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# Involvement of T-box genes *Tbx2-Tbx5* in vertebrate limb specification and development

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## SUMMARY

**We have recently shown in mice that four members of the T-box family of transcription factors (*Tbx2-Tbx5*) are expressed in developing limb buds, and that expression of two of these genes, *Tbx4* and *Tbx5*, is primarily restricted to the developing hindlimbs and forelimbs, respectively. In this report, we investigate the role of these genes in limb specification and development, using the chick as a model system. We induced the formation of ectopic limbs in the flank of chick embryos to examine the relationship between the identity of the limb-specific T-box genes being expressed and the identity of limb structures that subsequently develop. We found that, whereas bud regions expressing *Tbx4* developed characteristic leg structures, regions expressing *Tbx5* developed characteristic wing features. In addition, heterotopic grafts of limb mesenchyme (wing bud into leg bud, and vice versa), which are known to retain the identity of the donor tissue after transplantation, retained autonomous expression of the appropriate, limb-specific T-box gene, with no evidence of regulation by the host bud. Thus there is a direct relationship between the identity of the structures that develop in normal, ectopic and recombinant limbs, and the identity of the T-box gene(s) being expressed.**

**To investigate the regulation of T-box gene expression during limb development, we employed several other embryological manipulations. By surgically removing the apical ectodermal ridge (AER) from either wing or leg buds, we found that, in contrast to all other genes implicated in the patterning of developing appendages, maintenance of T-box gene expression is not dependent on the continued provision of signals from the AER or the zone of polarizing activity (ZPA). By generating an ectopic ZPA, by grafting a sonic hedgehog (SHH)-expressing cell pellet under the anterior AER, we found that *Tbx2* expression can lie downstream of SHH. Finally, by grafting a SHH-expressing cell pellet to the anterior margin of a bud from which the AER had been removed, we found that *Tbx2* may be a direct, short-range target of SHH. Our findings suggest that these genes are intimately involved in limb development and the specification of limb identity, and a new model for the evolution of vertebrate appendages is proposed.**

Key words: T-box gene, *Tbx2*, *Tbx3*, *Tbx4*, *Tbx5*, Limb development, Limb identity, Chick

## INTRODUCTION

One of the defining features of jawed vertebrates (gnathostomes) is the development of two sets of paired appendages at specific levels along the primary body axis: the pectoral and pelvic fins of fishes, and their derived homologs, the forelimbs and hindlimbs of tetrapods (Carroll, 1988). Classical embryological studies demonstrate that limb identity and early anteroposterior limb polarity are first established in flank (lateral plate) mesoderm (Hamburger, 1938; Zwilling, 1955). Signals emanating from the mesoderm induce the formation of an apical ectodermal ridge (AER) in surface ectoderm at specific axial levels for limb formation. The AER in turn provides signals that maintain high proliferation rates in the adjacent mesoderm, forming a progress zone that drives limb outgrowth along the proximodistal axis (Saunders, 1948; Summerbell et al., 1973). Signals emanating from the AER induce the formation of an organizer, the zone of polarizing activity (ZPA), at the posterior margin of the bud (Saunders and Gasseling, 1968; Rowe and Fallon, 1981). The ZPA then takes over the role of providing positional information along the anteroposterior axis, and also provides signals required to maintain the AER (Saunders and Gasseling, 1968; Tickle et al., 1975). Dorsoventral axis specification is sequentially controlled by signals from the somites, somatopleure and non-ridge ectoderm (MacCabe et al., 1974; Pautou, 1977; Michaud et al., 1997).

A great deal is now known about the molecular genetic

pathways involved in the outgrowth and patterning of developing appendages (reviewed by Tickle, 1995, 1996; Niswander, 1997; Shubin et al., 1997). However, far less is known about the genes responsible for initiating these pathways, how positional information is provided, so that appendages do not develop in inappropriate places, or how morphological differences between the forelimb and hindlimb are specified. *Hox* genes are clearly key regulators involved in determining the axial levels at which limbs and fins develop (Charité et al., 1994; Rancourt et al., 1995; Cohn et al., 1997) and Fibroblast Growth Factor-8 (FGF8) is known to be an important effector mediating the early inductive interactions between surface ectoderm and lateral plate mesoderm required to initiate limb outgrowth (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). However, *Hox* genes are transcription factors expressed in lateral plate mesoderm, and FGF8 is a secreted protein expressed in surface ectoderm only *after* the limb territories have been specified. It is therefore apparent that a signal transduction mechanism must exist to interpret the relevant *Hox* code and signal the adjacent ectoderm to express FGF8. FGF10 is a good candidate for a mesoderm-secreted factor that could signal the ectoderm to express FGF8 (Ohuchi et al., 1997), however transcription factors responsible for activating *Fgf-10* expression have not been identified.

We have recently identified a family of putative transcription factors, the T-box genes, shown to regulate a variety of inductive interactions during embryogenesis (reviewed by Papaioannou and Silver, 1998). Four of these genes, *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5*, are represented in the mouse genome as two cognate, linked gene pairs. These have evolved from a single ancestral locus by a two-step process in which an initial tandem-duplication event was followed by duplication of the derived gene pair (Agulnik et al., 1996; Ruvinsky and Silver, 1997), possibly during one of the complete genome duplications thought to have occurred near the root of the vertebrate lineage (Holland et al., 1994). We have found that all of these genes are expressed in developing mouse limbs, either at the time of limb field specification, during bud outgrowth, or both (Chapman et al., 1996; Gibson-Brown et al., 1996). Interestingly, *Tbx2* and *Tbx3* are expressed in similar spatiotemporal patterns in both limbs, whereas *Tbx4* and *Tbx5* expression is primarily restricted to the developing hindlimb and forelimb buds, respectively. Since *Tbx4* and *Tbx5* are expressed in lateral plate mesoderm at the time of limb field specification, well before bud formation, we have proposed that the limb-specific expression of these genes may play a role in the specification of limb identity (fore versus hind) and limb axis determination (Gibson-Brown et al., 1996). In this investigation, we examine the role of all four of these T-box genes in chick limb development, as the chick model system makes it possible to perform many different embryological manipulations.

It has recently been reported that provision of an exogenous source of FGF to medial somatopleure can induce the formation of additional, ectopic limb buds in the flank of chick embryos (Cohn et al., 1995, 1997; Ohuchi et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996), and that the identity of the limb that develops (wing, leg, or mosaic limb) is dependent on the specific axial level at which the FGF source is provided: FGF-soaked beads placed adjacent to somites 21-22 generally induce ectopic wings, beads next to somite 25 induce ectopic legs, and those placed in between induce either wings, legs or mosaic limbs, at approximately equal frequency (Cohn et al., 1995, 1997). We have cloned the chick orthologs of *Tbx4* and *Tbx5* to take advantage of this novel experimental system, and to investigate whether the identity of the ectopic limb that develops is related to the identity of the limb-specific T-box gene, or genes, being expressed. Since it has been shown previously, using heterotopic grafts of limb mesenchyme, that limb identity is a stable property of the mesenchyme (Cairns and Saunders, 1954; Saunders et al., 1957, 1959), we also examined *Tbx4* and *Tbx5* expression after grafting blocks of isolated leg mesenchyme beneath the AER of wing buds and blocks of wing mesenchyme beneath the AER of leg buds. In addition, we also obtained the chick orthologs of *Tbx2* and *Tbx3*, to investigate the regulation of these genes during limb development.

Three further experimental manipulations were employed in these studies. The AER was surgically removed from either wing or leg buds to investigate whether maintenance of T-box gene expression is dependent on the continued provision of AER/ZPA-derived signals. Sonic hedgehog (SHH)-expressing cells were grafted under the anterior AER, thereby generating an ectopic ZPA (Riddle et al., 1993; Chang et al., 1994), to investigate whether *Tbx2* expression in the posterior part of the wing autopod is dependent on, and therefore downstream of, SHH expression. Finally, SHH-expressing cells were grafted to the anterior margin of wing buds from which the AER had been removed, to investigate whether *Tbx2* expression in the autopod is directly dependent on SHH, or indirectly regulated through a dialogue between the ZPA and the AER.

