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Coordinated expression of *Hoxa-11* and *Hoxa-13* during limb muscle patterning

Masakazu Yamamoto[1,3], Yoshihiro Gotoh[1], Koji Tamura[2], Mikiko Tanaka[2], Atsushi Kawakami[1], Hiroyuki Ide[2] and Atsushi Kuroiwa[1,*]

[1]Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan
[2]Biological Institute, Tohoku University, Aoba, Sendai, 980-8578, Japan
[3]CREST, Japan Science and Technology Corporation (JST)
*Author for correspondence (E-mail: i45240a@nucc.cc.nagoya-u.ac.jp)

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SUMMARY

The limb muscle precursor cells migrate from the somites and congregate into the dorsal and ventral muscle masses in the limb bud. Complex muscle patterns are formed by successive splitting of the muscle masses and subsequent growth and differentiation in a region-specific manner. *Hox* genes, known as key regulator genes of cartilage pattern formation in the limb bud, were found to be expressed in the limb muscle precursor cells. We found that HOXA-11 protein was expressed in the premyoblasts in the limb bud, but not in the somitic cells or migrating premyogenic cells in the trunk at stage 18. By stage 24, HOXA-11 expression began to decrease from the posterior halves of the muscle masses. HOXA-13 was expressed strongly in the myoblasts of the posterior part in the dorsal/ventral muscle masses and weakly in a few myoblasts of the anterior part of the dorsal muscle mass. Transplantation of the lateral plate of the presumptive wing bud to the flank induced migration of premyoblasts from somites to the graft. Under these conditions, HOXA-11 expression was induced in the migrating premyoblasts in the ectopic limb buds. Application of retinoic acid at the anterior margin of the limb bud causes duplication of the autopodal cartilage and transformation of the radius to the ulna, and at the same time induces duplication of the muscle pattern along the anteroposterior axis. Under these conditions, HOXA-13 was also induced in the anterior region of the ventral muscles in the zeugopod. These results suggest that *Hoxa-11* and *Hoxa-13* expression in the migrating premyoblasts is under the control of the limb mesenchyme and the polarizing signal(s). In addition, these results indicate that these *Hox* genes are involved in muscle patterning in the limb buds.

Key words: *Hoxa-11*, *Hoxa-13*, *Pax7*, Homeoprotein, Limb bud, Myoblast, Muscle patterning, Retinoic acid, Somite, Chick, Quail

INTRODUCTION

In the developing limbs of vertebrates, individual muscles with characteristic size and shape are arranged in appropriate locations with specific origins and insertion points (Shellswell and Wolpert, 1977). The musculature of the limbs is composed of cells derived from the lateral half of the somites at the limb level (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992). After stage 15 of chick development, the presumptive limb mesenchyme interacts with the cells in the ventral dermomyotome to migrate into the limb bud (Chevallier, 1978; Hayashi and Ozawa, 1995). Migrating premyogenic cells proliferate and congregate in the dorsal and ventral muscle masses (Schramm and Solursh, 1990). Individual muscles are separated by sequential splitting of each muscle mass along the anteroposterior axis, and differentiation of the muscle fiber progresses in a position-specific fashion (Shellswell and Wolpert, 1977; Stockdale, 1992; Robson et al., 1994).

Molecules that are well known to control the migration of premyogenic cells into the limb bud are *Pax-3*, *Pax-7*, N-cadherin, HGF and its receptor c-Met (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994; Bladt et al., 1995; Brand-Saberi et al., 1996a,b; Epstein et al., 1996; Yang et al., 1996). *Pax-3* is expressed in the ventral part of the dermomyotome and is involved in controlling *c-met* expression. The cells expressing c-Met migrate into the limb bud by interacting with its ligand, HGF, expressed in the limb mesenchymal cells (Bladt et al., 1995; Brand-Saberi et al., 1996b). The cell adhesion molecule N-cadherin, which is expressed in both the myogenic cells and limb mesenchyme, is involved in migration of myogenic cells via homophilic interactions (Brand-Saberi et al., 1996a). Differentiation of myogenic cells is controlled by sequential expression of the *MyoD* gene family (Olson, 1992; Olson and Klein, 1994). The mechanism of limb muscle patterning is less well understood than that of cartilage patterning because the muscle pattern is more complex.

Pattern formation for the limb muscles is controlled by the interaction of myogenic cells with the limb mesenchymal cells (Chevallier and Kieny, 1982). For instance, transplantation of the zone of polarizing activity (ZPA) to the anterior margin of the limb bud induces duplication of the cartilage elements in

addition to mirror image transformation of anterior muscles to posterior muscles (Shellswell and Wolpert, 1977; Robson et al., 1994). *Wnt7a* is expressed in the dorsal ectoderm of the limb buds as a dorsalizing signal. Ectopic expression of *Wnt7a* in the ventral ectoderm induces transcription factor *Lmx1* expression in the ventral mesenchyme and results in transformation of the ventral muscle pattern to that of dorsal muscle (Riddle et al., 1995). In addition, disruption of the *Wnt7a* gene in mice causes ventralization of the dorsal muscles (Parr and McMahon, 1995). Thus, the signals from ZPA and from the dorsal ectoderm are required for determination of the polarity of both limb mesenchymal cells and muscle masses along the anteroposterior and the dorsoventral axes, respectively.

This positional information is interpreted by the cells in the muscle masses, then a genetic program encoding position-specific muscle pattern formation is induced. During limb development, *Hox* genes of the *Abdominal-B* (*Abd-B*) subfamily of the *HoxA* and *HoxD* cluster are expressed in a temporally and spatially coordinated manner in the limb mesenchymal cells (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Yokouchi et al., 1991b; Nelson et al., 1996). Ectopic expression and gene disruption experiments of these *Hox* genes revealed that they regulate proliferation, differentiation and adhesiveness of the limb mesenchymal cells to form the position-specific shape of each cartilage element (Morgan et al., 1992; Dollé et al., 1993; Davis et al., 1995; Yokouchi et al., 1995; Fromental-Ramain et al., 1996; Zákány and Duboule, 1996). In the case of *Drosophila*, segment-specific muscle pattern formation is shown to be determined by autonomous function of *Hox* genes in the mesoderm (Greig and Akam, 1993; Michelson, 1994; Roy et al., 1997; Roy and VijayRaghavan, 1997). Recently, it was demonstrated that *Hoxd-12* and *Hoxd-13* were expressed in the visceral mesoderm of the hindgut and regulate proper development of the anal sphincter (Kondo et al., 1996). Thus, it is possible that vertebrate *Hox* genes are also involved in tissue and cellular patterning of the limb muscles (Olson and Rosenthal, 1994).

To date, there has been no direct evidence of the expression of *Hox* genes in limb muscle precursor or limb muscle itself. Difficulty in discriminating between myogenic and mesenchymal cells make it difficult to detect the expression of *Hox* genes in myogenic cells in limb buds directly. The recent finding that migrating myogenic cells but not limb mesenchymal cells express *Pax-3*, *Pax-7*, c-Met and FREK facilitates identification of the myogenic cells in the limb bud (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994; Bladt et al., 1995; Marcelle et al., 1995; Yang et al., 1996). In this study, we analyzed the expression profile of two *Hox* genes, *Hoxa-11* and *Hoxa-13*, during limb muscle development. We found that HOXA-11 and HOXA-13 proteins were expressed in a temporally and spatially coordinated manner during limb muscle patterning. In addition, we also showed that HOXA-11 and HOXA-13 expression are under the control of the limb mesenchyme and the polarizing signal(s).

