Development 129, 211-220 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2763

Specification and determination of limb identity: evidence for inhibitory regulation of *Tbx* gene expression

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Accepted 3 October 2001

SUMMARY

Limb-type-specific expression of *Tbx5/Tbx4* plays a key role in drawing distinction between a forelimb and a hindlimb. Here, we show insights into specification and determination during commitment of limb-type identity, in particular that median tissues regulate *Tbx* expressions. By using the RT-PCR technique on chick embryos, the onset of specific *Tbx5/Tbx4* expression in the wing/leg region was estimated to be stage 13. Specification of the limb-type identity is thought to occur before stage 9, since all explants from stage 9 through 14 expressed the intrinsic *Tbx* gene autonomously in a simple culture medium. The results of transplantation experiments revealed that axial structures medial to the lateral plate mesoderm at the level of the wing region are capable of transforming leg identity to wing identity, suggesting that a factor(s) from the median tissues is involved in the limb-type determination. Nevertheless, the transplanted wing region was not converted to leg identity. The results of the transplantation experiments also suggested that wing-type identity is determined much earlier than is leg-type identity. Finally, we also found that inhibitory effects of median tissues mediate the specific expression of *Tbx5/Tbx4* in the presumptive wing/leg region. We propose a model for limb-type identification in which inhibitory regulation is involved in restricting one *Tbx* gene expression by masking the other *Tbx* expression there.

Key words: *Tbx5*, *Tbx4*, Limb bud, Specification, Determination, Chick

INTRODUCTION

Morphogenesis during development is often prearranged by the influence of localized determinants, by cell-cell interactions, and by inductive and repressive actions of organizing centers. One of the most important and intriguing issues regarding morphogenesis is when and how a particular structure (organ) is specified and determined from a group of cells in a specific region and how the structure can be formed in an appropriate site and position in a body. Limb development, particularly early limb initiation serves as a fascinating model system to investigate this issue. Classical experiments on limb identity (Saunders et al., 1957; Saunders et al., 1959; Stephens et al., 1989) and recent studies on functions of *Tbx* genes (*Tbx5* and *Tbx4*) (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999) have provided many insights into how two characteristic structures (forelimb and hindlimb) are committed from the lateral plate mesoderm in each particular position along the main body axis.

Limbs of a tetrapod emerge from four presumptive limb regions as four limb buds (a set of two forelimb and hindlimb buds). The forelimb and hindlimb buds arise at particular levels of the lateral region along the primary body axis at almost the same time. At the beginning of limb development, structural differences in forelimb and hindlimb buds are not obvious. In chick embryos, the shapes and sizes of forelimb and hindlimb buds appear identical for about 16 hours after the onset of budding. Each limb bud has common regions necessary for limb morphogenesis, such as the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER) (Saunders and Gasseling, 1968; Saunders, 1948; Saunders et al., 1976). Moreover, many genes involved in limb pattern formation are expressed at similar timings and regions in forelimb and hindlimb buds, suggesting that they share common mechanisms and signaling pathways through key molecules in order to construct the ‘limb’ structure. Nevertheless, structures of the forelimb and hindlimb in most tetrapods become completely different with the progress of development. In the chick, forelimb buds finally grow into wings with feathers, and hindlimb buds develop into legs with scales and claws as epithelial structures. The wings and legs also have distinct patterns of skeletal elements, muscles and tendon attachments as mesenchymal features. Many intriguing issues regarding limb-type identification during development remain unresolved: e.g., when the specification and determination of limb identity occurs, whether tissue interactions, inductions or inhibitions from other tissue(s) are involved in the establishment of limb-type identity, and, if so, which tissue(s) and what molecular mechanism are involved in it. Even the results of the following

recent interesting studies on *Tbx* genes showing the molecular nature of limb identity have not resolved these issues.

Limb-type-specific expression of *Tbx5/Tbx4* has been postulated to establish the forelimb/hindlimb identity. In normal chick development, *Tbx5* and *Tbx4* are exclusively expressed in the presumptive forelimb region (and later in the forelimb bud) and the presumptive hindlimb region (and in the hindlimb bud), respectively (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). Early expression of *Tbx5* or *Tbx4* in an ectopic limb bud induced by exogenous FGF corresponds with the later wing or leg phenotype of the additional limb. These limb-type-specific expressions of *Tbx5/Tbx4* are well conserved in other tetrapod embryos, including human (Li et al., 1997), mouse (Chapman et al., 1996; Gibson-Brown et al., 1996), *Xenopus* (Takabatake et al., 2000) and newt embryos (Simon et al., 1997). Paired fin buds in teleost fish have the same character of *Tbx* gene expression (Ruvinsky et al., 2000; Tamura et al., 1999). Furthermore, with chick manipulation systems, it has been demonstrated that wings with leg-like characters and legs with wing-like characters were observed when *Tbx4* and *Tbx5* were introduced into the presumptive hindlimb and forelimb regions, respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). These studies strongly suggest that *Tbx5* and *Tbx4* in the presumptive limb regions and limb buds are responsible for the distinct identities of limbs. However, the upstream mechanism restricting the *Tbx5/Tbx4* expression in each limb bud is not known.

We have examined conditions for the determination of limb identity by using transplantation methods and tissue culture systems with chick embryos. Interestingly, the medial tissues at the anterior level inhibit *Tbx4* expression in the forelimb region as well as activate *Tbx5* expression, and in the meantime, the posterior medial tissues are likely to have repressive action toward *Tbx5* expression in the hindlimb region. Our data presented here enabled us to predict the timing and conditions needed to specify, determine and exhibit each limb identity and the role of medial tissues for determination of the identity.

MATERIALS AND METHODS

Experimental manipulation and tissue explant culture

Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). The lateral plate mesoderm at the level of somites 15-20 and 26-32 was taken as the presumptive forelimb region and presumptive hindlimb region, respectively. Before the somites had formed in these regions, the presumptive regions were estimated according to the limb region map of Chaube (Chaube, 1959).

As graft tissues for transplantations, we used the lateral plate mesoderm of the presumptive limb region and the overlying ectoderm taken from stage 9-14 embryos. Methods of transplantation and relative positions of the host and the graft are shown in Fig. 3A. First, host dorsal medial ectoderm at the level of somite 15 (boundary between the neck and the forelimb) or somite 26 (boundary between the flank and the hindlimb) was cut and partially peeled toward the level of somite 19/20 or 30/31 so as to be separated from underlying tissues. The donor graft was inserted in the cavity of the host and placed between the dorsal medial ectoderm and the underlying neural tube and somites at the level of the forelimb or hindlimb region. After manipulations, the eggs were resealed and allowed to develop in an incubator at 38°C for subsequent analyses by in situ hybridization and skeletal pattern observation. For skeletal pattern observation, embryos were incubated for 7 days after the operation and then fixed in 10% formalin, stained with 0.1% Alcian Blue in 70% acid alcohol, dehydrated in ethanol, and cleared in methyl salicylate.

