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Monodactylous Limbs and Abnormal Genitalia Are Associated with Hemizygosity for the Human 2q31 Region That Includes the *HOXD* Cluster

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Summary

Vertebrates have four clusters of *Hox* genes (*HoxA, HoxB, HoxC,* and *HoxD*). A variety of expression and mutation studies indicate that posterior members of the *HoxA* and *HoxD* clusters play an important role in vertebrate limb development. In humans, mutations in *HOXD13* have been associated with type II syndactyly or synpolydactyly, and, in *HOXA13*, with hand-foot-genital syndrome. We have investigated two unrelated children with a previously unreported pattern of severe developmental defects on the anterior-posterior (a-p) limb axis and in the genitalia, consisting of a single bone in the zeugopod, either monodactyly or oligodactyly in the autopod of all four limbs, and penoscrotal hypoplasia. Both children are heterozygous for a deletion that eliminates at least eight (*HOXD3–HOXD13*) of the nine genes in the *HOXD* cluster. We propose that the patients' phenotypes are due in part to haploinsufficiency for *HOXD*-cluster genes. This hypothesis is supported by the expression patterns of these genes in early vertebrate embryos. However, the involvement of additional genes in the region could explain the discordance, in severity, between these human phenotypes and the milder, nonpolarized phenotypes present in mice hemizygous for *HoxD* cluster genes. These cases represent the first reported examples of deficiencies for an entire *Hox* cluster in vertebrates and suggest that the diploid dose of human *HOXD* genes is crucial for normal growth and patterning of the limbs along the anterior-posterior axis.

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Introduction

Homeodomain-containing homeotic genes (*Hox*) have been remarkably well conserved during animal evolution. Humans and all other vertebrates have four clusters of *Hox* genes (*HoxA*, *HoxB*, *HoxC*, and *HoxD*), which are homologues of the clustered homeotic genes in *Drosophila melanogaster* (McGinnis and Krumlauf 1992). *Hox* genes are expressed in discrete zones on the rostrocaudal axis of embryos and are required for diversification of the morphology within those zones. This is known from the homeotic transformations of axial structures that occur when *Hox* expression patterns have been altered. Sixteen genes located toward the 5′ end of the vertebrate *Hox* clusters have a high degree of homology with the *Abdominal-B* (*Abd-B*) gene in *Drosophila* (Zeltser et al. 1996). Many of these *Abd-B*-type genes have secondary functions during limb development in vertebrates.

The expression patterns and mutant phenotypes of these *Abd-B*-type genes in vertebrates indicate that members of the *HoxA* and *HoxD* clusters are involved in the growth and patterning of the limb in a signaling cascade involving Sonic hedgehog, fibroblast growth factors, and other factors (Duboule 1994; Tickle 1995). Developmental studies of mouse mutants suggest that *Hox* genes initially control the condensation of skeletal precursors in the limb bud and subsequently control the growth and elongation of these elements (Shubin 1986; Zákány 1996). In addition, the finding of anomalies along several axes of the limb bud in mutants for one or more *Hox* genes suggests a multiaxial patterning–function model, with complex interactions among members of the same cluster and paralogous members of different clusters. Their effect in controlling the size and number of digits seems to occur in a dose-dependent fashion (Zákány et al. 1998).

Mutations in only two human *Hox* genes have been

observed: an expansion of alanine codons in *HOXD13* is associated with type II syndactyly or synpolydactyly (Mugaraki 1995; MIM 186000), and a nucleotide substitution in *HOXA13*, which changes a Trp to a Stop codon, is associated with hand-foot-genital syndrome (Mortlock 1997; MIM 140000).

We have investigated two children with severe developmental limb defects along the anterior-posterior axis of the limbs. Both are heterozygotes for interstitial deletions in the long arm of chromosome 2. We demonstrate that these deletions involve the *HOXD* cluster, and we propose that the patients' phenotypes may be caused partially by haploinsufficiency for the *HOXD* cluster. These are the first deficiencies of an entire *Hox* cluster that have been reported in any vertebrate.

