Development 124, 4547-4556 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV2217

Dorso-ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb

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SUMMARY

We wish to understand how limbs are positioned with respect to the dorso-ventral axis of the body in vertebrate embryos, and how different regions of limb bud ectoderm, i.e. dorsal ectoderm, apical ridge and ventral ectoderm, originate. Signals from dorsal and ventral ectoderm control dorso-ventral patterning while the apical ectodermal ridge (AER) controls bud outgrowth and patterning along the proximo-distal axis. We show, using cell-fate tracers, the existence of two distinct ectodermal compartments, dorsal versus ventral, in both presumptive limb and flank of early chick embryos. This organisation of limb ectoderm is the first direct evidence, in vertebrates, of compartments in non-neural ectoderm. Since the apical ridge appears to be confined to this compartment boundary, this positions the limb. The mesoderm, unlike the ectoderm, does not contain two separate dorsal and ventral cell lineages, suggesting that dorsal and ventral ectoderm compartments may be important to ensure appropriate control of mesodermal cell fate. Surprisingly, we also show that cells which form the apical ridge are initially scattered in a wide region of early ectoderm and that both dorsal and ventral ectoderm cells contribute to the apical ridge, intermingling to some extent within it.

Key words: dorso-ventral patterning, AER, compartment, lineage restriction, chick embryo, limb development

INTRODUCTION

A fundamental question in the development of the vertebrate body plan is what regulates dorso-ventral pattern, and this is important with respect to limb number, type and position. Limb position must be specified in relation to both dorso-ventral and antero-posterior axes. Recently, we have shown that limb position along the antero-posterior axis appears to be related to patterns of *Hox* gene expression in lateral plate mesoderm (Cohn et al., 1997). Here, we begin to explore the mechanisms that position limbs along the dorso-ventral axis. Antero-posterior positioning is linked to specification of limb type but dorso-ventral positioning could have the same basis in both fore- and hindlimbs.

Formation of the apical ectodermal ridge is a central key-point for limb positioning and is also an important feature of limb ectoderm regionalisation, marking the interface between dorsal and ventral ectoderm (Saunders, 1948; Todt and Fallon, 1984). As the limb bud develops, the apical ridge comes to rim the tip and mediates outgrowth by production of fibroblast growth factors (FGFs) (Niswander and Martin, 1992; Niswander et al., 1994; Fallon et al., 1994; Mahmood et al., 1995a; Crossley et al., 1996; Vogel et al., 1996). At early pre-bud stages, the lateral plate mesoderm is a flat layer of cells covered by ectoderm. As the body wall tucks round to begin to close up ventrally, a thickened strip of ectoderm, which will give rise to the ridge, begins to develop over the slightly swelling mesoderm at the same dorso-ventral position for both fore- and hindlimbs. In chick embryos, the ridge consists of tightly packed columnar cells (Todt and Fallon, 1984; Jurand, 1965).

Signalling by pre-limb mesoderm is necessary for apical ridge formation (Kieny, 1968; Saunders and Reuss, 1974; Carrington and Fallon, 1984). When limb bud mesoderm is transplanted to flank, it can induce an apical ectodermal ridge and grow out to form a new limb. Positioning of the ridge with respect to the dorso-ventral axis appears to be the result of localised response of the ectoderm rather than localised production of mesoderm signals, as a discrete apical ridge still forms even when limb mesoderm is chopped in small pieces, reaggregated and then grafted in the flank (Kieny, 1960). There has recently been considerable interest in the idea that the ridge arises at a boundary region (reviewed in Zeller and Duboule, 1997). In insects, a boundary between *Fringe*- and non-*Fringe*-expressing cells positions the wing margin, which controls patterning of the wing (Irvine and Wieschaus, 1994). Rather remarkably, a member of the vertebrate *Fringe* family, *Radical Fringe*, has been shown to be expressed dorsally in chick limb bud ectoderm and the apical ectodermal ridge develops at the *Radical Fringe*/non-*Radical Fringe* boundary (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Expression of *Engrailed-1* (*En-1*), which is normally expressed in ventral ectoderm and the ventral part of the ridge (Davis et al., 1991; Gardner and Barald, 1992; Wurst et al., 1994; Logan et al., 1997; Rodriguez-Esteban et al., 1997), is able to repress *Radical Fringe* expression (Laufer et al., 1997; Rodriguez-Esteban et al., 1997) and ectopic mis-expression of *En-1* in

chick embryos leads to disruption of the ridge (Logan et al., 1997). Interestingly, in the chicken mutant *Limbless*, in which *En-1* is not expressed in limbs, no apical ridge is formed and the buds fail to grow out (Grieshammer et al., 1996; Ros et al., 1996). Altogether, these findings suggest that the apical ridge develops as a boundary specialisation.

Regionalisation of limb ectoderm into dorsal and ventral ectoderm domains is important in relation to dorso-ventral patterning of limb bud structures. The inversion of the dorso-ventral polarity of the limb ectoderm by rotation leads to limbs with changes in dorso-ventral polarity to correspond with that of the ectoderm (Pautou and Kieny, 1973; MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987; Akita, 1996).*Wnt-7a* is expressed in dorsal limb ectoderm from early stages (Dealy et al., 1993; Parr et al., 1993). It was shown to activate *Lmx-1* in dorsal mesenchyme and to be required for dorsal patterning (Riddle et al., 1995; Vogel et al., 1995). Transgenic mice in which *Wnt-7a* is functionally inactivated have paws with double ventral phenotype (Parr and McMahon, 1995). In contrast, when *En-1* is inactivated the tips of the limbs are bi-dorsal (Loomis et al., 1996). This mutant phenotype is also obtained after mis-expression of *Lmx-1* in chick embryo (Riddle et al., 1995; Vogel et al., 1995). In addition to *Radical Fringe* repression, *En-1* is also able to repress *Wnt-7a* expression (Logan et al., 1997). In *Limbless* mutants, *En-1* is not expressed in limb regions and *Wnt-7a* is expressed both dorsally and ventrally in the abortive limbs. Consequently, when the mutant limb phenotype is rescued by implantation of FGF-soaked beads, the growing limbs are double-dorsal (Grieshammer et al., 1996; Ros et al., 1996). Hence, inappropriate expression of molecules normally restricted to either dorsal or ventral ectoderm has serious consequences for limb patterning.

Recently, Michaud et al. (1997) mapped the origin of dorsal ectoderm, ventral ectoderm and apical ectodermal ridge in the wing, using quail-chicken chimeras. Here, we have used fluorescent cell tracers to study the behaviour of small patches of cells and mapped cell fate in the ectoderm covering the body in both limb and inter-limb regions. We present evidence that the ectoderm is composed of distinct dorsal and ventral compartments, in contrast to mesoderm, which is not compartmentalised. We relate the ectodermal compartment boundary to the position at which both normal and ectopic limbs develop. We also define the origin of cells that give rise to dorsal limb ectoderm, ventral limb ectoderm and apical ectodermal ridge. Apical ridge progenitors appear to be scattered within both presumptive dorsal and ventral ectoderm.

