. Consistent with such an 'en masse' duplication, a pair of paralogy groups, spanning no less than 5-6 cM, and centered on the T-box gene clusters, is found within the mouse genome (Ruvinsky and Silver, 1997). A phylogenetic analysis of the genes from these paralogy groups predicted that this duplication took place before the separation of the lineages leading to bony fish and tetrapods, a hypothesis since confirmed by the discovery of all four othologs in zebrafish (Ruvinsky et al., 2000a). This estimate predicts that the genomic arrangement of the two T-box clusters should be similar in all vertebrates, a hypothesis since supported by the discovery of two pairs of tightly linked genes in zebrafish (I. R. and M. Ekker, unpublished data). Maintenance of close linkage between genes in a cluster for long periods of evolutionary time may be indicative of selective pressure due to functional constraints, possibly as a consequence of the presence of shared regulatory elements. *Hox* genes represent a classical example of this situation, since

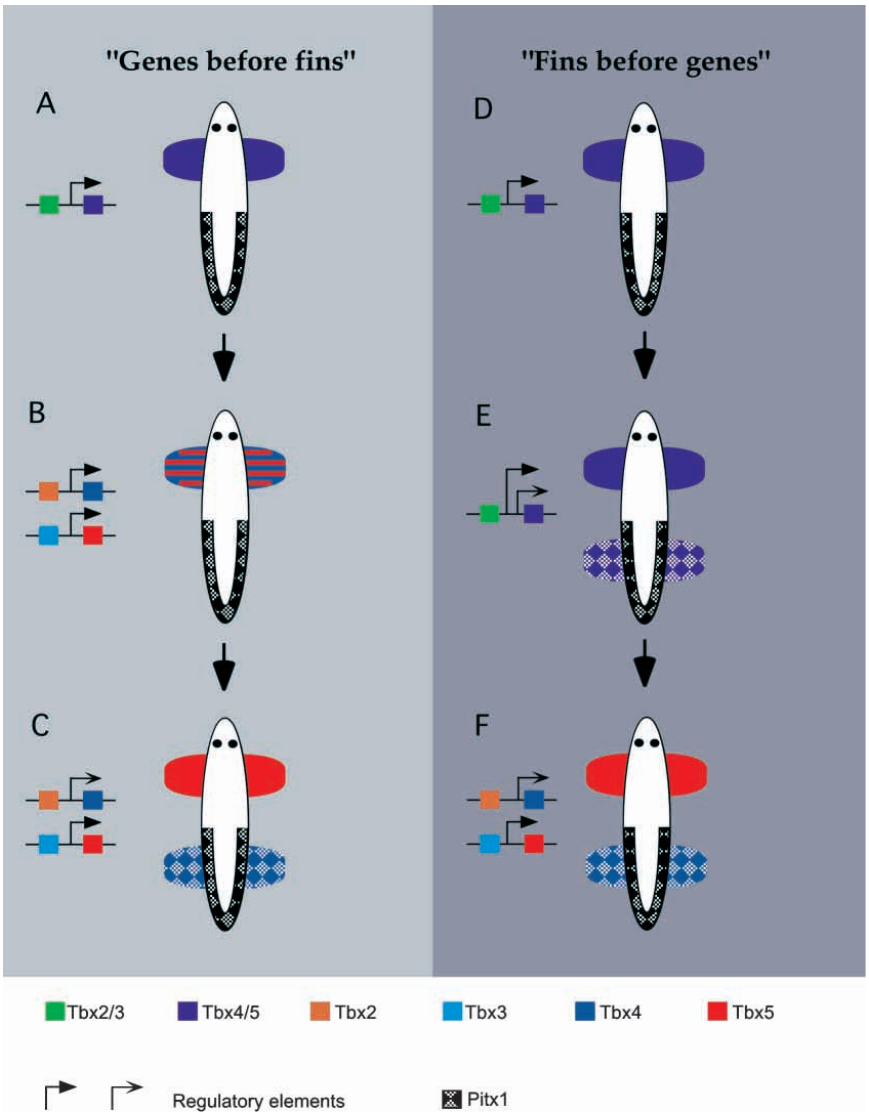

Fig. 6. A model for the evolution of distinct morphologies of pectoral and pelvic fins as a result of T-box gene coevolution with an asymmetrically expressed 'modifier', *Pitx1*. Stages (A-C) and (D-F) correspond to dorsal views of (B-D) and (F-H) from Fig. 5, respectively.

coordinate regulation of their colinear expression is dependent upon a number of *cis*-regulatory elements located both within and adjacent to the evolutionarily conserved clusters (Krumlauf, 1994; Duboule, 1998).

