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The mouse *Ulnaless* mutation deregulates posterior *HoxD* gene expression and alters appendicular patterning

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SUMMARY

The semi-dominant mouse mutation *Ulnaless* alters patterning of the appendicular but not the axial skeleton. *Ulnaless* forelimbs and hindlimbs have severe reductions of the proximal limb and less severe reductions of the distal limb. Genetic and physical mapping has failed to separate the *Ulnaless* locus from the *HoxD* gene cluster (Peichel, C. L., Abbott, C. M. and Vogt, T. F. (1996) *Genetics* 144, 1757-1767). The *Ulnaless* limb phenotypes are not recapitulated by targeted mutations in any single *HoxD* gene, suggesting that *Ulnaless* may be a gain-of-function mutation in a coding sequence or a regulatory mutation. Deregulation of 5′ *HoxD* gene expression is observed in *Ulnaless* limb buds. There is ectopic expression of *Hoxd-13* and *Hoxd-12* in the proximal limb and reduction of *Hoxd-13, Hoxd-12* and *Hoxd-11* expression in the distal limb. Skeletal reductions in the proximal limb may be a consequence of posterior prevalence, whereby proximal misexpression of *Hoxd-13* and *Hoxd-12* results in the transcriptional and/or functional inactivation of *Hox* group 11 genes. The *Ulnaless* digit phenotypes are attributed to a reduction in the distal expression of *Hoxd-13, Hoxd-12, Hoxd-11* and *Hoxa-13*. In addition, *Hoxd-13* expression is reduced in the genital bud, consistent with the observed alterations of the *Ulnaless* penian bone. No alterations of *HoxD* expression or skeletal phenotypes were observed in the *Ulnaless* primary axis. We propose that the *Ulnaless* mutation alters a *cis*-acting element that regulates *HoxD* expression specifically in the appendicular axes of the embryo.

Key words: *Ulnaless*, *HoxD*, limb, posterior prevalence, colinearity

INTRODUCTION

The vertebrate limb serves as an excellent experimental system to elucidate the molecular mechanisms underlying patterning of the embryo. A combination of embryological, molecular and genetic approaches have provided a framework for identifying genes associated with morphogenetic signaling centers that coordinate initial patterning along the three axes of the limb (Tabin, 1991; Cohn and Tickle, 1996). Translation of this initial patterning information into the final limb structure may be modulated through the action of *Hox* genes.

The mouse *Hox* gene family consists of 39 members, which are organized into four clusters, HoxA, HoxB, HoxC and HoxD, located on different chromosomes. All share a highly conserved DNA-binding motif, the homeodomain. Each cluster contains 9-11 genes transcribed in the same orientation (McGinnis and Krumlauf, 1992). The expression of a *Hox* gene in the embryo is colinear with its position in the cluster. In vertebrates, both temporal and spatial colinearity is observed: expression of 3′ genes precedes expression of 5′ genes and 3′ genes have more anterior limits of expression than 5′ genes (Duboule and Dolle, 1989; Graham et al., 1989; Izpisua-Belmonte et al., 1991). The most 5′ genes (groups 9 to 13) of the *HoxA* and *HoxD* clusters are related to the *Drosophila Abd-B* gene and are activated in the forelimb and hindlimb buds consistent with temporal and spatial colinearity, although their subsequent expression is dynamic and complex (Dolle et al., 1989; Haack and Gruss, 1993; Nelson et al., 1996). The molecular basis of colinearity remains an outstanding question. However, there is evidence to suggest that colinearity requires local regulatory interactions, such as enhancer sharing, coupled with higher order regulation (Gerard et al., 1996; van der Hoeven et al., 1996; Gould et al., 1997).

The colinear activation of *HoxA* and *HoxD* genes along the proximal-distal axis of the limb can be correlated with the prospective stylopod, zeugopod and autopod (Davis et al., 1995; Nelson et al., 1996). In the forelimb, the stylopod consists of the humerus, the zeugopod consists of the ulna and radius, and the autopod consists of the carpals and digits. Expression patterns of *HoxA* and *HoxD* genes within these regions are critical for their patterning because gene-targeted mutations in individual *HoxA* and *HoxD* genes lead to specific reductions of limb elements. The more 3′ proximally expressed genes (group 9) alter the stylopod, and the more 5′ distally expressed genes (groups 11, 12 and 13) alter the autopod. Combinations of double and triple loss-of-function mutations within the *HoxD* cluster, and between paralogous genes in the *HoxA* and *HoxD* clusters, have led to the conclusion that the overall dosage of *Hox* genes within a domain is important for patterning. However, some genes appear to have a more dominant role in the patterning of specific limb elements (Dolle et al., 1993; Small and Potter, 1993; Davis et al., 1995; Davis

and Capecchi, 1994, 1996; Favier et al., 1995, 1996; Fromental-Ramain et al., 1996a,b; Herault et al., 1996; Kondo et al., 1996; Zakany and Duboule, 1996).

In addition to mutations designed by gene targeting, the collection of existing mouse and chick limb mutants offers a parallel approach to elucidate the mechanisms of limb patterning. Characterization of these mutants can lead to the identification of novel genes, as well as structural or regulatory information for previously identified genes (Woychik et al., 1990; Schimmang et al., 1992; Storm et al., 1994). With respect to *Hox* genes, structural mutations in mice and human have recently been identified. The human synpolydactyly (SPD) mutation results in reductions, fusions and duplications of digits in hands and feet and is associated with a polyalanine expansion in the N terminus of the *HOXD-13* gene (Muragaki et al., 1996). This phenotype is similar to a targeted deletion of *Hoxd-11*, *Hoxd-12* and *Hoxd-13* in the mouse; therefore, the SPD mutation may result in loss of Hoxd-13 function, and a gain-of-function by suppressing Hoxd-11 and Hoxd-12 activity in the autopod (Zakany and Duboule, 1996). The *Hypodactyly* (*Hd*) mutation in mice and the hand-foot-genital (HFG) syndrome in humans lead to reductions in the autopods and are associated with coding mutations in the *Hoxa-13* gene (Mortlock et al., 1996; Mortlock and Innis, 1997). *Hd*/*Hd* forelimbs and hindlimbs resemble limbs of *Hoxa-13* and *Hoxd-13* double mutant mice; therefore, the *Hd* and HFG mutations may result in a primary loss of Hoxa-13 activity and a secondary loss of Hoxd-13 activity (Fromental-Ramain et al., 1996b; Mortlock et al., 1996).

Our prior genetic and physical mapping has suggested that the mouse mutation, *Ulnaless* (*Ul*), may represent an allele of the *HoxD* cluster (Peichel et al., 1996). *Ulnaless* is a semi-dominant, radiation-induced mutation resulting in reductions and delays in growth of limb elements, similar to targeted mutations in *Hox* genes (Davisson and Cattanach, 1990; Peichel et al., 1996). However, *Ulnaless* differs from loss-of-function mutations in single *Hox* genes because there are severe reductions of both forelimb and hindlimb zeugopods, and no axial skeletal defects. We show that *Ulnaless* does not result from a mutation in a *HoxD-coding* region. Rather, posterior *HoxD* gene expression is altered in *Ulnaless* limbs. In the prospective *Ulnaless* zeugopod, proximal misexpression of the 5′ *Hoxd-12* and *Hoxd-13* genes results in the inactivation of the more 3′ group 11 genes, which are required for the formation of the radius and ulna (Davis et al., 1995). In the prospective *Ulnaless* autopod, the reductions of *Hoxd-13*, *Hoxd-12*, *Hoxd-11* and *Hoxa-13* expression are consistent with digit reductions in *Ulnaless* limbs. Consistent with the absence of axial skeletal defects, expression of *HoxD* genes is unaltered in the primary axis of *Ulnaless* embryos. Taken together, these results suggest that the *Ulnaless* mutation identifies and alters a *cis*-acting regulatory element(s) that controls *HoxD* gene expression in the appendicular, but not the primary axis of the embryo.