MATERIALS AND METHODS

Preparation of antibodies

A DNA fragment encoding the N-terminal portion of HOXA-11 from amino acids 14 to 203 was prepared by PCR using a *Hoxa-11* cDNA clone and synthetic oligomers. The PCR fragment was joined to the glutathione S-transferase (GST; Pharmacia pGEX) coding sequence in frame, then the GST-*Hoxa-11* fragment was transferred to a vector carrying the T7 promoter (pET11d; Novagen). The GST-HOXA-11 fusion protein was produced in *E. coli* BL21(DE3) pLysS by standard methods (Sambrook et al., 1989) and the fusion protein was purified by glutathione-agarose column chromatography (Pharmacia). Immunization into guinea pigs and schedule for production of antibody followed standard methods (Coligan et al., 1991). HOXA-11-specific antibody was affinity-purified by maltose-binding protein (MBP)/HOXA-11 fusion protein/Sepharose 4B column chromatography. The same PCR fragment used for production of GST-HOXA-11 fusion protein was inserted into the MBP expression vector (pMal-c; NEB). The MBP-HOXA-11 fusion protein was produced and purified according to the manufacturer's instructions. Specificity of the antibody was determined by western blotting analysis and whole-mount immunohistochemistry. Preparation of anti-HOXA-13 antibody was described previously (Yokouchi et al., 1995). This anti-HOXA-13 antibody was cross-reactive with mouse HOXA-13 protein.

Operations

Chick and quail embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951), and incubated at 38°C. Transplantation of the somatopleure was performed as described previously (Hayashi and Ozawa, 1995; Tanaka et al., 1997). Graft fragments of somatopleure were excised from the presumptive wing region (corresponding to somite level 17-19) or flank region (somite level 23-25) of donor quail embryos (at stage 14-16). Grafts were transplanted into the flank region of host chick embryos (at stage 14-16, somite level 23-25) in the original anteroposterior orientation by replacement. The graft site was adjacent to the host flank somites. Operated host embryos were incubated until they reached developmental stage 20 or 25. Retinoic acid beads were implanted according to the standard method (Eichele et al., 1985; Tamura et al., 1990). Briefly, AG1-X2 ion exchange beads pre-soaked in 0.5 or 1 mg/ml retinoic acid (Sigma; diluted in dimethyl sulfoxide, DMSO) for 1 hour at room temperature were rinsed in a drop of culture medium (DMEM plus 10% FCS). A bead was placed on a slit made beneath the anterior side of the AER (at stage 19-20) and pushed into the slit with a tungsten needle.

Fixation of embryos

Embryos were treated with methanol/DMSO (4:1) at 4°C overnight, then incubated with methanol/DMSO/30% H_2O_2 (4:1:1) at room temperature for 5 hours. Fixed embryos were placed in 100% methanol and stored at −20°C. Embryos were rehydrated before staining. For detection of A223 chicken-specific antigen (Yokouchi et al., 1991a), embryos transplanted with lateral plate mesoderm were fixed with 4% paraformaldehyde in PBS and washed with PBS several times, then used immediately for staining.

Immunohistochemical staining of whole embryos

Whole-mount immunohistochemistry was performed as described previously (Yokouchi et al., 1995). For primary antibody reaction, rabbit anti-HOXA-13 IgG or guinea pig anti-HOXA-11 IgG were used at 1:3000 and 1:8000 dilutions, respectively. For secondary antibody reaction, HRP-conjugated goat anti-rabbit IgG (Zymed) or rabbit anti-guinea pig IgG (Zymed) was used at 1:500 dilution.

Immunohistochemical staining of sections

Cryosections 7 μm thick were prepared for sequential staining of PAX-7, HOXA-11 and/or HOXA-13, and for double detection of HOXA-11 and PAX-7, from embryos fixed with methanol/DMSO. For detection of A223 antigen, which is specific to the chick cells, cryosections were prepared from embryos fixed with 4% paraformaldehyde. Cryosections were washed with PBS and incubated with 3% BSA/PBT (PBS containing 0.1% Triton X-100) at

room temperature for 30 minutes. They were treated with guinea pig anti-HOXA-11 IgG (diluted 1:400) and mouse monoclonal anti-PAX-7 IgG (Kawakami et al., 1997) or A223 (diluted 1:500) at 4°C overnight. After several washes with PBS, sections were incubated with FITC-conjugated anti-guinea pig IgG (diluted 1:400; Cappel) and rhodamine-conjugated anti-mouse IgG (diluted 1:400; Sigma) for 2 hours. Sections were washed and sealed with glycerol, then photographed under UV illumination. After recording the distribution of HOXA-11 and PAX-7, staining of HOXA-13 was performed as described below.

Sequential staining with anti-HOXA-13 and anti-PAX-7

Staining of HOXA-13 protein was performed with Vectastain Elite ABC kit (Vector). The sections were washed with PBS then incubated with normal goat serum (diluted 3:200) in 3% BSA/PBT. Subsequently, they were incubated with rabbit anti-HOXA-13 IgG (diluted 1:400) in 3% BSA/PBT then washed with PBS three times for 5 minutes each. They were treated with biotinylated goat anti-rabbit IgG (diluted 1:200) in PBT for 30 minutes and washed, then incubated for 30 minutes with Vectastain Elite ABC reagent. After several washes, sections were treated with Konica immunostain HRP-1000 coloring mixture (Konica) at room temperature for about 3 minutes, and then washed with PBS to stop the coloring reaction. Sections were mounted in glycerol for observation. After recording the distribution of HOXA-13, the signal was cleared by treatment with 70% ethanol, then sections were washed with PBS. Incubation of the cleared sections with normal rabbit serum (diluted 2:300) in 3% BSA/PBT was followed by treatment with mouse anti-PAX-7 IgG (diluted 1:500) in 3% BSA/PBT for 2 hours. After washing with PBS, they were incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (diluted 1:100) (Zymed) for 2 hours and then washed with PBS. To visualize PAX-7 signals, sections were incubated with coloring solution (FAST Red tablets, Boehringer Mannheim) at room temperature for several hours. After stopping the coloring reaction, sections were mounted in glycerol and observed.

RESULTS

Whole-mount detection of HOXA-11 and HOXA-13 protein in limb bud

Hoxa-11 and *Hoxa-13* mRNA distribution profiles in the chicken limb bud have already been reported by detailed *in situ* hybridization analysis (Yokouchi et al., 1991b). However, to identify the functional domains of these genes precisely, it is necessary to determine the spatial gene expression at the protein level. *Hoxc-6* mRNA was detected in the mesenchymal cells of both wing and leg buds. On the other hand, HOXC-6 protein product was only detected in the wing bud, indicating the presence of a post-transcriptional control for HOXC-6 expression (Nelson et al., 1996). Thus, we analyzed the distributions of HOXA-11 and HOXA-13 proteins in chick limb buds by whole-mount immunohistochemistry and compared the protein distributions with those of the respective mRNAs.

Hoxa-11 mRNA is first detected in the medial region of the limb mesenchymal cells underlying AER at stage 19 (Nelson et al., 1996). Subsequently, the expression domain expands to the distal half of the limb bud, then becomes restricted in the zeugopod region by down-regulation in the presumptive autopod region (Yokouchi et al., 1991b; Nelson et al., 1996). HOXA-11 protein was first detected in the mesenchymal cells of both wing and leg bud at stage 17 in addition to the tail region (data not shown). HOXA-11 protein distribution was the same as that of the mRNA at stage 19 (Fig. 1A and Yokouchi et al., 1991b; Nelson et al., 1996). On the other hand, no HOXA-11 protein was detected in the flank or somites at the level of the prospective wing at this stage (Fig. 1A). By stage 23, HOXA-11 had disappeared from both the anterior and the

