DEVELOPMENTAL BIOLOGY **196,** 1–10 (1998)
ARTICLE NO. DB988856

Multiple Digit Formation in *Xenopus* Limb Bud Recombinants

Hitoshi Yokoyama, Tetsuya Endo, Koji Tamura, Hiroshi Yajima, and Hiroyuki Ide

Biological Institute, Graduate School of Science, Tohoku University, Sendai 980-77, Japan

We prepared recombinant limb buds of *Xenopus* tadpoles by grafting a mesenchyme mass of the hindlimb bud. The *Xenopus* recombinant limb buds with dissociated and reaggregated mesenchyme developed more than 30 digits with cartilage segmentation, while those with undissociated mesenchyme developed a limb with normal cartilage pattern. Before the formation of multiple digits, a patchy expression pattern of *fgf-8,* an AER marker, was observed in the distal region of recombinant limb buds. *shh,* a ZPA (zone of polarizing activity) marker, was expressed broadly in the distal region of recombinants. Recombinant limb buds with the reaggregated mesenchyme of anterior halves formed anterior digits with claws, and those with the mesenchyme of posterior halves formed posterior digits without claws. The temporal and spatial changes in the potency of multiple digit formation are discussed with reference to the regenerative capacity of *Xenopus* limb buds. © 1998 Academic Press

INTRODUCTION

Anuran amphibians are of interest because they exhibit strikingly different capacities for limb regeneration at different stages of their life cycle. In the case of *Xenopus laevis*, developing hindlimbs can completely regenerate prior to the onset of metamorphosis, but regenerative capacity declines gradually as metamorphosis proceeds. In froglets and adults, recognizable limb structures do not regenerate, and a hypomorphic outgrowth occurs following amputation (Dent, 1962). In addition to stage-specific changes, the regenerative capacity of *Xenopus* limb buds also varies depending on the proximodistal level of amputation, with regenerative capacity declining in a distal-to-proximal direction (Stocum, 1995). Thus, it is possible to examine both temporal and spatial changes in regenerative capacity in the same species. It has been shown that this difference in the regenerative capacity is due to intrinsic changes that occur in the limb bud rather than to systemic changes that occur as the larva undergoes metamorphosis (Sessions and Bryant, 1988).

One approach that has been widely used for studying the developmental potential of limb buds is the recombinant limb technique. This technique involves dissociating mesenchymal cells of chick limb buds and packing them into an epidermal jacket isolated from a limb bud of a similar stage. The resultant recombinant limb can be grafted onto a host embryo from which the developmental potential of mesenchymal cells can be determined. Studies on the chick limb buds indicate that recombinant limb buds have a restricted potential to form cartilage structure, forming a Y-shaped structure containing a pair of digits that lack anterior–posterior axial polarity (Zwilling, 1963; MacCabe *et al.*, 1973; Hardy *et al.*, 1995).

In this study, we modified the recombinant limb technique to study developmental and regenerative capacity of *Xenopus* limbs. Recombinant limbs were made from limb bud mesenchyme of varying developmental stages and also from different regions of the developing limb bud. Our results suggest that, unlike the chick limb bud, mesenchymal cells of the *Xenopus* have an extraordinarily high potential to form digits and that this potential is still present in cells derived from limb stages that have a restricted regenerative capacity.

MATERIALS AND METHODS

X. laevis tadpoles were allowed to develop until they reached the appropriate stage (stages 51/52, 55/56, 58; Nieuwkoop and Faber, 1967). In order to collect limb buds, the tadpoles were anesthetized in 1:5000 ethyl-3-aminobenzoate (Aldrich) dissolved in Holtfreter's solution, and hindlimb buds were amputated with an ophthalmological scalpel.

0012-1606/98 $25.00

Preparation of Recombinant Limb Buds

Collected limb buds were washed in Holtfreter's solution containing 2 μg/ml streptomycin and 100 units/ml penicillin and were treated with 0.2% trypsin and 0.2% collagenase dissolved in Holtfreter's solution (25°C, 2 h) to loosen the mesenchyme. After pipetting, these dissociated limb buds were filtered through lens papers (Kodak; four layers) to isolate mesenchymal cells from undissociated ectodermal tissues. The resulting suspension of cells was confirmed to be mesenchymal by microscopic examination. In some experiments, the ectoderm was mechanically removed by treatment with 0.05% EDTA in Ca, Mg-free Holtfreter's solution for 30 min before trypsin/collagenase treatment. The suspension of mesenchymal cells was counted using a hemacytometer, then pelleted by mild centrifugation in a 1.5-ml microfuge tube (Eppendorf). The microfuge tube containing the pellet was incubated for 20 h at 22°C so that cells could adhere to one another. The resulting pellet was removed from the microfuge tube and trimmed to a size and shape appropriate for grafting onto an amputated limb stump. Each pellet contained between 5.0 and 7.0 $\times$ 10^5 mesenchymal cells. This is equivalent to approximately five to seven limb buds of stage 51/52, two limb buds of stage 55/56, and one limb bud of stage 58. Each trimmed pellet was grafted onto a freshly amputated stage 56 hindlimb stump at knee level. The grafted pellet was held in place with a tungsten pin. After 24 h, each larva was examined visually. The larvae were reared in 30% Holtfreter's solution for 3 days and then in water. Five days after grafting, the pellets were examined histologically and were found to be covered by host epidermis (not shown). After 7 days, each larva was anesthetized and examined microscopically to confirm the survival of the grafted pellet. After metamorphosis, the resulting limbs were fixed overnight in 10% formalin in Tyrode's solution, stained with 0.1% Alcian blue in 70% ethanol with 0.1 N HCl at 37°C overnight, dehydrated, and cleared in methyl salicylate.

As a control, we grafted undissociated whole mesenchyme of stage 51 limb buds. Limb buds were excised and treated with 0.05% EDTA in Ca, Mg-free Holtfreter's solution for 30 min to remove the epidermis from limb buds. The limb bud mesenchyme was then grafted to a freshly amputated hindlimb stump at knee level of a stage 56 host tadpole. The alignment (AP, DV, and PD) of the graft was in accord with the host.