MATERIALS AND METHODS

Mice, genotyping and skeletal analysis

Ulnaless embryos were generated by one of four mating schemes: (*Ul*/+ × FVB/N) × FVB/N or C57BL/6J × (*Ul*/+ × MOLF/Ei) to generate +/+ and *Ul*/+ embryos; and (*Ul*/+ × MOLF/Ei) F_1 or (*Ul*/+ × FVB/N) F_1 intercrosses to generate +/+, *Ul*/+ and *Ul*/*Ul* embryos. For skeletal analysis, *Ulnaless* homozygote mice were produced from (*Ul*/+ × MOLF/Ei) F_1 intercrosses. For skeletal analysis of *Hoxd-11* *trans*-heterozygotes, an (*Ul*/+ × MOLF/Ei) F_1 female was mated to a *Hoxd-11*$^{+/-}$ male (Davis and Capecchi, 1994) and a resulting (*Ul*/+; *Hoxd-11*$^{+/-}$) female and (+/+; *Hoxd-11*$^{+/-}$) male were intercrossed. For skeletal analysis of *Hoxd-12* *trans*-heterozygotes, an (*Ul*/+ × MOLF/Ei) F_1 female was mated to a *Hoxd-12*$^{-/-}$ male (Davis and Capecchi, 1996), and a resulting (*Ul*/+; *Hoxd-12*$^{+/-}$) female and (+/+; *Hoxd-12*$^{+/-}$) male were intercrossed. All embryos and mice were genotyped at the *Ulnaless* locus (Peichel et al., 1996) and for the presence of the *Hoxd-11* and *Hoxd-12* targeted alleles (Davis and Capecchi, 1996) using established PCR assays on DNA from the yolk sacs or tails.

Embryonic, neonatal and adult skeletal stains were prepared as described (Selby, 1987; Jegalian and De Robertis, 1992; Peichel et al., 1996). Left and right forelimbs and hindlimbs of each animal were either measured on photographs taken at fixed magnification, or using an eyepiece micrometer on a Nikon dissecting microscope. Reductions in bone lengths were analyzed using the one-tailed *t*-test: two sample assuming unequal variances on Microsoft Excel 5.0. A *P*-value of <0.025 was considered significant.

Sequencing

PCR products were amplified from *Ul*/*Ul* and 101/H genomic DNA. All primer sequences and PCR conditions used are available on our laboratory homepage (www.molbio.princeton.edu/vogt/vogt.html). The products were cloned into the pCR2.1 vector and sequenced using the vector-specific primers TAF and TAR (Peichel et al., 1996).

Whole-mount in situ hybridization

Whole-mount in situ hybridization using digoxigenin-labeled probes was performed as described by Riddle et al. (1993). The probes were *Hoxd-1* (Frohman and Martin, 1992), *Hoxd-3* (Bedford et al., 1995), *Hoxd-9* and *Hoxd-10* (Duboule and Dolle, 1989), *Hoxd-11* and *Hoxd-12* (Izpisua-Belmonte et al., 1991), *Hoxd-13* (Dolle et al., 1991), *Hoxa-11* and *Hoxa-11*-as (Hsieh-Li et al., 1995), *Hoxa-13* (L. Post and J. Innis), *Hoxc-11* (Peterson et al., 1994) and *Shh* (Chang et al., 1994). The *Hoxd-4* probe was constructed by amplifying a 510 bp fragment in the homeodomain and 3′ UTR (Featherstone et al., 1988). The fragment was cloned into the pCR2.1 vector. To make a 348 bp antisense probe, the plasmid was linearized with *Bgl*II and transcription was performed with T7 polymerase.

RESULTS

Molecular analysis of the *Ulnaless Hoxd* cluster

The refined genetic mapping of the *HoxD* cluster to the *Ulnaless* locus and the similarities of the *Ulnaless* limb phenotype to gene-targeted mutations in *Hox* genes, prompted a molecular search for an alteration in *HoxD* cluster gene structure or expression (Fig. 1A,B; Peichel et al., 1996). A survey across the *HoxD* cluster of +/+, *Ul*/+ and *Ul*/*Ul* DNA by genomic Southerns showed no observable change in the structure of the locus (Peichel et al., 1996; data not shown). PCR amplification of the entire coding regions of *Hoxd-1*, *Hoxd-3* and *Hoxd-4* from genomic DNA of *Ul*/*Ul* and the 101/H parental chromosome revealed no alterations. The 5′ *HoxD* genes were examined for a structural change by DNA sequencing. Each exon and adjoining splice junctions of *Hoxd-8*, *Hoxd-9*, *Hoxd-10*, *Hoxd-11*, *Hoxd-12*, *Hoxd-13* and *Evx-2* were sequenced in both parental 101/H and *Ul*/*Ul* genomic DNA and no significant alterations were found (Fig. 1A).