MATERIALS AND METHODS

Embryos

Experiments were carried out on fertilised White Leghorn chicken embryos. Eggs were incubated at 38°C for appropriate times, then windowed and the vitelline membrane removed prior to experiments. To improve visibility, a small amount of Indian ink (Pelikan), diluted 1:10 in Tyrode solution, was injected under the blastoderm. Embryos were staged according to Hamburger and Hamilton (1951).

DiA and DiI application

Small deposits of the lipophilic dyes DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchloride, Molecular probes D-282) and DiA (4-Di-16-Asp, Molecular probes D-3883) at 3 mg/ml in dimethylformamide were iontophoresed via microelectrodes onto either body ectoderm or mesoderm, in ovo, using a 9 V battery (Cohn et al., 1997). This procedure labels a patch of tissue approximately 35 μm in diameter (Fig. 2A) containing about 10 cells. Microelectrodes, with a tip diameter of 3 μm, were filled at their tip with a small quantity of tracer and backfilled with 1 M lithium chloride. For embryos between stages 13 and 16, the most recently segmented level was labelled (see Fig. 1): in stage 13, the region at somites level 15-19; in stage 14, at somites 19-24; in stage 15, at somites 22-27; in stage 16, at somites 24-29. Embryos between stages 8 and 12 were labelled in the presumptive wing region prior to segmentation. Immediately after application, the size and the relative position of the spot with respect to the medial edge of the somites was measured using an epifluorescence microscope fitted with a micrometer. After 48-72 hours (depending on the initial stage of the embryos) of incubation at 38°C, the embryos were removed from the egg, dissected in phosphate-buffered saline (PBS) and fixed in 3.5% paraformaldehyde (PFA) overnight.

Analysis

Embryos were bissected through the neural tube and the labelled side viewed as a wholemount on a slide under a coverslip with a solution of PBS using a Nikon Optiphot 2 microscope with fluorescence attachment. Pictures were taken on Kodak Ektachrome 400X colour film. Some cases were further embedded in 20% gelatin, refixed in 3.5% PFA /0.1% gluteraldehyde solution, sectioned transversely at 50 μm on a vibratome and analysed by confocal microscopy (Leica TCS4D, laser scanning confocal microscope).

Whole mount in situ hybridisation

Whole mount RNA in situ hybridisation was performed using non-radioactive digoxygenin (DIG)-labelled RNA probe, as described in Logan et al. (1997). Specimens were refixed using 4% paraformaldehyde in PBS prior to storing or sectioning. Some cases were embedded in 0.45% gelatin/27% albumen/18% sucrose solution, sectioned transversely on a vibratome at 50 μm and further cleared in 90% glycerol in PBS.

FGF-2 bead implantation

Implantation of heparin acrylic beads (H5263, Sigma) of 100-150 μm diameter, soaked in FGF-2 (1 mg/ml, 133-FB-025, R&D System), was performed as described in Cohn et al. (1995). The slit in the ectoderm was made at different dorso-ventral and antero-posterior levels, allowing the insertion of the bead at the correct position onto the lateral plate mesoderm. After operation, eggs were reincubated at 38°C.

RESULTS

We systematically mapped the dorso-ventral fate of the ectoderm cells in presumptive limb and flank regions of chicken embryos. A single spot of DiA and/or DiI was applied to the ectoderm at early stages, at 25 μm intervals from the medial edge of the somites to the lateral border of the embryo. The fate map covers the region from somite 15 to somite 29 (Fig. 1). In chicken embryos, the presumptive wing region lies between somites 15 and 20, the presumptive leg region between somites 26 and 32, while the region between somites 21 and 25 gives rise to the flank region (inter-limb). The label was placed onto the ectoderm of the embryo before folding, when the embryonic body is still flat. As development and folding of the body progresses in an antero-posterior direction, mapping the entire region between somites 15 and 29 required

