THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Expression and regulation of Cek-8, a cell to cell signalling receptor in developing chick limb buds

Citation for published version:
Patel, K, Nittenberg, R, D'Souza, D, Irving, C, Burt, D, Wilkinson, DG & Tickle, C 1996, 'Expression and regulation of Cek-8, a cell to cell signalling receptor in developing chick limb buds', *Development*, vol. 122, no. 4, pp. 1147-55.

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Development

Publisher Rights Statement:
© The Company of Biologists Limited 1996

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

OPEN ACCESS

Download date: 19. Apr. 2024

Development 122, 1147-1155 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV4606

Expression and regulation of *Cek-8*, a cell to cell signalling receptor in developing chick limb buds

K. Patel[1,*], R. Nittenberg[2,3], D. D'Souza[2], C. Irving[1], D. Burt[4], D. G. Wilkinson[1] and C. Tickle[2]

[1]Division of Developmental Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
[2]Department of Anatomy, and [3]Department of Molecular Pathology, University College London Medical School, Windeyer Building, Cleveland Street, London W1P 6DB, UK
[4]BBSRC, Roslin Institute, Roslin, EH25 9PS, UK

*Author for correspondence

SUMMARY

The *Eph*-related receptor tyrosine kinase gene, *Cek-8,* is expressed in mesenchyme at the tip of chick limb buds, with high levels of transcripts posteriorly and apically but fading out anteriorly. Expression of *Cek-8* in distal mesenchyme is regulated by apical ridge- and FGF-polarising signals and retinoic acid, and is uniform across the antero-posterior axis in *talpid*^{3} mutants. These data indicate that *Cek-8* expression responds to regulatory signals during limb patterning and suggest that this receptor tyrosine kinase may have a role in coordinating responses to signals in the progress zone of early buds. Later on in limb development, *Cek-8* expression is associated with cell condensations that form tendons and their attachments to cartilage rudiments and then in developing feather buds.

Key words: patterning, limb, tendon, feather, retinoic acid, Sonic hedgehog, EPH, receptor tyrosine kinase, mouse, chick

INTRODUCTION

One of the outstanding problems in contemporary developmental biology is to understand the molecular basis of tissue patterning. Historically the vertebrate limb has been a popular structure for studying patterning because it is readily observed during development and accessible to manipulation and, as a result, the sets of cell interactions that lead to tissue patterning have been identified. A major challenge is to identify and understand the relationship between signals originating from mesenchyme and ectoderm. Mesenchymal signalling is based on the polarising region, and both the thickened ectodermal ridge and the non-ridge ectoderm also produce signals.

A signal from a small population of mesenchymal cells at the posterior margin of the limb, the polarising region, controls patterning along the anterior-posterior axis of the limb, and grafts of this region can lead to duplication of limb structures (Saunders and Gasseling, 1968). Retinoic acid (Tickle et al., 1982; Summerbell, 1983) and, more recently, cells expressing the Sonic hedgehog gene, *Shh*, (Riddle et al., 1993) have been shown to provide a polarising signal and induce additional digits. Interestingly *Shh* is also expressed in Hensen's node and floor plate of the neural tube (Riddle et al., 1993), both of which have been shown to have polarising activity (Hornbruch and Wolpert, 1986; Wagner et al., 1990).

Signals from the thickened ectodermal ridge that rims the bud are important for development of the proximodistal axis. However, unlike signalling by the polarising region, signalling by the apical ectodermal ridge (AER) does not directly pattern the limb but instead mediates bud outgrowth, which is accompanied by progressive laying down of structures along the proximal-distal axis. This has been elegantly demonstrated in experiments where the AER from young limbs is exchanged for its counterpart in older animals without any apparent change in pattern (Rubin and Saunders, 1972). The apical ridge maintains the progress zone, a region at the distal tip of the limb bud in which cells are sustained in a continuously dividing, undifferentiated state (Summerbell et al., 1973). Structures along the proximodistal axis are specified as cells leave the progress zone and the positional identity of the cells and their descendants is determined by the time spent in the progress zone, such that cells that spend the least time form proximal structures and those that remain longest form distal ones. Genes expressed in the mesenchyme at the tip of the limb include the homeobox containing genes *Msx-1* and *Msx-2*, which may be involved in maintaining the progress zone. Members of the FGF family have been shown to be able to substitute for the AER (Niswander and Martin, 1993a; Fallon et al., 1994) and *Fgf-4* transcripts are expressed in posterior apical ectodermal ridge (Niswander et al., 1994). A positive feedback loop is thought to maintain *Shh* and *Fgf-4* expression, thus stabilising signalling of polarising region and apical ridge as well as initiating the restricted expression of a number of genes, including 5′ genes in the *Hoxd* cluster and the gene encoding BMP-2 (Niswander et al., 1994; Laufer et al., 1994; Francis et al., 1994).

In this paper we show that Cek-8 *(*Sajjadi and Pasquale, 1993), a member of the Eph class of receptor tyrosine kinases,

