J. Anat. (2003) **202**, pp13–20

REVIEW

Sonic hedgehog: restricted expression and limb dysmorphologies

Robert E. Hill, Simon J. H. Heaney and Laura A. Lettice

MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK

Abstract

Sonic hedgehog, SHH, is required for patterning the limb. The array of skeletal elements that compose the hands and feet, and the ordered arrangement of these bones to form the pattern of fingers and toes are dependent on SHH. The mechanism of action of SHH in the limb is not fully understood; however, an aspect that appears to be important is the localized, asymmetric expression of *Shh*. *Shh* is expressed in the posterior margin of the limb bud in a region defined as the zone of polarizing activity (ZPA). Analysis of mouse mutants which have polydactyly (extra toes) shows that asymmetric expression of *Shh* is lost due to the appearance of an ectopic domain of expression in the anterior limb margin. One such polydactylous mouse mutant, sasquatch (*Ssq*), maps to the corresponding chromosomal region of the human condition pre-axial polydactyly (PPD) and thus represents a model for this condition. The mutation responsible for *Ssq* is located 1 Mb away from the *Shh* gene; however, the mutation disrupts a long-range *cis*-acting regulator of *Shh* expression. By inference, human pre-axial polydactyly results from a similar disruption of *Shh* expression. Other human congenital abnormalities also map near the pre-axial polydactyly locus, suggesting a major chromosomal region for limb dysmorphologies. The distinct phenotypes range from loss of all bones of the hands and feet to syndactyly of the soft tissue and fusion of the digits. We discuss the role played by *Shh* expression in mouse mutant phenotypes and the human limb dysmorphologies.

Key words acheiropodia; *Lmbr1*; pre-axial polydactyly; sasquatch; *Shh*.

Introduction

During development graded concentration profiles of signalling molecules provide information across a field of cells. The concentration of the molecule informs the cells where they are and what they should do; such molecules are known as morphogens. In limb development a focal, regulatory domain was postulated by Saunders & Gasseling (1968), which fits the definition of a signalling centre responsible for the production of a graded morphogen signal (Wolpert, 1969). This posterior localized signalling centre is the zone of polarizing activity (ZPA), and is responsible for the pattern of digits in our hands and feet or the limbs of all tetrapods. The ZPA is a biologically distinct region of cells which has the experimental attribute that it can be manipulated and transplanted to different sites in the limbud. The addition of the ZPA to the anterior edge of the limb opposite the normal ZPA gives rise to extra digits. In the chick wingbud, in which all (three) digits are unique and distinguishable, an additional ZPA gives digit duplications in a mirror image of the normal digit pattern; the number and identity of the digits are directly dependent on the number of ZPA cells transplanted (Tickle, 1981; see also reviews by Panman & Zeller, and by Sanz-Ezquerro & Tickle, in this volume).

The search for the morphogen responsible for the polarizing activity produced by the ZPA settled on a molecule called sonic hedgehog (SHH) (Echelard et al. 1993; Krauss et al. 1993; Riddle et al. 1993; Roelink et al. 1994). SHH satisfied the predicted criteria for the limb morphogen. First, SHH, which is a homologue of

Correspondence
Dr Robert Hill, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK. Tel. +44 (0)131 467 8410; fax: +44 (0)131 343 2620; e-mail: Bob.Hill@hgu.mrc.ac.uk

Accepted for publication *21 November 2002*

Table 1 *hemimelic-luxate* mutations which mis-express *Shh*

Mutation	Chromosome	Gene	Reference
Dominant hemimelia (*Dh*)	1	?	Lettice et al. (1999)
Luxate (*lx*)	5	?	Masuya et al. (1997)
Rim4	6	?	Masuya et al. (1995)
Strong's luxoid (*lst*)	2	*Alx4*	Masuya et al. (1997)
extra toes (*Xt*)	13	*Gli3*	Buscher et al. (1997)
X-linked polydactyly (*Xpl*)	X	?	Masuya et al. (1997)
Hemimelic extratoes (*Hx*)	5	*Lmbr1*	Clark et al. (2000)
Sasquatch *(Ssq)*	5	*Shh*	Sharpe et al. (1999)

the *Drosophila* morphogen hedgehog (HH), is a secreted factor. Secondly, the expression is highly restricted and co-localizes with the ZPA in vertebrate limb buds and thus is produced at a localized source. Both properties are essential for the initiation of a graded production of the putative morphogen.

