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RESEARCH ARTICLE

Characterization of *Xenopus* Digits and Regenerated Limbs of the Froglet

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***Xenopus* has 4 and 5 digits in a forelimb and hindlimb, respectively. It is thought that their limbs and digits develop in Xenopus by mechanisms that are almost conserved from amphibians to higher vertebrates. This is supported by some molecular evidence. The 5′*hoxd* genes are convenient marker genes for characterizing digits in the chick and mouse. The anteriormost digit is characterized by being *hoxd13*-positive and *hoxd12* (*hoxd11*)-negative in the chick and mouse. In this study, we revealed that the anteriormost digit of the *Xenopus* forelimb is *hoxd13*-positive and *hoxd11*-positive, that is, a more posterior character than digit I. The order of formation of digit cartilages also suggested that *Xenopus* forelimb digit identity is II to V, not I to IV. We have also been interested in the relationship between digit identity and *shh*. The anteriormost digit develops in a *shh*-independent way. A limb treated with cyclopamine (a *shh* inhibitor) has a gene expression pattern (*hoxd11*-negative) similar to that in *shh*-deficient mice, suggesting that a hindlimb treated with cyclopamine has a digit I character. However, a *Xenopus* froglet regenerate (spike), which lacks *shh* expression during its regeneration process, does not have such an expression pattern, being *hoxd11*-positive. We investigated *hoxd11* transcriptions in blastemas that formed in the anteriormost and posteriormost digits, and we found that the blastemas have different *hoxd11* expression levels. These findings suggest that the froglet limb blastema does not have a mere digit I character in spite of *shh* defectiveness and that the froglet limb blastema recognizes its positional differences along the anterior-posterior axis. *Developmental Dynamics 235:3316–3326, 2006.* © 2006 Wiley-Liss, Inc.**

Key words: *Xenopus*; digit identity; *hoxd11*; limb; regeneration and spike

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INTRODUCTION

Appendage regeneration is common among adults of many non-vertebrate organisms, but among adult vertebrates, it is unique to urodele amphibians (newts and salamanders). Urodela amphibians can also regenerate a missing part of the body, such as a lens, limb, tail, and brain, after amputation. The capacity to regenerate a limb is very high in amphibians but practically absent in other tetrapods despite similarities in developmental pathways and ultimate morphology of tetrapod limbs. Many studies have suggested that regeneration is the imitation of a developmental process. Some gene expression patterns support this idea. Bryant et al. divided the regeneration process into two phases: a preparation phase, which is unique to regeneration, and a redevelopment phase, which is similar to limb development (Bryant et al., 2002; Endo et al., 2004). Indeed, some unique phenomena and gene expression have been observed in the preparation phase. For instance, dedifferentiation was observed only during the preparation phase, and expressions of *hoxa13* and *hoxa9* do not follow the rule of spatial colinearity observed in developing limbs (Gardiner et al., 1995). The mechanisms of the preparation phase have been studied, but most of them are unclear. In the redevelopment phase, regeneration blastema expresses genes seen in developing limbs. For instance, a nested expression pattern of *hoxa13* and *hoxa9* recovered as seen in the developing limb (Gardiner et al., 1995).

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Limb regeneration is believed to be completed via redevelopment of the limb.

Limb development occurs along three axes: the anteroposterior (AP) axis, the dorsoventral aixs, and the proximodistal axis. In the case of the AP axis, *shh-gli3* signaling is essential for subsequent patterning (Capdevila and Izpisua Belmonte, 2001; Niswander, 2003). Several 5′*hoxd* genes are expressed in the posterior-distal domains of the early limb bud mesoderm. *Hoxd11–13* are also expressed in a *shh*-dependent fashion in the forming autopod of chickens and mice along the AP axis, and they play roles in the regulation of the digit number and the pattern downstream of *Shh-gli3* signaling (Dolle et al., 1989; Nelson et al., 1996; Zakany and Duboule, 1999). *Hoxd* genes are expressed with spatial and temporal colinearity along the AP axis. 5′*Hoxd* genes are expressed in overlapping, nested, posterior, and distal zones of the limb bud colinearly with their chromosomal order, and polarizing grafts induce mirrorimage duplicated *Hoxd* expression domains (Izpisua-Belmonte et al., 1991; Nohno et al., 1991). In the autopodium, *Hoxd13* was found to be expressed through digits I to V, whereas *Hoxd12* and *hoxd11* were not observed in digit I (Vargas and Fallon, 2005). Based on these observations, digit I identity was assumed to be *Hoxd13*-positive and *Hoxd12*-negative. The pattern of different digits (I to V) that form from the anterior (digit I, i.e., thumb) to posterior (digit V, i.e., little finger) is controlled by secreted Shh signals produced in the posterior limb bud mesoderm (Ingham and McMahon, 2001; Mariani and Martin, 2003; Tickle, 2003). *Shh* regulates both digit number and identity in a dose-dependent manner; increasing levels of *Shh* expanding digit-forming capacity and specifying to more posterior digit identities (Lewis et al., 2001; Yang et al., 1997). A comparison of *shh*-deficient mutant and wild-type expression patterns of *Hoxd11–13* suggested that digit I fate is independent of *shh* signaling (Chiang et al., 2001). Therefore, it can be assumed that digit I is formed by a mechanism that is slightly different from that by which other digits are formed. Whether digit I can be determined as having a *Hoxd13*-positive and *Hoxd12*-negative character has been argued on the basis of both morphological and molecular data. Little is still known about how 5′*hoxd* genes regulate AP pattern formation and then digit identities.

Xenopus laevis can regenerate a limb as a muscleless spike after metamorphosis (Dent, 1962; Satoh et al., 2005a). Whether this patternless feature is due to deformation of the three axes is unknown. Regarding the DV axis, *Xenopus lmx-1* is expressed in the dorsal mesenchyme of limb buds and is not detectable in a froglet blastema, suggesting that correct DV axis is not regenerated (Matsuda et al., 2001). In contrast, Tassava reported that a spike develops nuptial pad tissue only in the ventral side of reproductively mature males (Tassava, 2004). This suggests that the DV axis is not completely lost. The AP axis appears to be defective because *shh* transcription was not observed (Endo et al., 2000). *Hoxa13* and *Hoxa11* are both expressed from early stages of the blastema after amputation until redifferentiation begins in regenerating *Xenopus* limbs, suggesting that there is an attempt to reestablish the PD axis (Endo et al., 2000; our laboratory data). However, no skeletal structures supporting the existence of axes have been observed in a froglet spike. Forelimb and hindlimb blastemas of the *Xenopus* froglet express *tbx5* and *tbx4*, respectively (Suzuki et al., 2005; our laboratory data). Additionally, a hindlimb spike sometimes has a nail (Fujikura and Inoue, 1985). It is speculated that there is forelimb or hindlimb identity in the froglet blastema, although forelimb and hindlimb blastemas would regenerate quite similar structures (spikes). It is possible that the blastema has only forelimb or hindlimb identity and does not have other positional value, e.g., digit identity.

