INTRODUCTION

Vertebrate limb development requires an interacting network of signalling molecules to mediate patterning and growth (for review see Johnson and Tabin, 1997). A specialised thickened epithelium located around the distal tip of the limb, called the apical ectodermal ridge (AER), appears in the early stages of limb bud outgrowth and mediates development along the proximodistal axis (Saunders, 1948; Summerbell et al., 1973). This structure is a source of signaling molecules of the FGF family, which are implicated as the basis for AER activity (Niswander et al., 1993; Savage et al., 1993, Vogel and Tickle, 1993, Crossley et al., 1996).

\[ \text{Fgf8} \]

is expressed in the initiating limb bud in the distal epithelium, which subsequently gives rise to expression in the AER (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Fgf4 is expressed in a restricted region found in the posterior two-thirds of the AER (Niswander et al., 1993).

Previous work suggests that the expression of Fgf genes in the AER is induced and/or maintained by factors expressed in the limb mesenchyme. Production of Fgf10 in limb mesenchyme appears to induce expression of Fgf8, first in the distal epithelium and subsequently in the AER (Ohuchi et al., 1997). Fgf8, consequently, maintains the expression of Fgf10 in the progress zone (the underlying mesenchyme) (Ohuchi et al., 1997), which maintains the growth potential and the undifferentiated state of this region (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996).

The asymmetric expression of Fgf4 has been linked to signals that pattern the skeletal elements of the limb along the anteroposterior axis. Signals emanate from the posterior margin of the limb called the zone of polarising activity (ZPA) (Saunders and Gasserling, 1968; Tickle et al., 1975, Tickle, 1981). Sonic hedgehog (Shh) is the molecule responsible for polarising activity in the limb bud and its expression colocalises with the mapped polarising region (Riddle et al., 1993; Chang et al., 1994; Francis et al., 1994; Laufer et al., 1994). Fgf4 in the AER is expressed as a response to Shh and, conversely, was suggested to be the signal that maintains the polarising activity of the posterior mesenchyme (Niswander et al., 1993). A proposed positive feedback loop between Fgf4 and Shh suggests that FGF4 is the signalling molecule that is primarily responsible for the maintenance of Shh and, correspondingly, that SHH mediates the expression of Fgf4 (Niswander et al., 1994; Laufer et al., 1994).

Mutations in the mouse provide the basis for further
examination of limb developmental mechanisms and for identifying novel limb genes. A group of limb mutants classified as the preaxial hemimelia-luxate group (Gruneberg, 1963; Johnson, 1986) is characterised by two features; firstly, partial or complete loss of the tibia leading to luxation, or twisting, of the limbs and, secondly, a wide range of preaxial (anterior) digit abnormalities. Dominant hemimelia (Dh) is a semidominant mutation originally described by Carter (1954) and is a member of this group of mutations. Dh has pleiotropic effects (Searle, 1964; Green, 1967); the homozygous condition, similar but more severe than the heterozygote, is lethal at about the time of birth. The mutation affects development of the viscera, in particular splanchnic mesoderm, stomach, kidney and urogenital system, and all mutants are asplenic. Axial skeletal abnormalities include loss of a single lumbar vertebrae, and reduced numbers of ribs and sternabrae (Green, 1967; Suto, 1996).

In this study, we focus on the role of the Dh mutation on formation of the limb. Dh affects the preaxial side of the hindlimbs (Searle, 1964); the forelimbs are unaffected. In heterozygotes, the hindlimb phenotype is highly variable. In mild cases, only digit I (the hallux) is affected; defects range from broadening of this digit to triphalangy. In more overtly affected mice, a surprising contrast is seen ranging from polydactyly with the addition of up to two additional digits to oligodactyly (loss of digits) with associated partial loss of the tibia (hemimelia). In homozygous fetuses, the hindlimb is affected bilaterally and with high penetrance. These animals are missing one or two preaxial digits and show complete absence of the tibia, whereas the fibula is unaffected. We show here that the Dh mutation acts at or soon after the time of limb induction to affect the pattern of gene expression in the AER. Fgf4 and Fgf8 are expressed in anterior patterns in the absence of changes in the mesenchymal expression of Shh or Fgf10. Also we show the size, in particular the width, of the bud is significantly reduced. Narrow limb buds are associated with preferential loss of anterior mesenchyme; loss of this tissue is responsible for the severe preaxial defects observed.

