Limb outgrowth and limb identity (forelimb versus hindlimb) begins very early in development with the establishment of a special group of cells termed the limb field (Harrison, 1918). As development proceeds, precisely positioned limb buds appear, opposite each other, and at very distinct trunk levels, as a result of the coordinated proliferation of cells derived from the lateral plate mesoderm (LPM) (reviewed by Johnson and Tabin, 1997; Tickle, 1999; Capdevila and Izpisúa Belmonte, 2001). While the molecular mechanisms responsible for the initiation, positioning and identity of the limb field are still largely unknown, in the last few years several signaling molecules and transcription factors have been shown to be critical for these processes. Members of the fibroblast growth factor (FGF) family play central roles in limb initiation. When misexpressed in the LPM in a localized manner, several FGFs are capable of inducing an ectopic limb (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997). Furthermore, disruption of the mouse Fgf10 gene, specifically expressed in the LPM before any visible outgrowth has occurred (Ohuchi et al., 1997), results in complete loss of limbs (Min et al., 1998; Sekine et al., 1999). In addition to FGF, we have recently demonstrated that different members of the WNT family are differentially expressed in the LPM before limb outgrowth. Specifically, Wnt2b is expressed in the presumptive forelimb region while Wnt8c is expressed in the presumptive hindlimb region. Both Wnt2b and Wnt8c are capable of inducing Fgf10, which, in turn, maintains Tbx5 expression during limb outgrowth. Furthermore, our results indicate that Tbx5 and Wnt2b function together to initiate and specify forelimb outgrowth and identity. The molecular interactions governed by members of the T-box, Wnt and Fgf gene families uncovered in this study provide a framework for understanding not only limb development, but how outgrowth and identity of other tissues and organs of the embryo may be regulated.

INTRODUCTION

Limb outgrowth and limb identity (forelimb versus hindlimb) begins very early in development with the establishment of a special group of cells termed the limb field (Harrison, 1918). As development proceeds, precisely positioned limb buds appear, opposite each other, and at very distinct trunk levels, as a result of the coordinated proliferation of cells derived from the lateral plate mesoderm (LPM) (reviewed by Johnson and Tabin, 1997; Tickle, 1999; Capdevila and Izpisúa Belmonte, 2001). While the molecular mechanisms responsible for the initiation, positioning and identity of the limb field are still largely unknown, in the last few years several signaling molecules and transcription factors have been shown to be critical for these processes. Members of the fibroblast growth factor (FGF) family play central roles in limb initiation. When misexpressed in the LPM in a localized manner, several FGFs are capable of inducing an ectopic limb (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997). Furthermore, disruption of the mouse Fgf10 gene, specifically expressed in the LPM before any visible outgrowth has occurred (Ohuchi et al., 1997), results in complete loss of limbs (Min et al., 1998; Sekine et al., 1999). In addition to FGF, we have recently demonstrated that different members of the WNT family are differentially expressed in the LPM before limb outgrowth. Specifically, Wnt2b is expressed in the presumptive forelimb region while Wnt8c is expressed in the presumptive hindlimb region. Both Wnt2b and Wnt8c are capable of inducing Fgf10 and, subsequently, limb outgrowth (Kawakami et al., 2001).

Despite the orchestrated interactions needed between outgrowth and identity during development of tissues and organs, these phenomena, for ease of analysis, are often treated as distinct processes. The decision to become either a forelimb or a hindlimb is also made at the earliest stages of limb initiation. Several studies suggest the intriguing possibility that limb identity could be determined by the specific expression of a single transcription factor. Two members of the T-box family of transcription factors, Tbx4 and Tbx5 (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Ohuchi et al., 1998; Tamura et al., 1999; Begemann and Ingham, 2000) (reviewed by Ohuchi and Noji, 1999), and a member of the OTX-related subclass of paired-type homeodomain proteins, Pitx1 (Lanctot et al., 1997; Logan et al., 1998), show a restricted forelimb and hindlimb distribution in the LPM prior to limb budding. Tbx5 transcripts are expressed in the presumptive forelimb area in all tetrapods studied so far, and conversely, Tbx4 and Pitx1 transcripts are restricted to the
presumptive hindlimb identity. Furthermore, gain- and loss-of-function experiments in chick and mice lend support to the idea of limb identity being determined by a discrete set of molecular determinants (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999a; Szeto et al., 1999; Takeuchi et al., 1999).