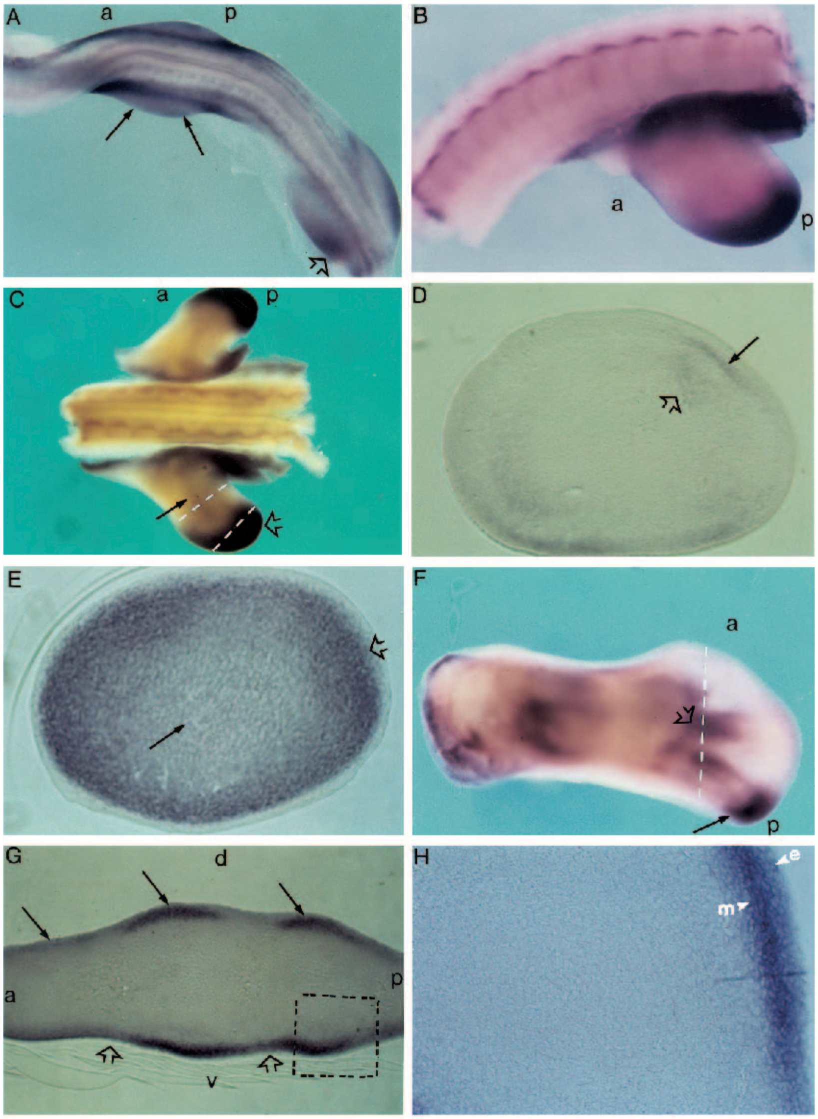

Fig. 1. Distribution of *Cek-8* expression during chick limb development. (A) Dorsal view of stage 18 embryo showing *Cek-8* expression throughout the wing bud (arrows) but restricted to posterior in leg bud (open arrow). (B) Stage 22/23 wing shown in dorsal view, showing strong expression distally with a sharp edge posteriorly but fading anteriorly. (C) Stage 24/25 wing shown in dorsal view. Weak expression is observed in proximal regions (arrow) and strong expression at tip (open arrow). The white dotted lines indicate the level of the transverse sections shown in D and E.
(D) Transverse section of the proximal part of a stage 24/25 limb. *Cek-8*-expressing cells were located subectodermally (arrow) and also at the part of dorsal and ventral edges of pre-cartilage cell condensations (open arrow). (E) Transverse section of the distal part of a stage 24/25 limb through the distal *Cek-8*-expressing part of the wing. A graded distribution of *Cek-8* transcript is seen, with reduced levels of transcripts in core mesenchyme (arrow) and high levels in the subectodermal mesenchyme (open arrow).
(F) Stage 27/28 leg in ventral view. High levels of transcript are observed in regions associated with metacarpal development (open arrow) and remnant of distal expression domain at posterior tip (arrow). The white dotted line indicates level of transverse section shown in (G). (G) Transverse section of metacarpals of a stage 27/28 leg. Dorsally (d): three regions of weak mesenchymal expression (arrows), ventrally (v): strong continuous mesenchymal expression but with zones of decreased expression (open arrows). Dotted lines indicate area shown at high magnification in H.
(H) High magnification of a transverse section of metacarpals of a stage 27/28 leg showing *Cek-8* transcripts in mesenchyme (m) and ectoderm (e). a, anterior; p, posterior; d, dorsal; v, ventral.

displays a restricted expression during limb development. Eph-related receptors comprise a large family of receptors (van der Geer et al., 1994) that bind membrane-anchored ligands (Bartley et al., 1994; Beckmann et al., 1994; Davis et al., 1994; Cheng and Flanagan, 1994) and may therefore mediate cell contact-dependent interactions. We show that *Cek-8* is expressed in distal mesenchyme of early chick limb buds with more pronounced expression posteriorly. Expression decreases as the limb develops but is then up-regulated in mesenchymal cell condensations associated with formation of tendons, including their attachment sites to cartilage rudiments. As tendons mature, *Cek-8* is down-regulated in these structures but up-regulated in other patterned sites, feathers and scales.

We have identified factors that influence early expression of *Cek-8* in the developing limb and demonstrate that signals originating from the AER are required for *Cek-8* induction and maintenance and that FGF-2 and FGF-4 can mimic these signals. Grafts of polarising region, retinoic acid or BMP-2 modulate the *Cek-8* expression domain. We also investigated *Cek-8* transcript distribution in limb buds of the polydactylous chick mutant *talpid*^{3} to gain insights into its position in the signalling cascade that governs patterning and outgrowth.

MATERIALS AND METHODS

Chick embryos

Fertilised chicken embryos were purchased from Poyndon Farm, Herts, England, and were incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951). Following surgical manipulation, embryos were dissected into PBS and processed for whole-mount in situ hybridisation.

FGF-2, retinoic acid and BMP-2 application on beads

FGF-2 was obtained from R and D Systems and BMP-2 was provided by the Genetics Institute, Cambridge, Massachusetts. All-*trans*-retinoic acid was obtained from Sigma (UK). FGF-2 and retinoic acid were applied to heparin acrylic and AG1X2 beads respectively, as described by Niswander et al. (1993) and Tickle et al. (1985). BMP-2 was applied to heparin beads as described by Francis et al. (1994).

Experimental manipulation of chick wings

All manipulations were performed at stage 20 unless otherwise stated.

