Development 121, 4329-4337 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV2024

Gene expression, polarising activity and skeletal patterning in reaggregated hind limb mesenchyme

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SUMMARY

The developing chick limb has two major signalling centres; the apical ectodermal ridge maintains expression of several important genes and outgrowth of the limb, and the polarising region specifies the pattern of skeletal elements along the anteroposterior axis. We have used reaggregated leg grafts (mesenchyme dissociated into single cells, placed in an ectodermal jacket and grafted to a host) to study patterning in a system where the developmental axes are severely disrupted. Reaggregates from different regions of leg mesenchyme developed correspondingly different digits, giving a system in which skeletal phenotype could be compared with the expression of genes thought to be important in patterning.

We found that posterior third and whole leg reaggregates gave rise to different digits, yet expressed the same combination of *HoxD*, *Bmp-2* and *shh* genes throughout their development. Anterior thirds initially only express the 3′ end of the *HoxD* cluster but activate the more 5′ members of the cluster sequentially over a period of 48 hours, a period during which *Bmp-2* is activated but no *shh* or *Fgf-4* expression could be detected. Our results suggest that there are two independent mechanisms for activating the *HoxD* complex, one polarising region-dependent and one independent, and that *shh* expression may not be necessary to maintain outgrowth and patterning once a ridge has been established.

Key words: limb development, *shh*, *Fgf-4*, *Bmp-2*, *HoxD*, polarising region, chick, apical ectodermal ridge

INTRODUCTION

The patterns of skeletal elements seen in the adult fore- and hindlimbs arise from the undifferentiated mesenchyme of the embryonic limb bud. The correct generation of these patterns is dependent upon signalling pathways both within the mesenchyme and between the mesenchyme and the surrounding epithelium (Saunders, 1948; Saunders and Gasseling, 1968; Summerbell, 1974). An important region of signalling in the mesenchyme has been shown to be the polarising region, situated posteriorly in the limb bud. Grafts of an additional polarising region to the anterior margin of the limb bud result in mirror-image duplications of the digits along the anteroposterior axis. The response appears to be graded such that digit 2 is specified at low levels of activity and digit 4 at high levels. The graded response to this polarising signal has led to the suggestion that the polarising region possibly acts by producing a graded concentration of a morphogen across the anteroposterior axis (for reviews see Tabin, 1991; Tickle and Eichele, 1994).

A recent candidate for the endogenous morphogen is the product of the gene *Sonic hedgehog* (*shh*; Riddle et al., 1993). *shh* is the vertebrate homologue of the *Drosophila hedgehog* gene (reviewed by Fietz et al., 1994), a segment polarity gene thought to encode a protein secreted by cells at the posterior of each segment and which determines cell fate within the segment (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). In the chick limb, *Sonic hedgehog* expression maps directly to the polarising region at all times when the region is active and disappears at the stage when activity is lost. Grafts of cells transfected with *shh* will produce mirror-image duplications comparable to those produced by a polarising region graft (Riddle et al., 1993). Retinoic acid implants at the anterior margin of the limb, previously shown to induce an ectopic polarising region, induce *shh* expression within 24 hours (Riddle et al., 1993).

Some of the genes of the *HoxD* complex (formerly Hox-4; Scott, 1992) are expressed in characteristically restricted domains along the anteroposterior axis of the limb (Izpisúa-Belmonte et al., 1991). These are strong candidates for responders to positional signals as their expression can be altered by grafts of either polarising region (Hunt and Krumlauf, 1992;

Izpisúa-Belmonte et al., 1992a,b) or *shh*-expressing cells (Riddle et al., 1993). It has been suggested that the combination of these genes expressed at a certain level could directly determine the digit type formed (Morgan et al., 1992; however, see Dollé et al., 1993). The best evidence for this has come from an apparent homeotic transformation of an anterior digit into a more posterior identity by the overexpression of a single *HoxD* gene (*Hoxd-11*; Morgan et al., 1992).

The apical ectodermal ridge (AER) has been shown to be important in maintaining both *shh* expression and polarising activity (Niswander et al., 1993, 1994; Vogel and Tickle, 1993; Laufer et al., 1994), and for maintaining outgrowth and patterning of the limb (Saunders, 1948; Summerbell, 1974). Removal of the apical ectodermal ridge leads to truncation of the limb (Saunders, 1948; Summerbell, 1974) and loss of gene expression (Niswander et al., 1994). Fibroblast growth factor-4 (*Fgf-4*) is a gene localised to the posterior half of the murine apical ectodermal ridge. Application of a bead soaked in FGF-4 protein to a limb denuded of its AER leads to complete outgrowth and maintenance of gene expression (Niswander et al., 1993, 1994; Vogel and Tickle, 1993; Laufer et al., 1994). This ability of FGF-4 to substitute for AER functions suggests that it may be the primary ridge signal.

We have examined a reaggregate system where leg mesenchyme is triturated to single cells before pelleting, placing in an ectodermal jacket and grafting onto a host chick wing. Despite the fact that the cells are completely disorganised, morphologically good digits can be produced. Recently, Ros et al. (1994) used anterior third wing reaggregates to study the expression of the *HoxD* complex with or without a localised polarising region present. They found that the *HoxD* genes were expressed uniformly across the anteroposterior axis of a reaggregate with no polarising region present, but that normal, nested domains were re-established with one present. This was reflected in the digits obtained, which were symmetrical in non-polarised reaggregates but polarised as expected when the polarising region was present.

Our study has used leg because we have found that reaggregates made from different regions of leg mesenchyme give characteristically different, recognisable, digits. This was not the case in wing where the presence of posterior mesenchyme severely inhibited morphogenesis (Crosby and Fallon, 1975; Frederick and Fallon, 1982). By making these reaggregated limbs from different regions of leg mesenchyme we have been able to study the relationship between polarising activity, the apical ectodermal ridge and the gene expression leading to different skeletal patterns.