The co-localization of factors shown to be capable of inducing limb outgrowth (WNT2b and WNT8c) and factors involved in limb identity (Tbx5 and Tbx4), raises the question of whether both processes are linked or take place independently of one another. In this study, we combine gain- and loss-of-function approaches in both zebrafish and chick embryos to analyze the molecular relationships occurring among Fgf10, Wnt2b and Tbx5 during initiation and specification of forelimb identity. Our results demonstrate that, in addition to its role governing forelimb identity, Tbx5 is both necessary and sufficient for forelimb initiation. We also demonstrate that Tbx5 is directly upstream of Fgf10 in the signaling cascade that directs limb outgrowth. In turn, a feedback loop is uncovered in which FGF signaling is required for both processes, and that Tbx5 and Tbx4 interact to specify the forelimb identity. Altogether, our results unveil the existence of a complex network of molecular interactions that establishes, propagates and maintains the determination and limb initiation are not independent processes, and that Tbx5 and Wnt2b function together to initiate and specify forelimb identity. These results indicate that both key events of limb development, namely limb identity determination and limb initiation, occur in a coordinated manner in zebrafish embryos.

MATERIALS AND METHODS

Cloning of zebrafish fgf10 and wnt2b genes

To isolate the zebrafish orthologue of Fgf10, we screened zebrafish genomic and 24 hpf cDNA λ phage libraries (Stratagene) under low stringency using chick Fgf10 cDNA as a probe. Several independent clones were obtained and sequenced. The zebrafish wnt2b gene was similarly obtained except that chick Wnt2b cDNA was used as a probe.

Morpholino injections

Morpholino oligonucleotides were designed by and obtained from GeneTools LLC (Eugene, Oregon). The zebrafish tbx5 morpholino lies from nucleotide position −5 to +18:

5′-GTGGTTACTGTTCCCTGCGTTTTCG-3′.

The zebrafish wnt2b morpholino sequence lies from nucleotide position −66 to −43 and targets the 5′ UTR:

5′-AATGCTATATATGTTACAGTACATG-3′.

The standard control oligonucleotide available from Gene Tools was used. The morpholinos were solubilized in 1× Danieau’s solution and injected into one-cell stage zebrafish embryos at a range of 2-10 ng/embryo.

heartstringsN21 (hst) mutant lines

The hst mutant was identified in an ENU-induced mutagenesis screen for perturbation of cardiac function in zebrafish. The mutation has been mapped to the tbx5 gene and introduces a nonsense mutation at codon 316 (Garrity et al., 2002).

RNA injections

The open reading frame, excluding the 5′ and 3′ untranslated sequences, of zebrafish fgf10, wnt2b and tbx5 were cloned into the pCS2 vector. Capped RNAs were synthesized from these constructs using the mMessage mMACHINE kit (Ambion). Seventy picogram of fgf10 mRNA and 100 pg of tbx5 and wnt2b mRNA was injected into one-cell stage zebrafish embryos.

Whole-mount in situ hybridization and Alcian Blue cartilage staining

Injected zebrafish embryos were scored for pectoral fin phenotypes at 30 hours post-fertilization (hpf) to 5 days post-fertilization (dpf) using a stereomicroscope. Further analysis was conducted at 24-48 hpf by whole-mount in situ hybridization, as described previously (Hammerschmidt et al., 1996), and at 5 dpf by Alcian blue staining as described previously (Schilling et al., 1996). Zebrafish riboprobes used were fgf8 (Furthauer et al., 1997), tbx5 (Tamura et al., 1999) and shh (Ekker et al., 1995). The zebrafish fgf10 riboprobe spans the entire ORF, and the wnt2b riboprobe contains 900 bp of the coding sequence corresponding to the N-terminal region. Viral injected and bead implanted chick embryos were examined by whole-mount in situ hybridization and Alcian Blue cartilage staining as described by Vogel et al. (Vogel et al., 1996). For the zebrafish studies, a minimum of 100 embryos was examined for each in situ hybridization.