Proof that the *Shh* gene participates in polarizing the limb came from the grafting of SHH-producing cells or beads impregnated with SHH protein into the anterior of limb buds. Both treatments were capable of producing mirror image digit duplications (Riddle et al. 1993; Chang et al. 1994; Lopez-Martinez et al. 1995). In addition, SHH production is 'turned-on' by retinoic acid, a known inducer of the ZPA (Riddle et al. 1993). The removal of *Shh* in knockout mice also reveals that SHH has a polarizing function (Chiang et al. 1996). Mice homozygous for the *Shh* knockout allele exhibit severe defects in many embryonic structures including the limb. Mutants have four limbs with recognizable A-P patterning in the stylopod (humerus/femur), but distal to the stylopod the A-P patterning is severely disrupted. The forelimb autopod is represented by a single distal cartilage element whereas the hindlimb autopod consists of a single digit (Chiang et al. 2001; Kraus et al. 2001). Thus SHH is vital for the activity of the ZPA, and for A-P patterning the zeugopod and autopod of the limb.

Shh misregulation leads to mouse limb abnormalities

What mechanisms control the restricted *Shh* expression in the posterior located ZPA of the limb bud? Tight control of transcription is required for the restricted ZPA production essential for a graded morphogen action. Indeed, realization of the restricted *Shh* expression led to investigations into the role of misregulation as the basis for some limb dysmorphologies. Work focused on a group of spontaneously derived mouse limb mutations which arose showing additional digits on the pre-axial (anterior) side of the limb: some mutants are affected on one set of limbs, some on both. These were classed as the *hemimelic-luxate* group of mutants (Table 1). In many of these mutants the extra-digit phenotype appeared as mirror-image duplications, reflecting the transplant experiments performed in the chick wing bud. Thus misdirected expression of *Shh* seemed a reasonable mechanism for generating polydactyly, and subsequently a number of these pre-axial polydactylous mice were shown to express *Shh* in the anterior margin of the limb.

The genetic bases for *Shh* misexpression for three of the *hemimelic-luxate* mutations are known, i.e. the genes responsible for *Strong's luxoid* (*lst*) and *extra toes* (*Xt*) have been identified (the third, *sasquatch*, is discussed below). There are three alleles of the *lst* mutation, all of which are genetic defects (one is a targeted mutation in ES cells) in the *Alx4* gene (Qu et al. 1997, 1998; Takahashi et al. 1998). *Alx4* is a paired-type homeobox-containing gene that is homologous to *Drosophila aristaless*. The *lst* phenotype is a result of loss of function of *Alx4* and is dosage dependent; as a heterozygote the hindlimbs are polydactylous whereas as a homozygote all four limbs are affected as well as displaying a broader phenotype. The suggestions are that *Alx4*, perhaps through interactions with the related gene *Cart1* (Qu et al. 1999), is important in repressing the anterior limb bud expression of the *Shh* gene, important in assuring asymmetric, posterior expression of the gene. *Gli3,* the kruppel-like zinc finger-containing gene, has been identified as the gene responsible for the *Xt* mutation (Hui & Joyner, 1993). As postulated for *Alx4*, *Gli3* may be a repressor of anterior *Shh* (Masuya et al. 1995; Buscher et al. 1997) such that reduction in gene dosage results in anterior *Shh* expression. (Other roles for *Gli3* in the limb are more fully discussed in this volume: see review by Panman & Zeller).