MATERIALS AND METHODS

Reaggregate limbs

Fertilised eggs of the *Ross White* strain of chicken were incubated at 38±1˚C and then windowed and staged according to Hamburger and Hamilton (1951). Reaggregates were made from stage 19-21 mesoderm and stage 21-24 ectoderm. Three types of reaggregate were made (Fig. 1A); those made from the whole leg mesenchyme, those made from only anterior third mesenchyme (i.e. opposite somites 26-27) and those made from only posterior third mesenchyme (i.e. opposite somites 31-32). Leg mesoderm and ectoderm were used because they gave the best morphogenesis of skeletal elements and provided identifiable digits from all types of reaggregate.

Leg buds were incubated at 4˚C in 2% trypsin for 10-15 minutes (mesoderm donors) or for 1 hour (ectoderm donors) and then washed in medium containing 10% foetal calf serum. The buds then had their ectoderms and mesoderms separated and the appropriate components discarded. Mesoderm donors were triturated to single cells by drawing them through a fine bore pipette and re-pelleted by mild centrifugation (6500 rpm for 4 minutes) before being recombined with mesoderm-free ectodermal jackets (Fig. 1B). The reaggregates were then allowed to consolidate for 1 hour at 37°C before being grafted to stage 20-24 host wing buds. Reaggregates were generally between 500 and 600 μm wide when grafted, significantly smaller than a normal limb bud.

The reaggregates were grafted either by placing them in a trough left by removing a cube of mesoderm from the proximoanterior region of the dorsal surface of the wing, or by pinning them to the anterior border of a wounded host wing. Both methods gave similar results but the pinning method was unsuitable for those specimens used for wax sectioning as removal of the pin often precipitated a blood blister. Reaggregates left to develop to ascertain skeletal pattern were incubated for a further 6 days before harvesting. They were then fixed in 5% aqueous trichloroacetic acid, stained with 0.1% Alcian Green, differentiated in 70% acid alcohol, dehydrated and cleared in methyl salicylate to visualise skeletal elements.

The criterion for identifying the digits of the normal foot is phalanx number (Fig. 1C). Furthermore, the phalanges have different shapes and sizes in different digits; those in digit **IV** consisting of five phalanges, for example, are shorter and smaller than those in digit **II** consisting of three phalanges. The shape of the phalanges in the best reaggregate digits corresponded with the digit identity deduced from phalanx number (Fig. 1D-G). For the majority of digits however, the reaggregation process resulted in proximodistal segmentation of the cartilage but poor morphogenesis of individual phalanges. In reaggregates, therefore, the number of phalanges in a digit can be determined, and digit identity assigned, only if the following criteria are met. The digit should have: an elongated metatarsal, to identify the basal phalanx; a terminal, claw-shaped phalanx, to ensure the digit is not distally truncated; and the phalanges in the digit should be proximodistally distinct from each other. Digits with 2, 3, 4 or 5 phalanges were scored as digit **I**, **II**, **III** or **IV** respectively. Sometimes, a joint swelling had developed between two phalanges but no joint space was visible in wholemounts (Fig. 1I). Since we could not be sure whether this was 1 phalanx with abnormal morphology or 2 phalanges fused, these cases were scored as **II**/**III** if there were 2 other phalanges in the digit, or **III**/**IV** if there were 3 other phalanges. Digits in which a phalanx count could not be made (Fig. 1H) were scored as **U** (unidentifiable).

Cases not giving rise to digits usually consisted of a small nodule of cartilage, lacking segmentation or recognisable morphogenesis, and are not considered further.

Determination of polarising activity

Whole leg and anterior third reaggregates were allowed to grow for 0-48 hours before being harvested and removed into PBS. They were then trypsinized as described above, the ectoderm removed and the distal tip of the reaggregate taken (the approximate progress zone). After allowing the mesoderm to compact, at 37°C for 1 hour, small pieces were grafted at the anterior margin of a host wing bud (stage 18-20), held in place by a loop made from the apical ectodermal ridge. The hosts were then returned to the incubator for a further 6 days before being harvested and stained for cartilage pattern as described above.

In situ hybridisation for mRNA

Reaggregates were fixed in 4% (w/v) buffered paraformaldehyde for 18-42 hours at 4°C. They were processed for wax histology as described by Davidson et al. (1988). Sections were cut at 8 μm and in situ hybridisation to tissue sections was carried out as described by