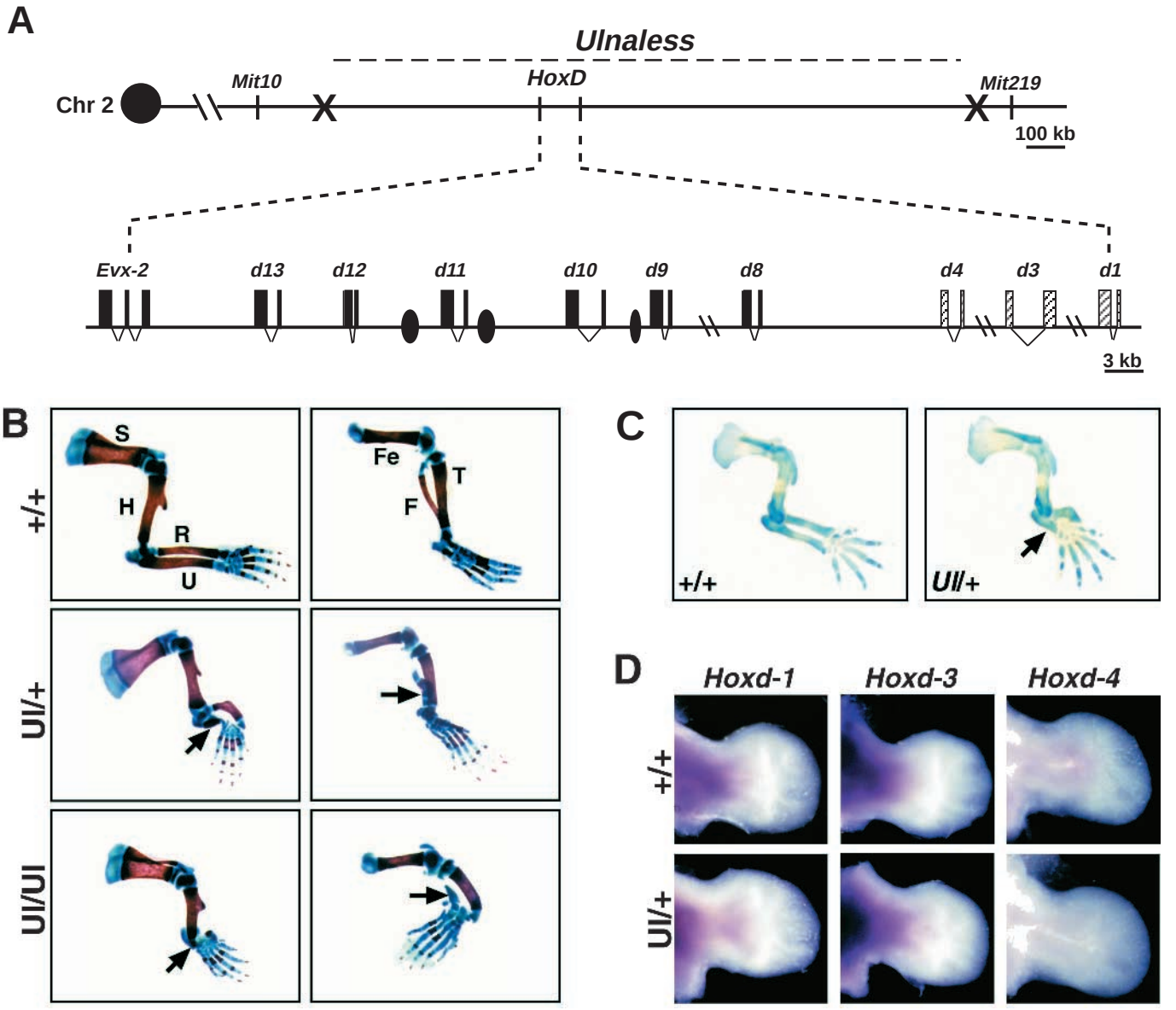

Fig. 1. Molecular and phenotypic analysis of the *Ulnaless* locus. (A) A schematic of the *Ulnaless* locus is shown at the top. The *Ulnaless* locus has been defined to a 0.4 cM genetic interval on mouse chromosome 2, flanked by proximal marker *Mit10* and distal marker *Mit219*. This genetic interval has been cloned and defines a 1.7 Mb physical region that includes the *HoxD* cluster (Featherstone et al., 1988; Peichel et al., 1996). A map of the *HoxD* gene cluster is shown below. The filled boxes depict the exons and adjoining splice junctions of the cluster that have been completely sequenced in *Ul*/*Ul* and 101/H DNA. No significant alterations were found between *Ul*/*Ul*, 101/H and the published sequence except in *Hoxd-8* and *Hoxd-10*. In our sequencing of the first exon of *Hoxd-8* from *Ul*/*Ul*, as well as wild-type 101/H and 129Sv/J genomic DNA, we find amino acids 118 to 122 are four prolines, followed by a cysteine. Izpisua-Belmonte et al. (1990) report these five residues as methionine, serine, tyrosine, glycine and glutamine. This region of the protein is not conserved between mouse *Hoxb-8*, *Hoxc-8* and *Hoxd-8* or between mouse and chick *Hoxd-8*. Our sequencing of the second exon of *Hoxd-8* was in agreement with previously published changes of Sadoul and Featherstone (1991) to the original sequence (Izpisua-Belmonte et al., 1990). In *Hoxd-10*, we find that amino acid 268, which is the second residue of the homeodomain, is a lysine rather than a glutamic acid (Renucci et al., 1992). A lysine in this position is conserved in all other homeodomains of *Abd-B* like *Hox* genes, including human, chick and zebrafish *Hoxd-10* (Burglin, 1994). The hatched boxes depict exons that have been shown to be unaltered in *Ul*/*Ul* DNA by PCR analysis. The filled ovals represent previously defined regulatory elements in the *HoxD* cluster (Zappavigna et al., 1991, 1994; Renucci et al., 1992; Gerard et al., 1993, 1996; Beckers et al., 1996) that have been sequenced in *Ul*/*Ul* DNA. (B) Alizarin red (bone) and alcian blue (cartilage) staining of neonatal forelimbs (left column) and hindlimbs (right column). The long bones of the forelimb are the scapula (S), humerus (H), ulna (U) and radius (R), and the long bones of the hindlimb are the femur (Fe), fibula (F) and tibia (T). The humerus of *Ul*/*Ul* forelimbs is reduced distally. There are reductions of the ulna and radius in *Ul*/+ and *Ul*/*Ul* forelimbs (arrows), and reductions of the fibula and tibia in *Ul*/+ and *Ul*/*Ul* hindlimbs (arrows). At this stage, there is not an ossification center present in the ulna of *Ul*/*Ul* forelimbs and the fibula of *Ul*/*Ul* hindlimbs (arrows). (C) Cartilage stains of embryonic day 14 +/+ and *Ul*/+ forelimbs. A reduction in the ulna and radius is apparent at this stage of development (arrow). (D) Whole-mount RNA in situ hybridization was performed on day 11 +/+ and *Ul*/+ embryos. Forelimb buds were then removed and photographed. Expression of *Hoxd-1* , *Hoxd-3* and *Hoxd-4* is detected in a similar pattern in the proximal limbs of both +/+ and *Ul*/+ embryos.

The absence of a structural change in *HoxD*-coding sequences suggested that the *Ulnaless* mutation may be regulatory. To date, three local *cis*-acting regulatory elements for 5′ *HoxD* genes have been defined (Zappavigna et al., 1991, 1994; Renucci et al., 1992; Gerard et al., 1993, 1996; Beckers et al., 1996). These elements were sequenced in 101/H and *Ul*/*Ul* DNA, with no differences (Fig. 1A). Despite these results, evidence of altered *HoxD* gene expression would support the notion that *Ulnaless* affects an undefined regulatory element(s) required for the temporal and spatial coordination of *HoxD* gene expression.

Because alterations of the *Ulnaless* limb chondrogenic structures are evident at embryonic day 14, we focused our expression analysis of *HoxD* genes on earlier embryological stages (Fig. 1C). Examination of the 3′ *HoxD* genes at embryonic day 11 in *Ul*/+ limb buds revealed that relative to wild type, expression of *Hoxd-1*, *Hoxd-3* and *Hoxd-4* was not altered (Fig. 1D).

Expression of 5′ *HoxD* genes is altered in *Ulnaless* limb buds

In the developing limb bud, the 5′ *HoxD* genes (*Hoxd-9* through *Hoxd-13*) are activated in a colinear fashion that reflects their relative position in the cluster (Dolle et al., 1989; Nelson et al., 1996). In wild-type forelimb and hindlimb buds, *Hoxd-13* expression is restricted to the distal region of the prospective autopod of the limb. Examination of *Ul*/+ and *Ul*/*Ul* embryos revealed that *Hoxd-13* expression is altered in two ways. Proximally, in the prospective zeugopod, there is ectopic *Hoxd-13* expression in mutant limb buds, such that the domain of *Hoxd-13* expression is diffusely expanded to proximal sites (Fig. 2A). This proximal limb bud expression is indicative of a *Hoxd-13* gain-of-function in the region that will give rise to the radius and ulna. Distally, in the prospective autopod, there is a reduction in the levels of *Hoxd-13* expression in mutant limb buds (Fig. 2A). This reduced distal limb bud expression is indicative of a *Hoxd-13* reduction-of-function in the region that will give rise to the digits.

Having detected an alteration in the most 5′ *HoxD* gene, we next extended our examination to include the other 5′ *HoxD* genes. In wild-type limb buds, *Hoxd-12* is strongly expressed in a distal domain, corresponding to the prospective autopod, with weaker expression in a proximal and posterior domain. Examination of *Hoxd-12* expression in *Ulnaless* embryos revealed it to be altered in a fashion similar to *Hoxd-13*. The *Hoxd-12* domain is expanded proximally and anteriorly in *Ul*/+ and *Ul*/*Ul* limb buds (Fig. 2B). Distally, there is a reduction in *Hoxd-12* expression in *Ul*/*Ul* limb buds (Fig. 2B). These alter-

