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Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding

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SUMMARY

Basic helix-loop-helix (bHLH) transcription factors control developmental decisions in a wide range of embryonic cell types. The HLH motif mediates homo- and heterodimerization, which juxtaposes the basic regions within the dimeric complex to form a bipartite DNA binding domain that recognizes a DNA consensus sequence known as an E-box. eHAND and dHAND (also known as HAND1 and HAND2) are closely related bHLH proteins that control cardiac, craniofacial and limb development. Within the developing limb, dHAND expression encompasses the zone of polarizing activity in the posterior region, where it has been shown to be necessary and sufficient to induce the expression of the morphogen sonic hedgehog. Misexpression of dHAND in the anterior compartment of the limb bud induces ectopic expression of sonic hedgehog, with resulting preaxial polydactyly and mirror image duplications of posterior digits. To investigate the potential transcriptional mechanisms involved in limb patterning by dHAND, we have performed a structure-function analysis of the protein in cultured cells and ectopically expressed dHAND mutant proteins in the developing limbs of transgenic mice. We show that an N-terminal transcriptional activation domain, and the bHLH region, are required for E-box-dependent transcription in vitro. Remarkably, however, digit duplication by dHAND requires neither the transcriptional activation domain nor the basic region, but only the HLH motif. eHAND has a similar limb patterning activity to dHAND in these misexpression experiments, indicating a conserved function of the HLH regions of these proteins. These findings suggest that dHAND may act via novel transcriptional mechanisms mediated by protein-protein interactions independent of direct DNA binding.

Key words: dHAND/HAND2, Limb, Zone of polarizing activity, Sonic hedgehog, DNA binding, Basic helix-loop-helix, Mouse

INTRODUCTION

Studies of the vertebrate limb have yielded important insights into the mechanisms of embryonic patterning along the dorsoventral, anteroposterior (AP) and proximodistal axes (Johnson and Tabin, 1997). Growth and patterning of the limb bud along the AP axis is controlled by the zone of polarizing activity (ZPA), a specialized region of mesoderm at the posterior margin of the limb bud. Grafts of the ZPA into the anterior margin of a recipient limb bud result in mirror-image duplications of the distal skeletal elements (Saunders and Gasseling, 1968). The secreted protein sonic hedgehog (SHH) is localized to the ZPA, and application of beads soaked in recombinant SHH to the anterior margin of the limb results in mirror-image digit duplications (Riddle et al., 1993). Limbs from *Shh* mutant mice also show severe defects in patterning along the AP and dorsoventral axes, and lack polarizing activity in grafting experiments (Chiang et al., 2001; Chiang et al., 1996).

While SHH is a key mediator of ZPA activity, several lines of evidence indicate that it is not the sole determinant of AP information within the limb field. The chick *limbless* mutant, for example, does not detectably express SHH, but displays nested AP expression of 5′ *Hoxd* genes within the limb (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). Limb buds from *Shh* mutant mice also retain graded AP expression of some components of the SHH/FGF feedback loop, and form autopodial structures with recognizable AP polarity (Chiang et al., 2001; Zuniga et al., 1999). In light of these findings, there has been intense interest in identifying factors that act upstream of SHH to establish the ZPA.

The basic helix-loop-helix (bHLH) transcription factor dHAND (HAND2 – Mouse Genome Informatics) is expressed in the posterior limb mesenchyme prior to SHH, and its expression pattern completely encompasses that of SHH at all later stages of limb development (Charité et al., 2000). Moreover, limb buds from mice and zebrafish lacking dHAND

fail to upregulate SHH expression (Charité et al., 2000; Yelon et al., 2000). Misexpression of dHAND in the anterior limb mesoderm of mouse or chick embryos also is sufficient to induce ectopic SHH and mirror image duplications of posterior skeletal elements (Charité et al., 2000; Fernandez-Teran et al., 2000).

A recent study has further defined the role of dHAND in prepatterning the AP axis of the limb bud (te Welscher et al., 2002). The transcriptional repressor GLI3 is expressed in the anterior limb mesenchyme, and plays an important role in positioning the ZPA at the posterior margin of the limb bud (Zuniga and Zeller, 1999). In the polydactylous mouse mutant Extratoes (Xt), in which *Gli3* is disrupted (Buscher et al., 1997; Hui and Joyner, 1993; Schimmang et al., 1992), dHAND (*Hand2* – Mouse Genome Informatics) expression persists in the anterior mesenchyme, demonstrating that GLI3 represses transcription of *dHAND* during limb bud outgrowth. Conversely, in *dHAND*$^{-/-}$ embryos, expression of *Gli3* and the *aristaless-like 4* (*Alx4*) gene, which encodes a transcriptional repressor, are expanded into the posterior limb mesenchyme. Misexpression of dHAND in the anterior mesoderm is also sufficient to induce the BMP antagonist Gremlin, an important component of the SHH/FGF feedback loop that maintains the ZPA (Zuniga et al., 1999). Therefore, it appears that dHAND acts both positively to induce ZPA formation, as well as negatively to repress expression of factors that inhibit ZPA formation. The fact that dHAND is required for the activation of *Shh*, *Hoxd11*, *Hoxd12* and *Bmp2*, and repression of *Gli3* and *Alx4*, which are key regulators of AP patterning (Charité et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002; Yelon et al., 2000), suggests that it may act as both a transcriptional activator and repressor. It remains unclear, however, which, if any, of these genes represent direct transcriptional targets of dHAND.

Members of the bHLH family of transcription factors regulate the specification and differentiation of numerous cell types during embryonic development (Massari and Murre, 2000). In general, tissue-specific (class B) bHLH factors form obligate heterodimers with ubiquitous bHLH proteins (class A), called E-proteins. Dimerization juxtaposes the basic regions of bHLH proteins to form a bipartite DNA binding domain that recognizes the E-box consensus sequence (CANNTG) with resulting transcriptional activation or repression of target genes.

dHAND and the closely related bHLH protein eHAND (HAND1 – Mouse Genome Informatics) are co-expressed in many embryonic cell types, although only dHAND is expressed in the developing limb bud (Charité et al., 2000; Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995). eHAND has been shown to bind a nonconsensus E-box sequence (NNTCTG) as a heterodimer with the E-protein E12. Reporter gene assays in tissue culture cells have suggested that eHAND may have dual functions as both a transcriptional repressor and activator (Hollenberg et al., 1995; Scott et al., 2000). However, the potential transcriptional activities of dHAND have not been reported (see note added in proof).

To further understand the mechanisms whereby dHAND regulates transcription during limb development, we performed a structure-function analysis of the dHAND protein in cultured cells and misexpressed dHAND mutant proteins in the developing limb buds of transgenic mice. Remarkably, our results show that only the HLH motif of dHAND is required to induce the formation of ectopic digits. This activity is independent of direct DNA binding and the basic region, as well as the N-terminal transactivation domain. Digit duplications are also induced by eHAND, but not by the related bHLH protein paraxis, indicating that the highly conserved HLH regions of dHAND and eHAND have unique functions not shared by other bHLH family members. These findings reveal an unanticipated specificity of the HLH region of dHAND as a regulator of tissue morphogenesis and patterning and suggest that dHAND may govern these processes through a mechanism independent of direct DNA binding.