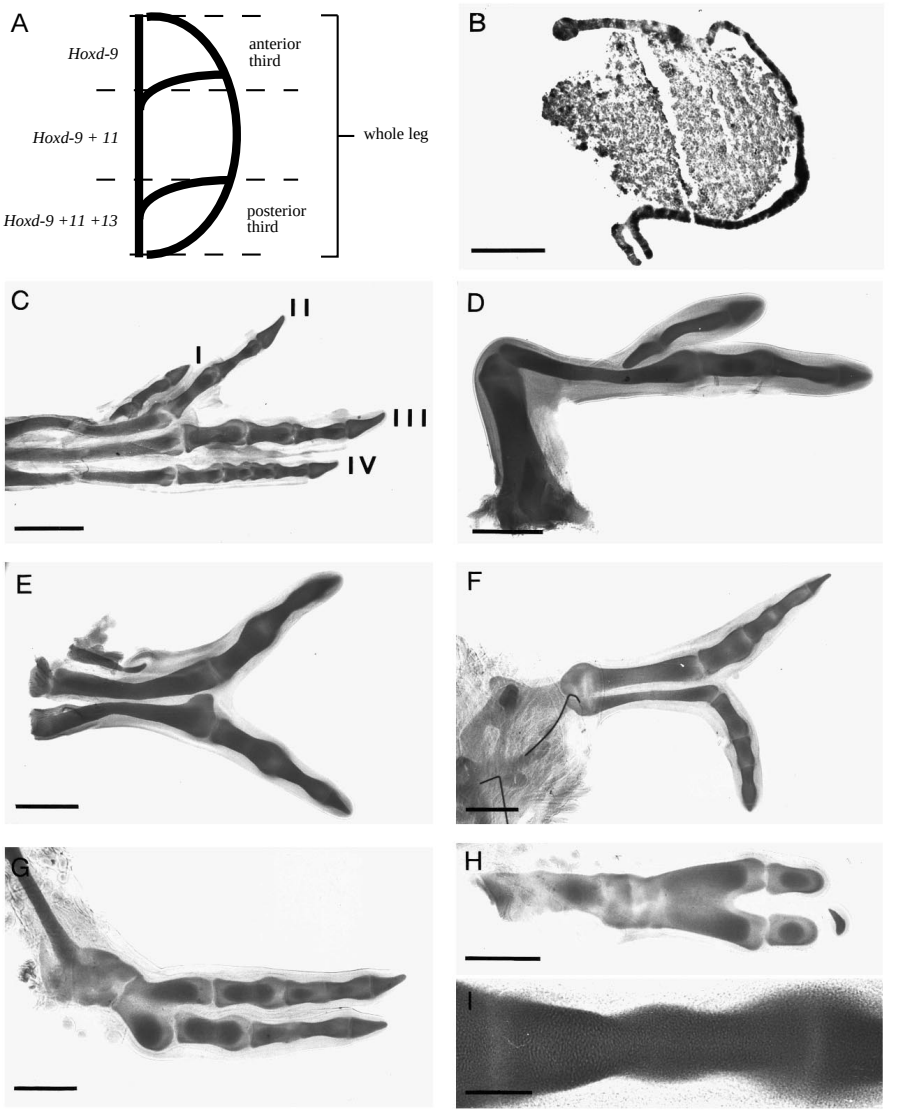

Fig. 1. (A) Schematic diagram showing the axial level of the mesoderm used for making reaggregates. A leg bud of approximately stage 20 is shown. Dotted lines indicate incisions made for producing either anterior reaggregates (upper lines) or posterior reaggregates (lower lines), with the bracket indicating the mesoderm used for whole-leg reaggregates. The approximate expression domains of the examined *HoxD* genes in these pieces of mesoderm is indicated by solid, curved lines. (B) Haematoxylin and Eosin stained paraffin section of freshly jacketed mesoderm. No mesenchyme has been carried over inside the jacket from the ectoderm donor. Scale bar, 150 μm. The following are whole mounts stained for cartilage and cleared (fixed 6-7 days after grafting). Proximal is left. (C) Normal chick foot, stage 37. Scale bar, 2 mm. Roman numerals indicate digit identity. (D) Anterior reaggregate with a pair of digits scored as **I**, **II**. Scale bar, 1 mm. (E) Anterior reaggregate with a pair of digits scored as **II**, **II**. Scale bar = 1 mm. **(**F) Whole-leg reaggregate with a pair of digits scored as **III**, **III**. Scale bar,1 mm. (G) Whole-leg reaggregate with a pair of digits scored as **IV**, **IV**. Scale bar, 1 mm. (H) Reaggregate scored as **U** (unidentified). Scale bar, 1 mm. (I) Two phalanges with a joint swelling indicated but no joint space visible on the whole mount, from a digit scored as **II/III**. Scale bar, 500 μm.

Rowe et al. (1991). Details of the *HoxD* probes and hybridisation conditions have been described previously (Izpisúa-Belmonte et al., 1991). Whole-mount in situ hybridisation of the *Sonic hedgehog* and *Fgf-4* genes were carried out as described by Izpisúa-Belmonte et al. (1993). For experiments on the Bone morphogenetic protein-2 (*Bmp-2*) gene, the details of the probe and hybridisation conditions were as described by Francis et al. (1994).

RESULTS

Digits obtained from reaggregates made from different regions of mesenchyme

71% of surviving reaggregate grafts (72 from 102) gave rise to digits. Of 106 reaggregate digits examined, 76 (72%) could be assigned an identity based on phalanx count (see Fig. 1C for number of phalanges in each digit). In reaggregates developing a pair of clearly identifiable digits, 11/12 showed an identical pair in which each digit had the same number of phalanges.

Of reaggregates made with whole leg-bud mesoderm, the majority (20/31) of identifiable digits were identified as digit**III**s

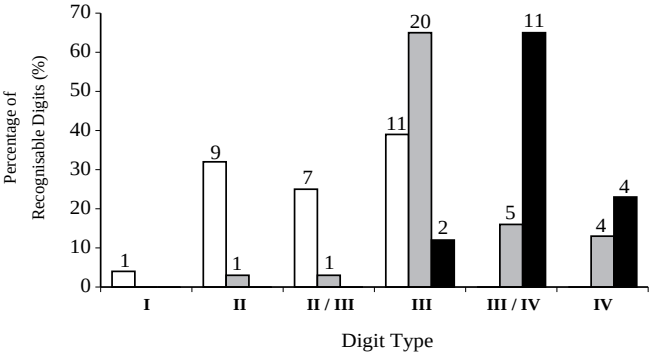

Fig. 2. Histogram showing the percentage distribution of digit types arising from anterior, whole and posterior leg reaggregates. Numbers above the bars indicate number of digits within total number of identifiable digits for each group. As reaggregates typically form 1 or 2 digits, the numbers refer to the number of digits and not the number of reaggregates. White bars indicate anterior reaggregates, grey bars indicate whole leg reaggregates and black bars indicate posterior reaggregates. Note that anterior reaggregates do not give rise to posterior digits and vice versa.

(Figs 1F, 2). Only 2 (out of 31) anterior digits (**II** and **II/III**) and a number (9/31) of posterior digits (**III/IV** and **IV**) developed.

Reaggregates made from posterior leg-bud mesoderm gave predominantly digits **III/IV** (11/17), with the rest being digits **III** (2/17) or **IV** (4/17; Figs 1G, 2). Posterior reaggregates gave rise to a higher proportion of single digits than the other types of reaggregate (singles:doubles = 15:5 in posterior thirds, 15:9 in anterior thirds and 9:19 in whole leg).

Reaggregates made with anterior leg-bud mesoderm gave rise to digits **II** (9/28; Fig. 1E), **II/III** (7/28; Fig. 1I) or **III** (11/28; Fig. 2). No posterior digits were seen. A single digit **I** developed, the only one ever seen, belonging to an unequal pair, the other digit being a digit **II** (Fig. 1D). In the normal limb, a splint-like metatarsal is associated with digit **I** and this was present in the reaggregate digit.

