Development 122, 2319-2330 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2083

The limb field mesoderm determines initial limb bud anteroposterior asymmetry and budding independent of *sonic hedgehog* or apical ectodermal gene expressions

Maria A. Ros[1], Alric López-Martínez[2], B. Kay Simandl[2], Concepción Rodriguez[3], Juan Carlos Izpisúa Belmonte[3], Randall Dahn[2] and John F. Fallon[2,*]

[1]Departamento de Anatomia y Biologia Celular, Universidad de Cantabria, 39011 Santander, Spain
[2]Department of Anatomy, University of Wisconsin, 1300 University Avenue Madison, WI 53706, USA
[3]The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA

*Author for correspondence (e-mail: jffallon@facstaff.wisc.edu)

SUMMARY

We have analyzed the pattern of expression of several genes implicated in limb initiation and outgrowth using *limbless* chicken embryos. We demonstrate that the expressions of the apical ridge associated genes, *Fgf-8, Fgf-4, Bmp-2* and *Bmp-4,* are undetectable in *limbless* limb bud ectoderm; however, FGF2 protein is present in the limb bud ectoderm. *Shh* expression is undetectable in *limbless* limb bud mesoderm. Nevertheless, *limbless* limb bud mesoderm shows polarization manifested by the asymmetric expression of *Hoxd-11, -12* and *-13, Wnt-5a* and *Bmp-4* genes. The posterior *limbless* limb bud mesoderm, although not actually expressing *Shh,* is competent to express it if supplied with exogenous FGF or transplanted to a normal apical ridge environment, providing further evidence of mesodermal asymmetry. Exogenous FGF applied to *limbless* limb buds permits further growth and determination of recognizable skeletal elements, without the development of an apical ridge. However, the cells competent to express *Shh* do so at reduced levels; nevertheless, *Bmp-2* is then rapidly expressed in the posterior *limbless* mesoderm. *limbless* limb buds appear as bi-dorsal structures, as the entire limb bud ectoderm expresses *Wnt-7a,* a marker for dorsal limb bud ectoderm; the ectoderm fails to express *En-1,* a marker of ventral ectoderm. As expected, *C-Lmx1,* which is downstream of *Wnt-7a,* is expressed in the entire *limbless* limb bud mesoderm. We conclude that anteroposterior polarity is established in the initial limb bud prior to *Shh* expression, apical ridge gene expression or dorsal-ventral asymmetry. We propose that the initial pattern of gene expressions in the emergent limb bud is established by axial influences on the limb field. These permit the bud to emerge with asymmetric gene expression before *Shh* and the apical ridge appear. We report that expression of *Fgf-8* by the limb ectoderm is not required for the initiation of the limb bud. The gene expressions in the pre-ridge limb bud mesoderm, as in the limb bud itself, are unstable without stimulation from the apical ridge and the polarizing region (*Shh*) after budding is initiated. We propose that the defect in *limbless* limb buds is the lack of a dorsal-ventral interface in the limb bud ectoderm where the apical ridge induction signal would be received and an apical ridge formed. These observations provide evidence for the hypothesis that the dorsal-ventral ectoderm interface is a precondition for apical ridge formation.

Key words: pattern formation, limb initiation, axial influence, dorsoventral compartments, Fibroblast Growth Factors, Hoxd genes, Sonic Hedgehog, Bone Morphogenetic Proteins, *Wnt-7a*, *C-Lmx1*, *Engrailed-1*

INTRODUCTION

The first indication of the chick wing bud is a condensation of somatopleural mesoderm opposite somites 15-20 at Hamburger and Hamilton stage 16 (Hamburger and Hamilton, 1951). The initial wing bud appears during stage 17 covered by a simple cuboidal epithelium (Todt and Fallon, 1984). During stage 18, the ectoderm at the distal margin of the bud differentiates into a pseudostratified columnar epithelium that is called the apical ectodermal ridge (Saunders, 1948; Todt and Fallon, 1984). Classical transplantation experiments have shown that the limb mesoderm induces the apical ridge in the overlying ectoderm and that the flank ectoderm also has the ability to form an apical ridge when in contact with limb field mesoderm (Kieny 1960, 1968; Saunders and Reuss, 1974; Carrington and Fallon, 1984a). If the apical ridge is surgically removed it does not regenerate and predictable proximodistal truncations result in the limb that develops (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). A variety of experiments demonstrate that the apical ridge gives a permissive signal that maintains the cells beneath it in

an undifferentiated and replicating state (Zwilling, 1955; Rubin and Saunders, 1972). The ridge then permits limb bud elongation and it is proposed that as cells leave apical ridge influence they differentiate (Summerbell et al., 1973).

Two other signaling centers corresponding to the anteroposterior and dorsoventral limb axes have been described. The anteroposterior limb axis is assumed to be controlled by a region of mesoderm along the posterior border of the limb bud; these cells will induce mirror-image duplications of the anteroposterior axis if grafted to the anterior margin of the wing (Saunders and Gasseling, 1968; Tickle et al., 1975). The third signaling center is the ectoderm that appears to mediate patterning of the dorsoventral axis (Pautou and Kieny, 1973; MacCabe et al., 1974; Riddle et al., 1995; Vogel et al., 1995).

Several genes have been identified whose products are related to the activities of these signaling centers. Members of the fibroblast growth factor (FGF) family are expressed by the apical ridge, including *Fgf-2* (Savage et al., 1993; Dono and Zeller, 1994; Savage and Fallon, 1995), *Fgf-4* (Suzuki et al., 1992; Niswander and Martin, 1992) and *Fgf-8* (Heikinheimo et al., 1994; Ohuchi et al., 1994; Mahmood et al., 1995; Crossley and Martin, 1995; Crossley et al., 1996; Vogel et al., 1996). Each of the three proteins functionally substitute for the apical ridge (Niswander et al. 1993; Fallon et al., 1994; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). The expression of *Sonic hedgehog* (*Shh*) in the limb bud spatially and temporally colocalizes with the mapped polarizing region (Riddle et al., 1993). Ectopic expression of *Shh* (Riddle et al., 1993; Chang et al., 1994) or application of N-SHH protein (López-Martínez et al., 1995) at the anterior border of the limb causes duplications comparable to polarizing region grafts. Consequently, *Shh* is envisaged as the endogenous polarizing region signal. Finally, WNT-7a has been proposed as the factor responsible for the action of the dorsal ectoderm on dorsoventral patterning through the induction of the LIM homeobox gene C-*Lmx1* (Parr and McMahon, 1995; Yang and Niswander, 1995; Riddle et al., 1995; Vogel et al., 1995). It is proposed that the three signaling centers act in feedback loops that maintain their own unique syntheses and activate or maintain downstream gene expression (Laufer et al., 1994; Niswander et al., 1994).

Recent approaches have focused attention on the limb field and how initiation or budding of the limb bud is accomplished. Application of FGF1, 2, 4 or 8 to the embryonic chick flank results in ectopic limb induction (Cohn et al., 1995; Mima et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996). This suggests that one or more of the FGFs may be the endogenous signal responsible for limb induction, and when applied to a responsive tissue, initiates a cascade of events that results in the development of an additional limb. Of the different FGFs, FGF2 may be a candidate for the initial signal because it is expressed in the ectoderm, although its expression is widespread (Savage et al., 1993; Savage and Fallon, 1995). FGF4 is unlikely to be the endogenous signal because it is expressed in the posterior apical ridge only after the limb bud is initiated and the apical ridge is formed (Niswander and Martin, 1992; Laufer et al., 1994). *Fgf-8* is expressed throughout the limb field ectoderm very early, before apical ridge formation, leading to the hypothesis that it is required for limb initiation (Heikinheimo et al., 1994; Mahmood et al., 1995; Crossley and Martin, 1995; Crossley et al., 1996; Vogel et al., 1996).

Useful information regarding gene expression associated with budding may be obtained by study of the *limbless* mutant. *limbless* is a simple Mendelian autosomal recessive mutation that in the homozygous condition is characterized by chicks that lack wings and legs (complete amelia, Prahlad et al., 1979). Although the mutation causes defects in no other organs, it is effectively lethal because the chicks are unable to hatch without legs (Carrington and Fallon, 1984b; Lanser and Fallon, 1984). Previous studies have shown that limb buds arise normally in *limbless* embryos and are morphologically indistinguishable from normal embryos until stage 19. However, no apical ridge forms and the entire limb bud mesoderm undergoes cell death beginning at stage 19-20, such that all signs of the buds have disappeared by stage 24 (Fallon et al., 1983). Experiments recombining *limbless* limb bud or limb field mesoderm with normal ectoderm and vice versa demonstrated that the *limbless* mesoderm was able to induce and maintain an apical ridge (Fallon et al., 1983; Carrington and Fallon, 1988). This and other evidence leads to the conclusion that the *limbless* defect is in the ability to form an apical ridge. The unique properties of *limbless* embryos provide an excellent model for dissecting molecular mechanisms governing limb initiation.

MATERIALS AND METHODS

Manipulation of chick *limbless* mutant embryos

limbless mutant and normal embryos were obtained from a heterozygous mating flock maintained at the University of Wisconsin Poultry Science Department (Madison, WI). Homozygous mutant embryos have the *limbless* phenotype while heterozygous and homozygous wild type embryos have the normal phenotype. Eggs were routinely incubated and opened with a dental drill and sealed with Scotch Magic Transparent tape. At the earlier stages of limb development (up to stage 19) the *limbless* phenotype is not recognizable. To analyze gene expression in *limbless* embryos before the phenotype is recognizable, we removed the right wing buds from embryos at the appropriate stages, and allowed the embryo to develop to show the phenotype. Alternatively, for whole-mount in situ hybridization, we used large numbers of embryos of the appropriate stage where approximately one quarter of these embryos should be homozygous for the *limbless* gene. These two methods always gave the same results.

