The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development

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SUMMARY

Limb outgrowth and patterning of skeletal elements are dependent on complex tissue interactions involving the zone of polarizing activity (ZPA) in the posterior region of the limb bud and the apical ectodermal ridge. The peptide morphogen Sonic hedgehog (SHH) is expressed specifically in the ZPA and, when expressed ectopically, is sufficient to mimic its functions, inducing tissue growth and formation of posterior skeletal elements. We show that the basic helix-loop-helix transcription factor dHAND is expressed posteriorly in the developing limb prior to Shh and subsequently occupies a broad domain that encompasses the Shh expression domain. In mouse embryos homozygous for a dHAND null allele, limb buds are severely underdeveloped and Shh is not expressed. Conversely, misexpression of dHAND in the anterior region of the limb bud of transgenic mice results in formation of an additional ZPA, revealed by ectopic expression of Shh and its target genes, and resulting limb abnormalities that include preaxial polydactyly with duplication of posterior skeletal elements. Analysis of mouse mutants in which Hedgehog expression is altered also revealed a feedback mechanism in which Hedgehog signaling is required to maintain the full dHAND expression domain in the developing limb. Together, these findings identify dHAND as an upstream activator of Shh expression and important transcriptional regulator of limb development.

Key words: dHAND, Hand2, Zone of polarizing activity, Sonic hedgehog, Limb development, Mouse

INTRODUCTION

Development of the vertebrate limb is an important paradigm for studying the regulation of growth and patterning in three dimensions, and both classical experiments and recent molecular approaches have provided significant insights into the mechanisms involved. These studies have revealed that growth and patterning along the three orthogonal axes (anteroposterior, dorsoventral, and proximodistal) of the limb are regulated by distinct but interdependent pathways, many aspects of which have been evolutionarily conserved (for reviews, see Johnson and Tabin, 1997; Martin, 1998; Schwabe et al., 1998; Vogt and Duboule, 1999).

Limb bud formation is initiated by interaction of a specific region of lateral plate mesoderm, the limb field, with the overlying flank ectoderm. Dorsoventral polarity of the limb is controlled by the ectoderm (MacCabe et al., 1974), in part through secretion of WNT7A by the dorsal ectoderm, which activates the mesodermal target gene Lmx1 (Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). Outgrowth of the limb along the proximodistal axis depends on functions of the apical ectodermal ridge (AER), a specialized epithelial structure induced at the interface of dorsal and ventral ectodermal domains through a complex series of interactions (reviewed by Irvine and Vogt, 1997). Limb skeletal elements are laid down in a proximal to distal sequence, and predictable distal truncations result from removal of the AER (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982), which can be rescued by local application of FGFs that are expressed in the AER (Niswander et al., 1993; Fallon et al., 1994; Vogel et al., 1996).

In contrast to the dorsoventral axis, anteroposterior polarity is an inherent property of the limb field mesoderm rather than the overlying ectoderm (Harrison, 1918; Hamburger, 1938; Zwilling, 1956; reviewed by Saunders, 1977). A key role in anteroposterior patterning of the limb is played by the zone of polarizing activity (ZPA), a region of mesoderm along the posterior margin of the limb bud that can direct growth and patterning when transplanted to the anterior side of a recipient limb bud, giving rise to mirror-image duplications of skeletal elements (Saunders and Gasseling, 1968). The secreted signaling molecule Sonic hedgehog (SHH) colocalizes with the ZPA and is sufficient to mimic its functions when ectopically expressed (Riddle et al., 1993). Maintenance of Shh expression depends on WNT7A signaling by dorsal ectoderm (Parr and McMahon, 1995; Yang and Niswander, 1995), and on FGF signaling by the AER (Laufer et al., 1994; Niswander et al., 1994). Conversely, maintenance of the AER depends on...
induction of the bone morphogenetic protein (BMP) antagonist gremlin by SHH, which requires the product of the \textit{formin} gene, that is disrupted by the \textit{limb deformity} mutation (Zúñiga and Zeller, 1999; see also Merino et al., 1999; Capdevila et al., 1999).