For explant culture, tissues were taken from stage 9-13 embryos. Regions dissected as explants were similar to those used for the transplantation experiments, as described above, and shown in Fig. 5A and Fig. 6A. They were then incubated in tissue culture dishes (Falcon 3037) for appropriate times (24 or 48 hours) in Ham's F-12 medium supplemented with 10% FCS and antibiotics at 37°C in the presence of 5% CO_2. In order to keep explants floating on the medium and in close contact with each other, they were placed on an Isopore Membrane Filter (Millipore) with a pore size of 1.2 μm.

Whole-mount in situ hybridization

For single staining, whole-mount in situ hybridization (Yonei et al., 1995) and section in situ hybridization (Yoshida et al., 1996) were carried out essentially as described previously. Antisense RNA probes for chick *Tbx5* and *Tbx4* (kind gifts from Dr Izpisua-Belmonte) were prepared as previously described (Isaac et al., 1998). For double staining, fluorescein-labeled and digoxigenin-labeled RNA probes were used, and they were detected by staining with Fast Red and BCIP for substrates of alkaline phosphatase, respectively.

Relative quantitative RT-PCR

RNA was isolated from embryos and explants using RNeasy total RNA isolation kit (Qiagen) and analyzed by RT-PCR essentially as described previously (Kimura and Ide, 1998). *Tbx5*- and *Tbx4*-specific primers yielding product sizes as indicated are: *Tbx5* (369 bp) [forward primer, 5′-AACCCCTACCC GATCTCCCAGG-3′ (22 mer); reverse primer, 5′-GTGAAGTGGGCAGAGAAATG-3′ (20 mer)], *Tbx4* (306 bp) [forward primer, 5′-GCTTCACTTATATGG-TACTCAG-3′ (22 mer); reverse primer, 5′-CACGGTCAA-TGGGGGAAGAAGG-3′ (22 mer)], and *β-actin* (167 bp) [forward primer, 5′-TCTGACTGACCGCGTTACTC-3′ (20 mer); reverse primer, 5′-CCAT CACACCCTGATGTCTG-3′ (20 mer)]. These primer sets were based on the chick *Tbx5* mRNA sequence (GenBank No. AF069396), *Tbx4* mRNA sequence (GenBank No. AF069395) and *β-actin* mRNA sequence (GenBank No. L08165), respectively. Southern blotting and detection were performed with respective DIG-labeled probes according to the procedure described in the manufacturer's instructions, *DIG System User's Guide for Filter Hybridization* (Roche). Signal detection was performed with anti-digoxigenin-AP Fab fragments and its substrate, CDP-star (Roche). For quantitative assay, chemiluminescence intensity of reacting CDP-star in each PCR product was estimated as photostimulated luminescence (PSL) using Molecular Imager System (GS525, BioRad).

To determine the appropriate cycle number for amplification of each gene in the RT-PCR assay, a series of PCRs with several cycles (15-36) was done with cDNA derived from the stage 13 presumptive wing region for *Tbx5* and *β-actin* and the stage 13 presumptive leg region for *Tbx4* (Fig. 1A). For quantitative comparison of these gene expressions, the proportions of the intensity of the band for *Tbx5* and *Tbx4* to that for *β-actin* were calculated from respective PSL values. On standard samples of each figure, the values of the ratio were fixed as 1. Gene expressions in samples were compared using Student's matched-pair *t*-test.

RESULTS

Expression profile of *Tbx5/Tbx4* in presumptive limb regions and limb buds

In previous studies using whole-mount in situ hybridization, *Tbx5* and *Tbx4* were inconsistently detected in developing

chick forelimb and hindlimb regions from stage 12/13/14/15 and stages 14/15, respectively (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). Firstly, using the RT-PCR technique, we investigated in more detail the commencing stage of *Tbx5/Tbx4* expression in order to determine when the forelimb/hindlimb identity is exhibited as *Tbx* gene expression. Total RNA was prepared from stage 9-23 (presumptive) forelimb and hindlimb regions, and the amounts of *Tbx5/Tbx4* transcripts were compared (Fig. 1B,C). Fig. 1D,E show relative expression of *Tbx5* and *Tbx4* when the amounts of *Tbx5/Tbx4* expressions in the stage 9 presumptive wing/leg region was fixed as a standard. *Tbx5* transcripts in the presumptive wing region began to increase at stage 13, and the amount of *Tbx5* transcripts then began to increase as the developmental stage proceeded (Fig. 1D). Similarly, *Tbx4* expression in the presumptive leg region began to rise at stage 13 (Fig. 1E). These data show that specific expression of *Tbx5/Tbx4* in each presumptive limb region begins at stage 13, suggesting that forelimb/hindlimb identity begins to be exhibited from this stage. Interestingly, *Tbx5* expression in the leg region was detectable at a low level in all stages we examined (Fig. 1D), whereas *Tbx4* expression in the wing region was only just detectable or not detectable throughout the stage (Fig. 1E).

Expression of *Tbx5/Tbx4* in cultured explants of the presumptive limb region

The results in Fig.1 demonstrate that *Tbx5/Tbx4* begins to be expressed at stage 13 in the presumptive forelimb/hindlimb region. Before limb identity is exhibited as expression of *Tbx5/Tbx4*, specification and subsequent determination of limb identity should occur. In order to examine when the specification of limb identity occurs, we next observed *Tbx5/Tbx4* expression in isolated and cultured limb-region explants by RT-PCR (Fig. 2A). Presumptive limb regions at stages 9-14 were excised to isolate them from surrounding tissues and cultured for 24 hours. The relative amounts of *Tbx5/Tbx4* in each sample were calculated and