Viral production and injections into chick embryos

Adenovirus expressing the mouse Axin gene was produced and injected as previously described (Kawakami et al., 2001). The full-length mouse Tbx5 cDNA and a truncated form of chick Tbx5 (amino acids 62-521), lacking the N-terminal region upstream of the T-box, were cloned into an RCAS retroviral vector to produce RCAS-Tbx5 and RCAS-Tbx5ΔN constructs, respectively. Subsequent transfection into chick embryonic fibroblasts and retroviral production were performed as described previously (Vogel et al., 1996). RCAS-Tbx5 was injected into stage 5-8 chick embryos in the LPM. RCAS-Tbx5ΔN was injected into stage 8-10 chick in the LPM. Staging of chick embryos was according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). An RCAS-alkaline phosphatase virus was injected as a control and no phenotypic changes in gene expression or limb morphology were observed.

Bead implantation

Beads soaked with the FGF receptor tyrosine kinase inhibitor SU5402 (Calbiochem), used at 1 mg/ml in DMSO, were implanted into stage-14 to -18 chick embryos as described previously (Rodriguez-Esteban et al., 1999b). Control beads were implanted at the same stage and no change in gene expression was observed.

RESULTS

Tbx5 is necessary for limb initiation

We, and others, have previously shown that the T-box genes Tbx5 and Tbx4 play key roles in specifying the identity of the vertebrate forelimb and hindlimb, respectively (Rodriguez-Esteban et al., 1999a; Takeuchi et al., 1999). The early onset of expression of Tbx5 genes in the LPM of the presumptive limb field suggests additional roles for these factors in early limb development. To further our understanding of the role of Tbx genes in limb development, we performed loss-of-function experiments in zebrafish by using morpholino antisense oligonucleotides injected into one-cell stage zebrafish embryos. We have focused on tbx5 since the initiation of hindlimb structures, or pelvic fins, and concomitant tbx4 expression in zebrafish embryos, does not take place until one month after fertilization (Tamura et al., 1999). While injection of a control morpholino at the one-cell stage gave no obvious phenotype (Fig. 1A,C), injection of a tbx5 morpholino gave a range of
altered fin phenotypes depending on the amount injected. With the highest non-toxic levels of injected morpholino (10 ng), the most common phenotype was complete loss of pectoral fins (95%, n=250; Table 1; Fig. 1B). Cartilage staining confirmed that these embryos lacked pectoral fin structures and the shoulder girdle (Fig. 1E). Milder phenotypes, including shorter pectoral fins, reduced number of rays and sometimes upturned orientation of the pectoral fins, were obtained by decreasing the amount of injected morpholino (to a minimum of 2 ng; Fig. 1D).

tbx5 morphants also exhibited an enlarged pericardium and a stretched heart tube that failed to loop (data not shown).

The results of the tbx5 loss-of-function experiments suggest that, in addition to its role in controlling limb identity, tbx5 plays a role in limb initiation and outgrowth. The range of phenotypes obtained also indicates that the function of tbx5 is dosage dependent. These results are in agreement with the recent Ahn et al. study which also showed that tbx5 is required for pectoral fin formation (Ahn et al., 2002).

The FGF family of signaling molecules is known to be required and sufficient for limb initiation (reviewed by Martin, 1998; Martin, 2001). Given the known role of T-box proteins as transcriptional regulators, we reasoned that tbx5 might function by regulating the expression of fgf10. To start addressing this, we screened several zebrafish cDNA libraries using chick fgf10 as a probe and cloned zebrafish fgf10 (Fig. 2A). As has been previously reported in mouse and chick embryos (Ohuchi et al., 1997), at 24 hpf, prior to initial budding of the pectoral fins, zebrafish fgf10 can be detected in the LPM of the presumptive pectoral fin field, in a pattern temporally and spatially similar to tbx5 (compare Fig. 2B,C). In later stages, zebrafish fgf10 is observed in the mesenchyme of the pectoral fin buds, overlapping tbx5 expression (compare Fig. 2D,F with 2E,G). Expression is also observed in the branchial arches, otic vesicle, heart primordium and tail bud (data not shown), as in the corresponding structures of other vertebrates (Ohuchi et al., 1997).