To summarize, two members of the T-box family of transcription factors, *Tbx5* and *Tbx4*, exhibit limb-specific expression patterns and have emerged as regulators of forelimb and hindlimb identity in vertebrates. Their close paralogs, *Tbx2* and *Tbx3*, are expressed at the anterior and posterior margins of both forelimbs and hindlimbs, and are also likely to play important roles in limb patterning. Essentially identical expression patterns of these genes in the mouse, chick, *Xenopus* and zebrafish (Fig. 2), strongly suggest that the last common ancestor of all jawed vertebrates possessed all four of these genes and used them to specify the identity, and regulate the patterning, of two sets of paired appendages. Since the estimated divergence time of *Tbx4* and *Tbx5* coincides with the period when key events in the evolution of vertebrate limbs occurred, there is the possibility of a causal connection between the evolution of these genes and the evolution of paired appendages in vertebrates. By combining our knowledge of T-box gene functions and evolution with evidence from the fossil record, two alternative scenarios can be proposed to account for the evolution of serially homologous paired appendages in vertebrates.

MOLECULAR MECHANISMS FOR THE EVOLUTION OF SERIALLY HOMOLOGOUS VERTEBRATE LIMBS

'Genes before fins'

According to this model (Fig. 5A-D), the evolution of genetic redundancy preceded, indeed served as a necessary prerequisite for, the origin of serially homologous limbs. Initially (Fig. 5A) a limbless ancestor of jawed vertebrates, an animal morphologically similar to amphioxus, possessed a single T-box cluster containing the *Tbx2/3* and *Tbx4/5* precursor genes. The transition to the next stage (Fig. 5B) was driven by the acquisition, by the *Tbx4/5* gene, of a novel expression domain within the lateral plate mesoderm at an axial level corresponding to the position of the pectoral appendages in modern vertebrates.

Whenever a gene gains a new expression pattern, one, or both, of two possible mechanisms can be responsible: it is either due to the origin of a novel regulatory element or to the modification of a pre-existing element. The term 'element' must be understood broadly in this context to include both the *cis*- and *trans*-regulators of the gene. In the cases discussed here, the changes can involve either mutations in the DNA sequences regulating the expression patterns of a particular T-box gene, or modification of the DNA-binding specificity of upstream regulatory (e.g. *Hox*) genes. Clearly, changes in either of these two interacting components can lead to the evolution of a novel gene expression domain. Equally clearly, once established, the two sides must coevolve if the functional cohesiveness of the interaction is to be maintained.

Regardless of the exact mechanism, the '*Hox* code' activating the expression of the *Tbx4/5* gene changed. The ectopic redeployment of *Tbx4/5* caused the activation (and/or repression) of a number of its original downstream target genes in a new location, thus reiterating at least a portion of a pre-existing genetic program in a new location (Niehrs and Pollet, 1999). Recruitment of 'pre-assembled modules' may be a common mechanism responsible for the evolution of novel

morphologies (Keys et al., 1999). Clearly, it is possible that some of the original downstream targets would not be activated/repressed upon redeployment of the module, since their transcriptional regulation would require the presence of additional cofactors, which are not expressed in the new location. Activation of the *Tbx4/5* gene in a rostrocaudally restricted subset of lateral plate mesoderm cells, which already expressed *Fgf10*, resulted in the establishment of a new regulatory interaction and led to the outgrowth of an appendage. Because misexpression of *Tbx4* or *Tbx5* in the inter-limb flank is not alone sufficient to induce an ectopic outgrowth (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), some additional interaction must be required for bud initiation. Other downstream 'appendage' genes also could have been activated as a result of *Tbx4/5* expression (e.g. *SnR*/*twist*; Isaac et al., 2000). The resulting animal, with a single pair of appendages at the pectoral level, would correspond morphologically to an osteostracan (Figs 1, 5B).