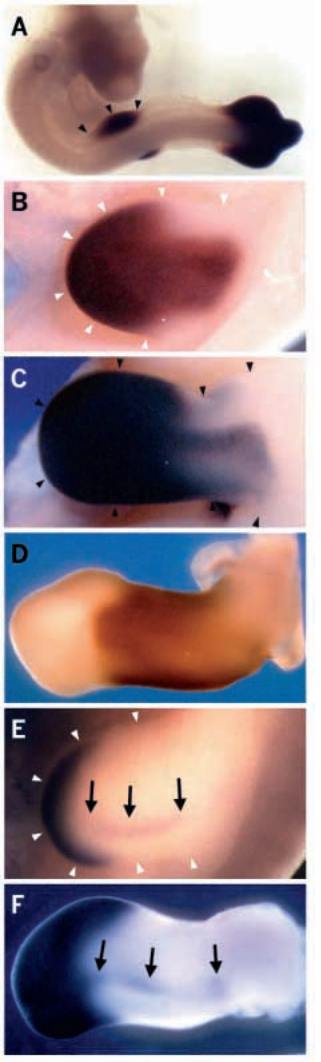

Fig. 1. Distributions of HOXA-11 and HOXA-13 proteins in chicken limb bud. Whole-mount detection of HOXA-11 (A-D) and HOXA-13 (E,F) in whole embryos (A) and wing bud (B-F). (A) HOXA-11 was expressed in the limb buds, the trunk of the leg bud region and the tail at stage 19. (B) At stage 23, HOXA-11 was expressed throughout entire region of the distal half and medial portion of the proximal half of the wing bud. (C) At stage 25, the expression domain of HOXA-11 became narrower in the proximal part of the wing. (D) At stage 28, HOXA-11 protein disappeared in the prospective autopod region as observed by *in situ* hybridization. (E) At stage 23, HOXA-13 was expressed in the distal mesenchyme and in the proximal-medial narrow region along the proximodistal axis (arrows) in the wing bud. (F) The expression domain of HOXA-13 expanded to almost the entire autopod region. A stripe-like expression domain of HOXA-13 at the proximal part was intermitted at the both stylopod-zeugopod and zeugopod-autopod boundaries (arrows). Neither HOXA-11 nor HOXA-13 were expressed in the ectoderm. The arrowheads show the margin of the forelimb bud. In B-F, distal is to the left and anterior is to the top.

posterior regions of the proximal half of the wing bud (Fig. 1B). HOXA-11 protein was detected in the medial region of the proximal half and entire region of the distal half of the wing mesenchyme, in a 'mushroom-like' pattern (Fig. 1B). HOXA-11 was expressed uniformly at the distal half of the wing bud, and the belt-like expression domain of HOXA-11 at the proximal half of the wing bud was narrowing at stage 25 (Fig. 1C). After stage 26, HOXA-11 protein disappeared from the prospective autopod, and the intensity of HOXA-11 expression in the stylopod region decreased leaving strong expression in the zeugopod (Fig. 1D).

Hoxa-13 mRNA is distributed in an autopod-specific manner in the developing chicken limb bud (Yokouchi et al., 1991b; Nelson et al., 1996). In the wing bud, HOXA-13 protein was detected in the posterior distal mesenchyme underlying the apical ectodermal ridge at stage 22, the onset of formation of the autopod region (Yokouchi et al., 1995). At stage 23, the expression domain of HOXA-13 expanded toward the anterior distal of the wing bud (Fig. 1E). In addition, stripe-like expression of HOXA-13 along the proximodistal axis was detected in the proximal posterior region of the wing bud (Fig. 1E). At this stage, each expression domain was separated clearly (Fig. 1E). At stage 26, the distal end of the stripe-like expression domain of HOXA-13 reached the autopod expression domain, but the stripe-like expression domain was intermitted at both stylopod-zeugopod and zeugopod-autopod boundaries (Fig. 1F). The intensity of the stripe-like domain was much weaker than that of the distal mesenchyme of the wing bud at all stages observed (Fig. 1E,F). HOXA-13 was expressed in the leg bud in the same manner as in the wing (data not shown).

By whole-mount immunohistochemical analysis we found that the distribution patterns of both HOXA-11 and HOXA-13 proteins were same as those of the respective mRNAs except for the following weak expression domains. Belt-like expression of HOXA-11 was detected in the prospective stylopod, and stripe-like expression of HOXA-13 was seen in the proximal region of the limb. These additional expression domains of both genes, in the proximal region relative to their mesenchymal expression domains, have not so far been reported in the chick limb buds by *in situ* hybridization analysis; this may be due to the problem of sensitivity to detection. These new expression domains were reminiscent of the distribution of myogenic cells migrating from the dermomyotome to the limb bud. However, expression of neither gene was detected in the trunk at the level of the wing or flank (Fig. 1A; data not shown).

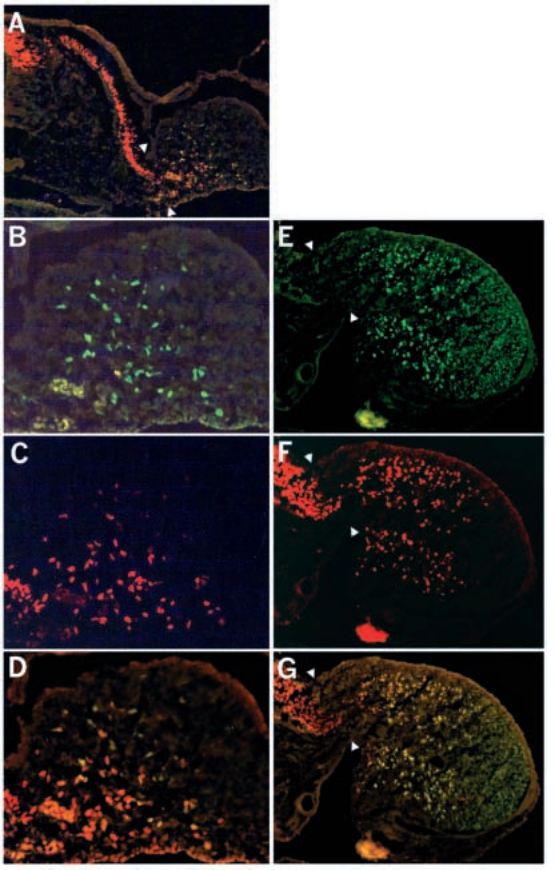

Fig. 2. HOXA-11 expression in the premyoblasts of the wing bud. Immunostaining of transverse sections at the level of the wing for HOXA-11 (green) and PAX-7 (red) as a marker of myogenic cells. HOXA-11/PAX-7 double-positive cells are shown as orange. (A-D) Stage 19. (A) Distributions of HOXA-11 and PAX-7. (B-D) Higher magnification of limb field of A; (B) HOXA-11, (C) PAX-7 and (D) HOXA-11 and PAX-7. Note that HOXA-11 was expressed in almost all PAX-7-positive cells in the limb bud but not those out of the limb field. (E-G) Stage 20. (E) HOXA-11, (F) PAX-7, (G) HOXA-11 and PAX-7. Note that PAX-7-positive cells were separated into the dorsal and ventral muscle masses (F) and expressed HOXA-11 (E). HOXA-11 was also expressed weakly in the distal mesenchyme of the limb. Proximal is to the left and dorsal is to the top. Arrowheads indicate the mesenchymal boundary between the trunk and the wing bud.

Detection of HOXA-11 in premyogenic cells in the limb bud

The belt-like expression of HOXA-11 suggested that myogenic cells express HOXA-11 as they invade the limb bud from the dermomyotome. To examine this possibility, cryosections were probed with anti-HOXA-11 antibody, and at the same time premyogenic cells were double-stained with an antibody against PAX7, a marker of the migrating premyogenic cell and myogenic cells in the limb buds.

