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Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity

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SUMMARY

The wing and the leg of the chick, although homologous structures, have characteristic patterns of skeletal elements, muscles, tendons, featherbuds and scales. Despite recent advances in understanding the common genetic pathways patterning the wing and leg, the molecular nature of the specification of limb-type identity has remained elusive.

Embryological experiments have indicated the existence of limb-specific territories in the flank. In the newt, deviation of nerves from the limb into the flank can induce ectopic limbs to form from this tissue. In the chick, Fibroblast growth factor (FGF)-soaked beads applied to the flank can induce ectopic formation of limbs from the surrounding tissue. In both cases, the type of limb that forms, either a wing/forelimb or leg/hindlimb, is dependent upon the location to which the limb-inducing signal is applied.

We have isolated and characterised three candidate genes for controlling limb identity in the chick. Two T-box transcription factors, *cTbx4* and *cTbx5*, are expressed in a restricted manner in the leg bud and wing buds, respectively. *cPtx1*, a member of the *Otx*-related subclass of paired-type homeodomain proteins, is expressed exclusively in the leg bud. Using FGF to induce ectopic limb buds of wing, leg and intermediate identity, we show that early expression of *cTbx5*, *cTbx4* and *cPtx1* in the induced limb buds correlates with later wing- or leg-type identity of ectopic limbs. We observe a general correlation between the location of an ectopic outgrowth induced by FGF and the identity of the resulting limb but, significantly, we report that there is no definitive rostral-caudal level that divides the ectopic wing and leg territories.

Key words: Limb development, T-box genes, Chick, Limb identity, *Ptx*, *Tbx*

INTRODUCTION

In recent years, rapid advances have been made in our understanding of the initiation and patterning of the chick limb bud, mostly focusing on genes that are expressed and have common roles in both wing and leg buds. Although homologous structures, however, each type of limb is distinct in its skeletal elements, tendon attachments, muscles, featherbuds and scales. In addition, the wing and leg buds are formed at particular axial levels along the main body axis.

Experiments have indicated the existence of limb-specific territories in the flank. Nerves deflected from the limb into the flank of the newt can induce ectopic limbs to form from this tissue. Whether a forelimb or hindlimb forms is dependent upon the location to which the nerve is moved (Guyenot et al., 1948; Guyenot and Schott, 1926). Similarly, forelimbs and hindlimbs are induced in the anterior or posterior of the flank, respectively, depending on the location of grafted otic vesicles (Balinsky, 1925; Filatow, 1927) and nasal placodes (Balinsky, 1957; Glick, 1931). More recently, similar results have been obtained using FGF-soaked beads applied to chick interlimb flank mesoderm (Cohn et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996).

The molecular nature of positional information responsible for the specification of limb-type identity has remained elusive. We reasoned that, if a gene is involved in the specification of limb-type identity, its expression is likely to be restricted to one or other limb type and that this restricted pattern would be maintained throughout the early stages of limb formation. While a number of genes of the HoxC cluster are differentially expressed in the hindlimb or forelimb (Simon and Tabin, 1993; Oliver et al., 1988; Nelson et el., 1996), none of these genes are expressed throughout the limb mesenchyme and their relatively late onset of expression in the limb bud indicates they do not play an early, formative role in specifying limb identity. Recently, three genes have been reported that fit these criteria for controlling limb identity. Two T-box family transcription factors, *Tbx5* and *Tbx4*, are expressed in the forelimb bud and hindlimb bud, respectively (Gibson-Brown et al., 1996; Simon et al., 1997). T-box genes are characterised by the presence of a highly conserved motif (T-box) that encodes a 180 amino acid DNA-binding domain, the T-domain. The prototype of this

family is the mouse T protein (Kispert and Herrman, 1993; Kispert et al., 1995) and its *Xenopus* homologue, Brachyury (Conlon et al., 1996). Members of this family are expressed throughout development and are important in tissue specification, morphogenesis and organogenesis (Papaioannou and Silver, 1998, Chapman et al., 1996; Chapman and Papaioannou, 1998). In addition, an *Otx*-related, paired-type homeobox transcription factor, *Ptx1* (also named *P-Otx*) has been described in the mouse that is expressed in the developing hindlimb and not the developing forelimb (Shang et al., 1997; Szeto et al., 1996).

The chick embryo is a powerful system for embryological manipulation. To examine the possible functional roles of these genes in limb-type specification, we cloned the chicken homologues of *Ptx1*, *Tbx4* and *Tbx5* and, in the process, also isolated homologues of *Tbx2* and *Tbx3*. As a first step toward evaluating the possible roles of these genes, we exploited the ability of FGF-soaked beads to induce ectopic limbs of wing, leg and intermediate identity and investigated the respective gene expression profiles. Our data are consistent with a role of *cTbx4*, *cTbx5* and *cPtx1* in limb-type specification.

MATERIALS AND METHODS

Degenerate PCR and library screening

Fertilised White Leghorn chicken embryos were obtained from SPAFAS, Norwich, CT. Eggs were incubated at 37.5˚C. RNA was extracted from whole embryos, dissected wings and dissected legs of stage 22-23 (Hamburger and Hamilton, 1951). Chick embryos using the Trizol reagent (Gibco, BRL) following the manufacturers instructions. 36-48 limbs were homogenised with 0.8 ml Trizol and the isolated total RNA resuspended in water. 5 μg of total RNA was used in a first-strand synthesis reaction using Superscript reverse transcriptase (Gibco, BRL) following the manufacturers protocol. The reaction was diluted in 400 μl 10 mM Tris pH 8, phenol extracted and ethanol precipitated. The DNA was resuspended in 10 μl of 10 mM Tris pH 8 and used as template in degenerate PCR reactions (50 μl PCR reaction containing 67 mM Tris pH 8.8, 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 33 μg/ml BSA, 400 μM NTPs, 10 μM primer, 2 μl template, 1 μl Taq polymerase (Boehringer Mannheim). The reaction was run for 40 cycles of 94˚C, 1 minute; 40˚C, 2 minutes; 60˚C, 3 minutes. Degenerate primers were designed from an alignment of T-box sequences: TbxFwd: CACATCGATGTAC/TATICAC/TCCIG-AC/TA/TC/GICC; TbxRev: CACCTCGAGTA/GTC/GA/GTTC/GT-GA/GTAIC/GCIGTIAC (I=deoxyinosine). PCR products of the expected size were gel purified (Qiaquick columns, Qiagen) and cloned into the pGEM T vector (Promega, Madison, WI) following the manufacturers' instructions. The resulting clones were sequenced (Sequenase kit, Amersham) and were compared to sequences in GENBANK using the BLAST network service, National Center for Biotechnology Information (NCBI). The chick sequences were most closely related to the mouse (U15566) and human (S81264/U28049) *Tbx2* clones. We used a *cTbx2* clone isolated from the degenerate PCR screen (cTbx2-13) and two mouse probes, *mTbx1* and *mTbx3* (a gift from Javier Capdevilla, Bollag et al., 1994) that span the T-box of each gene to screen a random primed plasmid library (Ausubel et al., 1987) prepared from chick stage 22 limb RNA (Johnson and Tabin, 1997). Filters were hybridised in 30% formamide; 10% dextran sulphate; 2× SSC, 1% SDS at 42˚C overnight and washed in 2× SSC, 0.1% SDS at 55˚C. Positive clones were sequenced manually (Sequenase, Amersham) and by automated sequencing (carried out by the Biopolymer Facility, HHMI, HMS using an ABI 373 automated sequencer), identifying partial clones for *cTbx2, cTbx3* and *cTbx5*. Clones of *cTbx4* were isolated in a separate screen. From the available mouse (*mTbx4*) and amphibian (*NvTbx4*) sequences primers 5TB-8Cla: GAGATCGA TAT/C AAA/G TTT/C GCI GAT/C AAT/C AAA/G TGG and 3TB-7Xho: CACCTCGAG CC C/TTT IGC A/GAA IGG G/ATT G/ATT T/CTC were designed and employed in degenerate PCR . Using the resulting PCR product as a probe we screened an oligo(dT)-primed chick limb (stages 18-24) Lambda Zap cDNA library (gift from Susan Mackem, NIH) as described above. Four clones were isolated and confirmed by sequencing.