Notwithstanding its crucial importance for outgrowth and patterning of the limb bud, \textit{Shh} expression is but one manifestation of the initial anteroposterior polarity of the limb field. This is clearly illustrated by chicken \textit{limbless} mutant limb buds, which do not elaborate an AER due to an ectodermal defect and consequently do not detectably express \textit{Shh}, yet do show asymmetric expression of \textit{Hox} genes (Griesshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). Furthermore, \textit{Shh}\textsuperscript{−/−} limbs, while ultimately severely truncated (Chiang et al., 1996), maintain remnants of polarized expression of some of the genes involved in the SHH/FGF feedback loop, at least at early stages of development (Zúñiga et al., 1999). It is thus of great interest to determine which factors determine the polarity of the limb field and are involved in anteroposterior signaling upstream of or independently of SHH.

Several genes uncovered by polydactylyous mouse and chick mutants are known to affect formation of the ZPA. Some of these encode products that are expressed anteriorly in the limb bud and appear to be negative regulators of ZPA formation, as loss of gene function results in formation of an ectopic \textit{Shh} domain at the anterior margin of the limb bud (Masuya et al., 1995; Büscher et al., 1997; Qu et al., 1997, 1999). It has been shown that for at least one of these genes, \textit{Gli3}, loss of function affects not only \textit{Shh}, but also results in ectopic expression of 5’ \textit{Hox} genes long before \textit{Shh} is ectopically expressed (Zúñiga and Zeller, 1999), suggesting that the primary polarity of the limb is affected.

\textit{Hox} genes are expressed in the lateral plate mesoderm and are foremost candidates for specifying the limb field. Several studies support this notion. In \textit{Hoxb5}\textsuperscript{−/−} mice, the position of the forelimb is shifted anteriorly (Rancourt et al., 1995), and indirect evidence for the involvement of other \textit{Hox} genes in positioning the limb field has been presented (Cohn et al., 1997). Furthermore, ectopic expression of \textit{Hoxb}8 in the flank and forelimb results in formation of ectopic limb outgrowths anterior to the forelimbs and formation of an ectopic ZPA at the anterior side of the forelimb (Charité et al., 1994). Correlative evidence in support of a role for \textit{Hoxb}8 in ZPA formation in chick has been presented as well (Lu et al., 1997; Stratford et al., 1997). However, although expression of \textit{Hoxb}8 in the flank and early forelimb bud is consistent with it specifying the posterior part of the forelimb field and competence to subsequently express \textit{Shh}, expression in the outgrowing limb bud does not colocalize with \textit{Shh} expression (Charité et al., 1994; Stratford et al., 1997). \textit{Hoxb}8 null mice have normal limbs (Van den Akker et al., 1999), which may be due to functional redundancy. 5’ \textit{Hoxd} genes, which are expressed posteroirly in the limb bud prior to \textit{Shh} in nested patterns that are subsequently modified by SHH signaling (see Johnson and Tabin, 1997), were originally thought to mainly control proliferation and differentiation of limb mesenchyme cells (Duboule, 1995). More recently, ectopic expression of \textit{Hoxd12} has been shown to result in formation of an ectopic \textit{Shh} domain (Knezevic et al., 1997), suggesting that these genes may also control establishment of the ZPA. However, mice homozygous for an engineered deficiency eliminating function of \textit{Hoxd11}, \textit{Hoxd12} and \textit{Hoxd13} do not exhibit limb truncations (Zákány and Duboule, 1996), suggesting that \textit{Shh} function in their limbs has not been abrogated. To date, no single gene product has been shown to be directly responsible for the spatially restricted induction of \textit{Shh}.

Here we report that the basic helix-loop-helix (bHLH) transcription factor \textit{dHAND/Hand2} (Srivastava et al., 1995), also known as \textit{Thing-2}/\textit{Hed} (Hollenberg et al., 1995; Cross et al., 1995), is expressed in a remarkably dynamic expression pattern throughout limb bud outgrowth and differentiation. At the onset of limb outgrowth, \textit{dHAND} expression is localized to the posterior region of the limb bud, and subsequently occupies a domain that, at all stages analyzed, is broader than, and encompasses, the \textit{Shh} domain. Mouse embryos homozygous for a targeted deletion of \textit{dHAND}, which die at mid-gestation from cardiovascular abnormalities (Srivastava et al., 1995), have small, underdeveloped limbs that fail to upregulate \textit{Shh}. Conversely, ectopic expression of a \textit{dHAND} transgene in the anterior region of the limb of transgenic mouse embryos results in ectopic expression of \textit{Shh} and \textit{SHH} target genes, as well as digit duplications. These results reveal a novel role for \textit{dHAND} as an upstream activator of \textit{Shh} expression and regulator of limb development and polarity.