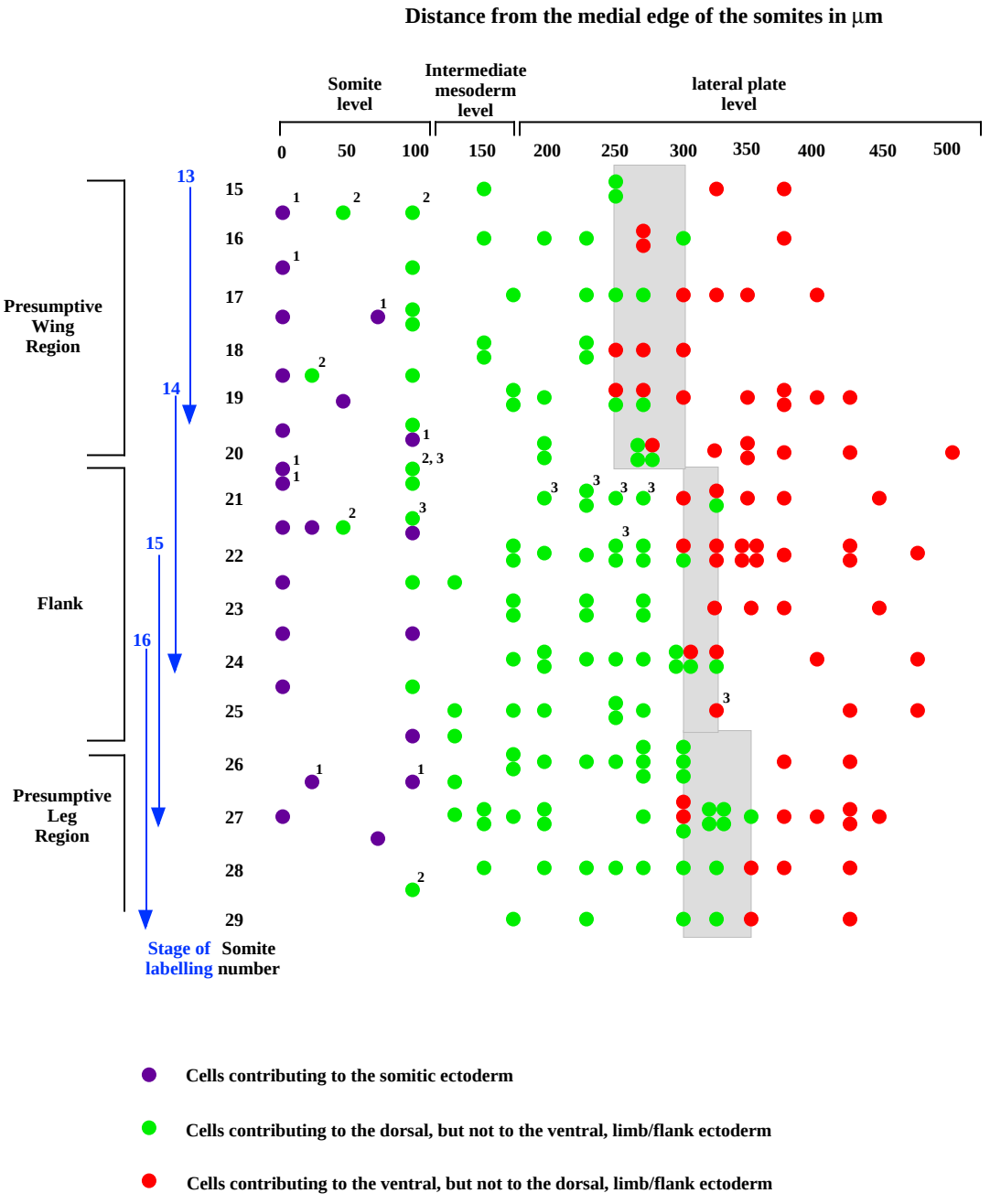

Fig. 1. Summary map of ectodermal fate in presumptive wing, flank and leg regions (derived from *n*=204 embryos). The dots represent individual spots of dye placed in the ectoderm at the position indicated. The color indicates the fate of the labelled cells in each experiment. Purple indicates contribution to somitic ectoderm; green, contribution to dorsal ectoderm; and red, contribution to ventral ectoderm. Further detailed information about cell contribution is noted in superscript: [1]cells contribute also to ectoderm immediately lateral to the somites; [2]cells contribute to both limb and somitic ectoderm; [3]cells from flank contributing to limb ectoderm. The grey outlined area represents the region of the invisible dorso-ventral compartment boundary. The reason that there are both green and red spots within this area is due to the fact each spot is obtained from an individual embryo and not to the mixing of prospective dorsal and ventral ectodermal cells. Note that the scale of the antero-posterior axis has been compacted.

the use of four different stages (HH stages 13 to 16; Hamburger and Hamilton, 1951) (Fig. 1). Progeny of labelled cells were analysed 48 hours later (HH stages 22-27). We found several categories of fluorescent clusters (Fig. 1): clusters contributing mainly to the somitic ectoderm (purple dots); clusters contributing to dorsal limb or dorsal flank ectoderm (green dots) and clusters contributing to ventral limb or ventral flank ectoderm (red dots).

Origin of the limb ectoderm

An extensive medio-lateral region of ectoderm gives rise to the ectoderm of the limb (Fig. 1). However, ectoderm covering the extreme medial edge of the somites is never found to contribute to the limb ectoderm. In contrast, cells situated just a little more laterally (25 µm from the medial edge of the somites) can contribute to the limb ectoderm, while cells originally lying over the lateral part of the somites and over the intermediate mesoderm routinely contribute to the ectoderm of the limb. In most of the cases, labelled clusters form a long proximo-distal strip covering most, if not the entire, length of the limb, whatever the initial position of the spot of dye (Fig. 2B,D). This strip runs perpendicular to the main axis of the body. The labelled strip also contains non-labelled cells, suggesting that cell mingling occurs with outgrowth (Fig. 2C).

Cells at limb levels only contribute to the limbs, but in the flank there is some displacement and cells can contribute to the limb ectoderm by extending either anteriorly into the wing or posteriorly into the leg (Fig. 2G).

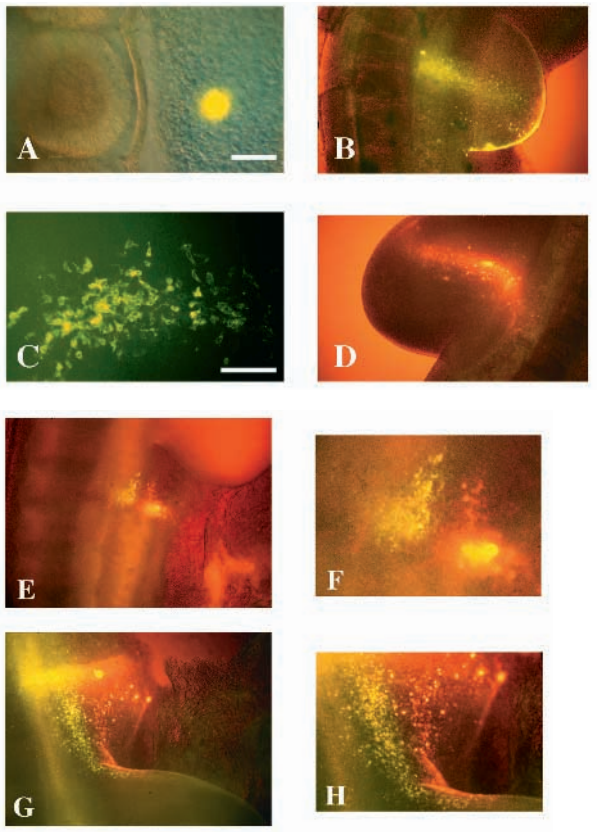

Fig. 2. Dorso-ventral cell fate of limb and non-limb ectoderm. (A) Small patch of labelled cells immediately after DiA application, at HH stage 14. Bar, 50 μm. (B) Distribution of fluorescently labelled cells in the dorsal limb ectoderm 72 hours after DiA application at HH stage 8. Both apical ridge and non-ridge ectoderm are labelled. (C) High power view of dorsal ectodermal cells labelled with DiA. Note mixing with non-labelled cells. Bar, 50 μm. (D) Distribution of fluorescently labelled cells in the ventral limb ectoderm 48 hours after DiI application at HH stage 13. (E,G) Double labelling of dorsal (DiA, green) and ventral (DiI, red) flank ectoderm. Cells appear to deviate towards wing (E) or leg (G) as they approach the dorso-ventral interface and generate right-angled clusters. High power views are shown in (F) and (H); some very bright DiI-labelled cells appear yellow due to photosaturation.