Subjects and Methods

Patient 1

The first patient was a 4-year-old male born to healthy nonconsanguineous parents after a normal pregnancy. Both parents and three healthy siblings were carefully examined, and no abnormalities were found. Abnormal features in the patient included severe growth retardation and developmental delay; microcephaly; abnormal genitalia, consisting of rugous scrotal skin with no palpable testes and a small phallus; and severe symmetric limb anomalies (fig. 1*A*). In both the upper and the lower limbs, the proximal segment, or stylopod, consists of a single thin bone (humerus and femur), the intermediate segment, or zeugopod, consists of a single poorly defined bone (a radius-like structure in the upper limbs and a small incurved unidentifiable bone in the lower limbs). The distal segment, or autopod, contains an unossified carpal space, a single metacarpal, and a biphalangeal digit in the upper limbs (fig. 1*C–E*) and, similarly, two tarsal bones (calcaneous-like and cuboid-like structures), a single metatarsus, and a biphalangeal digit in the lower limbs. Radiographs of the spine and a brain computed-tomography scan in patient 1 showed brain atrophy but no structural abnormalities.

Patient 2

The second patient was a newborn male born to healthy nonconsanguineous parents. Their first child was a male who died during the neonatal period, with multiple congenital anomalies (by report, including monodactyly). Abnormal features in the patient included severe growth retardation; multiple craniofacial anomalies, including hypertelorism, left micropthalmia with corneal clouding, cleft palate, and low-set ears; penoscrotal transposition with micropenis; and severe limb defects. The upper limbs had bilateral monodactyly. A single zeugopodal bone with radial morphology was seen in the left upper limb. In the right upper limb, the radius and ulna both appeared short. The lower limbs had two digits and several tarsal bones (fig. 1*B*). X-rays of the spine showed 12 ribs and normal vertebral structures; however, sacral detail was limited. Autopsy studies showed severe renal hypoplasia with multiple cysts, an atrial septal heart defect, and CNS structural defects including arrhinencephaly, ectopic periventricular gray matter, cavum septum pellucidum, and left optic-nerve hypoplasia.