Cell Marking

To analyze the cell contribution of graft and host to the structures formed, we labeled stage 51/52 dissociated limb bud cells with a PKH26 fluorescence staining kit (Zynaxis) as previously described (Ide *et al.*, 1994). Briefly, dissociated mesenchymal cells were washed with Holtfreter's solution and incubated in 1.6×10^{-6} M PKH26 (4 min at room temperature). The staining reaction was quenched with culture medium (MEM) containing 10% FCS. The PKH26-labeled mesenchymal cells were used to make recombinant limbs as previously described. After digit protrusions were observed in grafted labeled recombinant pellets (18 days), recombinants were fixed in 2% paraformaldehyde in phosphate-buffered saline and observed in whole mounts under a fluorescence microscope. Recombinant limbs were then cryosectioned at 20 μm, and the sections were mounted in cyanoacrylate ester glue and observed under a fluorescence microscope.

In Situ Hybridization

The method of whole-mount *in situ* hybridization of the *Sonic hedgehog* (*shh*) probe in the *Xenopus* limb bud has already been reported (Endo *et al.*, 1997).

The *Xenopus fgf-8* gene was cloned by RT-PCR from stage 51 hindlimb total RNA. Nested primers were designed against previously cloned *Xenopus fgf-8* (GenBank, Accession No. Y10312). The PCR product of about 430 bp was cloned into the pCR2.1 vector (Invitrogen) and sequenced. To synthesize antisense and sense RNA probes, the insert was subcloned into the *Eco*RI site of pBluescript II SK(+) vector (Stratagene). Digoxigenin-labeled Fgf-8 RNA probes were synthesized after the protocol of Boehringer Mannheim.

RESULTS

Multiple Digit Formation in Recombinants with Reaggregated Whole Mesenchyme

In control experiments, we grafted undissociated whole mesenchyme of stage 51 limb buds onto freshly amputated stage 56 hindlimb stumps. Within 1 day, the grafted mesenchyme appeared enclosed within a wound epidermis. Interspecific grafting between *X. laevis* and *X. borealis* indicated that the wound epidermis was of host origin (not shown). After metamorphosis, whole-mount cartilage staining revealed that the grafted limb bud had formed an ectopic limb with normal cartilage pattern, whereas the stump tissue had formed an incomplete regenerate (Fig. 1A). This result is not surprising, since ectopic limb formation after grafting has been previously reported (Sessions and Bryant, 1988). Thus, the grafting procedure used in these experiments does not influence normal limb development of grafted mesenchyme.

Stage 51–52. The recombinant limbs made from reaggregated stage 51–52 whole limb bud mesenchyme formed a large number of ectopic digits (Figs. 1B–1D). Fifteen days after grafting, the recombinant limb expanded in the PD and AP axes and was roughly twice the size of the original pellet. From the distal region of the recombinant limb, many small protrusions that appeared to be digit rudiments were observed (Fig. 1B). These digit rudiments appeared to be scattered randomly in the distal region of the graft, but each rudiment protruded distally. At 28 days, organization of the multiple digit rudiments along the PD axis was clearly evident, and each rudiment appeared to have developed into a digit (Fig. 1C).

Whole-mount cartilage staining of mature recombinant limbs revealed that each protrusion had developed a digit composed of segmented cartilage elements, including metatarsus, a varying number of phalangeal elements, and in some cases a terminal claw (Fig. 1D). The mean number of digits that formed was 13.0 digits/recombinant limb (five samples). Most digits were morphologically normal, although, in rare instances, digits were either truncated or fused. No digit bifurcation was observed. No distinct proximal structures such as femur, tibiofibula, or tarsus could be identified. The recombinant limb buds completely inhib-