In the identifiable digits, metatarsal elements were common and were sometimes truncated such that only the distal half was present. Structures proximal to this were represented only by mis-shapen masses of cartilage; we never saw good morphogenesis of elements corresponding to the femur, tibia or fibula.

All types of reaggregate gave rise to a number of unidentifiable digits (for example, Fig. 1H): anterior third reaggregates gave 6 out of a total of 34 digits, whole leg gave 16 out of 47, and posterior reaggregates gave 8 out of a total of 25.

Gene expression

HoxD genes

As some members of the *HoxD* gene complex have been implicated in the patterning of the limb, we examined their expression in the three types of leg reaggregate. In the leg at the stages used for reaggregation (19-21) expression of *HoxD* genes were comparable to published descriptions (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991; Mackem and Mahon, 1991). *HoxD* genes were expressed in a series of overlapping domains along the anteroposterior axis (see Fig. 1A to see how this related to the pieces of mesenchyme used for each type of reaggregate). At later stages (31-35) all *HoxD* genes examined were expressed in the perichondrial tissues, the interdigital regions and in the undifferentiated mesenchyme at the tips of the digits. All digits (**I-IV**) expressed all 3 *HoxD* genes examined at these late stages (data not shown).

Whole-leg reaggregates

At 0 hours (i.e. before grafting), *Hoxd-9*, *-11* and *-13* were

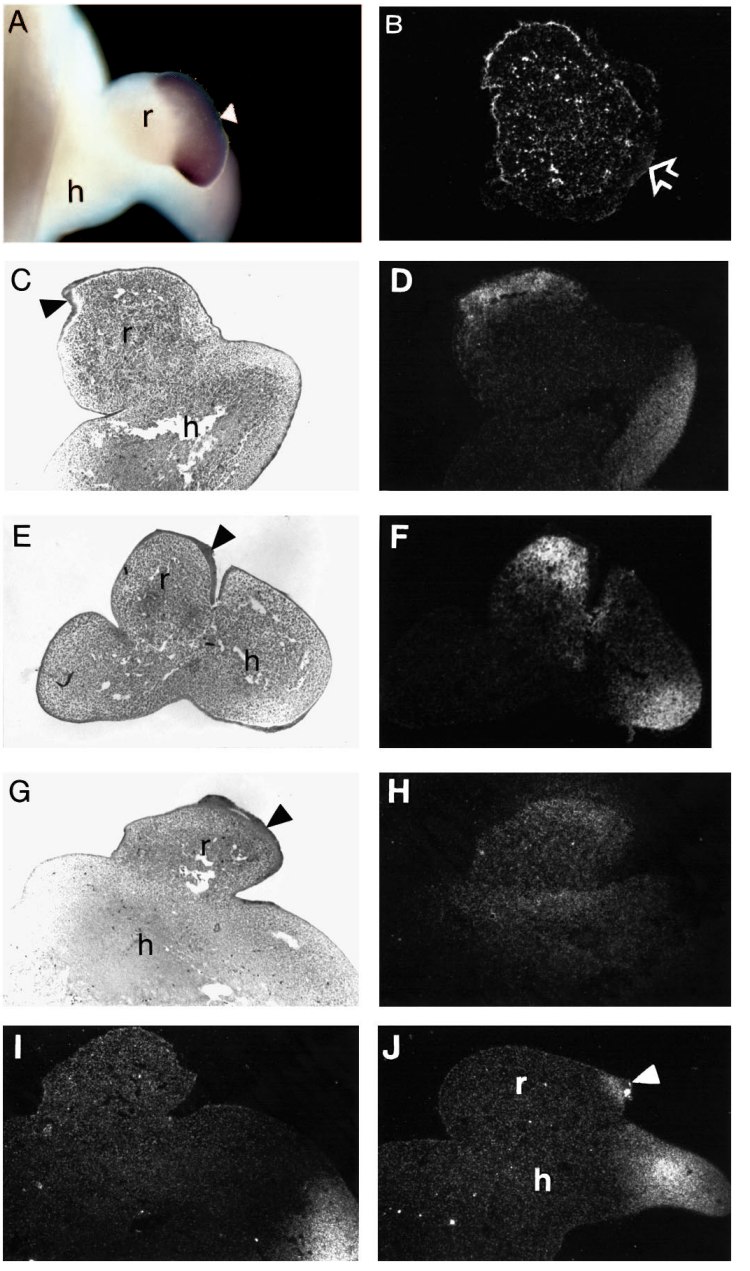

Fig. 3. In situ hybridisation studies for mRNA. Studies using sense probes showed no specific hybridisation (data not shown). h, host; r, reaggregate; arrowheads indicate AER. (A) Whole-mount in situ hybridisation of whole-leg reaggregate 48 hours after grafting showing distribution of *Hoxd-13* transcripts. Regions to which the probe has hybridised are darkly stained. Expression here is uniform with respect to the anteroposterior axis of the reaggregate, immediately subjacent to the AER, and is of uniform depth proximally from the AER. This pattern is representative of all *HoxD* gene expression seen in all types of reaggregate. (B) Whole leg reaggregate fixed after jacketing and before grafting to a host, hybridised with a probe to *Hoxd-13*. Note the expression of the gene in the mesenchyme and not the ectoderm. There is a strong edge-effect to the mesenchyme that does not represent specific hybridisation. (C,D) Tissue sections of whole leg reaggregate fixed at 24 hours and hybridised with a *Hoxd-13* radioactive probe. The reaggregate is sectioned along the plane of the AER to give an anteroposterior plane through the progress zone. (C) Bright-field; (D) dark-field illumination. Only *Hoxd-13* is shown because 5′ members of the *HoxD* cluster were never expressed until after the 3′ members (data not shown). (E,F) Posterior third reaggregate (24 hours). (E) Bright-field illumination. (F) *Hoxd-13* expression. **(**G-I) Anterior third reaggregate (24 hours). (G) Bright-field illumination. (H) *Hoxd-9* expression. (I) *Hoxd-13* expression. Note that the 5′ *HoxD* gene is not yet expressed in the region expressing the 3′ member. (J) Anterior third reaggregate (48 hours) probed for *Hoxd-13*. Note the expression now under the AER. This is a dorsoventral section through the AER at the anteroposterior mid-point of the reaggregate. Proximal reaggregate is left and distal right.

