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Unusual pattern of *Sonic hedgehog* expression in the polydactylous mouse mutant Hemimelic extra-toes

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ABSTRACT We have examined the dynamic expression of *Sonic hedgehog (Shh)* in limb buds of the *Hemimelic extra-toes (Hx)* mutant. An ectopic domain of expression appears in the limb bud at embryonic day 11.5, which is not restricted to the anterior mesenchyme as in other polydactylous mutants, but extends along the entire apical ectodermal ridge. No difference in expression was observed between heterozygotes and homozygotes. This ectopic expression domain forms later and is maintained longer than the normal one. We verified that the *Shh* signal is properly transduced in the ectopic expression domain by analysing the expression of downstream target genes and provide evidence that the ectopic domain is functional. Interactions between *Msx1* and *Hx* were investigated by constructing a double mutant strain. Embryos from this strain exhibit little difference in *Shh* expression compared to *Hx* simple mutants. However, homozygous *Msx1* / *Hx* double mutants exhibit a postaxial polydactyly at birth, demonstrating that the two genes interact.

KEY WORDS: *limb development, gene interactions, Msx1, Hoxd13, nubbin*

Introduction

Polydactylies in the mouse have received refined molecular interpretation with the emergence of sophisticated models for limb patterning (Pearse and Tabin, 1998; Capdevila and Izpisua-Belmonte, 2001). It appears that most of these defects are associated with an ectopic, anterior domain of Sonic hedgehog (*Shh*) expression, which leads to the duplication of the zone of polarising activity (ZPA). This phenotype is very similar to the duplications induced experimentally in chick by grafting onto the anterior limb mesenchyme cells from the ZPA, cells producing SHH, or by applying retinoic acid (Tickle, 1999).

Hx belongs to the luxoid family of polydactylous mutations. The *Hx* mutation causes preaxial polydactyly in all four limbs and a shortening of the tibia and radius. Fibula and ulna are normal in size but bow because of the reduction of their companion element, resulting in a luxation that is much more severe in the hind- than in the forelimbs. The autopod has six to seven metatarsals or metacarpals and six to eight digits (Knudsen and Kochhar, 1981; Heus *et al.*, 2001). *Hx* is a dominant mutation located on Chr 5, close to *Shh*, and it was hypothesised that it might affect a regulatory element for this gene (Chang *et al.*, 1994; Sharpe *et al.*, 1999). However, recently, another candidate gene has been proposed for *Hx* : Limb Region 1 (*Lmbr1*). This gene is located within 295 kb of *Hx* and, although no molecular alteration could be found in its structure, is downregulated in the *Hx* limb mutant at E11.5 (Clark *et al.*, 2000; Clark *et al.*, 2001).

In spite of the genetic relationship between *Hx* and *Shh*, limited attention has been given to *Shh* expression during *Hx* development (Masuya *et al.*, 1995). In this report, we describe the expression pattern of *Shh* and other genes related to polarising activity in *Hx*. We further explore the interaction between *Hx* and *Msx1*, using an *Msx1* mutant allele we have generated (Houzelstein *et al.*, 1997). From these results, we speculate on the mechanisms that might lead to the *Hx* phenotype.

Results

Shh *Expression Pattern in the* Hx *Mutant*

Shh expression was examined in normal mice and *Hx* mutants between E9.5 and E11.75. The expected posterior domain of expression could be detected at E9.5 in forelimb buds. From E9.5 to E10.5, no difference was observed in *Shh* expression between normal and mutant embryos (not shown). At E11.5, an anterior ectopic domain was observed in the four limb buds in the mutant (Fig. 1). At the same stage, a slight overgrowth of the anterior

Abbreviations used in this paper: AER, apical ectodermal ridge; E, embryonic day; Hx, hemimelic extra-toes; PCR, polymerase chain reaction; ZPA, zone of polarising activity.

