The dorsoventral polarity of the presumptive limb is determined by signals produced by the somites and by the lateral somatopleure

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SUMMARY

When it first appears at stage HH16, the wing bud is already polarized along the dorsoventral axis. To study the mechanisms leading to the establishment of its dorsoventral polarity, we decided to focus our attention on an earlier stage (HH13). Using the quail-chick chimera system, we first show that the presumptive wing mesoderm occupies the medial half of the somatopleure at the level of somites 15-20. The corresponding ectodermal area, however, will only give rise to the apical ectodermal ridge. The rest of the limb bud ectoderm originates from the ectoderm overlying the paraxial and the intermediate mesoderms for its lateral aspect and the lateral somatopleural mesoderm for its ventral aspect. We next used five experimental paradigms to show that the dorsoventral polarity of the presumptive limb is determined by its environment. Thus, presumptive limb regions flanked on two sides by rows of somites give rise to bidorsal limb buds, indicating that the somites produce a dorsalizing factor. In addition, insertion of filters laterally to the presumptive limb region also results in bidorsal limb buds, suggesting that the lateral somatopleure produces a ventralizing factor. We propose a model in which the polarizing activity of these two signals is mediated by the morphogenetic movements of the presumptive dorsal and ventral ectoderms, which carry the dorsoventral information over the limb bud mesenchyme.

Key words: limb bud, somites, dorsoventral axis, chick, quail, induction

INTRODUCTION

The vertebrate limb develops along three axes: anteroposterior (AP), dorsoventral (DV) and proximodistal. Most of the available information concerning the patterning of limb structures along these axes has come from studies carried out in chick embryos which can be easily manipulated in ovo (for a review, see Tickle and Eichele, 1994). The first indication of the development of the chick wing bud is a condensation of somatopleural mesoderm opposite somites 15-20 at stage HH16 (Hamburger and Hamilton, 1951). A few hours later, at the end of stage HH18, a thickening of the distal ectoderm, called the apical ectodermal ridge (AER), is induced by the mesoderm at the interface between the dorsal and the ventral sides of the wing bud (Kierny, 1960; Saunders and Reuss, 1974; Carrington and Fallon, 1978; Todt and Fallon, 1984). The AER is an important signaling center, which keeps the underlying mesenchyme undifferentiated and in a proliferative state (Summerbell et al., 1973). If the AER is surgically removed, growth stops and the limb is truncated along the proximodistal axis (Saunders, 1948). Before the appearance of the AER, the DV interface is not morphologically recognizable but can be defined by the expression of Fgf-8 detectable from stage HH16 in a strip of ectodermal cells which have been suggested to give rise to the AER (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996).

As early as stage HH15/16, dorsal and ventral aspects of the limb bud can be distinguished by different molecular markers. For instance, Wnt-7a (Dealy et al., 1993; Riddle et al., 1995) and En-1 (Davis et al., 1991; Gardner and Barald, 1992) are expressed in the dorsal and in the ventral sides of the limb bud ectoderm, respectively. The Wnt-7a expression domain is excluded from the AER but the En-1 expression domain reaches the apex of this structure up to its midline. The factors patterning the limb DV polarity have long been the subject of investigation. It was established in the 1970s that this process involves tissue interactions between the two initial limb bud ectodermal and mesodermal components. Experiments carried out in the chick embryo showed that from stage HH15/16 on, the ectoderm is able to impose its polarity on the limb mesodermal rudiment. Thus, when the limb bud mesenchyme is jacketed in limb bud ectoderm whose dorsoventral axis has been reversed, epidermal, muscular and skeletal components of the distal limb develop with the dorsoventral polarity of the ectoderm (MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987). Moreover, Wnt-7a and En-1 appear to play a key role in mediating the DV polarizing activity of the limb bud ectoderm, since in mice homozygous for a mutation in Wnt-7a, dorsal structures of the limb bud adopt a ventral identity (Parr and McMahon, 1995), whereas loss of En-1 function results in dorsal transformations of ventral paw structures (Loomis et al., 1996). Wnt-7a produces at least some of its effect by inducing Lmx-1 in the underlying dorsal mesenchyme (Riddle et al., 1995; Vogel et al., 1996).

