The role of *Engrailed* in establishing the dorsoventral axis of the chick limb

Cairine Logan, Amata Hornbruch, Iano Campbell and Andrew Lumsden*

Department of Developmental Neurobiology, UMDS, Guy’s Hospital, London SE1 9RT, UK

*Author for correspondence

SUMMARY

Expression and mutation analyses in mice suggest that the homeobox-containing gene *Engrailed* (*En*) plays a role in dorsoventral patterning of the limb. During the initial stages of limb bud outgrowth, *En-1* mRNA and protein are uniformly distributed throughout the ventral limb bud ectoderm. Limbs of *En-1*−/− mice display a double dorsal phenotype suggesting that normal expression of *En-1* in the ventral ectoderm is required to establish and/or maintain ventral limb characteristics. Loss of *En-1* function also results in ventral expansion of the apical ectodermal ridge (AER), suggesting that *En-1* is also required for proper formation of the AER. To further investigate the role *En* plays in dorsoventral patterning and AER formation, we have used the replication competent retroviral vector, RCAS, to mis-express mouse *En-1* in the early chick limb bud. We show that ectopic *En-1* expression in dorsal ectoderm is sufficient to repress the endogenous expression of the dorsal ectodermal marker *Wnt7a*, with a resultant decrease in *Lmx1* expression in underlying dorsal mesenchyme. Furthermore, the AER is disrupted morphologically and the expression patterns of the AER signalling molecules *Fgf-8* and *Fgf-4* are altered. Consistent with recent evidence that there is a reciprocal interaction between signalling molecules in the dorsal ectoderm, AER, and zone of polarising activity (ZPA), loss of *Wnt7a, Fgf-8* and *Fgf-4* expression leads to a decrease in expression of the signalling molecule *Shh* in the ZPA. These results strongly support the idea that, in its normal domain of expression, *En-1* represses *Wnt7a*-mediated dorsal differentiation by limiting the expression of *Wnt7a* to the dorsal ectoderm. Furthermore, our results provide additional evidence that *En-1* is involved in AER formation and suggest that *En-1* may act to define ventral ectodermal identity.

Key words: Engrailed, AER, dorsoventral axis, patterning, chick, limb

INTRODUCTION

During formation of the vertebrate limb, cells must be positionally specified along three axes; proximodistal (P-D), anteroposterior (A-P) and dorsoventral (D-V). Recent studies have identified a number of molecules involved in signalling along these axes and there is increasing evidence that the signalling systems interact with one another (reviewed in Tickle, 1996; Cohn and Tickle, 1996).

Patterning along the D-V axis is controlled by signals from the ectoderm. Tissue recombination experiments in chick have shown that presumptive limb ectoderm acquires D-V information which is imposed on the underlying mesoderm at Hamburger and Hamilton stage 15. Thus, rotation of limb bud ectoderm along the D-V axis induces a corresponding reversal in muscle and skeletal patterns (MacCabe et al., 1974; Geduspan and MacCabe, 1987, 1989; Akita, 1996). Rotations made at earlier developmental stages show a more complete reversal in D-V repatterning and distal regions are more labile than proximal ones. Akita (1996) has recently shown that dorsalisation appears to be initiated by dorsal ectoderm and affects mesodermal structures progressively towards the midline. Recent studies have provided information about the molecular cues that underlie dorsal limb patterning: signalling from dorsal ectoderm is mediated by a member of the Wnt family of secreted proteins, *Wnt7a* (Parr and McMahon, 1995), which is expressed uniformly throughout the dorsal ectoderm during initial stages of limb bud outgrowth in mouse (Gavin et al., 1990; Parr et al., 1993) and chick (Dealy et al., 1993) and is required for the acquisition of dorsal limb pattern (Parr and McMahon, 1995). Loss of *Wnt7a* function in homozygous mutant mice results in transformation of dorsal limb structures to a more ventral phenotype. Furthermore, *Wnt7a* appears to mediate dorsalisation of underlying limb mesenchyme through induction of the LIM homeobox-containing gene, *Lmx1* (Riddle et al., 1995; Vogel et al., 1995). In chick, both the onset and pattern of *Lmx1* expression in dorsal limb bud mesenchyme closely follows that of *Wnt7a* in the ectoderm. Removal of dorsal ectoderm leads to a corresponding loss of *Lmx1* expression in the underlying distodorsal mesenchyme which can be rescued in vitro when dorsal limb mesenchyme deprived of ectoderm is grown on *Wnt7a*-expressing cells. In addition, *Wnt7a* is sufficient to induce ectopic *Lmx1* expression in ventral limb mesenchyme and leads to dorsalisation of ventral mesoderm.