To isolate chick homologues of the *Ptx1/P-OTX* gene, we screened an oligo(dT)-primed library (as described above) with a mouse probe generated by PCR. Based on the sequence of *mPtx1/P-OTX* (Lamonerie et al., 1996; Szeto et al., 1996), we designed primers OTHinf: CCAGCGAATCGTCCGACGCT and OTXho: CAAGGCTCGAGTTGCACGTG. A 720 bp fragment PCR fragment (nucleotides 167-887) was generated using the One-Step RT-PCR kit (Life Technologies-a kind gift of Dr Jun Lee) following the manufacturers instructions and total RNA, extracted from a single E14.5-15 mouse head, as template. PCR conditions were 55˚C, 30 minutes, 94˚C, 2 minutes for the first-strand synthesis, followed immediately by PCR amplification, 94˚C, 15 seconds; 55˚C, 30 seconds; 72˚C, 90 seconds, 40 cycles, 72˚C, 5 minutes. A single band of the correct size was cloned into the pGEM T vector (Promega) following the manufacturers instructions. Sequencing confirmed that the clone contained a fragment of the mouse Ptx1/P-OTX gene.

Whole-mount and section in situ hybridisation analysis

Whole-mount in situ hybridisation was carried out essentially as described (Riddle et al., 1993). Probe templates were: cTbx2-15f (*Sal*I, T7 RNA polymerase), cTbx3-06d (*Sal*I, T7 polymerase), cTbx5-08c (*Sal*I, T7 polymerase), cTbx4-R1-Xba (*Eco*RI, T3 polymerase), cPtx1-OT17 (*Pst*I, T7 polymerase). All the probes span large portions of the respective open reading frames including the T-box/homeobox of each gene.

Section in situ hybridisation was carried out as previously described (Bao and Cepko, 1997).

FGF4-soaked bead implants and embryo incubation

Affi-Gel beads (150-300 μm; Bio-Rad) were washed in phosphate-buffered saline and then soaked in a solution of FGF4 (1 μg/μl, a gift from Vickie Rosen, Genetics Institute, Cambridge, MA) for at least 1 hour before use. All operations were done on stage 14-16 embryos. Using tungsten needle, a narrow cut was made in the surface ectoderm over the lateral plate mesoderm of the flank. With forceps, an FGF4-soaked bead was placed into direct contact with the flank mesenchyme by inserting the bead through the cut and by tucking it under the ectoderm. Embryos were harvested after incubation for either 1.5 or 8 days following implantation of a bead.

Limb transplants, explants and AER removals

The distal tip of the wing and leg buds were removed and transplanted essentially as described (Kieny, 1964). The distal 1/3 of either a wing or leg bud from a stage 22-23 embryo was removed using a tungsten needle. In most cases a reciprocal transplant procedure was carried out on a single embryo such that the distal 1/3 of the wing bud was transplanted onto the proximal stump of the leg bud and vice versa. The grafted tissue was held in place using staples made from small strips of platinum wire (Goodfellow, Cambridge, England). Wing- and leg-cell implants were done by making a deep incision into either the distal, wing or leg bud and removing a piece of tissue (with ectoderm intact), approximately 50 μm^3. The removed tissue was replaced with a similar-sized piece from either the leg or wing.

Prior to removal of the AER, 3-5 μl of dilute (~0.1%) Alcian Blue (Aldrich) solution was applied externally to improve visibility of AER. Small incisions were made between the ridge and underlying mesenchyme using a tungsten needle, being careful not to damage the

mesenchyme cells. By making a series of such incisions the AER was removed.

Skeletal preparations and analysis

Embryos were fixed overnight in 4% paraformaldehyde /phosphate-buffered saline at 4˚C, washed thoroughly in phosphate-buffered saline and eviscerated. The skeletal elements were stained in a fresh solution of Alcian Blue 8GX (C.I. 74240; Sigma) (20 mg in 70% ethanol/ 30% glacial acetic acid) for 6-12 hours. Embryos were then brought through an ethanol series into distilled water and then cleared in 0.5% KOH and a stepwise series of 0.5% KOH/glycerol solutions into 100% glycerol. Cartilage stains deep blue while calcified tissues do not take up the stain.

RESULTS

Cloning of four chick T-box genes and chick *Ptx1*

To identify chick genes differentially expressed in the forelimb or hindlimb buds, we screened cDNA libraries for clones related to the T-box and Ptx gene families. Multiple T-box cDNA isolates were sequenced. An alignment of the deduced T-domain sequences for *cTbx2*, *cTbx3*, *cTbx4* and *cTbx5* with the respective mouse and human sequences (Agulnik et al., 1996; Bamshad et al., 1997; Basson et al., 1997) indicated a remarkably high degree of conservation for each gene (Fig. 1A). The cTbx2 T-domain is 100% conserved between chick, mouse and human at the amino acid (a.a.) level (182 of 182 a.a.) and the T-domain of cTbx3 shares 95% (173 of 182 a.a.) and 97.8% (178 of 182 a.a.) identity to mouse and human, respectively. The T-domain of cTbx5 is 96.7% (178 of 184 a.a.) and 97.8% (180 of 184 a.a.) conserved to the mouse and human sequence, respectively. For cTbx4, only a mouse sequence is available for comparison and this has 99.4% identity to the deduced chick peptide differing only by the presence of an extra alanine residue (Fig. 1A, a.a. residue 68). A phylogenetic tree constructed based on the alignment of T-domain sequences confirmed the assignment of each chick gene as an orthologue of the mouse and human genes (Fig. 1B). The *Tbx2/3/4/5* genes constitute a subfamily within the larger T-box-containing gene family (Bollag et al., 1994). Within the subfamily, *Tbx2* and *Tbx3* are most similar to one another whilst *Tbx4* and *Tbx5* are more closely related (Bollag et al., 1994 and Fig. 1B). The crystal structure of the *Xenopus* Brachyury(T) T-domain (Muller and Herrman, 1997) confirmed previous suggestions that the T-protein binds DNA as a homodimer (Papapetrou et al., 1997) and identified specific amino acid residues within the T-domain as important in DNA-binding and dimerisation. These residues are almost completely conserved (Fig. 1A).