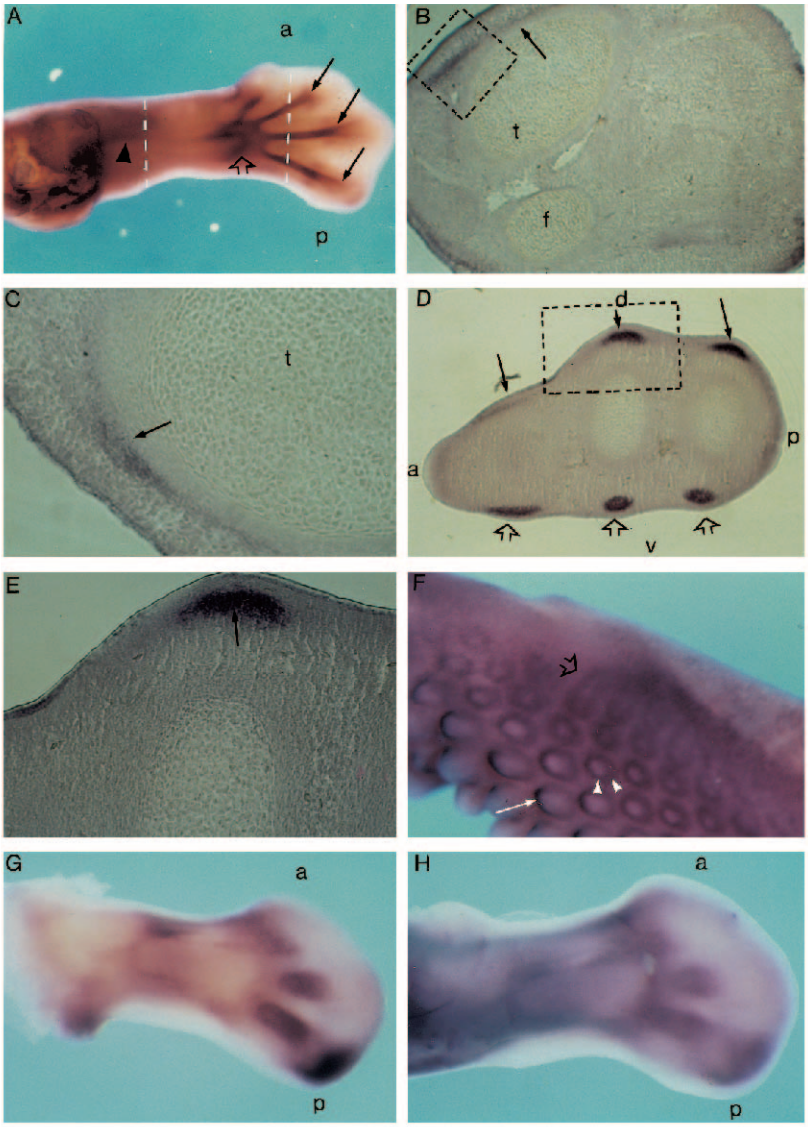

Fig. 2. Distribution of *Cek-8* expression during chick limb development and in tendons and feathers. (A) Stage 31 leg in ventral view. Strong expression is observed in stripes fanning out over metacarpals (open arrow) and phalanges (arrows) and also at more proximal regions (arrowhead). White dotted lines indicate level of transverse sections shown in (B-E). (B) Transverse section of the proximal part of a stage 31 leg. *Cek-8* transcripts are found adjacent to the part of tibia perichondrium (t) closest to the ectoderm and mark the tendon attachment site to cartilage element (arrow) – fibula (f). Dotted lines indicate area shown at high magnification in C. (C) High magnification of a transverse section of proximal part of a stage 31 leg. *Cek-8*-expressing cells are adjacent to tibia perichondrium (arrow). (D) Transverse section of a stage 31 leg at metacarpal level. Dorsally (d) expression is observed in three discontinuous sheets of mesenchymal and ectodermal tissue (arrows). Ventrally (v) tissue condensation is most advanced posteriorly (p) (open arrows). Dotted lines indicate area shown at high magnification in E. (E) High magnification of a transverse section of metacarpals of a stage 31 leg showing one of the dorsal zones of *Cek-8*-expressing cells. (F) Stage 33 wing shown in dorsal view. Expression is detected at low levels in ectoderm but higher levels in placode cylinders (open arrow). Placodes appear to bud away from the medial aspect of each cylinder (solid arrowhead). *Cek-8* expression is confined to posterior and proximal regions of the feather bud (arrows). G and H show matching distribution of *Cek-8* mRNA and CEK-8 protein respectively, of a right and left leg of the same embryo (stage 27, dorsal view). a, anterior; p, posterior; d, dorsal; v, ventral; t, tibia; f, fibula.

For AER removal, a fine tungsten needle was employed. Beads were secured at apical sites using a fine platinum wire staple or by placing them under the AER. Beads were applied to proximal sites by inserting them into tissue in which a fine slit had been cut with a tungsten needle. For polarising region grafts, tissue was dissected from the posterior margin of a stage 20 chick embryo and treated with trypsin in order to remove the ectoderm. The tissue was subsequently grafted under the anterior ridge of a stage 20 host chick embryo. One embryo from each series of manipulations was allowed to develop for 6 days in order to validate the treatment.

Grafting of mouse limb tissue to chick wing buds

Forelimb buds from 10.5-day-old C57/BL/Ha^t mouse embryos were dissected from the body wall and trypsinised for 20 minutes to remove the ectoderm. Mesenchymal tissue was dissected using tungsten needles and grafted proximally, as described above, or distally under the ridge of a stage 20 chick embryo wing.

Whole-mount in situ hybridisation

All chick embryos were washed in PBS and fixed overnight in 4% paraformaldehyde at 4°C. Preparation of digoxigenin-labelled RNA probes and protocol for whole-mount in situ hybridisation were as described by Nieto et al. (1995). A 420 bp antisense RNA probe corresponding to nucleotides 394-813 was used to detect *Cek-8* (Sajjadi and Pasquale, 1993). The entire coding region for *Sek-1* (3.5 kb; Gilardi-Hebenstreit et al., 1992) was used after size reduction to 700 bp. *Msx-1* probe was a kind gift from Dr S. Wedden (Brown et al., 1993).

Whole-mount antibody staining

Stages 26-28 chick embryos were bisected along the dorsal mid-line. One half was processed for RNA whole-mount in situ hybridisation and the other for whole-mount antibody staining with an affinity-purified anti-SEK-1 antibody that cross-reacts with its avian homologue (Irving et al., 1995). Briefly the dissected embryos were washed in ice-cold PBS and then fixed in 2% trichloroacetic acid for 2 hours at 4°C. They were then washed 3× 10 minutes in PBS and bleached with 0.05% H_2O_2 for 30 minutes at 4°C, followed by a 30 minute wash in PBS at room temperature and then incubation for 1 hour in 10% sheep serum to prevent non-specific protein binding. The embryos were then incubated with anti-SEK-1 antibody (1:1000) for 12 hours at 4°C. Subsequently embryos were washed 3× 1 hour with PBT before incubating for 12 hours with an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:200). The embryos were then washed 3× 10 minutes before visualising antibody binding with NBT/BCIP.

RESULTS

Expression of *Cek-8* in limb buds

During early embryogenesis, stages 6-20, *Cek-8* transcripts were detected in a number of sites that had previously been described in the mouse (Nieto et al., 1992), including presumptive somites and hindbrain. In addition, we detected expression in limb buds that has not been previously described. *Cek-8* was first detected in the posterior part of the emerging wing bud and in the body wall at stage 17. Subsequently weak expression of *Cek-8* extended throughout the wing bud (Fig. 1A). As the limb buds became more distinct, expression of *Cek-8* became confined to the distal tip by stage 22/23 and in a stripe at the base of the bud that extended into the flank (Fig. 1B). Dynamic expression of *Cek-8* was also observed in leg buds, although slightly delayed compared with wing buds. A similar pattern of expression has been observed for *Sek-1* transcripts (murine homologue of *Cek-8*) during mouse embryogenesis (data not shown). Examination of transverse sections through stage 22 limb buds showed that *Cek-8* was transcribed in mesenchyme cells but not ectoderm (data not shown). As the limb bud grew out further (stages 23-29), *Cek-8* expression was confined to the distal apical part of the bud, with a sharp posterior demarcation and fading anteriorly (e.g. see control side in Fig. 4A,B; this A-P gradient is obscured in strongly stained specimens). Sections revealed that higher levels of expression occur in peripheral mesenchyme, as shown by sections in Fig. 1D,E. During stages 24-29, transcripts at the bud apex gradually decreased in abundance but were still detectable up to stage 29 (Fig. 1F).

New sites of *Cek-8* expression were detected as tissues of the limb began to differentiate. *Cek-8* transcripts were observed at stage 24/25, in regions where cells were condensing to give rise to the humerus (Fig. 1C). In transverse sections of the proximal part of a stage 25 limb, *Cek-8*-expressing cells were located subectodermally and also near dorsal and ventral edges of pre-cartilage cell condensations (Fig. 1D). As development proceeded, *Cek-8* expression was up-regulated in distal structures and down-regulated proximally, although expression was also maintained between long bones in joint regions. By stage 27/28, expression was not observed over previously expressing long bones, but was now prominent in the developing hand and foot plates. In the foot plate, for example, the initial metacarpal-like zone of distal expression pointed towards the fading posterior distal site of *Cek-8* mesenchymal expression (Fig. 1F). *Cek-8* was initially up-regulated in posterior metacarpals, then in more anterior ones. *Cek-8* expression was detected earlier and was subsequently stronger ventrally in the foot plate compared to dorsally. By stage 31, the earlier posterior region of *Cek-8* expression was no longer detectable but there was expression of the gene over distal structures including developing phalanges (Fig. 2A).