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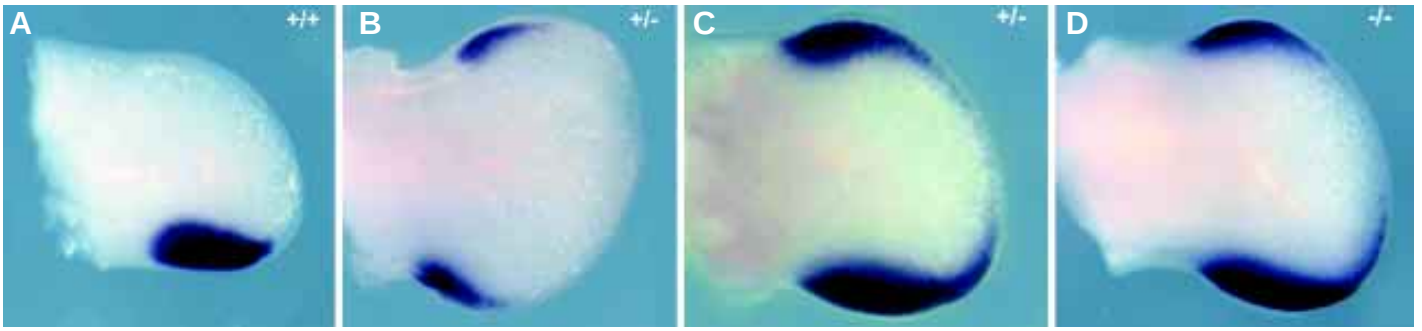

Fig. 1. *Shh* expression in the limb bud at E 11.5. *As in all figures in this paper, anterior is up.* **(A)** *A wild type forelimb: normal* Shh *expression takes place in the posterior mesenchyme of the limb bud.* **(B)** *A heterozygous* Hx / + *embryo forelimb: in addition to the normal posterior domain, a large anterior ectopic domain of* Shh *expression is present.* **(C)** *A heterozygous* Hx / + *embryo hindlimb: the posterior domain of* Shh *expression extends distally compared to the wild type, up to the ectopic domain of expression that has formed anteriorly.* **(D)** *A homozygous* Hx / Hx *embryo hindlimb: no difference is conspicuous compared to the heterozygous* Hx / + *embryo hindlimb. Note that at this stage, the shape of the mutant limb buds differs slightly from the normal one, due to overgrowth of the anterior part of the limb.*

region of the autopod is detectable, which becomes much more prominent from E11.75 (Fig. 2D). In contrast to what is observed in other polydactylous mutants such as Strong's luxoid (*lst*) (Chan *et al.*, 1995), Extra-toes (*Xt*) or luxate (*lx*) (Masuya *et al.*, 1995; Masuya *et al.*, 1997), the ectopic domain in *Hx* is as large as the normal one. Furthermore, the posterior domain is enlarged distally, such that it extends within the mesenchyme along the apical region of the limb bud. This is more pronounced in the hindlimb, where the apical domain rims the length of the apical ectodermal ridge (AER) and fuses with the anterior domain, than in the forelimb, where the most apical region of the ectopic domain is missing (Fig. 1 B,C,D; Fig. 2B). This pattern correlates well with the increased severity of the phenotype in the hindlimb.