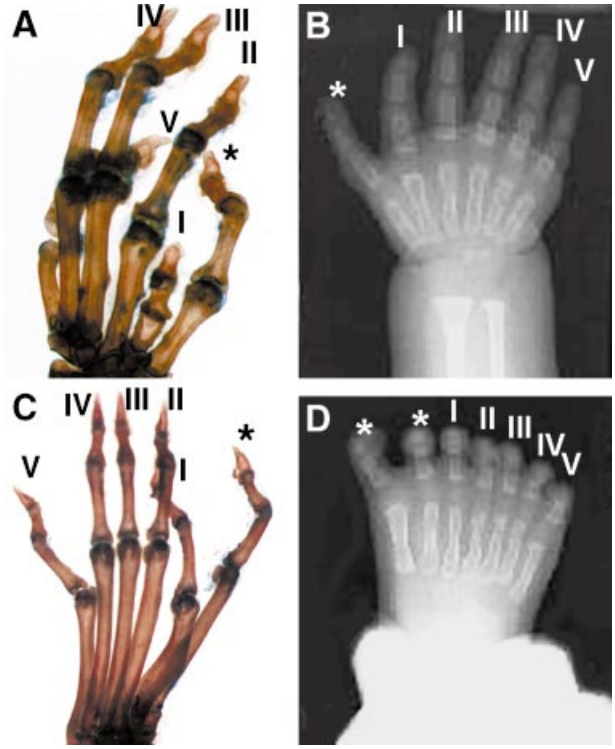

Fig. 1 Comparison of limbs seen in mouse and human limb dysmorphologies. Panels A and C show the bones of left fore (A) and hind (C) limbs from a homozygous *Ssq* mouse. Panels B and D show a radiogram of the right hand (B) and foot (D) of a patient with PPD. The roman numerals mark the positions of the normal digits and the asterisks mark the supernumerary digits.

Ssq: an *Shh* regulatory mutant

We have focused our work on the analysis of the *Ssq* polydactylous mutation. The *Ssq* mutation arose as the result of the random insertion of a *Hoxb1 human placental alkaline phosphatase* (*HPAP*) reporter construct (Sharpe et al. 1999). In adults heterozygous for the *Ssq* mutation the forelimbs are normal, but the hindlimbs display pre-axial polydactyly. Homozygotes (Fig. 1A,C) exhibit more extensive limb abnormalities, the hindlimb polydactyly is more severe, and the zeugopod displays hemimelia (reduction in the length of the tibia). Homozygous forelimbs also display pre-axial polydactyly and slight hemimelia. The semidominant nature of the *Ssq* mutation and the observation that hindlimbs are more severely affected than forelimbs is consistent with other members of the *hemimelia-luxate* group of mutants. Analysis of *Shh* expression in the developing *Ssq* limbuds showed, like other *hemimelia-luxate* mutants, ectopic anterior expression (Fig. 2A,B). Unlike most of the *hemimelia-luxate* group no other defects are seen outside of the limb.

Genetic analysis showed that the transgene insertion site on chr 5, which segregates with the polydactylous phenotype, was physically linked to the *Shh* gene but was situated almost 1 Mb away (Fig. 3A) (Sharpe et al. 1999). The transgenic insertion site was cloned and found to lie within an intron of a gene identified as