Polarizing activity assay

The *limbless* phenotype can be clearly recognized during stage 19. We assayed for polarizing activity in the posterior mesoderm of either wings or legs of stage 20 *limbless* embryos; both gave the same results. Two facts were noted: cell death begins in the mid-distal *limbless* mesoderm away from the posterior mesoderm; stage 20 *limbless* limb buds provided with a normal ectodermal jacket with a ridge will form limbs (Fallon et al., 1983). A small piece of *limbless* mesoderm from the region of maximal polarizing activity was grafted under the anterior apical ridge of stages 20-21 normal wing buds following the procedure of Tickle (1981). Similar pieces of mesoderm taken from the anterior margin of the limb bud were used as controls. After 7 more days of incubation the embryos were fixed, stained with Victoria blue and the digit pattern analyzed. The expression of *Shh* in the grafted tissue was analyzed 24 hours after the operation.

Polarizing activity was quantified by the method described by Vogel and Tickle (1993). An extra digit **4** was scored as 1.0, digit **3** as 0.67, digit **2** as 0.33 and an extra cartilage as 0.13. Only the most posterior digit was scored for each graft and the activity for a particular set was obtained by averaging.

Applications of FGF-soaked beads to *limbless* embryos

Affigel-blue beads (BioRad, 153-7301) or heparin acrylic beads (Sigma, H5263) were soaked in FGF1, FGF2 (0.5 mg/ml, and

1 mg/ml; gifts from B. B. Olwin), FGF8 (0.5 mg/ml; Vogel et al., 1996) or FGF4 (0.85 mg/ml; a gift from Genetics Research Institute, Cambridge, MA) for 1 hour. The beads were grafted mid-distally in the limb field of *limbless* embryos. The embryos were either allowed to develop for whole-mount cartilage staining, or fixed at succeeding times in 4% paraformaldehyde in phosphate-buffered saline for in situ hybridization.

In situ hybridization, whole mounts and tissue sections

Digoxigenin-labeled antisense riboprobes were prepared and used for whole-mount in situ hybridization according to the procedure of Nieto et al. (1995). ^{35}S-labeled riboprobes were prepared and the hybridization to tissue sections performed essentially as described by Wilkinson and Nieto (1993). The probes used were *Shh* (kindly provided by J. Dodd), *Fgf-4*, *Bmp-2*, *Bmp-4*, *Wnt-7a*, *Wnt-5a*, *Hoxd-11*, *Hoxd-12* and *Hoxd-13* (kindly provided by C. Tabin), *Fgf-8* and *C-Lmx1* (isolated by us), *En-1* (kindly provided by A. Joyner).

Immunohistochemical localization of FGF2

Performed as described by Savage et al. (1993) using monoclonal antibody 148.2.1.1. Briefly, paraffin sections were covered with primary antibody at a dilution of 1:1000 and incubated overnight in a humid chamber at 4˚C. After washing, biotinylated goat anti-mouse secondary antibody was applied. The slides were washed and incubated with streptavidin peroxidase according to the Vector protocol. Antibody localization was visualized using diaminobenzidine. As a control, the procedure was run without the primary antibody; these were always negative.

RESULTS

limbless ectoderm does not express apical ridge markers, but both dorsal and ventral ectoderm express *Wnt-7a* and FGF2 protein is present

In the normal chick limb bud *Fgf-4*, *Fgf-8*, *Bmp-2* and *Bmp-4* are expressed in the apical ridge. *Fgf-8*, *Bmp-2* and *Bmp-4* transcripts are detected throughout the length of the apical ridge while *Fgf-4* expression is restricted to the posterior apical ridge (Francis et al., 1994; Niswander and Martin, 1992; Laufer et al., 1994; Mahmood et al., 1995). *Fgf-8* is normally expressed in the ectoderm of the prospective limb territory and becomes progressively confined to the distal pre-ridge ectoderm and eventually to the ridge as it develops (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). We have analyzed the expression of these four apical ridge markers in *limbless* embryos from stage 16 through stage 20. The *limbless* phenotype is not discernible up to stage 19; consequently, for each of the above mentioned genes, we hybridized several dozen embryos of the *limbless* heterozygote matings before their phenotype was discernible. As the *limbless* mutation is only manifested in the homozygous condition, we expected that approximately 25% of the embryos would be homozygous and later display the *limbless* phenotype. Accordingly, we found that approximately 75% of the embryos exhibited the normal pattern of expression of *Fgf-4*, *Fgf-8*, *Bmp-2* and *Bmp-4* (Fig. 1; normal) while 21% (15 of

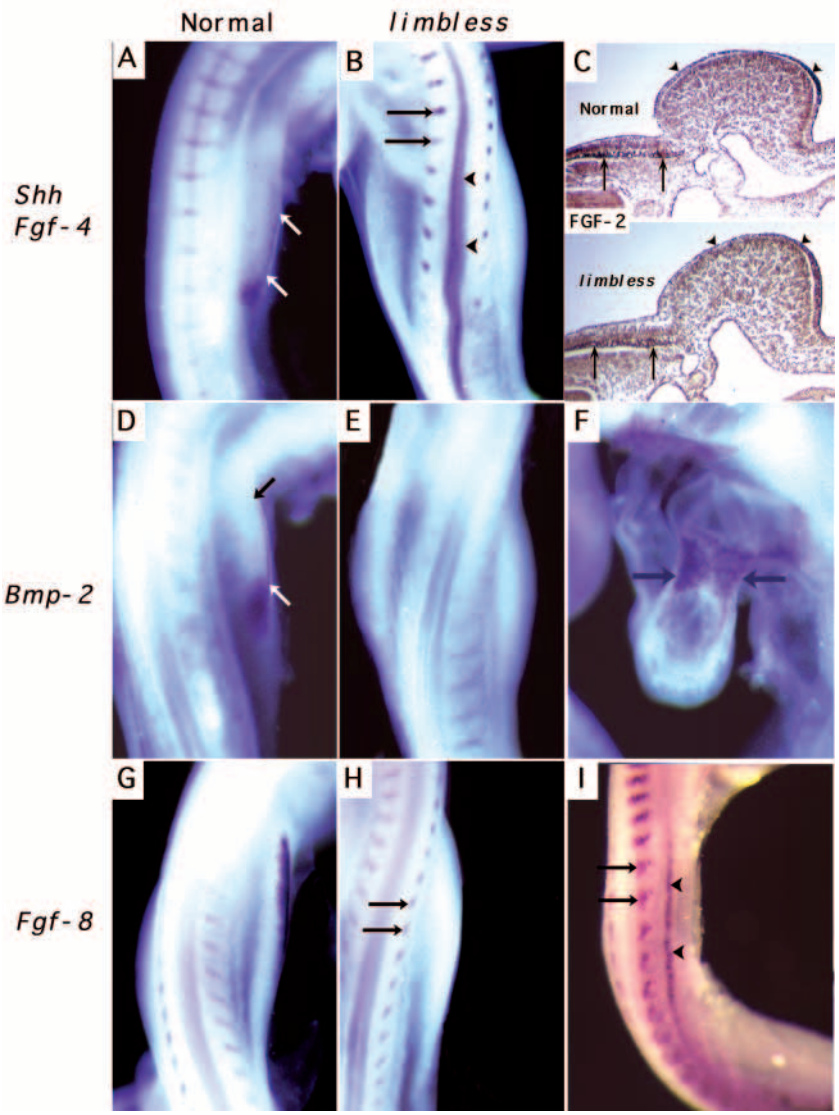

Fig. 1. Expression pattern of *Shh*, *Fgf-4*, FGF2, *Bmp-2* and *Fgf-8* in normal and *limbless* embryos. (A) Expression of *Shh* and *Fgf-4* in a normal chick embryo. The hybridization was performed for both genes with the same stain in order to allow better comparison of the results. Arrows indicate *Fgf-4* in the apical ridge. (B) *Shh* and *Fgf-4* pattern of expression in a *limbless* embryo. Note normal expression of *Shh* in floorplate (arrowheads) and of *Fgf-4* in somitic mesoderm (arrows) while there is no detectable expression of these genes at the level of the *limbless* wing buds. (C) Expression of FGF2 in normal and *limbless* embryos. Arrows indicate staining in the myotome and arrowheads indicate ectodermal staining. (D) Expression of *Bmp-2* in a normal chick embryo. Arrows indicate apical ectodermal ridge expression. (E) There is no detectable expression of *Bmp-2* in the *limbless* wing buds. (F) *Bmp-2* expression is normal on the growing atrio-ventricular cushions (arrows) of the same *limbless* embryo. (G) Expression of *Fgf-8* mRNA in a normal chick embryo. (H) Expression of *Fgf-8* in a *limbless* chick embryo showing no detectable expression at the level of the wing buds, while expression is normal in somitic mesoderm (arrows). (I) Expression of *Fgf-8* in a 29 somite *limbless* embryo showing normal expression in somites (arrows indicate somite 16 and 17) and intermediate mesoderm (mesonephros, arrowheads).

71) of the embryos lacked or showed altered expression of these genes in the developing limb (Fig. 1; *limbless*). Since this percentage corresponded to the predicted number of homozygous embryos and only the limb buds showed abnormal gene expression patterns, we inferred that the embryos lacking expression of these genes were the *limbless* embryos. Furthermore, the lack of expression of *Fgf-4, Fgf-8* and *Bmp-2* was confirmed in *limbless* wings isolated from the embryo in ovo and fixed at stage 17, 18 or 19 and the embryo allowed to develop to confirm the phenotype (see Materials and Methods). This failure of expression in the homozygous *limbless* embryos was restricted to the limb, since expression of these genes in all other embryonic structures was indistinguishable from normal embryos (Fig. 1). For example, for Fig. 1A,B we performed in situ hybridization for *Fgf-4* and *Shh* on the same embryos; the normal expression of *Fgf-4* in the somites (arrows) contrasts with the lack of expression in the limb of *limbless* embryos. Similarly, *Shh* is normally expressed throughout the embryo e.g. the notochord, floor plate of the neural tube, gut and developing brain (not shown).