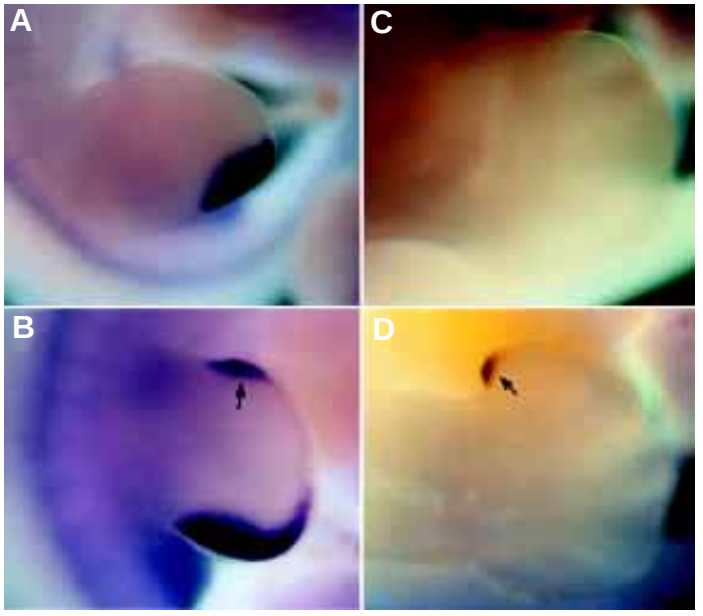

Fig. 2. Dynamic expression of *Shh*. (A) *At E11.5, normal* Shh *expression is observed in the posterior mesenchyme of a wild type hindlimb bud.* **(B)** *In a* Hx +/- *embryo hindlimb bud, an ectopic, apical and anterior domain of* Shh *expression is visible (arrow), in addition to the normal domain.* **(C)** *At E11.75,* Shh *expression is extinct in the normal, posterior domain of a normal embryo.* **(D)** *In a mutant embryo, the normal posterior domain of* Shh *expression is also extinct, but a small anterior, ectopic domain is still visible (arrow). Note that expression in this site is associated with a prominent overgrowth of mesenchyme.*

At E11.75, a reduced ectopic domain of *Shh* expression is still present anteriorly in the hindlimb bud, while the normal one is extinct both in normal and mutant embryos (Fig. 2 C,D). At this stage, the ectopic domain has disappeared in the forelimb bud, which develops more precociously. Therefore, the ectopic domain of expression forms later (E11.5 vs. E9.5) and is maintained longer than the normal one.

In order to determine whether *Shh* expression pattern is more severely affected in the homozygous mutant than in the heterozygote, we set up a cross in which we could identify the normal versus mutant allele. The *Hx* mutation arose originally in a B10.D2/nSn strain (Dickie, 1968; Knudsen and Kochhar, 1981). The strain we used was maintained by inbreeding on this background, and we could not detect any polymorphism between the normal and the mutated alleles in microsatellite markers around the mutation. To differentiate between normal and mutant alleles, simple sequence length polymorphisms (SSLPs) were identified in the *D5Mit387* and *D5Mit13* microsatellites that bracket *Hx* (Schimenti *et al.*, 2000) between C3H/He and B10.D2/nSn, and an F2 (B10.D2/nSn-*Hx*/+ X C3H/He) X (B10.D2/nSn-*Hx*/+ X C3H/He) was generated. The F2 offspring could be identified by PCR of *D5Mit387* and *D5Mit13* as normal, heterozygous or homozygous mutant at the *Hx* locus. At E11.5, the class distribution in the F2 did not depart significantly from a mendelian segregation (Table 1). No difference in the *Shh* expression pattern could be detected in *Hx*/*Hx* homozygotes versus the heterozygotes (compare Fig. 1 C,D).

Consistent with this analysis, very subtle or no difference could be observed in the morphology of *Hx* / *Hx* relative to *Hx* / + in the

TABLE 1

CLASS DISTRIBUTION OF THE F2 OFFSPRING FROM A (B10.D2/nSn-*Hx*/ + X C3H/He) X (B10.D2/nSn-*Hx*/ + X C3H/He) CROSS

Genotype	+ / +	*Hx* / +	*Hx* / *Hx*
Number of offspring	20	23	9

Statistical analysis shows that it does not depart from a mendelian distribution with a > 95% probability ($Chi^2 = 5.35$, < significance threshold: 5.99).

adult. This was investigated by setting up a progeny test for a number of mutants, to discriminate the homozygotes from the heterozygotes. Viability and fertility of the homozygous mutant appears severely impaired in the original B10.D2/nSn genetic background since only one out of 25 males analysed transmitted exclusively the *Hx* allele and thus was likely to be homozygous, when one third of the polydactylous studs are expected to be homozygous in our breeding scheme. This homozygote exhibited the same limb phenotype as its heterozygote littermates (not shown).

Is the Shh *Signal properly transduced?*

In some mutants, polarising activity can be dissociated from *Shh* expression (Francis-West *et al.*, 1995; Rodriguez *et al.*, 1996; Yang *et al.*, 1998). Therefore, expression of a number of markers associated with the ZPA was further analysed. We first investigated whether the *Shh* signal is properly transmitted. Patched1 (*Ptch1*) is one of the two homologues of Drosophila patched, the membrane receptor of hedgehog (*hh*) (Goodrich *et al.*, 1996). In mice as in fly, it is expressed in cells adjacent to cells expressing *hh* and repressed in cells expressing *hh* (Platt *et al.*, 1997). This profile is indeed observed in *Hx* limb buds in a way that can be expected for effective *Shh* signal transduction in the anterior zone of expression (Fig. 3B). Furthermore, *Fgf4*, which is activated by *Shh* (Laufer *et al.*, 1994; Niswander *et al.*, 1994), is expressed along the entire AER in the *Hx* mutant (Fig. 3 E,F). Therefore, we may conclude that *Shh* signals normally in its ectopic domain of expression.