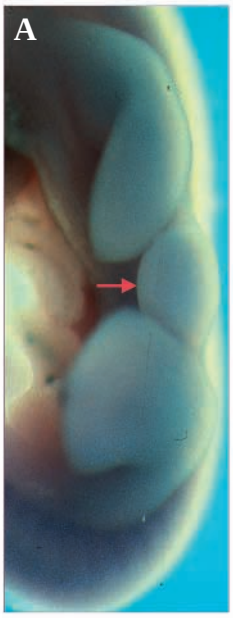

Fig. 3. Effects of beads soaked in FGF-2 implanted at various dorso-ventral levels, in the flank, on the eventual location of the additional limbs induced. (A) Additional limb produced by implantation of FGF-2 soaked bead in the most lateral region of the lateral plate (at approximately ⩾400 μm). The extra limb shown (red arrow) is in register with the normal limbs. (B) Table showing number of FGF-2 soaked beads implanted in different positions and location of the additional limbs induced. All the additional limbs are in register with the normal limbs whatever the initial location of the bead.

B

Effects of beads soaked in FGF-2 implanted at different dorso-ventral levels between somites 21 and 25

	Bead location		
Somite level	Next to the intermediate mesoderm	In the middle of the lateral plate	In the most lateral region of the lateral plate
21	1	1	1
21/2	–	1	3
22	1	1	3
22/3	1	2	–
23	2	–	1
23/4	–	1	–
24	1	1	1
24/5	1	–	2
25	–	–	1
Additional limb developed in register with normal limbs	7/7	7/7	12/12

In conclusion, limb ectoderm arises from an area approximately 500 μm wide that lies lateral to the medial quarter of the somites.

Dorsal and ventral compartments in limb ectoderm

Limb ectoderm was found to consist of two populations of cells in both fore- and hindlimb regions: one fated to be dorsal ectoderm and the other fated to be ventral ectoderm (Figs 1, 2B,D). In every case, labelled cell populations are strictly confined to either dorsal or ventral limb ectoderm and do not extend beyond the apical ridge. This fact is remarkable as, within these territories, labelled clusters usually extend for long distances, as already mentioned. At early HH stages 13-16, cells fated to be dorsal limb ectoderm are located between 25 μm and 300 μm from the medial edge of the somites for the forelimb, and between 25 μm and 350 μm for the hindlimb. Cells fated to be ventral are spread between 250 μm and 500 μm for forelimb; 300 μm and 450 μm for hindlimb (Fig. 1). Our measurements suggest that the border between dorsal and ventral compartments lies around 250-300 μm from the medial edge of the somites in the forelimb region and around 300-350 μm in the hindlimb region. Variations reflect slight differences from one embryo to another. As cells were never found in both dorsal and ventral limb ectoderm and as these cells arise from distinct early territories, this result shows that, at limb level, ectodermal cells are organized into compartments.

In order to determine when these compartments are established, we labelled ectoderm in embryos from HH stages 8 to 12 (Fig. 2B), in the presumptive wing region. After 48 or 72 hours, in all cases (n=25), labelled cells were again confined to either dorsal or ventral ectoderm: labelled cell populations never straddled the ridge. Thus, ectoderm compartmentalisation may be a very early process not directly dictated by limb formation.

Dorsal and ventral compartments in flank ectoderm

At flank level, in contrast with clusters at limb level, the predominant shape of the clusters is longitudinal, parallel to the body axis (Fig. 2E,G). Strikingly, like the limb, no clusters of fluorescent cells were found to cross a line drawn between the bases of wing and leg buds. In two specimens (Fig. 2E-H) in which double labelling was performed, both dorsally located DiA cells and ventrally located DiI cells appear to approach this dorso-ventral interface and then to change their dispersal pattern to spread along the length of the interface towards the nearest limb, thus generating right-angled clusters (Fig. 2F,H). At the time of labelling, the border between prospective dorsal and ventral cells lies around 300-325 μm from the medial edge of the somites (Fig. 1). This result demonstrates a continuous boundary of lineage-restriction running the entire length of ectoderm from wing to leg levels.

There is no morphological structure that marks this dorso-ventral interface in the flank, but ectopic limbs induced by FGF bead implants always appear to arise along this line (Cohn et al., 1995). In order to test whether ectopic limb position along the dorso-ventral axis always coincides with the position of the compartment boundary, a bead soaked in FGF-2 was placed in three different dorso-ventral sites: immediately next to the intermediate mesoderm (approximately at 200 μm-300 μm from the medial edge of the somites), in the middle of the lateral mesoderm (approximately at 300 μm-400 μm) or at the most lateral region of the lateral plate mesoderm (approximately ⩾400 μm). The implantations were done at different levels between somites 21 and 25. In all cases, irrespective of the position in which the FGF bead is implanted (n=26, Fig. 3A,B), the ectopic limb bud arises in register with the normal limb buds, suggesting that additional limbs can only arise at a particular dorso-ventral level that coincides with the boundary of lineage restriction.

Origin of the apical ectodermal ridge

Labelling of pre-limb ectodermal cells produces clusters containing both non-ridge cells and ridge cells (55/104), others containing only non-ridge cells (46/104) and a few containing only ridge cells (3/104) (Fig. 4). We sought to determine if there was a region of the early ectoderm that contributes mostly to the apical ridge. Very surprisingly, as shown in Fig. 4, apical ridge progenitors are widely spread in early ectoderm: from the ectoderm covering the intermediate mesoderm to the most lateral part of the ectoderm covering the lateral plate region (425 μm from the medial edge of the somites). Apical ridge progenitor cells of the forelimb come mainly from a region close to the somites (150 μm to 325 μm from the medial edge of the somites) while for the hindlimb, they originate more laterally (lateral to 225 μm from the medial edge of the somites) (Fig. 4). Note that the somitic ectoderm does not contain apical ridge progenitors. These results show that at stages 13 to 16, apical ridge progenitors are mingled with non-apical ridge progenitors and are not located only in the future apical ectodermal ridge region. In

Table 1. Distributions in individual limbs of dorsal and ventral cells within the apical ridge

Distribution of cells in the AER	Dorsal contribution	Ventral contribution
Up to 1/2 of the AER	0	10
Between 1/2 and 2/3 of the AER	3	0
Up to 2/3 of the AER	2	0
Throughout the AER	4	0
n total	9	10

AER, apical ectodermal ridge.
n, number of specimens analysed.
Numbers indicate the number of cases in which the particular cell distributions were seen.