Because it is known that *limbless* embryos do not form an apical ridge, we expected the lack of *Fgf-4* expression in the *limbless* limb ectoderm. However, it was not expected that *limbless* presumptive limb ectoderm would lack expression of *Fgf-8* because *limbless* embryos do initiate limb bud development. These results demonstrate that *Fgf-8* expression in the limb ectoderm is not required for limb bud initiation. Using a specific antibody, we found that the entire *limbless* limb bud ectoderm expressed FGF2 protein (Fig. 1C; Savage et al., 1993). *Bmp-2* and *Bmp-4* are also expressed in the normal limb apical ectoderm at very early stages (Francis et al., 1994). The absence of expression of *Fgf-8*, together with the absence of *Bmp-2* and *Bmp-4*, indicates that *limbless* embryos lack apical ridge markers from the earliest stages of limb development. It is important to note that *Fgf-8* expression was indistinguishable in normal and *limbless* mesonephros (Fig. 1I).

The dorsal ectoderm also plays an important role during limb development, controlling patterning along the dorsoventral axis. Its action appears to be mediated by *Wnt-7a* (Yang and Niswander, 1995; Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). *Wnt-7a* is normally expressed in the entire dorsal ectoderm of the developing limb, but not including the apical ridge (Dealy et al., 1993; Fig. 2A). In *limbless* embryos, *Wnt-7a* was expressed in both dorsal and ventral ectoderm (Fig. 2A; compare with 2B). Further, the expression of *C-Lmx1*, which is downstream of *Wnt-7a* and normally restricted to the dorsal limb bud mesoderm (Riddle et al., 1995; Vogel et al., 1995), spanned the whole *limbless* limb mesoderm (Fig. 2C, compare with 2D). This led us to investigate the pattern of expression of *En-1* in *limbless*. *En-1* is a limb ectoderm ventral marker and its protein product has been shown to localize to the ventral limb ectoderm both in mouse and chick (Davis et al., 1991; Gardner and Barald, 1992). We found that expression of *En-1* in *limbless* wing buds was undetectable while it was at the normal level in the ventral body ectoderm and mesoderm (Fig. 2E, compare with 2F). The expression of *Wnt-7a* throughout the *limbless* wing bud ectoderm and *C-Lmx1* throughout the mesoderm, together with the absence of *En-1* indicates there is a lack of a dorsal-ventral boundary in the *limbless* wing bud and that it is a bi-dorsal structure.

limbless posterior wing bud mesoderm does not express detectable levels of *Shh* in situ, but exhibits polarizing activity

In the normal wing bud, *Shh* mRNA is first detected in the posterior limb bud mesoderm of stage 17 embryos and colocalizes with the mapped polarizing region through stage 29 (Riddle et al., 1993). In contrast, *Shh* expression was never detected by in situ hybridization in the wing bud mesoderm of stage 16 to 24 *limbless* embryos analyzed both in whole mount and in sections (Figs 1A and 4A). As noted above, this lack of an endogenous *Shh* expression domain was specific to the limb bud.

The absence of *Shh* transcripts in *limbless* limb mesoderm

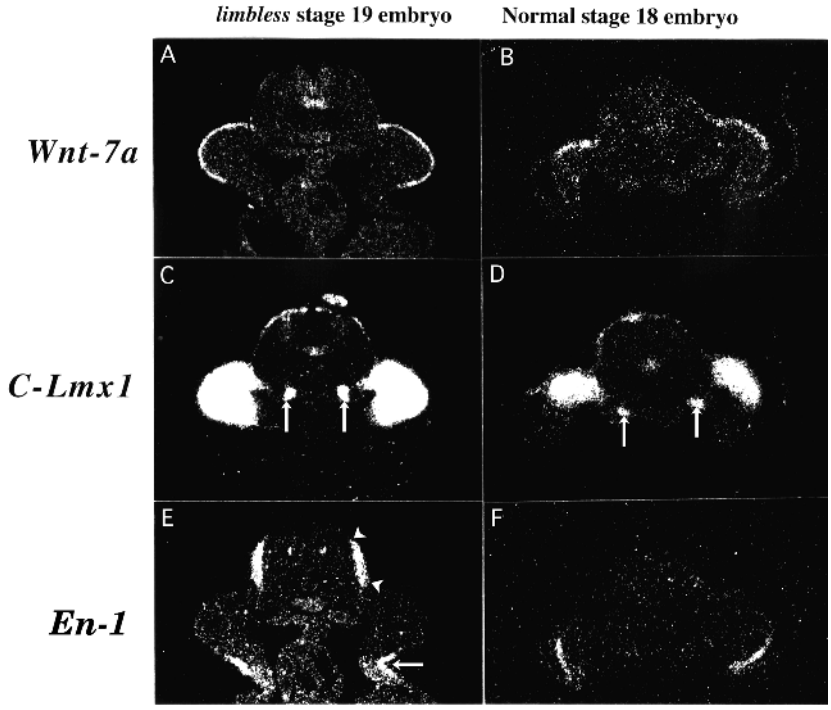

Fig. 2. Distribution of *Wnt-7a*, *C-Lmx1* and *En-1* transcripts in *limbless* and normal embryos. Dark-field micrographs of in situ hybridizations of adjacent cross sections of a *limbless* (left panels) and a normal embryo (right panels) at the level of the wings. The specific probe used is marked on the left. (A) Note that the expression of *Wnt-7a* in *limbless* embryo spans the entire limb ectoderm from the dorsal body wall to close to the ventral body wall; (B) while in the normal embryo expression is found only in the dorsal ectoderm. (C) Accordingly, *C-Lmx1* expression is found in the whole *limbless* limb mesoderm and (D) only in the dorsal mesoderm of the normal embryo. Arrows indicate *C-Lmx1* mRNA in the mesonephros of both the *limbless* and normal embryos. (E) *En-1* transcripts are undetectable in *limbless* ventral limb ectoderm; note the normal expression of *En-1* in the body wall ectoderm and mesoderm (arrow). Arrowheads indicate *En-1* mRNA in somites of the *limbless* embryo. (F) *En-1* is expressed in the ventral limb bud ectoderm of the normal embryo.

was a surprising result because it has been shown that posterior *limbless* mesoderm has polarizing activity (Fallon et al., 1983). To examine this further, we grafted *limbless* posterior limb bud mesoderm under the anterior apical ridge of stage 20-21 normal limb buds and scored the ability of the grafts to induce duplications, as described in Materials and Methods. We found that 100% of the grafts had polarizing activity with an average polarizing index of 0.62 (Fig. 3B). We tested for the presence of *Shh* mRNA in *limbless* posterior mesoderm grafted under a normal apical ridge by whole-mount in situ hybridization, and detected *Shh* transcripts in the grafted tissue 24 hours after the operation (100%, *n*=7). Control anterior *limbless* mesoderm did not express *Shh* under the same conditions (0%, *n*=4) (compare Fig. 3A and B). These studies confirm that the polarizing activity exhibited by posterior *limbless* mesoderm was most likely mediated by *Shh*.

Shh and apical ridge growth factor expression are not required for asymmetric expression of 5′ *Hoxd genes, Bmp-4* or *Wnt-5a*

It has been proposed that the expression of the most 5′ genes of the *Hoxd* cluster occurs downstream of *Shh* (Laufer et al., 1994). We have demonstrated that *limbless* wing bud mesoderm does not express detectable levels of *Shh;* however, *limbless* wing buds show sequential colinear activation of the most 5′ *Hoxd* genes (Fig. 4B-D). *Hoxd-11* and *Hoxd-12* expression was detected at normal levels in posterior *limbless* mesoderm (Fig. 4B,C). *Hoxd-13* expression was detected in the distal posterior mesoderm but always at significantly reduced levels relative to normal limb buds (Fig. 4D). This asymmetry is dramatically shown in the whole-mount in situ for *Hoxd-12* (Fig. 4G). This result clearly demonstrates that in the *limbless* limb mesoderm the *Hoxd-11, -12* and *-13* gene expressions are activated in the correct spatiotemporal fashion, and in the absence of *Shh* or the apical ridge. We have also observed that *Bmp-4* is expressed asymmetrically in the anterior *limbless* wing bud mesoderm (Fig. 4H).

The limb bud mesodermal expression of *Bmp-2* has been proposed to be downstream of *Shh* (Francis et al., 1994; Laufer et al., 1994). *Bmp-2* is normally expressed in the distal ectoderm and also in the posterior limb mesoderm, in an area overlapping, but extending further anteriorly and distally than *Shh* (Francis et al., 1994). As indicated above, and illustrated in Figs 1E and 4E, both ectodermal and mesodermal domains of expression of *Bmp-2* were absent in the *limbless* wing buds. However, the other endogenous domains of expression of *Bmp-2,* aside from the limb buds, were normal in *limbless* embryos (e.g. Fig. 1E). The concomitant lack of *Bmp-2* and *Shh* expression in *limbless* embryos supports the proposed hypothesis that *Bmp-2* is under the control of *Shh* expression (Laufer et al., 1994).

Finally, we have examined *Wnt-5a* expression in *limbless* wing buds. Normally *Wnt-5a* is expressed in the limb mesoderm in an anteroposteriorly graded fashion, with the highest expression biased posteriorly (Dealy et al., 1993). As can be seen in Fig. 4F, *Wnt-5a* expression in *limbless* limb buds was similar to the normal expression pattern. This result provided an additional demonstration of the inherent asymmetry of *limbless* wing bud mesoderm.