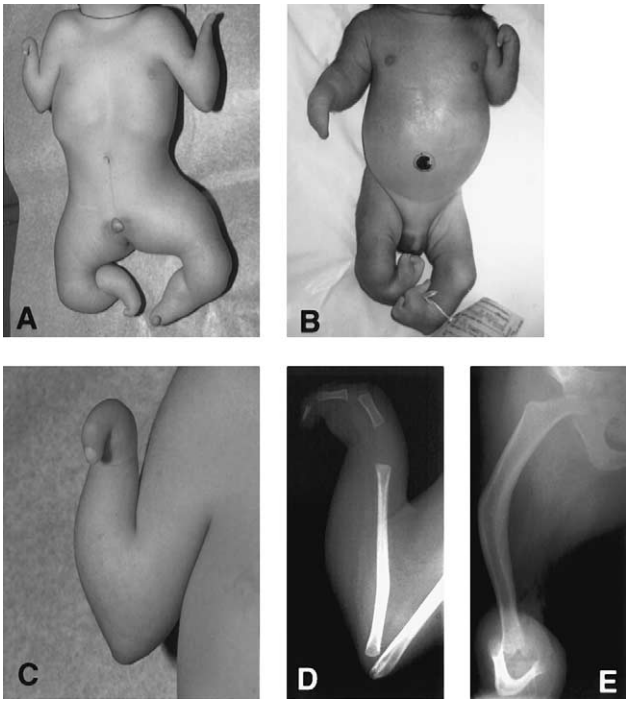

Figure 1 Photographs and radiographs of patients. *A,* Patient 1. *B,* Patient 2. Severe symmetric limb malformations are present in all four limbs. A proximal segment or stylopod, an intermediate segment or zeugopod, and a distal segment or autopod are present in each limb. Single digits with a dorsal nail are present in all limbs in patient 1; two digits are present in the feet in patient 2. In patient 1, the external genitalia are represented by a rugous scrotal skin, where no testes were palpable, and a small phallus with a distal urethral opening. Patient 2 has penoscrotal transposition and micropenis. *C,* Right upper limb of patient 1, showing contractures at the shoulders, elbows, and wrists, that separate the limb into 3 well-defined segments. *D,* Radiographs of left upper limb of patient 1, demonstrating a thin humerus, a single long bone in the zeugopod, an unossified carpal space, a single metacarpal, and two phalanges. *E,* Radiographs of right lower limb of patient 1, showing a long bowed femur and a single incurved long bone in the zeugopod phalanges.

Methods

PCR Amplification of Polymorphic Tetranucleotide-Repeat Markers

Highly polymorphic tetranucleotide-repeat markers

(Research Genetics) were chosen around the *HOXD* locus, according to their relative positions in the Genome Database (GDB) and the Cooperative Human Linkage Center (CHLC). PCR amplification of the patients' total DNA (extracted from blood) was performed for each pair of [γ-32 P]–end-labeled primers for the polymorphic markers, by use of DNA *Taq* polymerase (Qiagen) under the following conditions: 95°C for 3 min; then 31 cycles of 94°C for 30 s, 56°C–60°C (variable for each pair of primers) for 1 min, and 72°C for 1 min; and a final extension step of 72°C for 6 min. PCR products were analyzed in 6% polyacrylamide denaturing gels.

Southern Analyses for HOXD3 *and* HOXD13

Primers for amplification of a 942-bp fragment of *HOXD3* intron 1 from human genomic DNA were as follows: sense; 5′-TCCAGATTGACCCAAGGAAGCCTAGTCAGG-3′; and antisense; 5′-GCAAACCAGATCCCTGATAGCAAGCCAC-3′. Primers for amplification of a 710-bp fragment of *HOXD13*, exon 1, from human genomic DNA were as follows: sense; 5′-GGACCTCTGAGCGCACGGGCTCTTC-3′; and antisense; 5′-TTCACACGAACTGGAACAAATTTCCACTGGG-3′. PCR conditions with use of DNA *Taq* polymerase (Qiagen) were 95°C for 3 min; then 31 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min; and a final extension step of 72°C for 10 min. PCR products were run on a 0.7% agarose gel. The corresponding bands were excised from gels, and the DNA was reamplified by 15 additional PCR cycles with the original primer pairs under the same conditions. DNA was extracted from the resulting PCR products by a COMPASS kit (American Bioanalytical). These *HOXD3* and *HOXD13* DNA probes were labeled by [α-32 P]4dNTP mix according to a nick-translation protocol. Genomic DNA from each of the patients was extracted from blood, and 4 μg were digested in two separate samples, with *Eco*RI and *Hind*III. After the digestion, the absorbance of the DNA solution was measured at 260 nm, and the amounts of DNA loaded for each of the patients were equalized according to their absorbance values. The digested DNAs were loaded in a 0.7% agarose gel. The DNA was transferred to nylon membranes (Hybond N; Amersham) in a vacuum-transfer system. The blots were hybridized with the labeled probes overnight at 37°C, according to standard protocols (Sambrook et al. 1989), and were exposed to the PhosphorImager screen (Molecular Dynamics) for 48 h. The volume reports for each band were recorded by use of an "Object Average" method. These volume reports were corrected for the amount of DNA present in the digest, by image quantification of ethidium bromide–stained agarose gels that were transferred.

Genome Database accession numbers are D2S1395, 685557; D2S1776, 685566; D2S1238, 309229; D2S1244, 312398; D2S425, 686472; and D2S426, 686484. GenBank accession numbers are HoxD3, Y09980; and HoxD13, AF005219.