MATERIALS AND METHODS

Cell culture and transfections

10T1/2 and COS cells were maintained in Dulbecco's Minimal Essential Medium with 10% fetal bovine serum. Cells were transfected using FuGENE 6 reagent (Roche) with 200 ng of reporter (L8G4-luciferase or L8E6-luciferase) and expression constructs (Hollenberg et al., 1995). The pcDNA3.1 vector without a cDNA insert was used to equalize the amount of DNA in each transfection. To control for transfection efficiency, 50 ng of CMV-*lacZ* was included in all transfections. Cell extracts were prepared 48 hours after transfection and assayed for luciferase activity using the Promega Luciferase detection kit. Luciferase activities were normalized to β-galactosidase activity. Each experiment was performed in duplicate. Values from at least three independent experiments were averaged for each figure.

Coimmunoprecipitation and western blotting

Coimmunoprecipitation experiments were performed in COS-1 cells transiently transfected with 1 μg of each expression vector. Immunoprecipitation reactions were performed as previously described (McKinsey et al., 2000). Briefly, cell extracts were harvested in 500 μl of lysis buffer (1×PBS pH 7.4, 0.1% Triton X-100, 1mM EDTA, 1× Roche Complete protease inhibitor cocktail, 1 mM PMSF). Following incubation on ice for 10 minutes, extracts were sonicated briefly and centrifuged at 4°C for 10 minutes. Monoclonal anti-Flag antibody (Sigma) was added to the supernatant and rocked at 4°C for 1 hour. Protein G-sepharose beads (Zymed) were added and rocking was continued for another hour. The beads were then washed four times by brief centrifugation, and rinsing in lysis buffer. Protein was released from the beads by addition of SDS-PAGE loading buffer and boiling for 5 minutes.

SDS-PAGE and western blotting was performed using standard techniques (McKinsey et al., 2000). A 1:1000 dilution of rabbit polyclonal anti-Myc (Santa Cruz) antibody was used as a primary antibody, followed by a 1:10,000 dilution of donkey anti-rabbit horseradish peroxidase conjugated secondary antibody (Amersham).

Expression and transgene constructs

Mammalian expression vectors were generated from the dHAND cDNA by high-fidelity PCR using Pfu Turbo DNA polymerase (Stratagene) using standard techniques. Most cDNA fragments were cloned into a modified pcDNA3.1 vector containing an N-terminal Myc epitope tag (McKinsey et al., 2000). pSG424 and pM1 vectors used to generate GAL4 DNA-binding domain fusion proteins, and the L8G5E1b reporter have been described elsewhere (Lemercier et al., 1998). All cDNAs generated by high-fidelity PCR were cloned into the *Eco*RI cloning site of pcDNA3.1 or pSG424. The mouse p*rx1* promoter was used to direct the expression of dHAND cDNAs in transgenic mice. This promoter has been described previously (Martin

and Olson, 2000). p*rx1* transgenes were generated by cloning wild-type or mutant dHAND cDNAs into a *Bgl*II site in the 5′ untranslated region of the p*rx1* gene. Transgenes also contained a 3′ human growth hormone (hGH) polyadenylation signal. All dHAND mutations were confirmed by DNA sequencing. Sequences of PCR primers are available upon request.

Gel mobility shift assays

Gel mobility shift assays were performed as described previously (McFadden et al., 2000). In vitro translated proteins were generated using the Promega TNT kit, and 3 μl of lysate were used in each binding reaction. An oligonucleotide probe corresponding to the published eHAND/Thing1 E-box was used (Hollenberg et al., 1995): Th-1A, 5′-CTCGAGGGATCCAATGCATCTGGATCGGGGCA-3′; Th-1B, 5′-CTCGAGTGCCCCGATCCAGATGCAATGGATCC-3′. Oligonucleotides were annealed and radiolabeled as described (McFadden et al., 2000). DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel in 0.5×TBE and 2.5% glycerol.

Transgenic mice

Transgenic mice were generated as previously described (Cheng et al., 1993). F_0 embryos were harvested by sacrificing the foster mother at the desired embryonic time point. Embryos used for bone and cartilage staining were harvested, soaked in tap water overnight, skinned, eviscerated and dehydrated for 48 hours before staining with Alcian Blue and Alazarin Red (Hogan, 1994).

The genotypes of transgenic embryos were determined by Southern blotting. Yolk sacs were digested overnight in tail lysis buffer with 0.8 μg/μl proteinase K, followed by a second addition of proteinase K and further incubation for another 12-24 hours. Protein was extracted with phenol:chloroform (1:1), followed by chloroform. DNA was precipitated with an equal volume of isopropanol, followed by rinsing in 1 ml of 70% ethanol. DNA was resuspended in 100 μl deionized H_2O. DNA (15 μl) was digested with *Eco*RV overnight. Southern blotting was performed using standard techniques with [^{32}P] radiolabeled probes corresponding to dHAND-coding sequence used in transgene constructs.

In situ hybridization

In situ hybridization to embryo sections was performed as described previously, using the full-length dHAND cDNA labeled with ^{35}S-UTP (Shelton et al., 2000). Whole-mount in situ hybridization was performed as previously described (Riddle et al., 1993).

RESULTS

dHAND activates transcription through a consensus E-box

To begin to investigate the transcriptional properties of dHAND, we tested whether the in vitro translated protein was able to bind a consensus E-box identified in site selection experiments with eHAND (Hollenberg et al., 1995). dHAND alone did not show significant binding to this site in gel mobility shift assays, but in the presence of E12, a prominent DNA-protein complex was observed, reflecting the binding of dHAND-E12 heterodimers (Fig. 1A).

To determine the transcriptional consequences of dHAND binding to this site, we tested whether dHAND could activate a luciferase reporter linked to six copies of this E-box (Fig. 1B). dHAND alone evoked only a minimal increase in luciferase activity in transfected 10T1/2 cells. Similarly, E12 failed to substantially activate this reporter. However, co-transfection of dHAND and E12 expression vectors resulted in approximately 65-fold activation of the reporter. These results suggest that the level of expression of dHAND alone in transfected cells exceeds the level of endogenous E-proteins, therefore limiting activation of the reporter, and that providing