FGF-soaked beads permit elaboration of patterned skeletal development by *limbless* mesoderm

We have shown that the distal limb bud ectoderm in *limbless* lacks expression of *Fgf-8* and *Fgf-4*. Consequently, in an attempt to rescue the *limbless* phenotype and substitute for the action of the apical ridge, we exogenously administered FGFs. Preliminary experiments demonstrated that Affigel blue or heparin acrylic beads loaded with FGF1, 2, 4 or 8 inserted into stage 17 through 20 *limbless* wing or leg buds would permit further development and the formation of at least a partial long bone (humerus or femur) in all of the cases surviving to 10 days of development. Because the results were indistinguishable for all FGFs, we chose to work with FGF2 in the wing bud. A bead soaked in FGF2 (1 mg/ml, *n*=4; 0.5 mg/ml, *n*=18) was placed in the pre-limb mesoderm (opposite somites 15-20) or into the distal limb bud mesoderm at various times ranging from stage 14 to 19. After receiving the FGF bead, the embryos were allowed to develop until day 10 or sequentially fixed for analysis of gene expression. The FGF beads prevented the cell death that normally begins in *limbless* at about stage 19, and permitted elongation of the *limbless* wing bud with formation of the wing skeleton (Fig. 5).

FGF2, FGF4 and FGF8 have been shown to induce the development of extra limbs from the flank region (Cohn et al., 1995; Mima et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996), and these extra limbs exhibited a normal apical ridge. Consequently, the possibility existed that the FGF beads implanted in *limbless* limb territory could induce the formation of an apical ridge and subsequent development be dependent on the presence of such an apical ridge. The FGF2-induced *limbless* limbs were periodically examined and many were sectioned for in situ hybridization (*n*=20, see Fig. 6). The histological examination and the analysis for the expression of apical ridge factors always confirmed that these outgrowths lacked an apical ridge. We conclude that either the ectopic FGFs were not sufficient to induce an apical ridge in *limbless* limb ectoderm or *limbless* ectoderm was not able to respond to the inducing signal.

In the best cases, the *limbless* wing buds that developed after local FGF administration formed a humerus, one or two forearm bones and short distal elements (Fig. 5A,B,D). In some cases a humerus and two short forearm bones formed and in all the other cases at least a humerus developed (Fig. 5E). In 40% of the FGF-induced limbs in *limbless* embryos the development was to the autopod level, in 45% development was to the zeugopod level and in 15% to the stylopod level (*n*=13). The proximal cartilaginous elements that developed in the FGF-induced *limbless* wings more closely resembled normal morphology than the distal elements that formed. In many cases the humerus displayed a normal morphology (Fig. 5D,E). However, distal elements in the zeugopod and autopod were less clearly identifiable (Fig. 5A,B). In these limbs the normal rotation from the shoulder and elbow flexion did not occur. The pattern of feather distribution was not normal and in many cases it appeared that feathers covered the entire surface of the zeugopod and autopod (Fig. 5C). FGFs alone did not substitute for the apical ridge and permit the *limbless* mesoderm to produce a normal limb as in the recombinant experiments where *limbless* ectoderm was replaced with normal flank ectoderm (compare Fig. 5A and F and see Carrington and Fallon, 1988).

After apical ridge removal in normal limbs, FGF2 is able to substitute for ridge function; but in order to obtain a complete limb a continuous supply of FGF2 is needed (Fallon et al.,

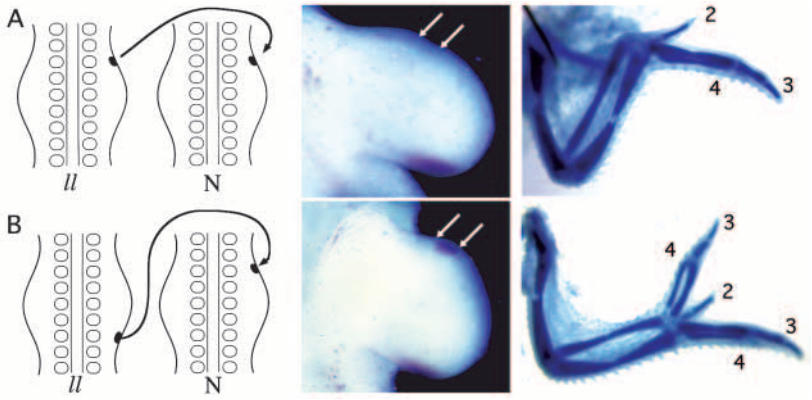

Fig. 3. Analysis of the effect of grafting posterior *limbless* mesoderm to anterior border of normal limb buds. The drawings at the left illustrate the experimental manipulation performed. The middle panels are whole-mount in situ hybridizations with *Shh* 24 hours after the experimental manipulation. The right panel is an example of the resulting limb bud at 10 days of development. When anterior *limbless* mesoderm was grafted under the anterior apical ridge of normal embryos, (A) *Shh* expression was not expressed in the graft (arrows) of anterior *limbless* mesoderm and no polarizing activity was manifested; example of resulting wing has digits **2-3-4**. However, posterior *limbless* mesoderm grafted under the anterior apical ridge of a normal embryo (B) showed *Shh* expression (arrows) within 24 hours, together with strong polarizing activity; example of resulting wing has digits **4-3-2-3-4**. The strength of the polarizing activity of the posterior *limbless* mesoderm was 62% (determined as described by Vogel and Tickle, 1993).

1994). As several of the FGF-induced *limbless* limbs were truncated at the level of the stylopodium or zeugopodium (Fig. 5) we wanted to provide *limbless* mesoderm with a source of FGF for longer periods; therefore, some *limbless* embryos received a second bead 24 hours after the first was grafted. Although several embryos with two sequential FGF2 beads were fixed for in situ hybridization, unfortunately only one survived to 10 days. The limb this embryo exhibited was no more complete than limbs receiving one bead suggesting that the two sequential beads had no additional effect.

Grafting of the beads, in some cases, was performed in the embryos of the *limbless* heterozygous cross at stages when the *limbless* phenotype was not distinguishable. Approximately three quarters of the embryos that received the beads had a normal phenotype and the beads ended up in normal developing limbs. It should be mentioned that in these cases the FGF beads strongly inhibited normal development, leading in many cases to truncated limbs (data not shown but similar to Cohn et al., 1995 and Vogel et al., 1996).

When FGF2-induced *limbless* wing buds were examined for *Shh* expression at 24 through 48 hours after the bead was grafted, generally no *Shh* transcripts were detected along the posterior border (Fig. 5G). However, in a percentage of cases (22%; 4 out of 18) we could detect *Shh* expression in posterior *limbless* mesoderm, although at low levels in comparison with normal limb buds (Figs 5H and 6A). This is a very important observation because it indicates that posterior *limbless* mesoderm is competent to express *Shh* in situ and that FGF2 is able to induce it at the exogenous doses administered here.

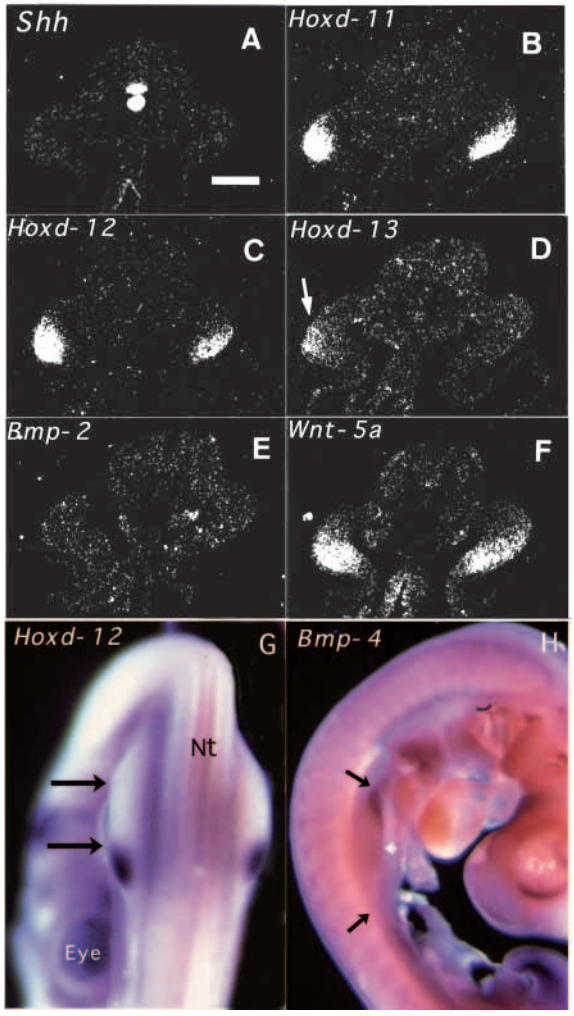

Fig. 4. Mesodermal gene expression in *limbless* limb buds. (A-F) Dark-field micrographs of cross sections through a stage 19 *limbless* embryo at the level of the wings. The specific hybridization is indicated in each panel. The level of each particular section varies slightly to show the most representative section for each gene analyzed. (A) Note the absence of *Shh* expression in *limbless* limb buds while the expression is normal in the notochord and floor plate. The expression of (B) *Hoxd-11* and (C) *Hoxd-12* are similar to normal. (D) The arrow points to the weak expression of *Hoxd-13* in *limbless* wing buds. (F) *Bmp-2* is undetectable in *limbless* limb buds while (F) *Wnt-5a* is expressed at a normal level in the posterior *limbless* limb mesoderm. (G) Whole-mount in situ hybridization showing asymmetric distribution of *Hoxd-12* in wing buds of a stage 19 *limbless* embryo. Note the flat distal aspect of the wing buds between the arrows, the first indication of the *limbless* phenotype. These wing buds do not have an apical ridge. Nt is neural tube and left eye is labeled. (H) Whole-mount in situ hybridization showing asymmetric expression of *Bmp-4* in the anterior *limbless* limb bud mesoderm. Arrows indicate anterior and posterior limits of the bud. White arrow indicates the apical ectoderm which has failed to express *Bmp-4*.