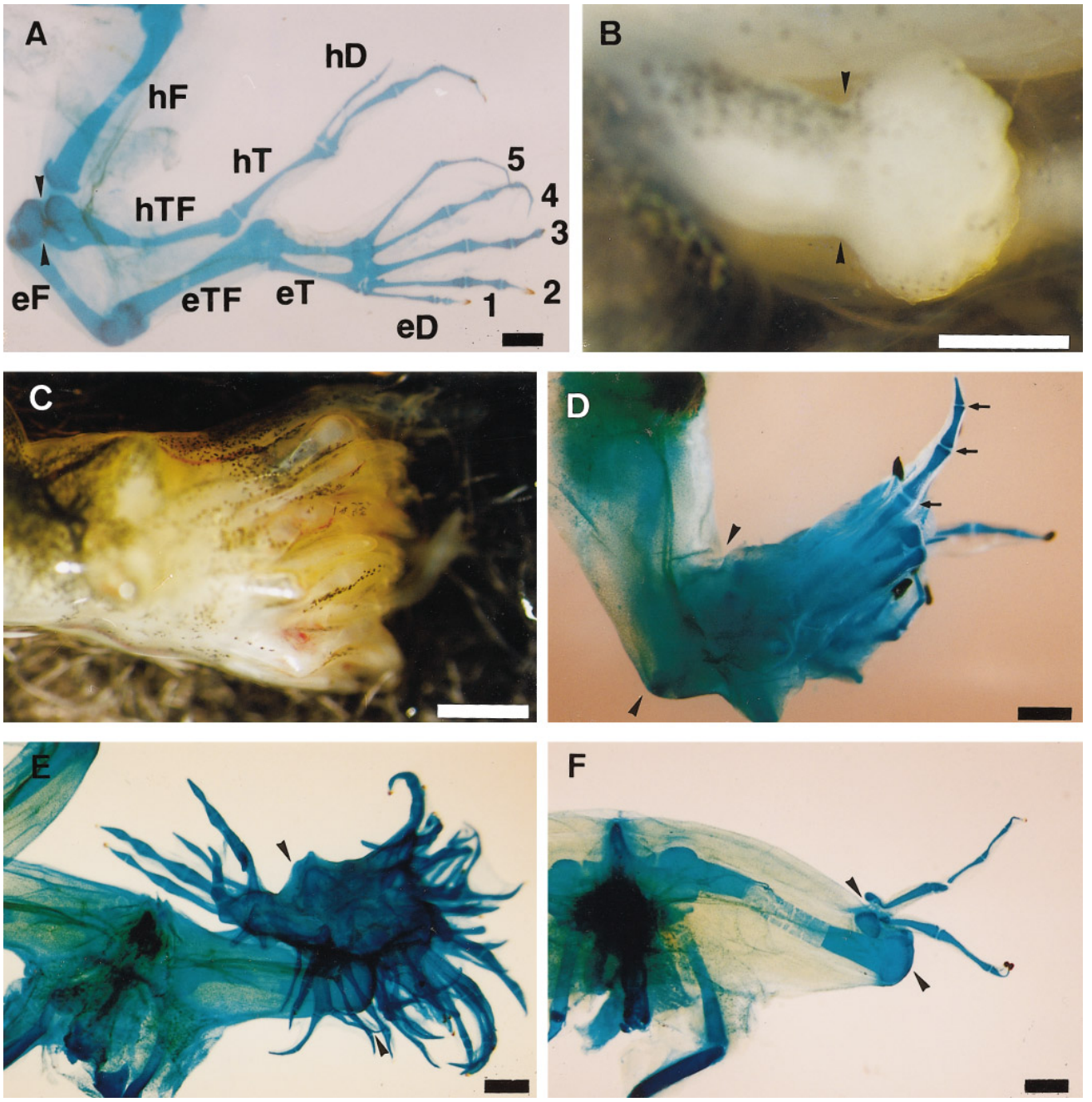

FIG. 1. Multiple digit formation with reaggregated whole mesenchyme. (A) Cartilage pattern of a recombinant with undissociated mesenchyme. Ectoderm-free limb bud mesenchyme at stage 51 was grafted onto the stage 56 limb stump amputated at knee level and allowed to develop for 30 days. An ectopic hindlimb with normal cartilage pattern was formed which included femur (eF), tibiofibula (eTF), tarsus (eT), anterior three digits with claw (eD1–3), and posterior two digits without claw (eD4–5). Host limb bud stump regenerated an incomplete pattern which included a femur (hF), a set of tibiofibula (hTF), a tarsus (hT), and two digits (hD). (B–D) Recombinant limbs with reaggregated stage 51–52 whole limb bud mesenchyme. (B) 15 days after operation. (C) 28 days after operation, before staining. Note the formation of many digits. (D) Alcian blue-stained multiple digits, 70 days after operation. Small arrows indicate cartilage segments in the digits. (E) Multiple digit formation in the recombinant with reaggregated stage 55–56 whole mesenchyme. Alcian blue staining. (F) Digit formation in the recombinant with reaggregated stage 58 whole mesenchyme. Alcian blue staining. Arrowheads show host–graft boundary. Bars, 1 mm.

ited the regeneration response from the stump, and the resulting recombinant limb appeared to develop autonomously at the graft site.

Stages 55–56 and 58. Recombinant limbs composed of reaggregated stage 55–56 mesenchyme formed dozens of digit-like structures similar to that observed from stage 51–52 mesenchyme. However, unlike stage 51–52 recombinant limbs, stage 55–56 recombinant limbs possessed digits that extended distally and radially from a proximal cartilage mass (Fig. 1E). The detailed structure of the proximal cartilage is unclear. The digits of stage 55–56 recombinant limbs were composed of segmented cartilage structure similar to that described for stage 51–52 recombinant limbs. While the majority of stage 55–56 recombinant limb digits were morphologically normal, the percentage of digits displaying abnormalities (i.e., digit truncation, fusion, or bifurcation) was higher than that of stage 51–52 recombinant limb digits. The mean number of total digits formed in stage 55–56 recombinant limbs was 37.8 digits/recombinant limb (six samples). To exclude the possibility that epidermal cells of dissociated limb buds remained in the reaggregated pellet and induced multiple digit formation, we removed the epidermis mechanically from stage 55–56 limb buds after EDTA treatment and made recombinant limbs. These recombinant limbs also formed dozens of digits, suggesting that epidermal cells of graft origin, if any, did not contribute to multiple digit formation.

Recombinant limbs formed from reaggregated mesenchyme of stage 58 limb buds failed to produce a large number of digits (2.0 digits/limb, four samples), although the digits that did form were segmented and proximal-distally complete (Fig. 1F). A regeneration response of the stump was inhibited as in the case of stage 51–52 and stage 55–56 recombinants, suggesting that production of multiple digits in recombinants did not directly inhibit regeneration response in the stump.

Cell contribution. To determine whether host cells participated in the formation of ectopic recombinant limbs, cells within the mesenchymal pellet were labeled prior to grafting. Most of the grafted mesenchymal cells remained in the recombinant (Figs. 2A and 2B), and few unlabeled cells invaded the graft. Analysis of histological sections revealed that almost all cells within each individual digit rudiment were derived from grafted mesenchyme (Figs. 2C and 2D). Thus, recombinant limb development seems to occur autonomously without significant cellular contribution from the host limb stump.

In situ hybridization. We examined the expression of *fgf-8* in recombinant limb buds as a molecular marker for the AER-like structure, which is known to be necessary for limb bud development (Ohuchi *et al.*, 1994). We prepared an RNA-probe of *fgf-8* for whole-mount *in situ* hybridization, which detected *Xenopus* AER (T. Endo *et al.*, in preparation) as it did mouse AER (Ohuchi *et al.*, 1994; Crossley and Martin, 1995). The probe detected *fgf-8* expression in the recombinant limbs 8 days after grafting, when recombinant limbs began to elongate. The expression of *fgf-8* was restricted to the distal region of the recombinant limb (Fig. 3A), and there were many patchy expression domains in the distal region, corresponding to the protrusion of digit rudiments (Fig. 3B).

Next, we examined *shh* expression in the recombinant limbs made from stage 51–52 limb mesenchyme and from stage 55–56 limb mesenchyme. In normal *Xenopus* limb buds, *shh* was expressed in the posterior margin (Endo *et al.*, 1997). In both recombinant limbs, *shh* was expressed 5 days after grafting, but the expression was more intense in stage 51–52 recombinants (Fig. 3C) than in stage 55–56 recombinants (Fig. 3D). The expression domain was observed throughout the recombinant, and there was no correlation between the expression domain and the AP polarity of the host limb bud.