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In contrast to what has been observed after the appearance of the limb bud at stage HH15/16, reversal of the dorsoventral orientation of the somatopleural ectoderm before that stage does not affect the dorsoventral polarity of the chick limb bud (Geduspan and MacCabe, 1989). This led to the contention that initially the dorsoventral information might reside in the mesoderm before being transferred to the ectoderm, which then gains the ability to impose its polarity on that of the mesoderm. However, the conclusion that the somatopleural mesoderm contains the DV information remains controversial. Saunders and Reuss (1974) have observed that, as early as stage HH12, presumptive wing bud mesoderm transplanted to the flank (i.e. the region between the two pairs of limb buds) gives rise to limbs having the dorsoventral polarity of the grafts. This indicates that the presumptive limb already contains the DV information at that stage. In contrast, using a similar approach, Kieny (1971) observed that when the dorsoventral axis of the graft is reversed, the resulting supernumerary limbs have the same DV polarity as ipsilateral, non-manipulated ones. This suggests that the environment polarizes the presumptive limb field. In view of these conflicting results, the mechanisms involved in the establishment of the DV polarity of the presumptive limb and of the limb bud ectoderm remain undefined.

For this reason we decided to further investigate when and how patterning of the dorsoventral axis of the presumptive wing bud takes place. Our purpose was to examine the relationship between the presumptive limb and its environment with respect to the establishment of the dorsoventral polarity. We first constructed a fate map of the different ectodermal and mesodermal territories which will compose the limb bud, at a stage preceding the limb bud’s appearance (stage HH13). An unexpected finding was that, at this stage, the ectoderm cells covering the entire presumptive limb region only give rise to the AER. Using the information provided by this fate map, we then designed a series of experiments with the goal of changing the environment of the presumptive wing region. We show that, at stage HH13, the dorsoventral polarity of the wing somatopleure is not yet determined. We provide evidence that the polarization of the wing somatopleure results from the production of a dorsalizing factor by the somites and of a ventralizing factor by the lateral somatopleure which operate between stage HH13 and HH15.

RESULTS

The respective origin of the AER and of the dorsal and ventral wing ectoderm

We used the quail-chick chimera system (Le Douarin, 1969) to map the mediolateral limits of the presumptive wing region at late stage HH13 (20 somites). This stage was chosen because it is the earliest at which the paraxial mesoderm of the wing region is segmented. The somites, the Wolffian duct and the somatopleure can therefore be clearly distinguished from each other. Strips of somatopleure, which had a width of 150 μm (approximately half of the somatopleure) and for which the medial limit was the lateral border of the Wolffian duct, were taken from the wing region of the chick and substituted by their quail counterparts (n=4) (Fig. 1A/experiment 1). The resulting limb buds were analyzed 40-44 hours later (stage HH21-22) for chimerism. The mesenchyme was almost completely composed of quail cells (Fig. 1B), except for the muscle precursor cells which arose from the chick lateral somites (Christ et al., 1974; Ordel and Le Douarin, 1992). Unexpectedly, in the ectoderm, the quail cells were strictly restricted to the AER (Fig. 1C). They occupied its dorsoventral midline, whereas its periphery was composed of chick cells. In order to verify whether this restriction of quail cells to the AER is also the case earlier in development, we collected the chimeras produced by experiment 1, 24 hours after the procedure (stage HH18). At that stage the AER begins to appear (Todt and Fallon, 1984). The mesenchyme of these early chimeric limb buds is mainly composed of quail cells (n=4; Fig. 1D). In the ectoderm, however, these cells occupied only the whole thickened region which gives rise to the AER.

We conclude from these results that the mesodermal somatopleural component of the limb bud is entirely contained in the area transplanted in this experiment. We will hereafter designate this as zone W (for wing). It thus appears that,
initially, at stage HH18, the DV interface is completely derived from the ectoderm overlying the presumptive wing region. With development of the AER, some cells, which originate either from the dorsal or the ventral ectoderm of the limb bud, are incorporated into the periphery of the AER.