In a separate screen, clones of the chick *Ptx1* (*cPtx1*) gene were isolated. Comparison of the complete deduced amino acid sequence with sequences of mouse *Ptx1* (*P-Otx*) and human *PTX1* (*backfoot*) (Semina et al., 1996, 1997; Shang et al., 1997) confirmed that the isolates represented a *Ptx1* orthologue (Fig. 1C). Within the paired-type homeodomain, the amino acid sequence is 100% conserved and, outside the homeodomain sequence, conservation is generally maintained.

To gain insight into their possible functions, the spatial expression patterns of *cTbx2*, *cTbx3*, *cTbx4* and *cTbx5* and *cPtx1* were first examined by whole-mount in situ hybridisation from the early stages of limb formation through to a time at which the limb buds are morphologically distinct (stages 17-29).

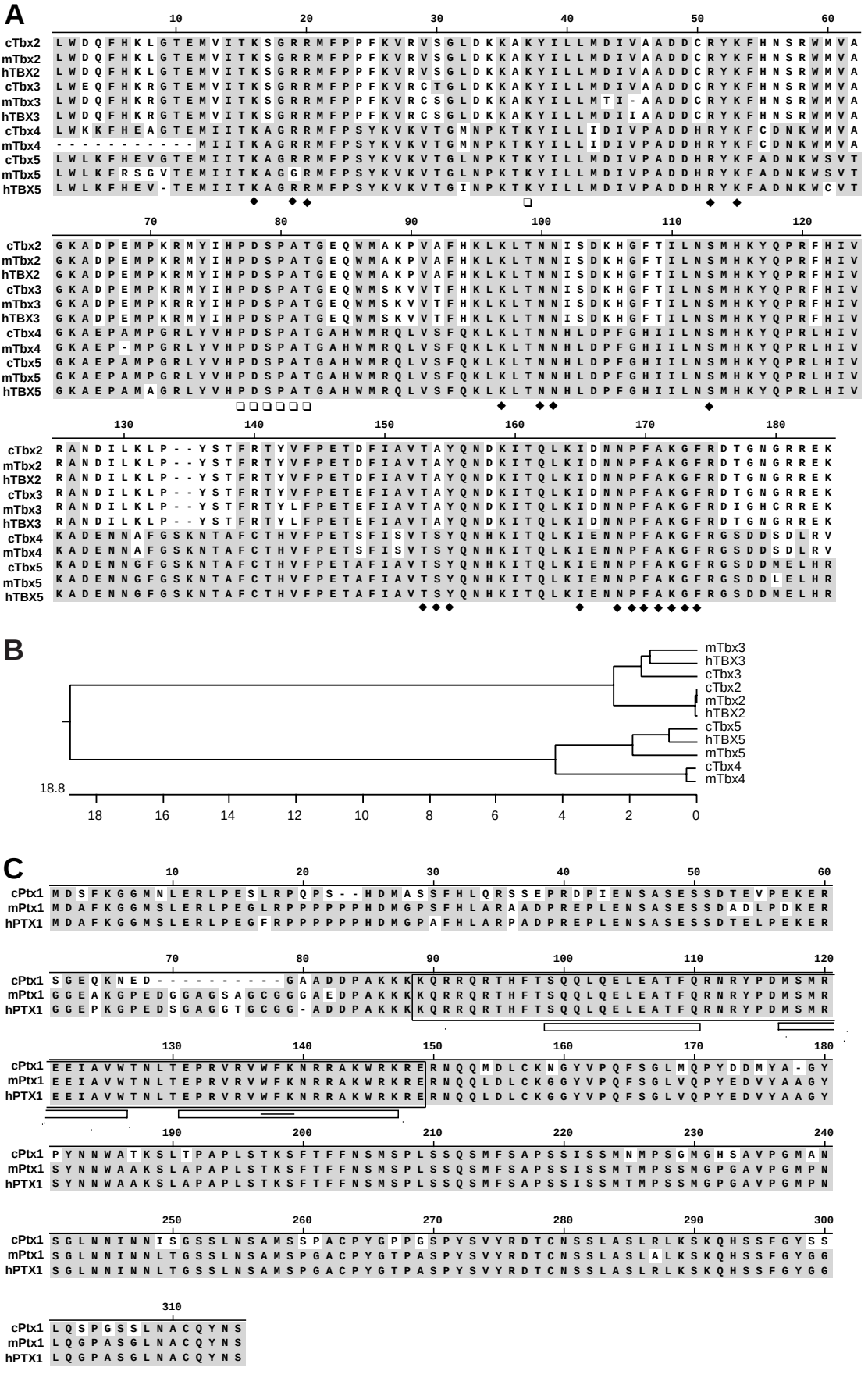
Expression patterns of *cTbx4*, *cTbx5* and *Ptx1* in the developing chick limb buds

At stage 17, expression of *cTbx5* is detected in the developing wing-bud mesenchyme (Fig. 2A) and, by stage 23, high levels of *cTbx5* expression are detected there. At no stage is expression detected in the leg bud (Fig. 2B). There is also transient expression of *cTbx5* in the flank, extending from the posterior of the wing bud to 2/3 of the interlimb flank region, from stage 22-24 (Fig. 2B and data not shown). Section in situ hybridisation analysis of the wing bud at stage 23 shows *cTbx5* expression throughout the wing mesenchyme, although at lower levels distally and dorsally (Fig. 2B). No expression was detected in the ectoderm or AER but high levels of expression were detected in the flank immediately under the wing bud (Fig. 2B inset). By stage 29, the distinct skeletal elements of both the wing and the leg are beginning to form. At this stage transcripts of *cTbx5* are detected throughout the limb mesenchyme, with high levels interdigitally and lower levels proximally and in areas of condensing cartilage (Fig. 2C).

In contrast to *cTbx5*, *cTbx4* is expressed throughout the early leg-bud mesenchyme but is not detected in the wing bud (Fig. 2D-F). At stage 29, however, a very low level of *cTbx4* expression is detected in the wing while high levels of transcripts are detected throughout the leg bud mesenchyme except at the very proximal and distal extremes (Fig. 2F). By section in situ analysis, transcripts are detected throughout the leg bud mesenchyme at stage 23 and, similarly to *cTbx5* in the wing, expression of *cTbx4* appears lower in the dorsal and distal extremes of the leg bud. Again, high levels of expression were detected in the flank mesenchyme directly underneath the leg bud (Fig. 2E inset). Unlike *cTbx5*, no expression of *cTbx4* was detected in the interlimb flank during stages 18-29 (Fig. 2D-F and data not shown).