## MATERIALS AND METHODS

### Isolation of chick *Tbx2-Tbx5*

A stage 20-24 chick limb cDNA library (provided by Dr Juan Carlos Izpisúa-Belmonte), a stage 17-18 chick leg library and a stage 18-24 chick limb library (both provided by Dr Susan Mackem; Ranson et al., 1995), were screened with $^{32}$P-labeled probes from the T-box portion of the mouse *Tbx4* and *Tbx5* genes (Agulnik et al., 1996). Low-stringency filter hybridization was performed at 50°C (Church and Gilbert, 1984). Filters were washed either at 65°C or 55°C in 2× SSC, 0.1% SDS, and positive clones recovered through secondary and tertiary screens. Following excision of the pBluescript phagemids from the λZAP vectors, positively hybridizing clones were sequenced by standard methods. These screens led to the identification of multiple cDNA clones containing portions of the chick *Tbx3*, *Tbx4* and *Tbx5* genes. A 197 bp PCR product from the T-box portion of chick *Tbx2* was generously provided by Dr Juan Carlos Izpisúa-Belmonte. Sequence comparisons were performed by FASTA and PILEUP routines in the Wisconsin GCG sequence analysis package (Genetics Computer Group, 1989).

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described by Wilkinson (1992), except that both hybridization and post-hybridization washes were carried out at 62°C (*Tbx2*) or 70°C (*Tbx3-Tbx5*) in the following solution: 50% formamide, 1.3× SSC, 5mM EDTA, 50 μg/ml yeast RNA, 0.002% Tween-20, 0.005% CHAPS, 100 μg/ml heparin, 2% Blocking Reagent (Boehringer Mannheim). Sense and antisense RNA probes including all or part of the T-box coding region were transcribed from linearized plasmids using T3 or T7 RNA polymerase

(Promega Biotech) in the presence of digoxigenin-UTP (Boehringer Mannheim) according to the protocols suggested by the manufacturers. The *Tbx2* plasmid consisted of a 197 bp portion of the T-box inserted at the *Sma*I site of pCR-Script; *Tbx3* and *Tbx5* plasmids consisted of 1.4 kb and 1.3 kb of the respective cDNAs for the genes inserted at the *Bst*XI site of pBluescript II KS (–); the *Tbx4* plasmid consisted of a 1.0 kb portion of the gene inserted at the *Eco*RI site of pBluescript II KS (–).

### Chick embryos and embryo culture

Fertilized White Leghorn chicken eggs (SPAFAS, CT) were incubated at 38°C in a humidified atmosphere, with rocking. Embryos were operated on between stages 13 and 21 (Hamburger and Hamilton, 1951, 1992), the eggs resealed with duct tape and returned to the incubator.

### FGF-2 bead implantation

Induction of ectopic limbs was achieved by modifying the technique described by Cohn et al. (1995). Embryos were operated on between stages 13 and 16. The vitelline membrane was torn open with the tip of an 18-gauge hypodermic needle to expose the embryo. A small transverse slit was made through the medial somatopleure with a sterile microscalpel at the level of somites 19-20. A single heparin-acrylic bead (H-5263, Sigma), 200 μm in diameter, which had been soaked in 1 mg/ml FGF-2 (133-FB-025, R&D Systems) in PBS for at least 1 hour at room temperature, was inserted through the slit into the coelom and pushed caudally to the desired axial level using the closed tips of a pair of fine forceps. Operated embryos were reincubated for a further 2 days before fixing for whole-mount in situ hybridization, or for a further 7 to 9 days before dissection for whole-mount skeletal preparation at stages 35-37.

### Heterotopic limb mesenchyme grafts

Donor and host embryos were operated on at stages 19-20. Wing and leg buds were dissected from donor embryos, rinsed in PBS, and placed in 2% trypsin in PBS for 10 minutes at room temperature. After washing once in PBS, the buds were placed in complete chick embryo fibroblast culture medium (Yang and Niswander, 1995) on ice, and the surface ectoderm teased away using fine forceps and discarded. Donor mesenchyme from the distal tip was cut into small blocks (100-200 μm in diameter, 1 to 3 blocks from each bud) and a single block heterotopically grafted under the AER of a recipient limb bud (wing mesenchyme from a donor being grafted to the leg bud of a host recipient, and vice versa) as described previously (Saunders et al., 1957, 1959). Recipient embryos were reincubated for a further 2 days before being dissected and fixed for whole-mount in situ hybridization.

### AER-removal surgeries

The AER of stage 20-21 wing or leg buds was teased away from the underlying mesenchyme using fine tungsten needles and then removed with fine forceps. Operated embryos were reincubated for 24 to 48 hours before dissection and fixation for whole-mount in situ hybridization.

### SHH-expressing cell implants

Pellets of chick embryo fibroblasts expressing the SHH protein were prepared as described previously (Yang and Niswander, 1995). The pellets were cut into small pieces (100-200 μm diameter) and either grafted under the anterior AER of stage 19-21 wing buds, or pinned with fine platinum wire to the anterior margin of wing buds from which the anterior AER had been surgically removed. Operated embryos were reincubated for a further 24 to 42 hours before dissection and fixation for whole-mount in situ hybridization, or for 7 or 8 days before dissection for whole-mount skeletal preparation.

### Whole-mount skeletal preparation

To visualize skeletal patterns and determine the identity of ectopic limbs, embryos were dissected at stages 35-37, stained with Alcian blue and cleared as described by Tickle (1993). These preparations were photographed and then ectodermal structures were visualized by placing the embryos in absolute alcohol overnight.

## RESULTS

### Cloning and phylogenetic comparison of chick *Tbx2-Tbx5*

Comparison of the T-box regions of the chicken genes, *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5*, with their corresponding mouse orthologs reveals a considerable degree of sequence conservation between the chicken and mouse genes. Orthologous genes are defined as direct decendants of a single ancestral gene that was present in the genome of the common ancestor of the two species under analysis. At the amino acid level the chicken and mouse *Tbx2*-*Tbx5* orthologs exhibit 98.5%, 95.6%, 99.4% and 99.4% identity, respectively, within the T-box domain (Fig. 1A). PILEUP comparison of these sequences against all other T-box genes for which putative orthologs have been identified in both mouse and chick clearly demonstrates the close degree of relatedness between the newly cloned chick genes and their corresponding mouse orthologs (Fig. 1B).

### Normal expression of chick T-box genes in developing limbs

We examined the expression patterns of all four of the newly cloned chick T-box genes during embryogenesis and limb development to compare these with the previously reported expression patterns of their mouse orthologs (Chapman et al., 1996; Gibson-Brown et al., 1996). Here we report expression in the limbs throughout limb specification and development. Expression in other parts of the embryo is reported elsewhere (Gibson-Brown et al., 1998).

#### *Tbx2*

*Tbx2* expression was detectable in flank mesoderm at stage 14 adjacent to somites 6-20 (Fig. 2A), a region encompassing, and extending beyond, the prospective wing field. Expression was also observed in lateral plate mesoderm adjacent to the caudal end of the segmental plate, corresponding to the region fated to form the anterior part of the leg bud. By stage 15 the entire flank between somites 4-24 was positive, with strongest expression in the wing and leg fields. At stages 17 and 18 expression was decreased in the medial part of the wing and leg buds, respectively (Fig. 2B). This pattern continued through later stages, such that only the anterior and posterior margins of the limbs expressed *Tbx2* (Fig. 2B-D). Beginning at stage 20-21, a marked asymmetry in expression became apparent in the anterior and posterior expression domains at the wing margins. In the posterior domain, expression extended from the point of limb articulation with the body wall to the distal tip of the bud whereas, in the anterior domain, expression did not extend so far distally (Fig. 2C,D). This asymmetry of expression was less apparent in the leg (Fig. 2D). Between stages 20 and 25, the AER of the wing, but not the leg, also expressed *Tbx2* (Fig. 2C). None of the other T-box genes examined were expressed in the AER of either limb at any stage.

