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Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway

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SUMMARY

dHAND is a basic helix-loop-helix (bHLH) transcription factor essential for cardiovascular development. Here we analyze its pattern of expression and functional role during chick limb development. *dHAND* expression was observed in the lateral plate mesoderm prior to emergence of the limb buds. Coincident with limb initiation, expression of *dHAND* became restricted to the posterior half of the limb bud. Experimental procedures that caused mirror-image duplications of the limb resulted in mirror-image duplications of the pattern of *dHAND* expression along the anterior-posterior axis. Retroviral overexpression of dHAND in the limb bud produced preaxial polydactyly, corresponding to mild polarizing activity at the anterior border. At the molecular level, misexpression of dHAND caused ectopic activation of members of the Sonic hedgehog (Shh) pathway, including Gli and Patched, in the anterior limb bud. A subset of infected embryos displayed ectopic anterior activation of Shh. Other factors implicated in anterior-posterior polarization of the bud such as the most 5′ *Hoxd* genes and *Bmp2* were also ectopically activated at the anterior border. Our results indicate a role for dHAND in the establishment of anterior-posterior polarization of the limb bud.

Key words: dHAND, Limb development, Pattern formation, RCAS-infection, Zone of polarizing activity, Chick embryo, Mouse embryo, Shh pathway

INTRODUCTION

The development of the vertebrate limb has proved to be a fruitful model for analyzing developmental processes. In the embryo, the first morphological evidence of the limb buds are small bulges emerging at the appropriate levels of the lateral body wall. In chick embryos this emergence occurs between stage 16 and 17 (Hamburger and Hamilton, 1951) and in mouse embryos at 9-9.5 days of embryonic development (Kaufman, 1992; Wanek et al., 1989). During limb growth, three regions are progressively specified: the proximal segment or stylopod with a single skeletal element (femur/humero); the medial segment or zeugopod with two skeletal elements (tibia-fibula/radius-ulna); and the distal segment or autopod with the skeletal elements of the hand or foot. As growth occurs, patterning is established in the three orthogonal axes of the bud under the direction of specialized signaling centers present along each axis (reviewed by Cohn and Tickle, 1996; Johnson and Tabin, 1997; Schwabe et al., 1998).

Three principal signaling centers have been identified in the limb bud including the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA), and the non-ridge ectoderm. Each signaling center is implicated in patterning primarily along one of the three orthogonal axes of the growing limb bud, however interdependent and coordinated action between the three centers is also essential (Laufer et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995; Zuñiga et al., 1999). The ectoderm rimming the distal edge of the bud constitutes the AER. It directs patterning and growth in the proximodistal axis (shoulder to fingertips) mainly through the production of one of several fibroblast growth factors (Fgfs) (reviewed by Martin, 1998). The action of the AER is permissive, maintaining the subjacent mesoderm, called the progress zone, in an undifferentiated proliferative state where progressively more distal fates are specified (Summerbell et al., 1973). The ZPA is a group of mesodermal cells located at the posterior border of the bud and is responsible for patterning along the anterior-posterior axis through the production of Sonic hedgehog (Shh) (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995). Finally, the non-AER ectoderm exhibits defined dorsal and ventral compartments of gene expression that control dorsoventral patterning. The dorsal ectoderm expresses *Wnt7a*, which controls dorsalization through the induction of the homeobox gene *Lmx1* in the dorsal mesoderm (Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). The ventral ectoderm expresses *Engrailed 1* (*En1*), which controls

ventral patterning by restricting Wnt7a expression to dorsal ectoderm (Logan et al., 1997; Loomis et al., 1996, 1998). Although numerous signaling pathways have been elucidated, the transcriptional basis for many events during limb development remains poorly understood.

Transcription factors containing a basic helix-loop-helix (bHLH) motif often function as regulatory molecules implicated in the determination and differentiation of specific cell types. Subfamilies of bHLH factors have been implicated in skeletal myogenesis and neurogenesis (Jan and Jan, 1993; Olson and Klein, 1994). Recently a subclass of tissue-specific bHLH factors consisting of the *dHAND/Hand2* and *eHAND/Hand1* was identified (Cserjesi et al., 1995; Cross et al, 1995; Hollenger et al., 1995; Srivastava et al., 1995). *dHAND*'s expression has been well studied at the level of the heart where it is implicated in chamber specific-growth (Srivastava, 1999), a function that has been confirmed by targeted mutation in mice (Srivastava et al., 1997). Mice mutant for *dHAND* have extreme hypoplasia of the right ventricle, pharyngeal arches and aortic arch arteries and die by embryonic day 10.5 (E10.5). Another prominent site of expression of *dHAND* during development is the developing limb, however no detailed characterization of the expression profile has been reported to date. Here we describe the pattern of expression of *dHAND* during limb development both in chick and mouse. We also analyze its function in limb development by experimental manipulations and gain-of-function experiments. Our results indicate a significant role for *dHAND* in anterior-posterior patterning of the limb bud.

MATERIALS AND METHODS

Embryos

Fertilized hen eggs were obtained from local commercial sources. For infection with retroviral vectors we used pathogen-free eggs (Intervet, Salamanca and CRIFFA, Barcelona, Spain). The eggs were routinely incubated, opened and staged according to Hamburger and Hamilton (1951). Mouse control embryos were obtained from Harlam Ibérica (Barcelona, Spain). *Shh* mutant mice were generously provided by C. Chiang, and *dHAND* mutant mice were generated as described previously (Srivastava et al., 1997).

Experimental manipulations

We performed a variety of experimental manipulations including AER removal, ZPA grafting and implantation of beads carrying specific molecules. Mostly the procedures used were as described by Ros et al. (1999). Briefly, for application of retinoic acid (RA; all-trans-retinoic acid, from Sigma), beads (AG1X2, Bio-Rad) were soaked in 0.1 mg/ml or 1 mg/ml RA in dimethyl sulfoxide (DMSO) (Tickle et al., 1985). Heparin acrylic beads (Sigma, H5263) were used as carriers of Fgf2 (1 mg/ml; a generous gift of Dr G. Giménez; Fallon et al., 1994). Fgf2 beads were also implanted in the lateral plate mesoderm of stage 13-15 embryos to induce extra limbs (Cohn et al., 1995). Affi-Gel Blue beads (Bio-Rad 153-7301) or heparin acrylic beads were used as carriers for Shh protein (1-3 mg/ml; R&D Systems). These beads were implanted at the anterior border of stage 20 limb buds under the AER.