Multiple Digit Formation in Recombinants with Reaggregated Proximal and Distal Mesenchyme

To clarify the mechanisms of multiple digit formation, we focused on the stage 55–56 limb buds because mesenchymal cells from limb buds of this stage formed many digits and also because its size provided enough material to easily investigate the role of position-specific differences. To investigate proximal–distal differences, the stage 55–56 limb bud was divided into three parts by cutting the limb at the presumptive knee and the presumptive ankle (Fig. 4A). Mesenchymal cells were recovered from distal autopodial fragments and from zeugopodial fragments and pelleted separately to make recombinant limbs. Recombinant limbs from the zeugopodial region formed many digits (21.3 digits/limb, three samples), which extended distally and radially, similar to that of recombinant limbs from whole limb buds at stage 55–56 (Fig. 4B). In contrast, recombinants from the distal region formed fewer digits (5.2 digits/limb, five samples), which were elongated only in a distal direction, similar to digits derived from stage 51–52 mesenchyme (Fig. 4C). There were no significant differences in digit size between them, but the percentage of digits displaying abnormalities (i.e., digit truncation, fusion, or bifurcation) was higher in proximal recombinant limbs than in distal ones.

Multiple Digit Formation in Recombinants with Reaggregated Anterior and Posterior Mesenchyme

In *Xenopus* hindlimbs, only the anterior digits (Nos. 1, 2, and 3) have a terminal claw, and digit identity can be determined based on the number of phalanges in association with whether or not a terminal claw is present (Cameron and Fallon, 1977). We divided stage 55–56 limb buds into anterior and posterior halves, and then recombinant limbs were formed from anterior versus posterior mesenchyme (Fig. 5A). Recombinant limbs derived from anterior mesenchyme formed an average of 13.0 digits, all of which possessed a terminal claw. Most of the digits were identified as digit 1 or digit 2 based on the number of phalanges (Fig.

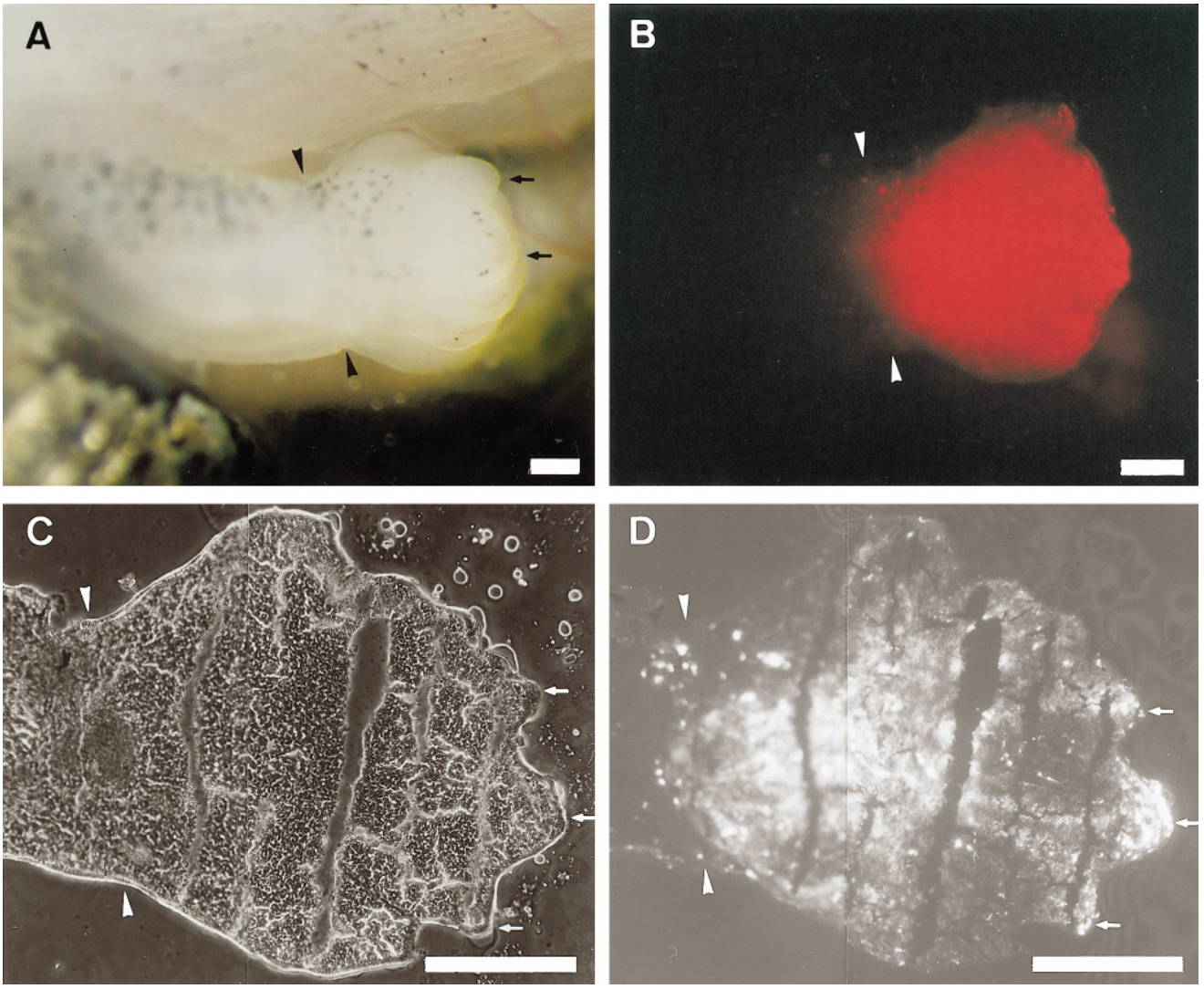

FIG. 2. PKH-26-labeled recombinant limb made from stage 51–52 mesenchyme. (A and B) Whole-mount preparation of a recombinant limb. (C and D) Cryosection of the same recombinant limb. (A and C) In ordinary light. (B and D) In fluorescent light. 18 days after operation. Small arrows indicate protrusions which later form digits. Note that the protrusion-forming regions are of graft origin. Bars, 250 μm.