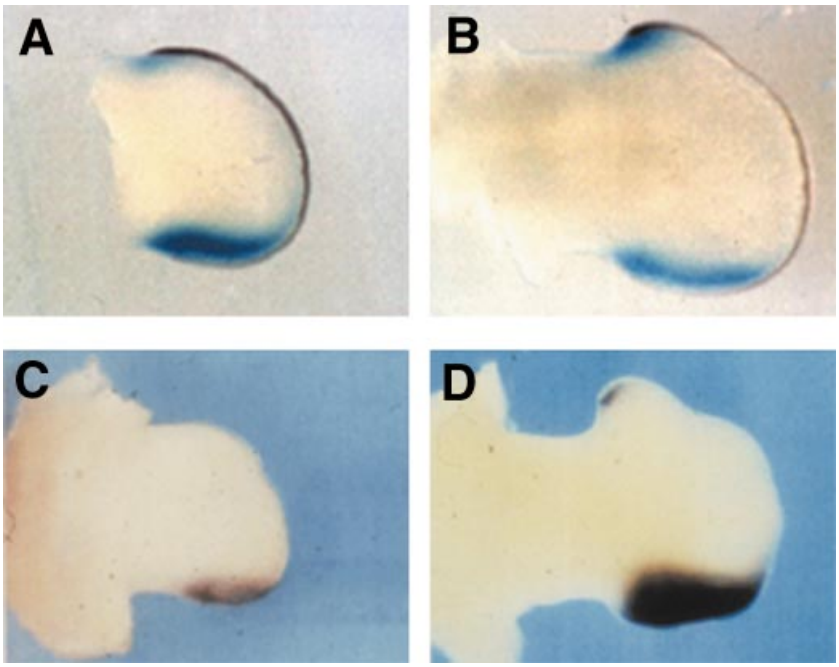

Fig. 2 Comparison of the expression patterns of the *Shh* gene and the HPAP reporter gene in limbs of the *Ssq* mouse. Panels A and B show *in situ* hybridization analysis of *Shh* expression in the hindlimb bud of an E10.5 embryo (A) and an E12.5 (B) embryo. The limbs are double labelled; Fgf8 expression in the AER is shown by the dark brown label and *Shh* expression by the blue. In all panels the anterior margin of the limb is situated at the top and the posterior margin is at the bottom. The ZPA expression is shown by the large blue area at the bottom of the panel, and the smaller blue domain at the top of the panel represents the ectopic expression. Panels C and D show the alkaline phosphatase expression from the HPAP reporter gene that composes the *Ssq* transgene. The black areas show regions of alkaline phosphatase activity in an area that overlaps the ZPA at E10.5 (C) and E12.5 (D) and the ectopic anterior domain at E12.5.

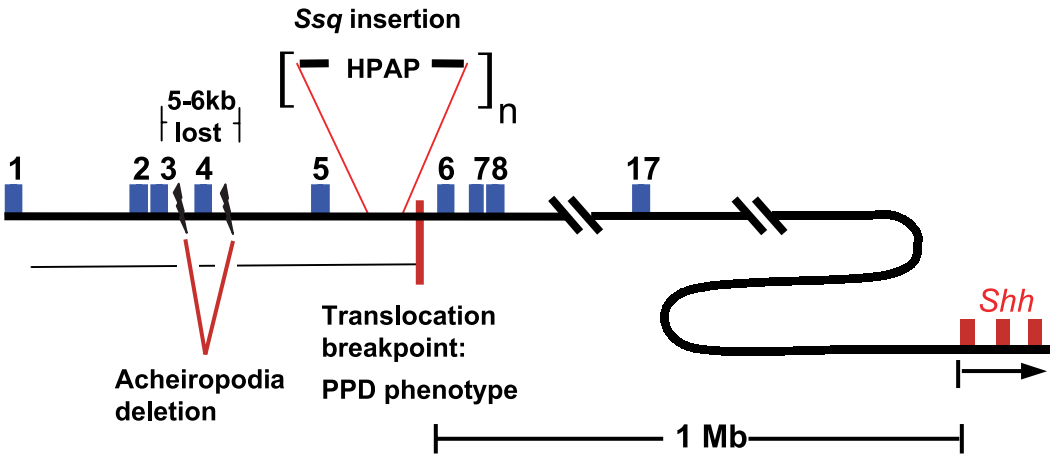

Fig. 3 A composite representation of human and mouse *Lmbr1*/*LMBR1* genes showing the relative positions of the limb mutations. The *Lmbr1* gene is composed of 17 exons represented by the blue rectangles and is 1 MB away from the *Shh* (exons in red) gene. The position of the Ssq insertion site in intron 5 of mouse and the close relationship to the human PPD translocation breakpoint in the corresponding human intron are represented. The brackets around the HPAP suggest that the transgene has incorporated multiple times ($n > 10$). The acheiropodia deletion is shown around exon 4; most of the 5–6 kb of deletion is intronic DNA.

