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Differential Expression of the Two Closely Related LIM-Class Homeobox Genes *LH-2A* and *LH-2B* during Limb Development

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We have isolated the chicken homeobox genes *LH-2A* and *LH-2B* encoding two related LIM domain-containing homeodomain proteins and examined the expression pattern during chick limb development. *LH-2A* is most closely related to human and rat *LH-2,* while *LH-2B* is less conserved. Although both *LH-2A* and *LH-2B* are expressed in the limb mesenchyme throughout stage 16 to stage 32, *LH-2A* transcripts are detectable in the distal limb bud and *LH-2B* transcripts are detectable in the anterior limb bud. Signals from the apical ectodermal ridge positively regulate *LH-2A* expression, since removal of the apical ectoderm resulted in the rapid reduction of *LH-2A* expression in the distal limb mesenchyme. Ectopic expression of the *sonic hedgehog* gene in the anterior margin of the limb bud resulted in the rapid reduction of *LH-2B* expression accompanying respecification of the positional value to the posterior phenotype. These results suggest that *LH-2A* and *LH-2B* play important roles in the determination and specification of the proximal-distal and anterior-posterior positional values, respectively.

Homeobox genes are implicated in determination of positional identity during limb development, and loss-of-function and gain-of-function studies suggest that the *AbdB*- class *Hox* genes are involved in proximal-distal pattern formation in the limb bud (1-6). However, anterior-posterior patterning of the digits could not simply be interpreted as the different but overlapping expression patterns of the *HoxD* genes, since knock-out or misexpression of the *HoxD* genes did not reproduce homeotic transformation of the digits, except *HoxD11* (7). Although anterior-posterior pattern is induced by the polarizing signal *sonic hedgehog* in the limb bud, no homeobox gene is known that shows consistent expression during digit pattern formation along the anterior-posterior axis.

In the present study, we have identified the LIM-class homeobox genes related to *Drosophila apterous*, and examined the expression pattern during limb development. One of the family called *Lmx-1* has been characterized as a determinant of dorsal-ventral patterning in the limb bud (8), and considered to represent a vertebrate counterpart of *apterous*. A member of the *LH-2*-subclass homeobox genes has been identified in the vertebrate (9,10). Two closely related *LH-2* genes, called *LH-2A* and *LH-2B*, revealed completely different expression pattern in the limb bud. *LH-2A* is expressed in the distal mesenchyme, whereas *LH-2B* expression is restricted to the anterior half of the limb mesenchyme. The different expression patterns suggest that *LH-2A* and *LH-2B* are involved in the specification of distal and anterior compartments, respectively, representing positional identity in the limb bud.

MATERIALS AND METHODS

Chick embryo was staged according to Hamburger and Hamilton (11). Total RNA was prepared from stage 20-23 chick embryos using ISOGEN (NipponGene), and poly(A) RNA was isolated using PolyA-Tract System (Promega). Complementary DNA was synthesized using Read-To-Go kit (Pharmacia) and used as a template for polymerase chain reaction (PCR) amplification with the following primers. Degenerate primers of 5′-CTNAARTGYTGYGARTGYAA-3′ and 5′-GCRTTYTGRAACCANACYTG-3′ encode LKCCECK sequence of the LIM domain and QVWFQNA sequence of the homeodomain on the complementary strand, respectively. PCR was carried out at an-

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nealing temperatures of 63 to 53°C, decreasing 0.5°C per cycle for 20 cycles, followed by additional 15 cycles at annealing temperature of 53°C with GeneAmp System 9600. The amplified cDNA of about 0.7 kb was cloned into pCR-Script SK(+) and identified by nucleotide sequencing with ABI Sequencer Model 373S. To determine full coding sequences, double-stranded cDNA was ligated at first with a *Bst*XI linker-adapter, then with *Bst*XI-digested pRc/CMV (Invitrogene), and used as a template for PCR. Primers were synthesized based on the partial cDNA sequences of *LH-2A* and *LH-2B*, and the vector sequence flanking *Bst*XI cloning site. Full-coding sequences of *LH-2A* and *LH-2B* were finally obtained by PCR using following primers: 5′-TCAGCGATGCTTTTCCACAG-3′ and 5′-CACTTGGAATTTCAACTAAG-3′ for *LH-2A;* 5′-GCCTCGGTGTAAACATTTTG-3′ and 5′-CGGCAATGTTAGAAAGGTT-3′ for *LH-2B*.