In the present investigation, we isolated the *Xenopus hoxd11* gene and investigated its expression pattern in the developing forelimb and hindlimb. *Hoxa13, hoxd13, hoxd11, tbx3*, and *sox9* expressions were detectable in both autopodiums, and their expression patterns suggested a unique idea about *Xenopus* digit identities. Based on Vargas and Fallon's insight (Vargas and Fallon, 2005), the expression pattern observed in the *Xenopus* developing forelimb suggested that identities of forelimb digits are II to V and that those of the hindlimb are I to V. We also investigated the AP axis in the froglet blastema, and we found a difference between gene expression in a spike and loss of *shh*-signaling phenotype of the *Xenopus* tadpole, indicating that spike formation is not merely a *shh*-deficient phenotype. Digit amputation experiments demonstrated that there was a difference along the AP axis. Anterior digit amputation resulted in the formation of a spike with a nail, and posterior digit amputation resulted in the formation of a spike without a nail. Gene expression indicated that anterior and posterior blastemas recognize their positions along the AP axis. These results provided a new insight into *Xenopus* limb regenerating blastema.

RESULTS

Hoxa13, d13, d11, tbx3, and *sox9* Expression Patterns in Both the Forelimb and Hindlimb

5′*Hoxd* genes are expressed along the AP axis in the chick and mouse (Tarchini and Duboule, 2006; Zakany and Duboule, 1999). However, the results of a PCR study have suggested that *Hoxd12* is absent in frogs and possibly other amphibians (Mannaert et al., 2006). We, therefore, investigated hoxd13 and hoxd11 expressions in Xenopus limbs. First, we tried to isolate the *Xenopus hoxd11* gene since the *hoxd11* sequence was not found in a database. By referring to genome information on *Silurana tropicalis*, we designed specific primers for *hoxd11* and then succeeded in isolating *hoxd11* by RT-PCR (Fig. 1A). *Xenopus Hoxd11* has a low sequence identity to others at the nucleotide level but has 61% amino acid identity to reported sequences for the axolotl and chick (Fig. 1B). Therefore, we decided that the gene we isolated is *Xenopus hoxd11*.

We used *hoxa13* expression as an autopodium marker. At the paddle stage of hindlimb bud development (stage 52 limb bud), *hoxa13* was detectable throughout the distal mesen-

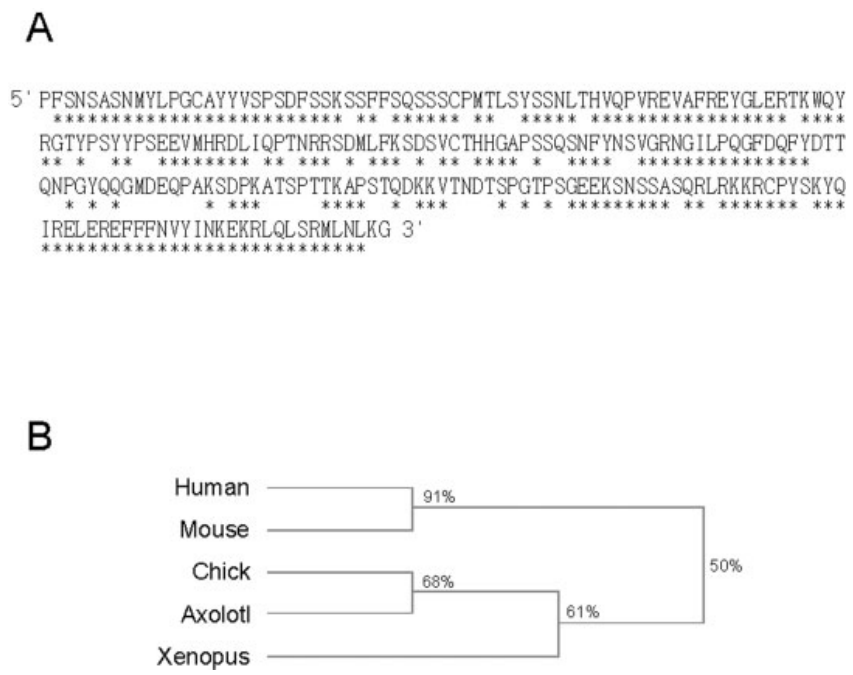


Fig. 1. **A:** Deduced amino acid sequence of *Xenopus* HoxD11. An asterisk marks amino acid residues identical with those in chicks, mice, and humans. **B:** A phylogenetic tree, constructed by the neighbor-joining method, illustrating the relationship of other HoxD11 proteins.

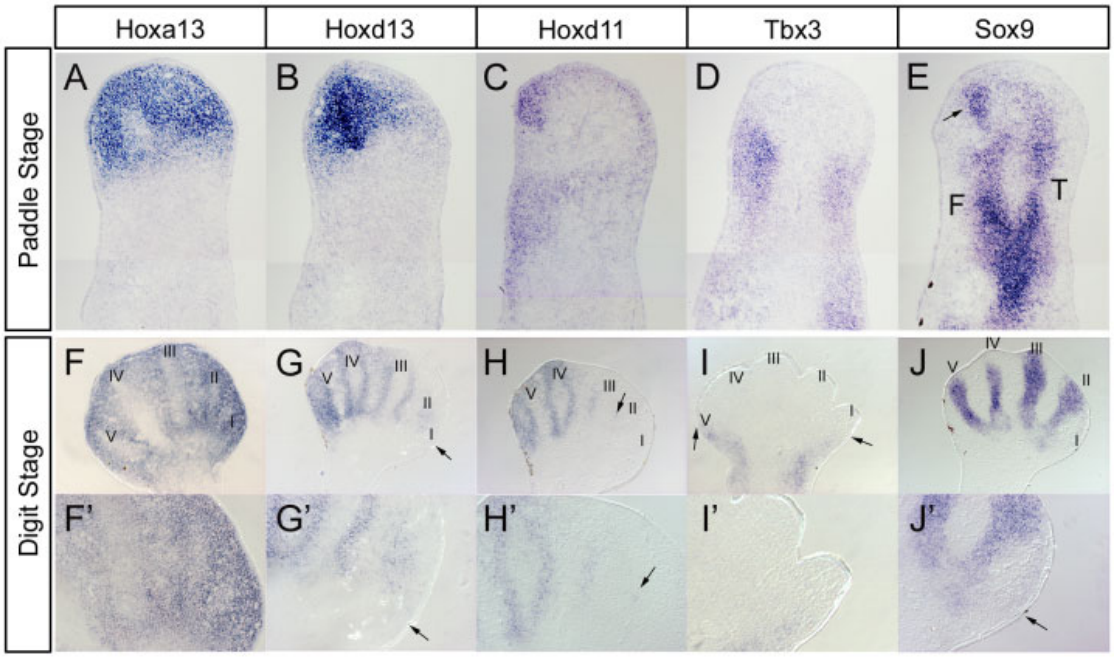
chyme as was reported for the chick and mouse (Fig. 2A)Haack and Gruss, 1993; Yokouchi et al., 1991). Expressions of *hoxd13* and *hoxd11* were detected and the signals of both *hoxd* genes were stronger in the posterior mesenchyme (Fig. 2B,C). Expression borders of *tbx3* appeared to coincide with the proximal end of digits I and V (Fig. 2D). *Tbx3* expression has already been reported by Takabatake et al. (2000). Comparing *hoxa13* and *tbx3* expressions, *hoxd13* transcription was detected in the putative digit I region. *Hoxd11* expression was also detected in the digit I region. However, the *hoxd11* expression pattern in the autopodium was different from the expression patterns in the chick and mouse. Subectodermal mesenchymal cells expressed *hoxd11* from the digit I to V region, and posterior expression seemed to be stronger than anterior expression. *Sox9* could be used as a cartilage marker gene (Fig. 2E) (Satoh et al., 2005b). It was observed that aggregation of sox9-expressing cells started in the autopodium at this stage. Differentiation of each digit cartilage starts and the first digit to be formed would be digit IV (Fig. 2E, arrow). Two cartilage elements, tibia-fibula and/or tibiale-fibulale, were observed in a more proximal region.