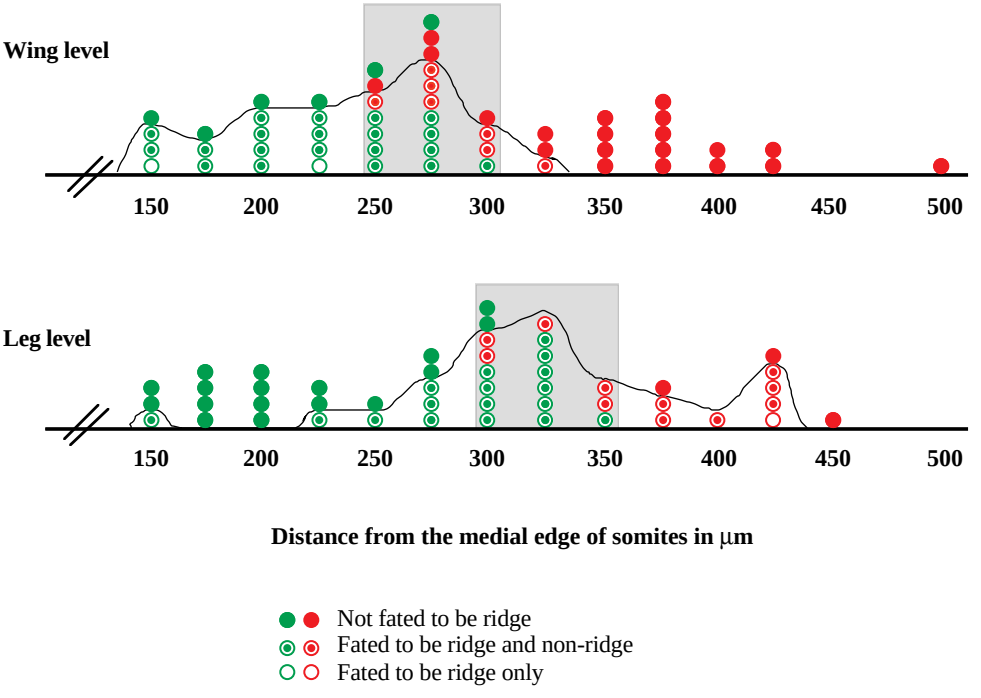

Fig. 4. Frequency distribution of apical ridge progenitors in early ectoderm. Data from Fig. 1, at wing and leg level, now showing in addition the contribution of labelled cell populations to the apical ridge. The plot emphasizes medio-lateral distribution of prospective ridge and non-ridge cells in wing (upper plot) and leg (lower plot). Most prospective ridge clusters (beneath the drawn line) also contribute to non-ridge ectoderm. Leg ridge cells have a more lateral origin than wing ridge cells. The most medial position, at which cell clusters that contribute to the apical ridge are seen, is at 150 μm from the medial edge of the somites (i.e. ectoderm above the intermediate mesoderm). Grey areas represent regions of approximate dorso-ventral compartment boundary. Green dots contribute to dorsal ectoderm and red dots contribute to ventral ectoderm.

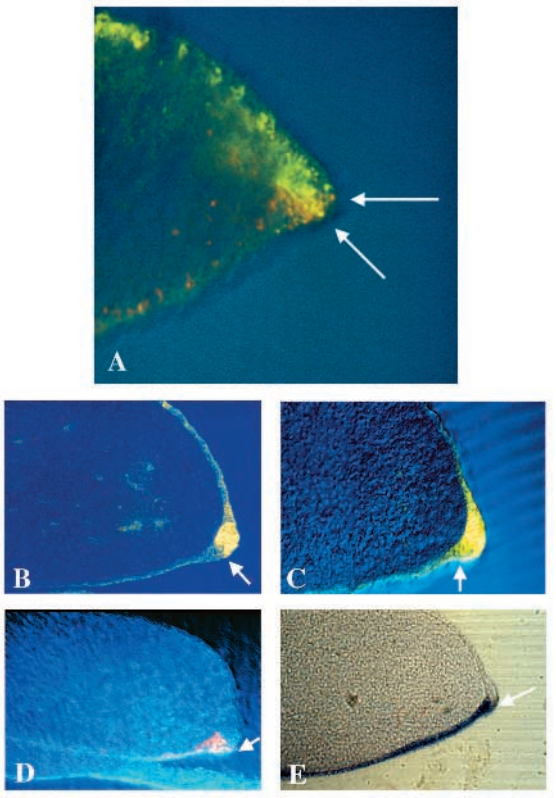

Fig. 5. Distributions of dorsal and ventral cells within the apical ridge. (A) Transverse section of double-labelled specimen analysed by confocal microscopy. Dorsally labelled cells (DiA, green) are seen up to 2/3 of the width of the ridge (bottom arrow). Ventrally labelled cells (DiI, red) are seen up to the midline of the ridge (top arrow). A few labelled cells are also observed in the mesenchyme. (B,C) Transverse section of dorsal cell clusters analysed by confocal microscopy. Arrow shows ventral limit of fluorescent cells within the ridge. Labelled cells occupy the dorsal 2/3 of the width of the ridge in B and the whole ridge in C. (D) Transverse section of ventral cell cluster analysed by confocal microscopy. Arrow shows dorsal limit of fluorescent cells coincident with mid-point of ridge, which is also the dorsal limit of *En-1* expression (E). In all cases, dorsal is up and ventral is down.

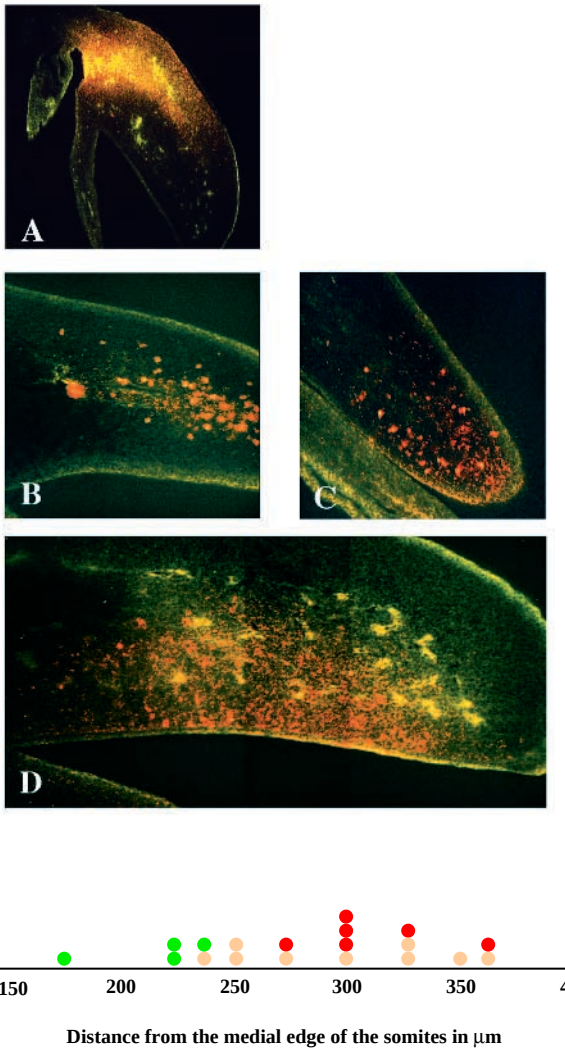

Fig. 6. Dorso-ventral cell fate in limb mesoderm. Transverse sections analysed by confocal microscopy. (A) Proximo-dorsal restricted labelled cluster. (B,C) Proximal and distal DiI-labelled clusters that contribute to both dorsal and ventral mesoderm. (D) Ventrally restricted DiI-labelled cluster. In both A and D capillary endothelial cells are visible due to autofluorescence. In all cases, dorsal is up and ventral is down. (E) Summary map showing origin of dorsal- (green), mixed dorsal and ventral- (orange) and ventral-fated (red) mesodermal cells.

addition, it demonstrates that there is no region that preferentially gives rise to ridge.