Whole-mount *in situ* hybridization was carried out as described (12) using following chicken cDNAs as probes. Digoxigenin-labelled cRNA probes for *LH-2A, LH-2B* and *Msx-1* were synthesized using T7 and SP6 polymerases as described previously (13-15). Corresponding sense probes were used to estimate signal specificity, and showed no significant hybridization signal.

Removal of the apical ectodermal ridge and implantation of the zone of polarizing activity were carried out using sharpened tungsten needles in the right limb bud at stages 20-21. Amino-terminal coding sequence of *sonic hedgehog* (SHH-N) was subcloned into the modified RCAS (A) retroviral vector (15). Virus-free chicken embryonic fibroblasts from line M embryo (Nisseiken) were transfected with the recombinant DNA using Lipofectin (GIBCO-BRL). Fibroblasts producing RCAS-SHH-N virus were implanted to the anterior margin of the right limb bud in the virus-sensitive chick embryos at stages 19-20. The embryos were fixed 24 to 72 hours after implantation to carry out whole-mount *in situ* hybridization.

RESULTS

We obtained two independent cDNAs, *LH-2A* and *LH-2B*. Comparison of the deduced amino acid sequence indicates that chicken *LH-2A* is 86% identical to rat and human *LH-2*, whereas chicken *LH-2B* is 71-72% identical to rat and human *LH-2* (9,10) (Fig. 1). Overall sequence of *LH-2A* and *LH-2B* are 74% identical within species, suggesting that rat *LH-2* and human *LH-2* are most likely to be cognates of chicken *LH-2A*, although the carboxy-terminal region and the joint region between the LIM domain and homeodomain are less conserved (Fig. 1).

Although *LH-2A* and *LH-2B* encode closely related LIM domain and homeodomain proteins, their expression patterns entirely differ. In the developing limb, the *LH-2A* gene is expressed in the distal mesenchyme throughout stages 16 to 32, whereas the *LH-2B* gene is expressed in the anterior half of the limb mesen-

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LH-2A (GG)  MLFHSLSGSEMHGVIDEMDRRTKTEAAAISSAIDRGETETQTMPSISSDRAALCAGCGGKISDRYYLLAVDKQWHMRCLK  80
LH-2B (GG)  ....GI..GHIQ.IME..E..S-KTESRLAKGGQMNGR..N-..PM.PEKP.....................L....  78
LH-2 (HS)   ........P.V..........-QERGSR........D...-.............G.....................  78
LH-2 (RN)   ........PQV.....Q....-QERGSG........D...-.....................................  78
                                                                 ============ LIM domain 1 ==

LH-2A (GG)  CCECKLNLESELTCFSKDGSIYCKEDYYRRFSVQRCARCHLGISASEMVMRARDLVYHLNCFTCTTCNKMLTTGDHFGMK 160
LH-2B (GG)  ......A........A.....................................ES....S.........T.......... 158
LH-2 (HS)   ..................................................................................... 158
LH-2 (RN)   ..........................................E.............................................. 158
            ================================  =============== LIM domain 2 ==================

LH-2A (GG)  DNLVYCRLHFETLIQGEYQVHFNHSDVA-AGKG------PALGAGSANTLGLPYYNGVGTVQKGRPRKRKSPGPGADLAA 233
LH-2B (GG)  .......A...S.L....PPQLSYTEL.-.---------------KSGG.A...F..T.............AL.V.IVN 222
LH-2 (HS)   .S.........A.L....PA....A..-Q.ARARAAAKSAG...AG..P.............................. 237
LH-2 (RN)   .S.........A.L....PP....A...R.AAAAEQLRVQDWAQLGL-.............................. 237
            ==========