In the digit stage of hindlimb bud development (stage 54 limb bud), *sox9* expression was observed in the digit region, where there are 4 obvious aggregations of *sox9*-expressing cells and 1 vague *sox9*-expressing domain (Fig. 2J,J'). *Hoxa13* transcription was downregulated in the putative digit cartilage and was maintained in the interdigital region throughout the autopodium (Fig. 2F,F'). *Hoxd13* was still expressed across the whole footplate except for the digital region (Fig. 2G); however, the posterior domain expressed *Hoxd13* at a higher level, as reported by Nelson et al. (1996) in chick limbs. Higher magnification shows the expression of *hoxd13* in putative digit I (Fig. 2G'). As for *hoxd11*, although *hoxd11* transcription was observed in the posterior domain, *hoxd11* was never observed in the putative digit I domain (Fig. 2H,H'). A vestigial signal of *hoxd11* expression was observed in only the digit II region (Fig. 2H', arrow). The border of tbx3 expression revealed the border of the autopodium and zeugopodium (Fig. 2I,I'), supporting the speculation that digit I of *Xenopus laevis* has a character of *hoxd13*-positive and *hoxd11*-negative.

Expression patterns in the forelimb

Fig. 2. Gene expression in the *Xenopus* developing hindlimb. A–E: Gene expression in the forelimb bud at the paddle stage (Nieuwkoop and Faber stage 52). **A:** *Hoxa13* expression. Digit cartilage started to differentiate and *hoxa13* expression was downregulated (compare with 3E). **B:** *hoxd13* expression. In the anterior domain, weak but clear expression of *hoxd13* was detected. **C:** *Hoxd11* expression. The expression pattern was similar to the pattern in the forelimb (compare with 2C, 3C). **D:** *Tbx3* expression. Expression of *tbx3* was present in both the anterior and posterior margins. **E:** *Sox9* expression. The first differentiating digit cartilage was observed (arrow). T; tibia (tibiale). F; fibula (fibulare). F–J: Gene expression in the forelimb bud at the digit stage (Nieuwkoop and Faber stage 54). **F:** *Hoxa13* expression. *Hoxa13* expression remained throughout the autopodium. **G:** *hoxd13* expression. In the anterior domain, weak but clear expression of *hoxd13* was still detectable (arrows in G,G'). **H:** *Hoxd11* expression. Arrows in H and H' indicate the expression border. **I:** *Tbx3* expression. Arrows in I indicate the expression border. **J:** *Sox9* expression. The cartilage of putative digit I was just starting to express *sox9* (arrow in J'). F'–J': Higher magnification of each figure. II–V are the digit numbers suggested by our results. Distal is to the top, and anterior is at the left.

Fig. 3. Gene expression in *Xenopus* developing forelimb. A–E: Gene expression in the forelimb bud at the paddle stage. **A:** *Hoxa13* expression. **B:** Hoxd13 expression. *Hoxd13* expression in the posterior region was stronger than that in the anterior region. **C:** *Hoxd11* expression. *Hoxd11* expression was detected in the autopodium. The expression domain in the autopodium was restricted to the subectodermal region. *Hoxd11* was detectable in the anteriormost region of the autopodium (arrow). **D:** *Tbx3* expression. Expression of *tbx3* was present in both the anterior and posterior margins. **E:** *Sox9* expression. R, radius; U, ulna. The first differentiating digit cartilage was observed (arrowheads). F–J: Gene expression in the forelimb bud at the digit stage. **F:** *Hoxa13* expression. The distal region of each digit still expressed *hoxa13* at this stage. **G:** *Hoxd13* expression. The *hoxd13* expression pattern was similar to the *hoxa13* expression pattern. **H:** *Hoxd11* expression. *Hoxd11* expression was detected in the anteriormost digit (arrows). **I:***Tbx3* expression. Arrowheads in I' indicate the expression border. **J:** *Sox9* expression. *Sox9* expression was detectable in digit cartilages. A'–J': Higher magnification of each figure. Arrowheads in I' indicate the *tbx3* expression border. II–V are the digit numbers suggested by our results. Distal is to the top, and anterior is at the left. Roman numbers in F–J represent putative digit numbers.


Hoxa13
Hoxd13
Hoxd11
Tbx3
Sox9
Paddle Stage
Digit Stage
A
B
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F'
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H'
I'
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Fig. 2.