In order to determine the respective contributions of dorsal and ventral cell populations to the apical ridge, we sectioned labelled limbs and analysed them using confocal microscopy. Cells coming from the dorsal compartment were found to extend beyond the midpoint of the apical ridge and to colonise the ventral apical ridge domain to various extents, depending on the specimen (n=9) (Table 1, Fig. 5A-C), whereas cells from ventral ectoderm occupy the ventral half of the apical ridge and were never seen beyond the midpoint of the apical ridge (n=10) (Table 1, Fig. 5A,D). The dorsal cell contribution fits with the described pattern of expression of *Radical fringe* encompassing the whole apical ridge (Rodriguez-Esteban et al., 1997). On the other hand, Fig. 5E shows that *En-1* is expressed in the ventral half of the ridge (see also Logan et al., 1997; Rodriguez-Esteban et al., 1997) and thus coincides with the contribution of ventral cells.

Origin of the limb mesoderm

Grafting experiments have shown that ectoderm determines the dorso-ventral identity of the underlying mesoderm (Pautou and Kieny, 1973; MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987, 1989; Akita, 1996). We examined cell fate of early mesoderm (HH stages 13-16) for both forelimb and hindlimb. Although some labelled mesodermal cells can be found as dorsally restricted or ventrally restricted clusters (Fig. 6A,D) at least 50% of labelled cell populations spread across the dorso-ventral midline either proximally (Fig. 6B) or distally (Fig. 6C). Therefore, unlike ectoderm, mesoderm at stages 13-16 does not contain a clear boundary between cells that will become dorsal and cells that will become ventral. Mesodermal cell fate is summarised in Fig. 6E. Note that

labelled clusters in the distal limb tip always occupy both dorsal and ventral territory, thus mingling could occur while the lateral plate mesoderm is a flat sheet of cell and/or after it has folded (see Fig. 7). Proximally, however, we also found cells straddling the dorso-ventral midline. Mingling between these dorsal and ventral cells could only occur after folding of the limb primordia since, at the time of the labelling, dorsal and ventral cells are far away from each other (Figs 6B, 7). All together, these results show that the early mesoderm does not contain lineage-restricted dorsal and ventral compartments.

DISCUSSION

Dorsal and ventral compartments in body ectoderm

Our fate map of body ectoderm in early embryos shows that dorsal and ventral cell-lineage restriction compartments exist in both presumptive limb and inter-limb regions. The compartment boundary appears to mark the position along the dorso-ventral axis of the body at which limbs develop. The body ectoderm is organised into dorsal and ventral compartments, at least from the anterior of the wing to the posterior of the leg. In normal development, the apical ectodermal ridge forms at the compartment boundary and furthermore, when ectopic limbs are induced in the inter-limb region by implantation of FGF-2 soaked beads, they also arise at this same dorso-ventral interface, irrespective of the initial dorso-ventral position of the bead. Thus this compartment boundary represents a fundamental feature of body plan organisation and limbs can be induced to form at any antero-posterior level along this interface.

Compartment boundaries

Cell lineage restriction compartments were first identified in insects (Garcia Bellido et al., 1973; Ingham and Martinez, 1992; Blair, 1995). Some have invisible boundaries, as in the wing (e.g. antero-posterior compartments), while others co-localize with morphological boundaries (e.g. segment boundaries). In the chicken embryo flank, the boundary of the ectoderm compartment is invisible, whereas in limbs the boundary is marked by the apical ectodermal ridge. Vertebrate compartments have been demonstrated in chick hindbrain and diencephalon (Fraser et al., 1990; Figdor and Stern, 1993). In hindbrain, compartment borders are also marked by a morphological boundary zone. Both this region and the apical ectodermal ridge in the limb are characterised by alterations in cell morphology, cell packing density (Todt and Fallon, 1984; Heyman et al., 1993) and expression of FGFs (Niswander and Martin, 1992; Mahmood et al., 1995a,b; Crossley et al., 1996; Vogel et al., 1996) and both can be disrupted by retinoic acid treatment (Tickle et al., 1989; Nittenberg et al., 1997). Recombination experiments show that both border specialisations can be ectopically generated by either confrontation of odd and even hindbrain cells (Guthrie and Lumsden, 1991) or dorsal and ventral limb ectoderm (Laufer et al., 1997; Tanaka et al., 1997). In the hindbrain, lineage restriction is probably established at the interface between prospective odd- and even-numbered cells by preferential adhesive properties, and specialised boundary zone cells appear later (Lumsden and Krumlauf, 1996). We have been unable to find an early time when prospective limb ectoderm cells contribute to both dorsal and ventral compartments; this suggests that lineage-restriction in limb ectoderm may also arise before morphological specialisation of the dorso-ventral interface.

Cell lineage restriction compartments in insects and hindbrain are marked by differences in gene expression (Blair, 1995; Lumsden and Krumlauf, 1996). At early stages of chick limb and flank development, none of the genes currently identified mark the ectoderm compartments that we have identified. However, at later stages, *En-1* is a possible candidate for main-

