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Tbx genes and limb identity in chick embryo development

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SUMMARY

***Tbx-2, Tbx-3, Tbx-4* and *Tbx-5* chick genes have been isolated and, like the mouse homologues, are expressed in the limb regions. *Tbx-2* and *Tbx-3* are expressed in anterior and posterior domains in wings and legs, as well as throughout the flank. Of particular interest, however, are *Tbx-5*, which is expressed in wing and flank but not leg, and *Tbx-4*, which is expressed very strongly in leg but not wing. Grafts of leg tissue to wing and wing tissue to leg give rise to toe-like or wing-like digits in wing and leg respectively. Expression of *Tbx-4* is stable when leg tissue is grafted to wing, and *Tbx-5* expression is stable when wing tissue is grafted to leg. Induction of either extra wings or legs from the flank by applying FGF-2 in different positions alters the expression of *Tbx-4* and *Tbx-5* in such a way that suggests that the amount of *Tbx-4* that is expressed in the limb determines the type that will form. The ectopic limb always displays a limb-like *Tbx-3* expression. Thus *Tbx-4* and *Tbx-5* are strong candidates for encoding 'wingness' and 'legness'.**

Key words: Limb development, *Tbx* genes, Chick embryos, FGF-2, Limb mesoderm

INTRODUCTION

As far back as the 1950s, it was shown in chick embryos that the mesoderm of the limb dictates limb type - either wing or leg - rather than the ectoderm. When the mesenchymal core of a chick leg bud was placed inside the ectodermal jacket of a wing bud this resulted in the formation of a leg, and vice versa (Zwilling, 1955). A classical experiment by Saunders et al. (1957, 1959) showed the importance of limb type in the interpretation of positional cues in developing limb buds. When prospective thigh leg mesoderm is grafted distally under the apical ridge of a wing bud it gives rise to toe-like structures in the wing. This shows that transplanted cells respond to their new location and form distal structures, but because they are leg cells, they form 'toes' rather than 'fingers' (Saunders et al., 1957, 1959).

In chick embryos, application of FGF-2 to the prospective flank (interlimb) region leads to the formation of additional limbs; the type of limb that forms depending on the position in the flank at which the bead is placed (Cohn et al., 1995; Ohuchi et al., 1995). When a bead coated with FGF-2 protein is placed in the flank opposite somite 21/22 the most likely outcome is an extra wing, whereas, when the bead is placed in the flank opposite somite 25 the most likely outcome is an extra leg (placement opposite somite 23/24 leads to either an extra wing or an extra leg). The cells that form ectopic limbs come from more or less the entire flank (Cohn et al., 1997).

Differential gene expression in limb mesenchyme could be a mechanism by which 'legness' and 'wingness' properties are established. From their expression patterns, members of the T-box family are good candidates to confer such limb identity properties. To date, 5 members of this family have been isolated in mouse (Bollag et al., 1994; Chapman et al., 1996; Gibson-Brown et al., 1996). In the mouse, *Tbx-2, Tbx-3, Tbx-4*, and *Tbx-5* genes are all expressed in the limbs, but of particular interest is the expression of *Tbx-5* which is found in forelimb and flank but absent from the hindlimb and *Tbx-4* which is expressed strongly only in the hindlimb. *Tbx-2* and *Tbx-3* are also expressed in stripes at the anterior and posterior margins of the limb buds and in flank. It is also of interest that some human syndromes have been shown to be caused by mutations in *Tbx* genes, and this provides a direct link between gene expression patterns in mice and function in humans. Mutations in *Tbx-5* are associated with Holt-Oram syndrome which leads to abnormalities of anterior elements of the upper limb such as the radius and carpal bones, and of the heart (Basson et al., 1997; Li et al., 1997). Mutations in *Tbx-3* are associated with Ulnar-mammary syndrome, the effects of which include abnormalities of posterior limb elements such as the ulna, metacarpals and phalanges (Bamshad et al., 1997). *Tbx-5* and *Tbx-3* are chromosomally linked and due to the phenotypes of the above syndromes, it is thought that they may have evolved complementary roles in limb development.

We have isolated the chick *Tbx* homologues of the mouse

Tbx-2, Tbx-3, Tbx-4, and *Tbx-5* genes. The expression patterns of the chick genes are remarkably similar to the mouse genes indicating a high degree of conservation of these genes during evolution. We have tested the idea that expression of these genes may encode limb type, by examining *Tbx* gene expression in leg grafts placed in wing and vice versa, and in ectopic limbs induced by FGF-2 application either anteriorly or posteriorly in the flank. The results of these manipulations are consistent with the idea that *Tbx* gene expression is related to limb type.

MATERIALS AND METHODS

cDNA cloning

PCR amplification using degenerate primers (YVHPDSP and FPETDF) within the T-box were used to amplify DNA fragments encoding the T box from stage 22 chick limb mRNA. Amplified fragments were cloned into pBluescript SK II and sequenced. Inserts corresponding to the T box from *Tbx-2* and *Tbx-5* were subsequently used to screen a stage 22-24 chick limb bud λzap cDNA library at low stringency (2× SSC, 50°C). Twenty-two clones were plaque purified, rescued and sequenced. Based on sequence homology it was determined that 3 overlapping cDNA clones corresponding to *Tbx-2*, 6 corresponding to *Tbx-3*, 4 corresponding to *Tbx-4*, and 9 corresponding to *Tbx-5* were obtained. Six additional clones for *Tbx-4* were obtained following screening of the same library with a 5′ probe isolated from the first round of screening. Partial coding sequences for chicken *Tbx-2* and *Tbx-3* and full-length coding sequence for chicken *Tbx-4* and *Tbx-5* have been deposited in the GenBank Database under accession numbers: cTbx-2, AF033668; cTbx3, AF033669; cTbx-4, AF033670; cTbx-5, AF033671. This information will be released on publication.

In situ hybridisation probes corresponded to sequences encoding amino acids 1-375 for *Tbx-2*; 1-300 for *Tbx-3*; 1-400 for *Tbx-4* and 1-412 for *Tbx-5*.