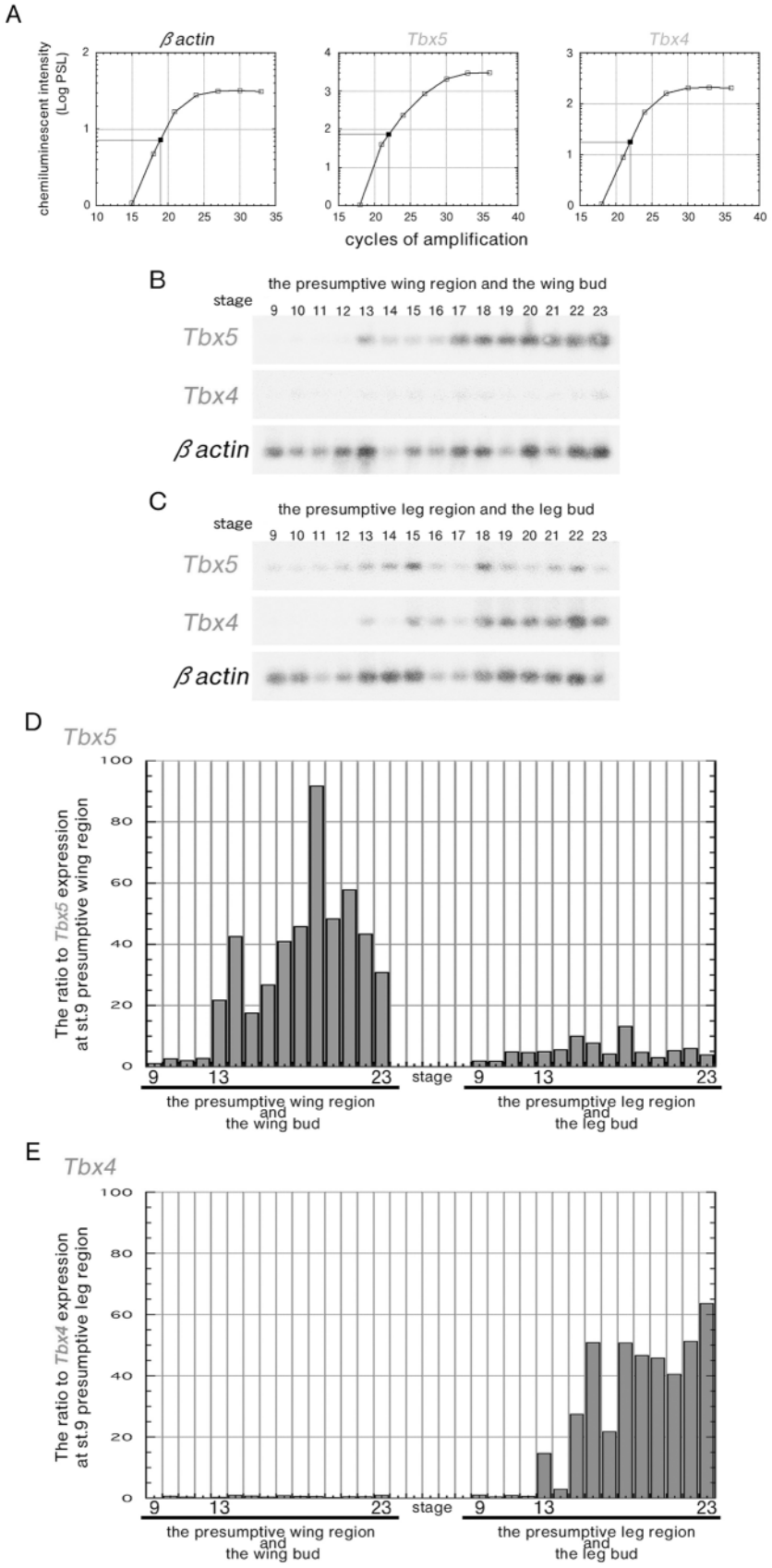

Fig. 1. (A) Amplification profiles for *Tbx5/Tbx4* and *β-actin*. The intensity of PSL of each band was measured using a molecular imager system and plotted on a semilogarithmic scale against the cycle number. The black square depicted in each graph indicates the cycle number used for quantitative analysis of each gene expression. Two independent sets of experiments using different RNAs gave essentially the same results. (B) *Tbx5/Tbx4* expression in the presumptive wing region and the wing bud from embryos of stages 9-23. (C) *Tbx5/Tbx4* expression in the presumptive leg region and the leg bud from embryos of stages 9-23. (D,E) The relative amount of *Tbx* expression after being standardized with *β-actin*. The expression of each gene in the presumptive limb region at stage 9 was taken as 1.0.

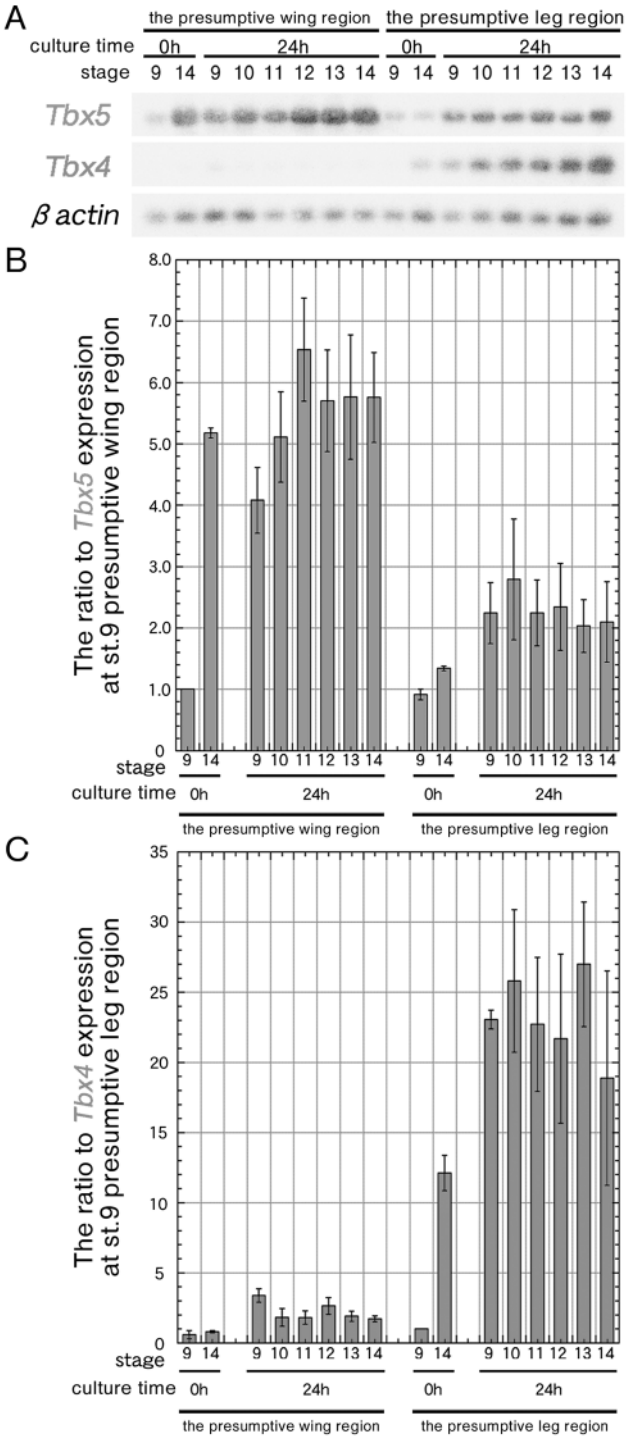

Fig. 2. (A) *Tbx5/Tbx4* expression in cultured explants. Lateral tissues at the level of the presumptive wing and leg regions were prepared from embryos at stages 9 to 14. After incubation for 24 hours, RT-PCR and Southern blot analysis with *Tbx5* or *Tbx4* probes were performed. The relative amount of each gene was plotted (B,C) after being standardized with *β-actin*. The gene expression in stage 9 non-cultured explants was taken as 1.0 for a control. Each experiment was repeated 5 times. Error bars indicate standard deviation.