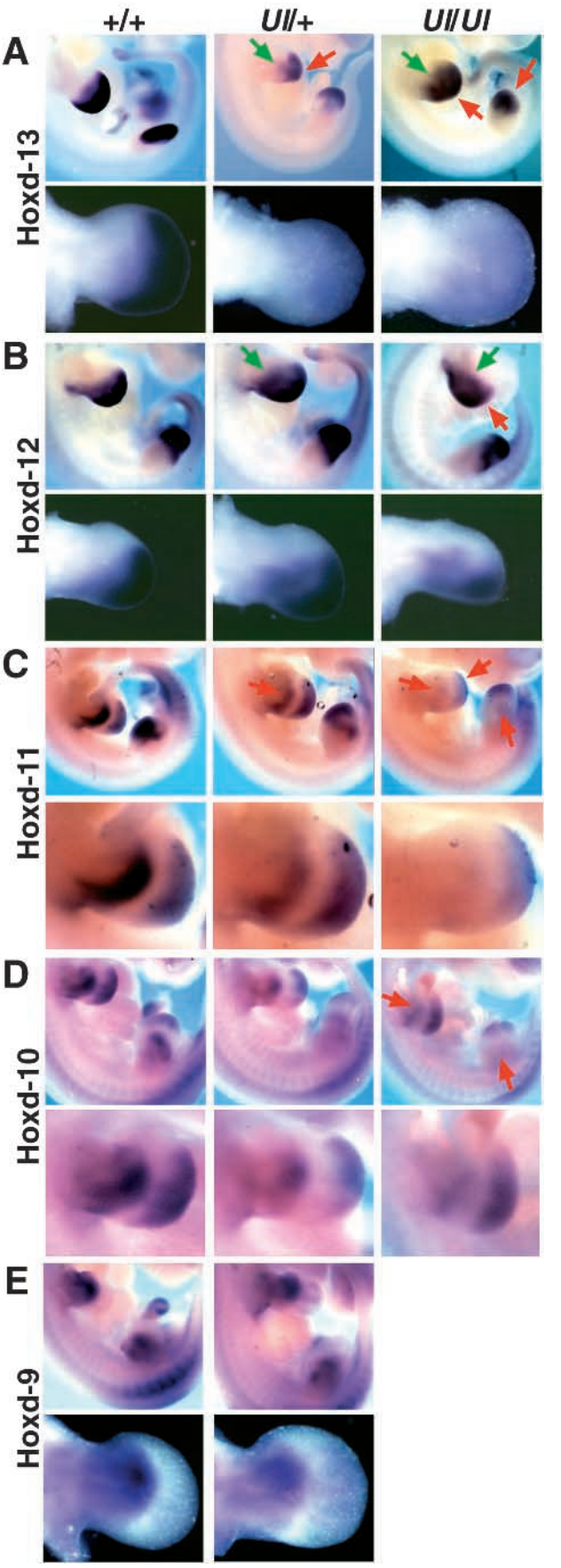

Fig. 2. RNA expression of 5′ *HoxD* genes is altered in *Ulnaless* limb buds. (A-E) Whole-mount RNA in situ hybridization was performed on day 11 embryos. Red arrows point to reductions in gene expression and green arrows point to ectopic gene expression. (A) *Hoxd-13* expression in +/+, *Ul*/+ and *Ul*/*Ul* embryos is shown above representative forelimbs. (B) *Hoxd-12* expression in +/+, *Ul*/+ and *Ul*/*Ul* embryos is shown above representative hindlimbs. (C) *Hoxd-11* expression in +/+, *Ul*/+ and *Ul*/*Ul* embryos is shown above a close-up of the forelimb of the corresponding embryo. (D) *Hoxd-10* expression in +/+, *Ul*/+ and *Ul*/*Ul* embryos is shown above a close-up of the forelimb of the corresponding embryo. (E) *Hoxd-9* expression in +/+ and *Ul*/+ embryos is shown above representative forelimbs.

ations in *Hoxd-13* and *Hoxd-12* expression are more readily discerned at later stages of limb development (Herault et al., 1997).

Gene targeting experiments suggested a primary role for group 11 genes in the patterning of the zeugopod (Davis et al., 1995). The *HoxD* group 11 paralogue, *Hoxd-11* is expressed in two domains, with a stronger proximal domain in the prospective zeugopod and a relatively weaker distal domain in the prospective autopod. We examined expression of *Hoxd-11* in +/+, *Ul*/+ and *Ul*/*Ul* embryos, and observed a reduction of *Hoxd-11* in the proximal domain of *Ul*/+ and *Ul*/*Ul* forelimbs and *Ul*/*Ul* hindlimbs. The distal domain of *Hoxd-11* is also reduced in *Ul*/*Ul* forelimbs (Fig. 2C). Therefore, the *Ulnaless* mutation appears to result in *Hoxd-11* reduction-of-function in both the prospective zeugopod and autopod.

In wild-type forelimbs and hindlimbs, *Hoxd-10* is expressed in a pattern similar to *Hoxd-11*, except the proximal and the distal domain are of equal intensity. In *Ul*/*Ul* forelimbs and hindlimbs, the proximal expression of *Hoxd-10* is reduced relative to the unaltered distal expression (Fig. 2D). In contrast to the more 5′ *HoxD* genes, *Hoxd-9* expression is indistinguishable in +/+ and *Ul*/+ proximal limb buds, in the region of the prospective stylopod (Fig. 2E).

Expression of *HoxA* genes is altered in *Ulnaless* limbs

The alterations in the expression of multiple *HoxD* genes in the limb buds of *Ulnaless* mutants may be due to a *cis*-acting mutation that affects each *HoxD* gene independently, or alternatively, a primary effect on one *HoxD* gene (e.g. *Hoxd-13*), with secondary consequences for the other *Hox* genes. These alternatives are difficult to distinguish by examining expression within the *HoxD* cluster. Therefore, we examined the potential *trans*-acting consequences of the *Ulnaless* mutation on the unlinked paralogous group 11 genes, *Hoxa-11* and *Hoxc-11*, which are also expressed in the prospective zeugopod. *Hoxa-11* is expressed in a proximal stripe in both forelimbs and hindlimbs. Expression in this domain is highest at its anterior and posterior aspects, and weaker in the middle of the limb. In *Ul*/+ and *Ul*/*Ul* forelimbs, *Hoxa-11* anterior expression is reduced and posterior expression is barely detectable (Fig. 3A, data not shown). Therefore, the *Ulnaless* mutation results in altered expression of an unlinked *HoxA* gene. Antisense transcription of the *Hoxa-11* gene in the distal limb has been reported (Hsieh-Li et al., 1995). There is no change in the pattern or levels of expression of antisense transcripts in *Ul*/+ limbs (Fig. 3B). The effects on *Hoxa-11* and *Hoxd-11* transcription in *Ulnaless* are consistent with the reported defects of radius and ulna in the *Hoxa-11*; *Hoxd-11* double mutant (Davis et al., 1995). However, the effects of *Ulnaless* on the hindlimb zeugopod are more severe than in the *Hoxa-11*; *Hoxd-11* double mutant, suggesting that *Ulnaless* may also interfere with *Hoxc-11* function in the hindlimb. In wild-type embryos, *Hoxc-11* is expressed in the proximal hindlimb, but not the forelimb. Unlike the effects of *Ulnaless* on *Hoxd-11* and *Hoxa-11* RNA distribution, *Hoxc-11* RNA expression appears unaltered in *Ul*/+ hindlimbs relative to wild type, suggesting that any effects of *Ulnaless* on *Hoxc-11* are most likely at the functional level (Fig. 3C).

Because we observe a reduction in the expression of *Hoxd-*

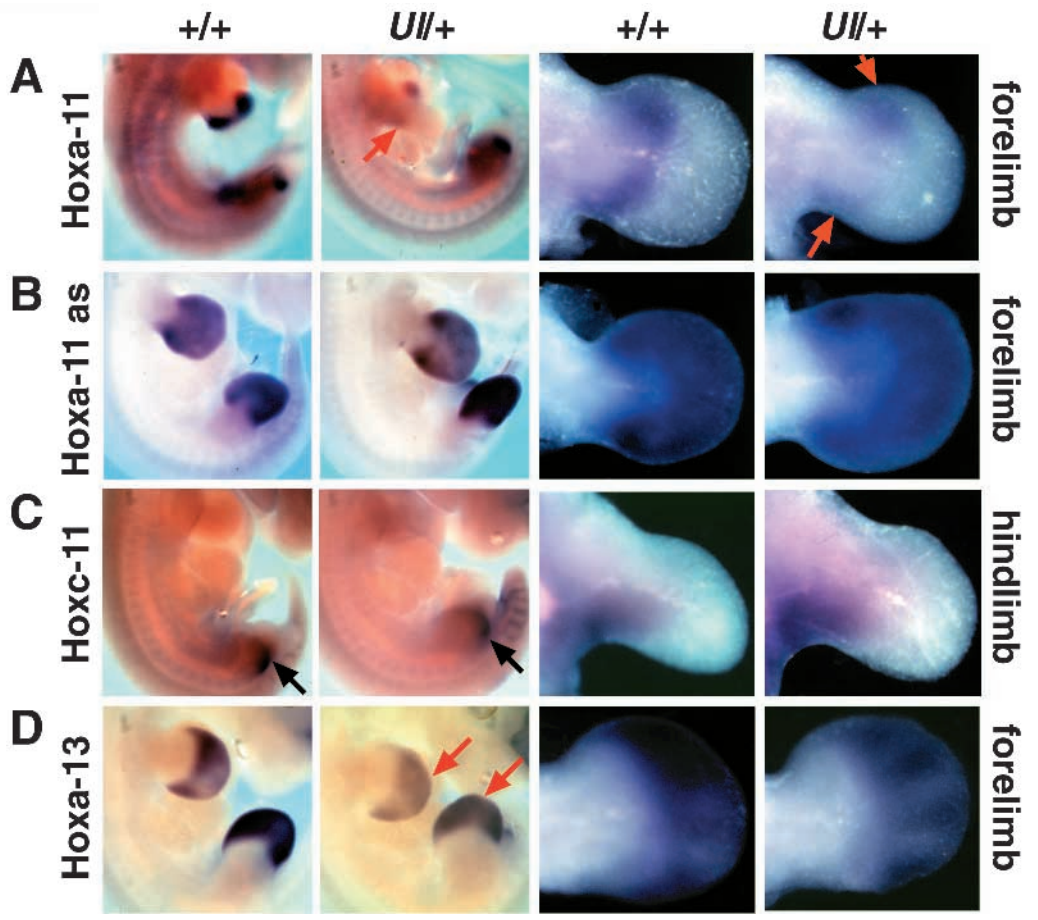

Fig. 3. RNA expression of *HoxA* genes is altered in *Ulnaless* limb buds. (A-D) Whole-mount RNA in situ hybridization was performed on day 11 +/+ and *Ul*/+ embryos. Red arrows point to reductions in gene expression. (A) Expression of *Hoxa-11* in +/+ and *Ul*/+ embryos is shown next to representative forelimbs. (B) Expression of antisense (as) transcripts of the *Hoxa-11* gene in +/+ and *Ul*/+ embryos is shown next to representative forelimbs. (C) Expression of *Hoxc-11* in +/+ and *Ul*/+ embryos is shown next to representative hindlimbs. Black arrow point to hindlimb expression in +/+ and *Ul*/+ embryos. (D) Expression of *Hoxa-13* in +/+ and *Ul*/+ embryos is shown next to representative forelimbs.