5B and Table 1), while only one digit was identified as digit 3 (1/39). Recombinant limbs derived from posterior mesenchyme formed an average of 19.3 digits with only a single digit possessing a terminal claw (1/58) (Fig. 5C and Table 1). In this case, 25 of a total of 58 digits could be clearly identified as digit 5. Some digits were unidentifiable because they had a fewer than normal number of phalanges, while other digits were unidentifiable because there were too many digits in one area to accurately observe the proximal regions of the digits. Unidentifiable digits were observed more often in posterior than in anterior recombinant limbs. The percentage of digits displaying abnormalities (i.e., digit truncation, fusion, or bifurcation) was higher in posterior recombinant limbs than in anterior ones. Based on the identification of digits in recombinant limbs, we conclude that dissociated and reaggregated mesenchymal cells retain their positional memory along the AP axis.

The number of digits in the posterior recombinant limbs (19.3 digits/limb, three samples) was larger than that in the anterior recombinant limbs (13.0 digits/limb, three samples); however, both anterior and posterior recombinant limbs formed few digits compared to whole stage 55–56 recombinant limbs (37.8 digits/limb, six samples). Since the total number of mesenchymal cells in each recombinant limb bud was almost the same, the difference in digit number cannot be attributed to differences in the size of the

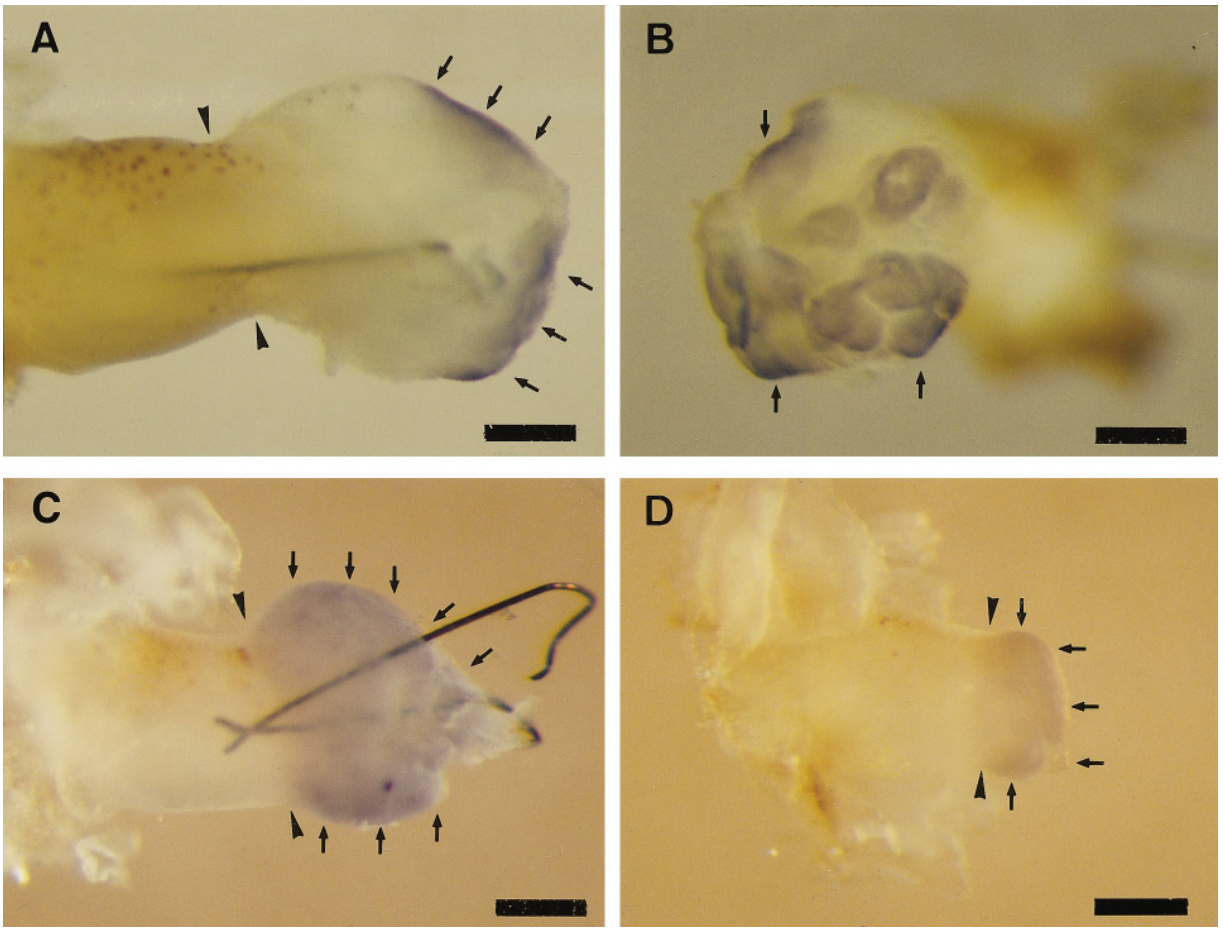

FIG. 3. Whole-mount *in situ* hybridization of *fgf-8* (A–B) and *shh* (C–D) in recombinant limbs. (A) Dorsal view of the recombinant made from stage 55–56 mesenchyme showing that *fgf-8* expression is restricted in the distal region of the recombinant. (B) Frontal view of the same recombinant as in (A), showing that there are multiple separate expression domains in the recombinant. (C) Dorsal view of the recombinant made from stage 51–52 mesenchyme showing widespread distribution of *shh*. (D) Dorsal view of the recombinant made from stage 55–56 mesenchyme showing *shh* expression in whole recombinants. Arrowheads show the host–graft boundary. Bars, 250 μm.

recombinant limb bud. Thus, we reaggregated equal numbers of anterior and posterior mesenchymal cells to reform whole stage 55–56 recombinant limb buds. After metamorphosis, these recombinants developed limb structures with a large number of digits (29.7 digits/limb) (Fig. 5D and Table 1). The combination of anterior and posterior mesenchymal cells seems to induce cell interactions that promote multiple digit formation. The possibility of interaction between the anterior mesenchymal cells and the posterior mesenchymal cells is further supported by the observation that the recombinant limbs with anterior and posterior mesenchyme made significantly more digits 3 (20/89 digits) than recombinant limbs derived from only anterior or only posterior mesenchyme (1/97 digits). Similarly, recombinant limbs with anterior and posterior mesenchyme developed a small number of digits 4 (3/89), which were not observed in recombinants with only anterior or only posterior mesenchyme, suggesting that interaction between the anterior and the posterior mesenchyme is necessary for the formation of digits 3 and 4 in recombinant limbs.