plotted in Fig. 2B and 2C. The amount of *Tbx5/Tbx4* expression in non-cultured presumptive wing/leg region at stage 9 was defined as the standard. In explants derived from stage 9-12 presumptive wing regions, in which normal *Tbx5* expression had not yet been up-regulated (see Fig. 1D), *Tbx5* expression become abundant after they were cultured for 24 hours, increasing to a level similar to that in non-cultured stage 14 wing explants (Fig. 2B). A high level of *Tbx5* expression in stage 13-14 presumptive wing regions was maintained for 24 hours in the culture system. In the same way, in explants *Tbx4* expression (cultured for 24 hours) in the presumptive leg region at all stages analyzed was much more abundant than that in the non-cultured stage 9 presumptive leg region (Fig. 2C), and each relative amount was comparable to that in non-cultured stage 14 leg explants. These results clearly show that the presumptive wing/leg regions after stage 9 have the inherent potential to express *Tbx5/Tbx4*, suggesting that each limb-type identity has already been specified by stage 9.

Expression of the other *Tbx* gene (*Tbx4* in the presumptive wing region and *Tbx5* in the presumptive leg region) in explants also changed after 24 hours of incubation (Fig. 2B,C). *Tbx4* expression in the presumptive wing region increased after 24 hours, although the relative amount was less than in the leg region cultured for 24 hours (Fig. 2C). In the 24-hour cultured presumptive leg region, the amount of *Tbx5* expression was nearly two-times greater than that in non-cultured stage 9 and 14 leg regions (Fig. 2B).

Transplantation of the presumptive limb region onto the dorsal midline

The next step after the specification of limb identity should be determination of the identity. After determination it is thought that the tissue identity cannot be altered, and the determined cell will follow its fate even when grafted into another part of the embryo.

We transplanted the presumptive wing or leg bud region onto the dorsal midline in median tissues, and we examined whether the surrounding tissues have the ability to alter *Tbx5/Tbx4* expression of the grafted presumptive limb region. The stage 10 presumptive wing/leg region was transplanted onto the dorsal midline at the level of the presumptive leg/wing region (Fig. 3A) and incubated for 48 hours, and *Tbx5/Tbx4* expression in the transplanted limb bud growing from the dorsal region were analyzed by whole-mount in situ hybridization. *Tbx5/Tbx4* expression in the tissue grafted to the same level (wing region to wing level and leg region to leg level) showed no change (all grafted wing buds having only *Tbx5* expression and grafted leg buds having only *Tbx4* expression, data not shown). When the stage 10 presumptive wing region was transplanted to the leg level, all limb buds expressed *Tbx5* (n=8/8, Fig. 3B) and none expressed *Tbx4* (n=7/7, Fig. 3C). However, all grafts of a stage 10 presumptive leg region into the wing level were *Tbx4* positive, (n=9/9, Fig. 3F) although the expression was sometimes weak. Surprisingly, most of the leg grafts in the wing level examined with the *Tbx5* probe showed significant *Tbx5* expression (n=5/8, Fig. 3E), suggesting that the grafted presumptive leg regions were converted to express *Tbx5*. To confirm that *Tbx5* and *Tbx4* are expressed in the same leg transplants at the same time, we performed double staining by whole-mount in situ hybridization on operated embryos after the same surgery. When the stage 10 presumptive wing region was transplanted