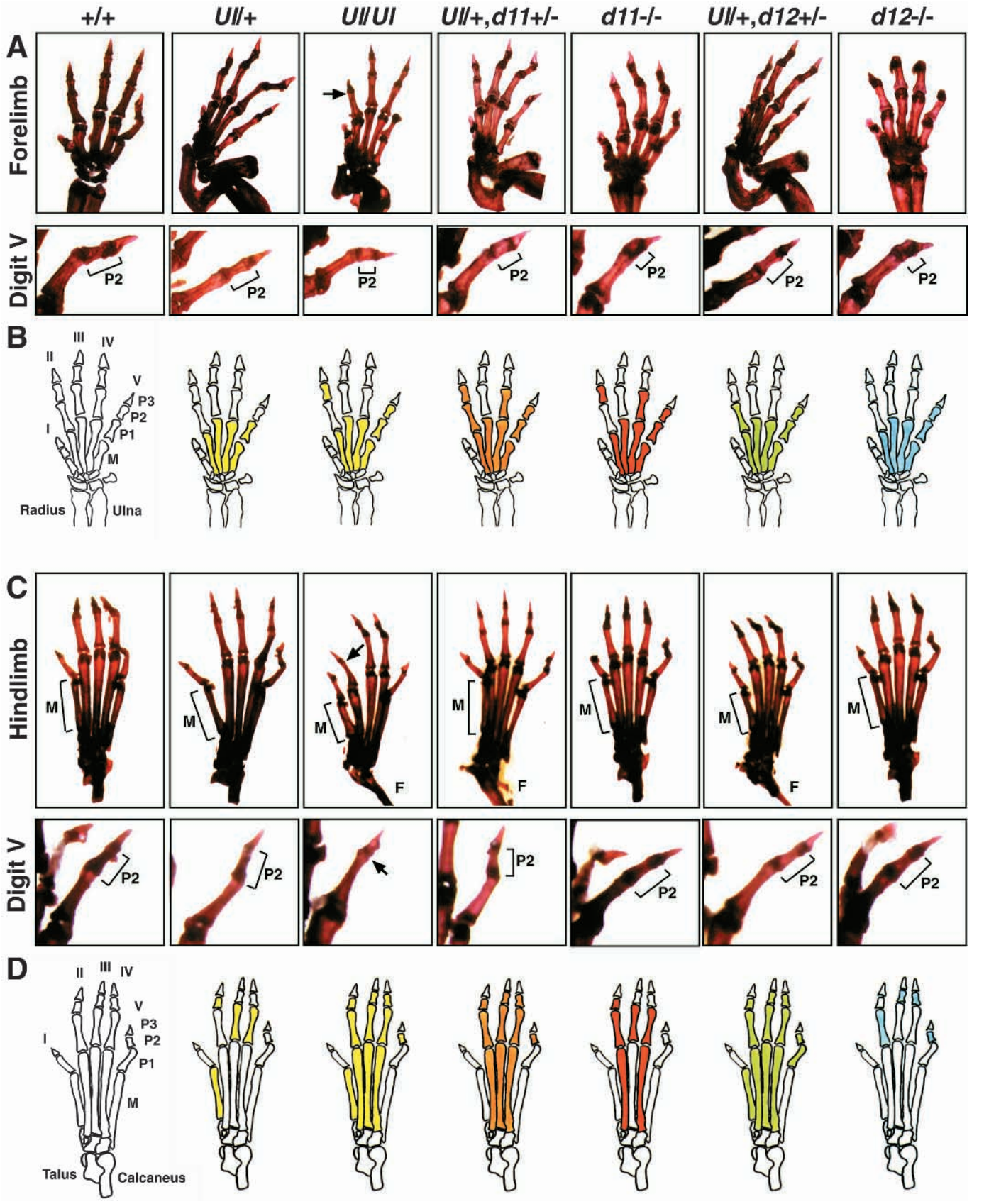
+/+
Ull+
Ull Ul
Ull+,d11+/-
d11-/-
Ull+,d12+/-
d12-/-
A
Forelimb
Digit V
P2
B
III
IV
II
V
P3
P2
I
P1
M
Radius
Ulna
C
Hindlimb
M
F
D
II
III
IV
V
P3
P2
I
P1
M
Talus
Calcaneus

Table 1. Digit lengths as a percentage of control

Genotype	Control	Bone	Forelimb digit II	III	IV	V	Hindlimb digit I	II	III	IV	V
Ul/+	+/+	Phalange 1	90	100	97	**89**	100	92	**93**	**93**	97
(*n*=20)	(*n*=6)	Phalange 2	100	100	109	97		**86**	101	94	97
		Metacarpal	**79**	**87**	**85**	**88**	**91**	99	98	100	100
Ul/*Ul*	+/+	Phalange 1	90	95	93	**83**	92	**79**	**87**	**88**	94
(*n*=10)	(*n*=6)	Phalange 2	**10**	94	91	**47**		**0**	**90**	**91**	**15**
		Metacarpal	**72**	**83**	**85**	**88**	**61**	**66**	**83**	**92**	94
Ul/+; *d-11*$^{+/-}$	+/+; *d-11*$^{+/-}$	Phalange 1	**87**	88	**93**	**93**	93	**88**	**86**	**89**	91
(*n*=10)	(*n*=6)	Phalange 2	**85**	92	102	**64**		**78**	**83**	**86**	**89**
		Metacarpal	**86**	**91**	**86**	**91**	96	**89**	**90**	**94**	96
+/+; *d-11*$^{-/-}$	+/+; *d-11*$^{+/-}$	Phalange 1	95	97	99	**91**	96	**95**	**96**	**96**	94
(*n*=12)	(*n*=6)	Phalange 2	**82**	102	104	**59**		**90**	104	98	98
		Metacarpal	**68**	**81**	**82**	**91**	98	**94**	97	**96**	94
Ul/+; *d-12*$^{+/-}$	+/+; *d-12*$^{+/-}$	Phalange 1	**92**	95	95	**89**	98	**91**	**94**	**93**	**92**
(*n*=14)	(*n*=12)	Phalange 2	92	94	89	**73**		**84**	**86**	**92**	**88**
		Metacarpal	**91**	**90**	**87**	**86**	**87**	**88**	**92**	**93**	97
+/+; *d-12*$^{-/-}$	+/+; *d-12*$^{+/-}$	Phalange 1	99	97	102	**93**	98	**93**	98	97	95
(*n*=12)	(*n*=12)	Phalange 2	98	106	89	**61**		94	**89**	**94**	**89**
		Metacarpal	**79**	**92**	**89**	**91**	99	97	96	98	97

Numbers in bold indicate that the reduction is significant (P<0.025) as determined by a one-tailed t-test.
The number of limbs analyzed for each genotype is indicated. Both left and right limbs of individual animals were measured.
Phalange 3 of all limbs, phalange 2 of hindlimb digit I, and digit I of the forelimb are not included as they were uninformative.
Metacarpal refers to the metacarpals of the forelimb and metatarsals of the hindlimb.

13, *Hoxd-12* and *Hoxd-11* in the prospective autopod of *Ulnaless* limb buds, we also examined expression of *Hoxa-13*, which is the only gene of the *HoxA*, *HoxB* and *HoxC* clusters expressed in the prospective autopod mesoderm (Haack and Gruss, 1993; Peterson et al., 1994; Nelson et al., 1996). In wild-type limb buds, *Hoxa-13* is expressed along the entire anterior-posterior length of the distal limb. In *Ul*/+ forelimbs and hindlimbs, the proximal extent of the *Hoxa-13* expression domain is unaltered relative to wild type; however, the levels of expression within the distal domain appear reduced in the prospective forelimb and hindlimb autopod (Fig. 3D).