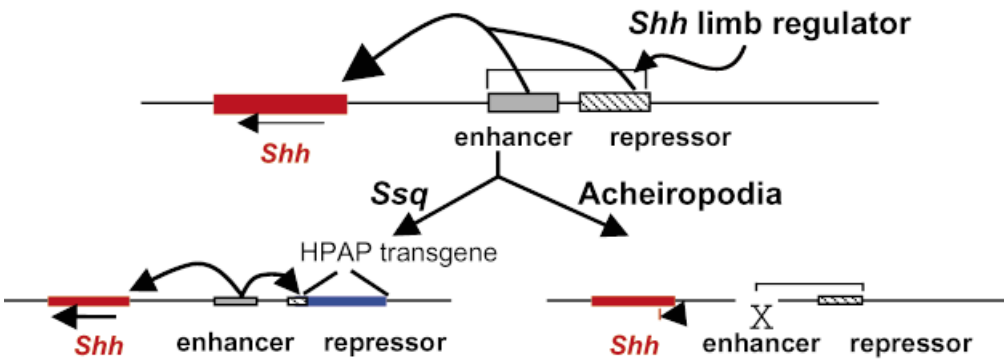

Fig. 4 *Shh* regulatory model for the generation of limb abnormalities in mouse and human. Multiple elements for a major, limb-specific regulator of *Shh* are postulated. These elements lie in or near the *Lmbr1* gene. At least two elements are necessary to generate the diverse limb phenotypes represented by *Ssq* (and PPD) and acheiropodia. First, an enhancer (grey box) is postulated which drives both the normal ZPA expression and the ectopic, anterior expression (the ectopic expression seen in Fig. 2A and B). Secondly, a repressor (cross hatched box) is present which down-regulates the ectopic, anterior expression. The *Ssq* transgene insertion (blue box) disrupts the repressor allowing anterior expression, and in the process the transgene has come under the influence of the enhancer (produces the pattern seen in Fig. 2D). The acheiropodia deletion removes and inactivates the enhancer such that there is no limb-specific expression of *Shh*.

Lmbr1 (Lettice et al. 2002). The insertion event led to duplication of the surrounding ~20 kb of the intron.

Human limb mutations mapped to 7q36

Preaxial polydactyly (PPD [MIM190605]), also referred to as pre-axial polydactyly type II (MIM174500), is one of the most frequently observed human congenital limb malformations. PPD is observed in sporadic cases, but most patients show an autosomal dominant mode of inheritance. The limb-specific phenotype varies markedly within families ranging from a simple addition of a phalanx in triphalangeal thumb to whole digit duplications and tibial aplasia. Genetic analysis of families showed that the PPD locus mapped to a 450-kb region on chromosome 7q36 (Heus et al. 1999) and all affected families described so far are linked to this locus (Heutink et al. 1994; Zguricas et al. 1994; Hing et al. 1995; Radhakrishna et al. 1997). PPD resides in a chromosomal region syntenic to the *Lmbr1* region of mouse chromosome 5 and is limb specific. The similarity in phenotype (Fig. 1, compare A, C with B, D) and

genomic location between *Ssq* and limb-specific PPD led us to suggest that *Ssq* is a mouse model for PPD. Interestingly, however, a thorough analysis of the LMBR1 structural gene failed to uncover any deleterious mutations (Lettice et al. 2002).

A major advance in investigating the genetic basis of PPD came from a young Japanese PPD patient who was found to carry a *de novo* reciprocal translocation t(5,7)(q11,q36) (Lettice et al. 2002). Fine mapping identified the location of the translocation breakpoint (Fig. 3) which lies within intron 5 of *LMBR1*, the corresponding mouse intron carrying the *Ssq* insertion.

The basis of the polydactylous mutations

Perturbations of the *Lmbr1* gene in mouse and human were originally thought to play a role in generating the polydactyly phenotype, perhaps by acting as a regulator of the *Shh* gene. *Lmbr1* encodes a 490 amino acid protein (LMBR1) (Clark et al. 2000) and possibly a 32 amino acid peptide by alternative splicing (LMBR1S). LMBR1 is hydrophobic and predicted to contain nine transmembrane domains. However, despite the efforts of several groups including our own, significant expression of *Lmbr1* could not be detected in a developmentally relevant pattern in the mouse limb bud by *in situ* hybridization (unpublished observations), suggesting that *Lmbr1* is expressed at low levels probably ubiquitously in the mouse. Secondly, and more importantly, no relevant mutations were found in the *Lmbr1* structural gene in human or mouse.

As discussed above, the *Ssq* mutation was generated by the random insertion of a transgene that included a *HPAP* reporter. Intriguingly, unlike the other seven lines generated with the same construct, when *Ssq* embryos were assayed for *HPAP* activity, they exhibited *HPAP* expression in the limb in addition to the typical rhombomere-4 pattern mediated by *Hoxb1* elements within the transgene (Sharpe et al. 1999). The limb pattern is first detected in the ZPA at E10.5 (Fig. 2C), and closely parallels that of endogenous *Shh* in the limb. *Ssq* heterozygous animals were also shown to exhibit *HPAP* activity in an overlapping anterior domain of the hindlimb buds that mirrors the anterior ectopic *Shh* (Fig. 2D). Homozygous embryos demonstrate anterior *HPAP* staining in forelimbs as well, consistent with the appearance of ectopic *Shh*. Thus, the HPAP reporter at the *Ssq* insertion site mirrors both normal and ectopic expression of *Shh* in the limb buds of *Ssq* heterozygotes and homozygotes.