Whole-mount in situ hybridisations

Single- and double-color whole-mount in situ hybridisations of chick embryos were performed as previously described (Izpisúa-Belmonte et al., 1993; Wilkinson and Nieto, 1993; Jowett and Lettice, 1994). Embryos were sectioned with a Vibratome as described by Altabef et al. (1997). The probes used are as described above.

Bead implantations into embryos in ovo

Heparin acrylic beads (Sigma H-5263) with a diameter of 125 mm were washed in PBS for 2 hours, and incubated in FGF-2 (1 mg/ml; R and D systems) for at least 1 hour at room temperature. A bead was inserted under the ectoderm in the lateral plate mesoderm of the flank of stage 13-15 chick embryos as shown in Fig. 4A, as described by Cohn et al. (1995). The eggs were then incubated further until they reached the desired stage.

Tissue grafts

Grafts of tissue from leg to wing and vice versa were carried out using an adaptation of the method described by Saunders et al. (1957). Donor limbs of stage 21 chick embryos were incubated in 2% trypsin at 0°C for 1 hour after which the ectoderm was removed. Small mesoderm grafts were dissected and in some cases stained lightly with Nile blue sulphate for ease of observation. The grafts were implanted either directly beneath the apical ridge (Fig. 1A) or inserted into the mesenchyme (Fig. 3H) of host limbs in stage 20 chick embryos. Operated embryos were incubated for a further 24-48 hours for in situ hybridisation or a further 6 days for observation of skeletal elements.

RESULTS

Identification of chicken homologues of Tbx-2, Tbx-3, Tbx-4 and Tbx-5

Degenerate oligonucleotides designed against conserved motifs in the T box were used to amplify DNA products that encoded the T box regions of Tbx-2 and Tbx-5. These fragments were then used to screen a chicken cDNA library made from stage 22-24 limb buds at low stringency. The twenty-two clones which resulted were analyzed and their sequence compared to different DNA databases. Conceptual translation of the cDNA clones indicated that in all cases the T box regions of chicken Tbx-2, Tbx-3, Tbx-4 and Tbx-5 were highly conserved compared with their mammalian counterparts (96%-100% identity; Fig. 1). The N-terminal regions of chicken Tbx-2 and Tbx-3 were also both very similar to the human and murine homologs (96% and 92% identity, respectively), although the amino terminus of cTbx-2 lacked 10 of the 12 alanines in a homopolymeric stretch found in both the human and mouse sequences (amino acids 50-62). The 70 amino acids immediately C-terminal of the T box in cTbx-2 and cTbx-3 were 69% and 77% identical to this region in human Tbx-2 and Tbx-3, respectively.

A full-length Tbx-4 clone has not yet been reported. The conceptual translation of the 10 overlapping cDNA clones obtained for cTbx-4 yielded a 488 amino acid protein; 66 amino acids N-terminal of the T box and 238 amino acids C-terminal of the T-box. The N-terminal regions of cTbx-5 was only 66% identical (78% conserved) to human Tbx-5, while the region immediately C-terminal of the T box, amino acids 246-417 of cTbx-5, was more highly conserved (88% identity).

Tbx-2, Tbx-3, Tbx-4 and *Tbx-5* expression during chick limb bud development

We have characterized the expression domains of *Tbx-2, Tbx-3, Tbx-4* and *Tbx-5* genes during chick limb development. Like their counterparts in the mouse (Gibson-Brown et al., 1996) we have found that the pair of genes *Tbx-2/Tbx-3* have similar spatiotemporal patterns during both forelimb and hindlimb development. In contrast, the pair *Tbx-4/Tbx-5* have very distinct and restricted expression domains in leg and wing respectively. Fig. 2 shows a summary of the transcript distribution for the four genes at various stages of chick limb development.

Tbx-5 and *Tbx-4* are first detected in the presumptive wing and leg mesoderm respectively at around stages 14-15 (Fig. 2A,E). As limb outgrowth proceeds, both genes are uniformly expressed throughout the mesoderm, with *Tbx-5* transcripts exclusively restricted to the wing and transiently also in the flank (Fig. 2A-D,P,T,X), and Tbx-4 transcripts restricted to the leg mesoderm (Fig. 2E-H,O,S,W). In a few cases, and when the proteinase K incubation time was extended (40 minutes), weak expression of *Tbx-4* was seen in the wing (data not shown). Differential expression of *Tbx-4/Tbx-5* throughout limb mesoderm is not detected until stage 24 when proximocentral regions start losing expression that leads, in later stages, to a very characteristic pattern of expression (Fig. 2S,T). *Tbx-4/Tbx-5* transcripts are, from this stage on, absent from medial regions of the limb and are localized in mesenchyme surrounding cartilage digit primordia as well as the most proximal elements (Fig.