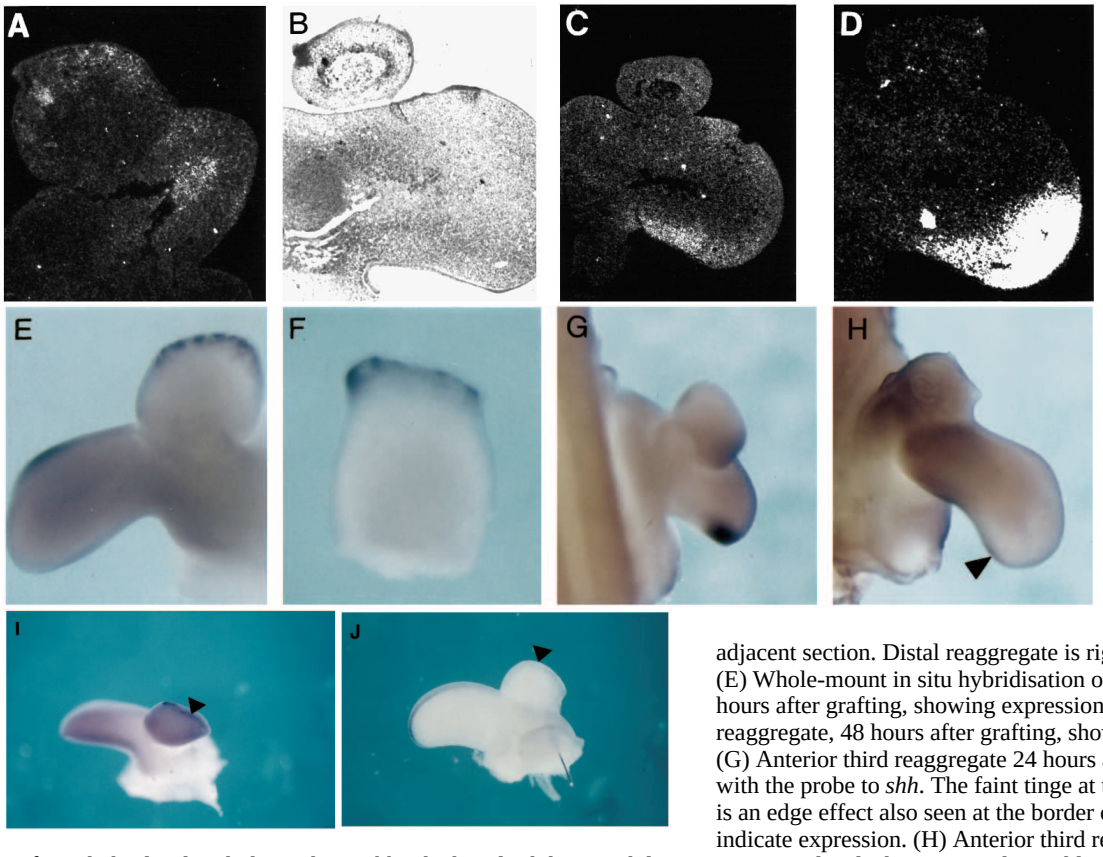

Fig. 4. In situ hybridisation for mRNAs on whole mounts and tissue sections. (A) Whole leg reaggregate (same reaggregate as in Fig. 3C,D) 24 hours after grafting, showing expression of *Bmp-2* transcripts. Note the expression is all the way along the AER but is not homogeneous. (B-D) Anterior third reaggregate 24 hours after grafting. (B) Bright-field illumination. (C) *Bmp-2* expression. (D) *Hoxd-13* expression. Note that the specimen is expressing *Bmp-2* but *Hoxd-13* is not yet expressed in an adjacent section. Distal reaggregate is right and proximal is left. (E) Whole-mount in situ hybridisation of a whole leg reaggregate, 24 hours after grafting, showing expression of *shh*. (F) Posterior third reaggregate, 48 hours after grafting, showing expression of *shh*. (G) Anterior third reaggregate 24 hours after grafting, hybridised with the probe to *shh*. The faint tinge at the edges of the reaggregate is an edge effect also seen at the border of the host wing and does not indicate expression. (H) Anterior third reaggregate 48 hours after grafting, hybridised with the probe to *shh*. The host limb has much lower expression levels that are just detectable (indicated by arrowhead). (I) Whole leg reaggregate 24 hours after grafting showing expression of *Fgf-4* in the AER (indicated by arrowhead). (J) Anterior third reaggregate 24 hours after grafting showing clear expression in the host AER but no expression in the reaggregate AER (indicated by arrowhead).

expressed uniformly in the reaggregated mesenchyme (for example Fig. 3B). 12 hours after grafting, transcripts were seen principally in the sub-ridge mesenchyme, although some expression persisted in core mesenchyme. Longitudinal sections through the midpoint of the reaggregate at 24-48 hours showed expression of all 3 *HoxD* genes in the sub-ridge mesenchyme, a region comparable with the progress zone. All genes were expressed across the full width of the reaggregate with none of the asymmetry in expression characteristic of their domains in the normal limb (Table 1; Fig. 3A,C,D). By 96 hours after grafting all genes examined were expressed in the mesenchyme surrounding the cartilage elements, the interdigital regions (where more than one digit was present) and in undifferentiated mesenchyme at the tip of the reaggregate, the same pattern seen in normal limbs (data not shown).

Posterior third reaggregates

At all stages, posterior reaggregates showed expression of the 3 *HoxD* genes examined (Table 1; Fig. 3E,F) in the sub-ridge mesenchyme. This is the same pattern observed in whole limb reaggregates.