We also examined the expression of *Sonic hedgehog* (*Shh*) in *Ulnaless* embryos, as perturbation of *Shh* expression would suggest that the primary defect in *Ulnaless* acts upstream of the *HoxD* genes (Riddle et al., 1993; Laufer et al., 1994; Chan et al., 1995; Parr and McMahon, 1995; Nelson et al., 1996). At all stages examined, no alterations in *Shh* expression in *Ul*/+ fore or hindlimbs were observed relative to wild type, consistent with the *Ulnaless* mutation altering limb patterning at the level of *HoxD* genes (data not shown).

Fig. 4. Genetic interactions between *Ulnaless* and *HoxD* genes alters digits. (A, C) Alizarin red stains of forelimb (A) and hindlimb (C) autopods are shown with a close-up of digit V below. (B, D) Schematic representations of the forelimb autopod (B) adapted from Dolle et al. (1993), and hindlimb autopod (D) adapted from Forsthoefel (1962), are labeled with digit number and bone identity: metacarpal/metatarsal (M), phalange 1 (P1), phalange 2 (P2) and phalange 3 (P3). Bones which show reductions are color-coded for each genotype: *Ul*/+ and *Ul*/*Ul* (yellow), *Ul*/+, *Hoxd-11*$^{+/-}$ (orange), *Hoxd-11*$^{-/-}$ (red), *Ul*/+, *Hoxd-12*$^{+/-}$ (green) and *Hoxd-12*$^{-/-}$ (blue). (A) There is a complete loss of P2 in digit II of *Ul*/*Ul* forelimbs (arrow). Close-ups of digit V show that P2 is reduced in *Ul*/*Ul*, (*Ul*/+, *Hoxd-11*$^{+/-}$), *Hoxd-11*$^{-/-}$, (*Ul*/+, *Hoxd-12*$^{+/-}$) and *Hoxd-12*$^{-/-}$ forelimbs. (C) There is a complete loss of P2 in digit II of *Ul*/*Ul* hindlimbs (arrow). The metacarpal of digit I is reduced in *Ul*/+, *Ul*/*Ul* and (*Ul*/+, *Hoxd-12*$^{+/-}$) hindlimbs. The fibula (F) of *Ul*/+ (not shown), *Ul*/*Ul*, (*Ul*/+, *Hoxd-11*$^{+/-}$) and (*Ul*/+, *Hoxd-12*$^{+/-}$) attaches directly to the calcaneus rather than the tibia, as in wild-type mice. Close-ups of digit V show that P2 is completely absent in *Ul*/*Ul* (arrow) and is slightly reduced in (*Ul*/+, *Hoxd-11*$^{+/-}$), (*Ul*/+, *Hoxd-12*$^{+/-}$) and *Hoxd-12*$^{-/-}$ hindlimbs.

Phenotypic consequences of altered *Hox* expression in *Ulnaless* limbs

Functional effects of the deregulation of *Hox* genes in *Ulnaless* limb buds on skeletal patterning were investigated in two ways: by comparing the skeletal defects in *Ulnaless* to those of known *Hox* mutations and by placing *Ulnaless* in *trans* to gene-targeted alleles of *Hoxd-11* and *Hoxd-12* (Davis and Capecchi, 1994, 1996).

In the zeugopod, the *Ulnaless* mutation appears to result in a gain-of-function. Misexpression of *Hoxd-13* in mouse by gene targeting and in chicken by retroviral infection also results in reductions of the zeugopod, although not as severe as in *Ulnaless* (van der Hoeven et al., 1996; Goff and Tabin, 1997). In addition, mice lacking both *Hoxa-11* and *Hoxd-11* have drastic reductions of the ulna and radius, and more subtle reductions of the fibula and tibia (Davis et al., 1995). Targeted mutations of *Hoxd-11* or *Hoxd-12* alone do not result in reductions of the zeugopod (Davis and Capecchi, 1994, 1996; Kondo et al., 1996). In (*Ul*/+, *Hoxd-11*$^{+/-}$) and (*Ul*/+, *Hoxd-12*$^{+/-}$) *trans*-heterozygotes, the *Ul*/+ zeugopod phenotype, not the more severe *Ul*/*Ul* phenotype, is observed (Fig. 1B; data not shown). Therefore, the *Ulnaless* zeugopod phenotype cannot simply be the result of a loss-of-function mutation in either

Hoxd-11 or *Hoxd-12*, suggesting that *Ulnaless* results in a gain-of-function in the zeugopod.

In contrast to the putative gain-of-function effects on the zeugopod, we found evidence for allelism of *Ulnaless* and *HoxD* loss-of-function mutations in the autopod. We performed alizarin red skeletal stains on adult limbs and measured individual bones of the digits of both forelimbs and hindlimbs (Table 1). Both *Ul*/+ and *Ul*/*Ul* limbs had reductions of specific bones of the digits, consistent with loss-of-function *Hox* phenotypes created by gene targeting (Fig. 4; Table 1; Dolle et al., 1993; Davis and Capecchi, 1994, 1996; Fromental-Ramain et al., 1996b; Kondo et al., 1996). There is a severe reduction or absence of phalange 2 (P2) of digits II and V in both the forelimbs and hindlimbs of *Ul*/*Ul* mice (Fig. 4A). These bones are the last to form in the limb (Shubin and Alberch, 1986) and they are reduced or absent in all *Hox* targeted mutations. In addition, there is a reduction in the overall size of the *Ul*/*Ul* hindlimb autopod (Fig. 4C; Table 1). In particular, there is a reduction of the metatarsal of digit I in both *Ul*/+ and *Ul*/*Ul* hindlimbs (Fig. 4C; Table 1). This is a bone that is specifically affected in *Hoxd-13*$^{-/-}$ hindlimbs (Dolle et al., 1993; Davis and Capecchi, 1996). Observation of *Hox*-like phenotypes in *Ulnaless* autopods suggest that the reduction of distal *Hox* RNA expression has phenotypic consequences. Both *Hoxd-11*, *Hoxd-12* and *Hoxd-13* triple mutant mice and *Hoxa-13*; *Hoxd-13* double mutant mice, created by gene targeting, have extreme reductions of forelimb and hindlimb autopods, consistent with *Ulnaless* resulting in partial loss-of-function of these genes (Fromental-Ramain et al., 1996b; Zakany and Duboule, 1996).

Analysis of the autopod phenotypes of (*Ul*/+, *Hoxd-11*$^{+/-}$) and (*Ul*/+, *Hoxd-12*$^{+/-}$) *trans*-heterozygotes is consistent with the *Ulnaless* mutation leading to a reduction of function in *Hoxd-11* and *Hoxd-12* in the autopod. There are two key features to note in both the forelimbs and hindlimbs of these mice. First, the reductions in *trans*-heterozygotes encompass all of the phenotypes observed in *Hoxd-11*$^{-/-}$ or *Hoxd-12*$^{-/-}$ limbs (Fig. 4B,D; Table 1). This suggests that the *Ulnaless* mutation results in a reduction of both *Hoxd-11* and *Hoxd-12* function in the autopod. Second, there are additional reductions in *trans*-heterozygotes that are not present in either *Ul*/+, *Hoxd-11*$^{-/-}$, or *Hoxd-12*$^{-/-}$ limbs alone (Fig. 4B,D; Table 1). Therefore, the *Ulnaless* mutation is likely to result in a partial loss-of-function of multiple *Hox* genes in the autopod, consistent with the observed reduction of *Hoxd-13* and *Hoxa-13* in the distal limbs of *Ulnaless* embryos.

The *Ulnaless* mutation alters appendicular but not axial expression of *HoxD* genes

Expression of *HoxD* genes in both limbs and genitalia appear to be coordinated (Beckers et al., 1996; van der Hoeven et al., 1996). Although both *Ul*/+ and *Ul*/*Ul* females are fertile, there is a reduction in *Ul*/+ male fertility and *Ul*/*Ul* males have not successfully bred (Peichel et al., 1996). The expression of *Hoxd-11* and *Hoxd-12* in genital buds is unaltered relative to wild type in *Ul*/+ and *Ul*/*Ul*; however, *Hoxd-13* expression is greatly reduced in *Ul*/+ and undetectable in *Ul*/*Ul* (Fig. 5). Consistent with this reduction in *Hoxd-13* expression in embryos, penian bones from adult *Ul*/+ males are reduced in width and clefted at the proximal end (Fig. 5D). The *Ul*/+ phenotype is reminiscent of penian bone defects in *Hoxd-13*$^{-/-}$ males (Dolle et al., 1993).