Results

Combining data from the cytogenetic breakpoints and from the use of six polymorphic tetranucleotide-repeat markers (fig. 2), we were able to determine that the deletions in both patients were likely to include the *HOXD* cluster. In patient 1, high-resolution–banding chromosome analysis detected a deletion eliminating the region 2q24.2-q31 of the long arm chromosome 2: karyotype 46,XY, del(2)(q24.2q31). Both parents had normal karyotypes. The proximal deletion breakpoint in 2q24.2 mapped centromeric to the *HOXD* cluster in 2q31.1, by G-banding. A single paternally inherited allele for marker D2S1244 mapped the distal deletion breakpoint telomeric to the *HOXD* cluster in 2q31.1. In patient 2, a cytogenetic deletion eliminating the region 2q31.1-q32.2 was present: karyotype 46,XY, del(2)(q31.1q32.2). The patient's father carried a deletion of the same cytogenic interval in the short arm of chromosome 2, balanced by its insertion in the long arm of the same chromosome. The mother had a normal karyotype. We presume that recombination in the paternal germline resulted in a chromatid with an unbalanced deletion that was passed to patient 2. The monodactylous sibling of patient 2 may have inherited a similar unbalanced-deletion chromosome, but DNA from the sibling was not available for testing. The distal deletion breakpoint in this case mapped telomeric to the *HOXD* cluster in 2q31.1, by G-banding. Single paternally inherited alleles for markers D2S1395 and D2S1776 mapped the proximal deletion breakpoint centromeric to the *HOXD* cluster in 2q31.1.

Intragenic fragments from two members of the *HOXD* cluster (fig. 3), *HOXD3* and *HOXD13*, were used as probes on Southern blots containing genomic DNA from the two patients and their parents. Quantification of Southern-band intensities in a PhosphorImager confirmed that both children had only half the amount of *HOXD3* and *HOXD13* coding sequences that was present in their parents, which is consistent with their possession of a heterozygous deletion for the *HOXD* cluster. As shown in figure 3, a signal of approximately one-half intensity is present in the child DNA (lanes C), when compared with either the father (lanes F) or the mother (lanes M). In patient 1, *HOXD3* signals were 38,302 (F), 19,967 (C), and 40,502 (M), and *HOXD13* signals were 27,107 (F), 13,614 (C), and 25,310 (M). In patient 2, *HOXD3* signals were 1,847 (F), 843 (C), and 2,272 (M), and *HOXD13* signals were 1,187 (F), 400 (C), and 971 (M).