MATERIALS AND METHODS

Production and genotyping of Dh embryos

Crosses were set up between Dh/+ males and females and the day the vaginal plug was detected was considered as embryonic day 0.5 (E0.5) of development. The Dh mutation is kept on a predominantly C57BL/6 background. Genomic DNA was isolated from fetal yolk sacs as described by Laird et al. (1991). Embryos were genotyped using PCR to amplify a polymorphic repeat mapping close to the Dh mutation (primers: 5'-GGAAGATGTTCTAGTTTCTA TGG-3' and 5'-AGCCTGGAGGCTTAACTAGCAC-3'). The PCR conditions were as follows; the DNA was initially melted at 94°C, for 3 minutes; followed by 30 cycles at a melting temperature of 94°C for 30 seconds; an annealing temperature of 57°C for 45 seconds; and an elongation temperature of 72°C for 45 seconds. The final cycle contained a final incubation at 72°C for 5 minutes.

Cartilage and bone staining

The protocol used for bone staining is modified from Kessel and Gruss (1991). Embryos aged E17.5 were dissected out and fixed in 100% ethanol for 3 days or longer. After removing the skin the embryos were transferred to 100% acetone for 3 days and then stained for 3

Fig. 1. Examination of hindlimbs in Dh mutant fetuses. External view of wild-type (A) and homozygous Dh (B) mice at E14.5. Brackets highlight the area that is affected; in the Dh limb the shaft of the lower leg is about half as wide as wild-type and two anterior (preaxial) digits are missing. The bones of the lower hindlimb (E17.5) are shown for wild type (C) and Dh/Dh (D). D shows that no remnant of the tibia is apparent and digits I and II are missing. Analysis of the bones of the hind foot in wild type (E) and Dh/Dh (F) in which two preaxial digits are missing. The bones of the Dh foot that do appear are unaffected and articulation is normal. The outlines of the chondrifying digits are seen in E12.5 embryos in wild type (G) and Dh/Dh (H) by in situ hybridisation to Hoxd12. The digits are numbered by Roman numerals; the preaxial side starts with digit I. Abbreviations used are tibia (ti), fibula (fi), calcaneous (ca), cuboid (cu), cuneiform 1,2 and 3 (c1,c2, and c3), and talus (ta). Scale bar, 200 mm.
Dominant hemimelia disrupts hindlimb development

Days in staining solution [1 volume of 0.3% alcian blue in 70% ethanol, 1 volume of 0.1% alizarin red S in 95% ethanol, 1 volume of 100% acetic acid and 17 volumes of 100% ethanol] at 37°C. After rinsing the embryos were kept in 1% aqueous KOH until the skeleton became visible 12-24 hours. The embryos were then placed in 1 volume of glycerol and 4 volumes of 1% KOH at room temperature until the tissue was clear. For storage, the specimens were transferred into aqueous 50% glycerol.

In vitro transcription

Linearised plasmid DNA was used as template for the incorporation of DIG or fluorescein into in vitro transcribed RNA probes. In vitro transcription was carried out using the DIG RNA Labelling Kit (fluorescein RNA labeling mix was substituted where appropriate) according to manufacturer’s instructions (Boehringer-Mannheim).

Whole-mount in situ hybridisation

Single-labelled and double-labelled whole-mount in situ hybridisations were carried out as described by Hammond et al. (1998) and Hecksher-Sørensen et al. (1998), respectively. The probes used are Msx1, Alx4, Bmp4 (B. Hogan), Fgf4 (L. Niswander), Fgf8 (I. Mason), Fgf10 (S. Bellusci), Hoxd-12 (P. Chambon), Shh (A. McMahon) and Sox9 (R. Lovell-Badge).