**A**

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          1                                                  50                                                 100
          **  *    * ** *** * * ***  **    *   * **** *  *  ***  ****    *  * *** * **  * * ***** ***  **    * * ******
ch-Tbx2   .......... .......... .......... .......... .......... .......... .......... .......... ..GEQWMAKP VAFHKLKLTN
 m-Tbx2   LWDQFHKLGT EMVITKSGRR MFPPFKVRVS GLDKKAKYIL LMDIVAADDC RYKFHNSRWM VAGKADPEMP KRMYIHPDSP ATGEQWMAKP VAFHKLKLTN
ch-Tbx3   LWEQFHKRGT EMVITKSGRR MFPPFKVRCT GLDKKAKYIL LMDIVAADDC RYKFHNSRWM VAGKADPEMP KRMYIHPDSP ATGEQWMSKV VTFHKLKLTN
 m-Tbx3   LWDQFHKRGT EMVITKSGRR MFPPFKVRCS GLDKKAKYIL LMTIVAADDC RYKFHNSRWM VAGKADPEMP KRRYIHPDSP ATGEQWMSKV VTFHKLKLTN
ch-Tbx4   LWKKFHEAGT EMIITKAGRR MFPSYKVKVT GMNPKTKYIL LIDIVPADDH RYKFCDNKWM VAGKAEPAMP GRLYVHPDSP ATGAHWMRQL VSFQKLKLTN
 m-Tbx4   .......... .MIITKAGRR MFPSYKVKVT GMNPKTKYIL LIDIVPADDH RYKFCDNKWM VAGKAEPAMP GRLYVHPDSP ATGAHWMRQL VSFQKLKLTN
ch-Tbx5   .......... .........R MFPSYKVKVT GLNPKTKYIL LMDIVPADDH RYKFADNKWS VTGKAEPAMP GRLYVHPDSP ATGAHWMRQL VSFQKLKLTN
 m-Tbx5   LWLKFRSGVT EMIITKAGGR MFPSYKVKVT GLNPKTKYIL LMDIVPADDH RYKFADNKWS VTGKAEPAMP GRLYVHPDSP ATGAHWMRQL VSFQKLKLTN

          101                                                150                                                184
          *  *  *  * **********  *** *  *          *  *  **** **  ** *** ** ***** **** *****
ch-Tbx2   NISDKHGFTI LNSMHKYQPR FHIVRA--ND ILKLPYSTFR TYVFPETNF. .......... .......... .......... ....
 m-Tbx2   NISDKHGFTI LNSMHKYQPR FHIVRA--ND ILKLPYSTFR TYVFPETDFI AVTAYQNDKI TQLKIDNNPF AKGFRDTGNG RREK
ch-Tbx3   NISDKYGFTI LNSMHKYQPR FHIVRA--ND ILKLPYSTFR TYVFPETEFI AVTAYQNDKI TQLKIDNNPF AKGFRDIGNG RREK
 m-Tbx3   NISDKHGFTI LNSMHKYQPR FHIVRA--ND ILKLPYSTFR TYLFPETEFI AVTAYQNDKI TQLKIDNNPF AKGFRDIGHC RREK
ch-Tbx4   NHLDPFGHII LNSMHKYQPR LHIVKADENN AFGSKNTAFC THVFPETSFI SVTSYQNHKI TQLKIENNPF AKGFRGSDDS DLRV
 m-Tbx4   NHLDPFGHII LNSMHKYQPR LHIVKADENN AFGSKNTAFC THVFPETSFI SVTSYQNHKI TQLKIENNPF AKGFRGSDDS DLRV
ch-Tbx5   NHLDPFGHII LNSMHKYQPR LHIVKADENN GFGSKNTAFC THVFPETAFI AVTSYQNHKI TQLKIENNPF AKGFRGSDDV ELHR
 m-Tbx5   NHLDPFGHII LNSMHKYQPR LHIVKADENN GFGSKNTAFC THVFPETAFI AVTSYQNHKI TQLKIENNPF AKGFRGSDDL ELHR
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**B**

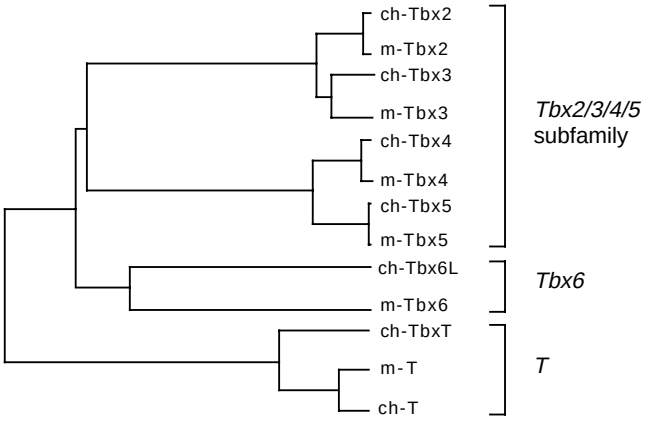



**Fig. 1.** Comparison of T-box domains from various mouse (m) and chicken (ch) T-box polypeptides. (A) Tbx2-Tbx5 sequences from both species aligned to maximize amino acid identity. Stars indicate conserved positions among all T-box domains where sequence is available. Amino acid differences between orthologs are indicated by black boxes. (B) PILEUP comparison of mouse and chicken T-box domains reveals the degree of divergence between the T-box 2/3/4/5 subfamily and other T-box subfamilies. The PILEUP program was used to produce a tree based on the percentage identity among amino acid sequences.

### *Tbx3*

Strong expression of *Tbx3* was detected in lateral plate mesoderm adjacent to somites 10-23 and the caudal end of the segmental plate at stage 14. At stage 15 expression in the lateral plate of the wing field had shifted caudally, now lying adjacent to somites 11-24 (Fig. 2E), and expression in the tail region lay adjacent to the caudal half of the segmental plate. By stage 19 limb expression was confined to mesenchyme in the posterior part of all limb buds (Fig. 2F), but by stage 21 was also detected in the anterior mesenchyme of all limb buds (Fig. 2G). Expression in the anterior and posterior limb margins persisted through stage 27, with the posterior domain extending further distally than the anterior domain (Fig. 2H). At stage 25 expression was also detected in the interdigital regions between wing digits **3** and **4**, and between leg digits **2**/**3** and **3**/**4** (Fig. 2H).

### *Tbx4*

Limb-associated *Tbx4* expression was confined to the hindlimb at all stages (Fig. 2I-L). It was first observed at stage 14 in medial somatopleure adjacent to the caudal end of the segmental plate (Fig. 2I). By stage 15 the anterior boundary of this expression lay adjacent to the border between somites 24/25. At stage 16 a posterior boundary appeared adjacent to the segmental plate at around the level from which somite 32 is destined to bud off; leg buds form between somites 26-32. By stage 19 expression was detected throughout the leg bud (Fig. 2J), persisting through stage 26 (Fig. 2K,L), with strong expression in the leg interdigits at stage 26.