Given the aforementioned tbx5 loss-of-function phenotypes, we decided to examine the expression of mesodermal [fgf10 and sonic hedgehog (shh)] and ectodermal (fgf8) markers involved in the early stages of limb development. As shown in

Fig. 1. Zebrafish tbx5 is required for pectoral fin initiation and outgrowth. All panels show the dorsal view of zebrafish embryos with anterior to the top. (A-E) Five dpf zebrafish embryos injected with control (A,C) or tbx5 (B,D,E) morpholinos. Embryos with 10 ng of tbx5 morpholino lack pectoral fins (B, asterisk) and Alcian Blue cartilage staining (E, asterisk), unlike control-injected embryos (A,C, arrows). Injection of a lower dose (2 ng, as in D) results in a milder fin phenotype such as a reduced pectoral fin blade (arrow in D).

Fig. 2. Amino acid sequence alignment of FGF10 proteins and comparison of fgf10 and tbx5 expression in the zebrafish embryo. (A) Alignment of deduced FGF10 protein sequences from zebrafish (Danio rerio, Dr), chick (Gallus gallus, Gg), mouse (Mus musculus, Mm), and human (Homo sapiens, Hs). Identical amino acids are indicated by dots, and gaps by dashes. The zebrafish sequence shows homology to that of chick (44.7%), mouse (42.7%) and human (41.7%). The GenBank accession number for zebrafish fgf10 is AF544025. (B-G) All panels show the dorsal view of zebrafish embryos with anterior to the top, excluding F and G. Whole-mount in situ hybridization staining of wild-type zebrafish embryos during pectoral fin development. fgf10 (B,D,F) and tbx5 (C,E,G) appear to be expressed in a similar spatial and temporal pattern during pectoral fin initiation and outgrowth. (B,C) fgf10 and tbx5 are expressed in the LPM of the presumptive pectoral fin bud (arrowheads) at 24 hpf. (D,E) At 30 hpf fgf10 is expressed in the branchial arches, otic vesicle, and pectoral fin bud (arrowheads), while tbx5 is expressed in the dorsal eye, heart tube and pectoral fin bud (arrowheads). (F,G) Lateral view of pectoral fin buds of 36 hpf embryos showing fgf10 and tbx5 expression throughout the mesenchyme. Anterior is to the left.
Table 1. Lack of pectoral fins in injected embryos

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<th>Injection sample</th>
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<th>% of embryos lacking pectoral fins</th>
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<tr>
<td>tbx5 MO (10.0)</td>
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<td>wnt2b MO (10.0) +tbx5 RNA (0.1)</td>
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MO, morpholino.

Wild-type zebrafish embryos were injected with tbx5 or wnt2b morpholinos and scored for the absence of pectoral fins at 5 dpf. In rescue experiments, morpholinos and capped RNA were co-injected into embryos and also scored for fin phenotypes.

Fig. 3A,B, injection of tbx5 morpholino, but not control morpholino, resulted in complete loss of fgf10 expression in the pectoral fin bud forming region of the LPM at 26 hpf, while expression in other regions remained unaffected. We could also not detect shh (data not shown) or fgf8 (a marker for the apical fold; compare Fig. 3C with 3D).

To complement the morpholino studies, we made use of the novel zebrafish heartstrings (hst) mutant. The hst mutation has been very recently mapped to a point mutation in the open reading frame of zebrafish tbx5, and generates an early terminated protein (Garrity et al., 2002). As with tbx5 morphant embryos, homozygous hst embryos do not develop pectoral fins. We examined the expression of fgf10 in hst mutant embryos, at the early stages of fin development, and observed a complete loss of expression (Fig. 3E). We have also examined whether the Fgf10 gene is a direct target of Tbx5 in vitro. A putative Brachyury/Tbx5 binding site is located approximately 400 base pairs upstream of the start site that is conserved in both human and mouse Fgf10 genes. Deletion of this site results in complete loss of activation of the fgf10 promoter by Tbx5, indicating that Tbx5 has the capacity to directly regulate fgf10 expression (data not shown). Taken together, our data indicate that tbx5 is required for fin initiation and suggest that it functions upstream of the FGF pathway that directs the early stages of fin outgrowth.

MO-injected embryos

wnt2b is necessary for limb initiation and regulates tbx5

We recently reported that the WNT signaling pathway is required for normal limb development in chick embryos, regulating very early stages of limb induction (Kawakami et al., 2001). We demonstrated that Wnt2b, which is expressed in the LPM of the forelimb field, regulates limb initiation through induction of Fgf10. Given the lack of Fgf10 expression in the Tbx5 loss-of-function experiments, we hypothesized that Tbx5 might interact with the WNT signaling pathway. To address this issue, we cloned the zebrafish wnt2b homologue and performed loss-of-function experiments.