All the experimental manipulations were performed in the right limb bud using the left limb bud as control. For each experiment, some embryos were allowed to develop up to 10-11 days to assess the effect of the manipulation in the skeletal pattern.

In situ hybridization in whole embryos and tissue sections

In situ hybridization was performed on whole mount and on tissue sections following standard procedures. We used specific chicken or mouse *dHAND* probes as described (Srivastava et al., 1995). Other probes used were *Shh* (kindly provided by T. Jessell), *Hoxd11, Hoxd13, Fgf4, Ptc, Gli, Gli3* and *Bmp2* (kindly provided by C. Tabin).

Retroviral construction and infection protocol

To construct a retrovirus with an adequate level of expression, the 5′ untranslated region of src from the adapter plasmid SLAX 12 NCO (Morgan and Fekete, 1996) was merged at the ATG with the chicken *dHAND* coding sequence (Srivastava et al., 1995). The fused sequence was excised with *Cla*I and subcloned into RCAS(BP)A. Transfection and growth of RCAS virus were performed as described (Morgan and Fekete, 1996). SPAFAS eggs were used for infections. Virus concentrated stock (titer 7.10^7) was used for injections and in general the injections were performed in the prospective wing region of stage 12-14 embryos. Another set of embryos were injected at the level of the prospective leg bud.

RESULTS

Expression of *dHAND* in the chick developing limb

From stage 8-9, *dHAND* expression was observed in the lateral plate mesoderm (Srivastava et al., 1995). At stage 14, prior to the emergence of the limb bud, *dHAND* was expressed along the whole anterior-posterior lateral plate mesoderm (Fig. 1A). At stages 16-17, coincident with the initiation of the limb buds, expression of *dHAND* was downregulated at the anterior of the limb buds so that a gradient of *dHAND* expression along the anterior-posterior axis of the bud was established with higher levels of expression at the posterior border (Fig. 1B). This pattern continued in subsequent stages (Fig. 1C), and was clearly appreciated when consecutive transverse sections through different levels along the anterior-posterior axis of the bud were studied. Fig. 1D-F show sequential sections along the anterior-posterior axis of a stage 18 wing bud (corresponding level of sections are indicated in Fig. 1C) in which increased levels of transcription can be observed from anterior (Fig. 1D) to posterior (Fig. 1F). At stage 19-20, expression of *dHAND* was greatly reduced in the anterior limb bud but persisted at high levels posteriorly (Fig. 1G). By stage 22-23 the level of expression at the anterior border was clearly not above background (Fig. 1H-K). Therefore, from the initiation of limb budding, there was a progressive polarization of *dHAND* expression towards the posterior part of the bud (Fig. 1I-K), resulting in a pattern of expression reminiscent of that of the 5′ *Hoxd* genes, particularly *Hoxd11* and *Hoxd12* (Nelson et al., 1996). From stage 23 to stage 25 an additional small domain of *dHAND* expression could also be detected at the most proximal-anterior location of the bud (Fig. 1H). The pattern of *dHAND* expression in the leg bud was similar to that described for the wing bud (Fig. 1).

At later stages, *dHAND* expression was very dynamic in the autopod. Fig. 2A-D show evolution of *dHAND* expression during sequential stages of the leg bud. From stage 26-27, a transverse band devoid of expression at the level of the tarsus-metatarsus (carpus/metacarpus in the wing) was observed (Fig. 2A). This pattern was coincident with that of *Hoxd11* (see Nelson et al., 1996 and Fig. 6G for comparison). The anterior limit of *dHAND* expression in the autopod was coincident with

the posterior limit of digit two (Fig. 2A,B). At around stage 28 (Fig. 2A) *dHAND* expression was downregulated from the digital chondrogenic regions while persisting in the interdigits (Fig. 2A,B). Expression in the interdigits faded from stage 30 and concentrated in the lateral borders of the developing digits (Fig. 2C), progressively encompassing the lateral aspects of the developing tendons (Fig. 2D). After stage 30, *dHAND* expression acquired a dorsoventral bias becoming undetectable in the dorsal side while being maintained in the ventral side, in the periphery of the developing ventral tendons (Fig. 2E).

We also analyzed *dHAND* expression during mouse limb development using a mouse specific *dHAND* probe. *dHAND* expression was detected in the posterior mesoderm of the limb buds and evolved in a pattern equivalent to that described in chick (Fig. 2F-J).

Based on *dHAND's* expression during limb development in chick and mouse, we hypothesized a role for *dHAND* in anterior-posterior patterning and performed a functional analysis of dHAND during chick limb development.

dHAND expression is dependent on AER signaling

Outgrowth and patterning along the proximodistal axis of the limb is dependent upon the action of the apical ridge (Martin, 1998). Removal of the AER leads to truncated limbs lacking distal elements, the level of truncation depending on the stage at which the AER was removed (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). To analyze dependence of *dHAND* expression on AER signaling, we removed the AER from the right wing bud of stages 19-21 embryos and analyzed *dHAND* expression after the operation. Removal of the AER caused a reduction in the level of *dHAND* expression, perceptible at 6 hours (Fig. 3A). However, 24 hours after the operation (Fig. 3B), no expression of *dHAND* was detectable in the limb lacking the AER.

Signaling through the ridge is mediated by Fgfs and a bead soaked in Fgf is able to substitute for AER function (reviewed by Martin, 1998). If, immediately after AER removal, a bead soaked in Fgf2 is put in its place, growth continues and there is maintenance of AER-dependent genes (Fallon et al., 1994). We checked for Fgf2's ability to maintain *dHAND* expression and implanted Fgf2 beads into the progress zone immediately after removal of the AER. Since expression of *dHAND* is mainly posterior, we tried different levels of placement of the Fgf2 beads along the anterior-posterior axis. When the bead was placed posteriorly, expression was maintained in a relatively normal pattern (Fig. 3C) although the anterior border of the limb narrowed as occurs after AER removal (arrow in Fig. 3C). However, when the Fgf2-bead was placed in the anterior progress zone, *dHAND* expression was also observed in the anterior mesoderm although at lower levels than in the posterior mesoderm (Fig. 3D).