Anterior third reaggregates

The early pattern of gene expression differed from that seen in whole-leg and posterior reaggregates in a number of respects. As with the other types of reaggregate, anterior third reaggregates expressed *Hoxd-9* at all times from 0 to 96 hours (Fig. 3G,H). However, *Hoxd-11* transcripts were detected in only 1 out of 2 cases at 0 hours (see Fig 1A to see that some *Hoxd-11* expressing tissue was included in anterior reaggregates), and in 5 out of 7 cases in the sub-ridge mesenchyme at 24 hours after grafting. Additionally, *Hoxd-13* was not detected at 0 hours (2/2 cases) or 24 hours after grafting (6/6 cases; Table 1; Fig. 3I). Only at 48 hours after grafting were *Hoxd-13* tran-

Table 1. *Hox-D* gene expression in reaggregates made from different regions of mesenchyme

Type of reaggregate	Hours after grafting	*Hox-D* gene expression		
		Hoxd-9	*Hoxd-11*	*Hoxd-13*
Whole leg	0	+ (8)	+ (8)	+ (8)
	6	+ (1)	+ (1) – (2)	+ (1) – (1)
	12	+ (3)	– (2)	+ (2)
	24	+ (7)	+ (7)	+ (7)
	36	+ (3)	+ (3)	+ (2)
	48	+ (1) – (1)	+ (3)	+ (3)
	96	+ (1)	+ (1)	+ (1)
Posterior third	0	+ (1) – (2)	+ (3)	+ (3)
	24	+ (2)	+ (2)	+ (2)
	48		+ (3)	+ (3)
	96		+ (2)	+ (2)
Anterior third	0	+ (2)	+ (1) – (1)	– (2)
	24	+ (7)	+ (5) – (2)	– (6)
	48		+ (1)	+ (4) – (2)
	96	+ (3) – (1)	+ (4)	+ (4)

+, hybridisation detected; –, hybridisation not detected. Numbers in parentheses indicate number of cases.

Table 2. *shh*, *Bmp-2* and *Fgf-4* gene expression in reaggregates made from different areas of mesenchyme

Type of reaggregate	Hours after grafting	Gene expression		
		shh	*Bmp-2*	*Fgf-4*
Whole leg	0		+ (6)	
	24	+ (2)	+ (7)	+ (5)
	48	+ (2)	+ (3)	+ (3)* – (1)
Posterior third	0		+ (2)	
	24	+ (2)	+ (2)	+ (1)
	48	+ (2)	+ (3)	+ (1)*
Anterior third	0		– (2)	
	24	– (2)	+ (3) – (2)	– (4)
	48	– (4)	+ (5) – (1)	– (5)
	72	– (3)		

+, hybridisation detected; –, hybridisation not detected. Numbers in parentheses indicate number of cases. *, expression hard to detect.

scripts seen (4/6 cases; Fig. 3J) and they were in the sub-ridge mesenchyme. At later stages anterior reaggregates showed the same pattern of expression of *HoxD* gene expression in undifferentiated and interdigital mesenchyme and perichondrium as the other types of reaggregate (Table 1) and the normal limb.

Bmp-2

All types of reaggregate were examined for the expression of the Bone morphogenetic protein-2 (*Bmp-2*) gene. Francis et al. (1994) showed this gene was expressed in the posterior mesenchyme of the normal limb in a domain similar, but not identical, to that of *Hoxd-13*. In whole leg reaggregates (Table 2), *Bmp-2* was expressed in the mesenchyme at 0 hours in all cases (6/6 cases) and in the sub-ridge mesenchyme at 24 and 48 hours (10/10 cases, for example see Fig. 4A). Posterior reaggregates (Table 2) showed the same pattern of expression as whole-leg reaggregates (7/7 cases). In anterior reaggregates, however, the gene was not detected at 0 hours in the mesenchyme (2/2 cases), but was present in about half the specimens at 24 hours (3/5 cases; Fig. 4B,C). At 48 hours the gene was strongly expressed in the sub-ridge mesenchyme in most cases (5/6 cases; Table 2). In all cases, *Bmp-2* was expressed across the width of the reaggregate with no anteroposterior asymmetry. However, unlike expression of the *HoxD* genes, the expression of *Bmp-2* often appeared clumpy (for example Fig. 4A). The pattern also differed from *shh* in that it extended further proximally and did not appear as punctate (see next section).

Sonic hedgehog

In whole leg and posterior third reaggregates *shh* was found to be expressed across the width of the reaggregate at 24 and 48 hours (8/8 cases; Table 2; Fig. 4E,F). The expression was very distal compared to the other genes examined, lying immediately subjacent to the AER. The expression of *shh* also differed in that it appeared punctate and was not homogeneous across its width as the *HoxD* gene expression was (compare Figs 3A and 4E).

In anterior third reaggregates, *shh* expression was not detected at 24, 48, or 72 hours (9/9 cases) in any part of the reaggregates (Table 2; Fig. 4G,H). This contrasts with other posterior genes in that *Bmp-2* is detected by 24 hours and *Hoxd-13* by 48 hours.

Table 3. Polarising activity in whole leg and anterior third reaggregates at different stages before and after grafting

Type of reaggregate	Hours after grafting	Identity of digits obtained			
		2234	**22234**	**23234**	No duplication
Whole leg	0	3	1	1	1
	24			1	1
Anterior third	0				3
	24				17
	48	1			15

Fgf-4

The *Fgf-4* gene is expressed in the posterior half of the murine apical ectodermal ridge during limb development (Niswander and Martin, 1992) and FGF-4 protein can functionally replace the ridge if applied on a bead (Niswander et al., 1993). It is thought it may interact with the *shh*, *Bmp-2* and *HoxD* genes (Niswander et al., 1993, 1994; Francis-West et al., 1995) to maintain polarising activity and outgrowth. Whole-mount in situ hybridisation was, therefore, performed on all types of reaggregate to assay its expression in this system. In whole leg and posterior third reaggregates, all cases showed expression of *Fgf-4* in the entire anteroposterior extent of the apical ectodermal ridge at both 24 and 48 hours (Table 2; for example, Fig. 4I), although expression levels were often difficult to detect at the 48 hour time point. In anterior reaggregates, however, expression was undetectable at both 24 and 48 hours (Table 2; Fig. 4J).

Assay for polarising activity

The distal tip of the reaggregate was used to assay polarising activity because in whole leg reaggregates *shh* expression was associated with the distal region.