Hoxd13 is expressed at early stages in a domain centred on the ZPA, and later in a transverse band over the whole extremity of the limb bud (Nelson *et al.*, 1996). In *Hx*, a broad apical domain was observed in all four limbs that extended proximally in the anterior region (Fig. 3 C,D). This may be related to the tibia/radius shortening observed in *Hx* (see Discussion).

Interactions between Hx *and* Msx1

The expression pattern of *Shh* in the *Hx* mutant overlaps with that of *Msx1* (Fig. 4). We therefore investigated whether the two genes may interact by introducing in the *Hx* background an *Msx1* mutant allele (*Msx1*^{nlacZ}) we produced previously (Houzelstein *et al.*, 1997). As the two genes are located only 5 cM apart (Robert *et al.*, 1994), once the *Hx* and *Msx1*^{nlacZ} alleles are associated in phase, the double mutant can easily be maintained through generations by inbreeding.

Shh expression profile in the *Msx1*^{nlacZ}/ *Msx1*^{nlacZ} homozygous embryos is normal (not shown). In the double *Hx Msx1*^{nlacZ} / + + mutant, the characteristic ectopic domain of *Shh* expression was observed and looked enlarged as compared to that of simple *Hx* mutant (Fig. 4A). This enlarged apical domain was reproducibly observed in double mutants as compared to simple ones. Furthermore, they did form an ectopic domain extending over the whole apical region also in the forelimb bud (Fig. 4A), while this is detected only in hindlimb buds in *Hx* simple mutants. As for *Hx* simple mutants (Fig. 1 C,D), the expression pattern did not differ significantly in double homozygotes (*Hx Msx1*^{nlacZ} / *Hx Msx1*^{nlacZ}) versus double heterozygotes (*Hx Msx1*^{nlacZ} / + +) (data not shown). Keeping in mind that *in situ* hybridisation is not a highly quantitative method, these results are nonetheless suggestive of genetic interactions between *Hx* and *Msx1*.

This is further supported by a morphological analysis of the double mutant. As previously described, the skeletal alteration in *Hx* is complex (Fig. 5C). The mutation causes a polydactyly on all four limbs and shortening of the tibia and radius. The autopod exhibits six to seven metatarsals or metacarpals and six to eight digits per paw. All these digits have three phalanxes so we could