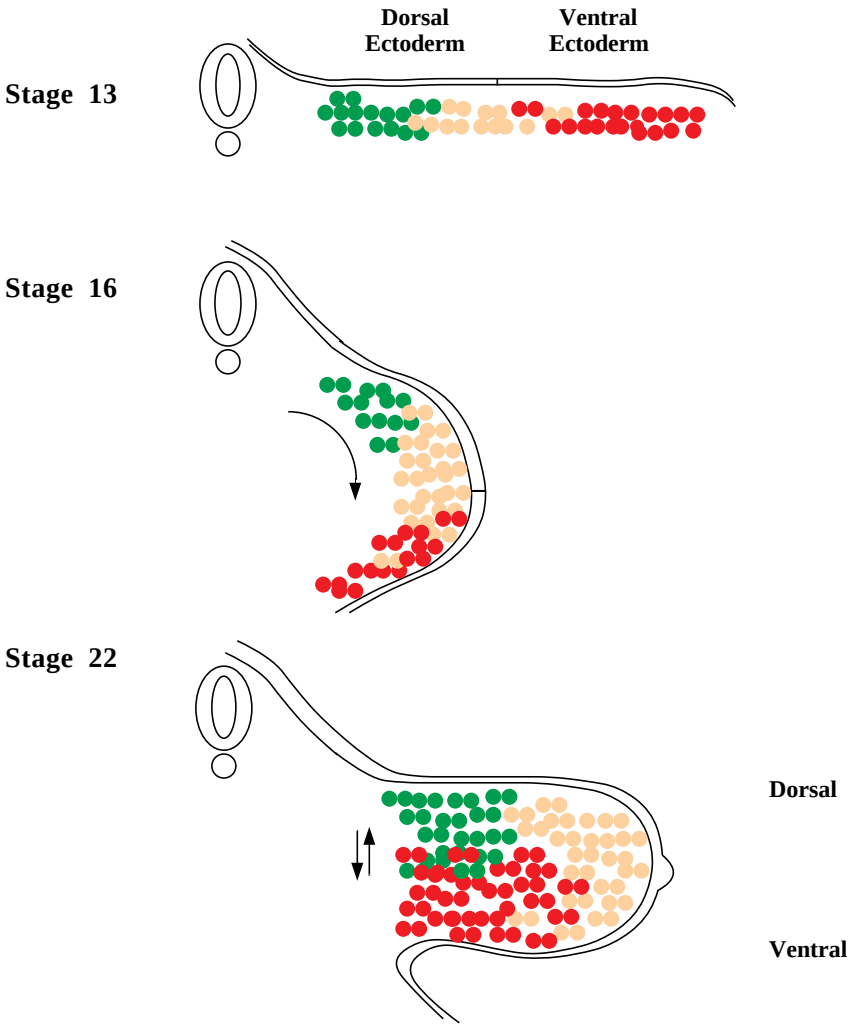

Fig. 7. Summary diagram to illustrate fate of medial (green), central (orange) and lateral (red) mesoderm cells in the lateral plate. At stage 16, arrow indicates folding of the lateral plate mesoderm to form the limb whereas at stage 22, arrows indicate cell movements across dorso-ventral interface.

taining the ventral compartment as it is expressed in both limb and flank regions. In *Drosophila* wing and leg imaginal discs, *Engrailed* is known to play a role in setting up cell lineage restriction compartment boundaries (Blair, 1992). In the dorsal ectodermal compartment of the chick, no suitable candidates are identified as the molecules already known are not concomitantly expressed in both limb and flank regions.

Apical ectodermal ridge formation

We had expected that the apical ectodermal ridge would originate only from cells lying at the dorso-ventral compartment boundary. We were surprised to find apical ridge progenitors intermingled with non-ridge progenitors in a wide area of ectoderm extending from intermediate mesoderm to a lateral region of the embryo, well beyond the small area that will be occupied by the apical ectodermal ridge later on. In recent work, Michaud et al. (1997), using quail-chicken chimeras, also found that the apical ectodermal ridge comes from a wide region of ectoderm. While their results suggest that the medial half of the ectoderm covering the lateral plate mesoderm gives rise to the ridge in the wing, our results differ in that we find this region contains both ridge and non-ridge progenitors and that ridge progenitors are also present ventrally.

The apical ridge contains densely packed cells that could arise as a result of compaction of a large area of loosely packed ectodermal cells containing ridge progenitors (Fig. 8A). However, our technique demonstrates that the prospective apical ridge region defined by Michaud et al. (1997) by grafting experiments is not fated to contribute to the ridge only, but to both ridge and non-ridge ectoderm. Consequently, the ridge cannot form by simple compaction.

Ectodermal cell rearrangements could result in cells from a wide area of ectoderm ending up at the boundary of the dorso-ventral compartment. Our cell lineage analysis shows that most of the long strips of labelled cells generated in the limb regions extend from bud base to tip, irrespective of the original position in which the spot of dye was placed. Furthermore, they always contain non-labelled intermingled cells. Altogether, this suggests bi-directional expansion along the medio-lateral axis in the limb-forming region accompanied by cell rearrangements and intercalations. Recent findings from a completely different direction also implicate cell migration in ridge formation. In *Limb Deformity* mutant mice, the apical ridge fails to form properly (Zeller et al., 1989). A study in yeast has shown that homologs of formin proteins (which are mutated in *Limb Deformity*) mediate regulation of actin cytoskeleton, a process that occurs during cell migration (Evangelista et al., 1997).

There are two possible ways in which cell rearrangements could lead to ridge formation. One possibility is that intercalation movements between cells within the ectodermal sheet accompany the distal extension of the limb bud. Some cells would then be determined to become ridge cells only following local interactions between dorsal and ventral cells that meet at the dorso-ventral interface (Fig. 8B). Recent work has shown that dorsal ectoderm cells express *Radical Fringe*, a vertebrate homolog of *Drosophila Fringe*, and that an apical ridge forms at boundaries between *Radical Fringe* and non-*Radical Fringe*-expressing cells (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Hence, it has been proposed that *Radical Fringe* signalling regulates ridge formation. Another possibility is that scattered ectodermal cells in presumptive limb regions could