LH-2A (GG)  YNAALSCNENDGDHLDRD-QQYPSNQKTKRMRTSFKHHQLRTMKSYFAINHNPDAKDLKQLAQKTGLTKRVLQVWFQNAR 312
LH-2B (GG)  .-NS-G....EA..M...Q.P..PS......A.............................................. 300
LH-2 (HS)   .TR........AE.....-.P...S.......................................................... 316
LH-2 (RN)   ..........AE.....-.P...S.......................................................... 316
                                  ********** Homeodomain *************************

LH-2A (GG)  AKFRRNLLRQENTGVDKTSDSTLQAGTPSGPASEISNASMSPSSTPTTLTDLTNPTMPTVTSVLTSVPGSLEVHESRSPS 392
LH-2B (GG)  ............G....ADGTS.-P-A.--.SAD-.-GALT.PG.A......---.N..I.-.V...TSN.DS..PG... 370
LH-2 (HS)   ................ST.AA..T.........L....L.............S..L............N..A-M.LTAP 395
LH-2 (RN)   ....................A...T.........L....L.............S..L............N..A-T.PTAL 395
            **********

LH-2A (GG)  QTTLTNLF------------------------                                                  400
LH-2B (GG)  ........------------------------                                                  378
LH-2 (HS)   HKR.LPT.SNDSQPPHP-T-IS-LKKKLSLV                                                  423
LH-2 (RN)   HKR.LPT.SNDSPPPSPLSPHDFFKKEIIFS                                                  426
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FIG. 1. Comparison of the deduced amino acid sequences of chicken *LH-2A* and *LH-2B* with human and rat *LH-2* cognates. Identical residues with chicken *LH-2A* are shown by dots, and spaces indicated by dashes are introduced to improve sequence alignment. Chicken *LH-2A* sequence has been deposited to the DDBJ/EMBL/GenBank Databases, while chicken *LH-2B* sequence is identical to that deposited in the Databases with accession number L35566.