RESULTS

Dh limb phenotype

The Dh/+ hindlimb phenotype is highly variable displaying a broad range of effects (Searle, 1964; Green, 1967). The least severely affected animals had either a slightly thickened or a tripalangeal digit I, while others show anterior (preaxial) polydactyly with one or two extra digits (data not shown). The most severely affected heterozygotes had lost anterior digits and the distal portion of the tibia. The homozygous Dh/Dh mice were much less variable. The majority of the fetuses had preaxial deletions displaying bilateral tibial amelia (complete loss of the tibia) and loss of at least one digit (Fig. 1A,B). The skeletal elements of the homozygous limb present at E17.5 were the femur, which may be shortened (not shown), and the fibula, which may be slightly bent but in all other respects was normal (Fig. 1C,D). In all cases examined, there was no remnant of the tibia detectable. The remaining bones in the oligodactylous foot appeared normal (Fig. 1E,F). The posterior tarsals such as the calcaneous, cuboid and the posterior cuneiforms were present, and articulated with the correct metatarsals. The distal elements such as the phalanges of the digits that appear (digits III, IV and V in Fig. 1F) were normal.

At earlier embryonic stages (E10.5), mutant limb buds were substantially narrower than wild type. Some Dh/+ buds were slightly but detectably smaller; whereas Dh/Dh buds were about half the width and the AER was accordingly shorter (compare Fig. 2A with B,C).

Relationship of Fgf4 and Shh expression domains

Shh is expressed in the distal posterior margin of the limb bud representing the zone of polarising activity (ZPA) (Riddle et al., 1993; Chang et al., 1994). Shh was first detected at about E10.5 (Fig. 2D), still present at E11.5 (Fig. 2J) but by E12.5 was undetectable. In the Dh/+ heterozygotes, Shh expression initiated at the appropriate time and continued to be expressed (E11.5) in the posterior domain. (Fig. 2E,K). In Dh/Dh limb

Fig. 2. Whole-mount in situ hybridisation comparing Fgf4 and Shh expression in the developing hindlimb. Embryonic limb buds from E10.5 (A-F), E11.5 (G-L) and E12.5 (M) were hybridised with probes for Fgf4 (A-C, G-I) and Shh (D-F, J-M). Posterior side of each limb is to the left. Fgf4 expression in wild-type limb buds (A,G) is in the apical ectodermal ridge (AER) covering the posterior two-thirds. In Dh/+ (B,H) and Dh/Dh (C,I), the expression has shifted to the anterior. In the Dh mutants at E11.5 (H,I), posterior expression is undetectable and Fgf4 expression is at the anterior extreme. The arrows in G-I represent the AER morphological boundary and the arrowheads the anterior extreme of expression. Shh expression (D-F, J-L) is found at the posterior mesenchymal margin. Notice by E11.5 (J-L) there is no association between expression of Fgf4 and Shh. Expression of Shh in the Dh/Dh limb (L) appears to extend apically; however, this is probably due to a slight curvature of the limb bud. At E12.5 a number of Dh/+ limb buds (M) show overgrowth of anterior tissue (marked by an asterisk) and anterior ectopic expression of Shh (arrow). Scale bar, 200 mm.
buds at E10.5 and E11.5. 

Both Fgf4 and Fgf8 are expressed in the AER of the limb and are factors that stimulate limb outgrowth (Niswander et al., 1993; Crossley et al., 1996; Vogel et al., 1996). Fgf4 expression was detected in the initial stages of AER production at E10.5 (Fig. 2A) and, as previously reported, overlies the expression of Shh (Niswander et al., 1994; Laufier et al., 1994). At E11.5, detection of Fgf4 was in the posterior two-thirds of the AER overlying the Shh domain (Fig. 3G). Fgf4 expression in Dh/+ embryos initiated at the appropriate stage but in contrast to the wild type, the normal posterior pattern had shifted to the anterior half of the AER (Fig. 2B). By E11.5, no Fgf4 expression was detected in the mutant AER directly underlying the Shh domain (Fig. 2H). The Fgf4 expression was anteriorised in the hindlimbs (arrowhead in Fig. 2H); Shh expression continued in the normal posterior domain. In Dh/Dh, the whole of the limb bud was narrow and the initial expression of Fgf4 was biased toward a small anterior AER domain (Fig. 2C). By E11.5, a small focus of expression was apparent at the extreme anterior end of the AER (arrowhead in Fig. 2I).