In contrast to the limbs and genitalia, we observed no alteration of *Hox* gene expression along the main body axis of the *Ul*/+ and *Ul*/*Ul* embryos (Figs 2, 3, 5, data not shown). This is consistent with the absence of any changes in the number or morphology of vertebrae along the axial skeleton of *Ul*/+ or *Ul*/*Ul* mice (data not shown). In addition, (*Ul*/+, *Hoxd-11*$^{+/-}$) and (*Ul*/+, *Hoxd-12*$^{+/-}$) *trans*-heterozygotes did not uncover any abnormalities of the axial skeleton (data not shown). However, in this genetic background in *Hoxd-11*$^{-/-}$ mice, we

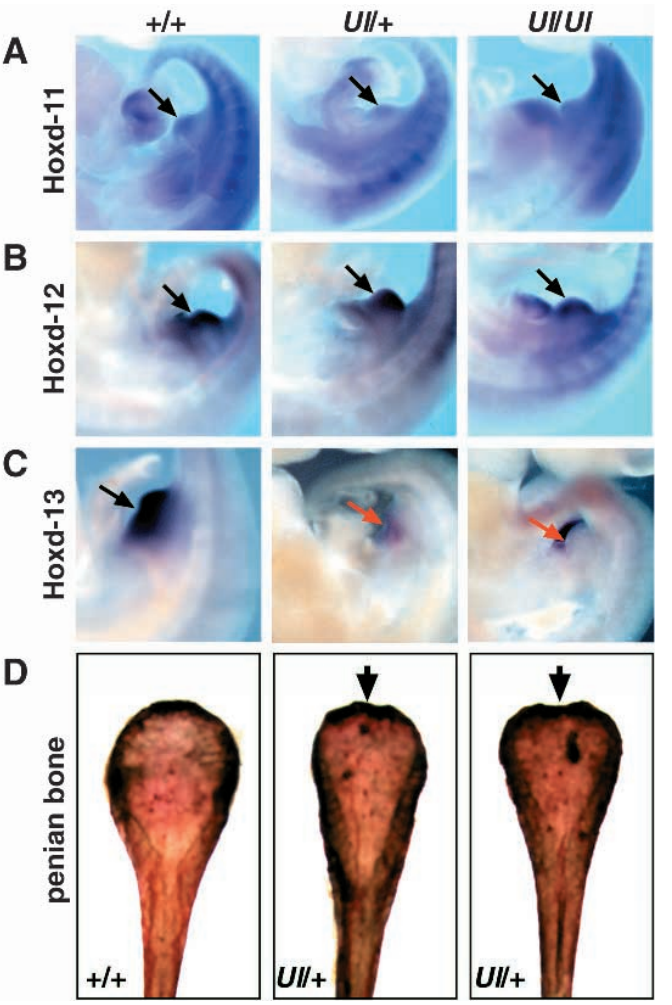

Fig. 5. *Hoxd-13* RNA expression is reduced in the genital bud of *Ulnaless* embryos and penian bone defects are observed in adult males. (A-C) Whole-mount RNA in situ hybridization was performed on day 11 embryos. (A) *Hoxd-11* is expressed indistinguishably in the genital bud of +/+, *Ul*/+ and *Ul*/*Ul* embryos (black arrows). (B) *Hoxd-12* is expressed indistinguishably in the genital bud of +/+, *Ul*/+ and *Ul*/*Ul* embryos (black arrows). (C) *Hoxd-13* is strongly expressed in the genital bud of +/+ embryos (black arrow), but is significantly reduced in *Ul*/+ embryos and undetectable in *Ul*/*Ul* embryos (red arrows). (D) Penian bones dissected from a +/+ male and two *Ul*/+ littermates at 5 months of age were stained with alizarin red. No change in the length of the bones was observed between +/+ and *Ul*/+ (data not shown); however, both *Ul*/+ penian bones are narrower, flatter and clefted at the proximal end (arrows).

find the previously reported homeotic transformations in the lumbar-sacral region (Davis and Capecchi, 1994).

DISCUSSION

The *Ulnaless* zeugopod phenotype is a result of posterior prevalence

The *Ulnaless* mutation provides insight into the complex interactions of *Hox* genes within the limb. We propose that a functional consequence of the ectopic misexpression of *Hoxd-13* and *Hoxd-12* in the presumptive zeugopod is the reduction of *Hoxd-10*, *Hoxd-11* and *Hoxa-11* transcripts (Fig. 6). A functional hierarchy of *Hox* genes has previously been observed both in *Drosophila* (phenotypic suppression) and vertebrates (posterior prevalence). Both transcriptional and functional suppression of anterior *Hox* genes by posterior *Hox* genes occurs in *Drosophila* (Bachiller et al., 1994; Duboule and Morata, 1994; Gould et al., 1997). However, there is evidence that transcriptional repression is a secondary effect of functional inactivation due to autoregulation (Bachiller et al., 1994; Duboule and Morata, 1994). Although we observe a reduction in *Hoxd-10*, *Hoxd-11* and *Hoxa-11* transcripts, we are unable to determine if it is due to a direct or indirect mechanism. In the accompanying paper, reductions in *Hoxd-11* and *Hoxd-10* transcripts are not observed in *Ul*/+ and (*Ul*/+, $HoxD^{Del/+}$) *trans*-heterozygote limb buds and *Hoxa-11* RNA levels were not examined (Herault et al., 1997). Differences in genetic constitution (*Ul*/+ versus *Ul*/*Ul* genotypes), background modifiers, or staging of embryos may be responsible for these results.

The reductions of the *Ulnaless* forelimb zeugopod resemble those obtained in *Hoxa-11*; *Hoxd-11* mutant mice (Davis et al., 1995). Reductions of the radius are less severe in *Ulnaless* forelimbs than in the *Hoxa-11*; *Hoxd-11* mutant forelimbs, which may be accounted for by the remaining *Hoxa-11* expression in the anterior of *Ulnaless* limb buds (Fig. 3A). In contrast to the forelimbs, reductions of the *Ulnaless* hindlimb zeugopod are more severe than in the *Hoxa-11*; *Hoxd-11* double mutants (Davis et al., 1995), an observation that we attribute to functional suppression of *Hoxc-11* by *Hoxd-13* and *Hoxd-12*.

We propose that the misexpression of *Hoxd-13* and *Hoxd-12* in *Ulnaless* results in suppression of group 11 genes, either through transcriptional repression or functional inactivation. These results are consistent with previous studies of *Hoxd-13* misexpression in the limb. Phenotypic reductions of the forelimb zeugopod were observed by misexpression of *Hoxd-13* in the *Hoxd-11* pattern through gene transposition in mice (van der Hoeven et al., 1996). In addition, retroviral expression of *Hoxa-13* or *Hoxd-13* throughout chick limb buds results in reductions of the zeugopod (Yokouchi et al., 1995; Goff and Tabin, 1997). In these studies, the phenotypic effects are hypothesized to result from functional inactivation of group 11 genes. However, the *Ulnaless* zeugopod reductions are more severe than those obtained by *Hoxd-13* misexpression through gene transposition or retroviruses (van der Hoeven et al., 1996; Goff and Tabin, 1997). This suggests that the timing, levels and patterns of misexpression in *Ulnaless* may be critical to cause the postulated transcriptional and functional inactivation, and for the severity of the zeugopod phenotype.

In *Ulnaless*, *Hox* targeted mutations and retroviral gain-of-function studies, altered *Hox* expression results in reduced and delayed growth of specific limb elements along the proximal-distal axis. In addition, X-irradiation of stage 20 chick limb buds depletes the overall cell population and the resulting limbs have reductions of the zeugopod, similar to *Ulnaless* (Wolpert et al., 1979). Therefore, a consequence of the *Ulnaless* mutation and other *Hox* mutations may be to reduce the number of cells available to condense into the cartilage precursors of the zeugopod and autopod and/or to alter the subsequent growth of these cells (Dolle et al., 1993; Davis and Capecchi, 1996; Zakany and Duboule, 1996; Goff and Tabin, 1997). Retroviral misexpression of *Hoxa-13* in chick limb buds appears to alter initial precartilage condensation by changing the adhesive properties of cells, resulting in reductions of the normal precartilage condensations and ectopic cartilage condensations (Yokouchi et al., 1995). In contrast, we do not observe similar ectopic cartilage condensations in *Ulnaless* limbs at a comparative stage (Fig. 1C).