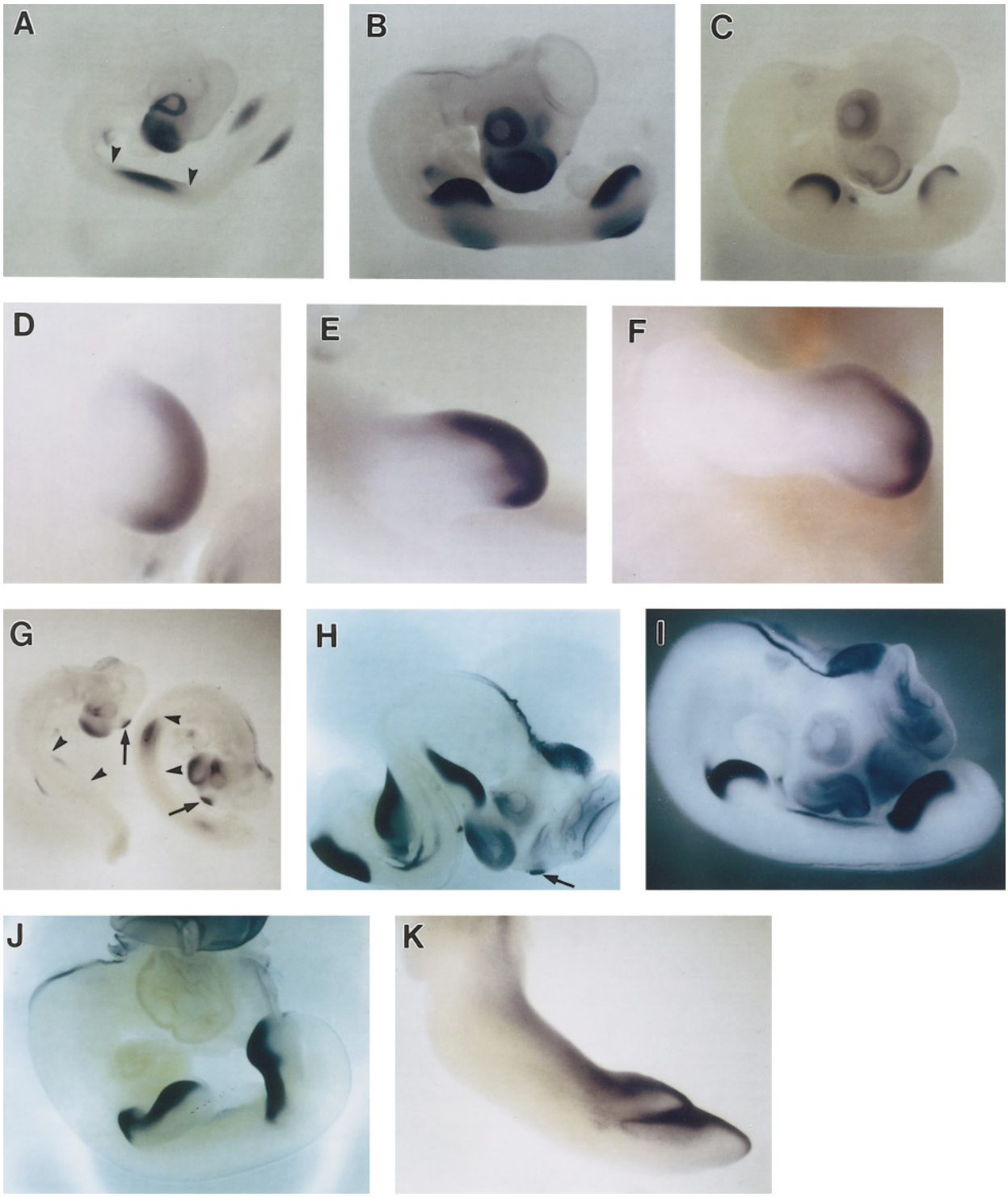

FIG. 2. Expression pattern of the *LH-2A* and *LH-2B* genes during limb development. (A-F) *LH-2A* expression at stage 18 (A), 20 (B), 23 (C,D), 25 (E), and 27 (F). Wing bud and leg bud showed similar expression pattern. (G-K) *LH-2B* expression at stage 15 (G, left), 17 (G, right), 20 (H), 22 (I), 26 (J), and 32 (K). Anteriorly restricted expression of *LH-2B* is observed equally in the wing and leg buds. In addition to the anterior limb bud, intense *LH-2B* signals are also detectable in the dorsal midbrain (shown by an arrow), forebrain, and dorsolateral region of the spinal cord and hindbrain (G-I). The prospective wing bud is indicated by arrowheads in A and G.