The expression pattern of *cPtx1* in developing limb is very similar to *cTbx4*. At stage 18 through stage 24, *cPtx1* is restricted to the leg-bud mesenchyme (Fig. 2G-I and data not shown). In later stage embryos, *cPtx1* expression is detected in cells at the most proximal extent of the leg and in the flank of the embryo at the level of the leg bud (Fig. 2I and data not shown). No expression of *cPtx1* is detected in the wing bud or in the ectoderm of the leg bud at any of the stages analysed. Section in situ hybridisation analysis at stage 23 confirmed that transcripts are present throughout the leg mesenchyme but are not present in the ectoderm or AER (Fig. 2H inset). Similar to *cTbx4*, high levels of expression are detected in the flank underneath the leg bud. However, unlike *cTbx4*, *cPtx1* is expressed at high levels in the dorsal and distal extremes of the limb bud and at lower levels ventrally.

To understand when these genes may be first acting in limb formation, we examined their expression at prelimb bud stages. *cTbx5* and *cTbx4* are, respectively, first detected in the presumptive wing- and leg-forming regions of the lateral plate mesoderm at stage 15 (data not shown). *cPtx1* expression is first detected at stage 14 in an area that extends from the level of the last somite to the tip of the tail and includes the prospective leg-forming regions (Fig. 2J). Therefore, transcripts for all three genes are present in the presumptive leg and wing mesenchyme many hours prior to overt outgrowth of


A
B
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mTbx4
18.8
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16
14
12
10
8
6
4
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cPtx1
mPtx1
hPTX1

Fig. 1. Comparison of the T-domain and *Ptx* peptide sequences from chick, mouse and human. (A) Alignment of the T-domain of chick (c) Tbx2/Tbx3/Tbx4/Tbx5 with the respective peptide sequences of the mouse (m) and human (h) genes. The amino acid residues conserved amongst the subfamily and each cognate pair are shaded. The residues shown to be important for DNA binding (black diamond) and dimerisation (open square) of the T-domain of *Xenopus* Brachyury are indicated. (B) Phylogenetic tree of the T-domain sequences shown in Fig. 1A. The sequences were compared using the MegAlign program (DNASTAR inc, Madison, WI). The length of each branch represents the relatedness of each sequence to the others. The scale beneath the tree measures the distance between sequences and the units beneath the scale indicate the number of substitutions between sequences. (C) Comparison of the entire coding sequence of chick (cPtx1) and mouse (mPtx1) and human PTX1(Bft) (hPTX1). Conserved amino acid residues are shaded. The amino acid residues that comprise the paired-type homeodomain are enclosed within a box. The positions of helix I, helix II and helix III are indicated by open boxes underneath the amino acid sequence. The conserved WFK motif in the third helix, characteristic of the Bicoid-class of homeodomains, is underlined. GenBank accession numbers for cTbx2, AF069393; cTbx3, AF069394; cTbx4, AF069395; cTbx5, AF069396; cPtx1, AF069397.

the limb and appear to identify the area of the lateral plate mesoderm destined to contribute to the limb. As such, they provide useful early markers to distinguish the presumptive wing- and leg-forming regions of the lateral plate mesoderm.

Expression patterns of *cTbx2* and *cTbx3* in the developing chick limb buds

Both *cTbx2* and *cTbx3* are initially expressed throughout the flank, lateral plate mesoderm at stage 18 (Fig. 3A,D). Expression levels are higher in the anterior and posterior domains of the nascent bud. At stage 23, *cTbx2* is expressed in two proximodistal bands of cells, one in the anterior and the other in the posterior of the limb, excluding approximately the medial two-thirds of the limb-bud mesenchyme (Fig. 3B). Section in situ hybridisation analysis indicates that *cTbx2* is excluded from the most distal limb bud (Fig. 3B and inset). At stage 29, *cTbx2* is still expressed in anterior and posterior domains but transcripts are not detected in the hand and foot plate (Fig. 3C). *cTbx3* is expressed in a similar pattern. *cTbx3* is expressed in two proximodistal bands of cells in the anterior and posterior of the limb bud mesenchyme (Fig. 3E). Section in situ hybridisation reveals that mesenchymal expression is restricted to proximal and dorsal parts of the limb bud (Fig. 3E inset). However, there are some important distinguishing features of the *cTbx3* expression pattern. Both the anterior and posterior bands of *cTbx3* expression are broader than those of *cTbx2*. Furthermore, the posterior domain of expression within the wing bud is broader than the posterior domain of *cTbx3*-expressing cells in the leg bud. At stage 29, *cTbx3* anterior and posterior expression is maintained (Fig. 3F). A broad posterior band of expressing cells extends into the most distal tip of the wing bud. In the leg, however, expression does not extend distally into the footplate but remains restricted proximal to the most posterior digit. A low level of *cTbx3* expression is also observed interdigitally in both the wing and the leg.

Expression of *cTbx5*, *cTbx4* and *cPtx1* is maintained in grafts of wing and leg buds

Classical experiments have provided information about some of the properties of limb-type specificity which can be used for evaluating potential molecular players in this process. For example, limb-type identity is specified by stage 14 (Stephens et al., 1989) and limb-type identity is independent of the limb ectoderm (Zwilling, 1956a,b). In agreement with these observations, *cTbx5*, *cTbx4* and *cPtx1* are expressed in the limb primordia by around stage 14 and expression of each gene is limited to the limb mesenchyme (Fig. 2 and data not shown). Since all three genes are expressed prior to the expression of *sonic hedgehog* (*shh*) in the zone of polarising activity (ZPA) and prior to the formation of an apical ectodermal ridge (AER),