It remained, therefore, to determine the origin of both the dorsal and the ventral ectodermal components of the wing. For this purpose, defined strips of ectoderm were isolated from the medial and the lateral sides of the zone W. In experiment 2, a strip of quail ectoderm covering the intermediate mesoderm and approximately the medial half of zone W was grafted isotopically in stage-matched chick embryos (Fig. 2A). In experiment 3, a strip of ectoderm which was 200 μm wide and for which the medial limit was approximately the middle of zone W was exchanged between quail and chick under the same conditions (Fig. 2D). The chimeras were analyzed at stage HH21-23. In experiment 2 (n=8), the quail cells were found on the dorsal side of the limb bud and occupied precisely the dorsal half of the ridge (Fig. 2B,C). The proximal third of the limb bud dorsal ectoderm was composed of chick cells derived from the ectoderm overlying the somites. In experiment 3 (n=5), the quail cells were found in the ventral ectoderm of the limb bud and reached the dorsoventral midline of the AER (Fig. 2E,F).

We conclude that the dorsal ectoderm is derived from the ectoderm overlying the paraxial and the intermediate mesoderm and that the ventral ectoderm is derived from the lateral somatopleural ectoderm. In view of the fact that there is a margin of error when grafting into a region without morphological boundaries, it is remarkable that in both experiments 2 and 3 the boundary between the chick and quail cells...
was always precisely the dorsoventral midline of the AER.

This observation is consistent with our finding that, at late stage HH13, the cells which will give rise to the AER occupy a wide territory. Results of experiments 1, 2 and 3 are summarized in Fig. 3.

The polarity of the dorsoventral axis is not determined at stage HH13

To determine whether the DV axis of the wing territory is established at HH13, we performed a 180° rotation of the ectoderm and mesoderm of zone W, which resulted in inversion of both the dorsoventral and anteroposterior axes (Fig. 4A). Manipulated embryos were collected 2 days later (stage HH21-22) and analyzed for the expression of Lmx-1, a marker of the limb bud dorsal mesenchyme. In all cases (n=5), Lmx1 was found to be expressed dorsally, in the same domain as in control limbs (Fig. 4B). The growth of the manipulated limbs was oriented anteriorly and Shh, a marker of the posterior mesenchyme, was found to be expressed anteriorly (n=5), showing that the anteroposterior polarity was reversed (Fig. 4C). We conclude that, at this stage, the dorsoventral polarity is not yet determined.

A presumptive limb region flanked by two rows of somites gives rise to a bidorsal limb bud

We hypothesized that the paraxial structures could play a role in the induction of the dorsoventral polarity of the presumptive limb region. To test this possibility, we flanked the presumptive limb region with two rows of somites. Strips of somatopleure were taken from late stage HH13 quail embryos between somites 15 and 20. Some of these strips entirely contained the W area (Fig. 5, type A; n=7); some others were taken more laterally, from a region overlapping the W area and the lateral somatopleure (Fig. 5, type B; n=11); and in a last series, they came from the region lateral to the W area (Fig. 5, type C; n=9). The strips were grafted into stage HH13 chick
embryos in place of the neural tube either at the level of the wing (n=5) or the flank region (n=22; Fig. 6A). Embryos were analyzed 48 hours later. In all cases, even in those in which the grafted strip came from a region lateral to the presumptive limb region, a limb bud with its AER developed (Fig. 6B-D). With the exception of the AER, the ectoderm was always composed of chick cells which are thus derived from the paraxial ectoderm. The AER was composed of quail cells on its midline and of chick cells laterally, strikingly reproducing the pattern obtained in experiment 1 (Fig. 6C). In the wider grafts (200 μm), the relative contribution of the quail cells to the composition of the AER was greater, with in some instances the quail cells occupying almost the entire AER. An important difference, however, from the limb buds obtained in experiment 1 (Fig. 6B-D) was that all the limb buds were bidorsal, as evidenced by the expression of Wnt-7a (Fig. 6E,G) or Lmx-1 (not shown) on both sides and the total absence of En-1 transcripts (Fig. 6F,H,I) whether the graft was implanted at the level of the wing or of the flank. These results confirm that the dorsoventral polarity of the presumptive limb region is not yet determined at late stage HH13 and suggest that the paraxial structures produce a dorsalizing factor.