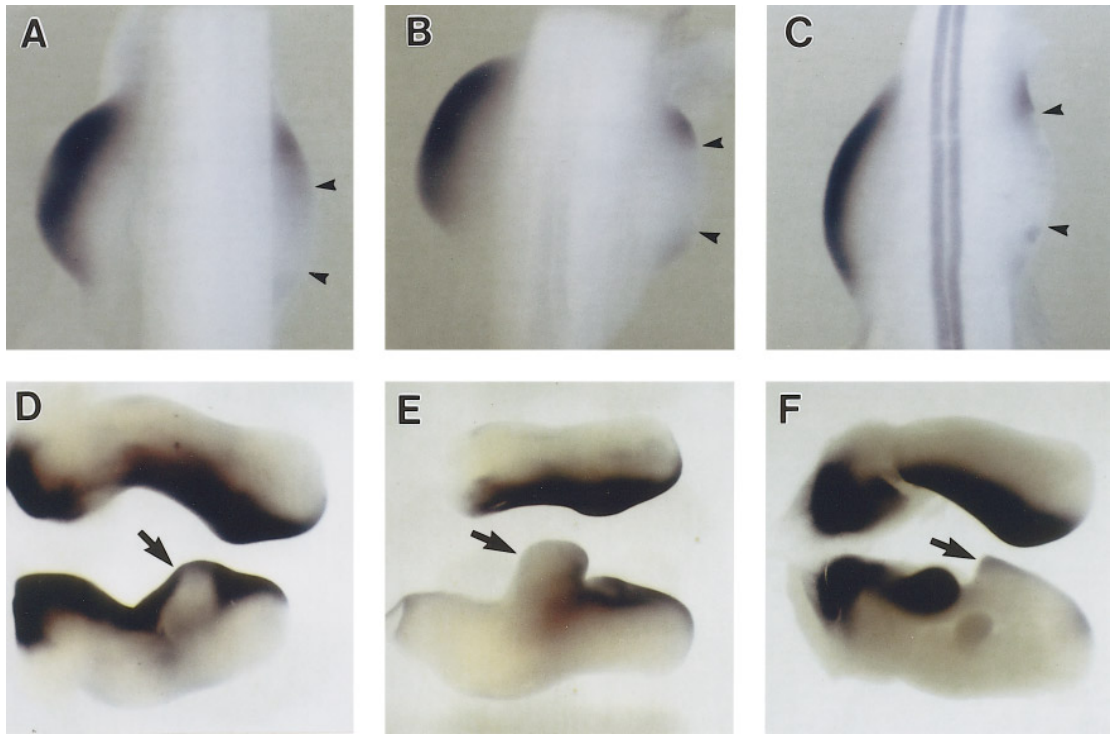

FIG. 3. (A-C) Dorsal views for the effect of apical ectoderm removal on *LH-2A* and *Msx-1* expression. Central region of the apical ectodermal ridge between arrowheads was removed from the right wing bud, and *LH-2A* expression was determined 6 (A) and 12 hours (B) after ridge removal. *Msx-1* expression was detected 6 hours after ridge removal (C). Contralateral left wing bud served as a control. (D-F) Dorsal views for the effect of polarizing signal on *LH-2B* expression. Fibroblasts producing RCAS-SHH-N retrovirus were implanted to the right wing bud, and *LH-2B* expression was detected 48 (D) and 72 hours (E) later. Native polarizing region was implanted to the anterior wing bud, and *LH-2B* expression was detected (F). An arrow indicates the anterior site of treatment in the right wing bud, while the control left wing bud on top of each panel is placed with the reversed anterior-posterior orientation.

chyme (Fig. 2). Both *LH-2A* and *LH-2B* signals were not detectable in the limb ectoderm including the apical ectodermal ridge, as determined by a section of the hybridized embryo (data not shown). Differential expression patterns of the *LH-2A* and *LH-2B* genes are observed as early as at stage 16 in the prospective wing bud, and also in the leg bud at stage 17. *LH-2B* expression is localized in the anterior half of the limb-forming region within the *LH-2A* expression domain. The expression pattern suggests that anterior-posterior identity of the limb bud is already determined before formation of the recognizable limb bud. *LH-2A* is expressed in the distal mesenchyme, whereas *LH-2B* expression is restricted in the anterior region of undifferentiated limb mesenchyme, and not observed in the central core region where mesenchymal condensation starts. *LH-2A* and *LH-2B* transcripts were detectable in the prospective limb bud at much earlier stages than the *sonic hedgehog* gene and also the *Abd-B* subclass of the *HoxA* and *HoxD* genes.

The *LH-2B* gene was intensely expressed in the developing neural tube, whereas the *LH-2A* gene was weakly expressed in this region (Fig. 2). At stages 15-18, *LH-2B* is expressed in the forebrain and anterior compartment of the dorsal midbrain. At stages 24-27, *LH-2B* transcripts were detected as a sharp longitudinal stripe in the dorsolateral region of the spinal cord and hindbrain. *LH-2A* transcripts were also detectable in the forebrain at stages 14-21.