The *Ulnaless* mutation deregulates posterior *HoxD* expression in the limb

We demonstrate that *Ulnaless* alters the regulation of *HoxD* genes in three domains: (1) in the proximal limbs, there is misexpression of *Hoxd-13* and *Hoxd-12*, (2) in the distal limbs, there is reduction of *Hoxd-13*, *Hoxd-12* and *Hoxd-11* expression and (3) in the genital bud, there is reduction of *Hoxd-13* expression. These observations lead us to propose that the *Ulnaless* mutation results in the loss-of-function of a *cis*-

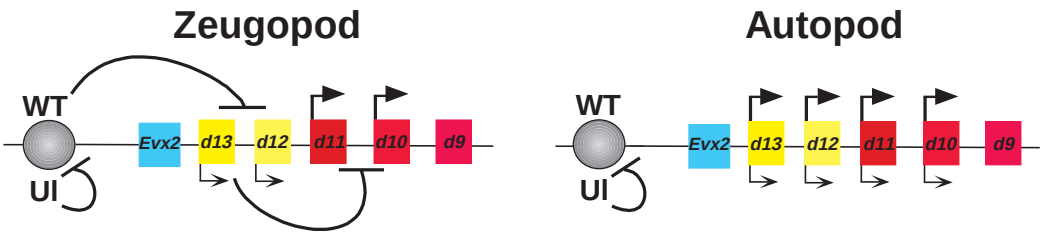

Fig. 6. A model for the deregulation of *HoxD* expression in *Ulnaless* limbs. We propose that the *Ulnaless* mutation alters the function of a distal *cis*-acting limb regulatory element (depicted as a grey circle), that is required for the proper control of *HoxD* expression. In the wild-type (WT) zeugopod, the limb regulatory element represses *Hoxd-13* and *Hoxd-12* expression, at the time and place of *Hoxd-11* and *Hoxd-10* expression. In the *Ulnaless* (Ul) zeugopod, the element is non-functional, and *Hoxd-13* and *Hoxd-12* are now expressed proximally. The ectopic expression of *Hoxd-13* and *Hoxd-12* in the zeugopod results in the transcriptional repression of more 3′ genes, *Hoxd-11* and *Hoxd-10*, and the unlinked *Hoxa-11* gene (not depicted). In the wild-type autopod, the same regulatory element is required for full activation of *Hoxd-13*, *Hoxd-12*, *Hoxd-11* and *Hoxd-10*. In the *Ulnaless* autopod, the element is non-functional, and expression of these genes is reduced. Arrows indicate relative levels of expression of an individual gene.

acting element(s) that regulates expression of the 5′ *HoxD* genes in the limbs and genitalia, but not the primary embryonic axis (Fig. 6). There are several lines of evidence to support this model. First, genes from both ends of the *HoxD* cluster do not recombine with the *Ulnaless* locus in 1564 N2 animals (Peichel et al., 1996), indicating that the *Ulnaless* mutation is tightly linked to the *HoxD* cluster. Second, in the *Ulnaless* background, there are no mutations in the coding sequence of *Hoxd-8* through *Evx-2*. In addition, there are no rearrangements of the *HoxD* gene cluster in *Ul*/*Ul* genomic DNA (data not shown; Peichel et al., 1996). Third, altered expression of 5′ *HoxD* genes (*Hoxd-10* through *Hoxd-13*), but not 3′ genes (*Hoxd-1* through *Hoxd-9*), is observed exclusively in the limbs and genitalia. Fourth, *Hoxd-13* and *Hoxd-12* are no longer expressed according to their position in the *HoxD* cluster. Although the initial activation of 5′ *HoxD* genes was not examined, we favor the model that the *Ulnaless* mutation leads to the deregulation of *HoxD* gene colinearity. Recent studies in the chick have shown that the later expression of *HoxD* genes in the limb is complex (Nelson et al., 1996; Vargesson et al., 1997). Therefore, it is possible that the misexpression of posterior *HoxD* genes in *Ulnaless* results from the alteration of a regulatory element required for a later phase of *HoxD* expression in the limb (Nelson et al., 1996), or from a failure of proximal cells to stop expressing *Hoxd-13* (Vargesson et al., 1997).

The nature and location of the elements required for regulation of the *HoxD* cluster in the axial and appendicular axes are undefined. Therefore, several possibilities exist to explain the deregulation of posterior *HoxD* expression caused by the *Ulnaless* mutation. There may be a distal control region that regulates all the 5′ genes in the *HoxD* cluster though long-range interactions, as in the case of the globin LCR (Hanscombe et al., 1991; Wijgerde et al., 1995; van der Hoeven et al., 1996). Alternatively, there may be intergenic chromatin domain boundaries that function to restrict the domains of expression of *Hox* genes, as in the *Drosophila* bithorax complex (Hagstrom et al., 1996; Zhou et al., 1996). We favor the first possibility for several reasons. First, the necessary elements for regulation of limb expression appear to lie outside of the *HoxD* cluster, downstream of *Evx-2*, because deletion of the region encompassing *Hoxd-11*, *Hoxd-12* and *Hoxd-13* results in proper expression of *Hoxd-10* and *Evx-2* in the limbs (Zakany and Duboule, 1996). Second, the region between *Hoxd-13* and *Evx-2* is not sufficient to direct limb expression of a *Hoxd-9* or *Hoxd-11* transgene (van der Hoeven et al., 1996). Third, our extensive Southern analysis of the genomic structure of the *Ulnaless* locus and sequencing of the intergenic regions (C. L. P. and T. F. V., unpublished data), has failed to yield any alterations of the *HoxD* intergenic regions.

Alternatively, it is possible that the *Ulnaless* mutation does not alter a *cis*-acting element in the *HoxD* cluster, but alters a yet unidentified tightly linked *trans*-acting gene that regulates expression of the *HoxD* cluster. We consider this less likely, in part because the alterations of *HoxD* expression in *Ul*/*Ul* limbs are stronger than in *Ul*/+ limbs, requiring a dosage effect of the putative *trans*-acting factor.

Recently, insight into the mechanism of *HoxD* regulation has been gained through transgenic mouse experiments. Random integration of *Hoxd-9* or *Hoxd-11* and their respective flanking sequences recapitulate many aspects of axial expression, but not appendicular expression. However, moving either *Hoxd-9* or *Hoxd-11* and flanking sequences to a new position within the *HoxD* cluster (between *Hoxd-13* and *Evx-2*) results in limb expression of these genes (Renucci et al., 1992; Gerard et al., 1993; Beckers et al., 1996; van der Hoeven et al., 1996). In their new positions, both *Hoxd-9* and *Hoxd-11* are initially repressed in the proximal limb, but later are expressed in their normal pattern. In addition, they are also expressed in the distal limbs and genitalia, unlike endogenous *Hoxd-9* or randomly integrated *Hoxd-11* transgenes (Gerard et al., 1993; Beckers et al., 1996). Therefore, expression of *HoxD* genes in limbs and genitalia is initially dependent upon position within the cluster and is controlled by a global regulatory mechanism. Local *cis*-acting elements are initially subservient to this higher order regulation and can be shared between genes (Gerard et al., 1996; van der Hoeven et al., 1996).

Morphological differences between organisms may in part correspond to changes in *Hox* gene expression (Carroll, 1995). In the *Ulnaless* mutation, deregulation of *HoxD* gene expression also leads to morphological changes and may therefore be acting as an agent of evolutionary change. A feature of the *Ulnaless* mutation is that it alters expression of *Hox* genes in the limbs, but not in the primary embryonic axis. This suggests that the mechanisms regulating *HoxD* expression differ in the primary and secondary embryonic axes, for example through the action of different members of the *Polycomb*- and *trithorax*-group genes (Cohn et al., 1997; Schumacher and Magnuson, 1997). Uncoupling of the regulatory mechanisms of *Hox* gene expression in these two regions of the embryo may have allowed for evolutionary diversity in the limbs. Therefore, *Ulnaless* provides a valuable opportunity to define the molecular mechanisms contributing to the regulation of *HoxD* gene expression, the uncoupling of the regulatory mechanisms for axial and appendicular patterning, and the hierarchy of *Hox* gene function.

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