**Fgf8 expression in the AER**

Fgf8 is detectable prior to the expression of Shh and, in agreement with others (Heikinheimo et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996), we detected expression in a stripe of epithelial cells at the apex of the bud at E10.5 (Fig. 3A). Fgf8 was expressed through the whole of the AER in the wild-type embryo but gradually decreased in level towards the posterior margin (Fig. 3D). In the mutant, similar to Fgf4, the Fgf8 expression pattern had shifted towards the anterior margin. At E10.5, Fgf8 already showed a significant anterior bias in epithelial expression in both Dh/+ and Dh/Dh (Fig. 3B,C) and, by E11.5, the domain of highest Fgf8 expression was in the anterior extreme (Fig. 3E,F). The limb buds in Fig. 3D,E are double labelled (Hecksher-Sørensen et al., 1998) to show expression of Shh (blue) in comparison to the Fgf8 (purple) domain. Dh/+ limbs, allowed to stain for an extended period, showed that Fgf8 was detectable in the posterior domain to the full extent of the AER overlying the Shh domain (Fig. 3E). The posterior AER is therefore capable of expression, albeit at a significantly reduced level. Reflecting Fgf4 expression, Fgf8 in the homozygote showed only a small anterior expression domain in the AER with significantly lower posterior expression (Fig. 3F). Thus from early stages of AER formation, the expression of both Fgf4 and Fgf8 was reset in the Dh mutants showing a clear anteriorisation of the pattern.

Not all gene expression in the AER was affected. At E10.5, Bmp4 was expressed in the posterior mesenchyme in a region that overlapped that of the Shh domain and in the AER, throughout most of the length (Fig. 3I). At the posterior margin of the limb bud, the AER expression overlapped that of the mesenchyme (arrow in Fig. 3J). In Dh/+ and Dh/Dh, there was no appreciable anterior shift in AER expression and the degree of overlap of Bmp4 in the AER and the mesenchyme remained unaffected (Fig. 3K,L). Thus the AER anterior shift of Fgf4 and Fgf8 in the mutant limb occurred in a background of normal expression for, at least, Bmp4 in the AER.

**Relationship of Fgf8 and Fgf10 expression**

Fgf10 was expressed in the mesenchyme at the earliest limb stages (Ohuchi et al., 1997; data not shown) and, by E12, was expressed in the distal region under the AER (Fig. 3G). In Dh/+ and Dh/Dh, the pattern of Fgf10 mesenchymal expression appeared to shift anteriorly accompanying the expression of Fgf8; i.e., Fgf10 expression is elevated in the anterior half of the hindlimb mesenchyme underlying the highest expression of Fgf8 (Fig. 3H). In contrast, in the homozygous mutant, mesenchymal expression is low throughout the width of the limb bud, which may reflect the low overall expression of Fgf8 in the AER. In Dh/Dh limb buds, the pattern of Fgf10 did not directly relate to the anteriorised expression of Fgf8 in the AER (Fig. 3I).

**Dh disrupts anterior mesenchyme formation**

Since Dh/Dh embryos have appreciably narrower limb buds and the latter fetal stages are lacking anterior digits, genes expressed in the anterior region of the limb were examined. Mxsl was expressed in the E10.5 embryo throughout the distal and lateral mesenchyme (Fig. 4A); the anterior expression level was higher than in the distal and posterior domains. In Dh/+ embryos, the anterior Mxsl expression was present; however, the domain appeared slightly narrower (13 embryos examined) (Fig. 4B). In contrast, no Dh/Dh embryo (5 embryos examined) showed an anterior expression domain; whereas, posterior expression appeared normal (Fig. 4C). The Alx4 expression is restricted to the anterior limb margin (Fig. 4D) (Qu et al., 1997, 1998; Takahashi et al., 1998), but in a narrower domain than Mxsl. In the Dh/+ limb buds, partial loss of the expression of this anterior marker is more obvious. In Dh/Dh animals, this expression is completely lacking (Fig. 4F). Thus the Dh mutation, in a dose-dependent manner, selectively deleted the anterior mesenchyme in the early bud stages.