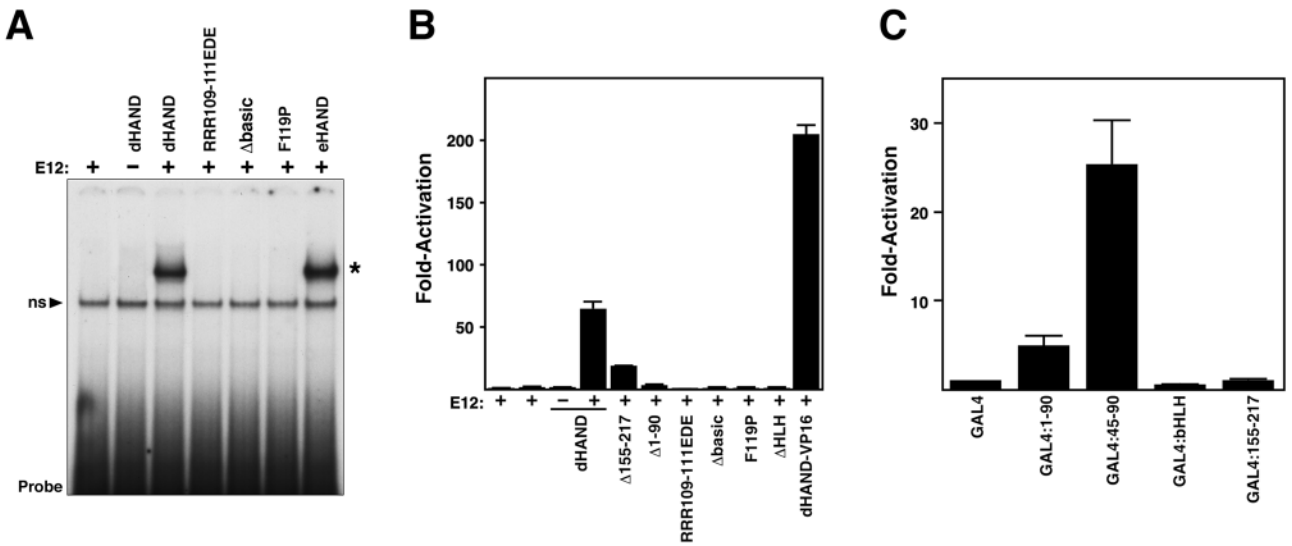

Fig. 1. DNA binding and transcriptional activity of dHAND mutant proteins. (A) Wild-type and mutant dHAND proteins were translated in vitro with or without E12, as indicated, and tested for DNA binding in gel mobility shift assays to a radiolabeled oligonucleotide probe containing the eHAND/Th1 E-box sequence. HAND/E12 complex mobility is indicated by an asterisk and a nonspecific complex is marked by ns. (B) 10T1/2 cells were transiently transfected with the L8E6-luciferase reporter and pcDNA3.1 expression vector encoding wild-type and mutant dHAND proteins as indicated (see Fig. 2), and luciferase activity was determined in cell extracts. Values are expressed as fold activation of the reporter gene relative to the level of expression with the reporter alone. (C) 10T1/2 cells were transiently transfected with the L8G4-luciferase reporter and the pGE1b-GAL4 expression vector encoding regions of the dHAND-coding region fused to the GAL4 DNA-binding domain and luciferase activity was determined in cell extracts. Values in B and C represent the mean±s.e.m. determined from at least three independent experiments.

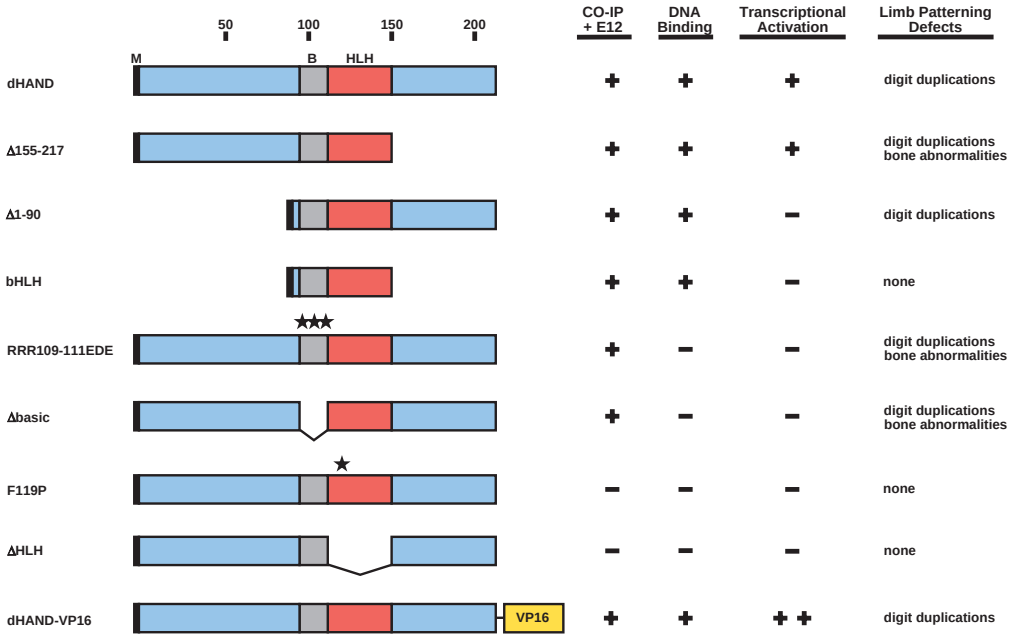

Fig. 2. Summary of the activities of dHAND mutant proteins. Mutant dHAND proteins and their activities are shown. All mutants contained an N-terminal Myc epitope tag. M, Myc epitope tag; B, basic region; HLH, helix-loop-helix.

additional E12 facilitates heterodimer formation and subsequent DNA binding and transcriptional activation.

In order to identify the regions of dHAND that contribute to its transcriptional activity, we generated a series of deletions of the dHAND-coding region (Fig. 1B, Fig. 2). All dHAND proteins contained an N-terminal Myc epitope tag that allowed their detection by western blotting. Mutant proteins were expressed at levels comparable with the wild-type protein (data not shown). To confirm that mutant proteins folded properly and were functional in vivo, each was also tested by co-immunoprecipitation with E12, and for DNA binding in gel mobility shift assays (Fig. 1A, Fig. 2, and data not shown).

Because the C-terminal amino acids of dHAND are highly conserved among all orthologs of HAND proteins (Angelo et al., 2000), we speculated that this region might be important for the transcriptional activity of dHAND in these reporter assays. Deletion of this region (Δ155-217) resulted in a threefold reduction of the transcriptional activity of dHAND. By contrast, deletion of amino acids 1-90 resulted in a near-complete loss of transcriptional activity. These findings suggested that the N-terminal region of dHAND acts as a transcriptional activation domain and is responsible for the majority of the transcriptional activity of dHAND, although the C- terminal residues appear to contribute to the transactivation potential of dHAND.

To confirm that amino acids 1-90 of dHAND can act as a transcriptional activation domain, we generated fusions of dHAND to the DNA-binding domain of the yeast GAL4 protein and tested these constructs for their abilities to activate a GAL4-dependent luciferase reporter (Fig. 1C). Residues 1-90 were able to stimulate transcription, whereas the bHLH region or C terminus (residues 155-217) was not. Further deletion of the first 90 residues revealed that the most potent activation function mapped to amino acids 45-90.

Transactivation-defective dHAND mutants induce ectopic digits in transgenic mice

Recently, we and others have shown that misexpression of dHAND in the anterior region of the limb buds results in ectopic expression of SHH at the anterior margin of the limb bud with resulting preaxial polydactyly at later stages of development (Charité et al., 2000; Fernandez-Teran et al., 2000). To determine if there was a correlation between the limb patterning activity of dHAND and the transcriptional activity detected in tissue culture, we expressed each of the mutant proteins described above in transgenic mouse embryos using regulatory sequences from the p*rx1* gene, which direct expression throughout the forelimb bud and in the anterior mesoderm of the hindlimb at E10.5 (Martin and Olson, 2000). We used the presence of ectopic digits at embryonic day 16.5 (E16.5) as a functional readout of the activities of these mutant proteins.