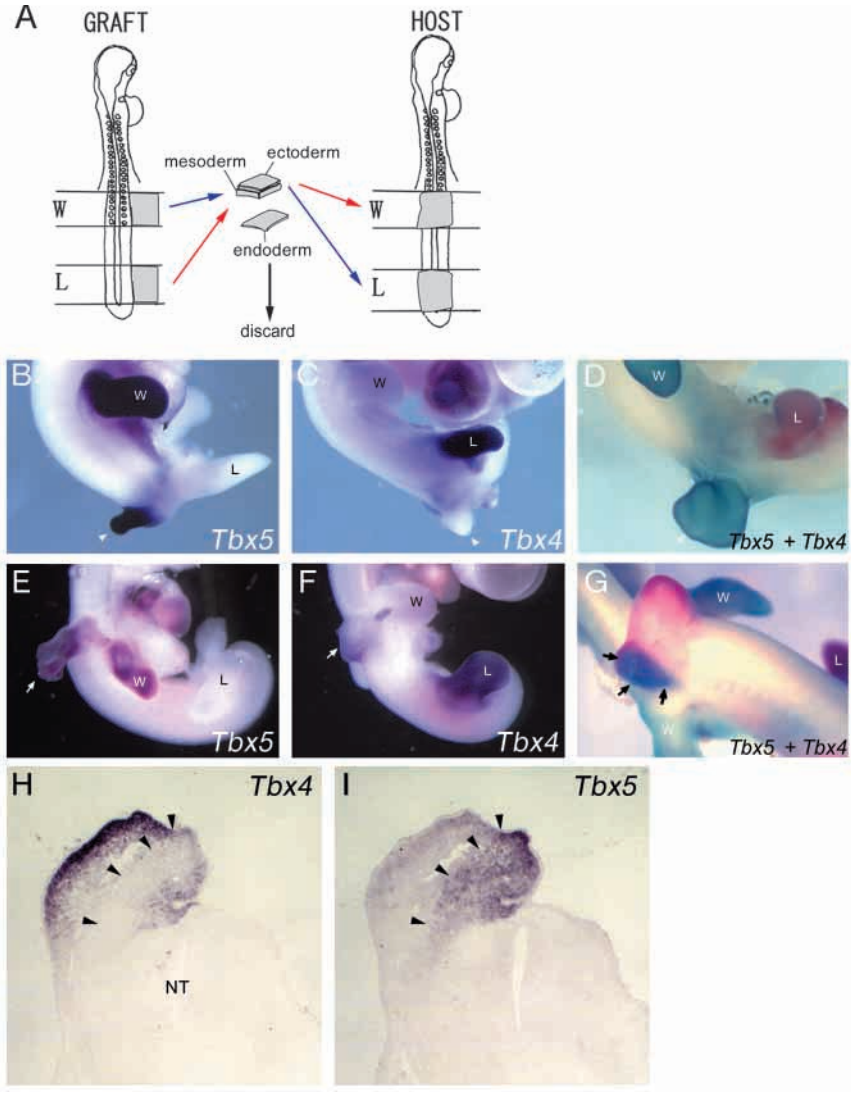

Fig. 3. (A) Schematic representation of the experimental procedure. As graft tissues for transplantations, the presumptive limb region was taken from wing level (W and blue arrows) or leg level (L and red arrows), and the lateral plate mesoderm with overlying ectoderm was separated from endoderm. Then, the donor graft was inserted in the cavity of the host and placed between the dorsal medial ectoderm and the underlying neural tube and somites at the level of the forelimb or hindlimb region. (B) An example of a wing graft in the leg-level median region. *Tbx5* is expressed in the grafted limb bud (white arrowhead) as well as in the host wing bud. (C) Another example of a wing graft in the leg-level median region. *Tbx4* is expressed in the host leg bud but is not detected in the grafted limb bud (arrowhead). (D) Expression of *Tbx5/Tbx4* in a wing graft at the leg level, analyzed by double in situ hybridization. *Tbx5* (turquoise) and *Tbx4* (red) are detectable in the host wing and leg bud, respectively. Only blue staining for *Tbx5* is obvious in the graft (arrowhead). (E) An example of a leg graft in the wing-level median region. *Tbx5* is clearly detectable in the grafted limb bud (arrow). (F) Another example of a leg graft at the wing level. *Tbx4* is detectable in the grafted limb bud. Note that *Tbx4* expression in the leg graft (arrow) appears weaker than that in the host leg bud. (G) Expression of *Tbx5/Tbx4* in a leg graft at the wing level. Chimeric staining with blue (for *Tbx5*) and red (for *Tbx4*) is apparent in the graft (arrows). (H,I) *Tbx5/Tbx4* expression in serial sections of a leg graft at the wing level. In the area to the right of the arrowheads *Tbx4* expression is much weaker than on the left of arrowheads, and the expression of *Tbx5* is reversed.

onto the presumptive leg level, all supernumerary limbs expressed *Tbx5* (in green, Fig. 3D; *n*=18/18), but *Tbx4* was negative in all specimen (in red, Fig. 3D). However, two types of gene expression pattern were observed in specimens of stage 10 presumptive leg region at the wing level. One was only *Tbx4* expression (*n*=4/16, see Table 1), and the other was *Tbx5* expression as well as *Tbx4* expression in a supernumerary limb (*n*=12/16, Fig. 3G) in which *Tbx5* and *Tbx4* seemed to have a mosaic expression pattern. To further examine the detailed distribution of the *Tbx5*-expressing region and *Tbx4*-expressing region in a graft, we prepared serial sections of a supernumerary limb bud derived from the presumptive leg region and stained them with *Tbx5* or *Tbx4*. The region in which *Tbx5* expression was induced in the supernumerary limb nearly corresponded to the region in which *Tbx4* expression was down-regulated (Fig. 3H,I).

We also observed the skeletal pattern of supernumerary limbs. In the case of transplantation of a presumptive wing region to the leg level, supernumerary limbs had a wing-type pattern (*n*=47/67, Fig. 4A) except for some specimens that had unidentified skeletal patterns (*n*=20/67). These unidentified patterns include defective skeletal patterns and wing-like patterns. When stage 10 presumptive leg regions were transplanted onto the wing level midline, half of the resulting supernumerary limbs had wing-type skeletal elements (*n*=6/12, Fig. 4B, see also Table 2). These results are consistent with the results of analysis of *Tbx* expression by in situ hybridization showing that some grafts of the presumptive leg region at the wing level also express *Tbx5*.

Table.1. *Tbx5*/*Tbx4* expressions in the supernumerary limb buds derived from the presumptive leg regions

	Stage of a graft					
Gene	st.9	st.10	st.11	st.12	st.13	st.14
Tbx5	0/14 (0)	0/16 (0)	0/19 (0)	0/19 (0)	0/15 (0)	0/14 (0)
Tbx5+Tbx4	14/14 (100)	12/16 (75)	13/19 (68)	12/19 (63)	2/15 (13)	0/14 (0)
Tbx4	0/14 (0)	4/16 (25)	6/19 (32)	7/19 (37)	13/15 (87)	14/14 (100)

The number in parentheses indicates percentage of expression pattern in grafts of same stage.

Table 2. Cartilage patterns in the supernumerary limb buds derived from the presumptive leg regions

	Stage of a graft				
Phenotype	st.10	st.11	st.12	st.13	st.14
Wing	6/12 (50)	7/22 (32)	1/20 (5)	0/12 (0)	0/13 (0)
Unidentified	2/12 (17)	5/22 (23)	12/20 (60)	8/12 (67)	10/13 (77)
Leg	0/12 (0)	0/22 (0)	0/20 (0)	0/12 (0)	0/13 (0)
No supernumerary limb	4/12 (33)	10/22 (45)	7/20 (35)	4/12 (33)	3/13 (23)

The number in parentheses indicates percentage of cartilage patterns in grafts of same stage.

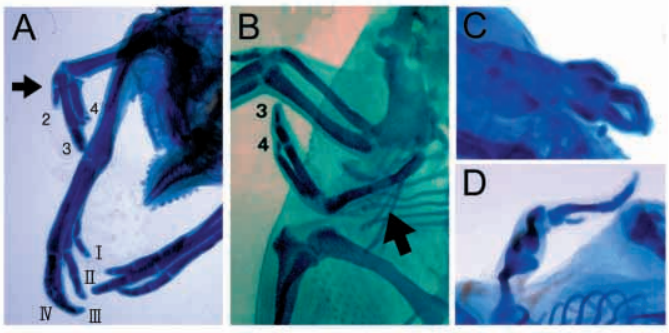

Fig. 4. Cartilage pattern of additional limbs derived from grafted presumptive limb regions. (A) A specimen of a wing graft (arrow) on the leg-level median region has a wing-type skeletal pattern that includes wing digits 2, 3 and 4. (B) A specimen of a leg graft (arrow) at the wing level has a wing-type pattern that includes wing digits 3 and 4. Compare them with leg digits (indicated by I, II, III, IV in A). (C,D) Specimens of a leg graft at the wing level have defective patterns that include leg-like digits. We classified these incomplete types of skeletal patterns (C,D) as unidentified in Table 2.