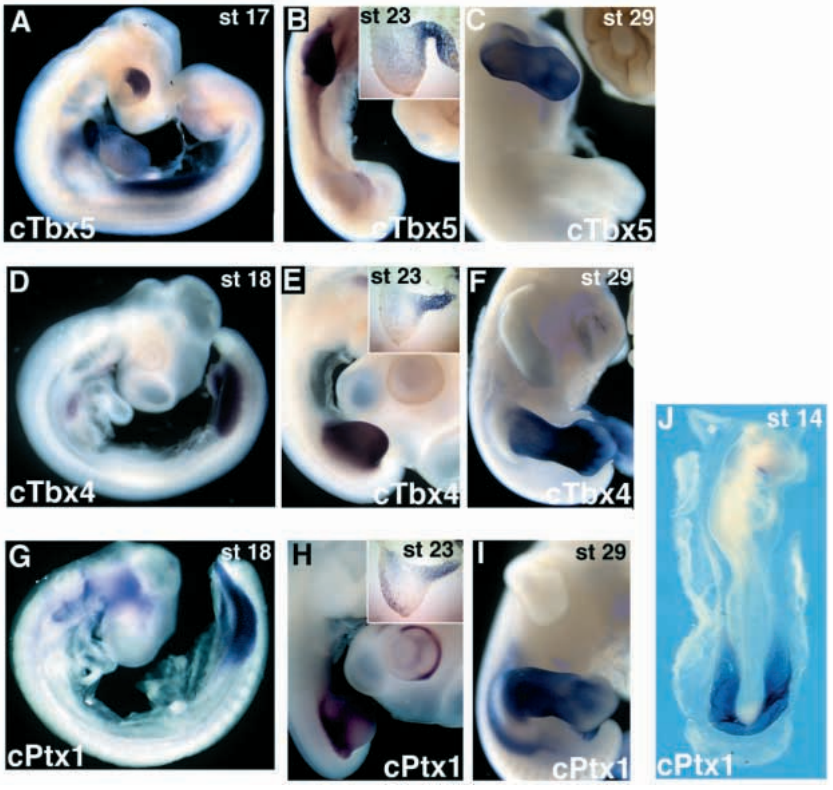

Fig. 2. Expression of *cTbx5*, *cTbx4* and *cPtx1* in developing limb buds. Expression of *cTbx5* (A-C), *cTbx4*, (D-F) and *cPtx1* (G-I) in chick wing and leg buds at developmental stages 17, 23 and 29 visualised by whole-mount in situ hybridisation. (J) *cPtx1* expression in the chick embryo at stage 14. Embryo orientated with head at top. Inset in B is a transverse section of a wing bud (stage 23) probed for *cTbx5*; inset in E is a transverse section through a leg bud (stage 23) probed for *cTbx4*; inset in H is a transverse section of a leg bud (stage 23) probed for *cPtx1*. The transverse sections of limb buds are oriented with the distal tip facing down and the dorsal side to the left. Specific, probe hybridisation was visualised with the NBT/BCIP stains which appears blue/purple or reddish/brown. Non-specific background staining was observed in the head of some examples (G).

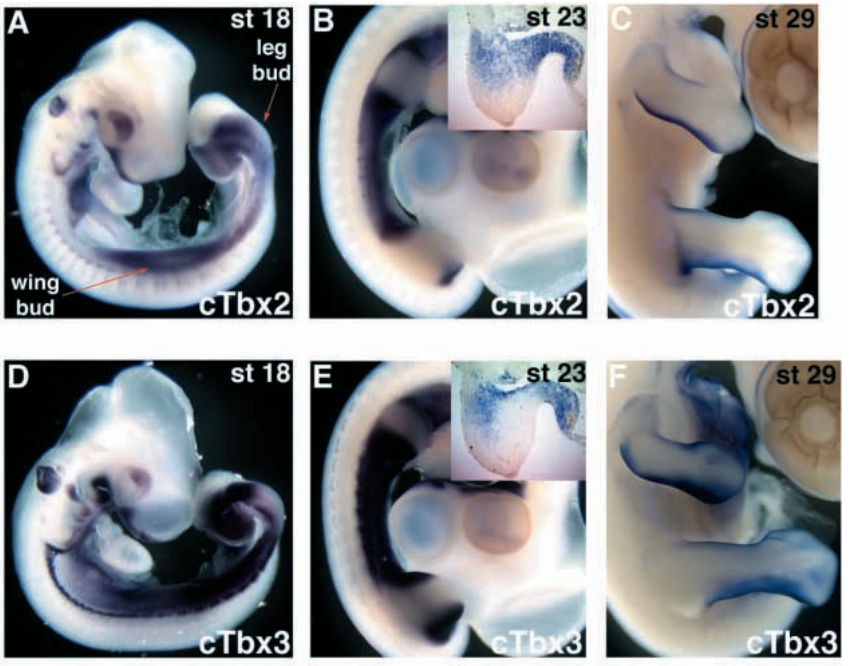

Fig. 3. Expression of *cTbx2* and *cTbx3* in developing limb buds. Expression of cTbx2 at stage 18 (A), stage 23 (B) and stage 29 (C). (A) The position of the nascent wing and leg buds are indicated. A small area of cells that do not express *cTbx2* is visible in both the medial wing and leg bud. The inset in B shows a transverse section through the posterior wing bud (stage 23). Expression of *cTbx3* at stage 18 (D), stage 23 (E) and stage 29 (F). The inset in E is a transverse section through the posterior of the leg bud (stage 23). The transverse sections are orientated with the distal tip of the limb bud facing down and dorsal side to the left.

the initial control of their expression must be independent of these signalling centres. Once these signalling centres form, a positive feedback loop is set up between FGFs produced in the AER and shh in the ZPA (Laufer et al., 1994; Niswander et al., 1994). Surgical removal of the AER removes the source of FGFs and results in a rapid down-regulation of *shh*. To test the potential role of *shh* and FGFs in the maintenance of *cTbx5*, *cTbx4* and *cPtx1*, we investigated their expression after AER removal. Expression of *cTbx5*, *cTbx4* and *cPtx1* is maintained throughout the limb bud up to 24 hours after the removal of the AER (Fig. 4A-F), indicating that maintenance of *cTbx5*, *cTbx4* and *cPtx1* expression is independent of *shh* and of signals from the AER.

It has also been shown that distal wing or leg tissue grafted to a leg-bud or wing-bud stump retains its original identity and develops, respectively, into distal wing or leg structures (Kieny, 1964). We tested whether expression of *cTbx5*, *cTbx4* and *cPtx1* is maintained in such grafts. The distal third of wing and leg buds were transplanted at stage 22-23 and experimental embryos were analysed 12 and 24 hours after the surgery. In all examples analysed, distal wing tissue grafted to a leg stump maintained expression of *cTbx5* (Fig. 4G) and grafted leg tissue maintained the expression of *cTbx4* (data not shown) and *cPtx1*