Duplication of the cluster containing the *Tbx2/3* and *Tbx4/5* genes, likely as a result of a whole-genome duplication (Ohno, 1970; Ruvinsky et al., 2000b), was the next crucial step in appendage evolution. The initial redundancy of the two resulting T-box clusters manifested itself in identical expression patterns of *Tbx4* and *Tbx5* (Fig. 5C). However, as is often the case following gene duplication (Li, 1997), *Tbx4* gained a novel expression domain, giving rise to the posterior (pelvic) appendages (Fig. 5D), and subsequently lost its original expression domain. The most plausible route by which this novelty was acquired was that a regulatory element of *Tbx4* coevolved with the posterior '*Hox* code', resulting in the acquisition of a novel posterior expression domain. Meanwhile, *Tbx5* maintained the original anterior expression domain of the *Tbx4/5* precursor gene. Following separation of the genes, each would have been free to acquire distinct downstream targets (Weatherbee et al., 1998, 1999). All of these evolutionary steps must have happened prior to the radiation of jawed vertebrates, since all members of this group examined so far share two sets of paired appendages with characteristic limb-specific patterns of T-box gene expression.

'Fins before genes'

In contrast to the above scenario, an alternative model can be proposed, in which the origin of serially homologous limbs predated the origin of genetic redundancy (Fig. 5E-H). The first two stages of this scenario are identical to the first two stages of the one detailed above (compare Fig. 5E,F to A,B). The next step, however, is dramatically different. While the original expression domain and function of the *Tbx4/5* gene in the pectoral appendages was maintained, its expression was reiterated more posteriorly at a level corresponding to modern pelvic fins (Fig. 5G). This resulted in an animal with two sets of serially homologous appendages. The next, and final step, was precipitated by a whole-genome duplication (Ohno, 1970; Ruvinsky et al., 2000b), following which the initial genetic redundancy decayed, such that eventually *Tbx4* and *Tbx5* were expressed in complementary patterns, each representing a subset of the original expression domain of the ancestral locus (Fig. 5H). A conceptual model for the evolution of distinct gene functions by complementary, degenerative mutations has recently been proposed by Force et al. (1999).

EVOLUTION OF MORPHOLOGICAL DIFFERENCES BETWEEN PECTORAL AND PELVIC APPENDAGES

The fossil record suggests that the morphology of pelvic appendages is primitively different from that of the pectoral pair (Carroll, 1988; Coates and Cohn, 1998, 1999). Since the two sets of appendages are serially homologous, what mechanisms can account for the observed differences? One way in which distinct morphologies likely evolve in serially homologous structures can be proposed as an extension of Lewis Wolpert's notion of 'positional nonequivalence', which emphasizes the fundamental differences between cells located at different positions within the embryo (Lewis and Wolpert, 1976). Applied to the case discussed here, once the limb outgrowth and patterning program was reiterated at a more posterior axial level, it would operate in a different molecular milieu from the one in its original location. These differences may be caused by the presence of molecules already expressed at the 'new' location. These modifying interactions would likely cause alterations to the original developmental program, resulting in the generation of a novel morphology.

An appealing candidate for a 'modifier' gene of this kind is a homeodomain-containing transcription factor, *Pitx1*. One of its expression domains is located within posterior lateral plate mesoderm (Szeto et al., 1996; Lanctot et al., 1997; Shang et al., 1997). The anterior boundary of this domain is positioned in such a way that, in modern vertebrates, *Tbx5*-expressing cells lie anterior to it, whereas *Tbx4*-expressing cells also express *Pitx1*. Furthermore, loss-of-function (Lanctot et al., 1999; Szeto et al., 1999) and gain-of-function (Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999) experiments strongly suggest that *Pitx1* is involved in the determination of hindlimb morphology, the same role assigned to *Tbx4*. It is important to note that initiation of expression of these two genes is independent of each other, and abolition of *Pitx1* function induces only partial hindlimb to forelimb transformations, which suggests that *Tbx4* and *Pitx1* act in concert in determining hindlimb morphology (Lanctot et al., 1999; Szeto et al., 1999; Takeuchi et al., 1999). These data, together with the fact that posterior mesendoderm expression of a *Pitx*-related molecule is an ancient feature characteristic of all chordates (Yasui et al., 2000), lead to the following hypothesis for the origin of morphological differences between the forelimbs and hindlimbs during vertebrate evolution (Fig. 6). The origin of a posterior expression domain of *Pitx* predated limb duplication (Fig. 6A,B,D). Reiteration of the limb outgrowth program in the *Pitx*-expressing domain provided an opportunity for the coevolution of this gene with the T-box genes in establishing the identity of the posterior appendage (Fig. 6C,E,F). Coevolution of several 'selector genes', all of which are required, but none alone is sufficient, for the proper specification of structural identity, may be a general feature of the evolution of distinct morphologies. For example, the cooperative interaction between *Distal-less* and *homothorax* in the specification of antennal identity in *Drosophila* (Si Dong et al., 2000), is remarkably similar to the interaction between *Tbx4* and *Pitx1* in the specification of hindlimb identity in vertebrates. Undoubtedly, other genes, both members of the 'limb-module' and those previously expressed in posterior lateral plate mesoderm, coevolved with these two regulators,

and with each other, to produce the diverse hindlimb morphologies seen today in modern vertebrates.