When stage 9-14 presumptive wing regions were used as grafts (implanted to the leg level), all supernumerary limbs expressed only *Tbx5* (*n*=59), and a leg-type skeletal pattern was not seen (*n*=67). In cases of stage 9-14 presumptive leg region transplantation, younger grafts from stage 9-12 tended to express both *Tbx5* and *Tbx4* together and have a wing-type skeletal pattern, whereas most older grafts from stage 13 and 14 expressed only *Tbx4* and never showed a wing-type skeletal pattern (Table 1, Table 2). However, we did not see any complete leg skeletal patterns but obtained many defective, partial or truncated limbs that were classified into unidentified patterns in Table 2 (see also Fig. 4C,D). It is possible that the wing level midline might affect outgrowth of leg.

In summary, we could alter leg-type gene expression and the skeletal phenotype to wing-type until stage 12, but wing-type gene expression and the phenotype were never converted to leg-type throughout the stages we examined. These results suggest that the presumptive leg region is determined as a leg at around stage 12 and that wing determination occurs very early (before stage 9).

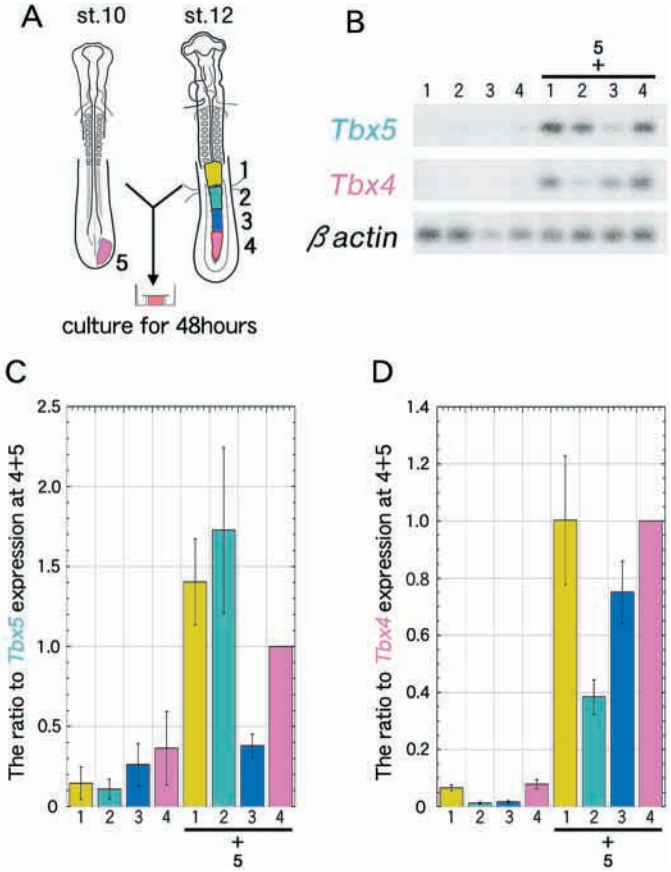

Fig. 5. (A) Schematic representation of the experimental procedure. Regions 1, 2, 3, 4 and 5 represent neck level- (yellow), wing level- (turquoise), flank level- (blue) and leg level- (pink) median tissues of stage 12 and the presumptive leg region of stage 10 (pink), respectively. (B) *Tbx5/Tbx4* expression in cultured explants. Lanes 1, 2, 3 and 4 represent *Tbx5/Tbx4* expression of each level-median tissue when regions 1, 2, 3 and 4 were cultured separately. Lanes 1+5, 2+5, 3+5 and 4+5 represent *Tbx5/Tbx4* expression of each level-median tissues and the presumptive leg region when these two regions were co-cultured. Forty-eight hours after incubation, RT-PCR and Southern blot analyses were performed. The relative amount of expression of each gene was plotted (C,D) after being standardized with *β-actin*. The amount of each *Tbx* gene expression in 2+5 explants was taken as 1.0. Each experiment was repeated 5 times. Error bars indicate s.d..

Effects of presumptive wing/leg level-median tissues on *Tbx5* and *Tbx4* expressions in the presumptive leg region

The results of transplantation experiments suggest that the environment of the midline seems to be able to regulate *Tbx5/Tbx4* expression in the presumptive limb region. In order to try to determine what controls this, we cultured stage 10 presumptive leg regions combined with tissues form various median levels (Fig. 5A, each level of the median tissue including a neural tube, notochord, somites, intermediate mesoderm, overlying ectoderm, and underlying endoderm) for 48 hours. *Tbx5* expression was higher in the combination of the presumptive leg region and presumptive wing level-median tissues (regions 2+5 in Fig. 5C) than in the case of

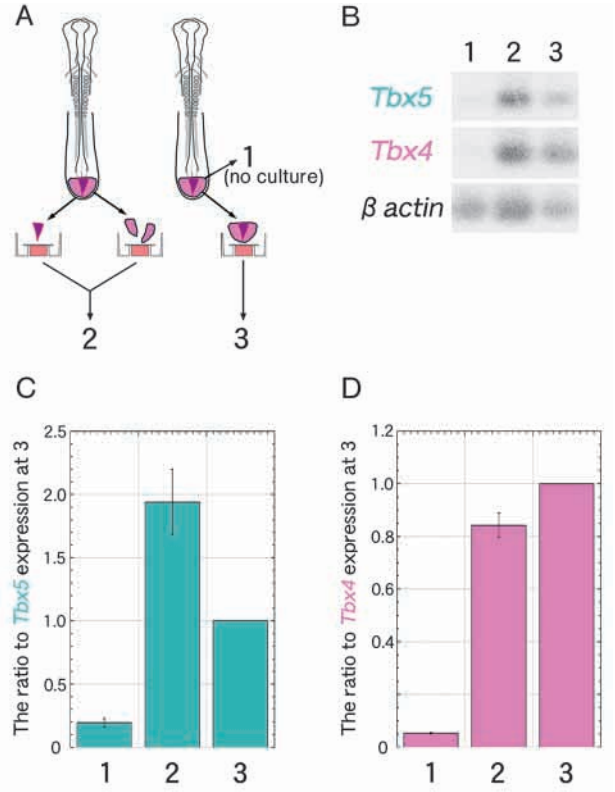

Fig. 6. (A) Schematic representation of the experimental procedure. Sample 1 represents a non-cultured 'unseparated' explant. Sample 2 is a 24-hour-cultured 'separated' explant. Sample 3 is a 24-hour-cultured 'unseparated' explant. (B) *Tbx5/Tbx4* expressions in non-cultured and cultured explants. Lanes 1, 2 and 3 correspond to *Tbx5/Tbx4* expressions in samples 1, 2 and 3, respectively. After incubation, RT-PCR and Southern blot analyses were performed. The relative amount of expression of each gene was plotted (C,D) after being standardized with *β-actin*. The amount of each *Tbx* gene expression in 1 was taken as 1.0. Each experiment was repeated 5 times. Error bars indicate s.d.