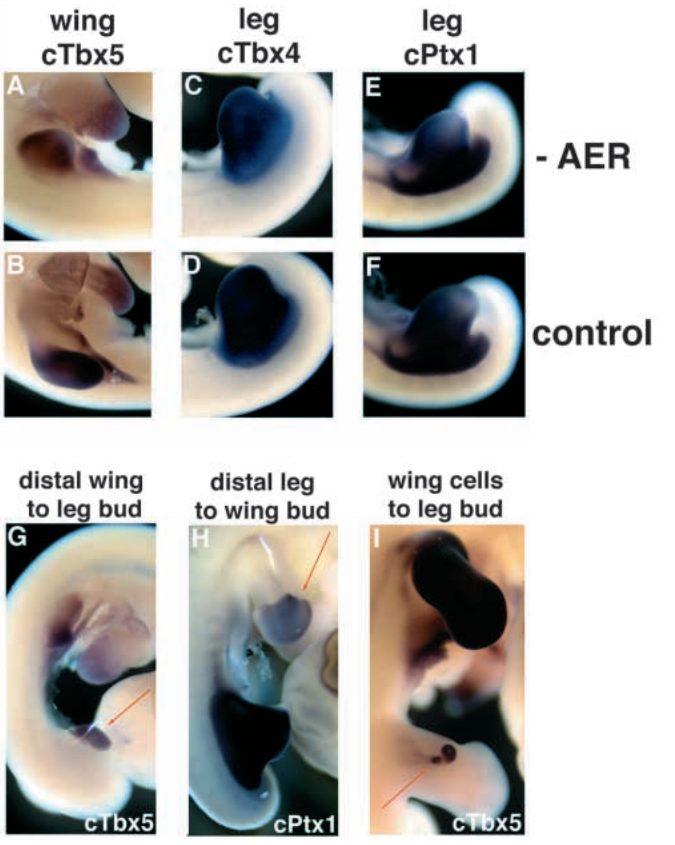

Fig. 4. Expression of *cTbx5*, *cTbx4* and *cPTx1* in manipulated limb buds. (A) Transcripts for *cTbx5* are detected in the wing bud 24 hours after removal of the AER. (B) Contralateral control, unoperated wing. Note the images of the contra-lateral control limb buds have been reversed in the horizontal plane for easier comparison. (C) Transcripts for *cTbx4* are detected throughout the leg bud 24 hours after the removal of the AER. (D) Contralateral control leg bud.(E) Expression of *cPtx1* is detected in the leg bud 24 hours after removal of the AER. (F) Contralateral control leg bud. (G-I) Grafted distal limb tissue continues to express genes characteristic of their original location. (G) The distal wing bud (red arrow) was removed and grafted to the proximal leg bud. After 12 hours, the distal wing graft maintained expression of *cTbx5* . (H) A distal leg bud (red arrow) grafted to the proximal wing bud maintains expression of *cPtx1* 24 hours after surgery. The platinum wire staples used to hold the graft in place are still present. (I) A transplant of wing-bud cells (red arrow) into the leg bud maintains expression of *cTbx5* up to 48 hours after transplantation.

(Fig. 4H). In all examples tested, the grafted tissue remained negative for *cTbx5* or *cTbx4*/*cPtx1* expression appropriate to the host site (data not shown). The ability of grafted tissue to retain the limb-type identity of its original environment was also tested with smaller cell-implants of wing and leg tissue. Prospective thigh mesoderm transplanted to the distal wing bud is capable of forming ectopic toes in the wing (Saunders, 1957). In similar cell transplant experiments, analysed 24 and 48 hours after the cell transplant, the grafted cells maintain gene expression corresponding to their origin (Fig. 4F and data not shown).

In summary, *cTbx5*, *cTbx4* and *cPtx1* are expressed and maintained in normal development and following surgical manipulation in patterns which suggests that these genes may play important roles in the specification of limb-type identity in the chick.

Expression of *cTbx4*, *cTbx5* and *cPtx1* in ectopic limbs

In order to gain insight into the possible roles of *cTbx4*, *cTbx5* and *cPtx1* in the developing limb we exploited the ability of an exogenous source of fibroblast growth factor (FGF) to induce ectopic limb buds. Beads soaked in FGFs, applied to the interlimb lateral plate mesoderm at stages 13 to 17, are capable of inducing ectopic limb outgrowths. Significantly, beads applied at levels closer to the presumptive wing bud generally give rise to wing-like structures whereas beads closer to the presumptive leg bud, generate ectopic leg-like outgrowths (Cohn et al., 1995, 1997; Crossley et al., 1996; Ohuchi et al., 1995; Vogel et al., 1996). We applied FGF4-soaked beads to the interlimb lateral plate mesoderm of stage 15 embryos at various axial levels and analysed the embryos after 2 and 8 days incubation. The early harvests were analysed by whole-mount in situ hybridisation with probes for *cTbx4*, *cTbx5* and *cPtx1* whereas the skeletal elements and featherbud patterns of later harvests were examined histologically. Some embryos destined for late stage harvest were photographed *in ovo* after 2 days so that the position of the early ectopic bud could be compared to the structures that formed.

Table 1. Ectopic gene expression

Position of ectopic outgrowth	*cTbx5* throughout	Mosaic (*cPtx5* or *cTbx4*/*cPtx1*)	*cTbx4*/*cPtx1* throughout	*n*
Anterior	7	2	0	9
Middle	2	10	0	12
Posterior	0	6	9	15

When an FGF4-soaked bead is placed close to the presumptive wing bud (approximately at the level of somite 21 and 22), an ectopic outgrowth is visible after 2 days, adjacent to the normal wing (Fig. 5A-C). *cTbx5* is most often expressed throughout such ectopic outgrowths (Fig. 5A; Table 1). However in some examples *cTbx5* is expressed only in the more anterior of the ectopic bud (Fig. 5B). Single and two probe in situ hybridisation with probes for *cTbx5* and/or *cTbx4*/*cPtx1* indicated that the cells in the posterior of the ectopic limb that did not express *cTbx5*, expressed *cTbx4* or *cPtx1* (Fig. 5C and data not shown). Conversely, when an FGF4-soaked bead is placed close to the presumptive leg mesenchyme (approximately at the level of somite 24/25), the resulting ectopic outgrowth forms adjacent to the normal leg bud (Fig. 5E-G). In these examples, commonly the ectopic limb buds are composed of cells expressing *cTbx4* and *cPtx1* (Fig. 5G; Table 1). In some cases however the outgrowth expresses *cTbx5* in the anterior portion and *cTbx4*/*cPtx1* through the remainder of the limb bud (Fig. 5E,F; Table 1, and data not shown). Placement of an FGF-soaked bead centrally between the prospective wing- and leg-forming regions usually gives rise to ectopic limb buds that arise from cells in the middle of the interlimb flank (Cohn et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996). These ectopic outgrowths often contain separate domains expressing *cTbx5* and *cTbx4*/*cPtx1* (Fig. 5D; Table 1 and data not shown). Interestingly the rostral-caudal boundary of cells that can be induced to ectopically express *cTbx5* or *cTbx4*/*cPtx1* is not fixed, but varies across a relative wide region within the interlimb flank (compare panels in Fig. 5).