DISCUSSION

In this study, we made recombinant limbs from dissociated and reaggregated mesenchyme of *Xenopus* hindlimb buds that showed stage- and position-dependent regenerative capacity (Dent, 1962; Stocum, 1995) to elucidate the relationship between morphogenetic potential and regenerative capacity.

Recombinant limbs made with the reaggregated mesenchyme of chick limb buds formed a single or Y-shaped sym-

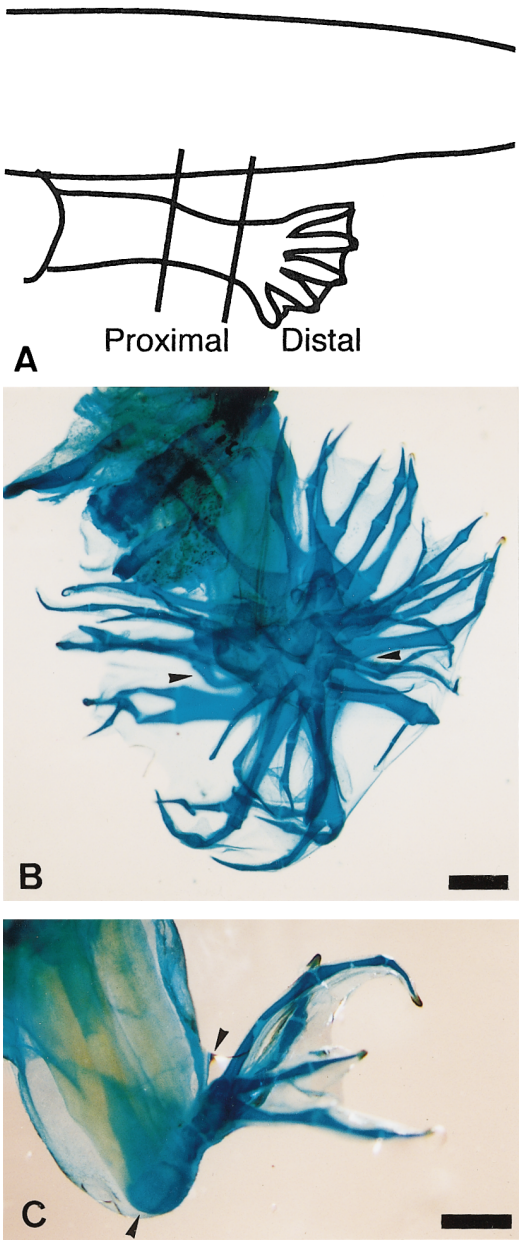

FIG. 4. Digit formation in the recombinants with proximal and distal mesenchyme. (A) Schematic diagram showing the limb bud at stage 55–56. Solid lines indicate incisions made for the preparation of proximal and distal mesenchyme. (B) Ventral view of the recombinant with multiple digits produced from proximal mesenchyme. (C) Dorsal view of the recombinant produced from the distal mesenchyme. Alcian blue staining. Note that the recombinant from the proximal mesenchyme developed many radially elongated digits, but that from the distal one developed a few digits. Arrowheads show the host–graft boundary. Bars, 1 mm.

metric digit-like structure (Zwilling, 1963; MacCabe *et al.*, 1973). However, in the present experiment, the *Xenopus* limb recombinants formed many more digits than those of chick limb recombinants. In an extreme case, about 50 digits were formed in a *Xenopus* recombinant with stage 55–56 mesenchymal cells that were derived from only about two hindlimb buds (Fig. 1E). This difference may be partially due to the procedure of recombinant preparation. In chick recombinants, mesenchyme was packed into an ectodermal jacket and grafted, and therefore the volume of the mesenchyme was limited. On the other hand, in *Xenopus*, the mesenchyme mass was directly grafted onto the stump and therefore the volume of the mesenchyme was not limited. It is extremely difficult to prepare chick-type recombinants with *Xenopus* limb buds because of the size, thickness, and fragility of ectoderm. Furthermore, *Xenopus*-type recombinants with a naked chick mesenchyme mass could not develop after grafting. Thus, it remains uncertain whether multiple digit formation depends entirely on the characteristics of the limb bud mesenchyme.

Why were so many digits formed in the *Xenopus* recombinant limb buds? To address this question, we can refer to several transplantation experiments on *Xenopus* limb buds. Cameron and Fallon (1977) reported that when a hindlimb bud tip was amputated, rotated 180° on the PD axis, and returned to the stump, one or two supernumerary limbs were formed from the site at which anterior and posterior limb bud tissues were apposed. Furthermore, Muneoka and Murad (1987) demonstrated that the graft and host cells contributed almost equally to the formation of supernumerary limbs. From these results, they considered that tip rotation to appose the anterior and posterior limb bud tissues made some gaps between different positional values along the AP axis and then supernumerary limbs were formed to fill the gaps by intercalation between the anterior and the posterior tissues.

These results suggest that *Xenopus* recombinants may also have such gaps in the positional values along the AP axis. Immediately after reaggregation, the mesenchymal mass is homogeneous. Widespread distribution of *shh* expression was actually observed in the recombinants. However, the mesenchymal cells seem to retain their positional values along the AP axis because the recombinant limbs derived from the anterior halves formed anterior digits with claws and those from the posterior halves formed posterior digits without claws. Thus, the intercalation between the cells with different positional values may occur in the recombinants to fill the gap of positional values along the AP axis, since the recombinants from the anterior *plus* posterior mesenchyme formed intermediate digits, i.e., digit 3 and digit 4 (Table 1), and they formed more digits than those of the recombinant limbs with only anterior or posterior mesenchyme (Table 1). It remains uncertain whether position-specific cell-sorting occurs in the recombinants as it does in the mesenchyme of chick limb buds (Ide *et al.*, 1994; Wada and Ide, 1994). We have to examine whether the reaggregated mesenchymal cells actually segregate in the recombinants and whether the mesenchymal cells actually intercalate along the AP axis.