**MATERIALS AND METHODS**

**Generation and analysis of transgenic embryos**

Construct BH4mutdHZe (see Fig. 3A) consists of dual independent transcription cassettes expressing \textit{dHAND} and \textit{lacZ}, respectively, each driven by a mutant \textit{Hoxb}8 promoter enhancer. The \textit{lacZ} expression cassette was based on construct 16m (a gift from J. Deschamps; see Charité et al., 1998) to the 5’ end of which three additional copies of the mutant BH1100 element (with four central CDX binding sites mutated; Charité et al., 1998) were added, yielding BH4mutZ. The \textit{dHAND} expression cassette was generated by excising the \textit{lacZ} gene and SV40 polyadenylation signal from BH4mutZ (utilizing a \textit{BglII} site in the \textit{Hoxb}8 5’-untranslated region), and replacing it with 3 kb of \textit{dHAND} genomic sequence, extending from the \textit{BssHI} site in the 5’-untranslated region to a \textit{BamHI} site downstream of the stop codon, followed by a 0.25 kb fragment containing the SV40 polyadenylation signal, yielding BH4mutdHZe. The \textit{dHAND} and \textit{lacZ} expression cassettes were then combined to yield BH4mutdHZ. This construct was linearized at the unique \textit{NcoI} site prior to microinjection.

To generate \textit{proxl-dHAND}, the \textit{dHAND} expression cassette was excised from BH4mutdHZ and inserted downstream of the \textit{proxl} promoter and limb enhancer (Martin and Olson, 2000), using a \textit{BglII} site in the 3’ \textit{UTR}. The \textit{proxl-dHAND} expression cassette was excised from the vector backbone with \textit{SalI} prior to microinjection.

DNAs were injected into C57Bl/6 \times \textit{Dba F1} \times \textit{F1} zygotes. Embryos were recovered from foster mothers at embryonic day (E)10.75 to E16.75, and BH4mutdHZe embryos were assayed for expression of the transgene by X-gal staining of either the whole embryo, or the head, or (for E16.75 embryos) a piece of the vertebral column removed from the embryo (to monitor expression in the spinal ganglia). Trunks of E10.5 to E12.5 embryos were used for whole-mount in situ hybridization fixed overnight in 4% paraformaldehyde (PFA) at 4°C, rinsed, dehydrated through a methanol series and stored at –20°C. E16.75 embryos were stained with Alcian blue and Alizarin red according to the method of Hogan (1994).