Since the *LH-2A* gene is expressed in the distal mesenchyme of the limb bud, *LH-2A* expression is possibly regulated by the signals from the apical ectodermal ridge. We therefore examined the effect of apical ectodermal ridge removal on *LH-2A* expression. Apical ridge removal resulted in decreased *LH-2A* expression in the adjacent distal mesenchyme, as revealed by whole-mount hybridization of the embryo at 6 to 12 hours after treatment (Fig. 3). However, *LH-2A* expression was not reduced so rapidly as observed for *Msx-1* expression, where *Msx-1* expression was almost completely disappeared within 6 hours after apical ridge removal.

To examine the role of *LH-2B* in limb pattern formation along anterior-posterior axis, polarizing region signal *sonic hedgehog* was ectopically expressed in the anterior margin of the limb bud. *LH-2B* expression in

the anterior-distal region was diminished at 48 to 72 hours after misexpressing *sonic hedgehog*, accompanying anterior expansion of the new digit-forming region (Fig. 3). Similar results were also obtained by grafting posterior limb mesenchymal cells that produce native polarizing signals. These results suggest that *LH-2B* expression in the limb bud is negatively regulated by *sonic hedgehog*.

DISCUSSION

Two closely related *LH-2* genes, *LH-2A* and *LH-2B*, are expressed in the different but overlapping mesenchymal region of the limb bud. Sharp boundary of the expression domain suggests the presence of anterior-posterior compartments defined by *LH-2B* and proximal-distal compartments defined by *LH-2A* in the developing limb bud. Since ectopic expression of the *sonic hedgehog* gene in the anterior limb bud resulting in respecification of positional identity to posterior phenotype concomitantly abolishes *LH-2B* expression in the anterior limb bud, anterior and posterior identity of the limb bud is probably represented by *LH-2B*-expressing and non-expressing domains, respectively. In contrast to *HoxD11-13*, which show different expression pattern between the wing bud and the leg bud (16,17), *LH-2B* exhibits identical expression pattern in the wing and leg buds throughout limb development. Therefore, *LH-2B* is most likely to represent positional values along anterior-posterior axis independent of the limb type.

On the other hand, *LH-2A* is consistently expressed in the distal mesenchymal region adjacent to the apical ectodermal ridge. Since *LH-2A* transcripts were detectable in the prospective limb mesoderm as early as at stage 15, *LH-2A* may play a role for formation and induction of the limb field. *LH-2A* expression is sustained in the distal mesenchyme until at later stages of limb formation, and correlates to the overlying apical ectodermal ridge. Therefore, *LH-2A* is considered to have an important function in maintenance of the apical ectodermal ridge. Similar expression pattern is known for *Msx-1*, but not for *Msx-2*, in the distal limb bud (13,18,19). However, *Msx-1* transcripts are detectable in the apical ectodermal ridge and underlying distal mesenchyme, whereas *LH-2A* is rather broadly expressed in the distal mesenchymal region of the limb bud and no signal is detectable in the apical ectoderm. Furthermore, *LH-2A* expression domain at later stages of limb development is similar but not identical to *HoxD13* and *HoxA13* (17), suggesting a distinct role for *LH-2A* in specifying distal phalangeal identity of the limb.

Signals from the apical ectodermal ridge have positive influence on the *LH-2A* expression, since ridge removal abolished *LH-2A* expression in the adjacent limb mesenchyme. Since BMP family and FGF-8 are known to be expressed in the apical ectodermal ridge (20-23), *LH-2A* expression may be induced or maintained by these growth factors. However, onset of *LH-2A* expression in the prospective limb bud is earlier than FGF-8 expression in the apical ectoderm, but temporally overlapping with FGF-10 expression in the limb forming region (24). *LH-2A* is therefore most likely to be regulated by mesenchymal expression of FGF-10 in initiation of the limb bud.

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