Whole leg reaggregate mesenchyme showed polarising activity at 0 (5/6 cases; Table 3) and 24 hours as might be expected with polarising region cells being included in the initial aggregate. The assay at 24 hours gave a **23234** duplication (1/2 cases; Table 3). Anterior reaggregates, however, exhibited very weak or no polarising activity. At 0 hours (0/3 cases) and at 24 hours (0/17 cases; Table 3), no evidence of polarising activity could be detected. At 48 hours, when *Hoxd-13* and *Bmp-2* are known to be expressed, again almost no activity was detected; only one of 16 grafts showed any evidence of polarising activity, inducing only an extra digit **2** (Table 3).

DISCUSSION

Reaggregates made from different regions of leg mesenchyme grow out and give characteristically different digits. Whole leg and posterior third reaggregates express the *HoxD*, *Bmp-2*, *shh* and *Fgf-4* genes throughout their early development. In contrast, anterior third reaggregates sequentially activate *Bmp-2* and the 5′ *HoxD* complex over a period of about 48 hours during which no *shh* expression, *Fgf-4* expression or polarising activity was detected. These results suggest that two mechanisms may be capable of activating the 5′ *HoxD* complex, one polarising region-dependent and one independent, and that *shh* expression may not be necessary to maintain outgrowth and patterning once a ridge has been established.

We have shown that recombinant limbs made from dissociated and reaggregated mesenchyme from different regions of the leg can grow, pattern and form identifiable, different, digits. Previous authors have reported growth and patterning of reaggregates (Zwilling, 1964; Singer, 1972; MacCabe et al., 1973; Crosby and Fallon, 1975; Frederick and Fallon, 1982; Ros et al., 1994), but studies on leg were only done with whole leg mesenchyme, and wing reaggregates only gave recognisable digits when anterior third mesenchyme was used. The presence of polarising region cells in wing reaggregates severely and deleteriously affected morphogenesis (Crosby and Fallon, 1975; Frederick and Fallon, 1982), making the present type of study impossible in wing.

HoxD genes in reaggregated leg mesenchyme

In the normal limb bud, the anteroposterior axis is thought to be controlled by the polarising region at the posterior margin of the bud. This region was automatically incorporated into reaggregates made from whole and posterior third leg mesenchyme but not into anterior third reaggregates. Whole leg and posterior third reaggregates continued to express all the 3′ and 5′ *HoxD* genes they were expressing at the time of the operation across their anteroposterior axis in the sub-ridge mesenchyme. Reaggregates made from anterior third mesenchyme, which are not normally expressing the 5′ members of the *HoxD* complex at the time the reaggregate is made, nevertheless sequentially activated the progressively more 5′ members (up to and including *Hoxd-13*) over a period of about 48 hours. This is in agreement with work done on anterior third wing reaggregates (Ros et al., 1994).

One model for the specification of the identity of the cartilaginous elements in general, and the digits in particular, is based on the combination of *HoxD* gene expression (Izpisúa-Belmonte et al., 1991; Yokouchi et al., 1991; Morgan et al., 1992; Davis et al., 1995). Posterior digits, for example, would be specified by the 5′ members of the complex. *HoxD* expression at the time the reaggregates are made provide a good predictor of the digits that develop. Posterior buds express all the limb *HoxD* genes. Reaggregates of these express all these genes from the time they are made and give rise predominantly to posterior digit **III/IV**s. Whole limb buds only express the 5′ *HoxD* genes in the posterior half. When reaggregates are made of these, again all the limb *HoxD* genes are expressed from the start, but they give rise predominantly to digit **III**s. This difference between posterior and whole leg reaggregates could possibly be due to the effects of dilution of the whole limb reaggregates by anterior cells. Anterior third reaggregates are not expressing the 5′ *HoxD* genes at the time they are made, they only express them at a later stage, and so develop more anterior digits.

Recently it has been found that a certain degree of functional redundancy may exist between *Hox* genes (Davis et al., 1995; Horan et al., 1995). The phenotype achieved if only individual genes are eliminated (for example *Hoxd-11*; Davis and Capecchi, 1994) is significantly different from that obtained if multiple paralogous genes are knocked-out (i.e. *Hoxd-11* and *Hoxa-11*; Davis et al., 1995). The former had raised serious doubts as to whether *Hox* genes would be involved in specifying cartilage rudiments in a combinatorial fashion, whilst the latter provided strong support for the idea. It remains to be seen whether this may hold true for digit specification in the limb where the elimination of individual *HoxD* genes has provided evidence against a combinatorial determination of digit phenotype (Dollé et al., 1993; Davis and Capecchi, 1994).

HoxD and *shh* expression in reaggregates

Recent work has shown that expression of the *HoxD* genes in the normal limb can be controlled by the expression of *Sonic hedgehog*, the putative polarising region signal (Riddle et al., 1993; Laufer et al., 1994; López-Martínez et al., 1995). We therefore assayed for expression of *shh* and polarising activity in our reaggregate system. A surprising finding was that, during the activation of the 5′ *HoxD* genes in anterior reaggregates, no *shh* expression or polarising activity could be detected. This would suggest that although the polarising region, probably through *shh*, can activate the *HoxD* complex, there may be other mechanisms in the limb that also share this capacity. One recent suggestion is that activation of these genes is an intrinsic property of rapidly dividing mesenchyme cells (Duboule, 1994; Ros et al., 1994). We would suggest an alternative, whereby the activation of the most 5′ *HoxD* genes at later stages is a consequence of the time a cell spends in the progress zone, activation of the complex being associated with cells becoming more distal (Morgan and Tabin, 1994). This implies that there are two separate mechanisms controlling activation of the *HoxD* complex, one polarising region-dependent and one independent.

Another interesting finding was the punctate nature of the *shh* expression directly underneath the AER (Fig. 4E,F). A likely explanation of why *shh* is expressed in this way could be that only cells that were expressing *shh* at the time the reaggregate was made, and that came by chance to directly underlie the AER, continue to express *shh* in the developing reaggregate. These expressing cells then divide under the influence of the AER, giving daughter cells that also express, but seem unable to induce their neighbouring cells to express the *shh* gene, thus giving the punctate pattern. There is evidence that the polarising region is unable to induce adjacent cells to be polarising region cells (Smith, 1979). Our results suggest that *shh*-expressing cells may be unable to induce their neighbours to express *shh*.