Transverse sections of stages 27-31 foot plate revealed that *Cek-8* expression was associated with development of tendons. Asymmetrical expression of *Cek-8* was initially observed in a broad domain in the ectoderm and its immediate underlying mesenchyme, with stronger expression ventrally than dorsally at stage 27 (Fig. 1G,H). As cartilage condensed, the broad domain of *Cek-8* expression concentrated into and became predominantly mesenchymal, first ventrally then dorsally. The ventral mesenchymal zone of *Cek-8*-expressing cells gradually became transformed into tight knots residing a few cell diameters under the ectoderm. Fig. 2D,E shows expression of *Cek-8* in the formation of tendons with relation to anterior-posterior and dorsal-ventral axes at stage 31. Ventrally, three zones of *Cek-8* expression were seen, with the posterior zone being associated with a tight mesenchymal condensation and the most anterior zone being more diffuse and including ectoderm and mesenchyme. Dorsally, two broad regions of expressing cells were found posteriorly, with a third anterior zone of cells only just beginning to express *Cek-8*. Eventually, tight knots of *Cek-8*-expressing cells were seen below the cartilage elements, whereas above, groups of *Cek-8* cells were more flattened, and these tendon-like structures became localised towards the centre of the limb. At proximal levels *Cek-8* transcripts were found adjacent to part of the tibia perichondrium, which is closer to the ectoderm and which marks the tendon attachment site to the cartilage element (Fig. 2B,C).

Between stages 29 and 37, *Cek-8* was also expressed in a restricted pattern in feather primordia (Fig. 2F). In the least differentiated anterior regions of the wing, *Cek-8* was uniformly expressed in the ectoderm but gradually became restricted, giving an appearance of hollow stripes of expressing tissue. Circular zones of cells seemed to bud away from the most posterior region of the *Cek-8*-expressing stripe and eventually formed a ring at the edge of each feather placode. As the feather appendage began to emerge, *Cek-8* expression was up-regulated at the posterior margin of the developing bud. At more advanced stages of feather development, *Cek-8* expression was down-regulated in the anterior region but markedly raised at the posterior region and confined to the base of the growing appendage. Expression was not sustained beyond stage 36. We also saw restricted expression of *Cek-8* in regions associated with scale formation (data not shown).

We compared the distribution of Cek-8 protein with that of *Cek-8* mRNA distribution in right and left limbs of embryos at stages 20, 24, 27 and 31. In all cases, the staining pattern of the antibody corresponded with the *Cek-8* transcript pattern (compare Fig. 2G with 2H).

Response of *Cek-8* expression to ridge removal

Removal of the apical ridge from early wing buds resulted in a dramatic down-regulation of *Cek-8* expression. 24 hours after the operation, wing buds were severely truncated and no *Cek-8* transcripts were detectable (n=2, Fig. 3A). A detailed time-course study revealed that *Cek-8* expression was only slightly reduced 3-4 hours after the operation (n=2) but lost completely between 5.5 hours (n=2) and 6 hours (n=2) after ridge removal (Fig. 3B,C). We further investigated localisation of the signal emanating from the ridge that is required for *Cek-8* expression, by removing various portions of the ridge along the anterior-posterior axis. Removal of anterior AER resulted in a general down-regulation of *Cek-8* expression in adjacent anterior mesenchymal tissue, but there was also a slight down-regulation of *Cek-8* expression in posterior mesenchyme (n=2, Fig. 3D). Similarly, removal of posterior ridge resulted in down-regulation of *Cek-8* in adjacent posterior mesenchyme but also, albeit to a lesser extent, in anterior mesenchyme (n=2, Fig. 3E). Neither manipulation on its own could mimic the ablation of the entire ridge.

Recent experiments have intimated that FGF-2 and FGF-4 can substitute for signalling activity originating from the ridge. We therefore investigated the relationship between FGF and *Cek-8* expression by applying FGF-2 to the limb after having removed the AER. Fig. 3F-G shows that application of FGF after ridge removal does indeed up-regulate and/or maintain *Cek-8* expression. The zone of *Cek-8* expression maintained by FGF was dependent upon the site of application. When FGF-2 was applied at the anterior margin of the bud, expression was predominantly induced around lateral and posterior rims of the bead (*n*=3, Fig. 3F), but when applied in more posterior regions *Cek-8* was expressed anteriorly, proximally and posteriorly to the FGF source (*n*=2, Fig. 3G). In both cases there was a zone of non-expressing tissue directly adjacent to the bead. These results imply that limb tissue displays an anterior-posterior gradient of competence in its ability to express *Cek-8* in response to FGF-2. We further examined this idea by placing beads soaked in FGF-2 into proximal regions of the bud where *Cek-8* is not normally expressed (*n*=11). We demonstrated that a gradient of competence to respond to FGF by expressing this receptor tyrosine kinase exists along both anteroposterior and proximodistal axes such that cells in the posteroproximal region can be influenced by FGF-2 to express *Cek-8* (Fig. 3H), whereas we were unable to elicit a response from cells in anterior-proximal regions.

In order to further investigate the ability of distal signals to induce cells to express *Cek-8*, we grafted proximal, non-expressing tissue from mouse forelimb into distal chick wing bud (*n*=3). 24 hours after grafting we found, using a mouse-specific probe for *Sek-1*, the murine homologue of *Cek-8*, that expression of the gene had been induced throughout the grafted tissue (Fig. 3I). In the complementary experiment where *Sek-1*-expressing distal forelimb tissue from mouse embryos was transplanted in proximal regions of the developing chick wing bud, no expression was detectable in the transplanted tissue after 24 hours (*n*=3; data not shown).

Effect of polarising signals on *Cek-8* expression

Expression of *Cek-8* in distal mesenchyme of the limb bud was asymmetrical across the anteroposterior axis, with a blunt edge posteriorly next to the polarising region and fading anteriorly. In order to determine whether the polarising region influences the shape of the expression domain of *Cek-8,* we grafted a polarising region to the anterior margin of a developing wing bud. Fig. 4A shows that transplantation of the polarising region led to anterior enhancement of *Cek-8* expression, resulting in symmetrical expression at tip of the bud (*n*=3), although expression did not appear to extend right up to the transplanted tissue. Interestingly the graft itself did not appear to express *Cek-8*.

Retinoic acid has been previously shown to mimic signalling of the polarising region and therefore we examined its effect on *Cek-8* expression by applying beads soaked in retinoic acid to the anterior margin of developing wing buds. Like polarising region grafts, retinoic acid beads, at 0.1 mg/ml, shifted the limit of *Cek-8* expression more anteriorly (*n*=3, Fig. 4B), but not immediately up to the bead. Thus, both polarising region signalling and retinoic acid applied anteriorly induce a very similar response with regard to *Cek-8* expression. When beads soaked in higher concentrations of retinoic acid (1 mg/ml, *n*=4) were applied anteriorly, *Cek-8* expression did not appear to be extended; instead the anterior limit of expression was shifted posteriorly (data not shown).