Expression of *shh* was observed in the recombinant limbs

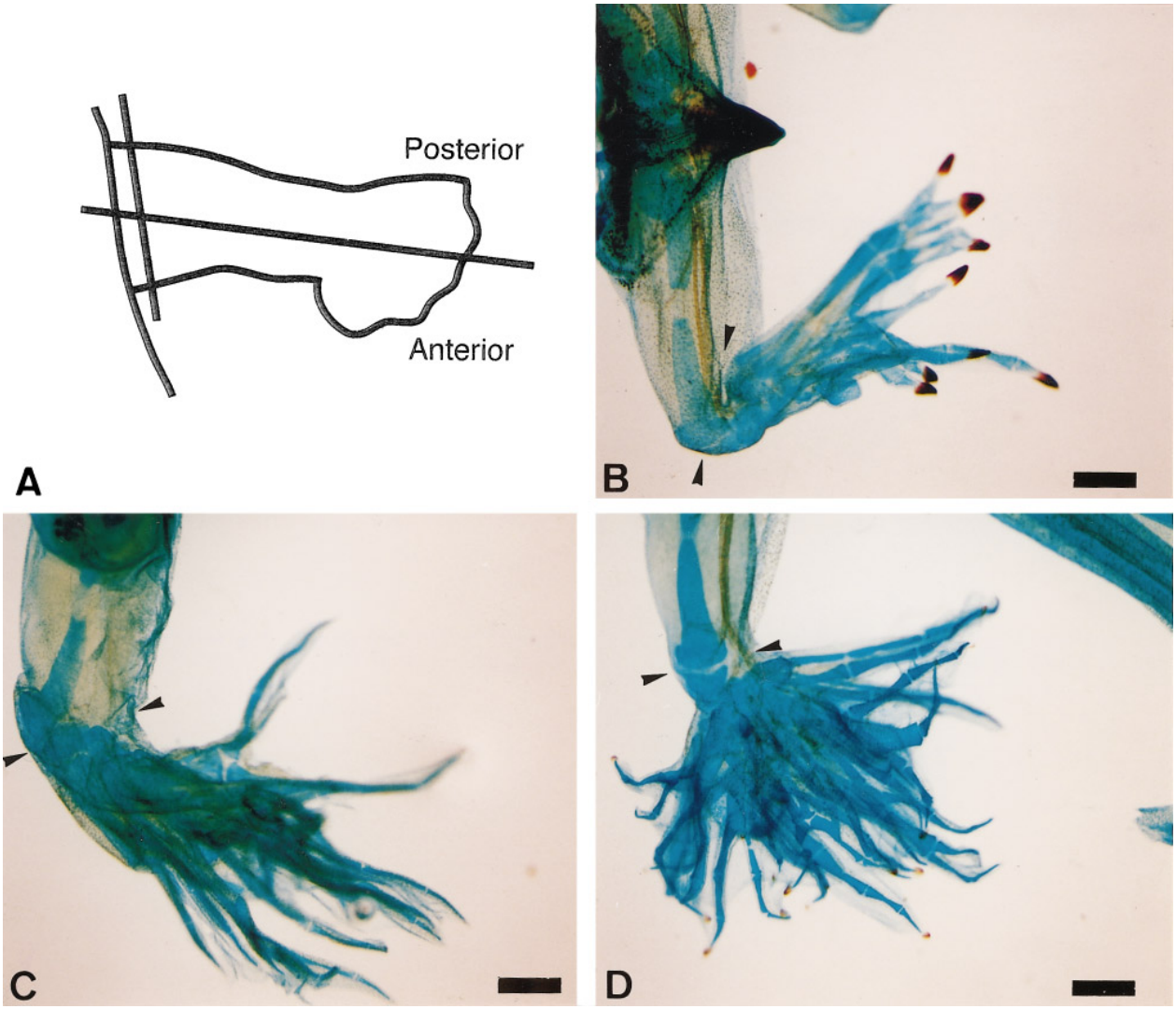

FIG. 5. Multiple digit formation in the recombinants with anterior, posterior, and whole mesenchyme. (A) Schematic diagram showing stage 55–56 limb buds used for the preparation of mesenchyme. The solid line indicates the incision dividing the limb bud into the anterior and posterior halves. (B–D) Dorsal views of the recombinants made from the anterior (B), posterior (C), and both anterior and posterior (D) mesenchyme. Note that all digits have a claw in (B) but no digits have a claw in (C), and some digits have a claw in (D). Arrowheads show the host–graft boundary. Bars, 1 mm.

made both from stage 51–52 mesenchyme and from stage 55–56 mesenchyme, but the expression in the latter was weaker than that in the former (Figs. 3C and 3D). Since *shh* expression disappears by stage 55 in normal limb bud development (Endo *et al.*, 1997), *shh* may be induced in reaggregated mesenchymal cells. Recombinant limbs made from stage 55–56 mesenchyme formed more digits than those from stage 51–52 mesenchyme; thus, there is no correlation between the strength of *shh* expression and the number of digits formed in recombinants. However, the *shh* expression pattern in recombinants is consistent with the *shh* expression pattern in regenerating blastemas: *shh* expression can be reinduced in the posterior margin of regenerating blastemas after amputation, and the expression in a stage 55 blastema is weaker than that in a stage 53 blastema (Endo *et al.*, 1997). In the recombinants, *shh* expression was not polarized but was distributed broadly in the mesenchyme. Dissociation and reaggregation of the mesenchyme seemed to induce weak but broad *shh* expression in the recombinant, which may support the multiple-digit formation.