The result shown in Fig. 3D may indicate induction of *dHAND* expression in the anterior mesoderm by the applied Fgf. However, it can also be interpreted as maintenance of expression since the experiment was performed at stages 19-20 HH when the anterior mesoderm may express low levels of *dHAND* (Fig. 1C,G). To analyze possible de novo induction of *dHAND* expression by Fgf, we implanted the Fgf2 bead in the anterior-distal mesoderm of stage 23-24 limb buds that does not express *dHAND* above background (Fig. 1I). This was done with and without previous removal of the anterior AER. In both circumstances we did not observe induction of *dHAND* expression around the bead (Fig. 3E) indicating that Fgf is probably not sufficient to induce de novo *dHAND* expression in the limb mesoderm, although it is required for maintenance of its expression.

dHAND expression in additional limbs induced at flank level

It has been shown that ectopic application of Fgf to the flank mesoderm of stage 13-15 embryos is sufficient to induce an extra limb (Cohn et al., 1995; Vogel et al., 1995; Ohuchi et al., 1997). At stage 13-15, the lateral plate mesoderm at the level of the flank expressed high levels of *dHAND* (Fig. 1A). During normal development, expression at flank levels decreased to a thin band of mesoderm along the dorsoventral boundary of the flank connecting the wing and leg bud (arrowheads in Fig. 1K). We have analyzed the pattern of expression of *dHAND* during development of the extra limbs induced in the flank by Fgf2. For this we applied Fgf2-soaked beads to the flank of stage 13-14 embryos and sequentially analyzed *dHAND* expression in the induced limbs. Fgf2-induced flank limbs developed with a pattern of *dHAND* expression similar to normal limbs. Clear downregulation of *dHAND* expression in the posterior border of the induced limb was seen from 40 hours after implantation of the bead. This is a pattern equivalent to control embryos (Fig. 3F) since the anterior-posterior axis is inverted in the flank induced-limbs (Cohn et al., 1995). This experiment further indicates that Fgf can maintain high levels of *dHAND* expression and that the development of the anterior-posterior axis of the induced limb bud is accompanied by downregulation of *dHAND* expression in the equivalent of the anterior mesoderm.

Regulation of *dHAND* expression by the ZPA, RA and Shh

The ZPA consists of a group of mesodermal cells located at the posterior border of the bud that was identified for its ability to cause mirror image duplications when transplanted to the anterior border of a control limb (Saunders and Gasseling, 1968; Tickle et al., 1975). Its action is mediated by the production of Shh, which becomes detectable at stage 17-18 and colocalizes with the ZPA (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995). The pattern of expression of *dHAND* during limb development is similar to that of the 5′ *Hoxd* genes (Nelson et al., 1996) which, because of their pattern of expression and modifications after duplication, are considered to be regulated by Shh (Riddle et al., 1993; Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Yang et al., 1997). Because of the similarity between *dHAND*'s pattern of expression and that of the 5′ *Hoxd* genes, we hypothesized that *dHAND* might also be regulated by Shh. To assess *dHAND*'s dependence on Shh signaling, we grafted the ZPA at the anterior border and subsequently analyzed *dHAND* expression. We found that the re-specification of the anterior mesoderm that occurs after ZPA grafts was always accompanied by ectopic expression of *dHAND* (Fig. 4A,B).

Retinoic acid (RA) has been shown to mimic the action of the ZPA through induction of a new ZPA when applied at the anterior border (Riddle et al., 1993; Wanek et al., 1991). Application of a RA-soaked bead to the anterior border of a normal wing bud leads to duplications mediated by Hoxb-8 (Lu

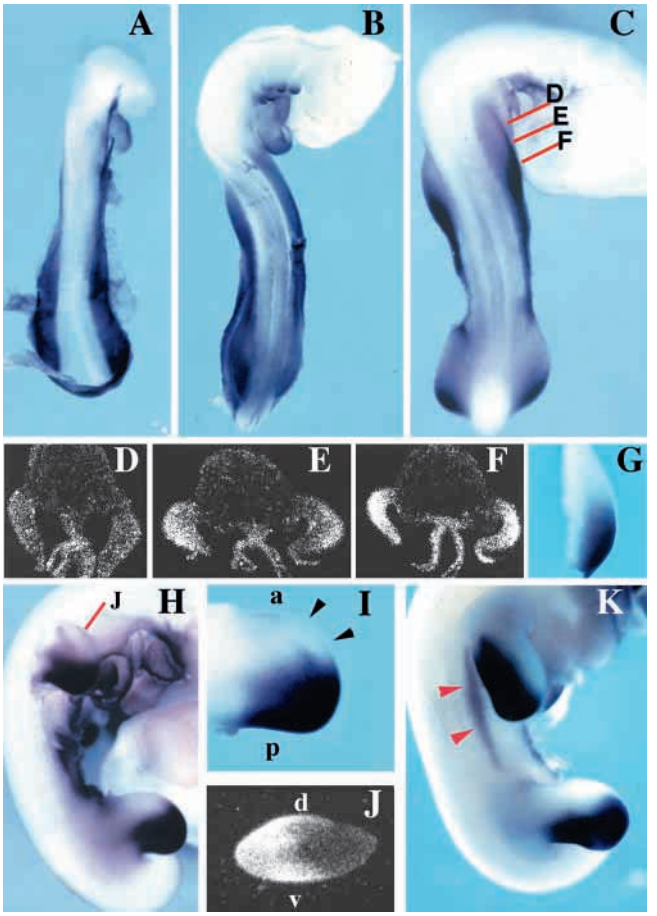

Fig. 1. *dHAND* pattern of expression during early chick limb bud development. (A) Stage 14 embryo showing expression of *dHAND* in the lateral plate mesoderm. (B,C) Expression of *dHAND* in stage 17 and 19 embryos respectively. Note the establishment of the gradient of expression along the anterior-posterior axis of the emerging limb bud. (D-F) Sequential transverse sections (level of section indicated in C by the red lines) clearly showing the highest level of expression posteriorly (F). (G) Stage 19-20 wing bud. (H) Expression of dHAND in stage 23 embryo. Transcripts are observed in the posterior half of both wing and leg. (I) Ventral view of a stage 22 wing bud showing dHAND expression. The arrowheads indicate the lack of expression in the AER. (J) Pattern of *dHAND* expression in a transverse section through the middle of a stage 23 wing bud (level of section indicated in (H) by the red line). (K) dHAND pattern of expression in a stage 26 embryo. Arrowheads indicate the domain of expression at the flank. A, anterior; p, posterior; d, dorsal; v, ventral.