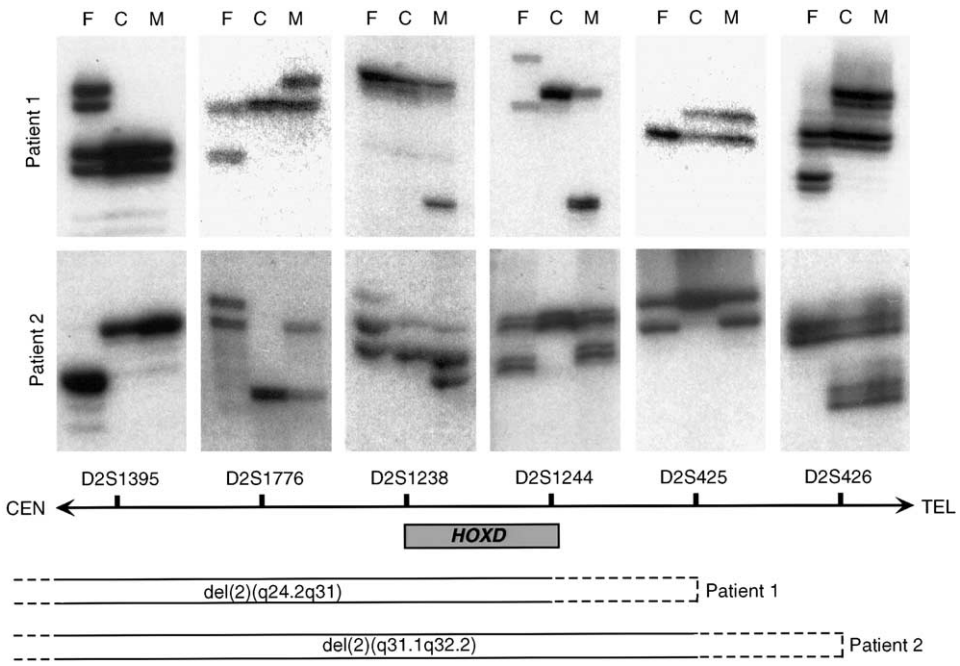

Figure 2 Denaturing gel images of PCR-amplified polymorphic tetranucleotide-repeat markers surrounding the *HOXD* cluster (*shaded box*). For each marker, the left lane corresponds to the father (F), the middle lane to the patient (C), and the right lane to the mother (M). The open rectangle below the markers represents the location of the deletion combining the cytogenetic and molecular findings. Dotted lines represent incompletely defined boundaries of the deletions. In both patients, the absence of paternal inheritance is demonstrated for one or more markers (D2S1244 for patient 1, D2S1395 and D2S1776 for patient 2).

These data indicate that the deletions remove at least eight members of the *HOXD* cluster—and, most likely, the entire cluster. We believe that the deletions in patients 1 and 2 in this study also include the proximally adjacent *HOXD*-complex gene *EVX2*, since the cytogenetic deletion in patient 1 extends to 2q24.2, and since the deletion in patient 2 includes the more proximal markers D2S1395 and D2S1776. Expression and mutant studies in mice have shown that *Evx-2* functions like a posterior *HoxD* gene in limbs, either directly or through the transactivation of posterior *HoxD*-gene expression (Herault et al. 1996). Another known pair of developmental regulatory genes, *DLX1* and *DLX2,* map proximally to the *HOXD* complex and are also expressed in the limb bud. We did not test for the dosage of these *DLX* genes in patients 1 and 2. It is possible that a lowered dosage of *DLX1* and *DLX2* plays a role in the limb phenotypes when other genes are also hemizygous, but this seems unlikely, since mice that are homozygous mutants for both genes have normal limbs (Qiu at al. 1997).

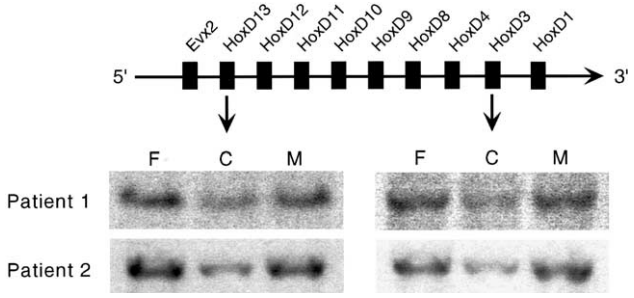

Figure 3 Southern blots confirming a deletion including the *HOXD* cluster in paternal chromosome 2 of patients 1 and 2. PhosphorImager signals (see text) from Southern hybridization for *HOXD3* and *HOXD13* confirmed hemizygosity for these genes.

Discussion

The monodactylous phenotypes associated with the overlapping deletions in these two patients indicate that genes exist within 2q31 that are crucial for the elaboration of pattern on the anterior-posterior axis of human limbs. At least 25 patients have been previously reported with different interstitial deletions that have a breakpoint in or near 2q31 (Shabtai et al. 1982; Ramer et al. 1989; Ramer et al. 1990; Boles et al. 1995; Nixon et al. 1997). The status of the *HOXD* complex is known in only one of these patients, who was heterozygous for a deletion that was cytogenetically indistinguishable from that of patient 1 in this study but who possessed a normal dose of the most proximal genes (*EVX2* and

HOXD13) of the *HOXD* cluster. The relatively mild limb defects in this *HOXD+* patient included camptodactyly, wide halluces, and missing second toes (Boles et al. 1995). This suggests that hemizygosity for one or more genes mapping proximal to the *HOXD* cluster may contribute to the extremely severe limb defects seen in patients 1 and 2. A large family described by Ramer et al. (1990) includes five patients who are hemizygous for deletions of 2q31-2q33. All five patients had limb abnormalities, and one member of this pedigree had a phenotype strikingly similar to those of patients 1 and 2, including a single digit in the autopod and a single long bone in the zeugopod. However, other affected second-degree relatives carrying the same cytogenetic deficiency had variable and milder limb defects. The status of *HOXD* dosage in those patients is, unfortunately, unknown.