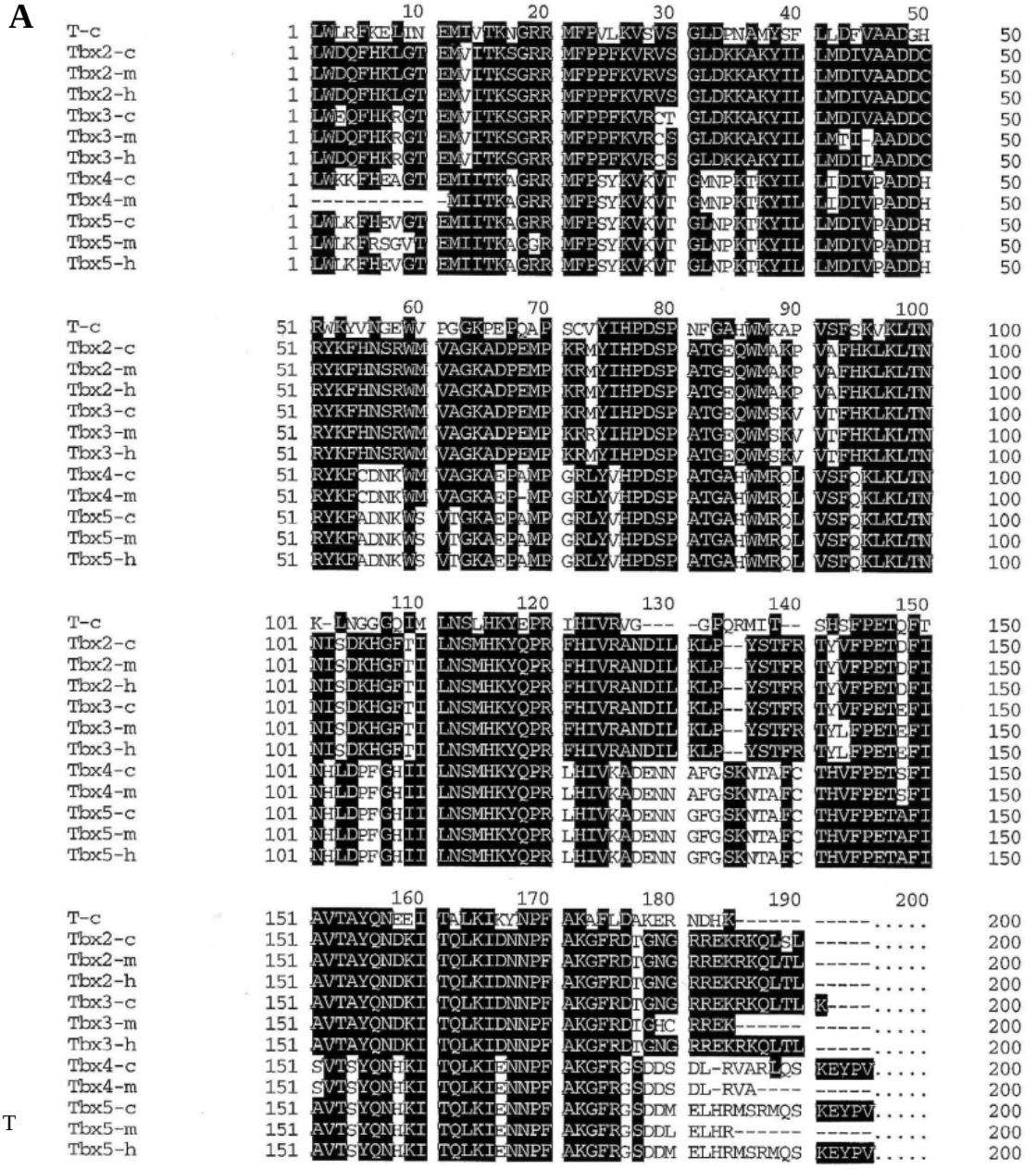
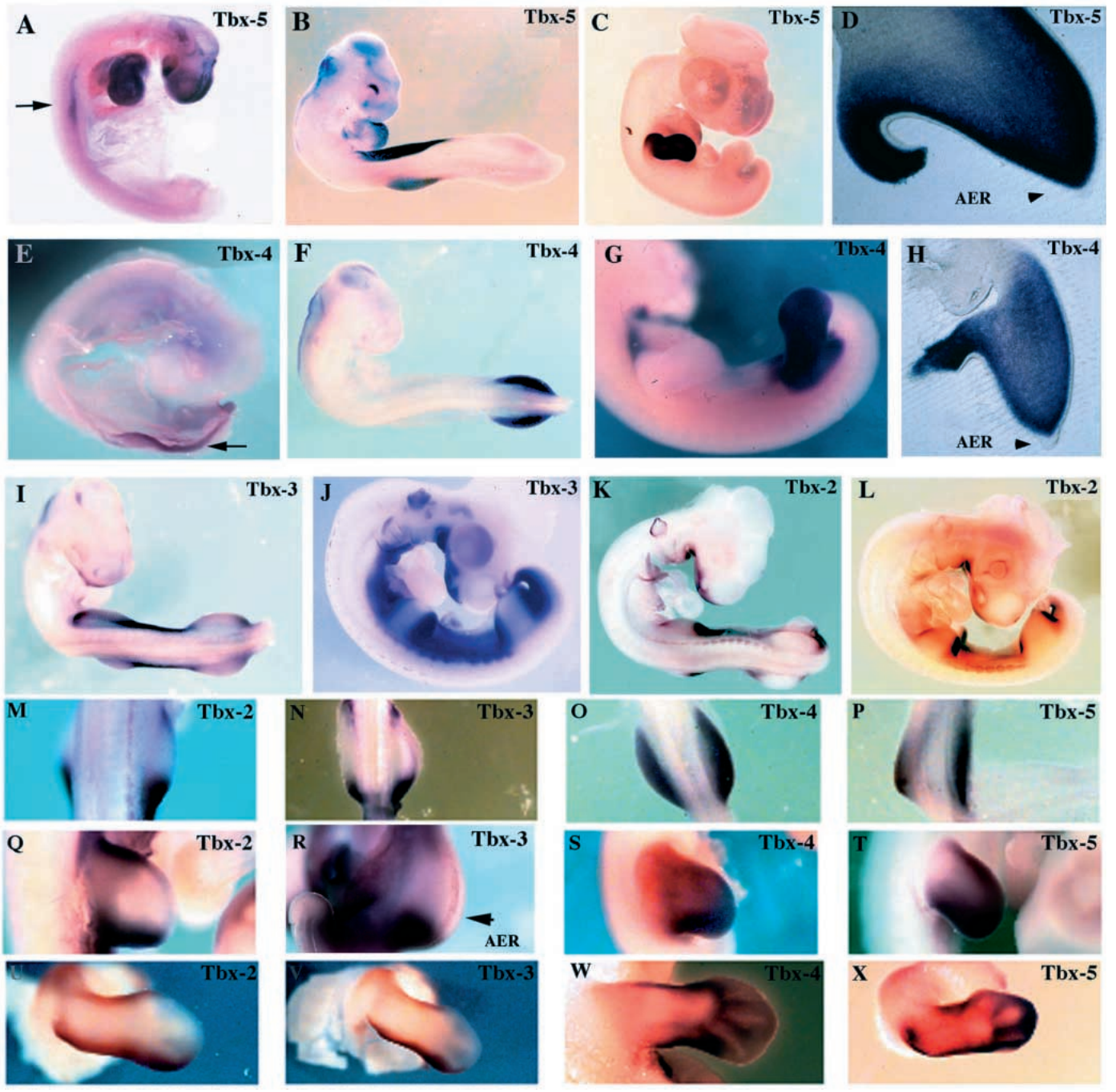