In the wing bud at stage 19, PAX-7-expressing cells were detected in the dermomyotome and the wing field (Fig. 2A,C). Scattered distribution of the migrating PAX-7-expressing cells was found at the proximal region of the wing bud and HOXA-11 was detected in the nuclei of the same PAX-7-expressing cells in the wing bud (Fig. 2A-D). On the other hand, HOXA-11 was not detected in the PAX-7-expressing cells in either the dermomyotome or the trunk-wing boundary at stage 19 (Fig. 2E). At stage 20, myogenic cells assembled as the dorsal and ventral muscle masses in the wing bud. HOXA-11 was detected in almost all PAX-7-positive myogenic cells in both dorsal and ventral muscle masses (Fig. 2E-G). HOXA-11 was also detected in the distal mesenchymal cells underlying AER of the wing bud, and signals of HOXA-11 in the myogenic cell were stronger than those in the limb mesenchymal cells (Fig. 2E). At stage 24, HOXA-11 levels decreased at the posterior halves of the both dorsal and ventral muscle masses (Fig. 3D-F; compare with 3A-C). Then HOXA-11 was no longer detected in myogenic cells of either muscle mass by stage 26 (data not shown).

These observations demonstrated that premyogenic cells in the dermomyotome, migrating myogenic cells in the trunk and the limb mesenchymal cells along the route of myogenic cell migration do not express HOXA-11, and HOXA-11 expression starts after the premyogenic cells invade the limb buds.

Expression of HOXA-13 in myogenic cells in the limb bud

The HOXA-13 expression domain at the proximal region of the limb bud was narrower than the distribution of migrating myogenic cells. This might reflect HOXA-13 expression in specific subsets of muscle mass. To address this issue, HOXA-13 and PAX-7 were sequentially detected by specific antibodies in the same cryosections of the wing bud.

In the wing bud at stage 24, HOXA-13 was found in the nuclei of the PAX-7-positive cells at the posterior region of both the dorsal and the ventral muscle masses (Fig. 3H,I compare with B,C). The proximal boundary of the HOXA-13 domain was more distal than that of HOXA-11 in both muscle masses (Fig. 3G,H compare with 3D,E). HOXA-11 was down-regulated in the posterior halves of both dorsal and ventral muscle masses at this stage. The HOXA-13 domain was narrower than the area where HOXA-11 has been down-regulated (Fig. 3D-I). At the anterior side of the dorsal muscle mass, in addition, a few myogenic cells that expressed both PAX-7 and HOXA-11 expressed HOXA-13 weakly (Fig. 3E,F,H,I). No PAX-7-expressing cells were found in the prospective autopod where mesenchymal HOXA-13 expression was prominent (data not shown). Localized distribution of HOXA-13 is the earliest indication of polarity in the muscle masses.

After stage 27, patterning of individual muscles proceeds by sequential splitting of both muscle masses. As the muscle patterns in the zeugopod and autopod region are easy to observe, we analyzed the expression of HOXA-13 in these regions after stage 27. In the proximal region of the zeugopod at stage 27, the ventral muscle mass started to split. HOXA-13 was expressed in the nuclei of the all PAX-7-expressing cells in the posterior one third of the posterior counterpart of the ventral muscle mass (Fig. 4A,B,D). HOXA-13 was not detected at the boundary between the posterior muscle mass and central muscle mass at this stage (Fig. 4B,D). HOXA-13 expression was detected before splitting of the dorsal muscle mass started in the myogenic cells of the posterior region (Fig. 4A,B,D). Weak expression of HOXA-13 was also found in a few PAX-7-expressing cells in the anterior region of the dorsal muscle mass (Fig. 4B). HOXA-13 was not detected in the non-PAX-7-expressing cells in the muscle masses (Fig. 4B,D).

By stage 28, the dorsal muscle mass had split into the anterior dorsal muscle mass and posterior dorsal muscle mass (Fig. 4E). At the dorsal side, HOXA-13 was found in the posterior two thirds of the posterior dorsal muscle mass and in a few myogenic cells in the anterior dorsal muscle mass (Fig. 4C,E). At the ventral side, HOXA-13 was detected in all the PAX-7-expressing cells in the posterior ventral muscle mass and in part of the central ventral muscle mass (Fig. 4C,E). In the zeugopod at stage 28, HOXA-13 expression in the posterior ventral muscle mass was stronger than in the other muscle masses (Fig. 4C). Splitting of the central ventral muscle mass started at stage 30. At this stage, HOXA-13 was expressed throughout the entire region of the central ventral muscle mass, but not in all the myogenic cells (data not shown). In the autopod region, where HOXA-13 was expressed in mesenchymal cells, splitting of the dorsal and ventral muscle masses had not yet started. HOXA-13 was hardly detected in the PAX-7-expressing cells of the autopod region (data not shown).

By stage 36, the splitting process was complete and individual muscles were distinct in the wing bud (Fig. 5A,E). In the zeugopod, strong HOXA-13 signals were detected in the nuclei of the PAX-7-expressing cells of FDS and FCU derived from the posterior ventral muscle mass (Fig. 5D,G) and EMR originated from the anterior dorsal muscle mass (Fig. 5C,F). As muscles showing strong HOXA-13 expression were derived from the region where HOXA-13 was found at stage 24, it is possible that HOXA-13 expression is maintained in this lineage (Figs 3H,I and 5C,D; also see Fig. 9). Almost all the PAX7-positive cells in FDS and FCU strongly expressed

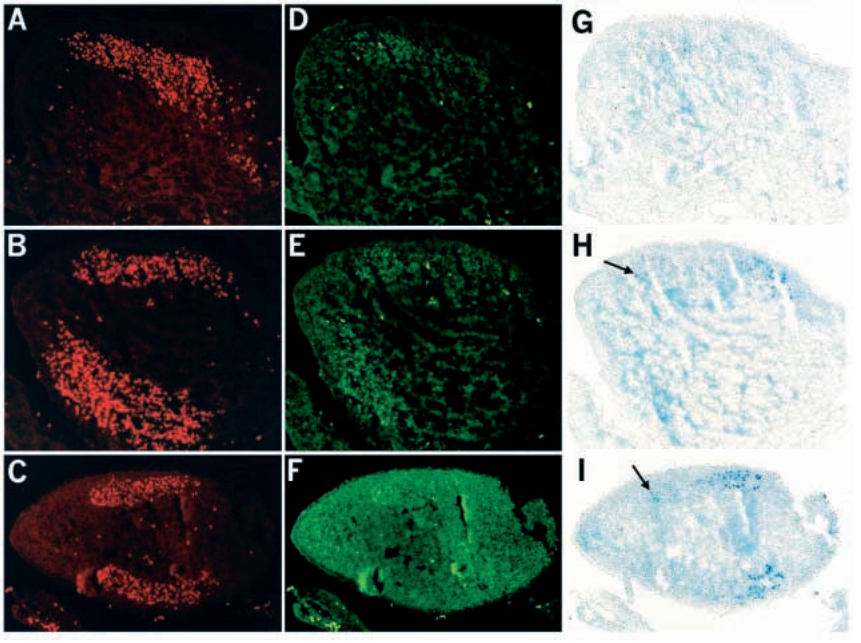

Fig. 3. HOXA-11 and HOXA-13 expression in the muscle masses in wing bud at stage 24. Cross sections of the wing bud were prepared by cutting perpendicularly to the proximodistal axis of the forelimb; (A,D,G) proximal level, (B,E,H) medial level, (C,F,I) distal level of the wing bud. The sections were stained for PAX-7 (red; A-C), HOXA-11 (green; D-F) and HOXA-13 (blue; G-I). Note that HOXA-11 was expressed in the anterior halves and was down-regulated in the posterior halves of both muscle masses (A-F). On the other hand, HOXA-13 was expressed strongly in all PAX-7-positive cells in the posterior region of both muscle masses and weakly in a few cells in the anterior region of the dorsal muscle mass (H,I; arrows). The proximal boundary of the HOXA-13 domain was more distal than the HOXA-11 domain in muscle masses (D,E,G,H). Dorsal is to the top and anterior is to the left.