It is well known that AER is necessary for limb development in both the chick (Saunders, 1948; Summerbell, 1974) and *Xenopus* (Tschumi, 1957). In chick limb recombinants

TABLE 1
Digit Formation in Limb Recombinants

Origin of mesenchyme	Recombinant	No. of digits								
		With claw (digit 1, 2, 3)				Without claw (digit 4, 5)				
		1 or 2	3	Unidentified	Total	4	5	Unidentified	Total	Total
Anterior	A1	11	1	2	14	0	0	0	0	14
	A2	10	0	0	10	0	0	0	0	10
	A3	11	0	4	15	0	0	0	0	15
Posterior	P1	0	0	1	1	0	12	6	18	19
	P2	0	0	0	0	0	1	20	21	21
	P3	0	0	0	0	0	12	6	18	18
Anterior plus posterior	AP1	4	5	7	16	0	5	5	10	26
	AP2	5	5	6	16	2	7	6	15	31
	AP3	3	10	4	17	1	7	7	15	32

with dissociated mesenchyme, AER remains at the tip of the intact ectodermal jacket, and some degree of limb development occurs (Zwilling, 1963; MacCabe *et al.*, 1973). In *Xenopus* recombinants with undissociated mesenchyme, limb development was normal (Fig. 1A), indicating that an AER-like structure regenerates from the stump ectoderm. However, in recombinants with dissociated and reaggregated mesenchyme, many digits were formed with spatial overlapping, suggesting that multiple AER-like structures may regenerate from the stump ectoderm, cover most of the recombinant mesenchyme, and allow the underlying mesenchyme to develop multiple digits. The expression pattern of *fgf-8* in the recombinant ectoderm suggests that multiple AER-like tip structures are actually formed in the recombinant limb, although the expression domain is not ridge-shaped but patch-shaped (Fig. 3). Since AER-like structures are formed only in the tips of recombinants, stump tissues may affect the recombinant ectoderm locally to inhibit the formation of the AER-like structures.

The regenerative capacity of *Xenopus* limb buds declines during development. After amputation, the hindlimb buds at stage 51–52 can regenerate completely, while one to three digits are formed at stage 55–56, and only a spike is formed in froglets (Dent, 1962). Their regenerative capacity also declines in a distal-to-proximal direction (Stocum, 1995). However, recombinants with stage 55–56 whole mesenchyme formed many more digits than those from stage 51–52 whole mesenchyme, and recombinants from the proximal region of stage 55–56 mesenchyme formed more digits than those from the distal region (Fig. 5). Recombinants made from stage 58 whole limb mesenchyme, in which the regenerative capacity is almost completely lost, developed only a few digits as was the case in chick recombinant limbs. This suggests that the highest capacity of multiple digit formation may be localized not in the mesenchyme with the highest regenerative capacity but in the mesenchyme with moderate capacity, which corresponds to stage 55–56. Also, along the PD axis, the highest capacity of multiple-digit formation was found not in the distal autopodial region but in the zeugopodial region of stage 55–56 limb buds. It is interesting that recombinants with zeugopodial mesenchyme of limb buds at stage 55–56 formed numerous digits, whereas the regenerative capacity of zeugopodial mesenchyme was relatively low and few digits were regenerated. After dissociation and reaggregation, the mesenchyme may restore its morphogenetic capacity for digit formation. Clarification of the detailed mechanism of restoration of morphogenetic capacity should provide us with the key to enable *Xenopus* limbs to regenerate completely at a later stage.

ACKNOWLEDGMENTS

We thank Professor K. Muneoka and Professor D. L. Stocum for critically reading the manuscript. We also thank Dr. Kosuke Tashiro for the gift of *Xenopus sonic hedgehog* cDNA. This work was supported by research grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

Cameron, J., and Fallon, J. F. (1977). Evidence for polarizing zone in the limb buds of *Xenopus laevis*. *Dev. Biol.* **55,** 320–330.

Crossley, P. H., and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121,** 439–451.

Dent, J. N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *J. Morphol.* **110,** 61–77.

Endo, T., Yokoyama, H., Tamura, K., and Ide, H. (1997). Shh expression in developing and regenerating limb buds of Xenopus laevis. *Dev. Dyn.* **209,** 227–232.

Hardy, A., Richardson, M. K., Francis-West, P. H., Rodriguez, C., Izpusia-Belmonte, J. C., Duprez, D., and Wolpert, L. (1995). Gene

expression, polarizing activity and skeletal patterning in reaggregated hindlimb mesenchyme. *Development* **121,** 4329–4337.

Ide, H., Wada, N., and Uchiyama, K. (1994). Sorting out of cells from different parts and stages of the chick limb bud. *Dev. Biol.* **162,** 71–76.

MacCabe, J. A., Saunders, J. W., Jr., and Pickett, M. (1973). The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm. *Dev. Biol.* **31,** 323–335.

Muneoka, K., and Murad, E. H. B. (1987). Intercalation and the cellular origin of supernumerary limbs in *Xenopus. Development,* **99,** 521–526.

Niewkoop, P. D., and Faber, J. (1956). ''Normal Table of *Xenopus laevis* (Daudin).'' North-Holland, Amsterdam.

Ohuchi, H., Yoshioka, H., Tanaka, A., Kawakami, Y., Nohno, T., and Noji, S. (1994). Involvement of androgen-induced growth factor (FGF-8) gene in mouse embryogenesis and morphogenesis. *Biochem. Biophys. Res. Commun.* **204,** 882–888.

Saunders, J. W., Jr. (1948). The proximo-distal sequence of the origin of the parts of the chick wing and the role of ectoderm. *J. Exp. Zool.* **108,** 363–403.

Sessions, S. K., and Bryant, S. V. (1988). Regenerative ability is an intrinsic property of limb cells in Xenopus. *J. Exp. Zool.* **247,** 39–44.

Stocum, D. L. (1995). ''Wound, Repair, Regeneration and Artificial Tissues.'' R. G. Landes, Austin, TX.

Summerbell, D. (1974). A quantitative analysis of the effects of the excision of the AER from the chick limb-bud. *J. Embryol. Exp. Morphol.* **32,** 651–660.

Tschumi, P. A. (1957). The growth of hind limb bud of Xenopus laevis and its dependence on the epidermis. *J. Anat.* **9,** 149–173 (1957).

Wada, N., and Ide, H. (1994). Sorting out of limb bud cells in culture. *Int. J. Dev. Biol.* **38,** 351–356.

Zwilling, E. (1963). Development of fragmented and dissociated limb bud mesoderm. *Dev. Biol.* **39,** 69–82.

Received for publication June 26, 1997
Accepted January 15, 1998