We screened several zebrafish genomic and cDNA libraries using chick Wnt2b as a probe and obtained a putative Wnt2b clone with a full-length open reading frame. Sequence comparison demonstrated that our positive clones represented zebrafish wnt2b (Fig. 4A). As reported in chick embryos (Jasoni et al., 1999; Kawakami et al., 2001), at 22 hpf, zebrafish wnt2b is expressed in the developing eye, and a localized mesodermal region medial to the LPM at the somite level where the pectoral fin buds will form (Fig. 4B-D). In later stage embryos, when the fin bud started to form, we could no longer detect wnt2b (data not shown). Thus, the expression pattern of zebrafish wnt2b appears to be conserved between zebrafish and chick embryos.

wnt2b loss-of-function experiments were performed using a wnt2b morpholino to downregulate endogenous wnt2b (10 ng per injection). This loss-of-function study resulted in a large portion (75%, n=230; Table 1) of embryos that lacked pectoral fins (Fig. 5B), indicating that wnt2b plays an important role during pectoral fin development. Cartilage staining confirmed that these embryos lacked pectoral fin structures and the shoulder girdle (Fig. 5E). By reducing the amount of wnt2b morpholino injected (to a minimum of 2 ng), we were able to obtain a broad range of fin phenotypes very similar to those described for the tbx5 morphants (data not shown). In 5 dpf wnt2b morphants, structures other than the pectoral fins developed similarly to wild type embryos (Fig. 5A,B,E). Thus, we exclude the possibility that the phenotypes observed were caused by general developmental arrest. These results indicate that the function of wnt2b is necessary for normal fin initiation, and is affected by gene dosage. Embryos injected with a control morpholino did not display any obvious abnormal phenotype (Fig. 5A,D).

To confirm the specificity of the morpholino antisense

Fig. 3. Zebrafish tbx5 is necessary for fgf10 and fgf8 expression in the pectoral fin budding region. All panels show the dorsal view of zebrafish embryos with anterior to the top. (A-D) Comparison of fgf10 and fgf8 expression patterns in embryos injected with 10 ng of control (A,C) or tbx5 (B and D) morpholino. (A and B) fgf10 expression in the pectoral fin bud region of 26 hpf (A, arrowheads) was not detected in the tbx5-morpholino injected embryo (B, asterisks). Note that expression remains unchanged in other regions of the embryo. (C and D) In 36 hpf tbx5-morpholino-injected embryos, fgf8 expression could not be detected in the region where the pectoral fin buds develop (D, asterisks), as compared to the control embryos (C, arrowheads point to the expression in the apical fold). fgf8 expression in the midbrain-hindbrain boundary remains unaltered in the injected embryos, as noted by the arrows. (E) fgf10 was not detected in the pectoral fin bud region of 24 hpf hst mutants (asterisks), but remained unaltered in other regions.
experiments, we attempted to rescue the \textit{wnt2b} loss-of-function phenotypes by co-injecting \textit{wnt2b} morpholin with \textit{wnt2b} RNA. As shown in Table 1, co-injection of the \textit{wnt2b} RNA completely rescued the fin phenotypes in more than half of the \textit{wnt2b} morphants (n=60).

Subsequent to morpholin injection, we analyzed the expression of early markers of limb development, as in the \textit{tbx5} morphants. We could not detect \textit{fgf10}, \textit{fgf8} or \textit{shh} expression in the fin field of embryos injected with the \textit{wnt2b} morpholin (compare Fig. 5L with 5M; and data not shown). These results are consistent with the observed phenotypes and our previous studies in chick embryos (Kawakami et al., 2001).