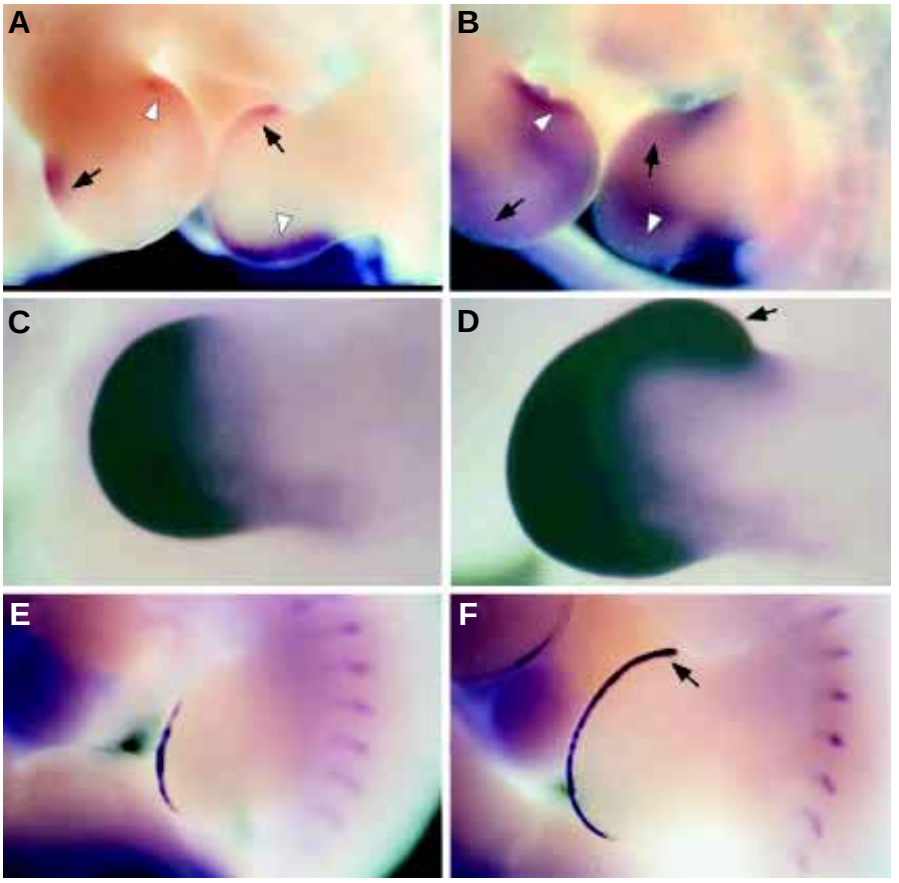

Fig. 3. Expression of genes in the *Shh* pathway at E11.5. (A) Shh *expression in a* Hx Msx1^{nlacZ} / Hx Msx1^{nlacZ} *embryo. The white arrowheads points to the normal, posterior domain of* Shh *expression, the black arrows to the ectopic anterior one.* **(B)** Ptch1 *expression in the contralateral limbs from the same* Hx Msx1^{nlacZ} / Hx Msx1^{nlacZ} *embryo. The normal domain of expression is indicated by white arrowheads and the ectopic anterior one by black arrows. These domains are adjacent to the* Shh *domains of expression seen in (A). (C,D)* Hoxd13 *expression in forelimb buds of* **(C)** *a wild type and* **(D)** *a* Hx / + *embryo. The anterior part of the limb bud has overgrown in the mutant and expresses* Hoxd13 *more anteriorly than the control limb bud. Note the proximal extension of the* Hoxd13 *ectopic anterior domain (arrow). (E,F)* Fgf4 *expression in hindlimb buds of* **(E)** *a wild type and* **(F)** *a* Hx / + *embryo. The AER normally expresses* Fgf4 *intensely in its postero-apical part (E). The expression domain in the mutant extends much more anteriorly (F, arrow).*

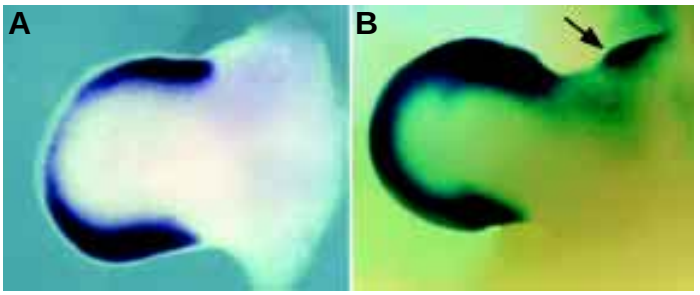

Fig. 4. Comparison of normal *Msx1* expression and ectopic *Shh* expression in a *Hx Msx1*^{nlacZ} / + + heterozygote. (A) *In the double mutant,* Shh *expression extends along the entire apical part of the limb bud, leaving no empty space between the two expression sites. This is to compare with* Msx1 *expression* **(B)** *in a normal embryo at the same stage. Expression covers the whole apical region of the limb bud with two larger sites of expression in the anterior and posterior parts. A site of expression for* Msx1 *that does not relate to the* Shh *expression domain is apparent in the presumptive shoulder (arrow).*