The observation that *Shh* pattern of expression in *limbless* limbs after the FGF2 application is restricted to posterior mesoderm reveals the earlier specification of a region with competence to express *Shh* in *limbless* mesoderm.

Another set of 20 *limbless* embryos that had FGF2-induced wing buds were sequentially fixed to analyze gene expression. We found that all of the outgrowths, independent of apparent *Shh* expression, developed with nested expression of the three most 5′ *Hoxd* genes. FGF2 permitted maintenance and subsequent upregulation of the expression of the three most 5′ *Hoxd* family members with *Hoxd-13* achieving a level of expression similar to normal (Fig. 6B). However, the pattern of expression of these genes was similar to normal only in some specimens while in others their expression appeared poorly developed. This may correlate with the variability in the morphogenesis obtained in the FGF2-induced limbs noted above. We stress that *Bmp-2* was expressed in the posterior mesoderm in every case analyzed, independent of detectable *Shh* expression (compare Fig. 6C with E). This result suggested that either expression of *Bmp-2* may be so sensitive to the presence of *Shh* that levels of *Shh* at or near background activate *Bmp-2* or that alternative ways of activating *Bmp-2* expression, independent of *Shh*, may exist.

The expression of *Wnt-7a*, *C-Lmx1* and *En-1* was also analyzed in FGF2-treated *limbless* wing buds. In these limbs *Wnt-7a* continued to be expressed both dorsally and ventrally, although the level of expression in the ventral ectoderm was decreased or down regulated (Fig. 6F). In accordance with *Wnt-7a* expression, *C-Lmx1* was expressed peripherally in the limb under dorsal and ventral ectoderm with the central part of the limb lacking expression of this gene (Fig. 6G). Finally, our analysis demonstrates that the FGF-induced outgrowths occurred without expression of *En-1* in what should become the ventral ectoderm (Fig. 6H). These data indicate that the FGF-induced limbs in *limbless* develop as bi-dorsal structures when the mutant buds elongate, which correlates with the pattern of feathers exhibited by some specimens and described above.

DISCUSSION

Although *limbless* homozygotes lack limbs, initial limb budding occurs normally in this mutant. Our analysis of gene expression during the initial formation of limb buds in *limbless* indicate that the emergence of the limb bud is independent of the expression of apical ectoderm associated genes such as *Fgf-4*, *Fgf-8* and *Bmp-2* and of *Shh*. In particular, *Fgf-8* expression by the limb field ectoderm appears to be dispensable for the initiation of the bud. However, FGF2 was present. Furthermore, the initial *limbless* limb bud shows mesodermal anteroposterior polarity as manifested by the asymmetric expression of the most 5′ *Hoxd* genes together with *Bmp-4* and *Wnt-5a*. Finally, *limbless* limb buds are bi-dorsal structures expressing *Wnt-7a* in the entire ectoderm and *C-Lmx1* in the entire mesoderm. We propose that the defect in this mutant is the lack of a dorsal-ventral ectodermal interface where the apical ridge would normally develop.

Asymmetry is intrinsic to limb mesoderm prior to *Shh* expression and apical ridge development

In normal wing buds *Shh* is first detected at stage 17 (Riddle et al., 1993) concomitant with initiation of expression of the most 5′ *Hoxd* genes (Izpisúa-Belmonte et al., 1991). Because of *Shh*'s ability to initiate ectopic expression of *Hoxd* genes when misexpressed on the anterior border, it has been assumed

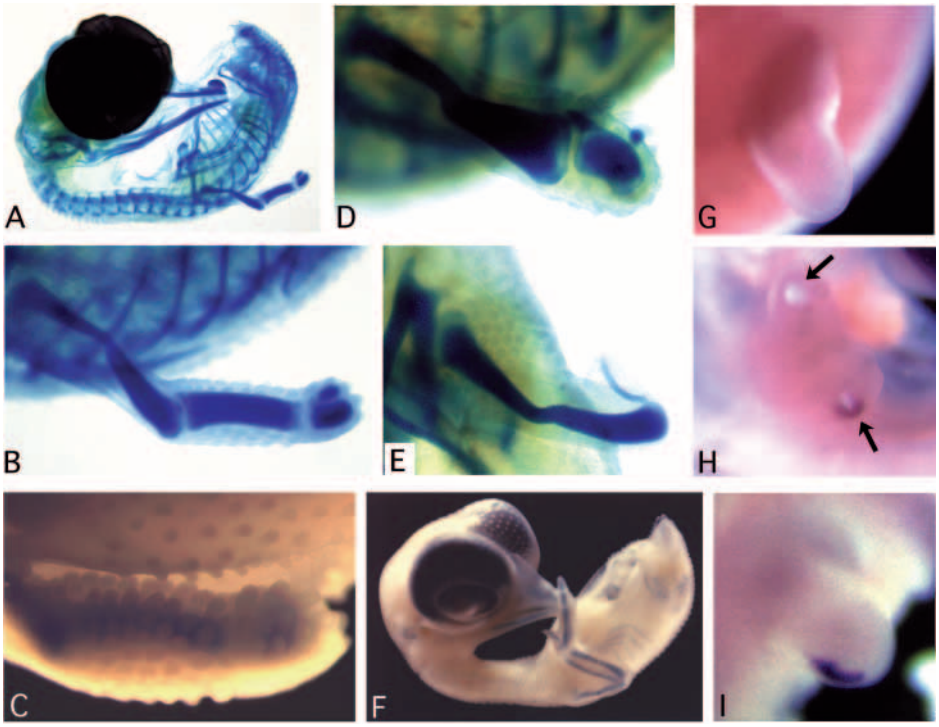

Fig. 5. FGF2-induced limbs in *limbless*. (A) *limbless* homozygous embryo that received an FGF2 bead at the wing level at stage 17. The limb that developed shows cartilage elements arranged in three proximodistal levels. This should be compared with the distally complete limb in F. (B) A higher magnification of the wing shown in A, note that proximal elements exhibit better morphogenesis than distal ones. (C) The feather patterns of the same limb indicate its bi-dorsal character. (D,E) Two different FGF2-induced limbs in *limbless* with less distal development than in the limb in A (in 40% of the FGF2-induced limbs in *limbless* the development was to the autopod level, in 45% development was to the zeugopod level and in 15% to the stylopod level). The humerus in both B and D clearly exhibits the condilar process. (F) A *limbless* homozygous embryo which received a graft of normal flank ectoderm to the wing field at stage 15. *limbless* mesoderm induced and maintained an apical ridge in the donor ectoderm and a normal limb developed including normal feather bud patterns. (G) Hybridization with *Shh* probe demonstrated that some of these FGF2-induced outgrowths developed without detectable expression of *Shh*. (H) Others had *Shh* expression, although at lower levels than in normal embryos (see also Fig. 6). This specimen received two sequentially placed FGF2 beads (arrows). The posteriorly placed bead shows weak induction of *Shh* around it. (I) For comparison, the expression of *Shh* in a normal wing bud that received an FGF2 bead is shown.

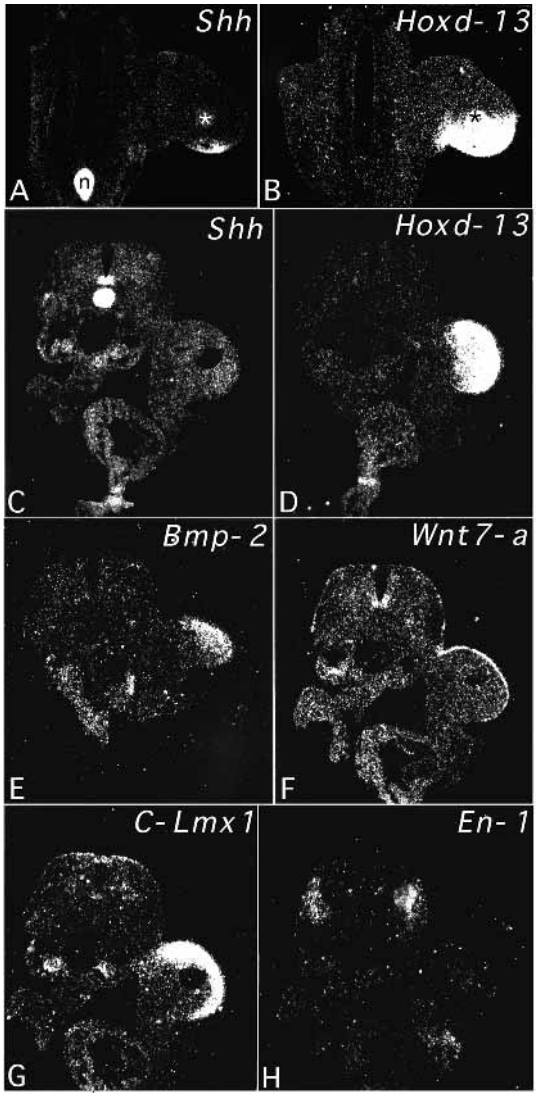

Fig. 6. Gene expression in FGF2 induced *limbless* limbs. Dark-field micrographs of in situ hybridizations of two FGF2-induced outgrowths 24 hours (A,B; coronal sections) and 18 hours (C-H; cross sections) after the application of the bead (indicated by the asterisk). (A) *Shh* has been induced by FGF in the posterior *limbless* mesoderm; N is the notochord**.** (B) *Hoxd-13* expression upregulated to normal levels. (C) *Shh* expression continues to be undetectable in this outgrowth while (D) *Hoxd-13* has been clearly upregulated to normal levels and (E) *Bmp*-2 is expressed. As the limb elongates the bi-dorsal character is maintained with (F) *Wnt-7a* being expressed in both dorsal and ventral ectoderm. (G) *C-Lmx1* is expressed in the mesoderm subjacent to *Wnt-7a*-expressing ectoderm and (H) *En-1* remains undetectable in what should be the ventral limb ectoderm. Note normal expression in the somites.