Cek-8 expression in the polydactylous mutant *talpid*^{3}

Homozygous *talpid*^{3} chickens have limbs with an increased number of morphologically identical digits. In early limb buds of *talpid*^{3} chickens, *Cek-8* expression was seen to extend anteriorly across the tip, reminiscent of the response of normal limb buds to application of either retinoic acid or a polarising region graft (Fig. 4C). In older *talpid*^{3} wing buds, we observed not only an anterior extension of the expression domain but also a weak signal throughout the bud, suggesting a possible disruption in distal as well as posterior restriction mechanisms (Fig. 4D).

Inhibitory action of retinoic acid on *Cek-8* expression

Retinoic acid did not induce *Cek-8* expression in directly adjacent tissue. To examine further this effect on *Cek-8* expression, we placed beads soaked in retinoic acid into the domain of expression at wing bud tips. When a retinoic acid soaked bead (0.1 mg/ml) was applied to bud apex, there was considerable down-regulation in *Cek-8* expression (*n*=2, Fig. 5A). However this down-regulation was not complete and there was residual expression both anterior and posterior to the bead. When a bead soaked in the same concentration of retinoic acid was placed posteriorly there was almost complete down-regulation of *Cek-8* expression (*n*=2, Fig. 5B), even anteriorly at the tip.

Response of *Cek-8* expression to BMP-2 application

Recently it has been shown that *Bmp-2* expression is activated by retinoic acid (Francis et al., 1994) and thus we examined whether this BMP-2 exerts the same effect on *Cek-8* expression as retinoic acid. When a bead soaked in 0.5 mg/ml of BMP-2 was applied to the anterior margin of the developing wing bud, *Cek-8* expression was down-regulated around it but, none the less, gene expression was maintained in more posterior regions of the bud tip (*n*=3, Fig. 5C). When BMP-2 was applied in the region corresponding to the polarizing region, it led to a complete down-regulation in *Cek-8* expression, similar to the effect induced by retinoic acid where inhibition of expression occurred over long distances (*n*=3, Fig. 5D). Thus BMP-2 can mimic the inhibitory effects of retinoic acid on *Cek-8* transcription. In addition, we determined the effect of anterior BMP-2 on expression of *Msx-1*, another gene whose expression is regulated by factors originating from the ridge. Unlike *Cek-8*, the expression profile of *Msx-1* was not altered by the application of BMP-2 to wing buds (*n*=4, Fig. 5E).

DISCUSSION

Expression profile of the receptor tyrosine kinase

The expression profile of the receptor tyrosine kinase Cek-8 suggests that the receptor has roles in both early and late chick limb development. Limb structures are generated in sequence from undifferentiated mesenchyme at the tip of the early bud and here we have shown that *Cek-8* is expressed in distal mesenchyme and expression is regulated by outgrowth and patterning signals. Later on, transcripts of the gene are associated with cell condensations that form tendons and their attachments to cartilage rudiments and with developing feather buds.

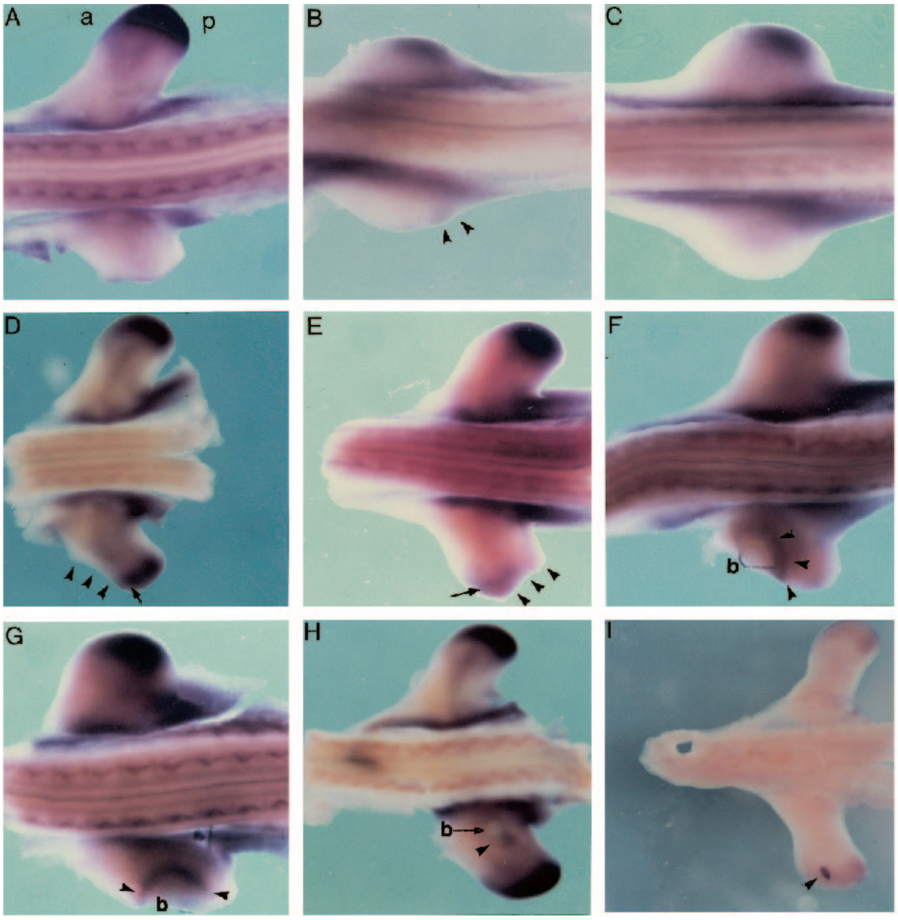

Fig. 3. Response of *Cek-8* expression to ridge removal and FGF-2. All manipulations were performed at stage 20. (A) Dorsal view of wing bud at 24 hours after ridge removal showing severe truncation of the limb and loss of *Cek-8* expression. (B) Dorsal view of wing 5.5 hours after ridge removal. Weak posterior expression is still detectable (arrowheads). (C) Dorsal view of wing bud, 6 hours after ridge removal. There is no detectable *Cek-8* expression. (D) Dorsal view of wing bud 24 hours after anterior ridge removal (area delineated by arrowheads). Slight decrease in *Cek-8* expression has occurred near the anterior edge of remaining ridge (arrow). (E) Dorsal view of wing 24 hours after posterior ridge removal (area delineated by arrowheads). *Cek-8* expression is down-regulated both adjacent to the site of ablation and anteriorly (arrow). (F) Anterior application of FGF-2. *Cek-8* expression is up-regulated proximally and posteriorly around the bead (arrowheads). (G) Posterior application of FGF-2. *Cek-8* expression is up-regulated both anteriorly and posteriorly (arrowheads). (H) Proximal application of FGF-2. Up-regulation of *Cek-8* expression distal to bead (arrowhead). (I) Ventral view of wing after proximal mouse limb grafted to the distal region of chick wing. Up-regulation of *Sek-1* is observed in grafted tissue (arrowhead). b, position of bead. FGF-2 was used at 1 mg/ml. a, anterior; p, posterior.