not identify any digit with a thumb identity. Fingers are severely affected in the anterior part of the footplate, with fusions and bifurcations of some phalanxes and formation of ectopic cartilage condensations, which are not linked to any proximal bone (Fig. 5C, arrow). Although the *Msx1*^{nlacZ} / *Msx1*^{nlacZ} mutation is lethal around birth (Houzelstein *et al.*, 1997), a number of homozygous double mutants (*Hx Msx1*^{nlacZ} / *Hx Msx1*^{nlacZ}) reached birth, suggesting that the two genes do not have additive effects on prenatal lethality. Homozygous *Hx Msx1*^{nlacZ} / *Hx Msx1*^{nlacZ} mutants show a preaxial polydactyly similar to that of *Hx* mutants in the forelimb (Fig. 5A), but in addition, exhibit a postaxial polydactyly in the hindlimb with the presence of a posterior non-ossified nubbin (Fig. 5B). The latter is never observed in either simple mutants, nor in *Hx Msx1*^{nlacZ} / + + heterozygotes (not shown). Therefore, our results demonstrate an interaction between *Msx1* and *Hx*.

Discussion

In this study, we report the analysis of expression of *Shh* and other genes associated with the ZPA in the *Hx* mutant. This mutation has been the subject of dedicated morphological (Knudsen and Kochhar, 1981; Heus *et al.*, 2001) and genetic (Martin *et al.*, 1990; Robert *et al.*, 1994; Heus *et al.*, 2001) studies. However, up to now, the molecular analysis of the developing limb in the mutant has been very limited (Masuya *et al.*, 1995). Our results bring important data to the understanding of how the *Hx* phenotype is elicited.

In most polydactylies analysed, the formation of extra digits is associated with the formation of an ectopic, anterior ZPA. This zone is limited to a small region in the anterior mesenchyme and in most cases, is associated with an ectopic domain of *Shh* expression (Chan *et al.*, 1995; Masuya *et al.*, 1995; Masuya *et al.*, 1997). In contrast, in *Hx*, a broad ectopic domain is formed which is as large as the posterior normal one and which further extends over the whole distal mesoderm of the bud. *Ptch1* expression is adjacent to sources of *Shh* signals (Platt *et al.*, 1997). In *Hx*, it was observed in the vicinity of both normal and ectopic *Shh* expression domains. Furthermore, the *Fgf4* expression domain extended along the whole AER. *Fgf4* is known to be induced by *Shh* in the AER (Laufer *et al.*, 1994; Niswander *et al.*, 1994), and this pattern correlates with ectopic *Shh* expression. These results indicate that the *Shh* signal is properly transmitted. *Hoxd13* is also associated with the ZPA (Dolle *et al.*, 1989; Nelson *et al.*, 1996); furthermore, it may be induced by *Shh* in conjunction with the AER (Laufer *et al.*, 1994; Niswander *et al.*, 1994). In *Hx*, its expression domain extends broadly anteriorly. This is in striking contrast with the expression profile of *Hoxd12*, the closest neighbour to *Hoxd13*, in the polydactylous mutant *Ist*, where it only forms a small ectopic anterior domain (Chan *et al.*, 1995). Therefore, we conclude that the ZPA is as widely extended as the *Shh* expression domain.

Preaxial polydactylies such as luxate (*lx*), Extra-toes (*Xt*), Pluridigite (*Pdt*) typically exhibit anterior triphalangeal digits which either appear anterior to the thumb or substitute for it (Carter, 1951; Johnson, 1967; I. Blanc *et al.*, in preparation). A striking morphological feature of *Hx* is that no thumb structure is ever observed. This suggests that a region of very low or null *Shh* expression level is required for the patterning of a digit with thumb identity during limb morphogenesis. In addition, these polydactylies usually consist of only one supernumerary digit anteriorly. In these polydactylies, limb buds exhibit a smaller ectopic domain of *Shh* expression