The loss of \textit{fgf} expression in both \textit{tbx5} and \textit{wnt2b} morphant experiments suggested that \textit{tbx5} and \textit{wnt2b} function in a common pathway with respect to limb development, and that this pathway lies upstream of the FGF signaling network that regulates limb initiation. The notion of a common pathway is further supported by the observation that \textit{tbx5} expression was significantly downregulated in the \textit{wnt2b} morphants (compare Fig. 5G,I with 5H,J,K). We reasoned that a key function of \textit{wnt2b} in the limb initiation process might be to regulate \textit{tbx5} expression. If this were the case, then forced expression of \textit{tbx5} in the \textit{wnt2b} morphants could rescue the fin phenotypes. To address this, we co-injected embryos with the \textit{wnt2b} morpholin and \textit{tbx5} RNA. As shown in Fig. 5C,F and Table 1, co-injection of the \textit{tbx5} RNA could rescue the \textit{wnt2b} morphant phenotypes, such that 18% fewer embryos had a no-fin phenotype (57% as compared with 75% of embryos injected with morpholin alone). Conversely, we could not rescue the \textit{tbx5} morphant phenotypes with \textit{wnt2b} RNA (data not shown). We note that LPM expression of \textit{wnt2b} at the time of fin initiation was normal in the \textit{tbx5} morphants (Fig. 5N). These results advocate a fin initiation pathway that is co-regulated by \textit{wnt2b} and \textit{tbx5}, and suggest that \textit{tbx5} lies downstream of \textit{wnt2b} in this process. We have also observed that a consensus binding site for LeF1, a transcription factor that interacts with \textit{b-catenin} (Roose and Clevers, 1999), is conserved in both human and mouse \textit{Tbx5} genomic sequences, approximately 7.3 kb upstream of the ATG. Deletion of this site results in a nearly 50% decrease in the activation of this promoter by \textit{Wnt2b}, indicating that WNT signaling through the canonical \textit{b-catenin} pathway has the capacity to activate the \textit{Tbx5} promoter (data not shown).

\textbf{\textit{tbx5} is necessary and sufficient for limb induction}

The above results indicate that \textit{tbx5} and \textit{wnt2b} function together to regulate limb initiation and outgrowth. We have previously shown that the WNT2b/\textit{b-catenin} pathway is required and sufficient for limb initiation, and that downregulation of this pathway is able to inhibit limb formation (Kawakami et al., 2001). To further examine the relationship between the WNT signaling pathway and \textit{tbx5}, as well as the evolutionary conservation of this process, we made use of chick embryos, which permit misexpression experiments in a temporally and spatially restricted manner. An adenovirus expressing \textit{Axin} was injected into the LPM of stage 8 chick embryos. \textit{Axin} is a potent, well-characterized negative regulator of WNT/\textit{b-catenin} pathway (Peifer and Polakis,
Tbx5 is conserved between chick and fish. We also observed that injection of the truncated truncations obtained with low levels of the truncations observed was comparable to the zebrafish fin the radius, and the absence of some anterior digits (compare hypoplasia or disappearance of zeugopodal elements, typically to be shared with the endogenous leg, whereas we obtained a range of extra zeugopodal and autopodal elements. The identity of the ectopic structures is difficult to determine because they appear as hybrid structures. However, our findings clearly demonstrate the inductive capacity of Tbx5 during limb outgrowth. We also observed that during the process of ectopic induction, Tbx5, Fgf10, and Wnt2b are led to a downregulation of Fgf10 expression in the treated embryos (Fig. 6C) (80%, n=50). Given these results, we postulate that since T-box transcription factors interact with other members of the transcription machinery to activate target genes (Brunet et al., 2001; Hiroi et al., 2001), removal of the potential interaction domain, but not the DNA-binding domain, may have resulted in a dominant negative form of Tbx5. Taken together, these results suggest that, as in zebrafish, downregulation of Tbx5 function in chick embryos inhibits limb outgrowth and that Tbx5 functions upstream of Fgf10.

The combined results from our zebrafish and chick experiments indicate that normal Tbx5 function is necessary for proper limb initiation. To determine if Tbx5 is sufficient for this process, we injected a retrovirus expressing Tbx5 in the LPM of chick embryos at stage 5-8. While embryos injected with a control virus did not display any obvious abnormal phenotype (data not shown), embryos injected with the Tbx5 retrovirus had additional limb bud-like structures (40%, n=89). These embryos were left to develop further, and the morphological features of the ectopic limb-like structures were examined after cartilage staining. As shown in Fig. 6G,H, Tbx5 overexpression induced additional cartilaginous elements. Stylopodial elements of the ectopic limb-like structure appear to be shared with the endogenous leg, whereas we obtained a range of extra zeugopodal and autopodal elements. The identity of the ectopic structures is difficult to determine because they appear as hybrid structures. However, our findings clearly demonstrate the inductive capacity of Tbx5 during limb outgrowth. We also observed that during the process of ectopic induction, Tbx5, Fgf10, and Wnt2b are...
induced (data not shown), suggesting a de novo deployment of the limb initiation program.