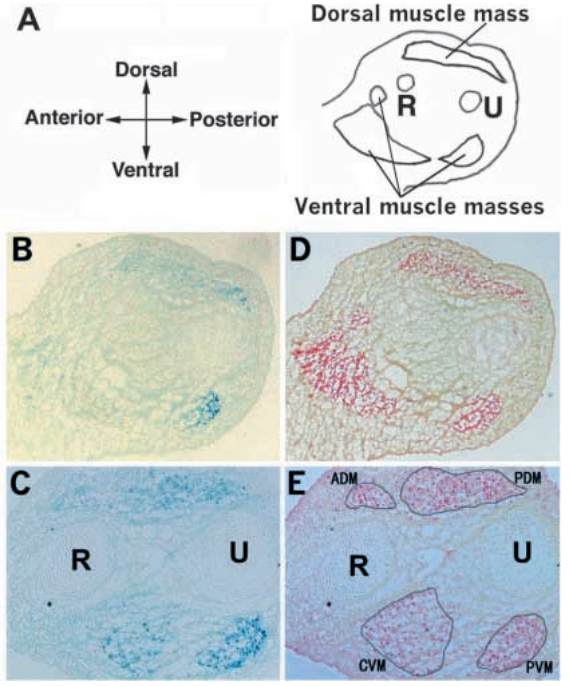

Fig. 4. Distribution of HOXA-13-positive cells in the zeugopod at stages 27 and 28. Cross sections sequentially stained for HOXA-13 (blue; B,C) and PAX-7 (red; D,E). (A,B,D) The proximal region of a stage-27 zeugopod. (A) Schematic view of the muscle pattern of the zeugopod at stage 27. HOXA-13 was expressed strongly in all PAX-7-positive cells in the posterior one third of the posterior ventral muscle mass and in the posterior region of the dorsal muscle mass (B). (C,E) Stage 28 zeugopod. At this stage, HOXA-13 was found in PAX-7-positive cells in the posterior ventral muscle mass, a part of the central ventral muscle mass and the posterior two thirds of the posterior dorsal muscle mass (C,E). At both stages, weak expression of HOXA-13 was detected in a few cells in the anterior region of the dorsal muscle mass (B,C). Dorsal is to the top and anterior is to the left. R, radius; U, ulna; ADM, anterior dorsal muscle mass; PDM, posterior dorsal muscle mass; CVM, central ventral muscle mass; PVM, posterior ventral muscle mass.

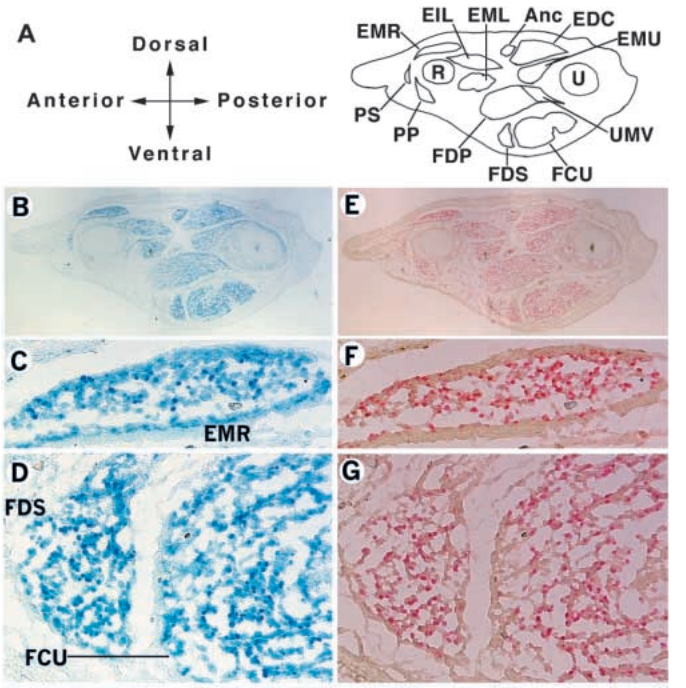

Fig. 5. Distribution of HOXA-13-positive cells in the zeugopodal muscle at stage 36. By this stage, the splitting process had finished. Cross sections stained sequentially for HOXA-13 (blue; B-D) and PAX-7 (red; E-G). (A) Schematic view of the muscle pattern of the zeugopod. Higher magnifications of B and E are shown in C,D and F,G, respectively. Strong expression of HOXA-13 was restricted in the nuclei of some PAX-7-positive cells in EMR (C,F) and in the most PAX-7 positive cell nuclei of FDS and FCU (D,G). Muscle identification and muscle nomenclature follow Shellswell and Wolpert (1977) and Robson et al. (1994). Dorsal is to the top and anterior is to the left. R, radius; U, ulna.

HOXA-13 (Fig. 5D,G). However, in EMR less than half of the PAX-7-positive cells expressed HOXA-13 and such cells showed a random distribution in the EMR (Fig. 5C,F). On the other hand, HOXA-13 was no longer detected in EMU and Anc, which originated from the posterior dorsal muscle mass that expressed HOXA-13 at stage 24 (data not shown). The expression pattern of HOXA-13 appeared not to be correlated with distributions of fast and slow muscle fibers that were described previously (Robson et al., 1994).

Formation of individual muscles proceeded in the autopod region at stage 36 (Fig. 6A,B,E). HOXA-13 was detected in the nuclei of the PAX-7-positive cells of EIB/Ad.I UMD/FDQ (Fig. 6B,D,E,G) and in the nuclei of the non-PAX-7-expressing cells of FI/Ab.I (Fig. 6C,F). At this stage, although weak HOXA-13 signals were detected in all muscles regardless of PAX-7 expression (Figs 5, 6), signals were not seen in the nuclei. As *Hoxa-13* encodes a transcription factor, functional HOXA-13 should be localized in the nuclei. It is likely that the weak cytoplasmic staining was a false signal, thus we ignored these cytoplasmic signals.

Ectopic induction of HOXA-11 in premyogenic cells

As HOXA-11 was detected in premyogenic cells in the limb bud but not in the somitic cells prior to invasion of the limb bud, it is possible that HOXA-11 expression in the premyogenic cells is dependent on the inductive interaction with the limb mesenchymal cells. To examine this possibility, the somatopleure of the prospective wing bud of stage 14-16 quails was transplanted into the flank region of stage 14-16 chick embryos, then expression of HOXA-11 in the ectopic limb bud was analyzed. Such replacement has been demonstrated to induce the migration of cells from flanking dermomyotome into the ectopic limb bud (Hayashi and Ozawa, 1995).

HOXA-11 was detected in the proximal region of the ectopic limb bud at stage 20 (Fig. 7A; both of 2 samples). As HOXA-11-positive cells were also detected with chicken-specific antibody A223, it is clear that they originated from the chicken somites (Fig. 7B). Similar results were obtained in the ectopic limb buds of transplanted animals allowed develop further to stage 25 (data not shown; both of 2 samples). On the other hand, transplantation of the flank somatopleure of stage 14-16 quail donors to the chick flank neither induced ectopic limb bud nor had an effect on HOXA-11 induction (Fig. 7C-E; all of 3 samples). These results suggest that HOXA-11 expression

in the myogenic cells was induced by the limb mesenchyme by inductive interaction when they invaded into the limb bud.

Ectopic expression of HOXA-13 by local application of retinoic acid

It was suggested that pattern formation of the limb muscles is under the control of the limb mesenchyme (Chevallier and Kieny, 1982). In particular, transplantation of ZPA to the anterior margin of the limb bud not only results in the mirror-image duplication of the autopodal cartilage and transformation of the radius to the ulna but also induces the symmetrical duplication of the posterior muscles in the anterior region (Shellswell and Wolpert, 1977; Tickle, 1991). The signal from ZPA can be reproduced by local application of retinoic acid and patterns of both cartilages and muscles are duplicated (Tickle, 1991; Robson et al., 1994). Positional signaling from ZPA might regulate the splitting processes of muscle masses and the specialization of each muscle through induction of the localized expression of HOXA-13 in the muscle masses. If this is the case, application of a posteriorizing signal at the anterior margin of the limb bud should result in HOXA-13 expression at the anterior region of the muscle masses. To address this possibility, we analyzed the expression pattern of HOXA-13 in the duplicated limb bud induced by the local application of retinoic acid.