Limbs buds resulting from the same types of grafts (A, B and C) were analyzed 14-24 hours following the operation to study the distribution of the quail cells with respect to the DV interface. This was performed by comparing expression of the QCPN epitope and Fgf-8 on adjacent sections, since at that stage, the AER is not yet visible or it is just beginning to appear (stage HH17-18; Todt and Fallon, 1984). Remarkably, in 14/18 cases, the quail cells and the Fgf-8-expressing cells were exactly the same, as shown in Fig. 7. In the four other embryos a few quail cells were distributed beyond the Fgf-8 domain. Therefore, as was the case in isotopic grafts of zone W analyzed after an incubation of 24 hours, the DV interface of these early chimeric limb buds is completely derived from the transplanted somatopleural ectoderm. In chimeric limb buds analyzed a few more hours after the graft (stage HH19), the AER was entirely composed of quail cells (n=3; Fig. 7C). In normal limb buds of early stages, En-1 is expressed in the ventral ectoderm and ventral half of the AER. (I) Dark-field photomicrograph of the same section as in F. En-1 expression is detected in the ventral body wall (BW) ectoderm and mesoderm. Bars, 250 μm (B), 25 μm (C) and 10 μm (bar in D for D-F; G-I).
domain (not shown). *En-1* expression was not detected in the experimental limb buds, even in the dorsoventral interface ($n=6$; not shown).

The fact that the explants of type C yield a limb shows that the capacity to produce a limb at the level of somites 15-20 exceeds the real limb bud presumptive territory. The existence of an actual limb morphogenetic field in the lateral plate is thus demonstrated. Moreover, this indicates that the lateral somatopleural ectoderm has the competence to form an AER. In order to determine whether this is also the case for the paraxial ectoderm, we transplanted strips of wing somatopleural mesoderm overlaid by paraxial ectoderm taken between somites 15-20 in place of the neural tube. This resulted in the growth of limb buds composed of an AER (not shown).

Therefore, at late stage HH13, the competence of the paraxial structures produce a dorsalizing factor, rows of brachial somites covered with their own ectoderm were taken from late stage HH13 quail embryos and grafted at different positions laterally to the presumptive wing region of stage matched chick embryos (Fig. 8A,B). Grafts of somites in a region of about 75 $\mu$m (zone I; Fig. 8B) located immediately laterally to the W region ($n=12$) induced a variety of abnormalities in the limb buds (atrophy, duplication along the dorsoventral axis, and duplication along the anteroposterior axis). One of these abnormal limb buds was bidorsal, as shown by the expression of *Wnt-7a*. In contrast, somites grafted more laterally (zone II, Fig. 8B) did not perturb the morphology of the limb buds, which in 6/7 cases had a bidorsal polarity (Fig. 8C-F). In order to determine whether there was a correlation between the presence of quail cells in the ventral ectoderm and the induction of *Wnt-7a*, the expression of the QCPN epitope and of *Wnt-7a* were compared on adjacent sections ($n=4$). Quail cells were found proximally in the ventral ectoderm and they always expressed *Wnt-7a*. However, *Wnt-7a* expression was not restricted to these cells, as it was also induced in the chick cells of the ventral ectoderm.
the limb buds resulting from these grafts were bidorsal (to the flank in a reversed dorsoventral orientation (Fig. 9C). All somatopleure, the mesonephros and the somites were grafted was always directed ventrally. In a last set of experiments, the polarity as the ipsilateral host limbs (not shown). The growth of the limb buds examined in these two series of experiments =7). The resulting limb buds also had the same dorsoventral information does not reside in the presumptive limb territory itself at that stage but is determined by its environment. We show that the polarization of the wing somatopleure results from the production of a dorsalizing factor by the mesoderm and of a ventralizing factor by the lateral somatopleure. The fact that permeable obstacles had an effect on the limb bud DV polarity suggests that this ventralizing signal is non-diffusible. Alternatively, the filters could interfere with a signal which acts over a short distance.