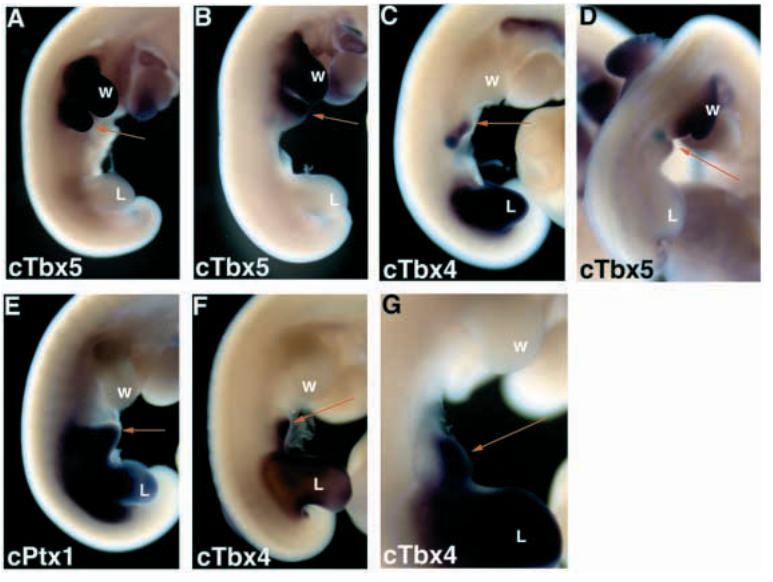

Fig. 5. Expression of *cTbx5*, *cTbx4* and *cPtx1* in FGF-induced ectopic limbs at stage 24. Ectopic limb buds that form adjacent to the normal wing (anterior, Table 1) bud commonly express *cTbx5* throughout (A). In some cases, the bud contains a region in the posterior of the ectopic bud that is not expressing *cTbx5* (B) and, in these cases, leg markers, for example *cTbx4* (C), are expressed in the posterior of the ectopic bud. (D) Ectopic limbs that arise from the cells in the middle of the interlimb flank (middle, Table 1) commonly express *cTbx5* in the anterior half. Ectopic limb buds that arise adjacent to the normal leg bud (posterior, Table 1) can be mosaic, and leg markers, *cTbx4* (E) and *cPtx1* (F), are expressed in the posterior portion of the outgrowth and non-expressing cells present in the anterior portion. In other examples, leg markers, such as *cTbx4* (G) were ectopically induced throughout the extra limb bud. The normal wing (W) and leg (L) buds are labelled in each example and the site of ectopic limb bud outgrowth marked with a red arrow.

Table 2. Type of ectopic limb

Position of ectopic outgrowth	Wing-like	Mosaic	Leg-like	*n*
Anterior	5	1	0	6
Middle	1	9	2	12
Posterior	0	2	10	12

Morphological examination of FGF-induced ectopic limbs

As a further test of the roles of *cTbx5*, *cTbx4* and *cPtx1*, we examined and compared ectopically induced limb buds for morphology after 8 days or for expression patterns after 2 days incubation, in parallel experiments. Those incubated for 8 days were first observed 2 days after bead implantation and a photographic record made of the location of the ectopic limb bud for comparison to those analysed by in situ hybridisation.

An anterior ectopic limb buds that formed adjacent to the normal wing bud, are generally wing-like, containing characteristic wing digits (Fig. 6A and inset; Table 2) and more proximal wing bones (not shown). In some cases, only the proximal and medial wing bones are present (data not shown). In a rare case, the ectopic limb bud developed into a structure with wing-like character proximally, consisting of a humerus, radius and ulna but, the single digit that formed is clearly toe-like (Fig. 6B).

Limbs that develop from the mid-flank almost always have a mosaic phenotype and with some skeletal elements characteristic of a wing and some of a leg (Fig. 6C,E; Table 2). Some limbs contain wing proximal elements (humerus and ulna) but distally, in the anterior, a wing digit forms while in the posterior, a leg digit 1 and 2 are obvious (Fig. 6C). Other examples have leg proximal elements (femur and tibia) while distally again the identity of the elements are split with anterior wing digits 4 and 3 and posterior leg digits 1 and 2. As previously described (Cohn et al., 1995; Crossley et al., 1996; Vogel et al., 1996), all ectopic skeletal elements form in mirror image of the normal wing and leg skeletal elements (Fig. 6C,E).

Posterior ectopic limb buds most often give rise to leg-like structures (Fig. 6D; Table 1), fused to the normal leg at the hip joint. Consistent with our observation that posterior ectopic limb buds occasionally contain small anterior areas of *cTbx5* expression (Fig. 5E,F and data not shown), some such buds gave rise to mostly leg-like structures that contain a single anterior wing digit 4 and/or a ridge of featherbuds on their anterior surface (data not shown).