Because our previous studies of dHAND misexpression in the limb used a *dHAND* genomic fragment containing the single intron within the coding region (Charité et al., 2000), we first tested whether expression of a cDNA encoding a dHAND protein with an N-terminal Myc-tag was capable of inducing ectopic digits. This dHAND cDNA effectively induced preaxial polydactyly of fore- and hindlimbs (Fig. 3A,B; data not shown). Polydactyly ranged from a single ectopic triphalangeal digit, to three ectopic digits replacing digit I. The severity and frequency of this limb phenotype were comparable to previous results using the *dHAND* genomic fragment linked to the p*rx1* promoter.

Surprisingly, preaxial polydactyly was also observed in 9/12 embryos with a deletion mutant lacking the N-terminal transcription activation domain (mutant Δ1-90, Fig. 3C). However, 3/12 embryos harboring this transgene showed small hypoplastic limbs with a reduced number of digits (data not shown), a phenotype we have not observed with the wild-type protein. Whether this phenotype reflects a weak dominant negative activity of the mutant protein or variability in transgene expression is unclear. Nevertheless, the presence of ectopic digits in the majority of mutant Δ1-90 transgenic embryos suggests that dHAND can function in the absence of the N-terminal transcriptional activation domain.

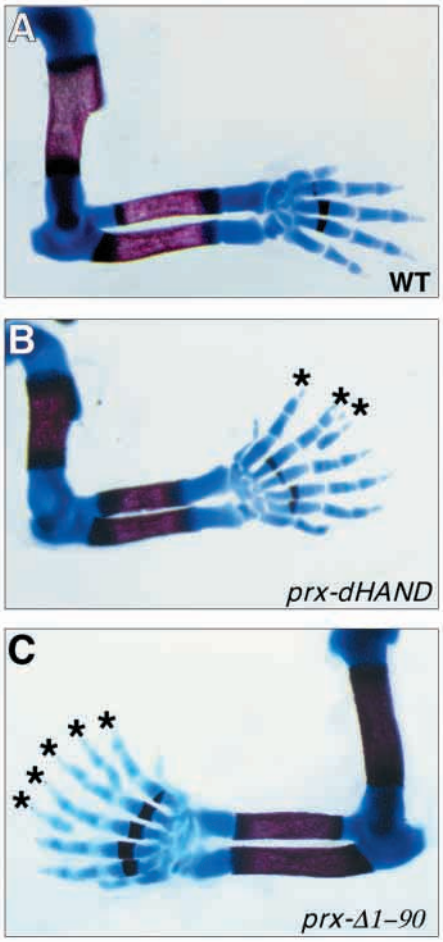

Fig. 3. Preaxial polydactyly induced by a dHAND mutant protein lacking the N-terminal transcription activation domain. F_0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. (A) Limb from a wild-type nontransgenic embryo. Embryos harboring p*rx-dHAND* (B) and p*rx-Δ1-90* (C) transgenes showed preaxial polydactyly. All panels show forelimbs. Ectopic digits are indicated with an asterisk.

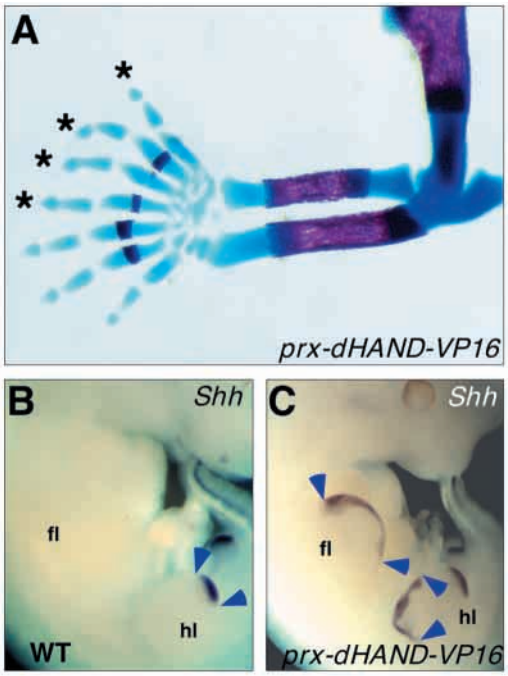

Fig. 4. Preaxial polydactyly and ectopic *Shh* expression induced by dHAND-VP16. F_0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. (A) Limb from an embryo harboring the p*rx-dHAND-VP16* transgene. This construct induced severe preaxial polydactyly. Note the presense of four ectopic digits that have similar morphology. Ectopic digits are indicated with an asterisk. (B,C) Detection of *Shh* transcripts by whole-mount in situ hybridization to wild-type and p*rx-dHAND-VP16* transgenic embryos, respectively, at E11.5. In wild-type embryos, *Shh* transcripts are localized to the ZPA at the posterior of the limb bud. In p*rx-dHAND-VP16* embryos, *Shh* transcripts are seen throughout the peripheral region of the limb bud. Blue arrowheads indicate *Shh* expression domain. fl, forelimb bud; hl, hindlimb bud.

Polydactyly in p*rx-dHAND-VP16* transgenic embryos

In principle, misexpression of dHAND could repattern the limb by acting as a transactivator to induce the expression of limb patterning genes, such as *Shh*, or by repressing a repressor of posterior identity in the anterior compartment of the limb. This was an especially interesting issue in light of our results, which indicated that the N-terminal transcriptional activation domain of dHAND was dispensable for activity of the protein in our misexpression assay. In addition, recent evidence suggests that dHAND may act as both a transcriptional activator and repressor during limb patterning. dHAND has been demonstrated to repress the transcription of *Gli3* and *Alx4* in the posterior limb mesenchyme prior to *Shh* expression (te Welscher et al., 2002). In addition, dHAND has been shown to be required for activation of *Shh*, *Hoxd11*, *Hoxd12* and *Bmp2* expression during limb and fin development (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000). Notably, eHAND has also been shown to act as a repressor or an activator, depending on the assay (Hollenberg et al., 1995; Scott et al., 2000).

To further assess the importance of transcriptional activity of dHAND for its digit-duplicating activity, we fused the viral coactivator VP16 to the C terminus, reasoning that the VP16 activation domain would interfere with this activity if dHAND acted as a transcriptional repressor. This dHAND-VP16 fusion protein was approximately threefold more active than the wild-type dHAND protein in reporter assays (Fig. 1B). Seven out of eight embryos harboring the p*rx-dHAND-VP16* transgene exhibited extreme preaxial polydactyly (Fig. 4A). This suggested that dHAND might act primarily through the activation of downstream target genes during limb development, although other interpretations are also consistent with this result.