**Fgf10 maintains Tbx5 expression during limb initiation**

The WNT and FGF signaling pathways interact and regulate each other to transfer inductive signals between tissues and cartilage staining revealed additional autopod- and zeugopod-like elements. Beads soaked in this inhibitor were applied to the LPM of stage 15-18 chick embryos. As shown in Fig. 7A, a significant downregulation of Tbx5 was observed in a broad area of tissue surrounding the bead (88%, n=43). Beads soaked in DMSO did not result in any alterations (data not shown). This indicates that FGF signaling is required to maintain expression of Tbx5 during limb outgrowth.

In agreement with the above results, the pattern of tbx5 expression in zebrafish hst mutants is also altered at 26 hpf, when fgf10 is normally expressed in the presumptive fin mesenchyme. In wild-type embryos, tbx5 is expressed in the presumptive fin field of the LPM by 26 hpf, when fin outgrowth initiates (Fig. 7B), and continues in the mesenchyme of the developing fin. In hst mutant embryos, however, tbx5 expression in the LPM is significantly decreased at 26 hpf (Fig. 7C) and is not detected by 48 hpf. Downregulation of tbx5 was also seen in tbx5 morphants (data not shown). Therefore, we examined the ability of fgf10 to maintain tbx5 expression, given the early loss of tbx5 expression in hst mutants. As shown in Fig. 7D, injection of 70 pg of zebrafish fgf10 mRNA into one-cell stage hst embryos resulted in a maintenance of tbx5 expression in embryos examined at 36 hpf (100%, n=213). However, we were unable to rescue fin outgrowth (data not shown), suggesting that functional Tbx5 is required for continuous outgrowth of the pectoral fins. These data further support our hypothesis that fgf10 is involved in maintaining tbx5 expression.

**DISCUSSION**

Vertebrate limb initiation involves signaling mechanisms that mediate the directional transfer of signals between different tissues, such as the intermediate mesoderm, LPM and surface ectoderm. Among these tissues, signals from the LPM trigger the onset of limb outgrowth, by acting on the surface ectoderm. Implantation of the prospective limb field into host LPM can result in an ectopic limb by inducing the AER (Saunders and Reuss, 1974). A mesenchymal signal that is essential for limb outgrowth is the product of the Fgf10 gene. Fgf10 is initially widely expressed in the LPM, but at the time of limb induction, becomes restricted to the prospective limb regions (Ohuchi et al., 1997). Localized expression of Fgf10 is a key factor for limb initiation. This is evidenced by the limbless phenotype of Fgf10−/− mice (Min et al., 1998; Sekine et al., 1999), and the ability of FGF10 to induce an ectopic limb in chick embryonic flank (Ohuchi et al., 1997).

In contrast to limb initiation, the process of limb identification appears to be regulated, at least in part, by the T-box family of transcription factors. Tbx5 plays a role in determining the identity of forelimbs, while Tbx4 determines the identity of hindlimbs (Rodriguez-Esteban et al., 1999a;
the activity is necessary not only for limb initiation, but also to by Ahn et al. (Ahn et al., 2002). The authors demonstrated that the expression of tbx5, could not rescue the loss of pectoral fins in tbx5. It is worth noting that downregulation of tbx5 tyrosine kinase activity in the chick embryo resulted in regulation of tbx5. In agreement with its ability to activate zebrafish, we demonstrate that tubx5 expression in the LPM. In support of this hypothesis, we have previously shown that Wnt2b functions upstream of Fgf10 in the LPM. The results presented here are consistent with the notion that wnt2b also functions upstream of tbx5. First and foremost, tbx5 mRNA can rescue the fin outgrowth phenotype of wnt2b loss-of-function morphants. In contrast, wnt2b mRNA cannot rescue the related phenotype of the tbx5 loss-of-function morphants. Second, although tbx5 expression is downregulated in the wnt2b morphants, wnt2b expression is unaffected in the tbx5 morphants. Third, a requirement for WNT/β-catenin signaling for Tb5x expression was demonstrated in chick embryos. Specifically, Axin, an inhibitor of WNT/β-catenin signaling, blocks Tb5x expression in the LPM. Not surprisingly, we identified a highly conserved Lef1 binding site in the Tb5x promoter, a known element required for β-catenin-dependent transcription (Roose and Clevers, 1999) (data not shown).