**Whole-mount in situ hybridization**

Embryos were rehydrated, washed in PBT (PBS, 0.1% Tween 20), treated with Proteinase K 10 µg/ml in PBT for 8, 15, 20, 40 or 50
Expression of dHAND during limb development was examined by whole-mount in situ hybridization of mouse embryos. Comparison of expression patterns of dHAND and Shh in E9.5 to E11.5 forelimb buds was done by double labeling; D,E,J,L and N show Shh-specific staining with BCIP, in turquoise, which was followed by detection of the dHAND probe with NBT/BCIP (purple staining) in the same embryos (C,F,K,M and O, respectively). Limb buds are oriented with the anterior side towards the top of the panels. The probe detected by the staining is indicated at the top right corner of each panel. (A) E8.5 embryo, ventral view, showing dHAND expression in lateral mesoderm (lm), heart (he) and allantois (al). (B) E9.0 embryo (16 somites; lateral view) showing strong expression of dHAND in the posterior flank (arrowheads) but downregulation more anteriorly, at the site of the prospective forelimb, adjacent to somites 7-12 (S7 to S12). (C) E9.5 embryo (23 somites) showing expression in the posterior part of the forelimb bud (green arrowhead), continuing in the flank (white arrowhead). (D) Left forelimb bud of embryo shown in C, showing lack of Shh expression. (E) Right forelimb bud of E9.75 embryo (28 somites), showing weak Shh expression at the posterior margin (arrowhead). (F) Same limb bud as shown in E, following detection of dHAND. Strong expression in the posterior part of the bud is indicated by the arrowhead. (G) Right hindlimb bud of the embryo shown in E and F. dHAND expression is downregulated in the center of the bud (arrowhead). (H) Right forelimb bud of an E10.25, 31-somite embryo, showing strong posterior expression of dHAND (arrowhead). (I) Right hindlimb bud of embryo shown in H, showing restriction of dHAND expression to the posterior part of the bud (arrowhead). (J) Right forelimb bud of an E10.75 embryo, showing an extended domain of Shh expression (arrowhead) along the posterior margin. (K) Same limb bud as shown in J, following detection of dHAND. The white arrowhead indicates a posterior domain of strong expression surrounding the Shh domain, the small black arrowhead indicates a domain of weaker expression proximally and anteriorly. (L,M) Expression of Shh and dHAND, respectively (arrowheads), in the E10.75 hindlimb bud. (N) Right forelimb bud of an E11.5 embryo. Shh is no longer expressed in the forelimb at a detectable level at this stage. (O) Same limb bud as shown in N, following detection of dHAND. In addition to the posterior and proximal-anterior domains of expression (white and black arrowhead, respectively) an apical domain of intermediate expression can be distinguished (green arrowhead). (P) Right hindlimb bud of the embryo shown in N and O, showing similar domains of dHAND expression (arrowheads as in panel O). (Q) Right forelimb of an E12.5 embryo. The white arrowhead indicates the posterior domain of dHAND expression, the black arrowhead indicates weaker expression anteriorly, and the green arrowhead indicates expression in the forming digits. Scale bars are 0.1 mm.
post coitus (E7.5 to E12.5). Two-color in situ hybridization was used to relate expression of dHAND to that of Shh in E9.5 to E11.5 limb buds. At E7.5, dHAND is expressed around the proximal perimeter of the embryo, including the precardiac mesoderm (data not shown). Expression at E8.5 is still in a circumferential pattern, including the heart, allantois and lateral mesoderm (Fig. 1A). At E9.0, after turning of the embryo is completed, expression remains high in the posterior lateral plate mesoderm (Fig. 1B, arrowheads), but gradually declines anteriorly over the region where the forelimb bud will develop, adjacent to somites 7 to 12. By E9.5 (23 somites), expression in the outgrowing forelimb bud is limited to the most posterior part (Fig. 1C, green arrowhead), extending posteriorly into the flank (Fig. 1C, white arrowhead). Shh expression is not detectable at this stage (Fig. 1D). In E9.75, 28-somite embryos, when weak Shh expression is detected at the posterior margin of the forelimb bud (Fig. 1E, arrowhead), dHAND expression in the limb is expanded and upregulated (Fig. 1F, arrowhead); dHAND continues to be expressed in the flank, but begins to be downregulated in the center of the outgrowing hindlimb buds (Fig. 1G, arrowhead). At E10.25 (31 somites), posterior domains of strong dHAND expression are observed in both fore- and hindlimb buds (Fig. 1H, I, arrowheads). By E10.75, the Shh expression domain has expanded distally with continued distal growth of the forelimb bud (Fig. 1J, arrowhead), and dHAND is expressed in a broad posterior domain roughly centered around the Shh domain (Fig. 1K, white arrowhead). Similarly, in the E10.75 hindlimb bud, the dHAND expression domain clearly encompasses that of Shh (Fig. 1L,M).

An additional, proximal-anterior domain of weak dHAND expression is observed from E10.75 onwards (black arrowheads in Fig. 1K,O-Q). At E11.5, Shh expression is no longer detected in the forelimb (Fig. 1N), but dHAND expression is maintained and a third, apical domain of expression (Fig. 1O, green arrowhead) can now be distinguished that appears to partly overlap with the posterior domain. dHAND expression in the hindlimb bud, development of which is now catching up with that of the forelimb, is very similar (Fig. 1P). At E12.5, strong posterior and weak anterior expression domains persist (Fig. 1Q, white and black arrowheads, respectively). Apical expression is now limited to the forming digits (green arrowheads, Fig. 1Q).

**Limb development in dHAND<sup>-/-</sup> embryos**

Embryos that are homozygous for a targeted deletion of the dHAND gene lack a distinguishable right ventricle of the heart and show abnormal development of the aortic arch arteries (Srivastava et al., 1997). They develop extreme edema by E9.75 (D. G. M., unpublished observations), most likely due to circulatory dysfunction, and die around E10.5. By this stage, they are grossly retarded relative to heterozygous littermates (Srivastava et al., 1997, see Fig. 2). We noted that the forelimb buds of dHAND null embryos were extremely small and variably malformed (Fig. 2A,B,D,E); they often appeared particularly deficient in the anteroposterior dimension, having their anterior margin at the level of somite 8 or 9 instead of somite 7 as in wild-type embryos. dHAND null embryos did not express Shh in the limb buds (Fig. 2B), whereas axial Shh expression was readily detected (Fig. 2C). Because these embryos form about 24 somites, we considered the possibility that limb development might not advance to a sufficient stage for Shh to be expressed. However, judged by their shape (Fig. 2A,C,E), dHAND null limb buds frequently were developed further than those of a wild-type embryo with the same number of somites; somites became progressively smaller towards the posterior end of the embryo, suggesting a progressive impairment of trunk development, which would preclude accurate determination of developmental stage from the number of somites formed.