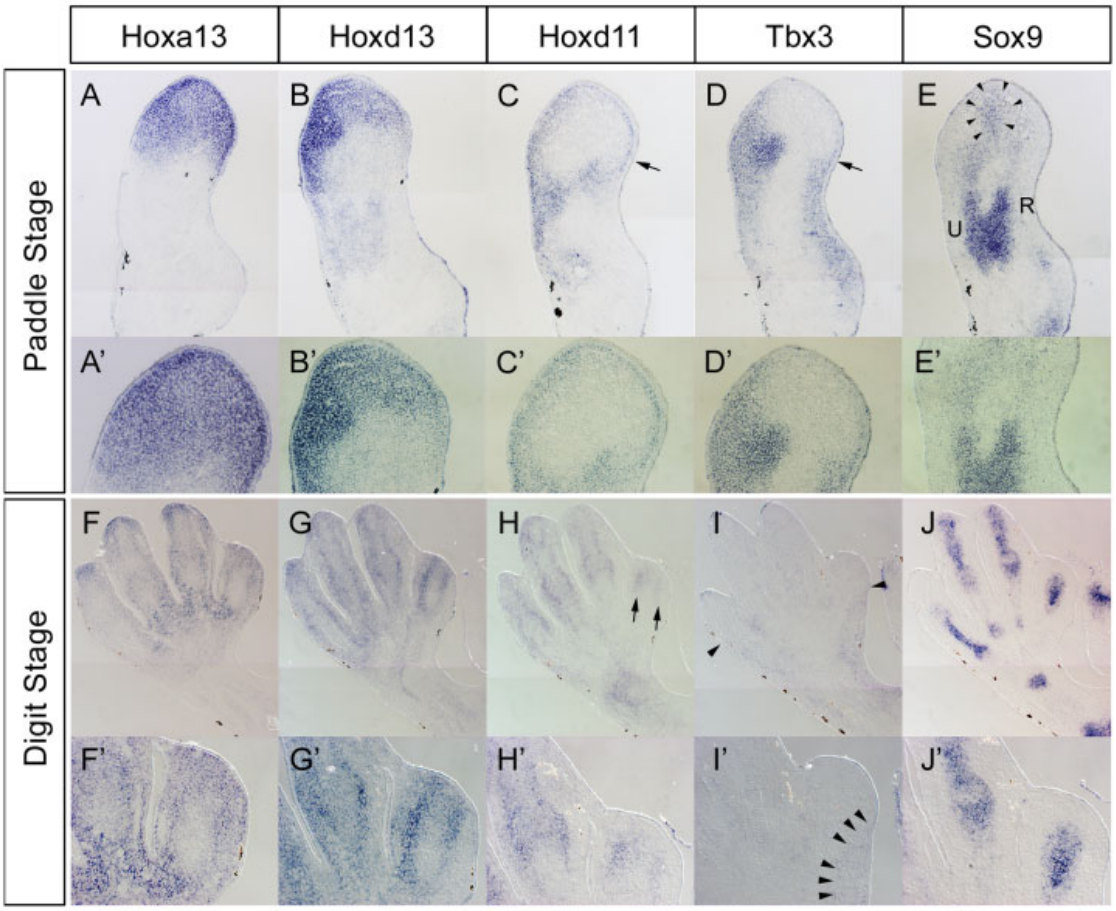
Hoxa13
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Fig. 3.

bud were slightly different from those in the hindlimb bud. In the forelimb at the paddle stage (stage 54), *hoxa13* and *hoxd13* expressions were detected throughout the autopodium (Fig. 3A,A',B,B'). Anterior expression of *hoxd13* seemed to be weaker than posterior expression. It is noteworthy that *hoxd11* expression was observed in the autopodium (Fig. 3C,C'). Subectodermal mesenchymal cells expressed *hoxd11*, but the putative metacarpal region, which is the medial region of the autopodium, did not express *hoxd11*. This expression pattern in the early limb bud seems to be unique in *Xenopus*. *Hoxd11* was detectable in the anteriormost region of the autopodium (Fig. 3C, arrow). *Tbx3* expression revealed the borders of both the anteriormost and posteriormost regions (Fig. 3D,D'). Cartilage was observed in the ulna and radius region but not in the autopodium at the paddle stage. However, *sox9* expression was observed in the autopodium (Fig. 3E,E'). The first digit to initiate cartilage formation seems to be the second digit from the posterior (Fig. 3E, arrowheads). By the digit stage, digit cartilages, which are aggregates of *sox9*-expressing cells, started to differentiate (Fig. 3J,J'). *Hoxa13* and *tbx3* were expressed as seen in the hindlimb (Fig. 3F,F',I,I'). Both *hoxd13* and *hoxd11* expressions were observed in the anteriormost digit (Fig. 3G,G',H,H'). These expression patterns indicate that the anteriormost digits of the hindlimb and forelimb have different digit identity because the anteriormost digit of the forelimb is *hoxd11*-positive and the anteriormost digit of the hindlimb is *hoxd11*-negative.

Anterior-Posterior Axis in Regenerating Froglet Blastema

Vargas and Fallon suggested that digit I has a *hoxa13*-positive and *hoxd12*-negative (*hoxd11*-negative) character (Vargas and Fallon, 2005). *Shh* mutant mice have posterior digit deficiency. The hindlimb digit of this mutant mouse was assumed to be digit I, in view of gene expression (*hoxd13*-positive and negligible levels of *hoxd11* and *d12*) (Chiang et al., 2001). Inhibition of *shh* by cyclopamine (a *shh* inhibitor) in the regenerating axolotl limb resulted in the formation of a spike-like structure in a dose-dependent manner (Roy and Gardiner, 2002). In *Xenopus*, it has been reported that a spike of *Xenopus* froglet limb regeneration has a *shh*-deficient feature (Endo et al., 2000). Therefore, it could be assumed that a *Xenopus* spike structure is caused, at least in part, by a *shh* defect.

To determine whether the spike phenotype in *Xenopus* is equivalent to the phenotype of *shh* inhibition, we performed a *shh* inhibition experiment and in situ hybridization in order to compare gene expression. First, we compared the cartilage patterns in the normal limb and spike (Fig. 4). Outwardly, the hindlimb and forelimb spikes showed similar forms. In the *shh* mutant mouse, the lengths of the forelimb and hindlimb were different. Furthermore, proximo-distal growth of the limb seemed to be inhibited in *shh* mutant mice compared with that in the normal mice (Chiang et al., 1996, 2001). However, the *Xenopus* spike had a well-developed structure in the proximo-distal direction (Fig. 4B,D) (Tassava, 2004). These findings suggest that the two phenotypes are not equivalent. To confirm this, we performed a *shh* inhibition experiment on a regenerating hindlimb of the *Xenopus* tadpole (Fig. 5). Tadpoles with limbs amputated at ankle level were raised in 2 μg/ml cyclopamine and fixed after 2 weeks. Growth along the AP axis was inhibited in cyclopamine-treated limbs (n = 5/5, Fig. 5A,B). In situ hybridization revealed that the cyclopamine-treated limbs expressed *hoxa13* (Fig. 5C). *Hoxd13* was also detected in the distal regions of the limbs, but the expression level was low (Fig. 5D). *Hoxd11* transcription was not detected by in situ hybridization (Fig. 5E). Cartilage differentiation was observed in the limbs (Fig. 5F). These findings suggest that the *shh* mutant phenotype and cyclopamine-treated limb in the *Xenopus* tadpole were similar in the gene expression pattern.

The expression patterns of *Hoxa13* and *sox9* in a froglet blastema have already been reported and are included here only for comparison (Christen et al., 2003; Lombardo and Slack, 2001; Satoh et al., 2005a,b). In the froglet blastema, *hoxa13* was observed (Fig. 6A). Both *hoxd13* and *hoxd11* were also detected (Fig. 6B,C). Tbx3 was observed in the froglet blastema, implying that correct AP and PD polarity was not established in the blastema (Fig. 6D). To confirm our results, we performed RT-PCR on cDNA prepared from hindlimb and forelimb blastema (Fig. 6F). Expressions of all *hox* genes were confirmed. The gene expression observed in the froglet blastema seemed to be different from those in both the *shh* mutant and cyclopamine-treated limb phenotype. The froglet blastema expressed *hoxd11*, but neither the *shh* mutant nor the cyclopamine-treated limb expressed *hoxd11*. Our results support the idea that the spike and *shh* mutant phenotype are not the same.