There is additional information that is consistent with an *HOXD*-dosage deficiency underlying the limb/genital defects in patients 1 and 2. The importance of gene dosage has long been known in *Drosophila* and *Tribolium,* in which heterozygosity for multiple *Hox* genes results in mild appendage- and/or genital-morphological defects (Lewis 1978; Kaufman et al. 1980; Stuart et al. 1991). The limb and genital defects in patients 1 and 2 correlate well with regions of expression and function of posterior *HOXD* genes in chick and mouse embryos but correlate less well with the mutant phenotypes that have been detected through hemizygosity for multiple mouse *HoxD* genes.

During the period when the zeugopod is being patterned, progressively more of the posterior *HoxD* genes are activated in posterior regions of the limb bud (Dollé et al. 1989; Ispizúa-Belmonte et al. 1991). The most anterior region of the zeugopod corresponds to the area where only one member of the *HoxD* cluster (*hoxd9*) is expressed at this early stage in mouse limb buds, in addition to members of the *HoxA* cluster. The radius, which developed relatively normally in patients 1 and 2, derives from the anterior region of the zeugopod, whereas the posterior bone (ulna), developing in a region where more *HoxD* genes are activated, is absent in both patients.

At later stages, when the limb autopod is being patterned, the spatial pattern of *Hox*-gene expression is altered. Notably, *Hoxa13* is expressed in the entire chick-embryo autopod, and posterior *HoxD* genes (*d10*, *d11*, *d12*, *d13*, and *Evx-2*) are expressed in overlapping posterior domains (Nelson et al. 1996). The presence of only two phalanges in the remaining single digits of patients 1 and 2 suggests that they represent the most anterior digit, which develops from an anterior limb bud region where no *HoxD* genes are activated. (We presume that the human patterns of expression mimic those in mouse limb development.) The single anterior zeugopodal bone and the biphalangeal digit could represent the elements that result from a normal proximal-distal patterning system that operates even when *HoxD* expression is at subnormal levels and for which the posterior members of the *HoxA* cluster, specifically *Hoxa11* and *Hoxa13*, could be essential (Yokouchi et al. 1991; Fromental-Ramain et al. 1996; Kondo et al. 1997).

The expression patterns and functions of the posterior genes of the mouse *HoxD* complex indicate that these genes are also involved in the patterning of the genital tubercle in mammals (Dollé et al. 1991; Kondo 1997). The abnormal phallus and scrotum seen in both patients are consistent with a requirement for normal *HOXD* dosage in normal development of human genital structures.

The study of mouse mutant phenotypes for the posterior *HoxD* genes suggests that complex interactions among members of the same cluster and among paralogous members of different clusters play a major role in the resulting limb abnormalities (Davis and Capecchi 1994; Favier et al. 1995; Davis and Capecchi 1996; Kondo et al. 1997). Although measurements of the length and thickness of the long bones are of limited value for comparison in patients 1 and 2 because of these patients' overall growth deficiency, the small sizes of the bone segments in these patients also suggest a global deficiency in bone growth, a common finding in animal models mutant for *HoxD* genes.