### *Tbx5*

In contrast to *Tbx4*, *Tbx5* was expressed only in the forelimb (Fig. 2M-P). It was detected in lateral plate mesoderm adjacent to somites 10-23 in the prospective wing field at stage 14. By stage 15, this expression had shifted caudally to lie adjacent to somites 11-24 (Fig. 2M). *Tbx5* was expressed throughout the wing bud from stage 17, after the bud has become morphologically defined, to stage 23, when expression becomes regionalized (Fig. 2N,O). Beginning at stage 23 expression was strongest at the anterior and posterior wing margins (Fig. 2O,P), and also transiently extended into the flank between the limb buds (Fig. 2O).

## The relationship of *Tbx4* and *Tbx5* expression to ectopic limb identity

Ectopic limb buds were induced in the flank of 69 chick embryos by implanting FGF2 beads at different axial levels between somites 21 and 26. Half of these were probed for *Tbx4* expression by whole-mount in situ hybridization, and half were probed for *Tbx5*. In rostally positioned buds, developing close to the normal wing, *Tbx4* was expressed only at the posterior margin of the bud, whereas *Tbx5* was expressed throughout all

or most of the bud (Table 1; Fig. 3A,B). Buds developing in the middle of the flank expressed *Tbx4* mainly in the posterior half of the bud, and *Tbx5* in the anterior half (Table 1; Fig. 3E,F). In caudally positioned buds, developing close to the normal leg, *Tbx4* was expressed throughout all or most of the bud, whereas *Tbx5* was only expressed in the anterior part of the bud (Table 1; Fig. 3I,J). In buds developing at all axial levels, there was a region of overlapping gene expression, such that the posterior boundary of *Tbx5* expression extended further caudally than the anterior boundary of *Tbx4* expression. This was confirmed by two-probe hybridization to an additional set of embryos in which ectopic buds had been induced at various axial levels in the flank ($n$=7). In all of these embryos, expression was detected throughout the ectopic buds, with no evidence of a gap between the expression domains of the two genes (data not shown). Thus, depending on the rostrocaudal position at which the bud developed, the region of overlapping expression shifted along the primary body axis: in rostral buds, the region of overlap lay adjacent to somite 22, in mid-flank buds, adjacent to somites 23-24, and in caudal buds, adjacent to somite 25.

To determine how the patterns of gene expression in ectopically induced limb buds are related to the identity of the limb that subsequently develops (wing, leg or mosaic limb), ectopic limbs were examined at stage 35-37. By this stage, it is possible to distinguish between the fingers and toes of distally complete limbs; the number of phalanges in a digit is characteristic of the identity of that digit within a hand or a foot, and wing digits develop feather buds along their margins, whereas toes develop scales. Out of 51 embryos which developed an ectopic limb, all but one developed an overtly mosaic limb (Table 2). The direction of limb mosaicism (wing-like versus leg-like) was directly related

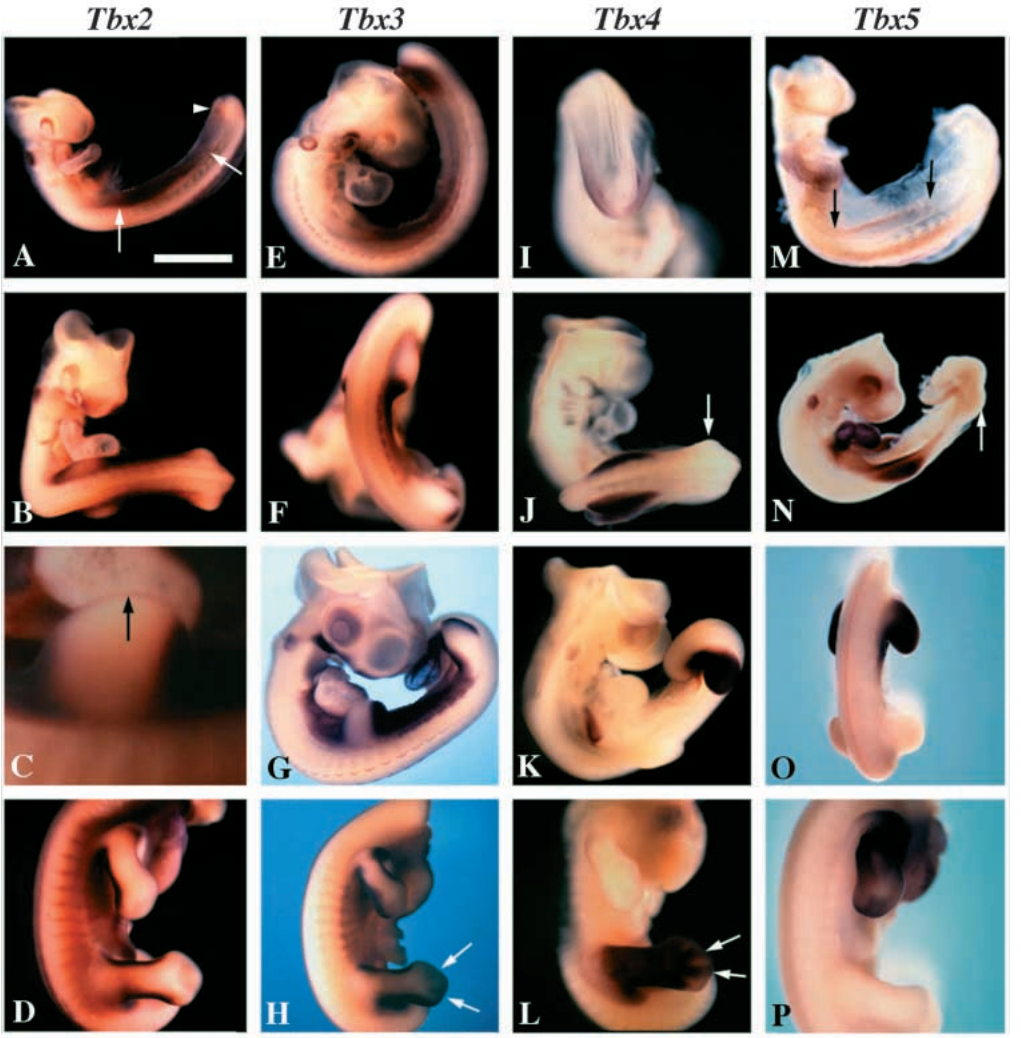
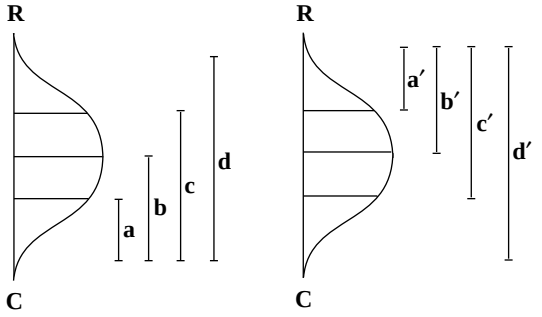