The absence of Shh in the limb buds of dHAND mutants did not appear to be attributable to a lack of mRNA integrity specifically in the limb buds, as transcripts for prxl/MHox, normally expressed from the onset of limb bud outgrowth (Cserjesi et al., 1992), were detected in the limb buds of all dHAND<sup>-/-</sup> embryos analyzed (Fig. 2D,E; see also Thomas et al., 1998). Gremlin expression was also observed in both fore- and hindlimb buds of dHAND null embryos (data not shown). Expression of Mxi1, Mxi2 and Dlx2 in dHAND null limb buds has been noted previously (Thomas et al., 1998). Thus, although dHAND null limb buds are severely dysmorphic, they...
express several genes involved in limb development. Because of the variability in limb morphology and because development of the entire embryo is compromised, precluding an accurate assessment of developmental stage, we did not feel confident in drawing conclusions with regard to possible anteroposterior polarity in these limb buds, or to their abnormal development being the result of the lack of \(\text{dHAND}\) function in the limb bud or, rather, secondary to the general morbidity caused by the cardiac insufficiency.

**Ectopic expression of \(\text{dHAND}\) in the limb results in polydactyly and mirror-image duplications**

In a complementary approach to investigate the possible functions of \(\text{dHAND}\) in limb development, a \(\text{dHAND}\) transgene (construct BH4mutdHZ; Fig. 3A) was used to ectopically express \(\text{dHAND}\) in the developing limbs, employing a novel Hoxb8 promoter-enhancer combination which, based on previous results (Charité et al., 1995, 1998), was expected to drive expression in migrating neural crest cells exclusively, but in addition showed expression in the limbs (see Fig. 3B). Construct BH4mutdHZ includes a lacZ reporter driven by the same promoter-enhancer combination as a means of assessing transgene expression. All analyses were done in F0 embryos isolated from foster mothers, each representing an independent transgene integration event. Analysis of lacZ expression by whole-mount staining revealed that transgene expression levels varied, but that ectopic activation by regulatory elements at the integration site occurred very rarely (data not shown).

One of two E12.5 BH4mutdHZ transgenic embryos expressing lacZ showed broadened footplates and slightly broadened handplates (Fig. 3B). In accordance with these observations, three out of four E16.75 transgenic embryos that correctly expressed the transgene as determined by X-gal staining of spinal ganglia, exhibited preaxial polydactyly of the hindlimbs, ranging from a single additional triphalangeal digit to replacement of digit I by three triphalangeal digits (see below), with or without agenesis or partial agenesis of the tibia. Fig. 3C shows an example of a BH4mutdHZ F0 transgenic hindlimb, in which digit I and its associated tarsal bones (see wild-type limb in Fig. 3D) are replaced by three ectopic digits, two of which are fused over most of their length. The ectopic digits have a more posterior identity than the digit they replace; they were classified as II/III based on the fact that they are triphalangeal as opposed to the biphalangeal digit I, on the length of their metatarsals and on the morphology of their tarsal bones, involving a replacement of the tibiale and the first
distal tarsal, the medial cuneiform, by an extension of the navicular bone and a distal tarsal similar to the second and third distal tarsals (the intermediate and lateral cuneiform, respectively; Fig. 3F, compare to E). Some mirror-image symmetry, with the axis of symmetry running through the center of digit II, is evident in these tarsal bones (Fig. 3C,F). No skeletal abnormalities were evident in the forelimbs of the four E16.75 transgenic embryos examined, which is likely to reflect the lower level of transgene expression in the forelimbs compared to the hindlimbs (see below).