Fig. 1. (A) Alignment of the T box of chicken Tbx-2-5 (c) with the murine (m) and human (h) homologues. The amino acid sequence of the chicken T gene is shown at the top of the figure. Identities are highlighted and gaps in the sequence are indicated by hyphens. (B) Phylogenetic tree of the Tbx-2-5 amino acid sequences. This consensus tree illustrates the most likely evolutionary relationship among the 12 sequences in the region of the T box. The values associated with the likelihood of branchpoints are indicated as percentiles.



2S,T,W,X). Transcripts of *Tbx-5* and *Tbx-4* were never detected in the AER (Fig. 2D,H).

Contrary to the uniform mesenchymal expression of *Tbx-4/Tbx-5*, transcripts for *Tbx-2/Tbx-3* show markedly differential expression at anterior and posterior side of both limbs from the first signs of limb budding (Fig. 2I-L,M,N). This expression is practically symmetrical until stage 23 when transcripts for *Tbx-3* start to disappear from the distal tip of the anterior domain (Fig. 2R,V). This recession does not seem to occur for Tbx-2, whose transcripts are symmetrically localized at anterior and posterior sides of the limbs (Fig. 2Q, U) until the latest stage examined (stage 30). Whilst transcripts for *Tbx-3* are present in the apical ectodermal ridge (Fig. 2R) they were never detected for *Tbx-2* in this tissue.

Tbx-4 and *Tbx-5* expression in tissue grafts between wings and legs

Tbx-5 is initially expressed exclusively in lateral plate mesoderm in the wing-forming region from stage 13, then, in addition, transiently in the flank at later stages, whereas *Tbx-4* is consistently expressed at high levels exclusively in the leg-forming region. If *Tbx-4* and *Tbx-5* encode limb identity, then expression of these genes should be stable when leg tissue is grafted into wing and vice versa. To test this prediction, grafts

Fig. 2. Normal expression of chick *Tbx-2, Tbx-3, Tbx-4* and *Tbx-5* in various limb bud stages by in situ hybridisation. (A-D) Expression of *Tbx-5* in stage 14-25 chick embryos. (A) Expression of *Tbx-5* is found in presumptive wing mesoderm (arrow) and the heart at stage 14. Staining in the head is due to trapping. (B) Expression of *Tbx-5* is found throughout wing bud and flank mesoderm as well as in lateral plate mesoderm anterior to the wing, the dorsal half of the eye and the heart. (C) Expression of *Tbx-5* is still strong in the wing bud mesoderm, but is now absent from the flank and eye at stage 25. (D) A transverse section through the wing bud of a stage 22 chick embryo, shows transcripts of *Tbx-5* are found throughout the mesoderm but are absent from the apical ectodermal ridge (AER) and ectoderm. (E-H) Expression of *Tbx-4* in stage 15-25 chick embryos. (E) Expression of *Tbx-4* is found in presumptive leg mesenchyme (arrow) at stage 15. (F) Expression of *Tbx-4* is found throughout the leg bud mesoderm of a stage 17 chick embryo. (G) Expression of *Tbx-4* remains throughout the leg bud mesoderm of a stage 25 chick embryo. Note that expression is also detected near the heart. (H) A transverse section through the leg bud of a stage 22 chick embryo, shows transcripts of *Tbx-4* are found throughout the mesoderm but are absent from the apical ectodermal ridge (AER) and ectoderm. (I-J) Expression of *Tbx-3* in stage 19-22 chick embryos. (I) Expression of *Tbx-3* is found in the anterior and posterior domains of the wing and leg and also throughout the flank of a stage 19 embryo. (J) Expression of *Tbx-3* remains in the anterior and posterior domains of the wing and leg and also throughout the flank of a stage 22 embryo. Note expression also in facial primordia. (K-L) Expression of *Tbx-2* in stage 19-22 chick embryos. (K) Expression of *Tbx-2* is found in the anterior and posterior domains of the wing and leg and also throughout the flank at stage 19. Note expression also in facial primordia. (The apparent expression in the leg apical ectodermal ridge is due to shadow). (L) Expression of *Tbx-2* remains in the anterior and posterior domains of the wing and leg and also throughout the flank. (M-P) Comparison of expression of *Tbx-2, Tbx-3, Tbx-4,* and *Tbx-5* in stage 19 chick embryos. (M) Higher magnification of a wing bud hybridised with *Tbx-2* showing anterior and posterior restriction, and lack of expression in the apical ectodermal ridge. (N) Higher magnification of a leg bud hybridised with *Tbx-3* also showing restricted expression anteriorly and posteriorly. *Tbx-3* transcripts are located in the apical ectodermal ridge. (O) Higher magnification of a leg bud hybridised with *Tbx-4* showing transcripts throughout. (P) Higher magnification of a wing bud hybridised with *Tbx-5* showing transcripts throughout. (Q-T) Comparison of expression of *Tbx-2, Tbx-3, Tbx-4,* and *Tbx-5* in stage 22-25 chick embryos. (Q) Higher magnification of a wing bud hybridised with *Tbx-2* at stage 22 showing absence of transcripts in the apical ectodermal ridge. Transcripts are still maintained in two domains anteriorly and posteriorly. (R) Higher magnification of a leg bud hybridised with *Tbx-3* at stage 23 showing the presence of transcripts in the apical ectodermal ridge (AER; arrow). Anterior domain does not extend to the limb tip. (S) Higher magnification of a leg bud hybridised with *Tbx-4* at stage 24. Transcript levels are now weaker in central regions. (T) Higher magnification of a wing bud hybridised with *Tbx-5* at stage 25. There is a clear reduction in transcripts in central bud regions. (U-X) Comparisons of expression of *Tbx-2, Tbx-3, Tbx-4* and *Tbx-5* in stage 26-28 chick embryos. (U) Higher magnification of a wing bud hybridised with *Tbx-2* at stage 26. Expression is maintained as two stripes along anterior and posterior margins. (V) Higher magnification of a leg bud hybridised with *Tbx-3* at stage 26. Anterior expression is now clearly reduced but the posterior stripe of expression is still present. (W) Higher magnification of a leg bud hybridised with *Tbx-4* at stage 28. Transcripts are localised around digit primordia and along anterior and posterior margins. (X) Higher magnification of a wing bud hybridised with *Tbx-5* at stage 28 showing expression around digit primordia and along anterior and posterior margins.