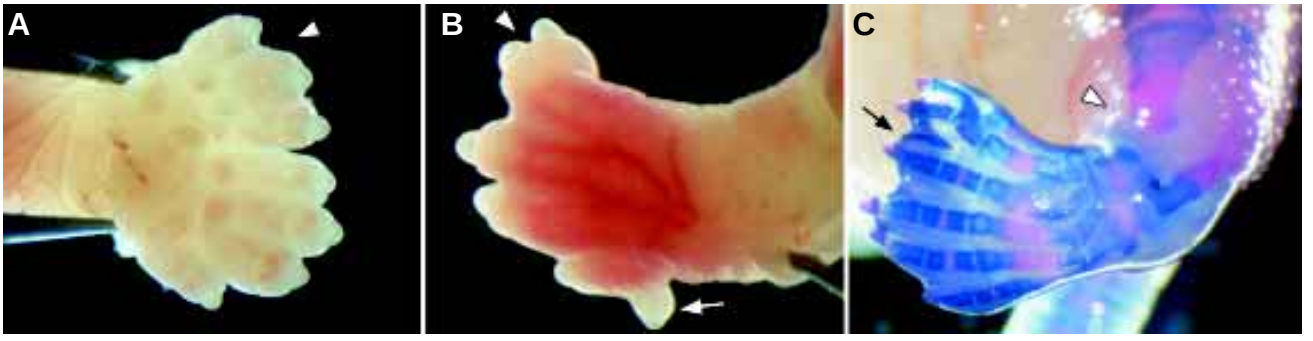

Fig. 5. Morphology of *Hx* mutant limbs. (A) *A forelimb paw from a* Hx Msx1^{nlacZ} / Hx Msx1^{nlacZ} *newborn. There are 7 digits clearly separated in two blocks, an anterior one where digits are malformed (arrowhead) and a posterior one. The presence of mutant alleles at the* Msx1 *locus does not further alter the phenotype (compare with panel C).* **(B)** *The posterior paw from the same animal. Note the nubbin on the posterior part of the limb, which is only observed in* Hx Msx1^{nlacZ} / Hx Msx1^{nlacZ} *double homozygotes (arrow).* **(C)** *Skeleton of a* Hx / + *newborn hindlimb. Cartilage is stained blue by Alcian blue and bone red by Alizarin Red. The tibia is shortened (arrowhead), which leads to the hemimelia characteristic of the mutation. Note the bifurcations and fusions of some of the phalanxes and an ectopic cartilage condensation, not linked to any proximal bone (arrow). In addition to the digits that are duplicated, there are six metatarsals that are proximally malformed. The tarsals also appear abnormal and fused.*

than in *Hx*. *Shh* dosage may therefore be related to the number of extra digits formed, as has been shown experimentally in the chick (Yang *et al.*, 1997). The polydactyly in *Hx* appears nonetheless less severe than expected, considering the extent and intensity of ectopic *Shh* expression, which might be expected to lead to duplication of the entire autopod. This probably relates to the late onset of ectopic *Shh* expression, which is detected two days after the normal domain has formed. This difference in timing remains to be explained.

In *Hx*, there is a broad extension of the *Hoxd13* expression domain, both anteriorly and proximally. In the Ulnaless mutant, *Hoxd12* and *Hoxd13* are expressed in a more proximal domain than in normal embryos (Herault *et al.*, 1997; Peichel *et al.*, 1997). The authors have proposed that misexpression of these *HoxD* genes proximally is not compatible with the development of a normal zeugopode. This hypothesis is supported by our observations in the *Hx* mutant. In the latter, the proximal part of radius and tibia, which differentiates anteriorly from the proximal region that expresses *Hoxd13* ectopically, is indeed affected, leading to the characteristic hemimelia of this mutant.

What is the primary alteration in *Hx* ? The *Hx* mutation has been located close to *Shh* on mouse Chr 5 and on this basis, *Hx* has been proposed to affect a regulatory element from this gene (Chang *et al.*, 1994; Sharpe *et al.*, 1999). With the progress of mouse genome sequencing, *Hx* may be evaluated to lie within 0.8 Mb of *Shh* (see http://www.ensembl.org). Recently, *Lmbr1* was identified as a potential candidate gene for *Hx*, since it maps at the same position and its expression is affected in the *Hx* mutant (Clark *et al.*, 2000; Clark *et al.*, 2001). Furthermore, deletion of the homologous gene in human also affects limb development (Ianakiev *et al.*, 2001). This proposition however is not easily reconciled with our observations on the expression pattern of *Shh* and other genes associated with the ZPA. *Shh* misexpression is likely to be directly involved in the polydactyly in *Hx.* In this mutant, *Shh* expression is altered as early as E11.5 and at this stage, it is properly signalling anteriorly (see above). This means that the *Shh* mRNA we detect by *in situ* hybridisation is already translated and the protein processed. Since *Lmbr1* mRNA does not appear to be downregulated before E11.5 (Clark *et al.*, 2000), this is unlikely to be responsible for the ectopic activation of *Shh*. *Hx* might be a mutation in a regulatory element acting in cis on both *Lmbr1* and *Shh*. In the HoxD complex, it has been proposed that a distant enhancer that regulates the expression of 5' genes in the cluster may affect the expression of any genes placed between the enhancer and *Hoxd13* (Herault *et al.*, 1999). It is noteworthy that another polydactylous mutation, Doublefoot, lies within 1.3 cM of Indian Hedgehog (*Ihh*) and has been proposed to affect a regulatory sequence for this gene (Yang *et al.*, 1998). Of course, it is difficult to determine whether the alteration of *Shh* is the primary defect in the *Hx* mutant, since the activation pathway of *Shh* is not completely elucidated yet, and a hypothetical regulatory gene at the *Hx* locus might be genetically upstream of both *Lmbr1* and *Shh*. However, the hypothesis of *Hx* being a mutation in a regulatory element of *Shh* remains to be investigated.