The most similar genotypes reported in mice are heterozygotes that retain only one dose each of either *hoxd12* and *hoxd13* or *hoxd11, hoxd12,* and *hoxd13*. Such heterozygotes for posterior *HoxD* genes possess truncations and mild morphological abnormalities in digits II and V of the autopod (Davis and Capecchi 1996; Zákány and Duboule 1996; Zákány et al. 1998), effects that resemble in kind, although not in severity, the limb abnormalities present in these *HOXD*-hemizygous patients. The limb defects present in mice have increasing severity as the number of inactive *HoxD* and *HoxA* genes increase, and they appear to be very severe only when multiple *Hox* mutations are homozygous. Evidence suggests that the posterior *HoxD* and *HoxA* genes control both the size and the number of murine digits in a dose-dependent fashion, rather than through a *Hox* code involving differential qualitative functions (Zákány et al. 1998). Because of the extensive overlap of function, the role of individual genes may not become apparent until they are combined with mutations in all members of the complex. Given both the severity of the defect found in patients 1 and 2 and the relatively modest effect of hetero- or homozygosity for multiple mouse *HoxD* genes, lower *HOXD* dosage might be the principal defect underlying the monodactylous phenotype in patients 1 and 2 only if human limbs are much more sensitive to *HoxD* dosage than are the murine limbs. This is pos-

sible, but we believe that the additive or synergistic effects of other hemizygous genes in the 2q31 region provide a better explanation for the discordance, in severity, between the phenotypes observed in mouse *HoxD* mutants versus patients 1 and 2.

Monodactyly is present in the mouse with the hypodactyly (*Hd*) phenotype, which is associated with a homozygous intragenic deletion and frameshift in *Hoxa13*. However, the *Hd* phenotype is different from—and, in some aspects, complementary to—the phenotype in patients 1 and 2. Homozygotes for *Hd* have forefeet in which the zeugopod is normal, and the single incompletely formed digit probably corresponds to digit IV (Mortlock et al. 1996).

Additional evidence that alteration in the functional dosage of several *HoxD* genes can lead to a dominant polarized phenotype is provided by both the mouse dominant mutation *Ulnaless* (*Ul*) (Davisson and Cattanach 1990) and the *HOXD13* semidominant mutation in human synpolydactyly (Mugaraki et al. 1995). The *Ul* mutation is tightly linked to the *HoxD* locus, and the modifications induced in expression of the posterior *HoxD* genes in *Ul*/+ mutants suggest that *Ul* is a regulatory mutation interfering with the function of multiple *HoxD* genes during limb development (Herault et al. 1997; Peichel et al. 1997). In addition, heterozygous phenotypes are present in *HOXD13* mutations that are associated with human synpolydactyly. The similarity of these phenotypes to those in which multiple mouse *HoxD* genes are present in only one dose suggests that the abnormal *HOXD13* product in human synpolydactyly may have a dominant-negative effect (Mugaraki et al. 1995), altering the expression or the function of other members of the *Hox* cluster.

Interestingly, a limb phenotype similar to those seen in patients 1 and 2 occurs in mice homozygous for a *Sonic hedgehog* (*Shh*) loss-of-function mutation (Chiang at al. 1996). In these mutants, only a single zeugopodal limb bone and a single digit bud are formed. It is known that *Shh* both activates and is activated by posterior *HoxD* genes in the mouse limb bud (Duboule 1992; Riddle et al. 1993; Tickle 1995; Knezevic et al. 1997). It is possible that heterozygosity for all of the posterior *HoxD* genes disables the positive-feedback loop with *Shh* to such an extent that the levels of expression of both *HoxD* and *Shh* decrease dramatically, explaining the similarity between the limb abnormalities in the mouse *Shh* mutants and those in patients 1 and 2.

An additional limb phenotype—mesomelic dysplasia, Kantaputra type (MIM 156232)—has recently been linked to the 2q24-q32 region (Fujimoto et al. 1998). This autosomal dominant mesomelic dysplasia is characterized by marked shortening of the ulnae, radii, carpal, and tarsal synostosis.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Cooperative Human Linkage Center (CHLC), http://www.chlc.org/

GenBank, http://www.ncbi.nlm.nih.gov/genbank (for the *HOXD3* and *HOXD13* genes)

Genome Database (GDB), http://gdbwww.gdb.org (for D2S1395, D2S1776, D2S1238, D2S1244, D2S425, and D2S426)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for synpolydactyly; hand-foot-genital syndrome; and mesomelic dysplasia, Kantaputra type).

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