that *Shh* is upstream of the most 5′ *Hoxd* genes (Riddle et al., 1993; Chang et al., 1994; Laufer et al., 1994; López-Martínez et al., 1995). However, asymmetric expression of the most 5′ *Hoxd* genes occurs in *limbless* mesoderm without apical ridge influence and without detectable *Shh* expression, suggesting that initial activation of *Hoxd-11, -12 and 13* is not downstream of *Shh* during normal limb bud development. The independence of *Hoxd-11, -12 and 13* from *Shh* is similar in recombinant limbs made with anterior mesoderm where 5′ *Hoxd* gene expression occurs without detectable levels of *Shh* (Ros et al., 1994; Hardy et al., 1995). This is also the case in *talpid*$_2$, *talpid*$_3$, *diplopodia* and *diplopodia*$_4$ chick mutants where *Hoxd* expression occurs at the anterior border of the mutant limb bud in the absence of *Shh* expression (Francis-West et al., 1995; Rodriguez et al., 1996). It has also been suggested that activation of *Hoxd-13* may be dependent upon *Bmp-2* expression (Francis et al., 1994). In *limbless, Hoxd-13* is expressed without detectable expression of *Bmp-2*, indicating that activation of this gene is independent of both *Shh* and *Bmp-2*. At present, the factor or factors responsible for the asymmetric initial expression of *Hoxd-11, -12 and -13* genes in limb mesoderm are unknown. It could be speculated that these are the same factors that define the competence to express *Shh*, although *Shh* activation would require additional influence, probably from the apical pre-ridge ectoderm and apical ectodermal ridge.

The reduced levels of expression of the most posterior gene of the complex, *Hoxd-13*, in *limbless* embryos also suggest that *Shh* and/or apical ridge factors, although not necessary for the activation of this gene, are needed for the elaboration of the normal pattern of expression. Regulation of the expression of the most 5′ *Hoxd* genes during stages subsequent to limb initiation are under the coordinated control of apical ridge and polarizing region signals (Izpisúa Belmonte et al., 1992; Laufer et al., 1994; Ros et al., 1994). In *limbless*, exogenous FGF permits maintenance and elaboration of the most 5′ *Hoxd* patterns of expression.

The demonstration that *limbless* limb bud mesoderm asymmetrically expresses the three most 5′ *Hoxd* genes without an apical ridge or a polarizing region strongly supports the hypothesis that expression of these genes is an intrinsic property of growing limb mesoderm (Ros et al., 1994; Duboule, 1994a). This suggests that proliferative signals resulting in the unique limb level cell cycle (Searls and Janners, 1971) must exist before the apical ridge develops. The inductions of the proliferative signals may originate axially (Pinot, 1970; Stephens et al., 1991) and likely involve the axial, paraxial and intermediate mesoderm. In particular, the mesonephros has been implicated recently in limb bud outgrowth. Insulin-like growth factors (IGF) (Geduspan and Solursh, 1993; Dealy and Kosher, 1995, 1996; Bhaumick and Bala, 1987; Streck et al., 1992) and FGF8 (Crossley et al., 1996; Vogel et al., 1996) have been proposed as candidate molecules. We stress here that the expression of FGF8 appears normal in the *limbless* mesonephros. However, there is no evidence one way or the other whether FGF8 from the mesonephros could by itself cause limb initiation (cf. Crossley and Martin, 1995; Vogel et al., 1996). We have not analyzed IGF and it remains to be seen if it is normally expressed in the *limbless* mesonephros (however, see Dealy and Kosher, 1996). Many other growth factors and signaling molecules are expressed in the axial and intermediate mesoderm and it is not known which, or more probably, which combination of them, are responsible for limb field specification and/or limb bud initiation.

limbless defect localizes in the ectoderm

Previous work performed with the *limbless* mutant demonstrated that the defect was in the limb ectoderm which is unable

to develop an apical ridge (Carrington and Fallon, 1988). In the present study we have confirmed and extended these results (Fig. 5F). The *limbless* limb ectoderm lacks expression of apical ridge genes such as *Fgf-4*, *Fgf-8*, *Bmp-2*, *Bmp-4* (this work) and *Msx-2* (Coelho et al., 1991; Robert et al., 1991). In contrast with *Fgf-4*, whose expression is restricted to the posterior apical ridge, *Fgf-2*, *Fgf-8*, *Bmp-2* and *Bmp-4* are normally expressed before a morphologically identifiable apical ridge has developed (Savage and Fallon, 1995; Francis et al., 1994; Mahmood et al., 1995; Crossley et al., 1996). The observation that *Fgf-8* was not expressed in *limbless* ectoderm was unexpected because it has been considered as the prime candidate to control initial limb bud outgrowth (Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). *limbless* embryos do initiate a limb and we can conclude that *Fgf-8* expression by the limb ectoderm is not required for limb bud initiation.

limbless limb bud mesoderm appears morphologically normal up to stage 19, when cell death begins. As discussed above, the expressions of the most 5′ *Hoxd* genes, anterior *Bmp-4* and of posterior *Wnt-5a* expressions are virtually normal in *limbless* mesoderm. However, *limbless* mesoderm does not express *Shh* or *Bmp-2*. Our data indicate that the lack of expression of these genes is not intrinsic to the mesoderm, since a normal apical ridge environment permits posterior *limbless* mesoderm to express *Shh* and exert polarizing activity. Similarly, *limbless* posterior mesoderm is competent to express *Shh*, in situ, and then sequentially *Bmp-2*, after exogenous FGF2 administration. In this context, Hornbruch and Wolpert's (1991) study on polarizing activity in pre-limb bud stages may be interpreted as a documentation of the spatial and temporal distribution of the competence to express *Shh*. Taken together, our data indicate that the lack of *Shh* expression in *limbless* posterior mesoderm is due to the lack of signals emanating from the ectoderm, that are not expressed in the *limbless* limb bud ectoderm. This supports the hypothesis that *Shh* expression in normal embryos depends on apical ectoderm signals, possibly *Fgf-8* (Crossley et al., 1996). It is clear that, at the concentrations administered here, FGF can mimic the endogenous signal**s** normally emanating from the pre-ridge ectoderm and responsible for normal *Shh* induction in the already specified region of high potential polarizing activity. It is likely that the maintenance of that expression involves WNT7a and FGF4 activities (Laufer et al., 1994; Niswander et al., 1994).

limbless limb buds are bi-dorsal structures

A secreted signaling molecule, WNT7a has been shown to be necessary and sufficient to regulate dorsoventral patterning (Riddle et al., 1995; Vogel et al., 1995). In normal limbs *Wnt-7a* is expressed by the limb dorsal ectoderm (Dealy et al., 1993; Riddle et al., 1995; Vogel et al., 1995) and induces the expression of C-*Lmx1* in the subjacent limb mesoderm. C-LMX1 is sufficient to impose dorsal pattern on limb mesoderm. In contrast, the entire limb ectoderm in *limbless* expresses *Wnt-7a* and consequently the whole mesoderm expresses *C-Lmx1*, indicating that these limbs are bi-dorsal structures. *En-1*, a marker of ventral limb ectoderm, is not expressed in the *limbless* limb bud ectoderm indicating that *Wnt-7a* and *En-1* ectodermal domains of expression may be mutually exclusive. We confirm the observation of Riddle et al. (1995) that *Wnt-7a* is not sufficient to induce *Shh*.

Of particular interest for this discussion is the conclusion drawn by Rosenquist (p. 94, 1971) that during stages 8 and 9 the 'premesoderm destined for the dorsal portion of each limb may be anteromedial to premesoderm destined for the ventral portion of each limb'. This is important since the available data indicate that the initial limb field dorsal-ventral information resides in the mesoderm prior to stage 14; this information is transferred to the limb field ectoderm during stages 14-16. In parallel, the limb field mesoderm loses the ability to transfer dorsal-ventral information to the ectoderm. Subsequently, it is the ectoderm that establishes dorsal-ventral polarity in the mesoderm of the elongating limb bud (Geduspan and MacCabe, 1987, 1989). The past history of the dorsal and ventral lateral plate mesoderm (e.g. Rosenquist, 1971) must account for their unique abilities to influence the overlying ectoderm. These observations combined with recent data on specific gene markers certainly implicate the compartmentalization of the ectoderm into regions expressing WNT7a, FGFs and EN-1. These compartments do not form in *limbless* wing buds. As the initiation of *Wnt-7a* and *En-1* expression by the pre-limb ectoderm occurs at stages prior to the initiation of expression of *Fgf-8* (compare Riddle et al., 1995 and Vogel et al., 1995 with Crossley et al., 1996 and Vogel et al., 1996) we suggest the existence of a distal ectodermal gene expressed prior to *Fgf-8* that delimits the boundary between dorsal and ventral ectoderm and this expression would be absent in *limbless*. The failure of *limbless* limb bud development can not be attributed simply to the bi-dorsal character of these limbs, since bi-dorsal limbs will grow and develop (Saunders et al., 1976; Carrington and Fallon, 1986; Robert et al., 1991; Riddle et al., 1995; Vogel et al., 1995; this report). It has been suggested on theoretical grounds that the dorsal-ventral juxtaposition in the chick limb bud ectoderm is a precondition for apical ectodermal ridge formation (Meinhardt, 1983, p. 122; see also Martin, 1995). Our data in *limbless* limb buds support this hypothesis and we propose the defect in *limbless* is the lack of a dorsal-ventral interface in the limb bud ectoderm where the ridge induction signal from the mesoderm would be received and an apical ridge formed. This failure of a functional ridge to form and the absence of its growth signals is the reason *limbless* limb buds regress.