Interestingly, in several p*rx-dHAND-VP16* transgenic embryos, the number and morphology of the ectopic digits appeared similar to the doublefoot (*Dbf*) mutant phenotype. This was also occasionally observed with other p*rx-dHAND* transgenes (for examples, see Fig. 3C and Fig. 4A). In contrast to other polydactylous mutants, *Dbf* mice generally display a greater number of ectopic digits that lack distinctive AP identity (Lyon et al., 1996). *Dbf* mutants also exhibit a broad domain of ectopic Indian hedgehog (*Ihh*) expression along the AP margin of the limb (Yang et al., 1998). This differs from other polydactylous mutants, as well as transgenic embryos

misexpressing *Hoxb8* and *Hoxd12*, which display a small ectopic domain of *Shh* at the anterior margin of the limb bud (Charité et al., 1994; Knezevic et al., 1997). It is believed that the broadened expression of *Ihh*, and subsequent activation of the hedgehog signaling pathway, is responsible for the increased digit number and altered morphology (Yang et al., 1998). Although we occasionally noted *Dbf*-like phenotypes in our experiments, this was most frequently observed in p*rx-dHAND-VP16* transgenic embryos.

In order to determine if a broadened ectopic *Shh* expression domain was a potential explanation for the *Dbf*-like phenotype, we examined expression of *Shh* in early stage p*rx-dHAND-VP16* embryos. Whole-mount in situ hybridization of transgenic embryos at E11.5 showed that this construct induced ectopic expression of *Shh* not only at the anterior margin of the limb bud, but also at intervening regions along the AP axis of the limb bud (Fig. 4B,C). Although the expression pattern directed by the p*rx1* regulatory elements becomes more complex at later stages of limb development, expression spans the AP dimension of the limb bud through to E10.5 (Martin and Olson, 2000). Therefore, the broad domain of ectopic *Shh* expression we detected one day later at E11.5 is consistent with the transgene expression at E10.5. The *Shh* expression pattern varied within individual p*rx-dHAND-VP16* transgenic embryos, and between different transgenic embryos (data not shown), suggesting that integration effects as well as transgene expression levels affect both the pattern and level of ectopic *Shh* expression. Nonetheless, the presence of *Shh* transcripts throughout the AP axis of the limbs provides a possible explanation for the *Dbf*-like phenotype occasionally observed in transgenic embryos.

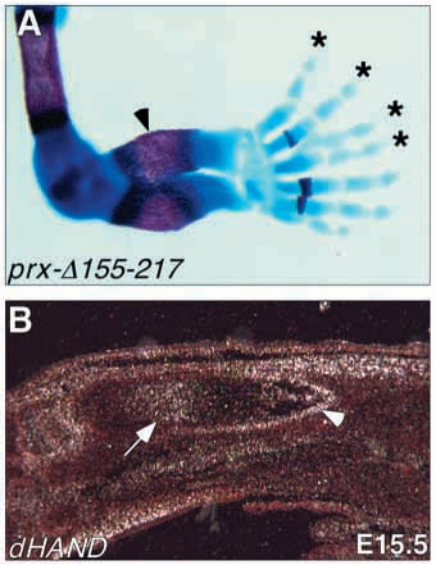

Fig. 5. Limb abnormalities induced by a dHAND mutant protein lacking the C-terminal region. F_0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. (A) A forelimb from an embryo harboring p*rx-Δ155-217*. This construct induced preaxial polydactyly, as well as truncation of the zeugopod (indicated by black arrowhead). (B) Detection of *dHAND* transcripts by in situ hybridization to a longitudinal section through the developing ulna of an E13.5 embryo. *dHAND* expression is detected in the chondrocytes and perichondrium (white arrowhead). Note highest levels of expression in the hyperplastic zone of the chondrocytes (white arrow). Ectopic digits are indicated with an asterisk.

Polydactyly and abnormal bone formation in p*rx-Δ155-217* transgenic embryos

The striking evolutionary conservation at the C terminus of all orthologs of HAND proteins suggested that these amino acids might be important for an activity of dHAND that was undetectable in transcriptional reporter assays. Therefore, we generated transgenic embryos expressing a mutant that lacked this region (mutant Δ155-217). These embryos also exhibited preaxial polydactyly (Fig. 5A). However, in contrast to the phenotype observed with the wild-type dHAND protein, bones of the zeugopod, in both forelimbs and hind limbs, were shortened and malformed. There was a direct correlation between the severity of the polydactyly and zeugopod truncation, suggesting that the variability in severity of these abnormalities reflected the expression level of the transgene.

It is interesting to consider how misexpression of dHAND mutants under the control of the p*rx1* promoter might result in zeugopod abnormalities, given that the p*rx1* promoter is not active in the zeugopod per se. At least two observations may relate to this phenomenon. First, p*rx1* is expressed throughout the early limb bud (Martin and Olson, 2000). Thus, it is possible that misexpression of dHAND mutants at this early stage results in abnormalities observed later in the zeugopod. Second, at later stages of limb development, endogenous p*rx1* is expressed in the periosteum surrounding the bones of the zeugopod (Cserjesi et al., 1992). The p*rx1* promoter fragment also directs expression in the periosteum and tendons at later stages of limb development (Martin and Olson, 2000). In support of these notions, bones of the zeugopod are malformed in p$rx1^{-/-}$ mice, suggesting that p*rx1* expression either throughout the early limb, or later in the periosteum, is required for normal bone morphogenesis, and that misexpression of proteins in these tissues can affect bone growth and morphogenesis (Martin et al., 1995).

The zeugopod phenotype observed with mutant Δ155-217 suggested that dHAND might play a role in bone growth or differentiation during late stages of limb development. We therefore examined the expression pattern of *dHAND* in developing limb bones of wild-type embryos from E12.5-E15.5 by in situ hybridization. As shown in Fig. 5B, *dHAND* was expressed at E15.5 in the chondrocytes and perichondrium. The highest levels of *dHAND* expression were noted in the hyperplastic zone of chondrocytes in the ulna. These findings raise the possibility that the phenotype of the zeugopod in p*rx-Δ155-217* transgenic embryos may reflect a dominant negative action of this truncated protein at some stage of bone development. However, as this mutant retained the ability to induce polydactyly with altered AP patterning, we focused on identifying the region of dHAND responsible for this activity.

DNA-binding independent functions of dHAND

In order to determine if direct DNA binding is required for dHAND activity in our transgenic assay, we generated mutations in the basic region that abolished DNA binding in

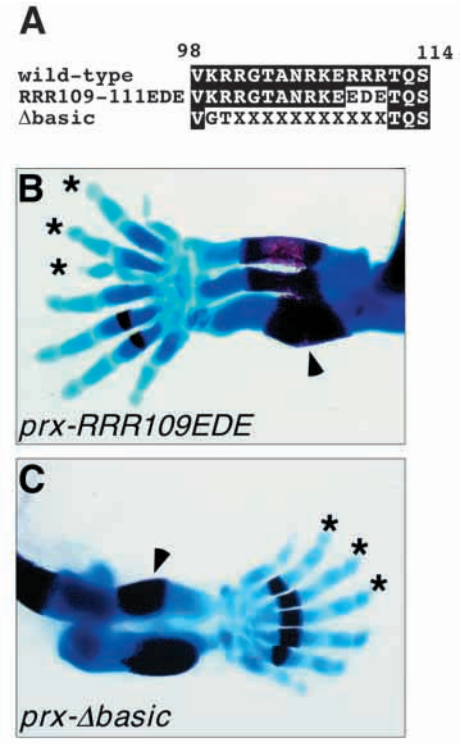

Fig. 6. Limb abnormalities induced by dHAND mutant proteins that fail to bind DNA. (A) Amino acid sequence of the basic regions of wild-type and mutant dHAND proteins. X designates residues that were deleted in the Δbasic mutant. (B,C) F_0 transgenic mouse embryos harboring p*rx-RRR109EDE* (B) and p*rx-Δbasic* (C) transgenes, respectively, were sacrificed at E16.5 and limbs were stained for bone and cartilage. Both constructs induced preaxial polydactyly, as well as truncation of the zeugopod. Both panels show forelimbs. Ectopic digits are indicated with an asterisk. Arrowheads indicate zeugopod abnormalities.