Another member of the *Eph* family, *Eck*, has also been found to be expressed in distal mesenchyme and in forming cartilage although, unlike *Cek-8*, *Eck* appears not to show a posterior restriction in distal mesenchyme (Ganju et al., 1994). Factors regulating *Eck* expression have not been examined, but Eck and Cek-8 may have overlapping or cooperative roles in the limb.

Expression in the early limb bud

Expression of *Cek-8* during early wing development overlaps with the expression domains of a number of genes that are thought to play pivotal roles in patterning of the limb. *Cek-8* expression overlaps at the posterior part of the limb bud tip with expression domains of both *Shh* (Riddle et al., 1993) and the gene encoding BMP-2 (Francis et al., 1994). *Shh* and *Bmp-2* transcripts remain confined to the posterior margin of the limb bud. In contrast, *Cek-8* expression extends much more anteriorly, probably up to but not into the anterior expression domain of the gene encoding BMP-4. *Cek-8* expression also overlaps at the distal tip with expression of three transcription factors, Msx-1, Msx-2 (Davidson et al., 1991) and Evx-1 (Niswander and Martin, 1993b), which are regulated by ridge factors.

Distal expression of *Cek-8* in apical and distal mesenchyme is modulated by ridge and polarising-region signals. When proximal cells are placed at the tip of the limb beneath the apical ridge, they can respond to AER signals by re-expressing *Cek-8*. Ridge removal results in the loss of detectable expression of *Cek-8* after 6 hours, but expression can be maintained by application of FGF. *Msx-1* expression is also maintained by FGF but is down-regulated very rapidly after ridge removal (Ros et al., 1992). One possibility suggested by these data is that *Cek-8* expression is downstream of *Msx-1*. However, with beads soaked in FGF, *Cek-8* expression apparently starts in tissue a few cell diameters away from the bead, whereas *Msx-1* can be expressed in cells immediately next to a bead soaked in the same concentration of FGF (Vogel et al., 1995). Furthermore when BMP-2 is applied anteriorly, expression of *Cek-8* and *Msx-1* are differentially affected.

Anterior application of retinoic acid or grafts of polarising region can also modulate *Cek-8* expression and extend the normal domain of *Cek-8* expression anteriorly. These manipulations would be expected to lead to anterior expression of *Fgf-4* (Niswander et al., 1994), which in turn could regulate *Cek-8*. Although application of FGF alone to anterior mesenchyme in the absence of the ridge does activate *Cek-8*, this occurs predominantly in mesenchyme posterior to the bead. Furthermore, only cells in the posterior two thirds of proximal limb bud and not anterior proximal cells can activate *Cek-8* in response to FGF. This suggests that another factor is present in the posterior part of the bud, which, together with FGF, regulates *Cek-8* expression. Recently Yang and Niswander (1995) have demonstrated that FGF-4 can induce *Shh* expression in proximal posterior regions of the bud. It is also possible that in addition to requiring inductive signals to activate and maintain the expression of *Cek-8* (and *Shh*) in posterior and

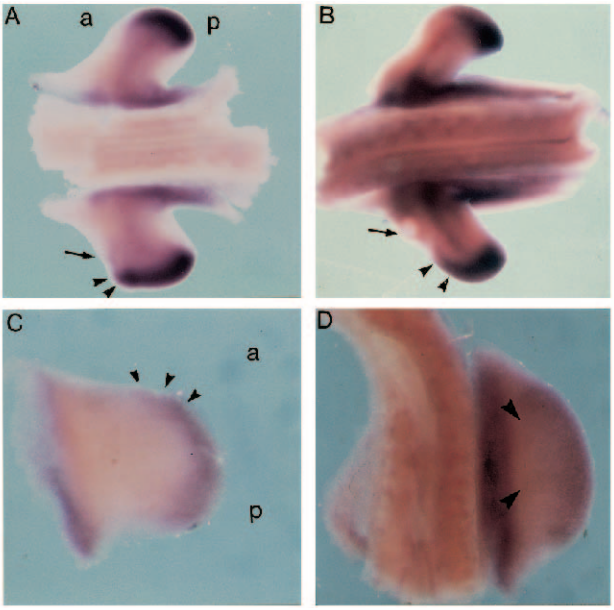

Fig. 4. Anterior extension of *Cek-8* expression induced by the polarising region and retinoic acid and in *talpid*^{3} mutants. All manipulations were performed at stages 19-21. (A) Anterior graft of the polarising region (arrow) or (B) anterior application of beads soaked in 0.1 mg/ml retinoic acid (arrow) result in an anterior extension of the normal expression pattern after 24 hours (arrowheads). (C) *Cek-8* expression in a stage 21 *talpid*^{3} wing showing an anterior extension (arrowheads). (D) *Cek-8* expression in a stage 24 *talpid*^{3} wing showing proximal (arrowheads) as well as anterior expression. All figures show a ventral view of wings. a, anterior; p, posterior.

distal regions, the anterodistal part of the limb is rich in inhibitory molecules that can be overridden by polarising signals. A fascinating finding not accounted for by these possibilities is that expression of *Cek-8* is always found distal to the FGF bead placed in posterior proximal mesenchyme. Yet when an additional bead is placed more proximally this mesenchyme is quite capable of expressing *Cek-8* (unpublished observations).

Several molecules are known to be regulated by cooperation between polarising and outgrowth signals, including transcripts of *Bmp-2*, *Bmp-7* and *Hoxd-13* genes. All of these molecules lie downstream of SHH signalling and are expressed uniformly across the anteroposterior axes of the broad limb buds of polydactylous *talpid*^{3} mutant embryos (Izpisua-Belmonte et al., 1992; Francis-West et al., 1995). We found that *Cek-8* is also expressed uniformly in early limb buds of this mutant, which, together with its restricted expression pattern, suggests that *Cek-8* expression is involved in coordination of responses to polarising and outgrowth signals.