Limb field specification and limb bud initiation

The analysis of the influence of axial, paraxial and intermediate mesoderm structures on the limb field dates back to seminal studies by Harrison (1918, 1921; reviewed by Stocum and Fallon, 1982) on the urodele limb. In the chick embryo, it is possible that sequential influences from Hensen's node (Stephens et al., 1991) the segmental plate (somites; Kieny, 1969, 1971; Pinot, 1970) and the intermediate mesoderm (mesonephros; Stephens and McNulty, 1981; Geduspan and Solursh, 1992, 1993) determine the limb field mesoderm and permit budding. In parallel, the evidence that limb field mesoderm initiates dorsal-ventral asymmetry (see above), likely reflects the past influences on the limb field mesoderm that distinguish dorsal from ventral. The sum total of these influences on the prospective limb field not only determines what Zwilling (1968) called 'limbness' but also specifies the asymmetry of the forming limb field, resulting in a specific

region of the somatopleure with potential for heterogeneous gene expression and specific patterning. The classic limb development signaling centers subsequently influence the pre-patterned limb bud mesoderm, stabilizing and augmenting the existing gene expressions and initiating new cascades of gene expressions that result in elongation of the bud and the patterned limb. This would mean that the initial steps of the 'limb plan' suggested by Duboule (1994b) would be set in the limb field; the signaling centers of the limb bud would build on that foundation. This view fits nicely with and extends evolutionary considerations of appendage development offered by Duboule (1994b) and others (e.g. Shubin and Alberch, 1986; Shubin, 1991; Coates 1993, 1994). This also directs attention to the fact that even prior to limb bud formation, the limb field mesoderm is determined to be forelimb or hindlimb (Hamburger, 1938; Rudnick, 1945; Lewis and Wolpert, 1976; Krabbenhoft and Fallon, 1989; Stephens et al., 1993). The axial influences and gene expressions that establish the forelimb-versus-hindlimb identity of the limb fields are not known.

This work was supported by a grant from FISS, 95/0576 to M. A. R; NIH Predoctoral Training Grant ST32GM07507 to A. L-M.; NSF grant number IBN- 95-13859 and a Basil O'Connor award from the March of Dimes to J. C. I-B; NSF Predoctoral Fellowship to R. D. and NIH grant # HD32551 to J. F. F. We are grateful to C. Tabin for helpful discussions and thank D. Stocum and S. Smith for reading the manuscript. We thank J. Dodd, C. Tabin and A. Joyner for providing some of the probes used in this work. We thank S and R Egg Farms Inc. Whitewater, WI for donating the White Leghorn flock. This paper is dedicated to the memory of G. Michael Fallon.

REFERENCES

Bhaumick, B. and Bala, R. M. (1987). Receptors for insulin-like growth factors I and II in developing embryonic mouse limb bud. *Biochem. Biophys. Acta.* **927**, 117-128.

Carrington, J. L. and Fallon, J. F. (1984a). The stages of flank ectoderm capable of responding to ridge induction in the chick embryo. *J. Embryol. Exp. Morphol.* **84**, 19-34.

Carrington, J. L. and Fallon, J. F. (1984b). Evidence that the ectoderm is the affected layer in the wingless mutant chick embryo. *J. Exp. Zool.* **232**, 297-308.

Carrington, J. L. and Fallon, J. F. (1986). Experimental manipulation leading to induction of dorsal ectodermal ridges on normal limb buds results in a phenocopy of the *Eudiplopodia* chick mutant. *Dev. Biol.* **116**, 130-137.

Carrington, J. L. and Fallon, J. F. (1988). Initial limb budding is independent of apical ectodermal ridge activity: evidence from a *limbless* mutant. *Development* **104**, 361-367.

Chang, D. T., López, A., Von Kessler, D. P., Chang, C., Simandl, B. K., Zhao, R., Selding, M. F., Fallon, J. F. and Beachy, P. A. (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* **120**, 3339-3353.

Coates, M. I. (1993). Ancestors and homology (the origin of the tetrapod limb). *Acta Biotheoretica* **41**, 411-424.

Coates, M. I. (1994). The origin of vertebrate limbs. *Development* **Supplement** 169-180.

Coelho, C. N. D., Krabbenhoft, K. M., Upholt, W. B., Fallon, J. F. and Kosher, R. A. (1991). Altered expression of the chicken homeo-box containing genes *GHox-7* and *GHox-8* in the limb buds of *limbless* mutant chick embryos. *Development* **113**, 1487-1493.

Cohn, M. J., Izpisúa-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.

Crossley, H. P. and Martin, G. R. (1995). The mouse *Fgf-8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Crossley, H. P., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation and maintenance of chick limb development. *Cell* **84**, 127-136.

Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an En-specific antiserum. *Development* **111**, 287-298.

Dealy, C. N., Roth, A., Ferrari, D., Brown, A. M. C. and Kosher, R. A. (1993). *Wnt-5a* and *Wnt-7a* are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsoventral axes. *Mech. Dev.* **43**, 175-186.

Dealy, C. N. and Kosher, R. A. (1995). Studies on insulin-like growth factor-I and insulin in chick limb morphogenesis. *Dev. Dyn.* **202**, 67-79.

Dealy, C. N. and Kosher, R. A. (1996). IGF-1, insulin and FGFs induce outgrowth of the limb buds of amelic mutant chick embryos. *Development* **122**, 1323-1330.

Dono, R. and Zeller, R. (1994). Cell type-specific nuclear translocation of fibroblast growth factor-2 isoforms during chicken kidney and limb morphogenesis. *Dev. Biol.* **163**, 316-330.

Duboule, D. (1994a). Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development* **Supplement**, 135-142.

Duboule, D. (1994b). How to make a limb. *Science* ***266,*** 575-576.

Fallon, J. F., Frederick., J. M., Carrington, J. L., Lanser, M. R. and Simandl, B. K. (1983). Studies on a *limbless* mutant in the chick embryo. In *Limb Development and Regeneration*, Part A (eds. J. F. Fallon and A. I. Caplan), pp. 33-43. New York: Alan R. Liss.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M., Olwin, B. and Simandl, B. K. (1994). FGF2: apical ectodermal ridge growth signal for the chick limb development. *Science* **264**, 104-107.

Francis, P. H., Richardson, M. K., Brickell, P. M. and Tickle, C. (1994). Bone morphogenetic proteins and a signaling pathway that controls patterning in the developing chick limb. *Development* **120**, 209-218.

Francis-West, P. H., Robertson, K., Ede, D. A., Rodriguez, C., Izpisua-Belmonte, J. C., Houston, B., Burt, D. W., Gribbin, C., Brickell, P. M. and Tickle, C. (1995). Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta^3) limb buds: their relationships in the signalling cascade involved in limb patterning. *Dev. Dyn.* **203**, 187-197.

Gardner, C. A. and Barald, K. F. (1992). Expression patterns of Engrailed-like proteins in the chick embryo. *Dev. Dyn.* **193**, 370-388.

Geduspan, J. S. and MacCabe, J. A. (1987). The ectodermal control of mesodermal patterns of differentiation in the developing chick wing. *Dev. Biol.* **124**, 398-408.

Geduspan, J. S. and MacCabe, J. A. (1989). Transfer of dorsoventral information from mesoderm to ectoderm at the onset of limb development. *Anat. Rec.* **224**, 79-87.

Geduspan, J. S. and Solursh, M. (1992). A growth-promoting influence from the mesonephros during limb outgrowth. *Dev. Biol.* **151**, 242-250.

Geduspan, J. S. and Solursh, M. (1993). Effects on the mesonephros and Insulin-like growth factor I on chondrogenesis of limb explants. *Dev. Biol.* **156**, 500-508.

Hamburger, V. (1938). Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. *J. Exp. Zool.* **77**, 379-399.

Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chick embryos. *J. Morphol.* **88**, 49-92.

Hardy, A., Richardson, M. K., Francis-West, P. H., Rodriguez, C., Izpisúa-Belmonte, J. C., Duprez, D. and Wolpert, L. (1995). Gene expression, polarizing activity and skeletal patterning in reaggregated hind limb mesenchyme. *Development* **121**, 4329-4337.

Harrison, R. G. (1918). Experiments on the development of the fore limb of amblystoma, a self-differentiating equipotential system. *J. Exp. Zool.* **25,** 13-461.

Harrison, R. G. (1921) On relations of symmetry in transplanted limbs. *J. Exp. Zool.* **32**, 1-136.

Heikinheimo, M., Lawshe, A., Shackleford, G. M., Wilson, D. B. and MacArthur, C. A. (1994). *Fgf-8* expression in the post-gastrulation mouse suggest roles in the development of the face, limbs and central nervous system. *Mech. Dev.* **48**, 129-138.

Hornbruch, A. and Wolpert, L. (1991). The spatial and temporal distribution of polarizing activity in the flank of the pre-limb-bud stages in the chick embryo, *Development* **111**, 725-31.

Izpisúa-Belmonte, J. C., Tickle, C., Dolle, P., Wolpert, L. and Duboule, D. (1991). Expression of the homeobox *Hox-4* genes and the specification of position in chick wing development. *Nature* **350**, 585-589.

Izpisúa-Belmonte, J. C., Brown, J. C., Duboule, D. and Tickle, C. (1992).

Expression of Hox-4 genes in the chick wing links pattern formation to the epithelial-mesenchymal interactions that mediate growth. *EMBO J.* **11**, 1451-1457.

Kieny, M. (1960). Role inducteur du mesoderme dans la différentiation précoce du bourgeon de membre chez l'embryon de Poulet. *J. Embryol. Exp. Morphol.* **8**, 457-467.

Kieny, M. (1968). Variation de la capacité inductrice du mésoderme et de la compétence de l'ectoderme au cours de l'induction primaire du bourgeon de membre chez l'embryon de Poulet. *Archs d'Anat. micr. Morphol. Exp.* **57**, 401-418.

Kieny, M. (1969). Sur les relations entre le mésoderme somitque et le mésoderme somatopleural avant et al course de l'induction primoire des membres de l'embryon de Poulet. *C. R. Acad. Sci. Paris* **268**, 3183-3186.