combinations with other level-median tissues (1, 3, or 4+5 in Fig. 5C). The relative amount of 2+5 was more than twice that of 4+5 if the amount of *Tbx5* in the median tissues themselves is considered (2 and 4 in Fig. 5C). In contrast, median tissue for the presumptive flank region (limbless space between the wing and leg, 3 in Fig. 5A) inhibited *Tbx5* expression (compare 3+5 with 4+5, with considering lanes 3 and 5 in Fig. 5C). The amount of *Tbx4* expression in the combination 2+5 is appreciably smaller than in any other combinations (Fig. 5D; $P<0.05$). We obtained similar results in samples cultured for 24 hours, although each amount was relatively low (data not shown). The results described above suggest that the presumptive wing level-median tissue has the ability not only to accelerate *Tbx5* expression but also to repress *Tbx4* expression. Although we tested the presumptive wing region with the same combinations, no significant differences were seen in the amounts of *Tbx5/Tbx4* transcripts in all samples (data not shown).

Finally, we investigated whether the presumptive leg level-median tissue has effects on the presumptive leg region. We compared the amount of *Tbx5/Tbx4* expression in cultured 'unseparated' explants (experiment 3 in Fig. 6A-D) that contained the presumptive leg region and leg level-median tissues with that in cultured 'separated' explants (experiment 2 in Fig. 6A-D. The presumptive leg region and leg level-median tissues were cultured separately and then, assayed together). As a control, we determined the amount of *Tbx5/Tbx4* expression in the same region before incubation (experiment 1 in Fig. 6A-D). When they were cultured for 24 hours, expression of both *Tbx5* and *Tbx4* were considerably stimulated in both conditions. The amounts of *Tbx5* expression in experiment 2 and 3 were significantly different (Fig. 6B,C; $P<0.05$). The relative amount of *Tbx5* expression in experiment 2 was about 1.9-times greater than that in experiment 3. These data suggest that the presumptive leg-level median tissues repress *Tbx5* expression.

DISCUSSION

A process by which a cell is committed to a certain fate precedes differentiation, which involves overt changes in morphology, cellular biochemistry and function. The process of the commitment can be divided into two phases (Slack, 1991; Wolpert et al., 1998). The first stage is a labile phase called specification. The fate of a cell or a tissue is considered to be specified when it is capable of differentiating autonomously when placed in a neutral environment such as a Petri dish. At this stage, the commitment is still reversible. The

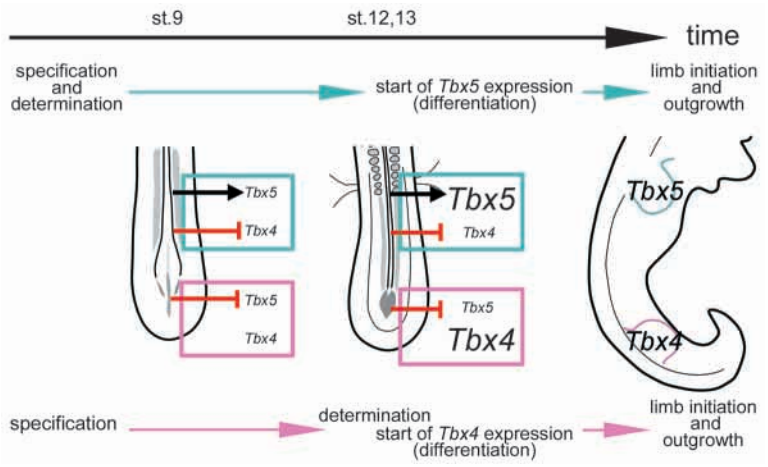

Fig. 7. A schematic model of wing/leg development. Turquoise and pink rectangles in the diagrams of the embryos represent the presumptive wing and leg region, respectively. The size of a letter indicates the degree of gene expression. In the presumptive wing region, wing-type identity is specified and determined before stage 9. This determination is maintained until stage 13, and this region begins to exhibit a wing phenotype, as *Tbx5* expression, at stage 13. In this process, the abilities to induce *Tbx5* and to repress *Tbx4* in the presumptive wing level-median tissues contribute to determination and maintenance of wing-type identity. In the presumptive leg region, leg-type identity is specified before stage 9 and determined at stage 12/13, and this region soon begins to differentiate into a leg bud with *Tbx4* up-regulation at stage 13. *Tbx5*-repressing and *Tbx4*-inducing abilities in the presumptive leg level-median tissues contribute to the determination of leg-type identity.

second stage is termed determination. A cell or tissue is said to be determined when it is capable of differentiating autonomously even when placed in another environment of the embryo. If it is able to differentiate according to its original fate even under these circumstances, it is conceivable that the commitment is irreversible. We discuss specification and determination, which are important concepts of developmental biology, during a process in which each presumptive limb region acquires unique limb-type identity. Insights into the identification of limb type would enable us to consider position-dependent commitment, which includes the differentiation of tissue to produce distinct morphologies and functions (depending on where each cell is positioned). One could see the similar concepts in position-dependent differentiation of motor neurons in the spinal cord [see a review by Sharma and Izpisua-Belmonte (Sharma and Izpisua-Belmonte, 2001)].

Limb-type specification, determination and *Tbx5/Tbx4* expression

Tbx5 and *Tbx4* in the presumptive limb regions and limb buds are responsible for the distinct identities of limbs (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), and hence these two genes can be viewed as 'selector genes' of limb-type identity in vertebrates [see review by Weatherbee and Carroll (Weatherbee and Carroll, 1999)]. In the present study, we have demonstrated that the expression of both *Tbx5* and *Tbx4* began to be up-regulated in each presumptive forelimb/hindlimb region at stage 13 (Fig. 1), soon after limb identity had been determined (see below). Taken together, the results suggest that *Tbx5/Tbx4* expression in each presumptive limb region is one of the earliest phenotypes representing limb-type identity. In other words, the process of commitment to limb-type is possibly complete at around stage 13. This possibility is supported by the results of transplantation experiments, showing the timing of determination of each region. It is likely that the presumptive wing region is determined before stage 9, since wing-type identity (*Tbx5* expression and wing-type cartilage pattern) was irreversible after stage 9 (Fig. 3, Fig. 4). However, the presumptive leg region is thought to be determined at a later stage, stages 12/13, because supernumerary limbs derived from the presumptive leg region between stages 9 and 12/13 were capable of ectopically expressing *Tbx5* and making a wing-type cartilage pattern, but the leg-type phenotype became irreversible after stages 12/13 (Fig. 3, Fig. 4 and Table 1, Table 2). In terms of limb-type specification, the fate of both regions seems to be specified before stage 9, because all presumptive limb regions isolated from stage 9-14 embryos and placed in culture dishes were capable of expressing innate *Tbx5/Tbx4* genes autonomously (Fig. 2). The timings of specification, determination, and *Tbx* gene expression for the commitment process of limb identity are shown in Fig. 7. The stage at which we suggest the presumptive leg region is determined only approximately corresponds with previous study (Stephens et al., 1989). They estimated the limb-forming potential of the presumptive neck-, forelimb-, flank- and hindlimb-level lateral plate mesoderm and suggested that stages 12-14 are characterized by the determination of the specific limb in the future wing and leg. However, they used a different transplantation system; they transplanted each presumptive wing/leg region into the coelomic cavity, where the effects of other tissues would be much less, and they did not estimate the stage by expressions of specific marker genes. Moreover, they did not clearly distinguish between specification and determination. These are thought to be the reasons why estimated stages in their study and those in the present study are somewhat different.