of presumptive thigh mesoderm were inserted beneath the apical ridge of a wing bud, or grafts of presumptive upper-wing mesoderm were inserted under the apical ridge of a leg bud (Fig. 3A). In all cases (10/10) the original gene expression is retained after 24-48 hours. This is indicated by a patch in the wing which expresses *Tbx-4* and not *Tbx-5* (Fig. 3D,E; *n*=8) or a patch in the leg which expresses *Tbx-5* and not *Tbx-4* (Fig. 3F,G; *n*=2). As previously reported by Saunders et al. (1957, 1959; see also Summerbell and Tickle, 1977) the leg grafts in the wing gave toe-like digits (Fig. 1B; *n*=4), and wing grafts into legs gave wing-like digits (Fig. 1C; *n*=1). These results indicate that the structures formed when tissue is grafted beneath the apical ridge depend on the donor limb type; once given limb identity, possibly by the *Tbx* genes, limb tissue cannot switch type. However, when the graft (presumptive thigh/upper-wing) is placed more proximally in either of the above scenarios, resulting in an apparently normal limb (Fig. 3I,J), expression in the graft is not always stable (*n*=13/26) 24-48 hours after grafting (Fig. 3K,M). Some of the grafts in the remaining cases can be identified in the host but do not express the gene (13/26).

Tbx expression in FGF2-induced limbs

The induction with FGF-2 of additional limbs of different types (either an additional wing or an additional leg) from the flank (Cohn et al., 1995) provides an opportunity to test whether Tbx expression is related to limb type. We implanted FGF-2-coated beads, at different positions in the flank of stage 13-15 chick embryos, and examined resultant *Tbx* gene expression patterns in ectopic buds 48 hours later. The results are summarised in Table 1. When an FGF-2 bead is placed opposite somite 21/22, the ectopic buds, which normally form wings, are predominantly *Tbx-5* positive, this expression always being in the anterior of the bud (Fig. 4B). Note that the *Tbx-5* expression is lost from the flank. The posterior part of such buds is *Tbx-4* positive (Fig. 4C). Comparison of such embryos suggests that there is little overlap of *Tbx-4* and *Tbx-5* expression patterns. In contrast, when an FGF-2 bead is placed opposite somite 25, the ectopic buds, which normally form legs, have a substantial *Tbx-4* positive domain posteriorly (Fig. 4H) and *Tbx-5* positive domain anteriorly (Fig. 4G). Comparison of the embryos probed for *Tbx-4* and embryos probed for *Tbx-5* suggests that the *Tbx-4* and *Tbx-5* expression domains overlap considerably. Ectopic buds induced by FGF-2 beads placed at the level of somite 23/24 are either approximately 50% *Tbx-5* positive or approximately 50% *Tbx-4* positive, *Tbx-5* being expressed anteriorly and *Tbx-4* being expressed posteriorly, with apparently small to medium amounts of overlap (Fig. 4E,F). Two-colour in situ hybridisations using both probes on the same specimen confirm that domains of *Tbx-5* and *Tbx-4* overlap (data not shown).

The above results show that inducing an ectopic bud at any level in the flank results in an anterior extension of *Tbx-4* expression into the ectopic bud and into the flank region (Fig. 4F). The flank, which normally expresses *Tbx-5* can therefore be either *Tbx-4* or *Tbx-5* positive. Thus, regardless of whether the ectopic limb is likely to be a wing or a leg, the bud contains some *Tbx-4* expression. Despite the overlap of *Tbx-4* and *Tbx-5* expression domains, which are normally mutually exclusive, ectopic limbs that result when the bead is placed opposite