Reestablished Digit Identities in Regenerated Digits of a Hindlimb

The gene expression pattern and structural features of a *Xenopus* froglet blastema suggest that it does not reestablish a correct AP axis. However, we speculated that blastema cells could not only reestablish the correct AP axis but had AP positional value in accordance with an amputation stump. To investigate this, we amputated hindlimb digits of the *Xenopus* froglet because there is a clear difference along the AP axis in froglet hindlimb digits: the anterior three digits each have a nail, whereas the posterior digits do not. If there is no identity, the blastema on the digit stump would not reform nails in the anterior three digits correctly because nails are induced in epidermis by mesenchymal-epidermal interaction (Hamrick, 2003; Kanzler et al., 1997; Yokoyama et al., 1998). We amputated digits at two levels, the phalanges level (amputated at the middle of second phalanges from distalmost) and metacarpal level. The same results were obtained for amputations at the two levels: the posterior two digits did not reform nails (Fig. 7A,B,F). These blastemas derived form the anterior three digit reformed nails (n = 15/15 at the phalanges level, 4/6 at the metacarpal level). Alcian blue staining revealed that the digit blastema makes a spike without any joints (Fig. 7C–E). This result indicates that reestablishment of

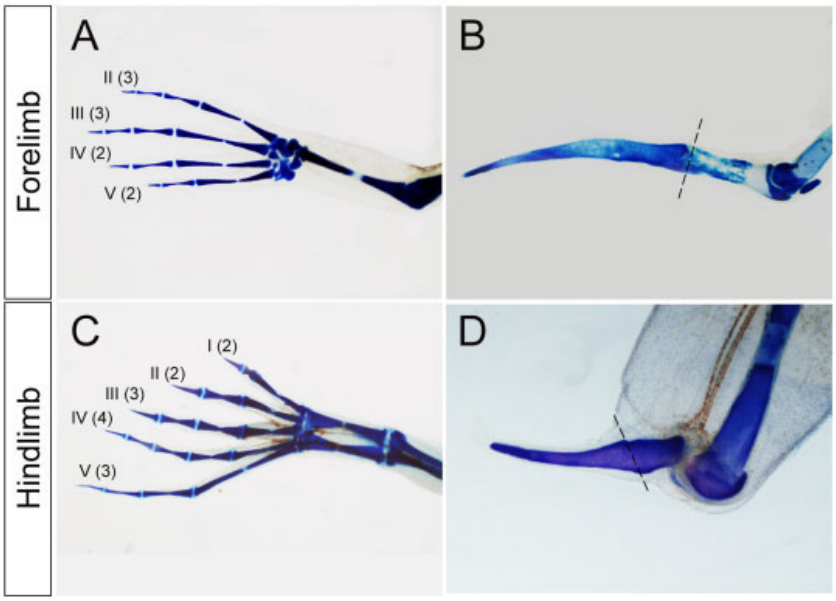


Fig. 4. Skeletal patterns of normal limb and spike. **A:** Normal forelimb of the froglet. **B:** Forelimb spike. **C:** Normal hindlimb of the froglet. **D:** Hindlimb spike. A comparison of forelimb and hindlimb spikes shows that they have similar structures. The numbers in parentheses are the numbers of phalanges. Broken lines in B and D indicate the amputation site.

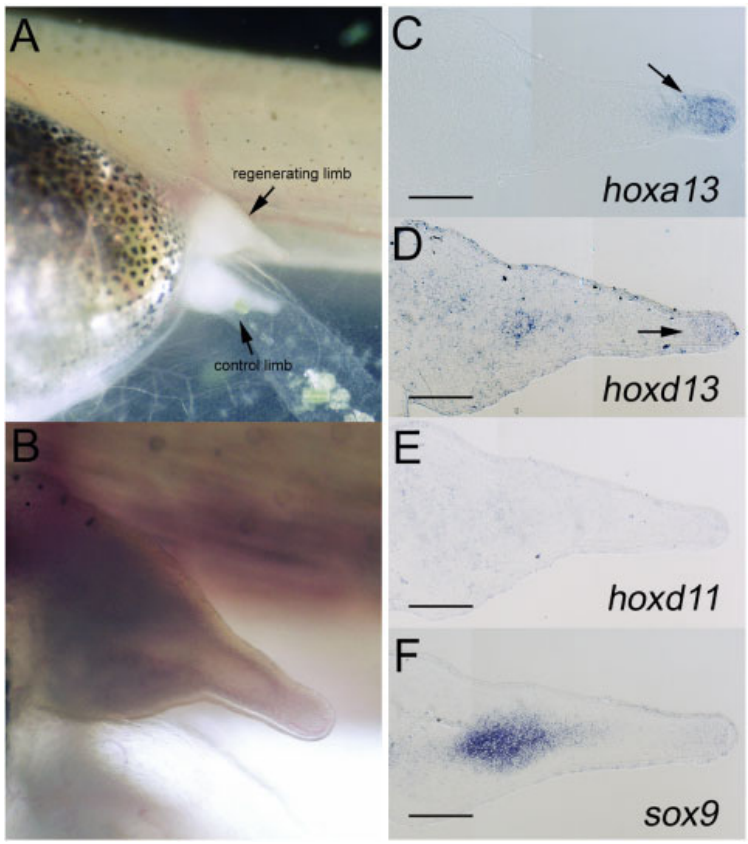


Fig. 5. Skeletal preparation of hindlimb regenerate and normal limb bud treated with cyclopamine. **A:** Lateral view of cyclopamine-treated limb bud and regenerate. Amputation was performed at the paddle stage (stage 52), and then samples were reared in 2 μg/ml cyclopamine. **B:** Higher magnificent of A. C–F: Gene expression of cyclopamine-treated blastema. **C:** *Hoxa13* expression was detected in the distalmost region (arrow). **D:** *Hoxd13* transcription was also weakly detected (arrow). **E:** *Hoxd11* expression was not observed in the cyclopamine-treated sample. **F:** *Sox9* expression. The expression profile of these seems to be similar to that in a limb of the *shh*-deficient mouse.

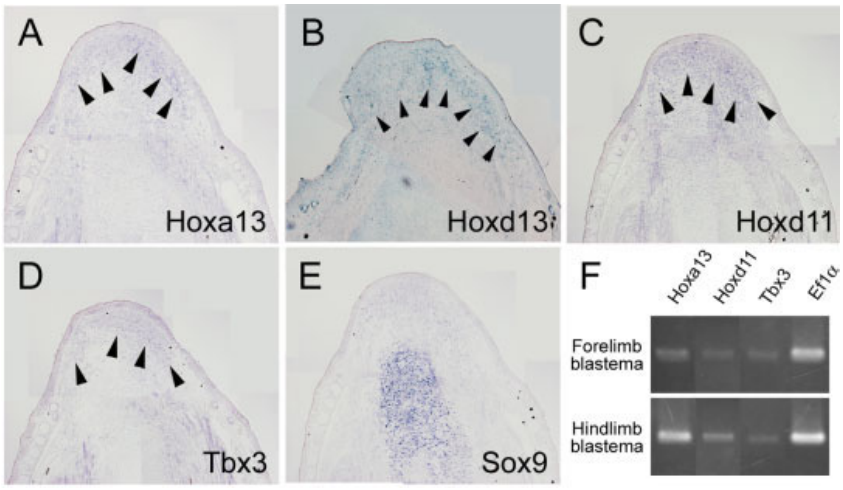
each identity along AP polarity occurs, at least at these two points.