Our data suggest that the roles of Wnt2b, Tb5x and Fgf10 are conserved in chick and zebrafish during limb initiation and identity. Yet, recent findings in mouse suggest the existence of a more complex WNT signaling network mediating limb initiation. We have generated mice bearing loss-of-function mutations in the Wnt2b gene and have observed no alteration in limb patterning or outgrowth. Further, we could not detect Wnt2b expression in the LPM of mouse embryos at a time when limb initiation begins (A. R. and J. C. I. B., unpublished data). Additionally, results from others show that Tcf1+/−/Lef1−/− mice lack a reduction in Tb5x expression (B. Bruneau, Hospital for Sick Children, Toronto, personal communication). However, it is possible that other Tcf family members could compensate for loss of the targeted genes. It should be noted that some other mouse Wnt genes are not expressed in comparable structures to those in chick and zebrafish. For example, wnt3a, a gene that is expressed in the apical ectodema ridge (AER) during the process of limb budding in zebrafish (Y. K. and J. C. I. B., unpublished data) and chick (Kengaku et al., 1998), and whose function is necessary for limb outgrowth in those organisms, is not expressed in the AER of mice (Parr et al., 1993). Thus, it is apparent that although the programs of limb initiation and identification are conserved in tetrapods, the molecular action of specific Wnts has diverged. As such, a different, uncharacterized WNT molecule might be expressed in the LPM, comparable to the expression pattern of Wnt2b in chick and zebrafish. Efforts are currently underway to identify and characterize this signaling molecule in mouse.

The results presented here lead us to propose that WNT/β-catenin signaling controls Tb5x expression in the LPM. Tb5x,
in turn, regulates the expression of Fgf10, leading to limb initiation. Last, Fgf10 appears to play a role in maintaining Tbx5 expression, indicating the existence of a feedback loop between these two factors. We cannot exclude the possibility that Tbx5 also regulates WNT signaling via a feedback loop. The interaction of Tbx5 and Fgf10 is well conserved in vertebrate limb development. In mice, loss of Tbx5 function results in both loss of Fgf10 expression and loss of forelimbs (B. Bruneau and M. Logan, National Institute for Medical Research, London, personal communications). While the forelimbs and hindlimbs of all tetrapods share many of the signaling pathways required for their outgrowth and patterning, so far no single transcription factor has been positioned in a molecular cascade that is specifically required for limb outgrowth. Our observations that Tbx5, currently regarded as a limb identity determination gene (Rodriguez-Esteban et al., 1999a; Takeuchi et al., 1999), is involved in the limb initiation process, provide significant insight into the tight linkage observed between limb initiation and limb identity. This may help us to further understand the orchestrated interactions needed during embryogenesis for the outgrowth and identity of other tissues and organs where T-box genes and the WNT and FGF signaling act in concert.

We thank Reiko Aoki, May Chu, Stefan de la Garza, Eduardo Díaz García, Ilir Dubova, Eva Fernández and Harley Pineda for excellent technical assistance and their expertise with zebrafish. We thank Gabriel Sternik for his expert advice in microscopy. We thank Ana Rojas for invaluable computational analyses, Henry Juguilon and Mike Downes for help with the luciferase assays, and Javier Capdevila for insightful comments on the manuscript. We thank Benoit Bruneau for comments and discussions and Benoit Bruneau, Malcolm Logan and Toshihiko Ogura for sharing unpublished data. We are deeply indebted to Marnie Halpern, Mary Mullins and Wolfgang Driever for their kindness and generosity in introducing us to the zebrafish. J. K. N. is partially supported by an NIH training grant and the Chapman Charitable Trust, A. R. is partially supported by a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte, Spain; T. I. is supported by a JSPS Postdoctoral Fellowships for Research Abroad, Japan; C. M. K. is supported by a postdoctoral fellowship from the Canadian Institutes of Health Research, Canada. This work was supported by grants from BioCell, March of Dimes, and the Fundação Calouste Gulbenkian, Fundação para Ciência e Tecnologia, the G. Harold and Leila Y. Mathers Charitable Foundation, and the NIH.

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