These observations suggested a second hypothesis, that the *HPAP* transgene at the *Ssq* insertion has come under the influence of a *cis*-acting gene regulator that drives expression in the limb bud. A possible scenario is that the *Ssq* insertion event has revealed the presence of this regulator required for driving *Shh* expression in the limb, and in addition has disrupted the activity, such that *Shh* becomes anteriorly expressed in *Ssq* limb buds. This hypothesis is attractive as it does not require the limb mutations mentioned above to affect the expression of *Lmbr1*. Instead, the various genetic lesions mapped to human chr 7q36/mouse chr 5 could act to disrupt *cis*-acting regulatory elements of *Shh*.

Genetic test to distinguish hypotheses

To examine the basis for pre-axial polydactyly a *cis-trans* genetic test was devised. The prediction tested was that an *Shh* regulator would function in a *cis*-acting manner, i.e. the *Ssq* mutation would affect only chromosomally linked *Shh*. In contrast, disruption of the *Lmbr1* structural gene which secondarily affected *Shh* expression would operate on both *Shh* alleles and therefore in *trans*. A mouse cross was devised to derive a recombinant chromosome 5 in which the *Ssq* mutation was located in *cis* to the targeted null allele of *Shh*. In the analysis of the phenotype, mice carrying the recombinant chromosome would exhibit extra pre-axial toes if *Ssq* is acting on *Shh* in *trans* and wildtype feet, i.e. suppression of the *Ssq* phenotype, if acting in *cis*.

Analysis of the limb phenotype in five recombinant mice showed no pre-axial polydactyly or other detectable limb phenotypes. Thus the data demonstrate that the *Shh* null allele inactivates the affects of the *Ssq* mutation when located in *cis* (but not in *trans*) on chromosome 5. It follows that the *Ssq* insertion is a dominant acting mutation that interferes with the limb-specific expression of *Shh*. The data are consistent with a long-range limb-specific regulator of *Shh* residing within or near the *Lmbr1* gene. In addition, the data are consistent with human PPD resulting from similar disruption of a long-range *SHH* regulator.

Acheiropodia: congenital abnormalites without hands or feet

Human chromosome 7q36 region is becoming recognized as a major locus for limb defects. In addition to PPD, other defects including acheiropodia (Ianakiev

et al. 2001), complex polysyndactyly (CPS) (Tsukurov et al. 1994) and acropectoral syndrome (distinct but related to F syndrome) (Dundar et al. 2001) map to the 7q36 region. In mouse two additional mutations map to the corresponding murine chromosome. The spontaneously derived mutation *hemimelic extra toe* (*Hx*) (Clark et al. 2000), a likely allele of *Ssq*, maps to the same locus but is distinct from *hammertoe* (*Hm*, a syndactyly phenotype) which may be the mouse counterpart of human CPS.

CPS in human occurs more rarely than PPD, and is typically bilateral, showing both pre- and post-axial polydactyly and syndactyly (fusion of the soft tissue between the digits) (Tsukurov et al. 1994). Acropectoral syndrome is similar, showing pre-axial polydactyly and syndactyly of the soft tissue: however, patients present with other skeletal defects such as a prominent upper sternum and blind-ending U-shaped sinus in the anterior chest wall. Compelling as the mapping relationship is, little is known about the molecular aetiology of either CPS or acropectoral syndrome. A recent report, however, showed that the autosomal recessive acheiropodia (Ianakiev et al. 2001) results from a small deletion within the *LMBR1* gene. Acheiropodia is a severe limb-specific phenotype and, in contrast to PPD, patients present loss of all bones of the hands and feet, and the tibia is truncated distally. Analysis of five families with acheiropodia (Ianakiev et al. 2001) identified a critical region for the mutation on 7q36 and subsequently affected individuals showed deletions in both *LMBR1* alleles that remove exon 4 and approximately 5–6 kb of surrounding genomic DNA (Fig. 3). These data were interpreted as suggesting that acheiropodia results from loss of function of the *Lmbr1* gene.