To determine whether the morphological difference along the AP axis is related to a molecular difference, we investigated the expressions of *hoxa13* and *hoxd11* (Fig. 8). As expected, *Hoxa13* was reexpressed in the regenerating blastema. This reexpression was observed in all digits at both day 5 and day 15 (Fig. 8A–D). Interestingly, cells in the interdigital region expressed *hoxa13* during regeneration. Mesenchymal cells in the interdigital region would be activated by amputation and be blastema cells. At day 5, *hoxd11* expression was detected in the anterior and posterior blastema (Fig. 8E,F). At day 15, *hoxd11* expression was still detectable in the anteriormost digit blastema (Fig. 8G). However, in some cases, the expression level in the anterior digits appeared to be low. To confirm this observation, we performed real-time RT-PCR with *hoxd11*-specific primers on the 15-day-old digit blastema (Fig. 9). *Hoxd11* expression level was higher in the posterior digit blastema than in the anterior digit blastema ($P < 0.01$). These results are consistent with reorganization of nails only in the anterior three digits.

DISCUSSION

Gene expression patterns in the *Xenopus* digit suggested that there are slightly different digit identities, especially in the forelimb. The anteriormost digit is *hoxd13*-positive and *Hoxd11*-negative. Blastema cells make a spike that expressed both *hoxd* genes. Therefore, the spike phenotype is different from the *shh* mutant mouse phenotype that has digit I in limbs. Furthermore, froglet blastema cells would have certain positional value based on the amputation stump. Depending on the information in the amputated stump, positional values would be reactivated.

Based on histological analysis, Korneluk and Liversage (1984) suggested that spike regeneration in *Xenopus* represents a "dominant tissue regeneration response" in contrast to the epimorphic regeneration seen in newts. This issue has been argued for a long time. We discuss the possibility that limb regeneration in the *Xenopus*



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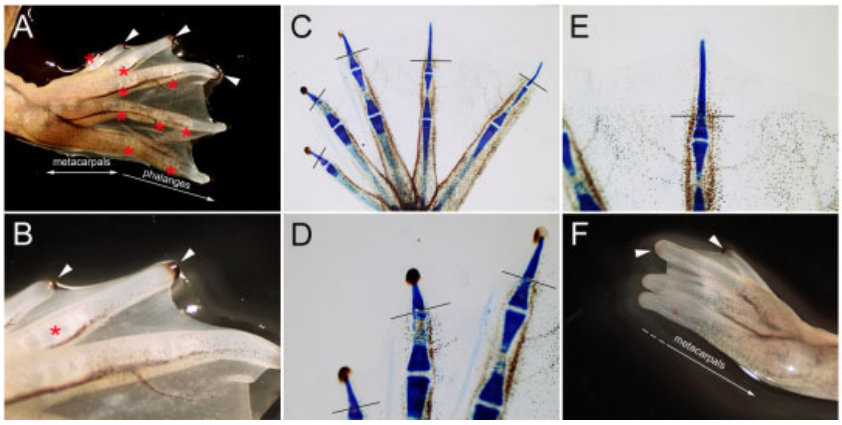

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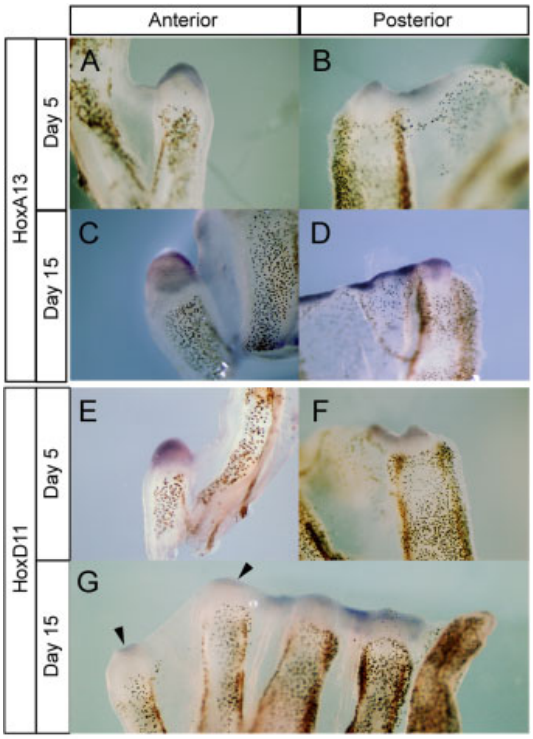

Fig. 8.

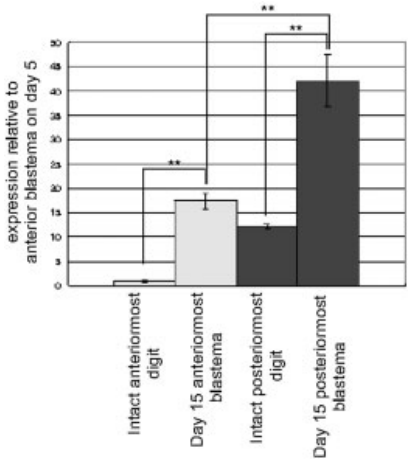

Fig. 9. Comparison of *hoxd11* gene expression levels in the anterior blastema and posterior blastema. Total RNA was extracted from intact digits and amputated digits 15 days after amputation, reverse-transcribed into cDNA, and then subjected to real-time RT-PCR analysis. *Hoxd11* expression level was measured by real-time RT-PCR using specific primers. Values were first normalized to *ef-1*α. Values represent the means of four independent experiments $\pm$ SD. $^{**}P < 0.01$ (*t*-test).

Fig. 6. Gene expression in regenerating froglet blastema. Section in situ hybridization was performed 2 weeks after amputation. **A:** *Hoxa13* was expressed in the froglet blastema (arrowheads). **B:** *Hoxd13* expression was also detected (arrowheads). **C:** *Hoxd11* expression was detectable (arrowheads). **D:** *Tbx3* expression. **E:** *Sox9* expression. **F:** RT-PCR analysis for forelimb blastema and hindlimb blastema of the froglet. A–E: Distal is to the top, and anterior is at the right.