vitro (Fig. 6A). Mutation of three conserved asparagine residues to acidic amino acids (RRR109-111EDE) completely abolished DNA binding activity of dHAND in vitro (Fig. 1A). Surprisingly, transgenic mouse embryos harboring a p*rx-RRR109-111EDE* transgene exhibited preaxial polydactyly, as well as malformed bones in the zeugopod, similar to those seen in p*rx-Δ155-217* transgenics (Fig. 6B, compare with Fig. 4A). To eliminate the possibility that the RRR109-111EDE mutant might bind DNA weakly or recognize undefined DNA binding sites in vivo, we deleted the entire basic region (mutant Δbasic). Embryos harboring the p*rx-Δbasic* transgene also exhibited polydactyly and malformed bones of the zeugopod; this phenotype was indistinguishable from those of p*rx-RRR109-111EDE* and p*rx-Δ155-217* transgenic embryos (Fig. 6C). Together, these results provide evidence that dHAND may not require, at least in some developmental contexts, direct DNA binding in order to modulate downstream target genes.

The role of the HLH region of dHAND

The above results suggested that the N-terminal region, the C-terminal region and the basic region were not required for dHAND to repattern the developing limb in our transgenic assay. To determine if the HLH motif was important for this activity, we deleted this region within dHAND. As predicted, this mutant protein failed to co-immunoprecipitate with E12 or bind DNA (data not shown). Ten out of ten p*rx-ΔHLH* transgenic embryos displayed normal limb morphology (Fig. 7A). This mutant protein was expressed at levels comparable with the wild-type protein, as determined by western blotting and immunostaining of transfected COS cells with anti-Myc antibody (data not shown).

To further examine the role of the HLH region, we mutated a highly conserved phenylalanine to a proline in the first helix (mutant F119P). A similar mutation has been shown to abolish dimerization of other bHLH proteins (Chakraborty et al., 1991; Davis et al., 1990). Co-immunoprecipitation assays confirmed that this mutant was unable to dimerize with E12, but it was expressed at levels comparable with wild-type dHAND in transfected cells (data not shown). Three of three p*rx-F119P* transgenic embryos exhibited completely normal limb morphology (Fig. 7B), confirming that a functional HLH motif is required for dHAND activity.

To test whether the HLH domain alone was sufficient to induce an ectopic ZPA, we expressed the dHAND bHLH under the control of p*rx* regulatory sequences. Fifteen out of 15 p*rx-dHAND-bHLH* transgenic embryos had normally patterned limbs (Fig. 7C). This suggests that although the HLH is required, it is not sufficient alone to induce an ectopic ZPA. This dHAND mutant protein was also expressed at levels equal to wild-type dHAND in transfected COS cells, although we cannot rule out the possibility that it might be expressed at reduced levels in transgenic animals.

In order to determine if early limb patterning activity is uniquely encoded by the dHAND HLH region, we tested two related bHLH proteins in this assay. The dHAND and eHAND proteins differ at only one amino acid in the HLH region, but eHAND is not expressed in the developing limb. As shown in Fig. 7D, a p*rx-eHAND* transgene was able to induce preaxial polydactyly that was indistinguishable from that of p*rx-dHAND* transgenic embryos. Paraxis, which is expressed in the developing somites, but not in the early limb buds , is 62% identical and 78% similar to dHAND within the HLH domain (Burgess et al., 1995). Transgenic embryos harboring a p*rx-paraxis* transgene consistently showed shortened limbs (Fig. 7E). However, only a single biphalangeal ectopic digit was observed among 11 embryos harboring the p*rx-paraxis* transgene (Fig. 7F). This suggests that although paraxis may be capable of recapitulating some aspects of dHAND function it is far less potent than dHAND in this assay. Together, these results suggest that HAND genes possess unique evolutionarily conserved functions that are not shared by other closely related bHLH proteins.

We also generated transgenic mice that expressed the inhibitory HLH protein Id under control of the p*rx1* promoter. Id proteins, which contain an HLH motif, but lack a basic domain, dimerize with E-proteins, thereby inhibiting the activity of class B bHLH proteins by sequestering their obligate dimerization partners (Benezra et al., 1990). Embryos harboring the p*rx-Id* transgene showed normal patterning of the digits, but exhibited severe malformations of the zeugopods (Fig. 7G). These findings raise the possibility that the limb patterning activity of dHAND does not require dimerization with E-proteins, although other interpretations are also possible.