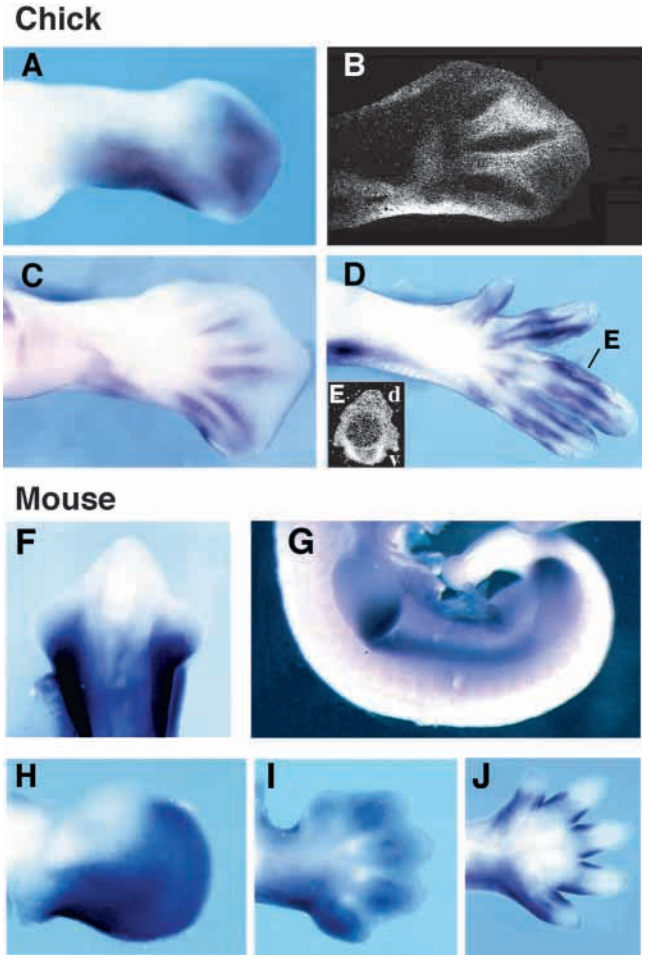

Fig. 2. *dHAND* expression at later stages of chick and mouse limb development. (A) *dHAND* pattern of expression in a stage 28 leg bud. Note the transverse band devoid of transcripts at the tarsus level. (B) Frontal section of a stage 30 leg bud showing expression in the interdigit area and in the periphery of the digital cartilages. (C) Subsequently, *dHAND* expression is downregulated in the interdigits but persists in the periphery of the digits. (D) At stage 35, *dHAND* expression encompasses the developing tendons with a marked ventral bias, better appreciated in section E. (F-J) *dHAND* expression during mouse limb development. (F) A dorsal view of the forelimbs of an E10.0 mouse embryo showing expression of *dHAND* in the posterior mesoderm. (G) Lateral view of an E11.0 day mouse embryo. (H-J) Ventral views of forelimbs of E12.0 (H), E13.0 (I) and E16.0 (J). In all the panels except E and G, anterior is up.

et al., 1997). We analyzed *dHAND* expression after ectopic application of RA at the anterior border. Expression of *dHAND* was observed at the anterior border 20 hours after the placement of a RA bead (Fig. 4D) and progressively evolved to a mirror image of the normal posterior pattern of expression correlating with the resulting duplication (Fig. 4E). Anterior expansion of the normal domain of *dHAND* expression was observed from 6 hours after RA application (not shown), although, as in the case of Fgf application, maintenance of residual anterior levels of expression may also occur. The kinetics of dHAND induction by RA is intermediate between early transient activation of Hoxb8 (Lu et al., 1997) and later Shh induction (Riddle et al., 1993). The two concentrations of RA used (1 mg/ml or 0.1 mg/ml) had the same effect.

We also checked whether there was activation of *dHAND* expression by Shh. A bead carrying Shh protein (1-3 mg/ml) was implanted at the anterior border of stage 20 limb buds. The phenotypes obtained were as reported for these doses (Yang et al, 1997). Ectopic *dHAND* expression was observed in the anterior mesoderm 24 hours after the application of the bead (Fig. 4F). During normal limb development Shh cannot be implicated in *dHAND* activation of expression since its expression is detected later than *dHAND* (Riddle et al., 1993). However, this does not rule out the possibility that Shh could

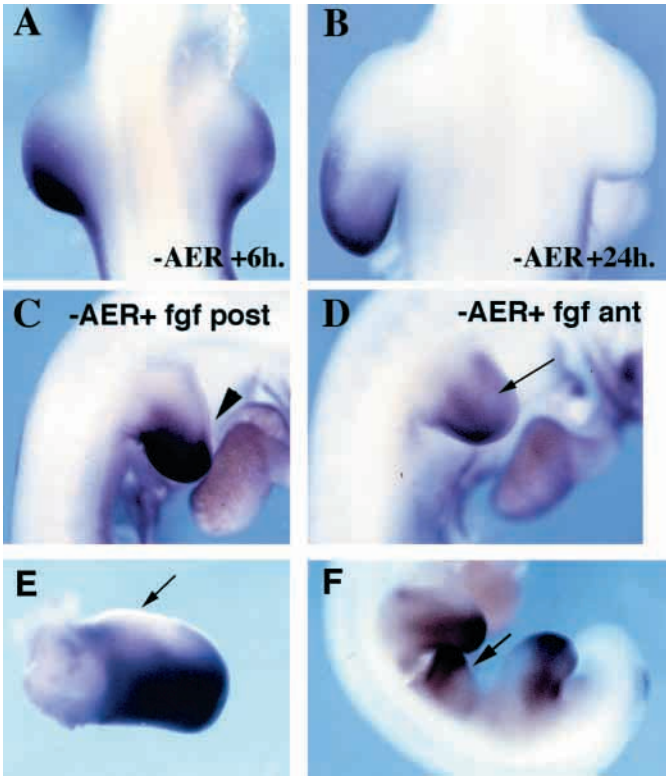

Fig. 3. AER and Fgf effects on dHAND expression. All the panels show hybridization with dHAND probe. (A) dHAND expression was perceptibly downregulated in the right wing bud of an embryo 6 hours after removal of the AER. (B) 24 hours after AER removal dHAND expression was undetectable in the operated right wing bud. (C) Placement of a Fgf2 bead into the posterior progress zone immediately after removal of the AER maintained *dHAND* expression. The arrowhead indicates the affected growth at the anterior border as a consequence of the AER removal. (D) When the Fgf2 bead (arrow) was placed into the anterior mesoderm *dHAND* expression was maintained at lower levels. (E) An Fgf2 bead inserted into the anterior mesoderm of a stage 23 embryo did not induce *dHAND* expression (arrow). (F) *dHAND* expression in an Fgf2-induced limb at flank level. Downregulation of *dHAND* expression at the posterior border of the induced limb (arrow) was observed 40 hours after the operation. Note reversal of anterior-posterior orientation in the induced limb compared to the normal limbs.