DISCUSSION

When it first appears at stage HH16, the wing bud is already polarized along the DV axis. To study the mechanisms leading to the establishment of this polarity, we decided to focus our attention on an earlier stage (HH13). Using the quail-chick chimera system, we first showed that the presumptive wing mesoderm occupies the medial half of the somatopleure, a zone that we designate as the W region. The corresponding ectodermal area, however, will only give rise to the AER. The dorsal limb bud ectoderm originates from the ectoderm overlying the somites and the intermediate mesoderm. The ventral limb bud ectoderm originates from the mesoderm overlying the somatopleure and of the intermediate mesoderm. The DV information does not reside in the presumptive limb territory itself at that stage but is determined by its environment. We show that the polarization of the wing somatopleure results from the production of a dorsalizing factor by the somites and of a ventralizing factor by the lateral somatopleure which operate between stage HH13 and HH15.

Delineation of the DV interface and involvement of the somites in patterning the dorsoventral axis of the limb bud

The induction of limb development depends on a signal produced by the somites and by the mesonephros around stage HH13/14 (Pinot, 1970; Kieny, 1971; Stephens and McNulty, 1981; Geduspan and Solursh, 1992; Stephens et al., 1993). IGF-1 and FGF-8, which are both expressed by these structures, appear to play an important role in this inductive process (Crossley et al., 1996; Dealy and Kosher, 1996). Geduspan and
Solursh (1992) have shown that, at stage HH14-16, the presumptive limb mesoderm occupies the medial half of the somatopleure and that its lateral half can also give rise to a limb when grafted immediately lateral to the mesonephros. Our fate map and our grafts of somatopleure in place of the neural tube support these results, showing that at these early stages the entire somatopleure located between somites 15 and 20 constitutes a morphogenetic field larger than the actual presumptive wing territory. Any region of this field can become a wing if subjected to the appropriate inducing influences. A similar conclusion has been reached for an amphibian (Amano, 1960).

The present work provides an important clue about how the DV interface is specified. The chimeric limb buds that we produced at late stage HH13 either by isotopic grafts of wing somatopleure or by grafting somatopleure strips in place of the neural tube, showed that, initially the DV interface, including the early stage AER, is entirely derived from the ectoderm overlying the presumptive limb mesoderm. With further developmental of the limb bud, some additional cells of the dorsal and ventral ectoderms are incorporated in the flanks of the AER. Classical studies have shown that the somatopleural mesoderm induces the AER (Kieny, 1960; Saunders and Reuss, 1974; Carrington and Fallon, 1984). Our data suggest that, at stage HH13/14, the mesoderm of zone W induced by the somites and the mesonephros specifies the entire overlying ectoderm to acquire its DV interface identity (Fig. 11A). One consequence of the induction by the mesoderm appears to be the onset of cell death (Vaahtokari et al., 1996) and/or a decrease in cell proliferation, as the prospective AER expands much less than do the ectoderms overlying the intermediate mesoderm and the lateral somatopleure, which will cover the dorsal and the ventral aspects of the limb, respectively.

A series of classical experiments carried out in the amphibian Amblystoma have shown that while the anteroposterior polarity of the presumptive limb region is determined as early as at the completion of gastrulation (Detwiler, 1933), its dorsoventral polarity is acquired much later, shortly before the appearance of the limb bud (Harrison, 1918, 1921; Ruud, 1926; Swett, 1927; Hollinshead, 1936). In the chick, there is also some evidence that the anteroposterior axis is determined early, possibly as early as stage HH10 (Hornbruch and Wolpert, 1991). However, the stage at which the presumptive limb acquires its DV polarity is controversial (Kieny, 1971; Saunders and Reuss, 1974).