Model for the role of Shh in limb defects

We predict that the inserted transgene in the *Ssq* genome, expression of which is activated in an *Shh*-like limb pattern, marks a proximate regulator which drives normal *Shh* expression. Thus the regulators responsible for normal as well as abnormal expression of *Shh* may reside in or near the *Lmbr1* gene. The analysis so far reported does not distinguish between regulation dependent on chromatin or chromosome structure or a traditional enhancer-like element. Clearly, a number of distinct regulatory activities are required to promote the long-distance regulation of *Shh* (Fig. 4). First, the regulator must convey an early limb bud signal over a distance of 1 Mb to initiate and drive *Shh* expression. Secondly, since *Lmbr1* is located within a cluster of tightly linked genes, none of which is expressed in the limb, the regulator must be able to control *Shh* expression without affecting that of the nearest genes. Thirdly, elements which drive *Shh* expression in the anterior margin of the limb bud exist but must normally be repressed.

Further predictions suggest that the long-distance *Shh* regulator is defective in the limb dysmorphologies that map to human chromosome 7q36. Disruption of different components of the regulator lead to the spectrum of dysmorphic phenotypes described. Pre-axial polydactyly, we submit, results from disruption of the proposed repressor of anterior limb bud expression. The location of the *Ssq* transgene insertion site and the PPD chromosomal translocation breakpoint suggests that the approximate genomic location of this repressor is in or near intron 5 of the *Lmbr1* gene. This repressor normally acts to suppress anterior expression; the genetic mutations, however, disrupt this repressor and enable the misexpression of *Shh*. Since PPD is relatively common in the human population (Heutink et al. 1994), it seems likely that a number of independent mutations interrupt the repressor element.

Furthermore we hypothesize that *LMBR1* is also incidental to the acheiropodia phenotype and propose an alternative rationale (Fig. 4). The ~5-kb acheiropodia deletion from within the *LMBR1* gene removes exon 4 and surrounding intronic sequences. Consistent with the proposal for generating polydactyly, we suggest that the acheiropodia mutation disrupts an *SHH* regulatory element; however, the mechanism is distinct from PPD in that normal ZPA expression of *SHH* is disrupted. These regulatory elements responsible for the phenotype may lie in the deleted region surrounding exon 4. Comparison of the acheiropodia phenotype with the mouse *Shh* targeted deletion (Chiang et al. 1996) is suggestive that loss of *Shh* plays a key role. In $Shh^{-/-}$ mutant mice the limbs show loss of all bones of the feet and truncations of the long bones, similar to that seen in the acheiropodia patients (Chiang et al. 2001; Kraus et al. 2001). We suggest that, in contrast to *Ssq* and PPD, acheiropodia results from a limb-specific loss of *Shh* expression. Thus, depending on the type of mutation, distinct phenotypes can arise. At present there is no hypothesis as to how CPS and acropectoral syndromes are caused. Molecular analysis of the proposed *Shh* regulators will be required for a clearer

understanding of normal regulation and the relationship to these limb dysmorphologies.

Conclusions

The door is now open for the molecular analysis of limb-specific regulation of *Shh* to help answer some of the outstanding questions presented by the genetics of these limb abnormalities. Foremost is the issue surrounding the nature of regulatory elements disrupted by the mouse and human mutations. These elements are located ~1 Mb away from the target gene. Thus are these elements the basis for moulding chromatin or chromosomal structure pertinent to *Shh* transcriptional activity? Alternatively, can enhancer elements act over such a distance of the chromosome to regulate a gene? It is difficult to understand why regulatory elements would reside at such a distance from the gene they regulate, and in the complicating circumstances of lying closer to unaffected genes. Such an arrangement of putative regulators along the chromosome may aid in pinpointing both transcriptional activators and silencers and the extreme distance may help in uncovering the chromatin interactions which presumably occur.

A second conundrum suggested by the analysis discussed above is the anterior ectopic expression of *Shh*. Speculatively, a molecular consequence of normal expression may be the ectopic anterior expression in the limb bud. To achieve the normal asymmetric pattern, the anterior expression must therefore be repressed. Interestingly, early tetrapods, such as *Acanthostega* and *Ichthyostega*, had large footplates composed of 7–8 digits (Kardong, 1998), perhaps a product of a primitive, unrepressed pattern of *Shh* expression. Candidate molecules involved in anterior repression of *Shh* are suggested by other mouse mutants of the *hemimelia-luxate* group, and presently the best candidate is *Alx4*. Further studies will possibly shed more light on both the normal regulation of *Shh* and the mechanisms for generating the disease phenotypes.

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