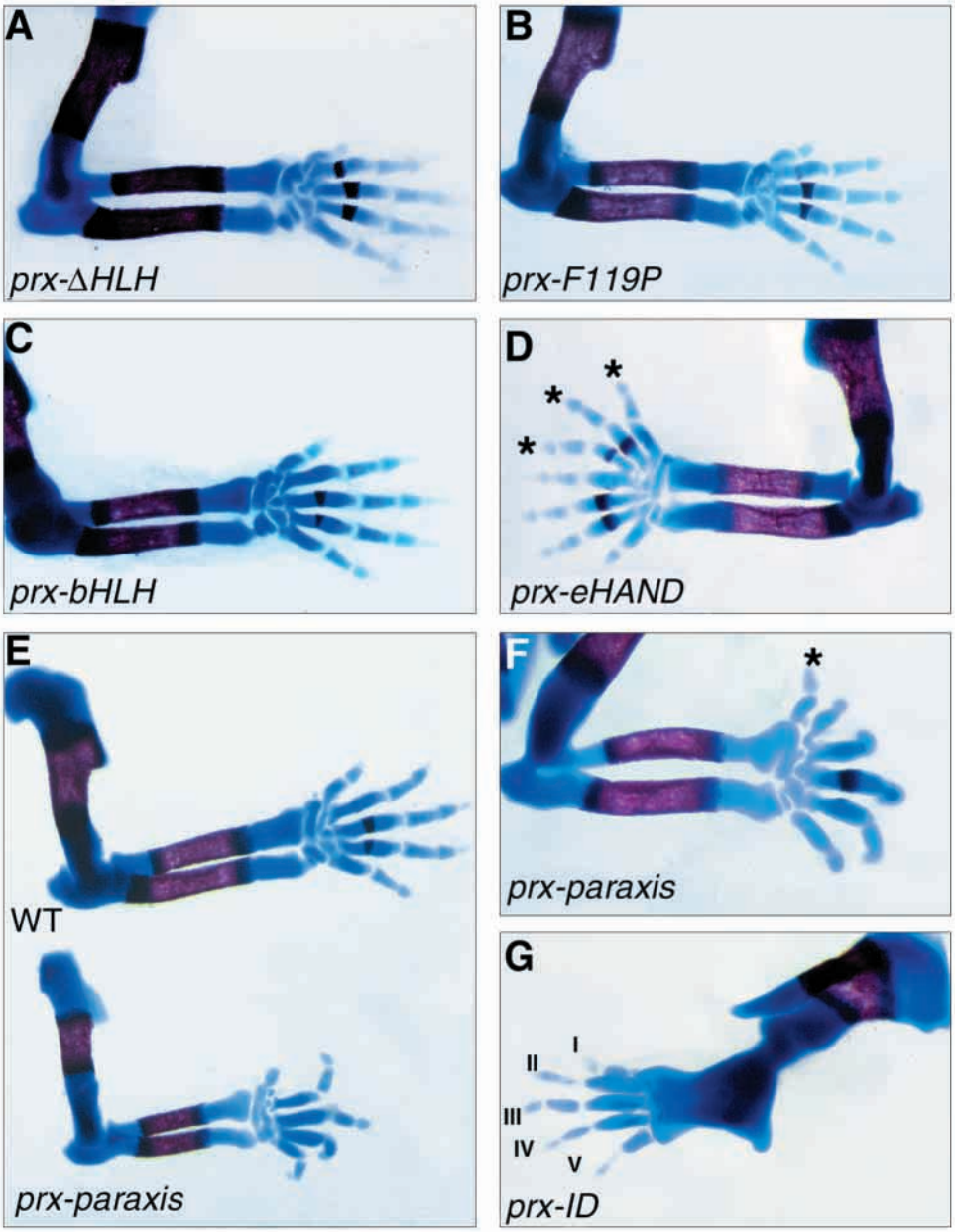

Fig. 7. Analysis of the role of the HLH region on limb patterning. F_0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. Transgenes are indicated in each panel. The p*rx-ΔHLH* (A), p*rx-F119P* (B) and p*rx-bHLH* (C) transgenes had no effect on limb patterning. The p*rx-eHAND* transgene (D) resulted in preaxial polydactyly indistinguishable from that resulting from p*rx-dHAND*. The p*rx-paraxis* transgene resulted in shortened zeugopodial bones (E) and only a single extra digit in one out of 11 transgenic embryos (F). (G) Embryos harboring p*rx-Id* transgenes displayed malformed zeugopodial structures and normally patterned digits that are labeled numerically. All panels show forelimbs. Ectopic digits are indicated with an asterisk.

DISCUSSION

We and others previously demonstrated a critical role for dHAND in patterning the AP axis of the limb and regulating *Shh* expression (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000). The results of this study extend those findings and lead to the unexpected conclusion that this activity of dHAND is mediated by the HLH region and involves a mechanism independent of direct DNA binding. eHAND, which shares high homology with dHAND, is also capable of repatterning the early limb, whereas the more distantly related bHLH protein, paraxis, lacks this activity. Our results suggest that the molecular mechanisms by which dHAND functions during development are more complicated than traditional models of bHLH protein function predict.

DNA-binding independent dHAND functions

Tissue-specific (class B) bHLH proteins are thought to act primarily as heterodimers with widely expressed E-proteins (Massari and Murre, 2000). Consistent with this notion, dHAND cooperates with E12 to bind DNA and activate transcription through the E-box consensus sequence in transfected cells. Mutations that abolish DNA binding or dimerization eliminate the transcriptional activity of dHAND in this assay. Similarly, deletion of the N-terminal transcription activation domain of dHAND severely impairs its ability to activate transcription. These results demonstrate that dHAND can act as a typical class B bHLH transcription factor.

The finding that the basic region, which is required for DNA binding to E boxes, is not required to induce ectopic digits in transgenic mice is unanticipated and suggests that dHAND may act in vivo through mechanisms other than those observed

in tissue culture reporter assays. In addition to mediating DNA binding, the basic regions of other bHLH proteins have been shown to participate in protein-protein interactions required for cell specification (Brennan et al., 1991; Davis et al., 1990). However, a dHAND mutant lacking the entire basic region was as active as the wild-type protein in transgenic mice, indicating that this region does not mediate association with transcriptional co-factors involved in this process.

Interestingly, the class B bHLH transcription factor SCL/Tal1 also exhibits DNA binding-independent activity in vivo. A mutant SCL protein lacking the basic region can rescue primitive hematopoiesis in SCL-null embryoid bodies and in zebrafish lacking *cloche*, the SCL/Tal1 homolog (Porcher et al., 1999). Similarly, a point mutant SCL protein that lacks DNA binding activity can induce leukemia in transgenic mice (O'Neil et al., 2001). This suggests that many class B bHLH proteins, like dHAND and SCL/Tal1, may operate through direct DNA-binding independent mechanisms.

Although misexpression experiments must be interpreted with caution, the present results provide evidence that dHAND can regulate target genes in the early limb bud without binding directly to DNA. While this activity could be considered to represent some sort of nonphysiological effect of ectopic expression, we do not believe this is the case for the following reasons. First, *dHAND* expression precedes the expression of *Shh* in the ZPA and encompasses the *Shh* expression domain at all stages of normal limb development. Second, mouse and zebrafish *dHAND* mutant embryos fail to express *Shh* in the ZPA and exhibit aberrant limb patterning (Charité et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002; Yelon et al., 2000). Third, altered limb patterning was only observed with ectopic dHAND and eHAND, but not with the related bHLH protein paraxis. This level of specificity would not be expected if this were a nonspecific effect of ectopic bHLH protein expression.

Potential roles for transcriptional activation in limb patterning by dHAND

Our results also demonstrate that either the N- or the C-terminal regions of dHAND can be deleted without a loss of digit-inducing activity. As the N-terminal region of the protein contains a transcription activation domain, but the C-terminal region of the protein does not exhibit measurable transcriptional activity when fused to the GAL4 DNA-binding domain, this suggests that the intrinsic transcriptional activity of dHAND is not required for limb patterning. However, a limitation in interpreting these results is that the bHLH region of dHAND alone was devoid of patterning activity. We favor the interpretation that this small region of the protein is unable to establish stable interactions in vivo with essential co-factors, owing to the possible absence of surrounding sequences to stabilize such interactions. However, because deletion of the C-terminal residues (Δ155-217) reduces the transcriptional activity of dHAND in tissue culture assays, we cannot rule out the possibility that a transcription activation domain is required for the limb patterning activity of dHAND and that the C-terminal region of the protein possesses transcriptional activity in vivo that is not detectable in GAL4-reporter assays.