HoxD and *Bmp-2* expression in reaggregates

It has been suggested that activation of *Hoxd-13* may be dependent, in part at least, upon the presence of *Bmp-2* (Francis et al., 1994), itself possibly dependent upon *shh*. In anterior reaggregates, *Bmp-2* was expressed at 24 hours (for example see Fig. 4C) at about the time *Hoxd-11* was activated, but before *Hoxd-13* (compare Fig. 4C with Fig. 4D). When expression of *Bmp-2* and *Hoxd-13* was examined in the same specimen, no expression of *Hoxd-13* was seen in any specimen not already expressing *Bmp-2*. Its expression in this system, before *Hoxd-13*, is therefore consistent with *Bmp-2* having a role in the activation of *Hoxd-13*.

The relationship between *Fgf-4* and *shh* in reaggregates

The expression of *Fgf-4* in this system is consistent with it having an interdependent relationship with *shh* (Laufer et al., 1994; Niswander et al., 1994). In whole leg and posterior reaggregates where *shh* expression is strong, *Fgf-4* is expressed in the ridge. In anterior reaggregates, where no *shh* expression is

detected, *Fgf-4* expression is also undetectable. This might suggest that the presence of *shh* is essential to maintain *Fgf-4* expression. The absence of *Fgf-4* expression in anterior reaggregates would also suggest that another ridge signal is responsible in these reaggregates for maintaining outgrowth. Strong contenders for this ridge signal would be other FGFs. Two obvious candidates would be FGF-8, which is localised throughout the ridge of normal limb buds (Crossley and Martin, 1995; Mahmood et al., 1995), and FGF-2, which is also expressed in the ridge (Savage et al., 1993) and has been shown to be able to direct outgrowth (Riley et al., 1993).

Reaggregates have similarities to known chicken mutations that affect the limbs

Several aspects of reaggregate development bear some resemblance to the development of limb buds in the polydactylous chicken mutant, *talpid*^{3} (*ta*^{3}; Francis-West et al., 1995). Both express genes that would normally be found anteroposteriorly polarised, all across their anteroposterior axis (for example, *HoxD* genes and *Bmp-2*) and give rise to a number of symmetrical digits (although in *ta*^{3} they are unrecognisable). There are, however, a number of differences. In *ta*^{3}, *shh* is localised to the posterior margin of the limb as normal, yet low polarising activity is found in anterior mesenchyme. It was suggested that this may be related to the presence of *Hoxd-13* and *Bmp-2* anteriorly (Francis-West et al., 1995). The lack of polarising activity in mesenchyme from anterior third reaggregates, which also express *Hoxd-13* and *Bmp-2*, would suggest that these genes are not responsible for the polarising activity found in the anterior of the *ta*^{3} limb. Also, the interdependence of *Fgf-4* and *shh* expression seen in reaggregates was not seen in *ta*^{3}, where *shh* is localised to the posterior and yet *Fgf-4* is expressed throughout the AER (Francis-West et al., 1995).

More recently, a study of the mutant *diplopodia*^{4} has provided further interesting observations. In this mutant the normal digits **II**, **III** and **IV** of the foot are present, and digit **I** is usually replaced by an approximately mirror image of these digits, resembling the phenotype obtained after grafting a polarising region to the anterior margin. However, at the anterior margin no evidence has been found of any *shh* expression, despite the fact that *Bmp-2* and the full complement of *HoxD* genes are expressed anteriorly in addition to their normal posterior domains (Concepción Rodriguez, Robert Kos, Ursula Abbott and Juan-Carlos Izpisúa-Belmonte, unpublished data). This contrasts with several published mouse mutants, where preaxial polydactyly is associated with ectopic *shh* domains at the anterior margin (Chan et al., 1995; Masuya et al., 1995). The activation of posterior genes in the absence of *shh* in *diplopodia*^{4} reflects, to some extent, the data from anterior reaggregates.

Concluding remarks

Recent models of limb development have suggested that a feedback loop is established relatively early between *shh* and *Fgf-4* expression, and that this is essential to co-ordinate the outgrowth and patterning of the limb bud (Laufer et al., 1994). Our data suggest that alternative pathways that are *shh* and *Fgf-4* independent may control growth and patterning anteriorly in the leg bud. We suggest that the activation of the 5′ *HoxD* genes in the anterior reaggregates may be affected as a consequence of cells spending time in the progress zone, giving a link between proximodistal and anteroposterior patterning also suggested by other authors (Laufer et al., 1994; Ros et al., 1994).

The growth and patterning apparent in anterior reaggregates suggests that *shh* and *Fgf-4* may not be necessary to allow outgrowth of the leg bud. This is in agreement with the observation that removal of the posterior half of the leg bud AER at an early stage results in the maintenance of the remaining AER and the anterior half growing to form anterior digits (Rowe and Fallon, 1981; A.H. unpublished observations). The same is not true of the wing, where a similar operation results in the necrosis of the anterior AER and the truncation of the limb (Rowe and Fallon, 1981). It may be, then, that *shh* is necessary to initiate the AER, in conjunction with an FGF (Cohn et al., 1995), but that it may not be required, in the leg at least, for maintenance of the AER. A contender for the maintenance factor might be BMP-2, which is expressed anteriorly later in the leg bud and is expressed in anterior reaggregates. A more intriguing contender might be BMP-7, another member of the BMP family expressed in different regions in the wing and leg (Francis-West et al., 1995; P. H. F.-W. unpublished observations). In the wing, *Bmp-7* is expressed posteriorly in the mesenchyme at early stages, whereas in the leg it is expressed initially in all limb mesenchyme and then slightly later in the anterior and posterior. This might explain the differences seen in anterior AER survival after extirpation of the posterior half.

The authors would like to thank Gail Martin and Lee Niswander for the *Fgf-4* probe. They would also like to thank Cheryll Tickle for helpful discussion and suggestions. A. H. is a recipient of an MRC studentship.

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(Accepted 12 September 1995)