We propose a model for the commitment process of limb identity and its mechanism. The results of co-culture experiments (see Fig. 6) showed that stage 10 presumptive leg level-medial tissues have the ability to repress *Tbx5* expression. This ability probably contributes to determination of leg-type identity in the presumptive leg region. Indeed, a low level of *Tbx5* expression in the leg region can be seen even during normal development (Fig. 1), and it was found to be upregulated in isolated and cultured conditions (Fig. 2). Since *Tbx5* seems to repress *Tbx4* expression (Takeuchi et al., 1999), the ability to repress *Tbx5* expression in the presumptive leg level-medial tissues may be essential for normal leg development. However, stage 12 presumptive wing level-medial tissues stimulated *Tbx5* expression and repressed *Tbx4* expression in co-culture experiments as shown in Fig. 5. Although it remains unclear whether the presumptive wing level-medial tissues directly repress *Tbx4* expression or whether some other molecular cascades are needed for the repression of *Tbx4*, there appears to be some capacity in the dorsal midline to change the fate of the presumptive leg region to the wing phenotype when the tissue is transplanted at the level of the presumptive wing (Fig. 3, Fig. 4). Since the presumptive wing region has already been determined to have wing-type identity by stage 9 as discussed above, it is assumed that these abilities have already been endowed in the presumptive wing level-median tissues earlier than stage 9 in order to determine wing-type identity and that these abilities still remain in later stages (maybe for maintaining the wing-type identity).

Fig. 7 shows a model of limb development at various stages. In the presumptive wing region, wing-type identity is specified and determined before stage 9. This irreversible determination is maintained until stage 13, and this region begins to exhibit a wing phenotype as *Tbx5* expression at stage 13. In this process, the abilities to induce *Tbx5* and to repress *Tbx4* in the presumptive wing level-median tissues contribute to determination and maintenance of wing-type identity. In the presumptive leg region, leg-type identity is specified before stage 9 but is not yet determined at this stage. Determination occurs at stages 12/13, and subsequently, this region begins to differentiate into a leg bud with *Tbx4* up-regulation at stage 13. *Tbx5*-repressing ability in the presumptive leg level-median tissues contributes to the determination of leg-type identity. The period from determination of leg-type identity to *Tbx4* up-regulation in the presumptive leg region is considerably shorter than the period in the presumptive wing region. It should be noted that the inhibitory regulation on the limb-type-specific expression of *Tbx* genes is the major factor in the determination of limb identity.

Possible molecular mechanisms concerned with regulation of limb-type identity

Although little is known about upstream regulators in the lateral plate mesoderm that control the specific expression of *Tbx5/Tbx4*, it is possible that the expression of *Tbx5/Tbx4* is regulated by some transcription regulatory factors in the

presumptive limb region. *Pitx1*, a bicoid-related transcription factor, is possibly an upstream regulator of *Tbx4*, because *Pitx1* expression precedes *Tbx4* expression in leg-region lateral plate mesoderm (Takeuchi et al., 1999), and misexpression of *Pitx1* in the chick wing bud induces ectopic *Tbx4* expression (Logan et al., 1998; Takeuchi et al., 1999). The other candidates for upstream regulators are Hox genes, which represent positional identity and determine the position-dependent morphologies of axial structures along the antero-posterior (AP) body axis. It is thought that each combination of Hox genes (Hox code) acts upstream of *Tbx5/Tbx4* in the determination of limb-type identity for the following reasons. A specific Hox code exists in each presumptive limb region at early stages (Gaunt and Strachan, 1994; Gaunt and Strachan, 1996), limb-type-specific expression patterns of Hox genes in ectopic limbs induced at the flank of the chick embryo mimic the endogenous expression patterns in the limb field (Cohn et al., 1997), and a loss-of-function mutation of a Hox gene, *Hoxb5*, in the mouse shifts the axial position of the limb bud (Rancourt et al., 1995). A recent study on Wnt signaling (Kawakami et al., 2001) demonstrated that new members of the Wnt family, *Wnt2b* and *Wnt8c*, are expressed in the presumptive forelimb and hindlimb regions of the lateral plate, respectively, and that they also have limb-forming ability, suggesting that these factors might control *Tbx* expression in a particular region of the lateral plate.

It should be questioned whether specific expressions of all these molecules for limb-type identification, Hox genes, *Pitx1* and *Tbx5/Tbx4* are regulated autonomously in the lateral plate mesoderm or whether some regulation from other tissues are involved in this process. The results of the present study are the first to suggest that median tissues can regulate *Tbx5/Tbx4* expression and that this regulation includes repressive factor(s) as well as inductive ones. These factors could be diffusible since they can act between discontinuous tissues in a culture condition, although the molecular nature of these regulators remains unknown. It is expected that these factors are specifically or differently expressed at the level of the wing or leg region in the median tissue and possibly regulated by another combination of *Hox* genes that acts to demarcate functional domains of the median tissues such as neural tissue, muscle structure, and skeletal elements along the primary body axis. The imaginary molecules may regulate *Pitx1* and a specific Hox code in the presumptive limb region, resulting in the specific expression of *Tbx5/Tbx4* and limb-type identity. It is possible that combinations of the above molecules may regulate the specific expression of Tbx genes and limb identity, and the possibility that unknown new molecules are also involved in this determination cannot be excluded. Based on the findings presented here, it is concluded that inhibitory regulation from the surrounding tissues is conducting the limb-type-specific Tbx expression.

This work was supported by research grants from Yamada Science Foundation, The Naito Science Foundation, Nissan Science Foundation, and the Ministry of Education, Science, Sports and Culture of Japan.

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