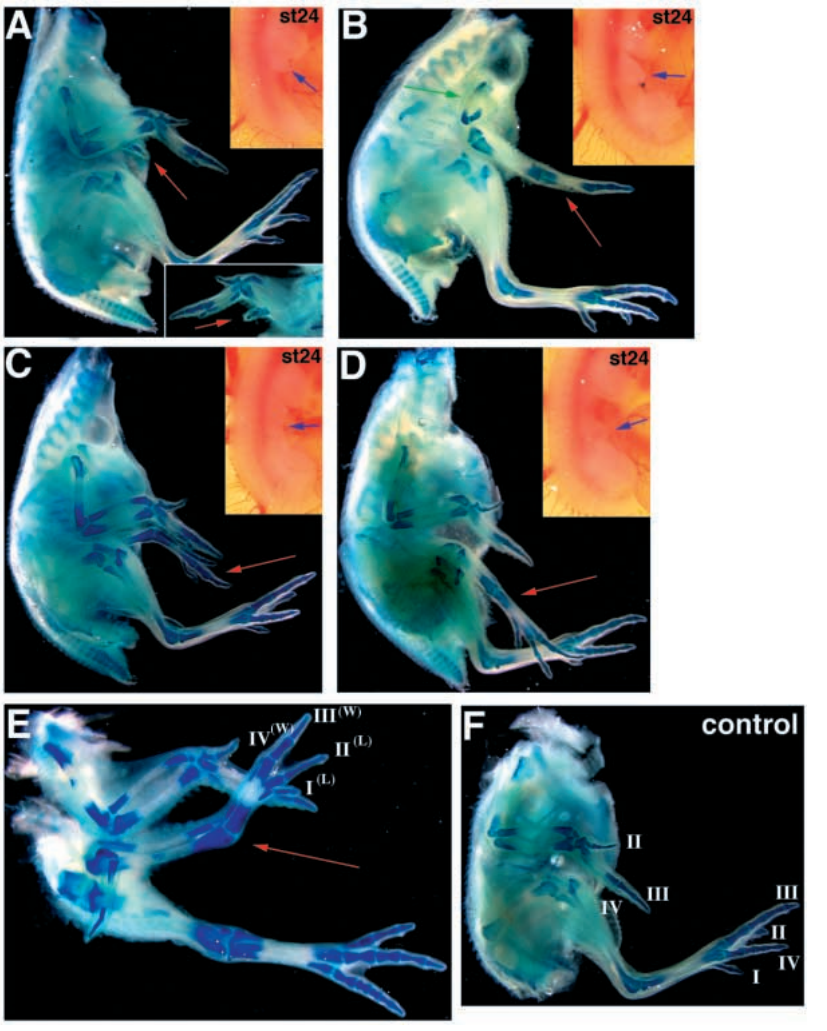

Fig. 6. Histological analysis of FGF-induced ectopic limbs at stage 36. (A-D) Examples of the same embryo photographed in ovo at stage 24 (inset) and at stage 36 after staining with Alcian Blue to examine the ectopic skeletal elements. The blue arrows indicate the site of the ectopic limb bud at stage 24; the red arrows indicate the ectopic limb structure that developed. (A) Example of the type of ectopic limb that can form by stage 36 from an ectopic limb bud that was visible in the anterior interlimb flank at stage 24 (upper inset). The ectopic wing-like outgrowth contains three wing-like digits (lower inset). (B) The anterior ectopic bud (stage 24, inset) gave rise to a wing-like ectopic limb that contains a toe-like digit element at its distal extreme. In this example, the ectopic bud appeared to interfere with formation of the normal wing (green arrow). Similar observations have been reported previously (Vogel et al., 1996). (C) An ectopic limb bud in the middle interlimb flank (stage 24) gave rise to a mosaic, ectopic limb (stage 36) that contains distal elements of both a wing and a leg. Leg digits I and II are present in the posterior and a wing digit IV has formed in the anterior. More proximally the skeletal elements are more wing-like, humerus and ulna bones. (D) A posterior ectopic bud (stage 24) developed into an almost perfectly formed leg by stage 36. (E) Example of a limb that developed from an ectopic bud that formed in the middle of the interlimb flank. In the posterior, a leg digit I and II are present and, more proximally, a tibia-like bone has formed. In the anterior, wing digits IV and III have formed and possibly a radius/ulna-like element. (F) A control embryo harvested at stage 36 and stained with Alcian blue. The characteristic wing and leg digits are indicated.

DISCUSSION

cTbx5, *cTbx4* and *cPtx1* expression correlates with limb identity

The most striking aspect of *cTbx5*, *cTbx4* and *cPtx1* expression is that each gene is highly restricted to either the wing or leg bud. The localisation of transcripts of these genes exclusively to the mesenchyme and not the ectoderm is consistent with evidence that the property of limb-type identity resides in the limb mesenchyme and is independent of the limb ectoderm (Zwilling, 1956a,b). All three genes are expressed in the prospective limb mesenchyme prior to the overt formation of a limb bud. The onset of expression of all three genes is coincident with the time at which the limb fields gain the ability to differentiate independently into either a wing or a leg when grafted to a host coelom (Stephens et al., 1989). Furthermore, the specific limb-type expression pattern of *cTbx5* and *cTbx4*/*cPtx1* is maintained in limb tissue grafted to a new location, consistent with the ability of such grafts to maintain their limb-type identity in a different environment.

Additional evidence supporting a role for these genes in limb-type specification is provided by the analysis of ectopic limbs induced by FGF. The type of ectopic structure that forms is related to the rostrocaudal position at which the source of FGF is applied (Cohn et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996 and reported herein). A bead placed adjacent to the wing commonly induces an ectopic wing-like structure, while a bead placed adjacent to the leg commonly induces an ectopic leg-like element. We observe that the type of gene induced in the ectopic outgrowths correlates well with the structures that generally develop from such outgrowths. Anterior, ectopic outgrowths contain *cTbx5*-expressing cells, posterior ectopic outgrowths contain *cTbx4*/*cPtx1*-expressing cells and outgrowths from the middle of the flank have mosaic wing/leg identity. Although these genes are expressed exclusively in the mesenchyme, they appear to have an effect on the formation of ectodermal derivatives. In mosaic buds, wing-like featherbud patterns are apparent on the anterior surface while the posterior ectoderm has more leg-like features.

cTbx5, *cTbx4* and *cPtx1* and limb initiation

Candidates for endogenous FGFs involved in limb bud initiation include *FGF8*, which is transiently expressed in the intermediate mesoderm, adjacent to the wing and leg fields, just prior to limb-bud outgrowth (Crossley et al., 1996; Vogel et al., 1996) and *FGF10*, which is expressed in lateral plate mesoderm at stage 14/15 (Ohuchi et al., 1997). In *Xenopus*, a regulatory relationship between the T-box gene *Brachyury* and embryonic FGF (eFGF) has been described (Schulte-Merker et al., 1994). A similar regulatory mechanism may exist in the lateral plate mesenchyme between FGFs and *cTbx5*, *cTbx4* and *cPtx1*. All three genes are initially expressed approximately coincident with the time at which FGF10 and FGF8 are first detected in the lateral plate mesoderm and intermediate mesoderm, respectively. It remains unclear what regulates the differential induction of *cTbx5* or *cTbx4*/*cPtx1* in anterior or posterior positions in response to FGF. Hox genes are expressed at defined rostrocaudal levels in the lateral plate mesoderm and their expression patterns in ectopic limbs change in response to FGF (Cohn et al., 1997). However, the border between *cTbx5*- and *cTbx4*/*cPtx1*-expressing cells in FGF-induced ectopic limb buds is not fixed on the rostrocaudal axis. Thus the molecular mechanism that specifies which of the limb-identity genes will be activated remains unclear.

Mutations in the human suggest roles for *TBX5* and *TBX3* in forelimb development

Direct evidence for a role of *TBX5* in the formation of the forelimb come from mutations in the human *TBX5* gene (Basson et al., 1997; Li et al., 1997) which cause Holt-Oram Syndrome (HOS), a pleiotropic, autosomal dominant, developmental disorder in man. The syndrome is characterised by skeletal abnormalities in the upper limbs and cardiac septation defects. The skeletal abnormalities can vary from a triphalangeal thumb to a range of reduction deformities which in the most severe cases include phocomelia, an almost complete absence of limb elements.

Mutations in human *TBX3* cause Ulnar-Mammary Syndrome (UMS), a pleiotropic disorder affecting limb, apocrine gland, tooth and genital development (Bamshad et al., 1997). Mutations characterised are predicted to cause haploinsufficiency of the TBX3 protein. This suggests that, similarly to TBX5, critical levels of this transcription factor are required for normal morphogenesis. Limb malformations in UMS are typically deficiencies, fusions or duplications of the posterior elements of the upper limb, including the ulna, metacarpals and phalanges (Bamshad et al., 1996, 1997). In contrast, limb malformations in HOS are most often observed in the anterior elements of the upper limb, primarily the radius, carpal and ulna bones. Thus TBX3 and TBX5 may play complimentary roles, patterning posterior and anterior elements, respectively. *cTbx3* is expressed in both posterior and anterior domains and, in the chick at least, the posterior domain correlates well with the position of the cells fated to form the more posterior elements. The cells in the anterior domain are not fated to form any of the skeletal elements and indeed many undergo apoptosis (Saunders, 1966), which may explain the lack of a visible phenotype in the anterior of the limb. Deformities observed in Ulnar-Mammary Syndrome indicate a role for TBX3 in forelimb formation whereas hindlimbs are mostly unaffected (Bamshad et al., 1996), consistent with the clear distinction between the chick wing and leg expression domains of *cTbx3* at later stages (Fig. 3F). In the developing wing, a much broader domain of expression for *cTbx3* is evident compared to the leg, where expression extends into the hand plate but is absent in the foot plate.

Our results strongly support the hypothesis that *Tbx5*, *Tbx4* and *Ptx1* are likely to have important functions in limb-type specification. Other, independent studies of *cTbx4* and *cTbx5* in normal and ectopic chick limbs have reached identical conclusions (Ohuchi et al., 1998; Isaac et al., 1998; Papaioannou et al., 1998). Further functional studies will help determine where in the hierachy of molecular regulatory pathways these transcription factors act to establish limb identity.

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