Fig. 7. Digit amputation experiment. **A:** Dorsal view of regenerated digits. **B:** Higher magnification of A. **C:** Skeletal pattern of regenerated digit. **D,E:** Higher magnification of C. Regenerates were observed as spikes. No joint was observed in the digit spikes. Nails were observed in the anterior three spikes but not in the posterior two spikes. **F:** Spikes with a nail were formed in metacarpal-level amputation. White arrowheads indicate the nail. A,B: Asterisks mark joints, and white arrowheads indicate the nail. Lines in C–E indicate the amputation site.

Fig. 8. *Hoxa13* and *hoxd11* expression in digit blastemas. **A–D:** *hoxa13* expression. *Hoxa13* expression was observed throughout the anteriormost blastema to the posteriormost blastema at 5 and 15 days after amputation. *Hoxa13* expression was also detectable in the web region. E–G: *Hoxd11* expression. **E,F:** At 5 days after amputation, *hoxd11* expression was detectable throughout the anteriormost blastema to the posteriormost blastema. **G:** At 15 days, *Hoxd11* was observed throughout the anteriormost blastema to the posteriormost blastema. However, weaker signals were detected in the anterior blastemas (arrowheads).

froglet is not mere "dominant tissue regeneration."

Digit Identities in *Xenopus* Forelimb

There has been much debate about digit identities, especially of the chicken wing (I, II, III or II, III, IV), depending on various molecular and histological results (Galis et al., 2005; Vargas and Fallon, 2005). Based on molecular data (Vargas and Fallon's insight), the character of digit I is thought to be *hoxa13*-positive and *hoxd12(11)*-negative. *Xenopus laevis* might not have *hoxd12* in its genome because *Silurana (Xenopus) tropicalis* does not have *hoxd12* (Mannaert et al., 2006). Overexpression of *hoxd11* in the chicken limb bud resulted in conversion of digit I morphology to digit II morphology (Goff and Tabin, 1997). Therefore, it seems appropriate to use *hoxd11* as a digit marker gene in the same way as *hoxd12*. The *Xenopus* hindlimb bud expressed *hox* genes as seen in the mouse and chick (Fig. 2). We showed that the anteriormost digit, digit I, did not express *hoxd11* but expressed *hoxd13* (Fig. 2G',H'). However, all of these *hox* genes were detectable in forelimb digits from the anteriormost digit to the posteriormost digit (Fig. 3). Based on these results, we hypothesize that the number of the anteriormost digit of forelimb is II or larger. In the view of skeletal pattern, the number of phalanges of the forelimb is 3,322 from anterior to posterior (Fig. 4). The largest number of phalanges was observed in the anterior two digits. In general, the number of phalanges of the anterior digit is less than that of the posterior digit. The *Xenopus* digit seems to be different from that. These observations indicate that digit II identity would be suitable for the anteriormost digit of the forelimb.

Some amphibians develop limbs and digits in specific ways; for example, a newt, *N. viridescens*, does not use interdigital apoptosis as the method of digit separation (Cameron and Fallon, 1977). Additionally, condensation of digit cartilage starts from anterior to posterior in the axolotl ((II-I)-III-IV in a forelimb and (II-I)-III-IV-V in a hindlimb) (Shubin and Alberch, 1986). In higher vertebrates, such condensation occurs from posterior to anterior. In general, the first digit to initiate cartilage formation is digit IV. This digit is also spatially in line with the ulna in the forelimb, allowing the conceptual description of a primary axis running from the ulna to digit IV (Shubin and Alberch, 1986). From this concept, *Xenopus* digit identities of a forelimb seem to be II to V since the first digit to initiate cartilage formation is the second digit from posterior (Fig. 3E). The notion that digit identities of *Xenopus* forelimb are II to V was first proposed by Oster et al. (1988). Our findings suggest that forelimb digit identities of *Xenopus laevis* are II-III-IV-V.

Characterization of the Froglet Blastema

Homology of the phenotype of the *shh*-mutant mouse limb and that of the cyclopamine-treated limb in the axolotl provides a valuable insight (Chiang et al., 1996, 2001; Roy and Gardiner, 2002). The spike of *Xenopus* has similarity with the cyclopamine-treated axolotl limb, and the spike does not express *shh*. There is no doubt that *shh* deficiency is one of the causes of the spike phenotype. However, the gene expression pattern suggests that the spike and *shh* inhibition phenotypes are not the same. The Shh inhibition phenotype in the *Xenopus* tadpole had profiles of gene expression similar to those of the *shh* mutant mouse (Fig. 5). In contrast, the spike did not have similar *hoxd11* gene expression (Fig. 6). Additionally, although the *shh* mutant mouse showed an inhibition of PD-directional growth in the forelimb, the forelimb spike elongated well (Fig. 4). Furthermore, the *shh* mutant has some joints in its limb, but the spike does not. These findings suggest that the spike is not simply a *shh*-deficient phenotype.

The pattern of different digits (I to V) that form from anterior to posterior is controlled by secreted Shh signals produced in the posterior limb bud mesoderm (Chiang et al., 1996, 2001; Ingham and McMahon, 2001; Mariani and Martin, 2003; Roy and Gardiner, 2002; Tickle, 2003). Shh regulates both digit number and identity in a dose-dependent manner; increasing levels of *Shh* expand digit-forming capacity and specify more posterior digit identities (Lewis et al., 2001; Yang et al., 1997). It is thought that digit I develops in a *shh*-independent-manner. Therefore, a digit of the *shh* mutant is thought to be digit I. Our findings indicate that a spike does not have a simple digit I character. At least, *hoxd11* expression implies that it is not similar to the digit I character. We speculate that a spike has a complex character; that is, a spike has not only a simple digit I but also more posterior digit identities.

Positional Value of Froglet Digit Blastema

We demonstrated that the anterior digit blastema could reform a nail and that the posterior digit blastema could not reform a nail in the hindlimb (Fig. 7). This seems to support the above idea that the blastemas simply reactivated their positional value following their position. However, *hoxd11* transcription does not seem to support this idea. *Hoxd11* transcription was not detected in the anteriormost digit at digit-stage during development (Fig. 2H,H'). If merely reactivation of positional value at the amputation site during regeneration occurred, it would be *hoxd11*-negative. However, *hoxd11* transcription was detectable in the anteriormost digit after amputation (Figs. 8G, 9). The expression level was lower than that in the posteriormost digit blastema (Fig. 9). We speculate that both anterior and posterior blastemas recapitulate a developmental program. In the case of limb development, as shown in Figure 2C,H,H', both are developed via a *hoxd11*-positive state, and then *hoxd11* expression is downregulated in the anteriormost digit and maintained in posterior digits. In the case of limb regeneration, reexpression of *hoxd11* occurred, but *hoxd11* expression did not disappear because fine positional value might not be established in the anteriormost blastema. Differences in *hoxd11* expression levels of digit blastemas indicate that the anterior blastema and posterior blastema have a different positional value.