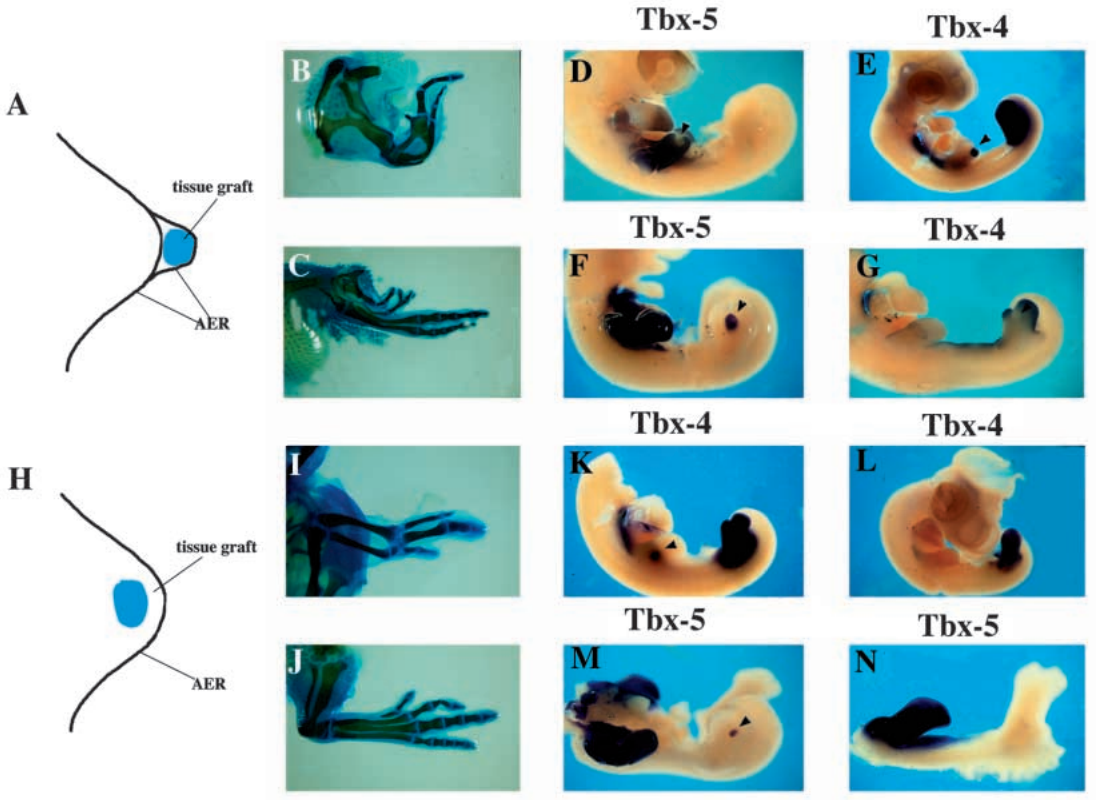


Fig. 3. Leg into wing and wing into leg grafts. (A) Diagram to show the position at which the graft from the donor embryo is positioned in the host embryo in B-G. (B,C) Grafts from wings or legs of stage 21 chick embryos were inserted either under the apical ridge (AER) of wings or legs of stage 20 chick embryos. Embryos were incubated for a further 6 days, then stained with Alcian green to show the skeleton. (B) A graft from presumptive thigh region of a donor leg was inserted beneath the apical ridge of a host wing. The resultant wing has extra toe-like digits distally. See I for comparison. (C) A graft from the shoulder region of a donor wing was inserted beneath the apical ridge of a host leg. The resultant leg has wing-like digits distally. See J for comparison. (D-G) Grafts from wings or legs of stage 21 chick embryos were inserted under the apical ridge of wings or legs of stage 20 chick embryos. Embryos were incubated for a further 24-48 hours, then hybridised with either the *Tbx-5* or *Tbx-4* RNA probe. (D) A graft from presumptive thigh region of a donor leg was inserted beneath the apical ridge of a host wing. The graft is *Tbx-5* negative (arrowhead) 24 hours after grafting. (E) A graft from presumptive thigh region of a donor leg was inserted beneath the apical ridge of a host wing. *Tbx-4* expression is retained in the graft (arrowhead) 24 hours after grafting. (F) A graft from presumptive shoulder region of a donor wing was inserted beneath the apical ridge of a host leg. *Tbx-5* expression is retained in the graft (arrowhead) 48 hours after grafting. (G) A graft from presumptive shoulder region of a donor wing was inserted beneath the apical ridge of a host leg. Graft is *Tbx-4* negative (arrowhead) 24 hours after grafting. (H) Diagram to show the position at which the graft from the donor embryo is positioned in the host embryo in I-N. (I) A graft from presumptive thigh region of a donor leg was inserted into the mesenchyme of a host wing. The resultant wing appears normal at this stage. (J) A graft from presumptive shoulder region of a donor wing was inserted into the mesenchyme of a host leg. The resultant leg appears normal at this stage. (K-N) Grafts from wings or legs of stage 21 chick embryos were inserted under the apical ridge of wings or legs of stage 20 chick embryos. The embryos were incubated for a further 24-48 hours, then hybridised with either the *Tbx-5* or *Tbx-4* RNA probe. (K) A graft from presumptive thigh region of a donor leg was inserted into the mesenchyme of a host wing. *Tbx-4* expression is retained in graft (arrowhead) 24 hours after grafting. (L) A graft from presumptive thigh region of a donor leg was inserted into the mesenchyme of a host wing. *Tbx-4* expression is lost in graft 24 hours after grafting. (M) A graft from presumptive shoulder region of a donor wing was inserted into the mesenchyme of a host leg. *Tbx-5* expression is retained in graft (arrowhead) 48 hours after grafting. (N) A graft from presumptive shoulder region of a donor wing was inserted into the mesenchyme of a host leg. The graft is *Tbx-5* negative 48 hours after grafting.

somite 25, appear to be normal legs (n=3; in this series of experiments).

Tbx-3 is expressed in an identical pattern in both normal wing and leg buds, and throughout the flank (Fig. 2I,J). When a bead is placed at any position in the flank (n=6), the expression of *Tbx-3* appears as an anterior and posterior stripe in the ectopic buds formed (Fig. 4D), with no expression within the central region of the ectopic bud, just as it appears in normal buds. As *Tbx-3* is normally expressed throughout the flank region this result suggests that Tbx 3 is switched off in the centre of the ectopic bud.

DISCUSSION

We have demonstrated here that *Tbx-2* to *Tbx-5* chick genes are extremely similar in sequence to those that have been isolated in mice (Chapman et al., 1996; Gibson-Brown et al., 1996) and humans (Bamshad et al., 1997; Li et al., 1997) and furthermore that the expression patterns of these genes in the chick are identical to those described in mice (Chapman et al., 1996; Gibson-Brown et al., 1996), and the expression of *Tbx-5* is also identical to that seen in human embryos (Li et al., 1996). In this study we have focused on the function that these

Table 1. Expression of *Tbx* genes in ectopic limb buds

Somite numbers opposite which FGF-2 bead placed in lateral plate mesoderm	Limb type	Gene expression in ectopic limb buds			
		Tbx-3 expression (*n*)	*Tbx-5* expression (*n*)	*Tbx-4* expression (*n*)	Amount of overlap of *Tbx-4* and *Tbx-5* expression
21/22	wg 80%(16)* lg 20%(4)	n+++(1)	+++(15)	+(15)	0/+
23/24	wg 50%(10) lg 50%(9)	n+++(1)	++(14)	++(15)	+/++
25	wg 10%(1) lg 90%(8)	n+++(4)	++(12)	+++(6)	+++