In order to address the question of the determination of the DV axis of the limb bud, we used several experimental procedures to modify the environment of stage HH13 presumptive limb mesoderm. When the presumptive wing region was rotated by 180° or when it was transplanted to the flank with a reversed dorsoventral orientation, a limb bud developed which had the dorsoventral polarity of a non-rotated or non-inverted control limb as shown by Lmx-1 expression. In other words, at stage HH13, the dorsoventral polarity does not reside in the presumptive limb mesoderm but is determined by its environment. To see whether the paraxial structures play a role in the induction of the DV polarity, we flanked the presumptive limb region by two rows of somites, either by transplanting it in place of the neural tube, by grafting a row of somites laterally in the lateral plate mesoderm or by inserting the somatopleure with the paraxial structures in a reversed dorsoventral orientation under the flank ectoderm. These manipulations resulted in the growth of bidorsal limb buds, suggesting that the somites produce a dorsalizing factor. Similar experiments performed in amphibians also showed that the paraxial structures play a role in the polarization of the dorsoventral axis (Swett, 1938). Therefore, the presumptive limb bud is patterned by at least two paraxial signals: the first signal, produced by the mesonephros and the somites, is responsible for limb induction and the second signal, provided by the somites, dorsalizes the limb bud. One possible explanation for Saunders and Reuss’ (1974) results is that portions of somites or of presomitic mesoderm were included in their graft.

**Role of the ectoderm in the dorsalization of the presumptive limb mesoderm**

Experiments carried out by a number of investigators have clearly indicated that from stage HH15/16 on, the ectoderm of the limb bud can impose its DV polarity upon that of the autologous mesoderm. The mechanisms by which the somites can polarize the presumptive limb ectoderm remain unclear. One possibility is that, between stage HH13 and HH15, the somites directly polarize the mesoderm of zone W, which could then transfer the dorsoventral information to the overlying ectoderm (Fig. 11B).

Another possibility is that, between stage HH13 and HH15, the somites specify the presumptive dorsal ectoderm, which overlaps the paraxial and the intermediate mesoderms, to acquire a dorsal identity (Fig. 11C1). The morphogenetic movements of this ectoderm layer would then carry the dorsal information over the wing mesoderm (Fig. 11C2). When we grafted quail somites and their overlying ectoderm laterally to the presumptive wing region of chick hosts, we observed no correlation between the presence of quail cells in the ventral ectoderm of the limb bud and the induction of Wnt-7a. Therefore, the paraxial mesoderm has induced the adjacent chick ectodermal cells to acquire a dorsal identity. The same explanation could account for the dorsaling effect of somites deprived of their ectoderm. These observations are consistent with a model which predicts that there are two dorsaling factors. First, a factor is produced by the somites, which induces the presumptive dorsal ectoderm to acquire a dorsal identity. This ectoderm then proliferates over the limb mesoderm and secretes the second factor, probably WNT-7a, which dorsalizes the wing bud.

Recombination experiments at stage HH14, in which dorsoventrally reversed ectoderm resulted in wings with mesodermal dorsoventral polarity, led Geduspan and MacCabe (1987, 1989) to propose that the presumptive limb mesoderm transfers the dorsoventral information to the overlying ectoderm at the limb budding stage. As these authors did not specify the boundaries of their grafts and in what orientation the recombinant tissues were grafted to the flank, it is difficult to interpret their results. Nevertheless, they raise the possibility that the presumptive ectodermal territories of the limb bud are not yet determined at stage HH14. The fact that a graft of somites laterally to the W region can program the presumptive ventral ectoderm to acquire a dorsal identity would also support this view.

In the limb regions, the ectoderm can be divided into two domains along the dorsoventral axis. (1) A dorsal one overlying the axial organs, the paraxial mesoderm and the
A Stage HH13

Induction of the limb mesoderm by axial structures and of the AER by the limb mesoderm (W).