The induced pattern alteration of cartilage and muscle was dependent on the concentration of retinoic acid. The wing bud treated with a bead soaked in 1 mg/ml retinoic acid developed two ulnae in the zeugopod and four digits in a 4334 pattern (data not shown). Digit 2 did not develop following application of either a high concentration of retinoic acid or a strong posteriorizing signal (Tickle, 1991). Ectopic expression of HOXA-13 was found in the anterior region of the muscle masses in the duplicated wing bud (Fig. 8A,C,D). In the zeugopod of the duplicated limb at stage 28, three muscle masses were arranged symmetrically at the ventral side (Fig. 8B). In addition to the cells in the posterior muscle mass, ectopic HOXA-13 expression was found in the nuclei of PAX-7-positive cells located in the anterior part of the anterior muscle mass at the ventral side (Fig. 8A). At the dorsal side, the number of HOXA-13-positive cells increased and at the same time the intensity of the HOXA-13 signal also increased (Fig. 8A). In the treated limb at stage 36, the posterior dorsal muscles EMU and EDC, and the posterior ventral muscles, ectopic FCU developed at the anterior side of the duplicated limb in place of EMR, PS and PP, as already reported (Robson et al., 1994) (Fig. 8C,D). HOXA-13 expression was found in

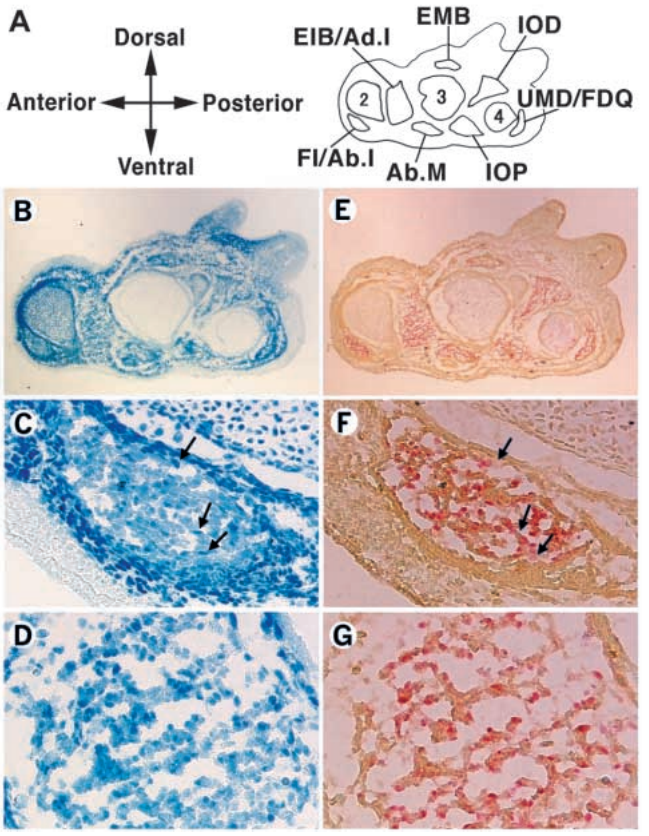

Fig. 6. Distribution of HOXA-13-positive cells in the muscles at the metacarpal level at stage 36. Cross sections of the autopod region sequentially stained for HOXA-13 (B-D) and PAX-7 (E-G). (A) Schematic view of the muscle pattern of the autopod. Metacarpal cartilages were labeled 2, 3 and 4. Higher magnification views of B and E are shown in C,D and F,G, respectively; (C,F) FI/Ab.I, (D,G) EIB/Ad.I. Note that HOXA-13 was expressed strongly in some PAX-7-positive cells of EIB/Ad.I and UMD/FDQ (B,D,E,G) and in some PAX-7-negative cells in FI/Ab.I (C,F; arrows). Muscle identification and nomenclature as in Fig. 5.

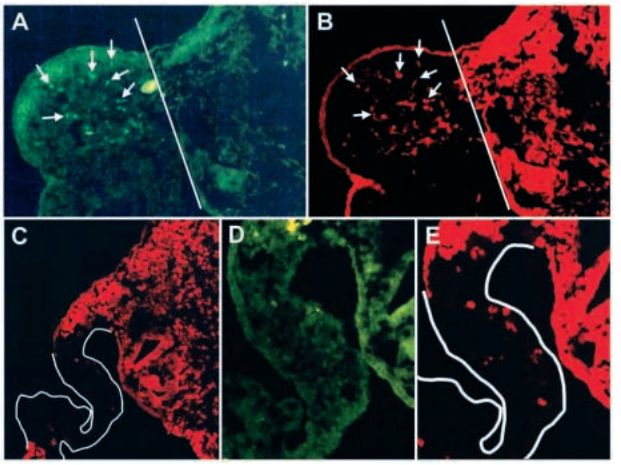

Fig. 7. HOXA-11 induction by transplantation of somatopleure. Quail lateral plate mesoderm from the prospective wing level (A,B) or the flank level (C-E) was grafted to the chick flank. Cross sections of stage-20 grafted embryos at the flank level were stained with anti-HOXA-11 IgG (green; A,D) and chicken-specific antibody A223 (red; B,C,E). (D,E) High magnification view of the section shown in C. Lateral plate mesoderm of presumptive wing bud grafted to the flank induced formation of the ectopic limb bud and migration of the somitic cells to the ectopic limb bud (B). Migrating chicken cells (arrows) expressed HOXA-11 in the ectopic limb bud (A,B). On the other hand, transplantation of lateral plate mesoderm of quail flank induced neither morphological change at the grafted site nor expression of HOXA-11 in the myogenic precursors (C-E). Lines in A and B indicate the boundary of quail and chick mesenchyme. Lines in C and E indicate the quail-derived tissues. Moderate staining with FITC in the dermomyotome and in the tip of limb bud was background. Dorsal is to the top and distal/lateral is to the left.

both intrinsic and induced FCUs (Fig. 8C-F). The number of cells expressing HOXA-13 in the ectopic anterior FCU was less than that of the original FCU, and the anterior FCU tended to be smaller than the original FCU (Fig. 8E,F). In the duplicated limbs, ectopic and symmetrical formation of both the posterior muscles, EDC, EMU and FCU, and the medial muscles, EIL, EML and FDP, occurred in the anterior region. On the other hand, Anc, FDS and UMV, normally located between the posterior and the medial muscles, often disappeared in the original posterior region, as already reported (Robson et al., 1994) (Fig. 8C,D).

A bead soaked in 0.5mg/ml retinoic acid had weaker posteriorizing activity than 1 mg/ml retinoic acid, and the manipulated wing bud had five digits in a 43234 pattern and apparently normal radius and ulna in the zeugopod (Fig. 8G). The limbs treated with a bead soaked in 0.5 mg/ml retinoic acid were apparently normal with respect to both the tissue pattern of the muscle masses and the expression pattern of HOXA-13 at stage 28 (data not shown). At stage 36, the expansion of the mesenchyme occurred in the manipulated limb but there were no differences in number or arrangement of the muscles (Fig. 8G). In this case, strong expression of HOXA-13 was detected in the nuclei of the myogenic cells in FDS and FCU as in normal development (Fig. 8G). However, the expression disappeared in EMR (Fig. 8G). Reduction in size of EMR was observed in this case. At the same time, reduction in size of EIL and EML was also observed (Fig. 8G).