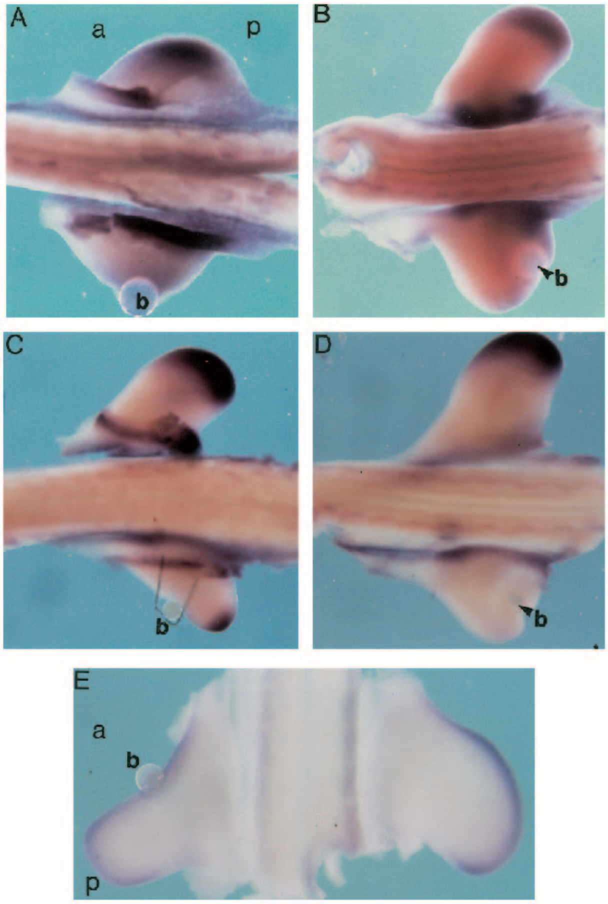

Fig. 5. Inhibitory effect of retinoic acid and BMP-2 on *Cek-8* but not *Msx-1* expression. All manipulations were performed at stage 20. (A) Ventral view. Apical application of a bead soaked in 0.1 mg/ml retinoic acid for 5 hours results in down-regulation of *Cek-8* expression, but slight expression remains in posterior regions. (B) Dorsal view after posterior application of retinoic acid results in almost complete down-regulation of *Cek-8* expression. (C) Ventral view after anterior application of BMP-2 for 24 hours results in down-regulation of *Cek-8* near the manipulation. (D) Dorsal view after posterior application of BMP-2 for 24 hours results in down-regulation of *Cek-8*, both in adjacent and anterior regions. (E) Ventral view of wing after anterior application of BMP-2 (25 hours) probed with *Msx-1*. BMP-2 did not affect *Msx-1* expression. b, position of bead. BMP-2 was used at 0.5 mg/ml. a, anterior; p, posterior.

Expression of *Cek-8* in mesenchymal condensations

Cek-8 is transiently expressed in condensing mesenchyme that will form tendons and their associations with the perichondrium of cartilage elements. Cellular localisation of *Cek-8* in developing tendons is first found ventrally and then dorsally, and this fits with anatomical observations (Wortham, 1948) that ventral tendons develop before dorsal ones. *Cek-8* is expressed in a sub-ectodermal layer of cells, which then condenses just below the ectoderm. Ventrally this condensation then becomes a discrete knot of cells expressing *Cek-8*. This progression appears to parallel the morphology of tendon development described by Hurle et al. (1989). *Cek-8* expression also bears a striking resemblance to the expression patterns of murine homologues of the *sine-oculus* genes originally isolated from *Drosophila* (Oliver et al., 1995). One gene member of this family, *Six-2*, shows a similar pattern of expression to *Cek-8*, with expression initially confined to a broad sheet under the ectoderm that rapidly condenses into a mesenchymal layer. One difference in expression patterns of the two genes is that distal expression of *Six-2* only extends as far as cartilage condensations, whereas *Cek-8* expression extends more distally and fans out from the digit primordia.

Expression of *Cek-8* in developing feather buds and scales

Cek-8 is expressed in ectodermal placodes that will form feathers and scales. Many of the same genes are expressed in both the early limb bud and the feather buds and the same signalling network may produce budding in both cases (Choung et al., 1990). Transcripts of *Cek-8* are localised to feather ectoderm whereas in early limb bud transcripts are found in mesenchyme. A similar switch in tissue expression is also found for Shh, which is mesenchymally expressed in the early limb bud but epithelially expressed in the feather buds (Nohno et al., 1995). In feather buds transcripts of *Cek-8* are found in epithelium associated with mesenchyme-expressing tenascin. It is intriguing that a similar association may also occur in developing tendons.

Potential role of *Cek-8* expression

Previous work has identified diffusible molecules, e.g. retinoic acid and retinoids, that potentially mediate signalling with a range of several to many cell diameters in the developing limb. Cek-8 is a member of the Eph family of receptor tyrosine kinases that recent work suggests are activated by membrane-bound ligands (Bartley et al., 1994; Davis et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994). Thus our finding that *Cek-8* is expressed in the chick limb bud suggests a role for contact-mediated signalling in limb patterning. In addition, cell-cell communication via gap junctions has been observed between limb bud cells (Coelho and Kosher, 1991) and thus all three mechanisms of signalling may operate in developing limbs.

Cek-8 may mark cells that have the ability to respond to short-range signals presented by neighbouring cells. A potential ligand, ELF-1, is known (Cheng and Flanagan, 1994), but since several ligands can bind to the same receptor in vitro (Beckmann et al., 1994; Davis et al., 1994), and expression patterns of ligands in limbs are currently unknown, it is unclear which ligand interacts with *Cek-8* in vivo. It is also not clear whether *Cek-8*-expressing cells constitute a population of mutually interacting cells or whether interactions only take place at the borders of *Cek-8*-expressing domains. In the first case, cells could express both receptor and ligand, and this would coordinate activities such as cell proliferation at the tip of the early limb bud or cell differentiation in developing tendon. On the other hand, if cells expressing *Cek-8* only interact with neighbouring cells at the boundaries of expression domains, interactions could occur at the proximal edge of the progress zone and at the sharp boundary of expression at the posterior margin of the progress zone adjacent to the polarising region. An interaction at the boundary of condensing cells in tendons could perhaps control formation of the tendon sheath and connection with skeletal elements.

Recent studies implicated ligands for receptor tyrosine kinases of the Eph family as good candidates for positional labels in the retinotectal system (Drescher et al., 1995; Cheng et al., 1995). Gradients of those ligands on the tectum provide positional information that is interpreted by the receptor tyrosine kinases on the axons to direct topographical projections. By analogy, *Cek-8* could perhaps be important in interpreting positional information in the limb progress zone.

Factors identified in this study that regulate *Cek-8* expression in the early limb bud could potentially modulate expression elsewhere in tendons, featherbuds and even the hindbrain. Key components (or related molecules) that modulate *Cek-8* expression in the limb are also found in the developing hindbrain; notochord is a source of retinoic acid as well as *Shh* (Riddle et al., 1993), *Fgf-3* is expressed in rhombomeres 5 and 6 (Wilkinson et al., 1989), and *Bmp-2* and *Bmp-4* as well as *Msx-1* and *Msx-2* are expressed in a restricted manner during embryogenesis (Graham et al., 1994). We are therefore currently investigating the relationship between these molecules and *Cek-8* expression in the rhombencephelon.

R. N. was in receipt of a Wellcome Prize Studentship. C. T. and D. D'S. are supported by the Wellcome Trust. We would like to thank Anne Crawley for help with the histology, and Professor Lewis Wolpert and Professor Paul Brickell for helpful comments on the manuscript.

REFERENCES

Bartley, T. D., Hunt, R. W., Welcher, A. A., Boyle, W. J., Parker, V. P., Lindberg, R. A., Lu, H. S., Colombero, A. M., Elliott, R. L., Guthrie, B. A., Holst, P. L., Skrine, J. D., Toso, R. J., Zhang, M., Fernandez, E., Trail, G., Varnum, B., Yarden, Y., Hunter, T. and Fox G. M. (1994). B61 is a ligand for the ECK receptor protein tyrosine kinase. *Nature* **368**, 558-560.

Beckmann, M. P., Cerretti, D. P., Baum, P., Van den Bos, T., James, L., Farrah, T., Kozlosky, C., Hollingsworth, T., Shilling, H., Maraskovsky, E., Fletcher, F. A., Lhotak, V., Pawson, T. and Lyman, S. D. (1994). Molecular characterisation of a family of ligands for eph-related receptor tyrosine kinases. *EMBO. J.* **13**, 3757-3762.