Possibility That *Xenopus* Froglet Limb Regeneration Is Not Merely Dominant Tissue Regeneration

We showed that the anteriormost digit blastema and posteriormost digit blastema expressed *hoxd11* differently following the positional value of an amputation stump. This indicates that a blastema cell recognizes its positional value based on an amputation stump. The anterior blastema and posterior blastema would have different positional values. This gives a new insight into *Xenopus* limb regeneration. It has not been determined whether other *hox* genes, such as *hoxa13* and *hoxa11*, are also expressed differently along the PD axis. Accumulation of more genetic information is needed in order to understand *Xenopus* limb regeneration.

Our previous studies (Satoh et al., 2005a,b, 2006) and the present study suggest that *Xenopus* limb regeneration has a feature of epimorphic regeneration rather than mere dominant tissue regeneration. We divided the problems of the *Xenopus* spike into two defects for convenience: (1) tissue deformities and (2) pattern defect. With regard to the former, some studies have suggested that it is improvable (Satoh et al., 2005a, 2006). The latter problem still remains unexplained. We will not know whether *Xenopus* can regenerate its limb completely unless the latter problem is solved. However, much evidence supports the idea that *Xenopus* limb regeneration is epimorphic but just incomplete.

EXPERIMENTAL PROCEDURES

Histology

Samples were fixed overnight in 10% formalin in Tyrode's solution and then the skin was carefully removed. Samples were stained with 0.1% Alcian blue in 70% ethanol with 0.1 N HCl at 37°C overnight and then cleared for 1–3 days in 4% KOH. Finally, the tissues were cleared in 50% glycerin overnight and stored in 100% glycerin.

Manipulation and Cyclopamine Treatment

Xenopus tadpoles were allowed to develop until they reached appropriate stages (Nieuwkoop and Faber, 1956). For manipulation of limb buds, the tadpoles were anesthetized with 1:5,000 ethyl-3-aminobenzoate (Aldrich) dissolved in Holtfreter's solution.

Presumptive ankle (according to the fate map by Tschumi, 1957) regions in the hindlimb buds were excised and washed with Holtfreter's solution. The tadpoles operated on were reared in 2 μg/ml cyclopamine (BIOMOL), and then the water was refreshed every 2 days until they regenerated. After limb regeneration, the limbs were fixed overnight in 10% formalin.

Gene Cloning and In Situ Hybridization

A partial cDNA encoding *Xenopus hoxd11* was obtained by RT-PCR with mRNA extracted from *Xenopus* limb buds. Primers 5′-CAGCAACAGTGCGTCCAATATGTATCTGCC-3′ (forward primer) and 5′-TGAGGTTCAGCATCCTGGACAACTGTAACC-3′ (reverse primer) were designed. *Hoxd13* was also cloned by RT-PCR. Primers 5′- ACTTTGGCAACGGATACTA(C/T)AG(C/T)TG(C/T)-3′ (forward primer) and 5′-TCCGCCTGGTTTAGCGCAACATCTC-3′ (reverse primer) were designed. The PCR products were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced by an ABI sequencer.

To synthesize an antisense RNA probe, templates were synthesized by PCR with KOD DNA polymerase (TOYOBO) and transcribed with T7 RNA polymerase (for *hoxa13*; GIBCO BRL) or SP6 RNA polymerase (for *hoxd13, hoxd11, tbx3*, and *sox9*; Ambion). In situ hybridization of sections was performed as described previously (Endo et al., 2000; Satoh et al., 2005b). Proteinase K treatment was performed at a concentration of 3.75 μg/ml for the tadpole's limb and at a concentration of 5 μg/ml for the froglet blastema. For whole-mount in situ hybridization, a modification of the protocol described by Kumasaka et al. (2003) was used. Briefly, specimens were fixed overnight at room temperature (RT) in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) and were then dehydrated in ethanol/PBT (PBS, 0.1% Tween-20) and 100% ethanol at RT. Then the samples were bleached by treatment with 6% H_2O_2 in ethanol. The embryos were rehydrated in PBT and treated with proteinase K (13.3 μg/ml) at RT for 30 min. To stop the proteinase K treatment, samples were treated with 2 mg/ml glycine for 5 min, washed with PBT, and acetylated with 0.5% acetic anhydride in 0.1 M triethanolamine (pH 7.8) for 10 min. Refixation was performed with 4% paraformaldehyde/0.2% glutaraldehyde. The samples were prehybridized for 2 h at 60°C and then hybridized in a solution containing RNA probes at 60°C overnight. After hybridization, the embryos were first washed with PBT for 5 min at RT. Excess probe was then removed by electrophoresis (100 mV, 60 min). Blocking was performed with 2% blocking reagent (Roche) for 30 min. The samples were then incubated with ½,500 diluted AP-conjugated anti-DIG antibody (Roche) overnight at 4°C. The samples were washed five times with MAB for 1 hour. The staining reaction was performed using BM purple (Roche) as a substrate for AP.

RT-PCR and Real-Time RT-PCR

Total RNA was prepared using an RNeasy mini kit according to the protocol of the manufacturer (Qiagen, Chatsworth, CA). Total RNA was prepared from the froglet blastema with the exclusion of stump tissues as much as possible. Total RNA was prepared from the digit blastema including some stump tissues because of the difficulty in isolating only blastema from a regenerating digit. cDNA was prepared with Super Script III (Invitrogen) following the manufacturer's protocol. Primers for RT-PCR were as follows: 5′-TCCCAGAGACTAAGGAAAAAGAGG-3′ (forward primer) and 5′-TCGATCCCTGTTAAGTTTCTTTTC-3′ (reverse primer) for *hoxd11,* 5′-GGACGAGTCCTCTAGTGAAC-3′ (forward primer) and 5′-TCAAATGGTCGATGCTCAGG-3′ (reverse primer) for *tbx3*, 5′-CAGAT-

TGGTGCTGGATATG-3′ (forward primer) and 5′-ACTGCCTTGATGACTCCTAG-3′ (reverse primer) for *ef-1*α, 5′-CTTCAAGTTCGGAGACGTGC-3′ (forward primer) and 5′-CAGAATAGTACTGCAGGCGG-3′ (reverse primer) for *Hoxa13*.

Two microliters of reverse-transcribed cDNA was used for real-time PCR with the fluorescent dye SYBR Green I to monitor DNA synthesis (SYBR Premix Ex Taq, Takara Bio.) using specific primers designed for *Xenopus hoxd11* (forward primer, 5′-ACAACTCGGTGGGCAGGAAT-3′; reverse primer, 5′-TGGTGGCTTTGGGGTCAGAT-3′) and *ef-1*α (forward primer, 5′-GTGAATTTGAAGCTGGTATCTC-3′; reverse primer, 5′-ATAGGTACAAAGGCAACAGTG-3′). PCR was carried out using a Light Cycler system (Roche) using the following cycling protocol: a 95°C denaturation step for 10 seconds followed by 45 cycles of 95°C denaturation (5 s), 55°C annealing (20 s), and 72°C extension (15 s). Detection of the fluorescent product was carried out at the end of the 72°C extension period. Gene expression was normalized to the housekeeping gene *ef-1*α. The PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified using Light Cycler software.

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