To further confirm these results, we created a transgene (construct prxl-dHAND) in which dHAND is under transcriptional control of regulatory sequences of prxl that direct expression throughout the developing limb bud (Martin and Olson, 2000). The expression pattern of these sequences at E10.5 driving a lacZ reporter is shown in Fig. 4A; β-galactosidase (β-gal) expression is detected from the onset of limb outgrowth through E14.5, when it becomes more restricted to the limb bud. This restricted expression was not observed (data not shown). Ectopic expression of dHAND in an E16.75 prxl-dHAND transgenic embryo resulted in complete mirror-image duplications of posterior elements of both autopod and zeugopod of the forelimbs (Fig. 4C, compare to B), as well as polydactyly in the hindlimbs, which was more similar to the BH4mutHZ phenotype (data not shown). In both forelimbs, the radius was replaced by a mirror-image duplication of the ulna as indicated by the presence of the olecranon (Fig. 4C,D). Digits I and II were replaced by four ectopic digits with a posterior identity based on their morphology (Fig. 4C). In addition, the ectopic digits III' and IV'/V' were associated with ectopic carpal bones resembling a mirror-image of the capitae and hamate, respectively (Fig. 4F).

Ectopic expression of dHAND induces an ectopic ZPA

The BH4mutHZ and prxl-dHAND limb phenotypes closely resemble those of limbs in which an ectopic zone of polarizing activity (ZPA) is generated anteriorly (Charité et al., 1994; and see for example Qu et al., 1999). This was confirmed by molecular analysis of transgenic limb buds. Whole-mount in situ hybridization of an E12.5 BH4mutHZ transgenic hindlimb showing an ectopic lobe of tissue preaxially, confirmed the posterior character of the ectopic tissue as indicated by the expression of Hoxd11 (Fig. 5A, compare to 5B). Analysis of E11.5-E12.0 BH4mutHZ transgenic embryos revealed, in one out of three embryos exhibiting reasonably strong transgene expression as judged by X-gal staining of the heads, an ectopic domain of Shh expression at the anterior margin of the hindlimb buds (Fig. 5C, compare to 5D), which was associated with an ectopic domain of Hoxd11 expression (Fig. 5E, compare to 5F). Together with the skeletal analyses, these results demonstrate unequivocally that ectopic expression of dHAND results in generation of an ectopic ZPA.

Reciprocal control of dHAND expression by Hedgehog signaling

While dHAND expression in the limb precedes expression of Shh, and dHAND can induce Shh expression ectopically, the spatial relationship between the posterior domain of strong dHAND expression and the Shh domain suggests that the reciprocal interaction may occur as well. To investigate this possibility, we analyzed dHAND expression in two mouse mutants in which Hedgehog signaling in the limb is altered in complementary ways. First, we examined dHAND expression in E10.5 Shh<sup>−/−</sup> embryos (Chiang et al., 1996; a gift from P. Beachy), and observed a dramatic reduction of dHAND expression in both fore- and hindlimb buds, expression being limited to a very small, posterior domain (Fig. 6C, compare to 6B, and data not shown).

Next, we investigated dHAND expression in the Doublefoot (Dbf) mutant mouse, which exhibits severe polydactyly of both

Fig. 4. Mirror image duplication in forelimbs of prxl-dHAND transgenic mice. (A) Expression of prxl-lacZ reporter in an E10.5 mouse embryo stained with X-gal. Note high levels of β-galactosidase expression throughout both forelimb (flb) and hindlimb (hlb) buds. (B) Wild-type E16.75 forearm stained with Alcian blue and Alizarin red for cartilage and bone, respectively, showing the normal morphology of the digits (I-V), radius (r) and ulna (ul). (C) Forearm of a prxl-dHAND transgenic littermate showing mirror image duplication of the posterior elements of both autopod and zeugopod. Normal digits III-V were associated with ectopic carpal bones resembling a mirror-image of the capitae and hamate, respectively (Fig. 4F). (D) Elbow joint of the right forelimb contralateral to the limb showing articulation of the humerus (hu) with the two olecranon processes (ol) of the ulna. (E) Forearm of a prxl-dHAND transgenic littermate showing mirror image duplication of the posterior elements of the autopod and zeugopod. Normal digits III-V are adjacent to four ectopic triphalangeal digits (III', III', IV' , and V'). The radius has been replaced by an ectopic mirror-image ulna (ul'); note the olecranon (ol) at the proximal end of the ectopic ulna. (D) Closeup view of the elbow joint of the right forelimb contralateral to the limb shown in C, showing articulation of the humerus (hu) with the two olecranon processes (ol) of ulnas ul and ul'. (E) Dorsal view of the wild-type hand shown in B, showing the normal anatomy of the carpal bones. Note the hamate bone (h), which articulates with digits IV and V, and the capitae (c'), which opposes the third digit. Also note the triquetral (tq). (F) Dorsal view of the hand shown in C, showing duplication of the posterior carpal bones. Note the ectopic capitae (c') and hamate (h') which articulate appropriately with the ectopic digits III' and IV'. In addition, a single, elongated, symmetrical carpal bone (tq') replaces the lunate and triquetral bones.
fore- and hindlimbs (Lyon et al., 1996; Hayes et al., 1998b). This polydactyly is preceded by precocious and ectopic expression of Indian hedgehog (Ihh; Yang et al., 1998) and ectopic activation of components of the Hedgehog signaling pathway (Hayes et al., 1998a; Yang et al., 1998) along the distal margin of the limb buds at E11.0-12.5. Normally, Ihh is not expressed in the limb until much later in development when cartilage differentiates (Vortkamp et al., 1996). In E11.5 Dbf/+ embryos (gift from M. Lyon), Ihh and dHAND were both found to be ectopically expressed along the distal margin of the limb bud (Fig. 6E,F compare to 6D).