The Sox9 gene is expressed in the condensing mesenchyme of the early bone primordia of the limb (Wright et al., 1995). At E11.5, the chondrogenic condensations that constitute the digital arch, and the long bones of the zeugopod (tibia and fibula) and the stylopod (femur) were apparent (Fig. 4G). Dh/Dh limb buds were not significantly different from the wild type (Fig. 4H). However, the number of embryos examined (n=5) may not reflect the full range of heterozygous abnormalities. In Dh/Dh embryos (n=4), Sox9 was expressed in a pattern that suggested that the condensing mesenchyme for the femur and only one of the long bones of the zeugopod was present (Fig. 4I). Based on the skeletal elements that appear later, it was likely that the posterior long bone, the fibula, was the long bone that formed. Also the width of the digital arch was compromised, predictive of the loss of digits. At E12.5 the presumptive digital elements are highlighted by the expression of Hoxd12 (Fig. 1G,H) showing the formation of the posterior digits in Dh/Dh. Thus loss of the anterior skeletal elements in Dh/Dh was not due to degeneration or to a developmental delay but rather resulted from the lack of anterior tissue from which the skeletal elements form.

**Ectopic ZPA relates to extra anterior digits**

A proportion of Dh heterozygous mice are born with extra preaxial digits. Analysis of Shh at E12.5 (a time at which normal, posterior expression of Shh was undetectable) showed, in a number of embryos, an ectopic region of Shh expression at the anterior margin of the limb bud. The ectopic expression
was associated with an additional anterior mass of mesenchyme (Fig. 2M). A number of Dh heterozygotes did not show ectopic Shh expression (10 of 24 hindlimbs examined). Shh ectopic expression and the anterior mesenchymal mass was most likely associated with the proportion of Dh/+ embryos that would develop polydactyly. Associated with the ectopic Shh domain was expression of Fgf4 in the overlying ectoderm and expression of the HoxD limb genes (Laufer et al., 1994) in the ectopic mass (data not shown). The homozygotes at the stages investigated (at E12-12.5, 12 embryos examined) never showed ectopic expression at the anterior margin or an additional mass of mesenchyme. This, again, relates to the digit phenotype of the homozygous mutant in which not only are there no supernumerary digits but in all cases anterior digits are missing.

DISCUSSION

Alterations in AER organisation

Expression patterns of both Fgf4 and Fgf8 in the AER were anteriorised in the Dh mutant hindlimb buds. In the Dh/+ embryos, the polarity of Fgf4 in the AER was reversed such that the anterior and not the posterior half expressed. Similarly, Fgf8 expression was high in the anterior half and lower but, in contrast to Fgf4, detectable in the posterior half. The posterior persistence of Fgf8 in Dh/+ showed that indeed the AER extended to the domain that overlies SHH. In addition, the Bmp4 expression pattern was normal in the mutants further indicating that the integrity of the AER was not grossly affected by the mutation. Other mutants of the hemimelia-luxate group (Chan et al., 1995; Masuya et al., 1995, 1997; Büscher et al., 1997) have previously been examined. These mice show anterior expression of Fgf4 and Fgf8 (when examined) in the AER, but only at late stages in the presence of normal posterior expression and in conjunction with the production of ectopic anterior tissue and Shh expression. The altered Fgf expression domains in the Dh limb buds are not ancillary to normal expression but rather represent re-specification of the initial expression patterns.

Association of Fgf4 and Fgf8 with mesenchymal expression in Dh mutants

Regulation of Shh in the polarising region and Fgf4 in the outlying AER is postulated to be coupled in a co-ordinately regulated positive feedback loop (Niswander et al., 1994; Laufer et al., 1994). In chick, exogenous addition of SHH induces and maintains expression of Fgf4; whereas removal of the outlying AER results in downregulation of native Shh. In these studies, Shh expression is maintained if Fgf4 is reintroduced. In mouse, genetic evidence of a link is supported by the ld (limb deformity) mutant, which fails to maintain limb Shh expression and in accord Fgf4 in the AER is not detected (Haramis et al., 1995; Kulman and Niswander, 1997).

In Dh mutant embryos, a direct association between Fgf4 and Shh was not apparent. Shh expression persists in the normal posterior domain in the absence of outlying Fgf4. Conversely, Fgf4 was expressed in the anterior half of the AER in the absence of detectable ectopic Shh. The postulated Fgf4- and Shh-positive feedback loop was uncoupled by the Dh mutation.