These results suggest that expression of the HOXA-13 in the posterior muscle masses is regulated by the posteriorizing signal. In addition, there was a correlation between the ectopic expression of HOXA-13 at the anterior side of the muscle masses and the formation of mirror-image duplication of the muscle pattern.

DISCUSSION

In this study, we first showed that limb mesenchymal expression patterns of *Hoxa-11* and *Hoxa-13* mRNA were the same as the distributions of the respective protein products. Then, we demonstrated direct evidence of the expression of *Hox* genes in the precursors of the limb skeletal muscle in vertebrates. Our results suggest that HOXA-11 is the earliest marker of the myogenic cells in the limb bud and that the expression of HOXA-13 is the first signal of polarization of the limb muscle masses. It was suggested previously that the pattern of *Hoxa-11* expression is reminiscent to the migratory process of the myogenic cells in the mouse limb bud (Haack and Gruss, 1993). Thus *Hox* gene expression in the myogenic cells seems to be common among vertebrates.

Induction of HOXA-11 in the invading premyogenic cells to limb bud

At the onset of limb muscle development, the premyogenic cells at the ventral dermomyotome are induced to migrate into the limb field by signals from the limb mesenchyme (Chevallier et al., 1977, 1978; Christ et al., 1977; Ordahl and Le Douarin, 1992; Hayashi and Ozawa, 1995). HOXA-11 was never detected in the dermomyotome at the level between the wing and the flank or in the premyogenic cells immediately after detachment from the dermomyotome. However, HOXA-11 was expressed in the premyogenic cells when they invaded into the limb field. The expression profile of HOXA-11 suggests two possible regulatory mechanisms for the initiation of *Hoxa-11*. Firstly, the extracellular signal(s) derived from the

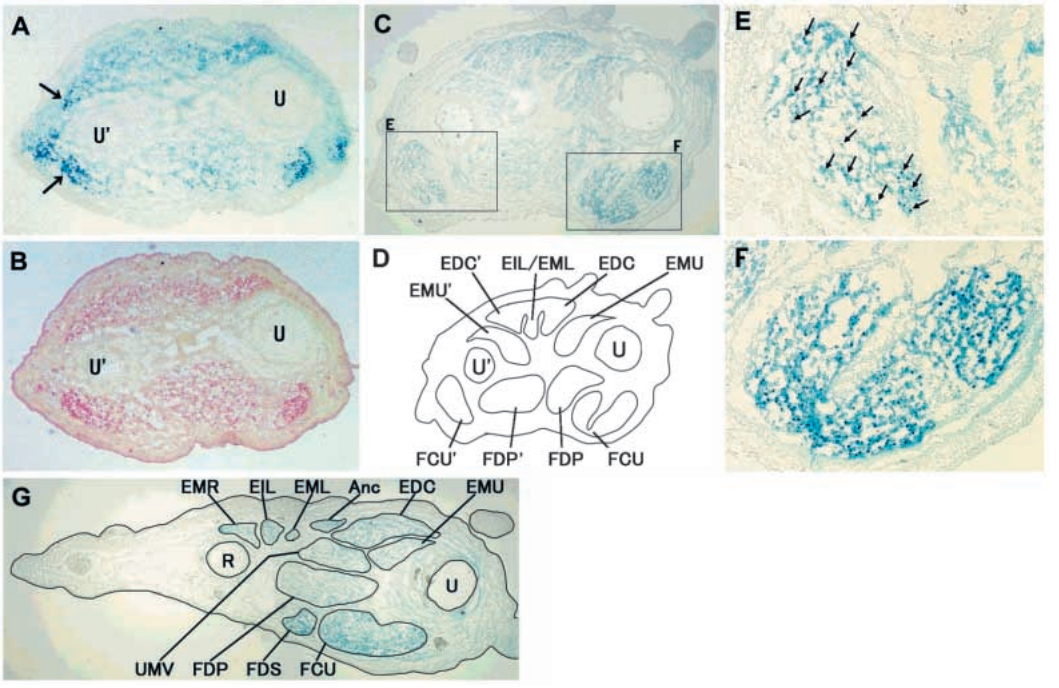

Fig. 8. Effect of local application of retinoic acid (RA) on expression of HOXA-13 in the musculature. (A,B) Cross sections of stage-28 zeugopod implanted bead soaked in 1 mg/ml RA at the anterior margin; (A) HOXA-13, (B) PAX-7. Strong expression of HOXA-13 was detected in the anterior region of the muscle masses ectopically (arrows; A,E). (C) Distribution of HOXA-13 in the stage 36 zeugopod treated with 1 mg/ml RA. Boxes show regions magnified in (E) and (F). (D) Schematic view of the muscle pattern in (C). At stage 36, EMU, EDC, FDP and FCU developed in the anterior side symmetrically as the posterior side (C,D), and strong expression of HOXA-13 was found in intrinsic and induced FCUs (E,F). (G) Expression of HOXA-13 in the stage-36 zeugopod treated with 0.5 mg/ml RA, together with the outlined muscle pattern. There were no differences in the number or arrangement of the muscles. In addition, HOXA-13 was detected in FDS and FCU as in normal development, but HOXA-13 expression disappeared in EMR (G). Dorsal is to the top and anterior is to the left. R, radius; U, ulna; Ectopically formed cartilage and muscle are indicated as U′, EMU′, EDC′, FDP′ and FCU′.

limb mesenchyme may induce the invading premyogenic cells to express *Hoxa-11*. Alternatively, according to the positional values distributed through the trunk, the ventral dermomyotomal cells at the wing level may be determined to express *Hoxa-11* autonomously after invading into the limb bud. Transplantation of the quail prospective wing mesenchyme into the chick flank induced the flank somitic cells to express *Hoxa-11* in the ectopic limb buds. This indicates that the somitic cells at the level of both the wing and the flank are competent to express *Hoxa-11*. As transplantation of the quail flank somatopleure into the chick flank failed to induce *Hoxa-11* in chick flank somitic cells, it is obvious that the premyogenic cells require the influence of limb mesenchyme to express *Hoxa-11*. Thus, it is suggested that expression of *Hoxa-11* in premyogenic cells is not programmed at the somite level but is induced by the extracellular signal derived from the limb mesenchyme. HGF expressed in the limb mesenchyme is thought to stimulate the premyogenic cells to migrate into the limb bud through its receptor, c-Met, expressed in the ventral dermomyotome (Bladt et al., 1995; Brand-Saberi et al., 1996b; Epstein et al., 1996; Yang et al., 1996). It is possible that HGF induces not only migration of the premyogenic cells but also the initiation of *Hoxa-11* expression. The results of analyses with mouse myogenic cell lines suggested that the FGF family, TGF and BMP-2, regulate cell proliferation and differentiation during myogenesis (Olson, 1992; Katagiri et al., 1997). As these signaling molecules are expressed in the limb mesenchyme during early limb development, they may be involved in the expression of *Hoxa-11*.

Induction of position-specific HOXA-13 expression in the limb muscle mass

In the limb bud, the myogenic cells congregate into the dorsal and ventral muscle masses, and then individual muscles are formed by the sequential splitting of the muscle masses (Shellswell and Wolpert, 1977). Fig. 9 shows schematically the splitting process of the zeugopodal muscle masses in the wing bud (Fig. 9A) and the expression pattern of HOXA-13 in myogenic cells of the zeugopod (Fig. 9A) and the autopod (Fig. 9B). The myogenic cells began to express HOXA-13 strongly at the posterior regions of both dorsal and ventral muscle masses and weakly at the anterior region of dorsal muscle mass before muscle splitting occurs. As the local application of retinoic acid to the anterior margin of the limb bud induced HOXA-13 expression at the anterior region of the muscle masses, expression of HOXA-13 expression in the myogenic cells appears to be regulated by the posteriorizing signal derived from ZPA (Shellswell and Wolpert, 1977; Robson et al., 1994). Sonic hedgehog is one such signaling molecule, although it is not known whether the myogenic cells are competent to respond to this signal (Riddle et al., 1993). BMP-2 may also be involved in the signaling pathway for *Hoxa-13* expression because *Bmp-2* expression is induced at the posterior region of the limb bud by the posteriorizing signal (Francis et al., 1994). In addition, application of BMP-2 at the anterior margin resulted in induction of another *Hox* gene, *Hoxd-13*, in the limb mesenchymal cells (Duprez et al., 1996). It was suggested that *Wnt7a* and *Lmx1*, expressed in the dorsal limb ectoderm and in the dorsal limb mesenchyme, respectively, are involved in the dorsalizing process of muscle mass (Parr and McMahon, 1995; Riddle et al., 1995). These factors may be involved in controlling the differences in *Hoxa-13* expression between dorsal and ventral muscle masses.