B

DV polarization of the limb via the wing mesoderm.

C

1

2 Stage HH16

DV polarization of the wing via the wing ectoderm.

Fig. 11. Signals patterning the presumptive limb bud. Schematic transverse sections of late stage HH13 (A-C1) and of stage HH16 (C2) embryos. (A) At stage HH13/14, the mesonephros and the somites induce the mesoderm of zone W (in blue) which then specifies the overlying ectoderm to acquire a DV interface identity. (B, C) Alternative pathways to specify DV axis in the limb bud. (B) The dorsalizing and ventralizing signals act on the mesoderm of zone W. (C1). The somites and the lateral somatopleure act on the future dorsal and ventral limb ectoderm. (C2) The induced ectoderms grow over the budding limb and carry their dorsal and ventral identity that they secondarily impose to the limb mesoderm.

Specification of the limb bud ventral identity

The insertion of filters laterally to the W region gave rise to bidorsal limb buds. These filters are likely to interfere with the propagation of a signal produced by the lateral somatopleure. In the absence of this ventralizing factor, the presumptive ventral limb bud would acquire a dorsal identity. Therefore, polarization along the DV axis would result from the antagonistic actions of two signals: a dorsalizing one produced by the somites and a ventralizing one by the lateral somatopleure. These two signals could act through similar mechanisms, namely by directly polarizing the mesoderm of zone W or by inducing their respective overlying ectoderms (Fig. 11B,C). In the latter case, the filters would produce bidorsal limb buds by interfering with the morphogenetic movements of the lateral somatopleural ectoderm.

Relationship between the establishment of the limb bud DV polarity and the formation of the AER

In the chick mutant limbless, limb buds develop normally until stage HH19. After that stage, no AER forms and the whole bud degenerates (Prahlad et al., 1979; Carrington and Fallon, 1988). Fgf-8 and En-1 transcripts are not detectable in these limb buds and Wnt-7a is expressed in the entire limb bud ectoderm (Ros et al., 1996; Noramly et al., 1996; Grieshammer et al., 1996). Meinhardt (1983) proposed that the juxtaposition of a dorsal and of a ventral domain is a prerequisite for the formation of the AER. The fact that in limbless the limb buds are characterized by the absence of both a dorsoventral juxtaposition and an AER supports this hypothesis. Our finding that an AER can form in a bidorsal context (see also Carrington and Fallon, 1986) would instead suggest that establishment of DV polarity and AER formation occur independently. It is possible, however, that the main function of the markers used in this study, En-1, Wnt-7a and Lmx-1, is to confer a dorsal or a ventral identity to the tissues in which they are expressed and that AER specification results from the dorsoventral juxtaposition of other genes in the ectoderm and/or mesoderm of zone W. Our results also do not necessary imply that DV interface specification is solely based on the interaction between the ectoderm and the mesoderm of zone W. For instance, signaling between the presumptive DV interface and the adjacent ectoderms could contribute to the delineation of the limb ectoderm territories.

We could not detect En-1 transcripts in the AER of bidorsal limb buds. This might indicate that En-1 expression in the ventral AER is dependent upon the presence of an adjacent ectoderm which has a ventral identity. In limb buds of En-1 mutant mice, the ventral border of the AER is shifted ventrally and proximally, whereas the dorsal border is located normally (Loomis et al., 1996). It is noteworthy that the morphology of the AER of bidorsal limb buds produced in our experiments was normal even though En-1 expression was not detected. The basis for this discrepancy remains unclear.

We are particularly grateful to Olivier Pourquié for interesting discussions at the beginning of this project and for his critical reading of the manuscript. We also thank Chen-Ming Fan, Anne Grapin and Domingos Henrique for their comments on the manuscript. We are indebted to Dr Gail Martin for the generous gift of the chick Fgf-8 in situ probe, to Dr Cliff Tabin for Wnt-7a and Lmx-1 and to Dr Cairine Logan for En-1. We are grateful to Françoise Viala, Hélène San...
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(Accepted 3 February 1997)