**Fig. 2.** Comparison of *Tbx2-Tbx5* expression in the limbs of developing chick embryos by whole-mount in situ hybridization. (A-D) *Tbx2*. (E-H) *Tbx3*. (I-L) *Tbx4*. (M-P) *Tbx5*. (A,E,I,M) Stages 14-15. (B,F,J,N) Stages 18-19. (C,G,K,O) Stages 20-23. (D,H,L,P) Stages 25-27. (A) *Tbx2* is expressed in the prospective wing field (between arrows) and adjacent to the caudal part of the segmental plate (arrowhead) at stage 14. (B) Expression decreases in the medial part of both limb buds at stage 18. (C) *Tbx2* expression in limbs is confined to the anterior and posterior limb margins, and the AER of the wing (arrow), at stage 21. Anterior is to the left, posterior to the right. (D) At stage 26 *Tbx2* is expressed symmetrically at the anterior and posterior leg margins (bottom), but expression in the wing extends further distally in the posterior margin. (E) *Tbx3* is expressed in the prospective wing field and adjacent to the caudal part of the segmental plate at stage 15. (F) *Tbx3* expression is confined to the posterior margin of both limb buds at stage 19. (G) At stage 21 expression is also present in the anterior margin of both limbs. (H) *Tbx3* is asymmetrically expressed in the anterior and posterior margins of both limbs at stage 26. Note also the interdigital expression observed in the leg at this stage (arrows). (I) *Tbx4* is expressed in lateral plate mesoderm adjacent to the caudal part of the segmental plate at stage 14. Dorsal view of the region indicated by an arrowhead in A. (J) *Tbx4* is expressed throughout the leg bud at stage 19. Note complete absence of expression in the wing bud (arrow). (K) Expression of *Tbx4* in the leg bud at stage 20. (L) At stage 27 expression is seen in the leg interdigits (arrows). (M) *Tbx5* is expressed in medial somatopleure in the prospective wing field (between arrows) at stage 14. (N) *Tbx5* is expressed throughout the wing bud at stage 19. Note complete absence of expression in the leg bud (arrow). (O) In the limbs, expression is confined to the wing and rostral flank at stage 21. (P) *Tbx5* expression is regionalized in the wing at stage 26, with strongest expression at the proximal limb margins and in the distal autopod. Scale bar, 400 μm in (I,C), 1 mm in (A,E,M), 2 mm in (B,F,J,N), 2.5 mm in (G,K,O), 3 mm in (D,H,L,P).

**Table 1. *Tbx4* and *Tbx5* expression in ectopically induced limb buds in relation to the axial level at which an FGF2 bead was implanted**

| FGF2 bead axial level (Somite No.) | No. of embryos | Position of ectopic bud | | | *Tbx4* expression | | | | *Tbx5* expression | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Near wing | Mid flank | Near leg | Region a | Region b | Region c | Region d | Region a′ | Region b′ | Region c′ | Region d′ |
| 21 | 19 | 17 | 2 | 0 | 9 | 1 | 0 | 0 | 0 | 1 | 1 | 7 |
| 22 | 12 | 6 | 5 | 1 | 3 | 2 | 1 | 0 | 0 | 1 | 4 | 1 |
| 23 | 6 | 4 | 2 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 2 | 0 |
| 24 | 10 | 2 | 3 | 5 | 0 | 1 | 4 | 1 | 0 | 2 | 2 | 0 |
| 25 | 7 | 1 | 3 | 3 | 0 | 0 | 3 | 1 | 1 | 1 | 1 | 0 |
| 26 | 15 | 1 | 5 | 9 | 0 | 1 | 2 | 4 | 4 | 3 | 1 | 0 |
| Totals | 69 | 31 | 20 | 18 | 12 | 7 | 11 | 6 | 5 | 9 | 11 | 8 |

The diagrams below indicate the bud regions that were scored for expression of *Tbx4* and *Tbx5*, respectively. R, rostral; C, caudal.




to the axial level of bead implantation, as reported previously (Cohn et al., 1995, 1997). Thus while anteriorly placed beads induced mosaic limbs, most of whose elements consisted of wing-like structures (Table 2; Fig. 3C,D), the more posterior the bead, the more leg-like the limb (Table 2; Fig. 3G,H,K,L). The single exception was an ectopic limb that was morphologically indistinguishable from a normal leg (4 toes, each containing the appropriate number of phalanges, and no feather buds). This leg developed in an embryo that had received a bead adjacent to somite 26, at the caudal end of the region shown previously to be capable of forming ectopic limbs (Cohn et al., 1995).

### *Tbx4* and *Tbx5* expression in heterotopic limb mesenchyme grafts

2 days after blocks of leg mesenchyme were grafted beneath the AERs of recipient wing buds ($n$=15), *Tbx4* expression was clearly observed in a characteristic wedge-shaped inclusion in all of the host wings (Fig. 4A,B). Likewise, 2 days after blocks of wing mesenchyme were grafted beneath the AERs of recipient leg buds ($n$=12), *Tbx5* expression was clearly observed in a similar inclusion in all of the host legs (Fig. 4C,D). The stability of this limb-specific T-box gene expression in the grafted tissue is in accordance with the stability of graft identity demonstrated previously (grafted wing mesenchyme autonomously differentiates into fingers, grafted leg mesenchyme autonomously differentiates into toes; Cairns and Saunders, 1954; Saunders et al., 1957, 1959).

### T-box gene expression in the absence of AER/ZPA-derived signals

1 or 2 days after wing or leg AER removal, *Tbx2* expression was not reduced in either of the wing ($n$=6) or leg ($n$=7) margins (Fig. 5A,B). Likewise, *Tbx3* expression was not reduced in these regions following wing ($n$=6) or leg ($n$=7) AER removal (Fig. 5C,D). Similarly, expression of *Tbx4* was maintained all the way to the distal tip of the truncated leg bud ($n$=9, Fig. 5E), and *Tbx5* expression was maintained to the distal tip of the truncated wing bud ($n$=9, Fig. 5F) at least 48 hours after AER removal. Thus maintenance of T-box gene expression in the developing limbs is not dependent on the continued provision of AER/ZPA-derived signals.

### Roles of SHH and the AER in regulating *Tbx2* expression in the posterior autopod

*Tbx2* expression is not present in the anterior autopod but extends to the distal tip of the posterior wing margin and may therefore be regulated by signals emanating from the ZPA. We generated an ectopic ZPA, by grafting a SHH-expressing cell pellet under the wing anterior AER, to examine whether *Tbx2* expression is regulated, directly or indirectly, by SHH. One ($n$=12) or 2 ($n$=11) days after grafting a SHH-expressing cell pellet, expression of *Tbx2* could be detected in the mesenchyme of the anterior autopod close to the implant immediately underlying the AER (data not shown). In control embryos, which had also received a SHH-expressing implant under the anterior AER but were allowed to develop to stages 35-36, digit duplications ranged from partial (**3-2-2-3-4**) to full (**4-3-2-3-4**) in all cases ($n$=5; data not shown), indicating successful generation of an ectopic ZPA.

**Table 2. Identity of ectopic limbs induced in the flank by FGF2 beads**

| FGF2 bead axial level (Somite No.) | No. of embryos | Ectopic limb identity* | | | | |
|---|---|---|---|---|---|---|
| | | Wing-like | ← | | → | Leg-like |
| | | a | b | c | d | e |
| 21 | 11 | 0 | 10† | 1 | 0 | 0 |
| 22 | 7 | 0 | 1 | 5 | 1 | 0 |
| 23 | 15 | 0 | 0 | 13 | 2 | 0 |
| 24 | 10 | 0 | 0 | 4 | 6 | 0 |
| 25 | 2 | 0 | 0 | 1 | 1 | 0 |
| 26 | 6 | 0 | 0 | 1 | 4 | 1 |

*Scoring criteria for each category were as follows: a, three digits, all possessing feather buds; b, three digits, feather buds on the first two only; c, two or three digits (fused or unfused), with feather buds on rostral digits but none on caudal digits; d, three or four digits, feather buds on the first digit only; e, four digits, none possessing feather buds.

†In one case, no ectopic limb was induced, but the endogenous wing became mosaic (digits identified as **2-3-toe**, based on the presence of an extra phalangeal bone and absence of feather buds in the third digit).