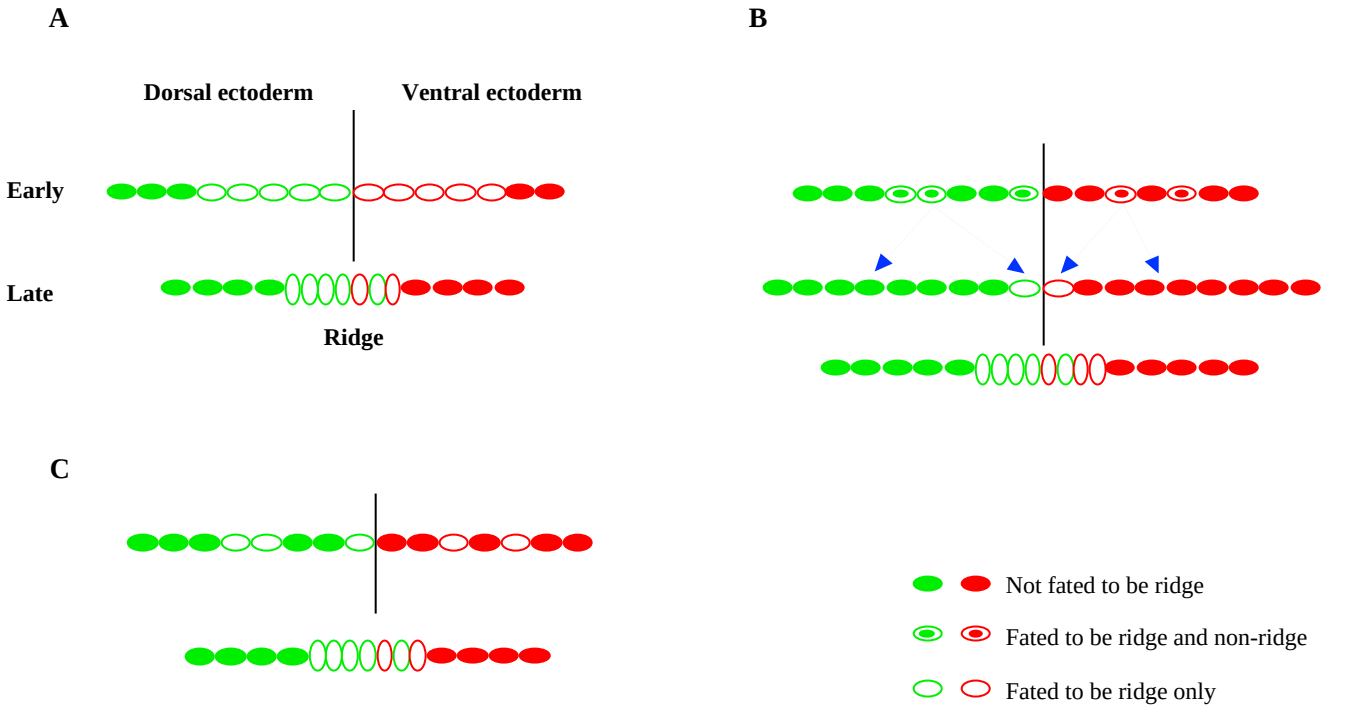

Fig. 8. Three alternative models of ridge formation. In (A) the ridge forms by simple compaction of midline ectodermal cells. This does not match our cell fate data. (B) shows early mixing of non-determined cells. Only those that reach the dorso-ventral interface will interact to form ridge. In contrast, in (C) pre-determined ridge cells are initially spread randomly throughout dorsal and ventral ectoderm and subsequently migrate to the dorso-ventral interface.

first become specified as ridge and then migrate to the correct position (Fig. 8C). *Fgf-8* transcripts, which are eventually confined to the limb apical ridge, initially exhibit a patchy pattern in the ectoderm that could reflect this early distribution of committed cells (Mahmood et al., 1995a; Crossley et al., 1996; Vogel et al., 1996). The dorso-ventral compartment boundary would mark the position at which ridge cells assemble. This model, in which committed cells move to the correct position, is suggested by analogy with early chick embryo development, in which epiblast cells become determined at random to form mesoderm and endoderm, and then migrate into the primitive streak (Stern and Canning, 1990).

It is well known from grafting experiments that mesodermal signals in limb regions induce apical ectodermal ridge formation in the ectoderm (Kieny, 1968; Saunders and Reuss, 1974; Carrington and Fallon, 1984). Both limb and flank ectoderm are competent to produce a ridge when recombined with limb mesoderm. One possibility is thus that mesodermal signalling activates *Radical Fringe* in dorsal limb and *Radical Fringe* expression then dictates ridge position. Another possibility is that mesodermal signalling induces apical ectodermal ridge precursors independently of *Radical Fringe* activation. A good candidate for providing such signalling functions is FGF-10, a mesenchymal factor, expressed specifically in prospective limb regions (Ohuchi et al., 1997).

Apical ectodermal ridge as a compartment boundary

The apical ectodermal ridge is derived from cells from both dorsal and ventral compartments. In addition, our results show that there is a greater number of ridge progenitors in the dorsal compartment than in the ventral compartment (Fig. 4). We also find that dorsal and ventral derived cells are, to some extent, intermingled within the ridge, in contrast with other results (Michaud et al., 1997). Dorsal cells extend further than the midline and colonise part or all of the ventral half of the ridge, whereas ventral cells occupy the ventral half of the ridge. Cell-lineage restriction therefore appears to have been released in the boundary region, at least ventrally.

Strikingly, two recent independent studies presented similar data at the molecular level (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). *Radical Fringe* is expressed in dorsal limb ectoderm at early stages and in dorsal and ventral regions of the ridge later on, while *En-1* is expressed in the ventral limb ectoderm and in ventral, but not dorsal, apical ridge (Rodriguez-Esteban et al., 1997; Logan et al., 1997; see also this paper, Fig. 5E). *En*-1 has been shown to repress *Radical Fringe*; however, the fact that there are both expressed in the ventral ridge presents an anomaly (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Taken together with our results, this suggests that expression of *Radical Fringe* and *En-1*, within the ridge, could be determined by cell lineage and that expression domains overlap in the ridge due to mingling of dorsally and ventrally derived cells.

Origin of ectoderm and mesoderm

Our results show that the ectoderm covering limbs is not completely derived from ectoderm overlying lateral plate mesoderm at early stages. Dorsal limb ectoderm comes from ectoderm covering the lateral edge of somites, intermediate mesoderm and medial third of lateral plate, while ventral limb ectoderm originates from the remaining lateral 2/3. Medio-lateral displacement of ectoderm at wing level was also recently shown, at similar stages, by Michaud et al. (1997) using quail-chicken chimeras, but they described the entire dorsal limb ectoderm as coming from ectoderm overlying somites and intermediate mesoderm. We found medio-lateral displacements in the flank region too, and ectoderm originally over lateral somite comes to lie over the body wall. There is little antero-posterior displacement at limb level in non-somitic ectoderm but, at flank level, antero-posterior and postero-anterior displacements contribute to wing and to leg ectoderm, respectively. In contrast, somitic ectoderm comes only from ectoderm covering the somites.

Our results show that limb mesoderm comes from the medial 2/3 of the lateral plate (see also Geduspan and Solursh, 1992; Michaud et al., 1997) but, in contrast to the ectoderm, the mesoderm is not organised into compartments. This lack of mesoderm cell lineage restrictions may be related to its three-dimensional structure. Limb ectoderm has been shown to impose dorso-ventral polarity on underlying mesoderm (Pautou and Kieny, 1973; MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987; Akita, 1996). The function of ectodermal compartmentalisation could precisely confine the source of ectodermal signals. This would serve to accurately pattern the mesoderm, which undergoes considerable growth and morphogenetic movements as limbs form.

We are grateful to C. Logan for stimulating discussions and for the gift of *En-1* probe. We thank A. Lumsden and J. Parnavelas for their hospitality, and K. Whitley for help with the confocal images. The confocal facilities are supported by the Wellcome Trust. This work was funded by BBSRC grant to C.T. and the Wellcome Trust to J.D.W.C. M.A. was supported by a fellowship from the Association pour la Recherche contre le Cancer (ARC).

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(Accepted 8 September 1997)