Brown, J. M., Wedden, S. F., Milburn, G. H., Robson, L. G, Davidson, D. R. and Tickle, C. (1993). Experimental analysis of the control of expression of the homeobox-gene Msx-1 in the developing limb and face. *Development* **119**, 41-48.

Cheng, H.-J. and Flanagan, J. G. (1994). Identification and cloning of Elf-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* **79**, 157-168.

Cheng H.-J., Nakamato M., Bergemann A. D and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 359-370.

Chuong, C.-M., Oliver, G., Ting, S., Jelalian, B., Chen, H. M. and De Robertis, E. M. (1990). Gradient of homeoproteins in developing featherbuds. *Development* **110**, 1021-1030.

Davidson, D. R., Crawley, A., Hill, R. E. and Tickle, C. (1991). Position-depentent expression of two related homeobox genes in developing vertebrate limbs. *Nature* **352**, 429-431.

Coelho, C. N. and Kosher, R. A. (1991). A gradient of gap junctional communication along the anterior-posterior axis of the developing chick limb bud. *Dev. Biol.* **148**, 529-535.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V. and Pawson, T. Goldfarb, M. and Yancopoulos, G. D. (1994). Ligands for EPH-related receptors that require membrane attachment or clustering for activity. *Science* **266**, 816-819.

Drescher, U., Kremoser, C., Handwerker, C., Loeschinger, J., Noda, M. and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa Tectal protein related to ligands for Eph receptor tyrosine kinases, *Cell* **82**, 359-370.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B. and Simandl, K. (1994). FGF-2: Apical ectodrmal ridge growth signal for chick limb development. *Science* **264**, 104-106.

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

Francis-West, P. H., Robertson, K. E., Ede, D. A., Rodriguez, C, Izpisua-Belmonte, J. C., Houston, B., Burt, D. W., Gribben, C., Brickell, P. M. and Tickle, C. (1995). Expression of genes encoding Bone Morphogenetic Proteins and Sonic Hedgehog in Talpid (ta^3). limb buds: Their relationships in the signalling cascade involved in limb patterning. *Dev. Dynamics* **203**, 187-197.

Ganju, P., Shigemoto, K., Brennan, J., Entwistle, A. and Reith, A. D.

(1994). The Eck receptor tyrosine kinase is implicated in pattern formation during gastrulation, hindbrain segmentation and limb development. *Oncogene* **9,** 1613-1624.

Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. -G., Chestier, A., Wilkinson, D. G. and Charnay, P. (1992). An Eph-related receptor tyrosine kinase gene segmentally expressed in the developing mouse hindbrain**.** *Oncogene* **7,** 2499-2506.

Graham, A., Francis-West, P., Brickell, P. M. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephelic neural crest. *Nature* **372**, 684-686.

Hornbruch, A. and Wolpert, L. (1986). Positional signalling by Hensen's node, when grafted to the chick limb bud. *J. Embryol. Exp. Morph.* **94**, 257-265.

Hamburger,V and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 105-125.

Hurle, J. M., Hinchliffe, J. R., Ros, M. A., Critchlow, M. A. and Genis-Galvez, J. M. (1989). The extracellular matrix architecture relating to myotendinous pattern formation in the distal part of the developing chick limb: an ultrastructural, histochemical and immunocytochemical analysis. *Cell Diff. Dev.* **27**, 103-120.

Irving, C.,. Neito, M., DasGupta, R., Charnay, P. and Wilkinson, D. G. (1995). Progressive spatial restriction of *Sek-1* and *Krox-20* gene expression during hindbrain segmentation. *Dev. Biol*. (in press).

Izpisua-Belmonte, J.-C., Ede, D. A., Tickle, C. and Duboule, D. (1992). The mis-expression of posterior Hox-4 genes in *talpid (ta*^{3}*).* mutant wings correlates with the absence of anteroposterior polarity. *Development* **114**, 959-963.

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

Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116,** 1137-1150.

Nieto, M. A., Patel, K. and Wilkinson, D. G. (1995). In situ hybridisation analysis of chick embryos in whole mount and tissue sections. In *Methods in Avian Embryology* (ed. M. Bronner-Fraser). San Diego: Academic Press (in press).

Niswander, L. and Martin, G. **R.** (1993a). FGF-4 and BMP-2 have the opposite effects on limb growth. *Nature* **361**, 68-71.

Niswander, L. and Martin, G. R. (1993b). FGF-4 regulates expression of *Evx-1* in the developing mouse limb. *Development* **119**,287-294

Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579-587.

Niswander, L., Jeffery, S., Martin, G. and Tickle, C. (1994). A positive feedback loop co-ordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609-612.

Nohno, T., Kawakami, Y., Ohuchi, H., Fugiwara, A., Yoshioka, H. and Noji, S. (1995). Involvement of the sonic hedgehog gene in chick feather formation. *Biochem. Biophys. Res. Comm.* **206**, 33-39.

Oliver, G., Wehr, R., Jenkins, N. A., Copeland, N. G., Cheyette, B. N. R., Hartenstein, V., Zipursky, S. L. and Gruss, P. (1995). Homeobox genes and connective tissue patterning. *Development* **121**, 693-705

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

Ros, M. A., Lyons G., Kosher R. A., Upholt W. B., Coelho C. N. and Fallon J. F. (1992). Apical ridge dependant and independent domains of GHox-7 and GHox-8 expression in chick limb buds. *Development* **116**, 811-818.

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

Sajjadi, F. G. and Pasquale, E. B. (1993). Five novel avian eph-related tyrosine kinases are differentially expressed. *Oncogene* **8**, 1807-1813

Saunders, J. W. Jr. and Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. In *Epithelial-Mesenchymal Interactions* (ed. R. Fleischmajer and R. E. Billingham), pp. 78-97. Baltimore: Williams and Wilkins.

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

Summerbell, D. (1983). The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morph.* **78**, 269-289.

Tickle, C., Alberts, B. and Wolpert, L. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarising region. *Nature* **296**, 564-566.

Tickle, C., Lee, J. and Eichele, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick limb development. *Dev. Biol.* **109**, 82-95.

van der Geer, P., Hunter, T. and Lindberg, R. A. (1994). Receptor protein tyrosine kinases and their signal transduction pathways. *Ann. Rev. Cell. Biol.* **10**, 251-337.

Vogel, A., Roberts-Clarke, D. and Niswander, L. (1995). Effect of FGF on gene expression in chick limb buds in vivo and in vitro. *Dev. Biol.* **171**, 507-520.

Wagner, M., Thaller, C., Jessell, T. and Eichele, G. (1990). Polarising activity and retinoid synthesis in the floor plate of the neural tube. *Nature* **345**, 819-822.

Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1989). Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. *Development* **105**, 131-136.

Wortham, R. A. (1948). The development of the muscles and tendons in the lower leg and foot of chick embryos. *J. Morphol.* **83**, 105-148.

Yang, Y. and Niswander, L. (1995). Interaction between the signalling molecules Wnt-7a and shh during vertebrate limb development: Dorsal signals regulate anteroposterior patterning. *Cell* **80**, 939-947.

(Accepted 10 January 1996)