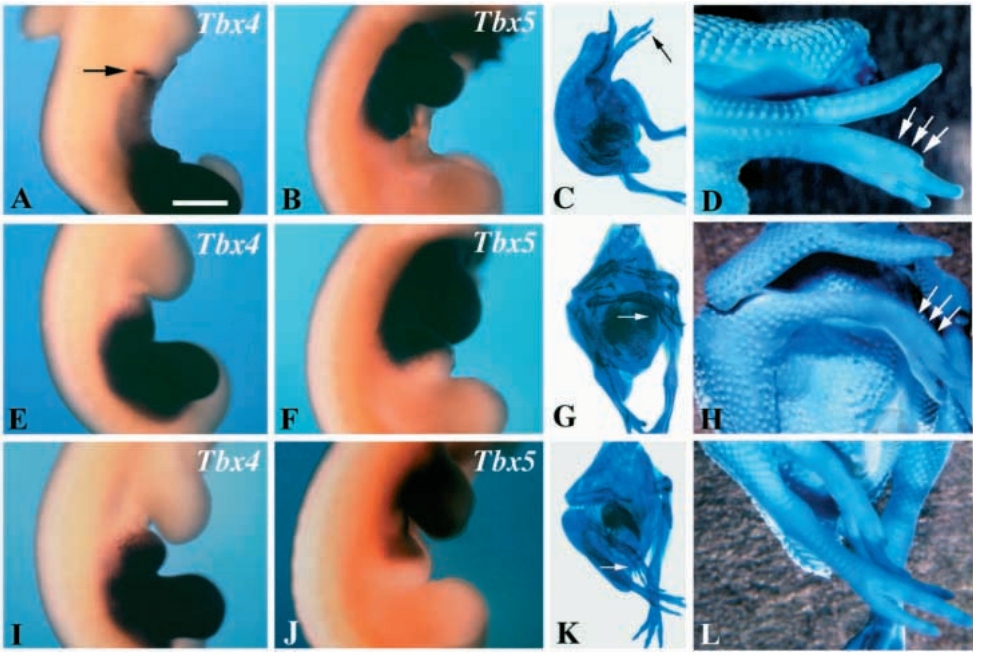



**Fig. 3.** Relationship between *Tbx4* and *Tbx5* expression in ectopically induced limb buds and the identity of the limb structures that subsequently develop in rostrally developing ectopic buds (A-D), mid-flank buds (E-H), and caudally developing buds (I-L). (A) *Tbx4* is only expressed at the posterior margin of a rostrally developing ectopic bud (arrow). (B) In contrast, *Tbx5* is expressed almost throughout. (C) Wing-like mosaic limb is induced following implantation of an FGF2 bead in the rostral flank. Although the anterior two digits are wing-like, the posterior digit is toe-like, based on the number of phalangeal bones (arrow). (D) Feather buds (arrows) are present along the anterior margin of the first digit in the ectopic limb shown in (C). The second and third digits do not possess feather buds and are therefore toe-like. All three digits possess feather buds in a normal wing at this stage. (E) *Tbx4* is expressed in the posterior half of an ectopic bud developing in the middle of the flank. (F) In contrast, *Tbx5* is expressed in the anterior half of a mid-flank bud. (G) Highly mosaic limb (arrow) induced following implantation of an FGF2 bead in the middle of the flank. Note that only three digits are present (a wing-like configuration). (H) Ectopic limb shown in G has feather buds along the anterior margin of the first digit only (arrows). The posterior digits are distinctly toe-like. (I) *Tbx4* is expressed almost throughout an ectopic bud developing in the caudal flank, close to the endogenous leg (bottom). (J) In contrast, *Tbx5* is only expressed in the anterior half of a similarly positioned ectopic bud. (K) Leg-like limb (arrow), possessing four digits, that formed in an embryo which had received an FGF2 bead in the caudal flank. (L) No evidence of feather buds was seen on any of the digits in the specimen shown in (K). Scale bar, 1 mm in (A-B,E-F,I-J), 2.5 mm in (D), 5 mm in (H,L), 1 cm in (C,G,K).

Since SHH-induced expression of *Tbx2* was detected only in mesenchymal cells close to the implant, SHH-expressing implants were grafted to the anterior margin of wing buds from which the anterior AER had been removed to determine whether gene expression is directly induced by SHH, or indirectly induced via interactions between the ZPA and AER. No *Tbx2* expression could be detected distal to the implant either 1 or 2 days after grafting a SHH-expressing cell pellet onto the anterior side of a wing bud from which the AER had been removed, ($n$=14). However, in all cases where the cell pellet could clearly be seen in the limb margin after incubation, cells in direct contact with the pellet did express *Tbx2* (Fig. 6A). Wing buds that received control pellets of chick embryo fibroblasts that did not express the SHH protein ($n$=16) did not exhibit any signal (Fig. 6B). The results of these two experiments suggest that expression of *Tbx2* in the posterior autopod lies downstream of SHH, and that *Tbx2* may be a direct, short-range target of SHH.

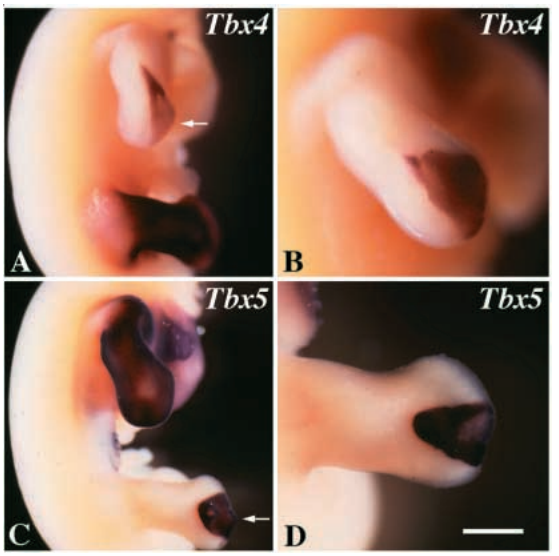



**Fig. 4.** Maintenance of *Tbx4* (A,B) and *Tbx5* (C,D) expression in heterotopic grafts of limb mesenchyme 2 days after the operation. (A) *Tbx4* expression in leg-derived mesenchyme cells which have been incorporated into the endogenous wing (arrow). (B) Higher magnification view of another wing bud which received a leg mesenchyme graft. (C) *Tbx5* expression in wing-derived mesenchyme cells which have been incorporated into the endogenous leg (arrow). (D) Higher magnification view of another leg bud which received a wing mesenchyme graft. Scale bar, 1 mm in (A,C), 500 μm in (B,D).

## DISCUSSION

### Differences in T-box gene expression between chick and mouse limbs

Expression of the chick *Tbx2-Tbx5* genes is generally very similar, both spatially and temporally, to the patterns previously reported for their mouse orthologs (Gibson-Brown et al., 1996, this report). However, mouse *Tbx3* is expressed in the AER of both the forelimb and hindlimb buds, whereas, in

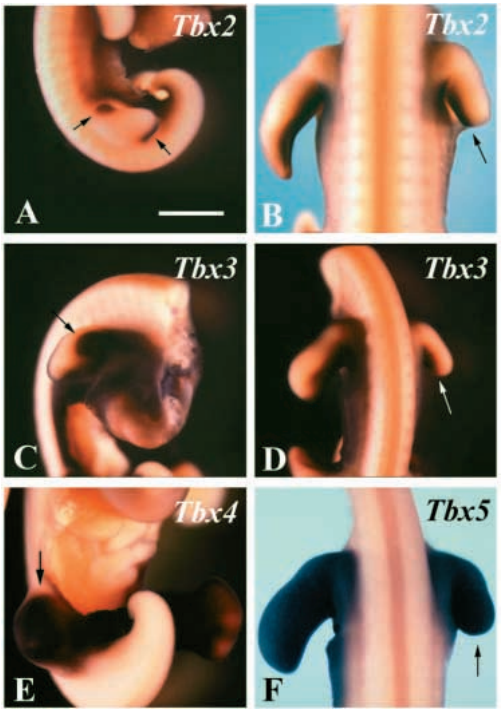



**Fig. 5.** Maintenance of *Tbx2* (A,B), *Tbx3* (C,D), *Tbx4* (E), and *Tbx5* (F) expression in truncated limb buds 2 days after removal of the AER. Arrows indicate expression in the truncated buds. Contralateral, unoperated-control limbs are visible in (B,D-F). Scale bar, 2 mm in (A,C-D), 1.5 mm in (B), 1 mm in (E-F).