**DISCUSSION**

The data presented here identify dHAND as a positive regulator of ZPA formation: expression of dHAND precedes expression of Shh in the posterior limb bud and continues to overlap and encompass the Shh domain during subsequent stages, and, most importantly, ectopic expression of dHAND is sufficient to induce formation of an ectopic ZPA as evidenced both by expression of Shh and by the resulting effects on limb patterning. Similar results have been obtained following ectopic expression of dHAND in chick limb buds (Fernandez-Teran et al., 2000).

**Control of ZPA formation by dHAND**

The mechanisms involved in ZPA induction by dHAND remain to be determined. In principle, dHAND, which binds
the E-box consensus sequence (CANNTG) and has the potential to activate transcription (D. G. M. and E. N. O., unpublished), could directly activate Shh transcription. A direct test of this possibility must await identification of the regulatory sequences required for expression of Shh in the developing limb. Alternatively, dHAND might repress, either directly or indirectly, negative regulators of ZPA formation such as Alx4 (Qu et al., 1997) and Gli3 (Büscher et al., 1997), although the evolution of posteriorly restricted dHAND expression from an initially ubiquitous flank expression domain rather suggests that dHAND itself is repressed in the anterior part of the limb bud. Genetic analysis of the epistatic relationships between dHAND and these negative regulators is required to resolve this issue.

The expression pattern of dHAND in the limb bud suggests that it may have functions in addition to regulating Shh. While the relevance of the proximal-anterior domain of weak expression is unclear, the apical expression domain, which appears to evolve into a digit-specific pattern, suggests that dHAND may be involved in morphogenesis or differentiation of the digits. Furthermore, the observation that posterior expression of dHAND continues long after Shh expression at the posterior margin of the limb subides, also suggests that dHAND has a role at more advanced stages of development. It is possible that agenesis of the tibia as observed upon ectopic expression of dHAND, while clearly one of several possible phenotypic consequences of ectopic ZPA activity (e.g. Charité et al., 1994; Qu et al., 1999), reflects a more direct interference of ectopic dHAND with specification or development of the tibia, related to a later function of dHAND in the posterior part of the limb.

In all likelihood, dHAND performs the same functions in all four limbs, as dHAND expression in fore- and hindlimbs is very similar, taking into account the relative delay in development of the hindlimbs. Furthermore, ectopic expression of dHAND caused polydactyly in fore- as well as hindlimbs. Differences in severity and frequency of the phenotype between fore- and hindlimbs are most likely attributable to expression characteristics of the promoter-enhancer combinations used. For example, analysis of lacZ expression in BH4mutdHZ transgenic embryos suggested that expression from the BH4mut promoter in the forelimb is weaker and/or more sensitive to inhibitory influences from the integration site than expression in the hindlimb (data not shown), thus providing a straightforward explanation for the prevalence of hindlimb phenotypes in BH4mutdHZ embryos. In addition, ectopic expression of dHAND using the strong prxl limb enhancer caused extreme mirror-image duplications in the forelimb that were similar in severity and proximodistal extent to those induced by ectopic expression of Hoxb8 (Charité et al., 1994). It will be of interest to determine whether dHAND and Hoxb8 regulate one another or act in parallel.