Kieny, M. (1971). Les phases d'activité morphogène du mésoderme somatopleural pendent de développement précoce du membre chez l'embryon de Poulet. *Ann. Embryol. Morphol.* **4**, 281-298.

Krabbenhoft, K. M. and Fallon, J. F. (1989). The formation of leg or wing specific structures by leg bud cells grafted to the wing bud is influenced by proximity to the apical ridge. *Dev. Biol.* **131**, 373-382.

Lanser, M. E. and Fallon, J. F. (1984). Development of the lateral motor column in the *limbless* mutant chick embryo. *J. Neurosci.* **4**, 2043-2050.

Laufer, E., Nelson, C., Johson, R. L. Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and *Fgf-4* act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.

Lewis, J. H. and Wolpert, L. (1976). The principle of non-equivalence in development. *J. Theor. Biol.* **62,** 479-90.

López-Martínez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A. and Fallon, J. F. (1995). Limb-patterning activity and restricted posterior localization of the amino terminal product of Sonic hedgehog cleavage. *Curr. Biol.* **5**, 791-796.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin. P., Lumsden, A., Dickson, C. and Mason, I. (1995). A role for FGF8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* **5**, 797-806.

Martin, G. R. (1995). Why thumbs are up. *Nature* **374**, 410-411.

MacCabe, J. A., Errick, J. and Saunders, J. W. (1974). Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Dev. Biol.* **39**, 69-82.

Meinhardt, H. (1983). A boundary model for pattern formation in vertebrate limbs. *J. Embryol. Exp. Morphol.* **76**, 115-137.

Mima, T., Ohuchi, H., Noji, S. and Mikawa, T. (1995). FGF can induce outgrowth of somatic mesoderm both inside and outside of limb-forming regions. *Dev. Biol.* **167**, 617-620.

Nieto, M. A., Patel, K. and Wilkinson D. G. (1996). In situ analysis of chick embryos in whole mount and tissue sections. In *Methods in Cell Biology* (ed. M. Bronner-Fraser), vol. 51, pp. 219-235. New York: Academic Press.

Niswander, L. and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114**, 755-768.

Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993). FGF4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579-587.

Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994). Positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609-612.

Ohuchi, H., Yoshioka, H., Tanaka, H., Kawakami, Y., Nohno, T. and Noji, S. (1994). Involvement of androgen-induced growth factor (FGF8) gene in mouse embryogenesis and morphogenesis. *Biochem. Biophys. Res. Com.* **204**, 882-888.

Ohuchi, H., Nakagawa, T., Yamahuchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Mikawa, T., Nohno, T. and Noji, S. (1995). An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochem. Biophys. Res. Com.* **209**, 809-816.

Parr, B. A. and McMahon, A. P. (1995). Dorsalizing signal *Wnt-7a* required for normal polarity of D-V and A-P axes in mouse limb. *Nature* **374**, 350-353.

Pautou, M. P. and Kieny, M. (1973). Interaction ecto-mesodermique dans l'establishment de la polarité dorso-ventrale du pied de l'embryon de poulet. *C.r. hebd Séanc. Acad. Sci., Paris D.* **277**, 1225-1228.

Pinot, M. (1970). Le role du mésoderme somitique dans la morphogenèse précoce des membres de l'embryon de Poulet. *J. Embryol. Exp. Morph.* **23**, 109-151.

Prahlad, R. B., Skala, G., Jones, D. B. and Briles, W. S. (1979). *Limbless*: A new genetic mutant in the chick. *J. Exp. Zool.* **209**, 427-434.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M. and Tabin, C. (1995). Induction of the LIM homeobox gene *Lmx1* by *Wnt7a* establishes dorsoventral pattern in the vertebrate limb. *Cell* **83**, 631-640.

Rodriguez, C., Kos, R., Abbott, U. K. and Izpisua Belmonte, J. C. (1996). Gene expression, polarizing activity, growth and patterning during the development of the polydactylous talpid$_2$, diplopodia$_1$ and diplopodia$_4$ mutant chick limb bud. *Dev. Gen.* In press.

Robert, B., Lyons, G., Simandl, B. K., Kuroiwa, A. and Buckingham, M. (1991). The apical ectodermal ridge regulates *Hox-7* and *Hox-8* gene expression in developing chick limb buds. *Genes and Dev.* **5**, 2363-2374.

Ros, M. A., Lyons, G. E., Mackem, S. and Fallon, J. F. (1994). Recombinant limbs as a model to study homeobox gene regulation during limb development. *Dev. Biol.* **166**, 59-72.

Rosenquist, G. C. (1971). The origin and movement of the limb-bud epithelium and mesenchyme in the chick embryo as determined by radioautographic mapping. *J. Embryol. Exp. Morphol.* **25**, 85-96.

Rowe, D. A. and Fallon, J. F. (1982). The proximodistal determination of skeletal parts in the developing chick leg. *J. Embryol. Exp. Morphol.* **68**, 1-7.

Rubin, L. and Saunders, J. W. Jr. (1972). Ectodermal-mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. *Dev. Biol.* **28**, 94-112.

Rudnick, D. (1945). Limb-forming potencies of the chick blastoderm: including notes on associated trunk structures. *Trans. Conn. Acad. Arts Sci.* **36**, 353-377.

Saunders, J. W., Jr. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.* **108**, 363-403.

Saunders, J. W., Jr. and Gasseling, M. T. (1968). Ectoderm-mesenchymal interaction in the origins of wing symmetry. In *Epithelial-Mesenchymal Interactions*, (eds. R. Fleischmajer and R. E. Billingham) pp. 78-97. Baltimore: Williams and Wilkins.

Saunders, J. W., Jr. and Reuss, C. (1974). Inductive and axial properties of prospective wing-bud mesoderm in the chick embryo. *Dev. Biol.* **38**, 41-50.

Saunders, J. W. Jr., Gasseling, M. T. and Errick, J. E. (1976). Inductive activity and enduring cellular constitution of a supernumerary apical ectodermal ridge grafted to the limb bud of the chick embryo. *Dev. Biol.* **50**, 16-25.

Savage, M. P., Hart, C. E., Riley, B., Sasse, J., Olwin, B. B. and Fallon, J. F. (1993). Distribution of FGF2 suggests it has a role in chick limb bud growth. *Dev. Dyn.* **198**, 159-170.

Savage, M. P. and Fallon, J. F. (1995). FGF2 mRNA and its antisense message are expressed in a developmentally specific manner in the chick limb bud and mesonephros. *Dev. Dyn.* **202**, 343-353.

Searls, R. L. and Janners, M. Y. (1971). The initiation of limb bud outgrowth in the embryonic chick. *Dev. Biol.* **24**, 198-213.

Shubin, N. H. (1991). Implications of the 'Bauplan' for development and evolution of the tetrapod limb. In *Developmental Patterning of the Vertebrate Limb*. (eds. J. R. Hinchliffe, J. M. Hurle and D. Summerbell), pp. 411-422. Plenum Press: New York.

Shubin, N. H. and Alberch, P. (1986). A morphogenetic approach to the origin and basic organisation of the tetrapod limb. *Evolutionary Biol.* **20,** 318-390.

Stephens, T. D. and McNulty, T. R. (1981). Evidence for a metameric pattern in the development of the chick humerus. *J. Embryol. Exp. Morphol.* **61,** 191-205.

Stephens, T. D., Spall, R., Baker, W. C., Hiatt, S. R., Pugmire, D. E., Shaker, M. R., Willis, H. J. and Winger, K. P. (1991). Axial and paraxial influences on limb morphogenesis. *J. Morphol.* **208,** 367-379.

Stephens, T. D., Baker, W. C., Cotterell, J. W., Edwards, D. R., Pugmire, D. S., Roberts, S. G., Shaker, M. R., Willis, H. J. and Winger, K. P. (1993). Evaluation of the chick wing territory as an equipotential self-differentiating system. *Dev. Dyn.* **197**, 157-68.

Stocum, D. L. and Fallon, J. F. (1982). Control of pattern formation in urodele limb ontogeny: a review and a hypothesis. *J. Embryol. Exp. Morphol.* **69,** 7-36.

Streck, R. D., Wood, T. L., Hsu, M. S. and Pintar, J. E. (1992). Insulin-like growth factor I and II and insulin-like growth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic and fetal limbs. *Dev. Biol.* **151**, 586-96.

Summerbell, D., Lewis, J. H. and Wolpert, L. (1973). Positional information in chick limb morphogenesis. *Nature* **224**, 492-496.

Summerbell, D. (1974). A quantitative analysis of the effect of excision of the apical ridge from the chick limb bud. *J. Embryol. Exp. Morphol.* **32**, 651-660.

Suzuki, H. R., Sakamoto, H., Yoshida, T., Sugimora, T., Terada, M. and Solursh, M. (1992). Localization of Hst1 transcripts to the apical ectodermal ridge in the mouse embryo. *Dev. Biol.* **150**, 219-222.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.

Tickle, C., Summerbell, D. and Wolpert, L. (1975). Positional signaling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199-202.

Todt, W. and Fallon, J. F. (1984). Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morph.* **80**, 21-41.

Vogel, A. and Tickle, C. (1993). FGF4 maintains polarizing activity of posterior limb bud cells in vivo and in vitro. *Development* **119**, 199-206.

Vogel, A., Rodriguez, C., Warnken, W. and Izpisúa Belmonte, J. C. (1995). Dorsal cell fate specified by chick *Lmx1* during vertebrate limb development. *Nature* **378**, 716-720.

Vogel, A., Rodriguez, C. and Izpisúa Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737-1750.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissues sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Yang, Y. and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* **80**, 939-947.

Zwilling, E. (1955) Ectoderm-mesoderm relationship in the development of the chick embryo limb bud. *J. Exp. Zool.* **128**, 423-442.

Zwilling, E. (1968). Morphogenetic phases in development. *Dev. Biol. Supplement* **2**, 184-207.

(Accepted 23 May 1996)