Interactions between *Msx1* and *Hx* are supported by the formation of an ectopic, postaxial finger in homozygous mutants for the two loci. This polydactyly is limited to the formation of a posterior non-ossified nubbin. Such a nubbin has been reported for the *Gli1* / *Gli2* double mutant (Park *et al.*, 2000). *Gli* genes encode transcription factors activated by *Shh*, suggesting that *Msx1* might play a role in *Shh* signalling. Indeed, *Msx1* is a component of the *Shh* pathway in tooth bud formation, although in a manner independent of *Gli* (Zhang *et al.*, 1999). It is noteworthy that posterior nubbins form in the limbs of *Msx1* / *Msx2* compound mutants which retain a single functional allele for the two genes (Y. Lallemand, M.A. Nicola and B. Robert, unpublished data).

Hx has been proposed to be embryolethal in the homozygous condition at E8-E9 (Knudsen and Kochhar, 1981). In the B10.D2/nSn background, we confirm that very few *Hx* / *Hx* animals reach the adult stage. However, we did not observe a significant reduction in the number of homozygotes at E11.5, in a mixed background. Furthermore, *Hx* / *Hx* homozygotes have been shown to be viable and fertile on C3H or castaneus inbred backgrounds (Clark *et al.*, 2000; Heus *et al.*, 2001). Lethality in *Hx* thus appears to be very dependent on the genetic background on which the mutation is carried.

Materials and Methods

Mouse Strains and Embryos

Hx mice were purchased from the Jackson laboratory as a B10.D2/nSn-Hx/+ stock, and were maintained by intercrossing. *Hx*/+ embryos were obtained by crossing *Hx* males and C57BL/6 females (IFFA-CREDO, France). C3H/He mice (IFFA-CREDO, France) were crossed to B10.D2/nSn-Hx/+ mice to produce a F1, and polydactylous offspring were intercrossed to produce F2 embryos that were genotyped by PCR. *Msx1*^{nlacZ} mice were maintained in a C57BL/6 background and genotyped by PCR as previously described (Houzelstein *et al.*, 1997).

Typing of Embryos

Simple sequence length polymorphisms (SSLPs) were identified for *D5Mit13* and *D5Mit387* microsatellites between the C3H and the B10.D2/nSn strains by PCR. Amplification was done for 35 cycles with annealing temperature of 55°C. *D5Mit13* produces a 194 bp product with B10.D2/nSn DNA and a 176 bp product with C3H/He, *D5Mit387*, a 174 bp product with B10.D2/nSn DNA and a 182 bp product with C3H/He, respectively. The amplification fragments were resolved in 3% agarose gels (Gibco).

Embryo Staining

In situ hybridisation probes were prepared from the following templates: *Shh* was a 640 bp cDNA fragment (Echelard *et al.*, 1993), *Ptch1* a 841 bp cDNA fragment (Platt *et al.*, 1997), *Fgf4* a 620 bp full length cDNA (Hebert *et al.*, 1990) and *Hoxd13* a 1.2 kbp full length cDNA (Dolle *et al.*, 1989). Whole mount *in situ* hybridisation, skeleton preparations and Xgal staining were performed as described previously (Houzelstein *et al.*, 1997).

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