+, small portion of ectopic limb expresses the gene; ++, approximately 50% of ectopic limb expresses the gene; +++, large proportion of ectopic limb expresses the gene; n+++, expression is as in the normal limb; *n*, the number of specimens; wg, wing; lg, leg. *The bracketed numbers in column 2 refer to all the experiments of this kind carried out in this laboratory.

genes may have in limb development. The expression of *Tbx-4* and *Tbx-5* is retained in tissue that has been grafted from wing to leg and vice versa. The induction of an extra limb (wing or leg) in the flank region results in a limb bud which expresses both *Tbx-5* and *Tbx-4* in varying degrees, such that an additional wing expresses mainly *Tbx-5*, but an additional leg often expresses *Tbx-4* and *Tbx-5* to equal extents. These additional limbs also express, in a limb-like pattern, *Tbx-3*, suggesting that expression may be important for establishment of correct anterior and posterior boundaries of the limbs. The highly conserved expression patterns of these genes, data from human studies, and our results from chick manipulation experiments are all consistent with the idea that the *Tbx* genes may encode limb identity.

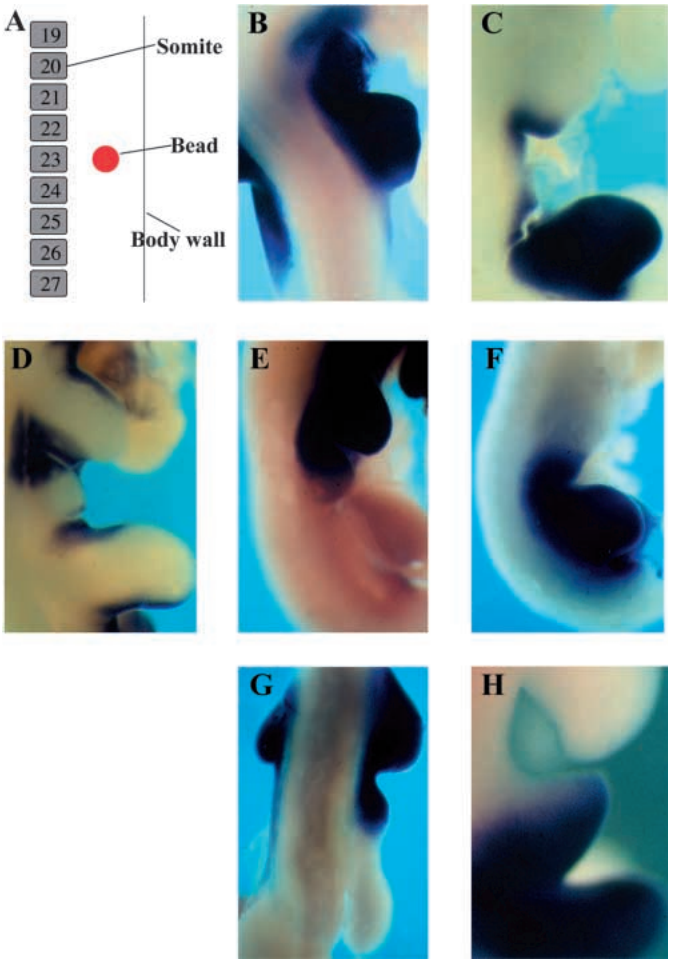


Fig. 4. FGF-2 induction of extra limb buds. (A) Diagram to show implantation of FGF-2-coated beads in the lateral plate mesoderm of presumptive flank, which lies opposite somites 21-25 inclusive. (B) An FGF-2 bead was positioned opposite somite 21 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-5* RNA probe. Expression of *Tbx-5* is found throughout most of the ectopic bud, which is fused to the normal bud. Note that posterior-most part of ectopic bud and remaining flank is *Tbx-5* negative. (C) An FGF-2 bead was positioned opposite somite 21 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-4* RNA probe. Expression of *Tbx-4* is found in the most posterior part of the ectopic bud and is patchy throughout the remaining flank, extending its expression in an anterior direction. (D) An FGF-2 bead was positioned opposite somite 23 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-3* RNA probe. Expression of *Tbx-3* is found as an anterior and posterior stripe in the ectopic limb, and expression is absent in the centre. This is true for all specimens (*n*=4), although the extent of expression at anterior and posterior boundaries of extra limbs varies between specimens. Expression of the posterior stripe (which will end up at the anterior of ectopic limb) is slightly less than that of the anterior stripe in ectopic limbs of this specimen. (E) An FGF-2 bead was positioned opposite somite 23 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-5* RNA probe. Expression of *Tbx-5* is found in the anterior-most half of the ectopic bud. (F) An FGF-2 bead was positioned opposite somite 23 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-4* RNA probe. Expression of *Tbx-4* is found in the posterior-most half of the ectopic bud and extends up the flank. (G) An FGF-2 bead was positioned opposite somite 25 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with a *Tbx-5* RNA probe. Expression of *Tbx-5* is found throughout most of the ectopic bud, extending its normal expression in a posterior direction. Note that the posterior-most part of the ectopic bud and the flank are *Tbx-5* negative. (H) An FGF-2 bead was positioned opposite somite 25 of a stage 14 chick embryo. The embryo was incubated for 48 hours followed by hybridisation with *Tbx-4* RNA probe. Expression of *Tbx-4* is found throughout most of the ectopic bud, extending its normal expression in an anterior expression. Note that the anterior-most part of the ectopic bud is *Tbx-4* negative.