After stage 28, the expression domain of HOXA-13 changed markedly in the zeugopodal musculature (Fig. 9A). These observations suggested that the potential of the limb mesenchyme for induction and maintenance of HOXA-13 expression in the muscle masses and/or competence of the myogenic cells to respond to these signaling vary between region and lineage during limb muscle development.

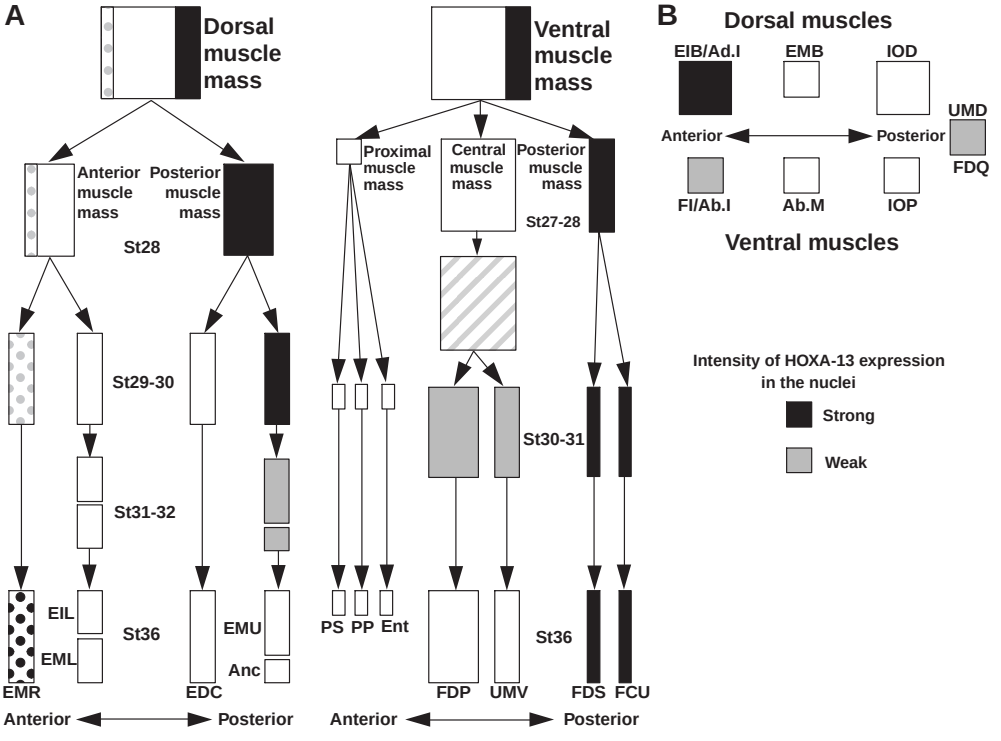

Fig. 9. Schematic diagrams of the splitting patterns of the muscle masses, superimposed with the expression pattern of HOXA-13 in the zeugopod (A) and in the autopod (B). Note that HOXA-13 was expressed in less than half of the PAX-7-positive cells in the anterior dorsal muscle mass and in its derivative, EMR (dotted box). In addition, HOXA-13 was expressed weakly in part of the central ventral muscle mass at stage 28 (hatched box).

Spatio-temporal colinearity of expressions of HOXA-11 and HOXA-13 in the myogenic cells

The musculature expression domains of both HOXA-11 and HOXA-13 are different from the mesenchymal expression domain in the limb bud. In the limb mesenchyme, expression of both *Hoxa-11* and *Hoxa-13* began in the distal-posterior region underlying AER (Yokouchi et al., 1991b; Rogina et al., 1992; Nelson et al., 1996). When HOXA-13 expression was prominent in the prospective autopod, mesenchymal HOXA-11 expression disappeared in this region (Yokouchi et al., 1991b and this study). Thus, the rule of temporal colinearity and the mutually exclusive manner of these gene expressions resulted in spatial colinearity of these gene expression domains along the proximodistal axis. On the other hand, musculature expression of HOXA-11 and HOXA-13 exhibited independent regional specificity from mesenchymal expression along the proximodistal axis in the limb.

However, the spatio-temporal relationship of expression profiles between HOXA-11 and HOXA-13 in the myogenic cells was similar to that in the limb mesenchyme. In both tissues, HOXA-13 was expressed after HOXA-11 expression in the region where HOXA-11 has been expressed (Yokouchi et al., 1991b; Nelson et al., 1996 and this study). This spatio-temporal colinearity is conserved in the expression profiles of almost all *Hox* genes (McGinnis and Krumlauf, 1992). In addition, activation of *Hoxa-13* is accompanied by down-regulation of *Hoxa-11* in both tissues. One characteristic point in musculature expression is that HOXA-13 expression was observed in the particular region where HOXA-11 was down-regulated. This would be due to the signal(s) for *Hoxa-13* activation in the muscle mass and in the limb mesenchymal cells being different, both in their nature and the position of their expression. At the same time, systems for receiving and/or interpreting these signals are assumed to be different between the mesenchymal cell and the muscle precursor cells in the limb buds.

Roles of *Hoxa-11* and *Hoxa-13* during limb muscle development

Individual muscles are formed by sequential splitting of the muscle masses and have characteristic size, shape, origin, insertion and pattern of muscle fibers (Shellswell and Wolpert, 1977; Stockdale, 1992; Robson et al., 1994). *Hoxa-13* was expressed in the posterior region of both the dorsal and the ventral muscle masses and in the anterior region of the dorsal muscle mass. Such localized expression of *Hoxa-13* is maintained in the prospective EMU/Anc until stage 30 and in the prospective FCU, FDS and EMR until the splitting process is completed at stage 36. Application of retinoic acid at the anterior margin of the limb bud induces ectopic expression of *Hoxa-13* in the anterior region of the muscle masses followed by mirror-image duplication of the muscles. The duplicated muscles always had ectopic FCU and EMU in which *Hoxa-13* was expressed during normal development. These results indicate the involvement of *Hoxa-13* in limb muscle patterning.

In *Drosophila*, *Hox* genes expressed in the muscle precursor cells play a crucial role during muscle patterning in an autonomous fashion (Greig and Akam, 1993; Michelson, 1994; Roy et al., 1997; Roy and VijayRaghavan, 1997). On the other hand, the experimental biology of vertebrates tells us that the pattern of the musculature appears to be controlled by the environment, for instance the connective tissue (Chevallier and Kieny, 1982; Lance-Jones, 1988; Grim and Wachtler, 1991; Ordahl and Le Douarin, 1992). At the same time, however, there are experiments suggesting that myoblasts have heritable, cell-autonomous information about their positional identity in vertebrate (Donoghue et al., 1992; Grieshammer et al., 1992; DiMario et al., 1993). Detailed analysis of the limb muscle pattern in *Hoxa-11* and *Hoxa-13* mutant mice and misexpression experiments via replication-competent retrovirus in chick that we are now addressing, will provide definitive answers on the role of *Hox* gene in limb muscle patterning.

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