There is no evidence that Fgf8 and Shh in the limb are co-ordinately regulated. Studies in the ld mouse mutant support this suggestion and show that Fgf8 continues to be expressed in the absence of Shh (Haramis et al., 1995; Kulman and Niswander, 1997). Ohuchi et al. (1997), however, suggest the possibility that Fgf8 may act to maintain expression levels of Shh. The Dh mutation enabled examination of this possibility since (as discussed above) posteriorly expressed Fgf4 is absent. Fgf8 expression in Dh/+ embryos, although anteriorised, persists in the posterior AER at appreciable but lower levels. However, in Dh/Dh embryos, the posterior expression level is further reduced and in most, but not all, cases examined is undetectable. In this environment of significantly reduced levels of Fgf8 and undetectable levels of Fgf4, Shh expression continues at a readily detectable level.

Furthermore Fgf8 is suggested to participate in a positive feedback loop with Fgf10 in the mesenchyme. Fgf10 is expressed in the distal limb mesenchyme of the progress zone and is essential for limb outgrowth (Sekine et al., 1999). In Fgf10-deficient mice, there is neither Fgf8 ectodermal expression nor AER formation. In later stages of development, Fgf10 expression is downregulated upon removal of the AER and is induced by implantation of Fgf8-expressing cells (Ohuchi et al., 1997). However, in the Dh/Dh mutant embryo, the anteriorised Fgf8 expression pattern in the AER occurred in the absence of a concomitant shift of Fgf10 in the underlying mesenchyme. Again, Dh appears to uncouple proposed epithelial-mesenchymal interactions in the limb, in this case expression of Fgf8 and Fgf10, suggesting that no reciprocal regulatory loop exists between these two genes.

How does Dh alter the pattern of AER expression in the hindlimb? The data suggest that Dh is acting as an intermediate that links the expression of the two Fgfs. However, it is unlikely that Dh is acting directly to regulate both Fgf4 and Fgf8 since these two genes are independently expressed in the limb. Since expression of Fgf4 and Fgf8 is altered by Dh from the earliest stages analysed, we suggest that Dh is operating at an early, fundamental level in a process that is specific for the hindlimb.

The mechanism of initiating and positioning the limb along the flank of the embryo differs between forelimbs and hindlimbs. For example, genes within the Hox clusters are involved in positioning initial limb outgrowth, a different combination operating for forelimb and hindlimb placement (Burke et al., 1995; Cohn et al., 1997). The lack of the BMP-related growth factor Gdf11 results in the posterior displacement of the hindlimbs without disturbing the forelimb (McPherron et al., 1999). Dh is also important in this process, since the Dh mutation alters hindlimb positioning resulting in an anterior shift equivalent to the distance of 2-3 somites (Searle, 1964; Green, 1967; personal observations). The observations, reported here, that the initiating limb bud is narrower in the mutant supports the suggestion that Dh has an early, key role in limb development. We suggest that specification of AER gene expression occurs at the time of limb initiation and may be subject to the mechanism that defines the position of the limb bud.

Polydactyly in Dh heterozygous embryos

In some Dh/+ embryos, the autopod is as large as wild type and often contains a prominent ectopic anterior mass of tissue. Presumably, the ectopic tissue is the source for the preaxial
skeletal expansion that results in triphalangial digit I and the frequent addition of supernumerary digits. Ectopic Shh occurs at approximately E12.5 and is associated with the appearance of the ectopic mass of tissue. The ectopic expression is presumably responsible for organisation of the enlarged autopod into preaxial skeletal elements. In contrast, other hemimelia-luxate mutants analysed, such as \( Xt \), \( Hx \), \( lst \) and \( Rim4 \) (Chan et al., 1995; Masuya et al., 1995, 1997; Büscher et al., 1997), give rise to supernumerary preaxial digits, but show ectopic production of Shh at an earlier stage (at least 24 hours earlier) that precedes the appearance of an enlarged autopod. This group of mutations is predicted to act by de-repressing an anterior Shh expression domain that is subsequently responsible for extra digit formation. In this