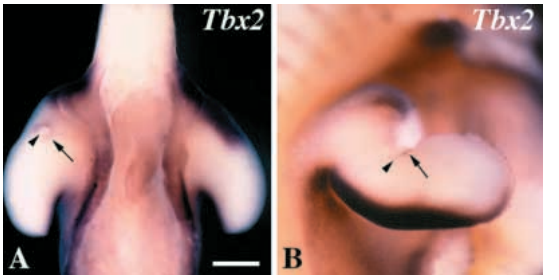



**Fig. 6.** *Tbx2* expression in the anterior autopod 2 days after grafting a SHH-expressing cell pellet to the anterior margin of a wing bud from which the anterior AER had been removed. (A) *Tbx2* is only expressed by cells (arrowed) in direct contact with, or very close to, the pellet (arrowhead). Contralateral control limb shows the normal pattern of *Tbx2* expression in a wing bud with an intact AER. (B) No *Tbx2* expression is detected around a control pellet of chick embryo fibroblasts which do not express the SHH protein (arrowhead). The position of the pellet can clearly be identified by the position and orientation of the wire pin used to secure the graft in place (arrowed). Scale bar, 1 mm in A, 800 μm in B.

the chick, only *Tbx2* is expressed in the AER and, in this case, expression is transient and confined to the forelimb (wing) only. The functional significance of this observation is not yet clear, but it might prove instructive to examine expression of these genes in the fin folds of fish (the ectodermal structures homologous to the AER of tetrapods) to determine which of these patterns is ancestral and which is derived.

### T-box gene expression and the specification of limb identity

*Tbx4* and *Tbx5* are first expressed in lateral plate mesoderm within clearly defined territories at the time the prospective limb fields are being specified by *Hox* genes (Gibson-Brown et al., 1996, this study; Cohn et al., 1997). *Hox* genes may therefore be responsible for regulating expression of these T-box genes within the limb fields. *Fgf-10* expression is also initiated in lateral plate mesoderm around this time, and FGF10 is a good candidate for the mesodermal factor that initiates limb outgrowth and signals the adjacent ectoderm to express FGF8 (Ohuchi et al., 1997; Xu et al., 1998). This makes *Tbx4* and *Tbx5* prime candidates to encode transcription factors, directly or indirectly regulated by *Hox* genes, required for the initiation of bud outgrowth and makes *Fgf-10* a possible downstream target in the mesoderm (Fig. 7).

In addition to being implicated in determining the specific axial levels at which limbs develop, the results of our experiments with ectopic limbs and limb mesenchyme grafts indicate that *Tbx4* and *Tbx5* are also involved in the patterning of skeletal elements and determination of the surface structures that form during later stages of development. The identity of limb elements developing in ectopic limbs was directly related to the position and rostrocaudal range of the limb-specific T-box genes being expressed. Rostral buds expressed *Tbx4* minimally and *Tbx5* extensively, and developed into wing-like mosaic limbs. Mid-flank buds expressed *Tbx4* posteriorly and *Tbx5* anteriorly, and developed into highly mosaic limbs. Caudal buds expressed *Tbx4* extensively and *Tbx5* minimally, and developed into leg-like mosaic limbs (Tables 1,2; Fig. 8). Similar findings have recently been reported by Ohuchi et al. (1998). Thus bud regions expressing *Tbx4* develop toes and scales, whereas regions expressing *Tbx5* develop wing digits and feather buds. Patterning of skeletal elements is controlled by *Hox* genes differentially expressed during different phases of limb outgrowth (Nelson et al., 1996), probably by the differential regulation of local cell proliferation rates and cell adhesion properties (Dollé et al., 1993; Yokouchi et al., 1995; Goff and Tabin, 1997). *Tbx4* and *Tbx5* could therefore be interacting with *Hox* genes during these later phases to regulate the precise position and pattern of the bones that develop. Determination of ectodermal structures (feather buds versus scales) is also controlled by the underlying mesenchyme (Cairns and Saunders, 1954; Saunders et al., 1957, 1959; Saunders and Gasseling, 1968). As in the limb fields, *Tbx4* and *Tbx5* may therefore regulate the expression of secreted molecules needed to instruct the adjacent ectoderm to develop the appropriate structures.

These observations raise the question of functional equivalence between the *Tbx4* and *Tbx5* gene products. Wings and legs are serially homologous structures that use the same basic set of signalling molecules to control their outgrowth and patterning (Tickle, 1996; Shubin et al., 1997). It seems likely, therefore, that initiation of wing and leg outgrowth is also effected by common signalling molecule(s). The mutually exclusive, limb-specific expression of *Tbx4* and *Tbx5* provides an exception to this general paradigm, which could be explained if the two gene products are biochemically equivalent, regulating expression of the same downstream

targets (Fig. 7). In contrast to limb bud initiation, however, the nature of the skeletal and ectodermal structures that form at later stages of development appears to be directly related to the specific identity of the T-box gene being expressed, implying non-equivalence of the *Tbx4* and *Tbx5* gene products. When surface ectoderm from either the wing or the leg is heterotopically transplanted to a host bud from which the ectoderm has been removed, the ectoderm assumes the identity of the host mesenchyme (Saunders and Gasseling, 1968). This shows that the ectoderm is competent to respond appropriately to signals emanating from the mesenchyme, and that these signals must be different in each limb. If T-box genes are involved in sending signals to the ectoderm that determine the identity of ectodermal structures, their downstream targets in the mesenchyme should be different in each limb, suggesting that *Tbx4* and *Tbx5* have acquired unique downstream targets and lost biochemical equivalency. Alternatively, since genes of the *HoxC* cluster are also known to be differentially expressed in the two limbs (reviewed by Nelson et al., 1996), the different context within which the two T-box genes are operating could provide a basis for the differential regulation of downstream targets. Clearly these issues will require both gain- and loss-of-function experiments before being resolved.

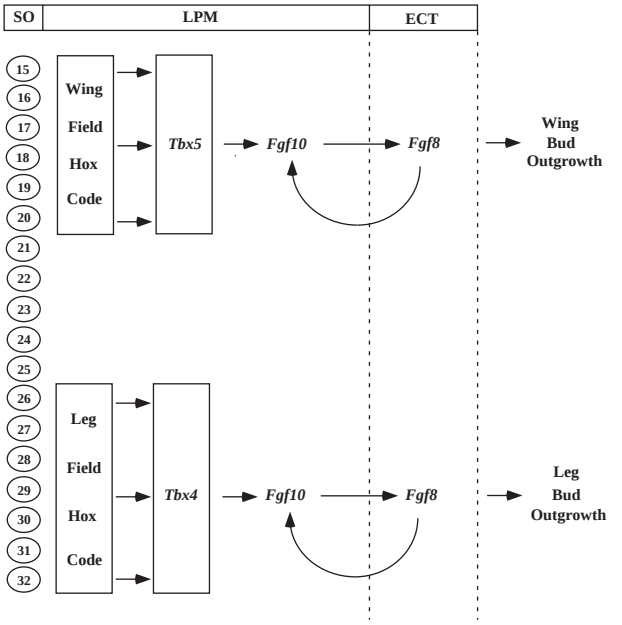



**Fig. 7.** A model for the roles of *Tbx4* and *Tbx5* in the initiation of normal limb bud outgrowth. Hox genes expressed within the lateral plate mesoderm (LPM) specify the positions at which wings and legs will develop. This positional (axial) information leads to limb-specific T-box gene expression within the prospective limb fields. Subsequently, Tbx4 and Tbx5 activate the same FGF10/FGF8 positive feedback loop shown previously to initiate the outgrowth of both limbs (Ohuchi et al., 1997; Xu et al., 1998). In the experimental situation, where ectopic limbs are induced in the flank by implanting FGF-soaked beads, we concur with the opinion of Cohn et al. (1997) that ectopic FGF probably acts indirectly to reprogram the interlimb flank, by shifting the position of Hox gene expression boundaries, such that rostral flank assumes a wing field identity and expresses *Tbx5*, and caudal flank assumes a leg field identity and expresses *Tbx4*. For an alternative model, which proposes FGF10 as the direct, proximal inducer of T-box gene expression in the limb fields, see Ohuchi et al. (1998). Abbreviations: SO, somites; LPM, lateral plate mesoderm; ECT, ectoderm.