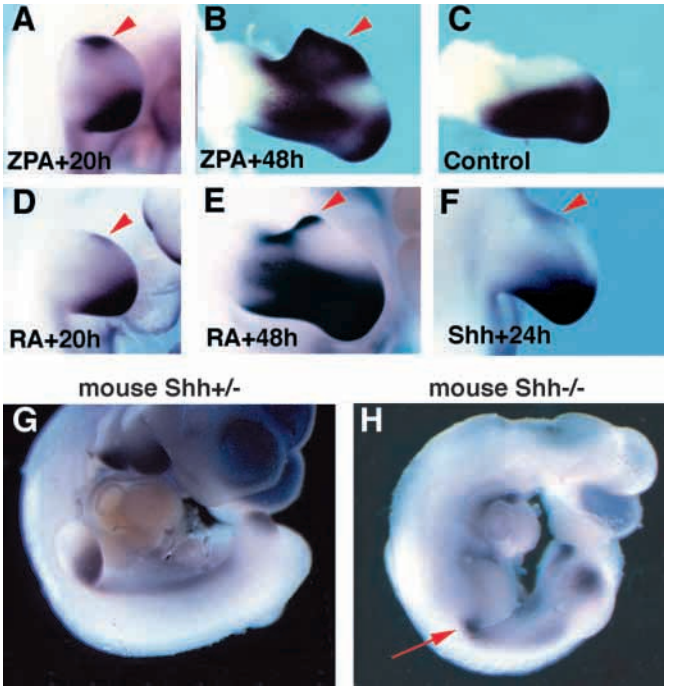

Fig. 4. Modification of *dHAND* expression during duplication of the anterior-posterior axis of the limb bud. (A-F) Hybridization with *dHAND* probe in chick. Red arrowheads indicate ectopic expression. (A) Ectopic *dHAND* expression at the anterior border 20 hours after a ZPA graft. (B) Duplication of the limb caused by the ZPA graft is accompanied by mirror-duplication of *dHAND* expression. (C) *dHAND* expression in the contralateral left (flipped) limb is shown for comparison. (D,E) Application of RA to the anterior border caused ectopic activation of *dHAND* at the anterior border 20 and 48 hours after the operation. (F) Shh ectopic application to the anterior border induced *dHAND* expression as shown here 24 hours after the operation. (G,H) dHAND expression in the *Shh* mutant mouse. (G) Heterozygote $Shh^{+/-}$ embryos have a normal pattern of *dHAND* expression in both fore and hindlimb. (H) Homozygous $Shh^{-/-}$ embryo showing a reduced domain of *dHAND* expression restricted to the posterior border of the forelimb (arrow) while expression is normal in the hindlimb.

induce or modulate later phases of *dHAND* expression as demonstrated for the 5′ *Hoxd* genes (Nelson et al., 1996).

To further explore the relationship between Shh and dHAND, we analyzed *dHAND* expression in the limbs of *Shh* mutant mice (Chiang et al., 1996). The limbs in *Shh* homozygous mutants are distally truncated and lacking digits. Interestingly, *dHAND* expression was affected in the mutant forelimb, exhibiting a very reduced area of expression in the posterior border of the bud (arrow in Fig. 4H), however the pattern of expression in the mutant hindlimb was similar to normal (Fig. 4H, compare with Fig. 4G for the heterozygous and Fig. 2G for the control). Thus, neither *dHAND* expression in the limb bud nor its polarization of expression required Shh signaling although Shh was required for the establishment of the normal domain of expression, at least in the forelimb. The polarization of *dHAND* expression in the *Shh* mutant limb confirms the existence of an anterior-posterior polarization in the limb bud independent of Shh (Ros et al., 1996; Grieshammer et al., 1996; Noramly et al., 1996). The differential alteration of *dHAND* expression in the fore versus the hindlimb of the *Shh* mutants indicates a differential requirement for Shh in the two types of limbs.

dHAND misexpression results in preaxial polydactyly

To further analyze the role of *dHAND* in limb development, we constructed a RCAS encoding the complete coding sequence of the chicken *dHAND*. We infected the prospective right wing at stages 12-14 and allowed the embryos to develop up to 10-11 days. The success of the infection was analyzed by hybridization with *dHAND* and an antiviral probe (not shown). Interestingly, the great majority of the infected embryos (95%) showed varying degrees of duplication of the right wing bud (Table 1 and Fig. 5). The variability in the phenotype was probably due to varying levels of *dHAND* misexpression. In

Table 1. Digit patterns following *dHAND* misexpression

Pattern	*n*	%
Normal (234)	2	4.5
Anterior cartilage (blib,234)	6	13.6
Forked digit 2 (f234)	10	22.7
Duplicated digit 2 (2234)	10	22.7
Posteriorized digit 2 (334)	12	27.3
Posteriorized digit 2 and cartilage (3blib3**4**)	4	9.1
Total	44	

increasing severity, the phenotypes observed were: extra cartilage at the anterior border of the limb (Fig. 5B), forked digit 2 (Fig. 5C), complete duplication of digit 2 (Fig. 5D), and transformation of digit 2 into digit 3 giving a pattern 334 (Fig. 5E). In the chick wing, both digit 2 and digit 3 have two phalanges, but these two digits differ in the shape of their phalangeal and metacarpal elements (Fig. 5A shows the skeletal pattern of a control wing). The most anterior digit of RCAS-dHAND infected limbs were frequently shaped more like normal digit 3 (Fig. 5E) indicating a posterior transformation. Finally, in some cases we observed an additional digit 3 and a cartilage between this digit and the normal wing digit 3 thus giving a pattern 3-cartilage-34 (Fig. 5F). More complete mirror duplications like those obtained after ZPA grafts or high doses of Shh (Tickle, 1981; Yang et al., 1997) were never found after dHAND misexpression. The limbs with stronger duplications were also abnormal at the zeugopod level showing elements thicker than normal whilst the digital elements were also thicker than expected (Fig. 5E,F). A subset of embryos were injected with the RCAS-dHAND in the prospective leg bud and the phenotype obtained was equivalent to that described for the wing bud (Fig. 5H, compare with the control in Fig. 5G).