TESTING THE MODELS: FUTURE DIRECTIONS

There are two major difficulties in testing hypotheses which seek to explain events that happened in the distant evolutionary past. First, numerous mutations in DNA sequences, which have accumulated since the event in question, tend to obscure the picture by increasing the noise-to-signal ratio. Second, intermediate taxa essential for the falsification of the proposed hypotheses have often become extinct, rendering direct tests impossible. Both of these problems are acute in the case of vertebrate limb evolution. The last common ancestor of extant jawed vertebrates lived over 450 million years ago (Kumar and Hedges, 1998), by which time both sets of paired appendages had already evolved. Furthermore, the first steps of the above scenarios (Figs 5 and 6) may have happened as far back as 550-600 million years ago (Hedges, 2000). Finally, both osteostracans and the most basal jawed vertebrates are extinct. Despite these difficulties, several proposals for discriminating between the two scenarios can still be made.

The first scenario suggests that duplication of the T-box gene cluster had already occurred prior to the origin of jawed vertebrates, and perhaps even earlier, in osteostracans. In contrast, the second scenario places gene cluster duplication after the origin of jawed vertebrates, with two sets of paired fins already present. Finding an animal that possesses two T-box clusters but primitively does not have paired appendages would support the first model (‘genes before fins’). On the other hand, identification of a jawed vertebrate with a single *Tbx2/3*, *Tbx4/5* gene cluster would support the second scenario (‘fins before genes’). In this regard, analysis of the T-box gene complements in a lamprey and a shark would prove most instructive, as they represent an agnathan and a basal gnathostome, respectively.

Another potentially promising line of research will be to use reporter constructs in transgenic mice to characterize the *cis*-regulatory elements responsible for limb-specific T-box gene expression. Once these elements have been identified, similarities and differences between the *Tbx4* and *Tbx5* loci can be elucidated. Additionally, comparative analyses of the regulatory regions of the T-box genomic loci in a variety of different species might allow the reconstruction of the evolutionary modifications responsible for the origin, and subsequent duplication, of paired appendages in vertebrates.

Understanding the genetic components involved in the initiation of limb bud outgrowth (Fig. 3) contributed to development of the models of vertebrate limb evolution presented here (Figs 5 and 6). It will therefore be important to test further the functions of *Tbx4* and *Tbx5* in limb development. This can be achieved by generating knock-out mice bearing null-mutations in these genes. Additionally, generating knock-in mice in which the endogenous *Tbx5* locus has been replaced with *Tbx4*, and vice versa, can be used to test for biochemical equivalence between the two gene products in vivo. Whatever the outcomes of these experiments the results are eagerly anticipated as they will greatly enhance our understanding of the genetic networks involved in the specification of limb identity.

CONCLUDING REMARKS

Two alternative scenarios can be proposed that integrate genetic, developmental and paleontological data to account for the evolution of paired appendages in vertebrates. Both models underscore the importance of coevolution between the ‘*Hox* code’ (i.e. the axial level at which the limbs are positioned) and the T-box genes, which act as ‘limb-selector’ genes. In both scenarios the limb outgrowth program was first assembled at an anterior (pectoral) axial level and subsequently reiterated at a more posterior (pelvic) level. This two-step process would account for the serial homology evident between the two sets of limbs. The morphological differences between the forelimbs and hindlimbs, a feature shared by all jawed vertebrates, can be explained by coevolution between the T-box genes and asymmetrically distributed modifying factors, including *Pitx1*.

The ideas presented here have broader implications for the evolution of developmental programs in general. First, the evolution of serially homologous structures can be understood in terms of the redeployment of preassembled genetic modules in novel locations. Second, the evolution of distinct morphologies in such reiterated structures can be explained as a consequence of the evolution of genetic interactions between the reiterated modules and the endogenous molecular milieu of the new location. Thus it appears that the cooperative action of several ‘selector genes’ may be a common mechanism for the establishment of organ identity during development. These principles underscore the importance of considering the coevolution of multiple components of interacting genetic networks for understanding the evolution of developmental complexity.

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