A possible dHAND-Hedgehog regulatory loop
Our data also demonstrate that elaboration of the dHAND expression domain in the limb is in turn, directly or indirectly, dependent on SHH signaling. Ihh is not expressed in the early limb buds of wild-type embryos, but is expressed throughout the apical region of the limb bud in Dbf mutants. The expression pattern of dHAND in Dbf mutant limb buds may suggest that activation of the Hedgehog signaling pathway is sufficient to induce dHAND expression in the anterior part of the limb. As the molecular basis of the Dbf mutation is unknown, the possibility that dHAND is, rather, a mediator of the Dbf phenotype and induces Ihh cannot be formally excluded, given the temporal coexpression of dHAND and Ihh at the stage analyzed. However, we consider this unlikely since dHAND (Cross et al., 1995) and Dbf, which is located at some distance from the Ihh locus (Yang et al., 1998), map to different chromosomes, and the dHAND expression pattern, at stages when Ihh is normally expressed (Vortkamp et al., 1996), does not suggest that it is involved in regulating Ihh (data not shown).

It is noteworthy that dHAND retains a posteriorly restricted, though severely reduced, expression domain in Shh−/− limb buds. This extends previous observations that asymmetric expression of limb developmental control genes is maintained, at least for a limited time, in the absence of SHH signaling (Griesserhammer et al., 1996; Noramly et al., 1996; Ros et al., 1996; Züüiga et al., 1999), and further demonstrates that dHAND is upstream of Shh. The factors responsible for this SHH-independent expression of dHAND remain to be identified. It is of interest that morphological development of dHAND−/− limb buds is much more severely affected than that of Shh−/− limb buds. This implies that dHAND has important functions in early limb development besides its function as a regulator of Shh induction.

While our results reveal overlapping expression domains of dHAND and Shh in the limb and show that dHAND is sufficient to induce Shh in the anterior domain of the limb, additional factors, either positive or negative, must influence the ability of dHAND to activate Shh expression, because dHAND is expressed in other sites in the embryo (e.g. heart, branchial arches and lateral mesoderm) where Shh is not expressed. Conversely, Shh is expressed in regions such as the notochord and the floor plate of the neural tube where dHAND is not expressed.

A well-known requirement for expression of Shh in the limb bud is the presence of a functional AER (Laufer et al., 1994; Niswander et al., 1994). Interestingly, the potential to exhibit ZPA activity in the permissive environment of the limb bud is not restricted to the limb field but present within a broad and dynamic domain in the lateral mesoderm (Hornbruch and Wolpert, 1991). Extrapolating Hornbruch and Wolpert’s mapping data from the chick to the mouse suggests that latent ZPA activity may be contained within, but more restricted than, the dHAND expression domain (cf. Hornbruch and Wolpert, 1991, and Fig. 1A,G).

It is intriguing that ectopic Shh expression in polydactylous mutant limb buds is, when present, restricted to the anterior margin of the limb bud in all cases examined (Chan et al., 1995; Masuya et al., 1995, 1997; Büscher et al., 1997), although activation of the Hedgehog signaling pathway may occur throughout the width of the bud, as demonstrated by the talpid2 and Dbf mutants (Hayes et al., 1998a; Yang et al., 1998; Caruccio et al., 1999). Anteriorly restricted ectopic Shh expression was also observed following ectopic expression of Hoxd12 (Knezevic et al., 1997) and Hoxb8 (Charité et al., 1994). Together, these data suggest that there are factors acting within the limb bud, in parallel or in concert with the AER, that restrict expression of Shh to the anterior and posterior side. This may explain why expression of dHAND throughout the
limb buds of BH4mutdHZ and prx1-dHAND embryos results in formation of a discrete anterior Shh domain, as we have shown for BH4mutdHZ and infer from the perfect mirror-image duplications in prx1-dHAND limbs, which contrast with both the Dbf (Hayes et al., 1998b) and talpid² (Dvorak and Fallon, 1991) phenotypes.

In view of the prevalence of feedback control in limb development, some caution must be exercised when interpreting the results of ectopic expression experiments. Nevertheless, the data presented here, including the early posterior restriction of dHAND expression in the flank and the outgrowing limb bud, its overlap with Shh expression, the fact that dHAND expression remains posteriorly restricted in the absence of Shh function, and the ability of dHAND to ectopically induce a ZPA, strongly support the notion that dHAND is an important factor in establishment of the ZPA. Considering that many genes affect establishment of the ZPA and its restriction to the posterior margin of the limb bud, it seems unlikely that the highly restricted expression of Shh is controlled by the activity of a single transcription factor. However, the possibility of dHAND being a key determinant of the elusive ‘posterior activity’ necessary for initiating the Formin-dependent SHH/FGF4 feedback loop (Zúñiga et al., 1999) is an attractive hypothesis.

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Note added in proof