Misexpression of *dHAND* gave phenotypes that parallel low/middle polarizing activity. These phenotypes correlated with low-mid doses of Shh at the anterior level (Yang et al., 1997). Consequently, we looked for ectopic Shh activation at the anterior margin of the dHAND-infected limb. We hybridized the dHAND-infected limbs sequentially after infection with a Shh antisense riboprobe. As expected, the infected bud was broader than the contralateral control bud from 2 days after injection. Curiously, we could only detect ectopic activation of Shh at the anterior border in a subset of infected limbs (2 out of 18; Fig. 6A,B). However, we cannot rule out the possibility that, in the remaining of specimens, Shh was activated at levels below detection limits or in a short time-window that we were missing. We also analyzed *Fgf4* expression in the AER of the infected limbs and found that accompanying the broadening of the limb bud, *Fgf4* expression expanded to the anterior AER (Fig. 6A). Frequently, the AER in the infected limbs was irregular in shape (Fig. 6A).

In an attempt to find clues for a possible low activation of Shh by dHAND at the anterior border, we analyzed the expression of *Gli* and *Patched* (*Ptc*), two genes that are components of the Shh pathway and are also targets for transcriptional control by Shh (Marigo et al., 1996a, b; Ruiz i Altaba, 1999). We found ectopic activation of these two genes at the anterior border in all the infected limbs analyzed (*n*=6; Fig. 6C,D). Taken together, our results indicate ectopic activation of the Shh pathway at the anterior border of RCAS-dHAND infected limbs.

It has also been shown that Gli3 and Shh have mutually exclusive domains of expression, with Gli3 being downregulated in the presence of Shh expression (Büscher et al., 1997; Marigo et al., 1996c; Masuya et al., 1997). The *extra toes* (*Xt*) mutation in mice, which results in preaxial polydactyly, is caused by a mutation in *Gli3* (Schimmang et al., 1992; Hui and Joyner, 1993). We observed downregulation of *Gli3* expression at the anterior border of the majority of dHAND infected limbs (4 out of 6; Fig. 6E) in a region overlapping the ectopic Shh expression observed (Fig. 6B). It was interesting that the specimens in which downregulation of *Gli3* was not observed were the youngest. During normal chick limb development *Gli3* is first expressed all along the anterior-posterior axis and only after activation of *Shh* expression is *Gli3* downregulated from the posterior border (Marigo et al., 1996c). Downregulation of *Gli3* may correspond to low levels of *Shh* expression along the anterior border of dHAND-infected limbs.

We also searched for ectopic activation of genes considered to be major downstream mediators of Shh, such as *Bmp2* and the 5′ *Hoxd* genes (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995; Duprez et al., 1996). *Bmp2* expression was activated anteriorly (100%, *n*=6; Fig. 6F) within 2 days after infection. Activation of *Hoxd11* (100%, *n*=6, Fig. 6G) and *Hoxd13* (100%, *n*=6, Fig. 6H) at the anterior border always accompanied the duplications caused by the overexpression of *dHAND*. Thus, our results indicate that overexpression of *dHAND* has a consistent effect at the anterior of the limb bud generating changes that mimic mild polarizing activity both in phenotype and gene expression.

Targeted disruption of *dHAND* results in heart and branchial arch malformations that cause death by embryonic day 10.5 (Srivastava et al., 1997). *dHAND* null mice develop rudimentary limb buds thinner than normal that express some limb transcription factors such as Msx1 and Msx2 (Srivastava et al., 1997; Thomas et al., 1998). We tried to molecularly characterize the mutant limbs but unfortunately the analysis was inconclusive since the embryos die around the stage in which activation of *Shh* and *Hoxd* genes occurs. However, preliminary studies indicate that *Hoxd11* may not be expressed in the mutant limb bud although further investigation is required to confirm this result.

DISCUSSION

Expression of dHAND in the developing limb

dHAND is expressed in the whole lateral plate mesoderm during early stages of chick development (Srivastava et al., 1995). When the limb buds emerge, its expression becomes restricted to the posterior border of the bud in a broad posterior-distal domain reminiscent of the pattern of expression of some of the 5′ *Hoxd* genes (Nelson et al., 1996). At later stages expression was restricted to the posterior border of the zeugopod and to the posterior autopod. In the autopod, *dHAND* was expressed in a dynamic manner affecting the interdigital regions, the lateral borders of the digits and eventually encompassing the developing ventral tendons. The difference in *dHAND* expression between developing dorsal and ventral tendons most likely reflects differential timing of differentiation as seen with other tendon markers (Oliver et al.,

1995) rather than implication in dorsoventral specification. In the mouse, *dHAND* expression during limb development paralleled that described for the chick.

The restriction of *dHAND* expression to the posterior half of the limb bud that occurs during early stages of limb development could be explained by the localization of positive regulators to the posterior border. The posterior restriction of *dHAND* expression was coincident with Shh activation of expression and therefore Shh could be implicated in regulating this process (Riddle et al., 1993). Although Shh is able to induce *dHAND* expression when ectopically applied to the anterior limb bud, *Shh*$^{-/-}$ mutant mice show a reduced but normally polarized expression of *dHAND* in the limbs. This indicates that Shh is not required for the initial activation or polarization of dHAND expression but may be involved in establishing a broader expression in the forelimb while expression in the hindlimb is normal in absence of Shh. Since *dHAND* has distinct temporal and spatial domains of expression, it remains possible that Shh could control later phases of *dHAND* expression, as has been shown for the 5′ *Hoxd* genes (Nelson et al., 1996).