Fig. 3. Expression of Fgf8 in the AER and comparison to gene expression in the underlying mesenchyme. Expression of Fgf8 is shown at E10.5 (A-C) in the whole of the AER in wild type (A) and shifted anteriorly in Dh/+ (B) and Dh/Dh (C). Double-label in situ hybridisations show Fgf8 expression in the AER (purple) in combination with expression of Shh (blue) at E11.5 (D-F) and Fgf10 (blue) at E12 (G-I). Posterior side of each limb is to the left and the arrows mark the posterior margin of the AER. Fgf8 expression in wild type (D,G) extends throughout the AER at E11.5 and at E12 is most highly expressed at the apex. Shh is seen at the posterior margin; Fgf10 expression underlies the AER in the distal mesenchyme. In Dh/+ embryos (E,H), Fgf8 has shifted anteriorily showing a higher concentration in the anterior AER. At E11.5 (E), Fgf8 expression is detected extending to the posterior limit of the AER (shown by arrow). At E12 in the Dh/Dh limb buds (F,I), Fgf8 expression resides at a small anterior focus (arrowhead). Shh and Fgf10 mesenchymal expression is retained and does not appear to respond to the Fgf8 shift. In wild type, Bmp4 (J) is expressed in the posterior mesenchyme and broadly throughout the AER. The posterior limit of Bmp4 AER expression and the anterior limit of the mesenchyme expression (indicated by arrowheads) overlap. The degree of AER overlap with the mesenchyme expression is similar in wild type compared to Dh/+ (K), and Dh/Dh (L). Scale bar, 200 mm.

Fig. 4. Analysis of anterior mesenchymal expression in Dh mutant hindlimbs. Msx1 (A-C) and Alx4 (D-F) at E10.5 in wild-type limbs (A,D) show strong expression in the mesenchyme at the anterior margin. Msx1 is also expressed at a lower level in the posterior and distal mesenchymal margins. (Posterior margin of each limb is situated toward the bottom.) In a number of Dh/+ embryos (B), the anterior domain of Msx1 (indicated by a white arrowhead) is detectably smaller. In the Dh/Dh limb (C), the anterior domain of expression is lacking; whereas, posterior and distal expression are unaffected. The Alx4 domain in the Dh/+ limb (E) is reduced in size and the distal limit (arrowhead) appears more proximal. In Dh/Dh (F), the anterior expression is lacking; a small domain of proximal expression remains. Sox9 expression marks the condensing mesenchyme that will form the skeletal elements of the limb. In wild-type limb buds (G) at E11.5, the two presumptive long bones, the tibia (indicated by the arrow) and the fibula (indicated by the arrowhead) are apparent. Dh/+ embryos (H) also show a similar expression pattern; whereas, Dh/Dh (I) shows only half the wild-type pattern with one of the presumptive long bones (the fibula) apparent (indicated by the arrowhead). Also the digital arch is reduced in size. Scale bar, 200 mm.
respects, the formation of supernumerary digits in Dh/+ does not conform with other members of the luxoid group. We suggest that the ectopic growth of anterior tissue results from the increased concentration of Fgf4 and Fgf8 in the anterior AER from which the production of Shh follows.

Oligodactyly and tibial hemimelia in Dh/Dh

In Dh/Dh embryos, the predominate phenotype is loss of anterior digits associated with complete loss of the tibia. When compared to wild-type littermates, Dh/Dh embryos have narrower hindlimb buds that are approximately half normal width with a correspondingly shorter AER. Analysis of Alx4 and Msx1 gene expression in the limb mesenchyme shows that anterior tissue is lacking in preference to posterior tissue. Furthermore the expression of Sox9 in the early stages of chondrogenesis shows that anterior skeletal structures are not produced; whereas, development of the posterior elements proceeds normally.

The width of the limb is dependent on the dosage of the Dh allele. In Dh/+ embryos, although some limb buds appear of normal size, a number examined are significantly narrower than wild type, but less so than Dh/Dh. These limbs show a partial loss of the anterior markers Msx1 and Alx4. It is these heterozygous limbs that will probably show the loss of preaxial skeletal elements. It is evident that Dh has a role in hindlimb initiation, disruption of the gene reducing the size of the budding limb bud. The limb width subsequently determines the number of preaxial elements that develop. Dh may operate either by directly regulating the width of the bud such that anterior tissue is preferentially lost or by specifying anterior tissue and thereby reducing limb size. The posterior skeletal elements develop normally in this altered environment showing that neither interactions with anterior tissue nor the correct localisation of genes in the AER are important for skeletogenesis to occur.

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REFERENCES


Saunders, J. W., Jr. (1948). The proximo-distal sequence of origin of the parts
of the chicken wing and the role of the ectoderm. 