Polydactyly in mice harboring the p*rx-dHAND-VP16* transgene suggests that although intrinsic transcriptional activity of dHAND may not be required to induce ectopic digits, fusion of a potent transcriptional activation domain to dHAND, effectively creating a super-activating form of dHAND, also does not abrogate this activity. This finding is consistent with the notion that dHAND acts as part of a transcriptional activation complex. The requirement of dHAND for the expression of *Shh*, *Hoxd11*, *Hoxd12* and *Bmp2* in mice and fish also is consistent with dHAND acting as a transcriptional activator. However, te Welscher et al. (te Welscher et al., 2002) have recently shown that dHAND is required for the repression of *Gli3* and *Alx4* in the posterior limb mesenchyme prior to *Shh* expression. This suggests that dHAND may have dual functions as both a transcriptional repressor and activator, although it remains possible that this apparent repressive activity of dHAND reflects the initial activation of a target gene that encodes a repressor of *Gli3* and *Alx4*. Because viral expression of dHAND in the anterior compartment of the limb does not repress endogenous *Gli3* or *Alx4* expression, but is sufficient to induce ectopic *Shh* and gremlin, it has been proposed that dHAND repression may require an as yet unknown bHLH partner expressed exclusively in the posterior compartment. However, it remains unclear which, if any, genes dysregulated in *dHAND*$^{-/-}$ limbs represent direct transcriptional targets. Considering that dHAND is required for the activation and repression of multiple genes at the same time and place in development, we favor the notion that at least some of these genes represent indirect transcriptional targets of dHAND.

doublefoot mutant mice ectopically express *Ihh* along the entire AP dimension of the early limb bud, with resulting severe (six to eight digits) polydactyly (Yang et al., 1998). It has been proposed that high levels of hedgehog signaling throughout the limb bud cause increased growth of the distal mesenchmye. This increased growth is reflected in formation of a greater number of ectopic digits. In addition, these ectopic digits display symmetric AP identity, as a result of the disrupted hedgehog signaling gradient. We observed a similar phenotype in a subset of transgenic animals, most frequent noted in p*rx-dHAND-VP16* embryos. Accordingly, we also detected a broad domain of ectopic *Shh* expression in p*rx-dHAND-VP16* embryos. This suggests that dysregulated *Shh* signaling may be responsible for *Dbf*-like phenotypes observed in our transgenic embryos.

It is possible that increasing the transcriptional activation potential of dHAND by fusion to VP16 accounts for the broadened expression of *Shh*. At high levels of transactivation, which may also be dependent on transgene copy number and integration effects, dHAND may override the endogenous mechanisms that restrict ectopic *Shh* expression to the anterior margin. The higher frequency of *Dbf*-like phenotypes that result from expression of *dHAND-VP16*, which displays the highest transcriptional activity in tissue culture, supports this notion. It is interesting to note that the dependence of *Shh* expression on FGF-mediated signals from the AER does not seem to be affected, as ectopic *Shh* is always restricted to the distal mesenchyme underlying the AER (Laufer et al., 1994; Niswander et al., 1994). However, because we did not examine expression of *Shh* in transgenic embryos generated with other dHAND mutant proteins, it is also possible that the broad ectopic expression domain of *Shh* is unique to the p*rx-dHAND-VP16* transgenic line.

Potential roles of the HLH region in limb patterning by dHAND

Mutations affecting the HLH domain of dHAND fail to induce ectopic digits in transgenic embryos. This is not unexpected, and suggests that protein interactions mediated by the HLH are required for activity of dHAND in vivo. However, overexpression of Id in the limb bud, which would be expected to sequester E-protein dimerization partners for dHAND, did not affect digit number or pattern. This finding raises the possibility that the dHAND HLH associates with partners other than E-proteins to regulate limb pattern.

Previous studies with eHAND have provided evidence that HAND proteins may indeed have multiple dimerization partners. Scott et al. (Scott et al., 2000) have demonstrated that eHAND forms homodimers and, interestingly, E-proteins are downregulated in trophoblast tissue at a time when eHAND is believed to function. This provides circumstantial evidence that eHAND acts either as a homodimer or with other unknown partners in this tissue. In addition, the eHAND HLH domain is required for transcriptional repression and activation in tissue culture reporter assays, suggesting that the transcriptional functions of eHAND are modulated by the choice of HLH partners (Hollenberg et al., 1995; Scott et al., 2000). Two-hybrid and biochemical pull-down experiments have also shown that eHAND and dHAND homodimerize as well as heterodimerize with one another and with members of the hairy-related (HRT) family of bHLH transcription factors (Firulli et al., 2000). In light of these studies, the work of te Welscher et al. (te Welscher et al., 2002) and the results presented here, it will be especially interesting to identify the transcriptional partners of dHAND in the developing limb and to determine how these interactions modulate dHAND transcriptional activity.

A possible role for dHAND in bone morphogenesis

Although we focused on digit number and morphology in our transgenic misexpression assay, we also noted severely malformed zeugopodial structures in embryos that expressed dHAND mutants lacking the basic and C-terminal regions (mutants Δbasic, RRR109-111EDE and Δ155-217). Interestingly, these dHAND mutants exhibit reduced or no activity in transfection assays. Thus, it is possible that during zeugopod development, dHAND binds DNA and requires protein-protein interactions mediated by the highly conserved C-terminal residues. Mutations in the DNA-binding domain or the C terminus may therefore generate dominant-negative dHAND molecules that interfere with the activity of the wild-type protein at this stage. The expression of *dHAND* in the perichondrium and hyperplastic chondrocytes of the ulna, coupled with the lack of forearm abnormalities in transgenic embryos overexpressing wild-type dHAND, support this notion and suggest that dHAND plays a role in bone maturation. Because we were interested primarily in early limb patterning by dHAND, a complete analysis of *dHAND* expression at all stages of bone development was not undertaken. However, based on our limited results, we favor the hypothesis that dHAND plays a role in the development of a subset of bones in the posterior zeugopod, rather than acting as a global regulator of bone growth and morphogenesis.

Because dHAND regulates expression of *Shh* during limb patterning, it is interesting to speculate that dHAND may also regulate *Ihh* expression during bone maturation. It will be interesting to address this and other issues in future experiments to determine the role of dHAND during bone development.

Functional redundancy of HAND proteins

The expression patterns of *dHAND* and *eHAND* overlap in several tissues during mouse and chick development and studies with anti-sense oligonucleotides in chick embryos suggest that these two factors play redundant roles during cardiac development (Srivastava et al., 1995). By contrast, the zebrafish genome appears to encode only a single HAND protein, which is most similar to dHAND (Yelon et al., 2000). The zebrafish *hands off* mutation, which disrupts the *dHAND* ortholog, shows a more severe cardiac phenotype than the mouse *dHAND* knockout mutation (Yelon et al., 2000). Because no *eHAND* ortholog has been identified in zebrafish, this suggests that dHAND and eHAND play redundant roles in early heart development in higher vertebrates.

Although our transgenic results do not demonstrate genetic redundancy, they show that dHAND and eHAND proteins can function similarly when misexpressed in the anterior compartment of the developing limb. These findings suggest that dHAND and eHAND may act in a redundant fashion in other tissues during development, including heart, pharyngeal arch neural crest and sympathetic neurons. Tissue-specific deletion of these genes should bypass global embryonic abnormalities and provide further insight into this issue.

Cumulatively, these and other studies suggest novel mechanisms of action of class B bHLH proteins. Although many bHLH factors, including dHAND and eHAND, act predictably according to these models in transfected cells, it appears that the mechanisms of action of these factors in vivo are more complex. Because of the crucial roles played by bHLH transcription factors during embryonic development, determining the molecular mechanisms by which these proteins operate should facilitate the identification of novel transcriptional targets that execute the developmental decisions controlled by bHLH proteins.

Note added in proof

While this manuscript was in review, Dai and Cserjesi (Dai and Cserjesi, 2002) reported similar transcriptional activation potential and DNA-binding specificity of dHAND in tissue culture cells.

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