Alternatively, negative signals at the anterior border of the limbs could be invoked to restrict dHAND to the posterior border. In this context, a set of negative regulators at the anterior border have been shown to restrict *Shh* expression to the posterior border. Those are *Gli3* (Büscher et al., 1997; Marigo et al., 1996c; Masuya et al., 1995, 1997) and *Alx4* (Takahashi et al., 1998), whose domains of expression in the limb bud are complementary to that of Shh. Interestingly, mutations in both Gli3 and Alx4 (Schimmang et al., 1992; Hui and Joyner, 1993; Qu et al., 1997, 1998) give a phenotype similar to overexpression of *dHAND*. It remains to be determined whether Gli3 or Alx4 could also be involved in polarization of dHAND expression.

During limb bud stages of development, *dHAND* expression appears to be modulated by the AER. Removal of the AER is followed by a rapid downregulation of *dHAND* expression. Application of Fgf, known to substitute for AER function, prevents downregulation of *dHAND* expression after AER removal. However, application of Fgf to mesoderm not expressing dHAND (anterior mesoderm from stage 23) does not result in activation of expression, indicating that Fgf is sufficient to maintain but not to induce dHAND. Fgf4 from the AER and Shh expression in the ZPA reciprocally activate one another (Laufer et al., 1994; Niswander et al., 1994). Removal of the AER causes a rapid downregulation of Shh expression, making it difficult to dissect the specific role played by the AER in *dHAND* expression (Laufer et al., 1994).

The expression of dHAND is differently affected in forelimb and hindlimb of *Shh* homozygous mutant embryos. Expression in the mutant forelimb is reduced to a small area in the posterior border while expression in the mutant hindlimb remains normal. In addition to the recent identification of a series of limb-specific transcription factors (Graham and McGonnell, 1999), other genes present subtle differences between their pattern of expression in developing fore and hindlimbs (Nelson et al., 1996; Mackem and Mahon, 1991). Furthermore, the requirements to induce a ZPA are different in fore versus hindlimbs (Stratford et al., 1997). Interestingly, hindlimbs of *Shh*$^{-/-}$ mutant are less affected than forelimbs, and expression of 5′ *Hoxd* genes is also less affected in the mutant hindlimbs than in forelimbs (Chin Chiang and John Fallon, personal communication). Thus, it is possible that *dHAND*'s pattern of expression is dependent on Shh based on the type of limb.

dHAND's role in anteroposterior polarization of the limb

The posteriorly restricted pattern of *dHAND* expression during limb development, together with the modifications observed after the experimental manipulations performed, suggest a role in anteroposterior patterning. Furthermore retroviral misexpression of dHAND in the limb bud led to preaxial polydactyly or transformation of digit 2 into a digit 3, inappropriate for the anterior most position. In the stronger phenotypes, we observed shortening and broadening of the long bones of the forearm and digits. This latter effect has been reported to occur with misexpression of Hoxd13 (Goff and Tabin, 1997), and thus it is possible that it is mediated by the activation of 5′ *Hoxd* genes.

The phenotypes of dHAND overexpression are similar to those obtained after low-mild polarizing activity at the anterior border. The same types of duplications have been reported in gain-of-function experiments with some of the 5′ *Hoxd* genes (Knezevic et al., 1997; Mackem and Knezevic, 1999). The luxoid/luxate group of mouse mutants also has similar phenotypes with varying degrees of digital duplications (Qu et al., 1997, 1998; Masuya et al., 1995, 1997). All these phenotypes can be easily explained by activation of Shh at the anterior border and indeed it has been shown to occur in the case of *Hoxd12* misexpression (Knezevic et al., 1997) and in the luxoid/luxate mutants (Qu et al., 1997, 1998; Masuya et al., 1995). Thus, we were expecting that *dHAND* misexpression at the anterior border of the limb bud would correlate with ectopic activation of Shh. Accordingly, we detected anterior ectopic *Shh* expression in a small subset of dHAND-infected limbs while components of the Shh pathway such as Gli and Ptc were detected in all of the specimens analyzed. Expression of Gli3, a negative regulator of Shh, appeared downregulated in a small anterior area correlating with the area of ectopic Shh activation. Ectopic anterior expression of other potential components of anterior-posterior patterning such as *Bmp2* and 5′ *Hoxd* genes was also observed in the totality of the specimens analyzed. Thus, overexpression of dHAND resulted in the activation of genes of the Shh pathway at the anterior border. A simple explanation for this is that dHAND misexpression leads to ectopic activation of Shh at the anterior border but that, in the majority of cases, it is below the detection level of in situ hybridization. Alternatively Shh induction may take place for a short period of time that would make it difficult to detect. We think this interpretation is likely and fits well with the mild duplications obtained that would only require low doses or a few hours of Shh activation (Yang et al., 1997). If this were the case, the ectopic activation of gene expressions, and the phenotypes observed after dHAND misexpression, would be mediated by Shh, and dHAND attributed a role similar to the most 5′ *Hoxd* genes participating in a positive feedback loop to reinforce Shh signal in the posterior limb bud (Knezevic et al., 1997; Mackem and Knezevic, 1999) (possibility A in Fig. 7). We consider this possibility most likely since Shh is a potent molecule and low levels of expression, even below the detection limit of in situ hybridization, could be sufficient to