Expression of *Tbx* genes

Comparisons between the amino acid sequences of Tbx 2 to 5 from human, mouse and chick show there is strong conservation, suggesting selective pressure to maintain the function of these genes during evolution. Although very similar to T/ Brachyury in sequence (Fig. 1), the expression patterns of these *Tbx* genes and *Brachyury* do not overlap. *Brachyury* is expressed early, in the streak and node, followed by expression in the notochord, pre-somitic mesoderm and tailbud (Wilkinson et al., 1990; Kispert et al., 1995), whereas these *Tbx* genes are never expressed in these areas (Chapman et al., 1996; Gibson-Brown et al., 1996). Other chick *Tbx* genes have been isolated that are expressed in the streak and node and therefore do overlap in expression with *brachyury* (Knezevic et al., 1997). *Tbx-2* and *Tbx-3* are expressed in both wings and legs as an anterior and posterior stripe, as well as throughout the flank region. *Tbx-5* and *Tbx-4* are expressed throughout the wing and leg regions respectively, from pre budding stages; *Tbx-5* is also expressed transiently throughout the flank at later limb bud stages. All four genes are expressed in mesoderm and *Tbx-3* is also expressed in the apical ectodermal ridge. Overall, these studies reinforce the idea proposed by Agulnik et al (1996) based on phylogenetic and mapping studies, that the *Tbx 4/5* gene pair evolved apart from the *Tbx-2/3* pair. The 4 genes could have arisen by an initial duplication of a unique ancestral gene to form a two-gene cluster: the *Tbx-2/3* and *Tbx-4/5* prototypes. This cluster was subsequently duplicated in tandem and dispersed into two chromosomal locations, giving rise to the actual gene configuration (*Tbx-2/4* in chromosome 11 and *Tbx-3/5* in chromosome 5). The tight chromosomal linkage of *Tbx 3/5* has led Bamshad et al. (1997) to suggest that *Tbx-3* and *Tbx-5* compensate for each other in the upper limb, leading to a reduction of abnormality caused by mutation in one of these genes. It is possible that the same would occur with mutations in *Tbx-2* and *Tbx-4* in the lower limb.

Limb identity depends on *Tbx* gene expression

If *Tbx-5* and *Tbx-4* are responsible for encoding limb identity of the wing and leg respectively, tissue expressing one gene should retain expression when removed from its normal limb environment. Saunders et al. (1957, 1959) showed that when mesodermal tissue from the leg bud is grafted beneath the apical ridge of the wing bud, toe-like digits will form in the wing. We have shown that this tissue retains the expression of *Tbx-4*, and that when wing mesoderm is grafted beneath the apical ridge of the leg bud, this graft retains *Tbx-5* expression. The extent of leg structures that form in the wing relates directly to the amount of mesoderm along the proximodistal axis of the bud that is transferred (Kieny, 1964); a complete leg will form in the wing region if the entire leg bud mesoderm is grafted in place of the wing mesoderm (Zwilling, 1955). It is likely therefore that the type of limb structures that form relates directly to the amount of *Tbx* expression. However, if mesoderm of one limb is grafted into more proximal regions of the other limb, away from the apical ectodermal ridge, we find that the graft does not always retain expression of the *Tbx* gene. In some cases the grafted tissue may have lost *Tbx* expression because, as development proceeds, expression is reduced in proximal regions of the limbs (Fig. 2W,X). It is not clear why other proximal grafts did not retain *Tbx* expression. The skeleton in most of our chick limbs following proximal grafts also appeared normal suggesting that the donor cells are entrained into the host limb pattern. The development of a normal skeleton following such operations has also been noted previously (Saunders et al., 1957, 1959; Krabbenhoft and Fallon, 1989), however, when operated limbs have been left to develop further, changes in skin appendages have been reported (Saunders et al., 1957, 1959). This shows that limb identity is still preserved in proximal grafts and is revealed when these contribute to the dermis.

Another prediction that follows from the idea that *Tbx* genes encode limb identity is that extra limbs that can be formed from the flank region (Cohn et al., 1995; Ohuchi et al., 1995) will have an altered *Tbx* expression pattern. By the time an ectopic bud is visible, the flank region would normally express *Tbx-5* and formation of an ectopic bud which is likely to form a leg shows very little anterior regression of *Tbx-5*. Whether the extra limb bud will be a wing or a leg appears to depend on the amount of *Tbx-4* expression, the gene normally expressed more posteriorly along the body axis. A prospective wing which forms when FGF-2 is applied anteriorly in the flank has only a small amount of *Tbx-4*, whereas a prospective leg which forms from posterior application has a large amount of *Tbx-4*. In no case did we see the complete absence of *Tbx-4* expression in the ectopic bud. Recently, Ohuchi et al. (1998) have also reported changes in *Tbx* expression in the flank when additional limbs are produced. Fate mapping of normal limb buds has shown that digits form from posterior mesenchyme (Vargesson et al., 1997). As the extra wing or leg forms with the opposite polarity to the original wing or leg (Cohn et al., 1995; Ohuchi et al., 1995), it might be expected that the *Tbx* expression found at the anterior of the extra bud, where digits will form, will determine the type of limb that forms. In the case of an extra wing, this region of the bud is *Tbx-5* positive and *Tbx-4* negative, but in the case of an extra leg, *Tbx-5* alone is also expressed in this region. It may be significant that in extra legs induced by FGF-2, digit IV, the most posterior digit of normal legs, is often incomplete (Cohn et al., 1995). An alternative possibility is that the decision to become either a wing or a leg is a democratic one depending on which *Tbx* gene has a majority. However, the transplantation experiments show that even small pieces of tissue appear to maintain identity when grafted to the limb of opposite type.

The results here are consistent with the idea that *Tbx-5* and *Tbx-4* expressions are related to ‘wingness’ and ‘legness’. However, definite proof that *Tbx* genes encode limb identity will require the interchange of *Tbx-4* and *Tbx-5* expression without physically moving tissue, by molecularly replacing one gene with the other, and showing that wings can be transformed into legs and vice versa. It should be noted that these *Tbx* genes are also expressed in non limb forming regions of the lateral plate mesoderm. The flank at later stages in normal embryos expresses *Tbx-5*, and in those embryos treated with FGF-2, the flank can express both *Tbx-5* and *Tbx-4*. Therefore, expression of *Tbx* genes on their own is probably not sufficient for limb development. A newly isolated homeobox gene, *Backfoot*, has been found to be expressed in leg and not forelimb (Shang et al., 1997) and later on in limb bud development, genes of *Hoxa* and *Hoxd* clusters have been shown to be expressed in different patterns in the wing and leg buds (Nelson et al., 1996). It will be interesting to find out how

expression of these genes together with the *Tbx* genes contribute to differences in wing and leg pattern.

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