## Regulation of T-box gene expression during limb development

*Tbx2* and *Tbx3* are expressed in the posterior part of both limbs prior to the onset of *Shh* expression at stage 17 (Riddle et al., 1993). In the forelimb, this corresponds to the region expressing *Hoxb-8* (Charité et al., 1994; Chan et al., 1995), which has been shown to be important for development of the ZPA (Charité et al., 1994). *Tbx2* and/or *Tbx3* may therefore be involved in establishment of the ZPA and the induction of *Shh*. After formation of the ZPA, however, both of these genes continue to be expressed in the posterior limb margin and, at least in the case of *Tbx2*, we have shown that this expression can lie downstream of SHH. This observation is consistent with the recent observation that *omb*, the *Drosophila* ortholog of *Tbx2*, may be a direct target of *hedgehog*, the *Drosophila* ortholog of vertebrate *Shh*, during patterning of the abdominal segments in the adult fly (Kopp and Duncan, 1997). It therefore appears that, depending on the phase of limb development, expression of these two genes can lie either upstream or downstream of SHH. This observation is similar to previous findings that *Hox* gene expression is differentially regulated during different phases of limb development (Nelson et al., 1996).

In contrast to the effect on all other genes previously implicated in limb outgrowth and patterning, removal of the AER does not lead to loss of expression of *Tbx2-Tbx5*. The

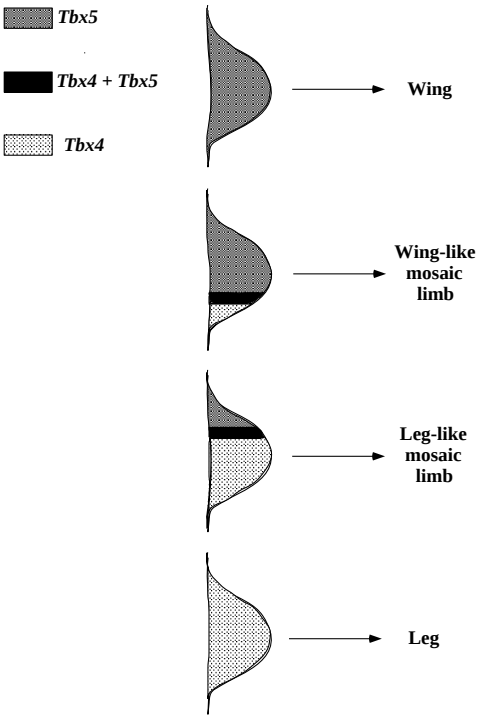



**Fig. 8.** Diagram summarizing the relationship between *Tbx4* and *Tbx5* expression and the identity of the limbs, either endogenous or ectopic, that develop. The area of overlapping *Tbx4* and *Tbx5* expression in ectopic limbs is indicated. Ectopic limb buds express both genes and develop into limbs that possess wing elements rostrally and leg elements caudally.

functional significance of this observation is not yet clear, however it does imply that, once established, some sort of autoregulatory loop might be maintaining T-box gene expression in the absence of external inductive signals. The autoregulatory loop demonstrated for *T* (*Brachyury*) (Schulte-Merker and Smith, 1995) provides a precedent for this type of control in the T-box gene family.

### T-box genes and specification of the anteroposterior limb axis

Since *Tbx2*-*Tbx5* are all expressed in lateral plate mesoderm before the appearence of a morphologically defined bud, beginning at the time of anteroposterior axis specification, we have proposed that these genes could all be involved in establishment of the limb axes (Gibson-Brown et al., 1996). Recently it has been found that mutations in the human orthologs of two of these genes (*TBX3* and *TBX5*) are responsible for two dysmorphic developmental syndromes. *TBX3* mutations are responsible for causing ulnar-mammary syndrome, in which postaxial limb defects, including loss of digit **5** and reduction or loss of the ulna, are found (Bamshad et al., 1997). *TBX5* mutations are responsible for causing Holt-Oram syndrome, in which preaxial limb defects, including loss of the thumb, reduction of the radius and phocomelia, are found (Basson et al., 1997; Li et al., 1997). These heterozygous phenotypes support our hypothesis that both of these genes are involved in establishment of the anteroposterior limb axis. Interestingly, both of these syndromes result from haploinsufficiency of the respective gene products, and because no nullizygous patients have been reported, it is possible that a more severe, possibly embryonic-lethal, phenotype might result from complete loss-of-function.

Holt-Oram syndrome patients exhibit forelimb defects only, which is not surprising as limb-associated *Tbx5* expression is confined to the forelimb (Gibson-Brown et al., 1996; Li et al., 1997). Ulnar-mammary syndrome patients usually exhibit forelimb defects only, although considerable variation in expressivity is found, with several cases reported in which there are also subtle postaxial defects in the hindlimb (Schinzel et al., 1987). *Tbx3* is expressed in both forelimbs and hindlimbs, and the general absence of a hindlimb phenotype in ulnar-mammary syndrome suggests at least partial functional redundancy for TBX3 in the hindlimb. Since *Tbx2* and *Tbx3* are expressed in very similar patterns in both limbs, it is possible that TBX2 can compensate for the loss of TBX3 function in the hindlimb in most ulnar-mammary syndrome patients. So far, no human developmental syndromes have been identified as candidates for *TBX2* or *TBX4* mutations. It will be interesting to see whether mutations in *TBX2* and *TBX4* have similar phenotypic consequences in the hindlimb as mutations in *TBX3* and *TBX5* exhibit in the forelimb.

### Evolutionary implications

The first vertebrates to develop paired appendages, the osteostracan fishes, appeared in Devonian seas around 400 million years ago (Carroll, 1988; Maisey, 1996). However, these jawless fishes only possessed paired pectoral appendages; no evidence of pelvic fins has ever been discovered in a fossil or extant agnathan. *Tbx2* and *Tbx3* are expressed in very similar patterns in both the forelimb and hindlimb (Gibson-Brown et al., 1996, this study), and are both derived from a common ancestral locus (the *Tbx2/3* locus) that was duplicated at a very early point along the vertebrate lineage (Agulnik et al., 1996; Ruvinsky and Silver, 1997). *Tbx2* and *Tbx3* are linked, respectively, to *Tbx4* and *Tbx5*, which were also derived from a common ancestral locus (the *Tbx4/5* locus) in the same duplication event (Agulnik et al., 1996; Ruvinsky and Silver, 1997). This raises the possibility that the ancestral *Tbx2/3*, *Tbx4/5* gene pair was involved in development of the paired pectoral fins of ancient agnathans, and that evolution of paired pelvic fins may only have been possible following duplication of these genes and establishment of the two cognate gene pairs (*Tbx2*/*Tbx3* and *Tbx4*/*Tbx5*). According to this model, *Tbx4/5* gene function was conserved by *Tbx5* for specification and development of the pectoral appendages, whereas *Tbx4* was then available to be recruited (co-opted) into serving an analogous role in specifying novel structures, the paired pelvic fins, at a different level along the primary body axis. Elaboration of the *Tbx2*-*Tbx5* subfamily may therefore have been an important element in the evolution of gnathostome appendages.

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