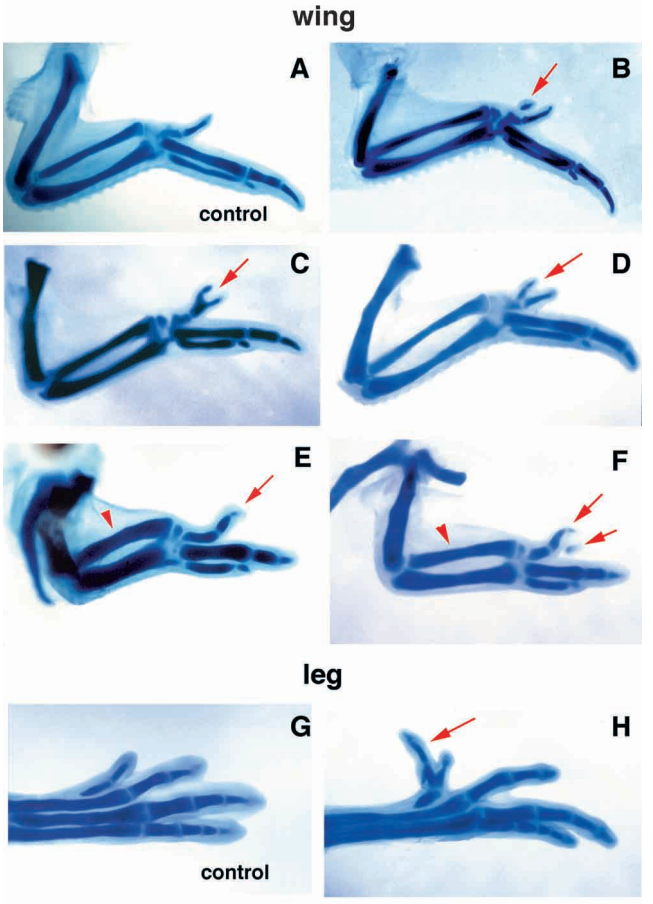

Fig. 5. Anterior digit duplication by dHAND misexpression in developing chick limbs. (A) Skeletal pattern of a control wing shown for comparison. (B) Mildest phenotype consisting of an ectopic cartilage anterior to digit 2 (arrow). (C) Forked digit 2. (D) Duplication of digit 2. (E) Anterior digit exhibiting a shape more appropriate of digit 3 (arrow). (F) Transformation of digit 2 into digit 3 plus a cartilage (arrows). Arrowheads in E and F indicate broadening of skeletal elements. (G) Skeletal pattern of a control leg. (H) Anterior extra digit 2 (arrow) in an infected leg. All panels are dorsal views and anterior is up.

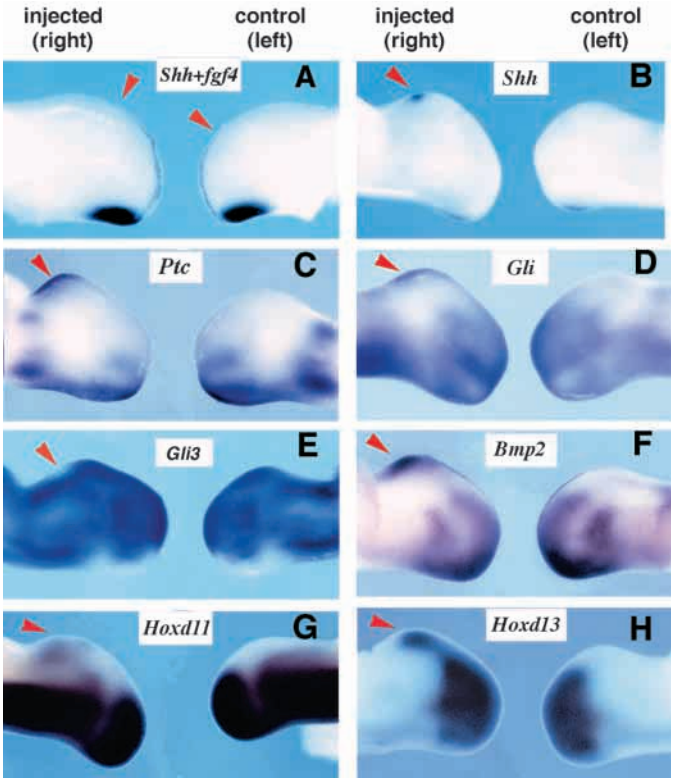

Fig. 6. Molecular analysis of RCAS-dHAND infected chick limbs. All panels show infected right wing bud and contralateral left wing as control as indicated at the top of the figure. The specific probe or probes used in the hybridization is indicated at the top of each panel. (A) Shh expression is undetectable at the anterior border of infected wing bud while Fgf4 expression is anteriorly expanded (arrowheads indicate the anterior limit of Fgf4 expression both in experimental and control wing). (B) Another specimen showing activation of Shh expression at the anterior border (arrowhead). (C) Ectopic Ptc expression is detected at the anterior border of infected wing (arrowhead). (D) Ectopic Gli expression is detected at the anterior border of infected wing (arrowhead). (E) An area devoid of Gli3 expression is detected at the anterior border of the infected wing (arrowhead) correlating with the presumptive area of Shh activation of expression. (F-H) Ectopic Bmp2 (F), Hoxd11 (G) and Hoxd13 (H) expression at the anterior border (arrowheads) of infected limbs.

induce target genes. However, other possibilities should be considered.

dHAND overexpression could activate the Shh pathway without induction of Shh itself. dHAND could participate in establishing the anterior-posterior polarity preexisting in the developing limb bud previous and independent of Shh (Ros et al., 1996; Grieshammer et al., 1996; Noramly et al., 1996; Neumann et al., 1999) (possibility B in Fig. 7). dHAND could be the factor implicated in the initial activation of Ptc and Gli

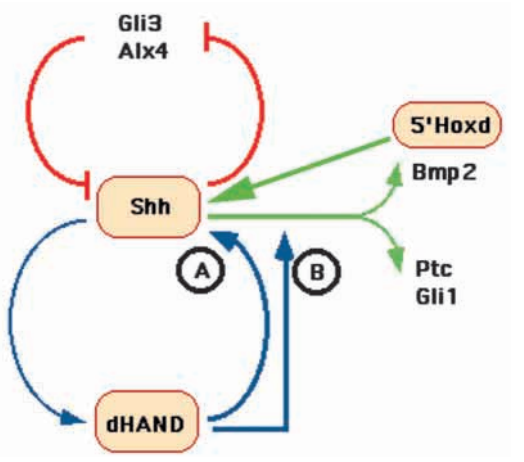

Fig. 7. Model for dHAND participation in anterior-posterior patterning. Green arrows and red lines indicate positive and negative regulation respectively. The relationship between dHAND and Shh pathway is shown in blue. Two possibilities, marked as A and B in the scheme are considered. A considers dHAND participation in the network that positions and polarizes Shh expression to the posterior distal limb bud. B considers dHAND participation in establishing the anterior-posterior polarity of the developing limb by activating the Shh pathway but not Shh itself.

to establish Shh in the posterior of the limb (Riuz i Altaba, 1999). According to this hypothesis, the ectopic activation of Shh observed occasionally by overexpression of dHAND would be secondary to the activation of *Hoxd* genes. Recently, a possible bifurcation in the Shh pathway has been suggested (Lewis et al., 1999; represented in our model by the bifurcated green arrow). dHAND would probably be situated before the branch point since it is able to induce targets in both pathways.

Taken together, our results support dHAND as a factor implicated in anteroposterior polarization of the developing limb. It may participate, together with the 5′ *Hoxd* genes in the positive feedback loop that reinforces Shh in the posterior of the bud or be an introductory factor required for the initiation of Shh signaling.

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