Less, however, is known about molecular cues that underlie ventral limb patterning. Mutational analysis in mice has shown that the homeobox-containing gene, *En-1*, is involved in pattern-
En-1 is expressed throughout the ventral ectoderm of the developing limb bud in mouse (Davis et al., 1991) and chick (Davis et al., 1991; Gardner and Barald, 1992). Loss of En-1 function in mice results in transformation of ventral pap structures to a more dorsal phenotype and, in both mouse En-1<sup>hd/hd</sup> (Loomis et al., 1996) and chick limbless (Ros et al., 1996; Grieshammer et al., 1996) mutant embryos, results in ventral expression of Wnt7a.

Along the P-D axis, expression in the apical ectodermal ridge (AER) of different members of the fibroblast growth factor (FGF) family including Fgf-2, Fgf-4 and Fgf-8 are thought to control proximodistal outgrowth and patterning. Signals from the AER maintain underlying mesenchyme cells in an undifferentiated and proliferative state (Saunders, 1948; Summerbell et al., 1973). Indeed, all three Fgf proteins can functionally substitute for the AER (Niswander et al., 1993; Fallon et al., 1994; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Along the A-P axis, pattern is established by signalling from the posterior mesenchyme cells or zone of polarising activity (ZPA). The product of the Sonic hedgehog (Shh) gene mimics the activity of the ZPA (Riddle et al., 1993).

There is increasing evidence that signalling systems along all three axes of the limb interact with one another. For example, Wnt7a expression in dorsal ectoderm, together with Fgf-4 expression in the AER, are required to maintain Shh expression in posterior mesenchyme (Parr and McMahon, 1995; Yang and Niswander, 1995). Moreover, there is a positive feedback loop between the expression of Shh and Fgf-4 (Lauffer et al., 1994; Niswander et al., 1994).

Transplantation experiments have shown that ectoderm at the distal margin of the developing limb bud differentiates into the AER, a pseudostratified columnar structure (Saunders, 1948), as a result of the inductive influence of the underlying mesoderm (Saunders and Reuss, 1974; Todd and Fallon, 1984). Meinhardt’s (1983) ‘boundary model’ suggested on theoretical grounds that a D-V ectodermal interface is preconditional for apical ridge formation. Recent molecular studies have provided support for this idea: in limbless, where ectoderm at the distal margin fails to respond to the inductive influence of the underlying mesenchyme and no AER is formed, Wnt7a is uniformly expressed throughout limb ectoderm and En-1 expression is lacking ventrally (Ros et al., 1996; Grieshammer et al., 1996). Hence the primary defect in this mutant may be due to the lack of the normal D-V interface in the ectoderm. Mutation analysis in mice has also shown that En-1 is required for proper formation and/or maintenance of the AER (Loomis et al., 1996): loss of En-1 function results in a ventral expansion of the AER as defined by both morphological and molecular criteria.

To directly test the role En plays in both establishing D-V polarity in the developing limb and in formation of the AER, we have used the replication competent avian retroviral vector RCAS (Hughes et al., 1987) to mis-express mouse En-1 in the developing chick limb bud in vivo. Here we show that ectopic En-1 expression in the dorsal ectoderm is sufficient to repress the endogenous expression of Wnt7a and results in a decrease in Lmx1 expression in underlying mesenchyme. Ectopic expression of En-1 across the D-V ectodermal interface also results in the loss of a morphologically and molecularly defined AER at the distal margin. In addition, as might be expected given the recent evidence that there is a reciprocal interaction between signalling molecules in the dorsal ectoderm, AER, and ZPA, loss of Wnt7a together with Fgf-8 and/or Fgf-4 expression leads to a corresponding decrease in Shh expression in the ZPA. These results provide further evidence in support of the idea that, in its normal domain of expression, En-1 acts in part to repress Wnt7a-mediated dorsal differentiation by limiting the expression of Wnt7a to the dorsal ectoderm and may play an additional role in AER formation by the induction and/or maintenance of a D-V boundary.

**MATERIALS AND METHODS**

**Retroviral construction and infection**

Construction of RCAS(BP)A and RCAN(BP)A retroviral vectors containing the entire coding region of the mouse En-1 cDNA has been described previously (Logan et al., 1996). Chick embryo fibroblasts and concentrated viral stocks were prepared and titred as described by Fekete and Cepko (1993). Viral titres ranged from 2x10<sup>8</sup> up to 10<sup>9</sup> cfu/ml. Concentrated viral supernatant was injected under the vitelline membrane and either layerd on top of the blastoderm of stage 4/5 embryos or on top of the ectoderm in the presumptive forelimb and/or hindlimb region of stage 8-10 embryos. All experimental manipulations were performed using Ross White chick embryos provided by Poyndon Farm (Hertfordshire, UK). Embryos were staged according to Hamburger and Hamilton (1951).

**In situ hybridisation and sectioning**

Whole-mount RNA in situ hybridisation of intact embryos or dissected limb buds was performed using non-radioactive digoxigenin- (DIG) or fluorescein- (FITC) labelled RNA probes (see below) as previously described (Logan et al., 1996). Specimens were refixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) prior to sectioning and/or storing.

Selected embryos or dissected limb buds were embedded in gelatin/albumin and 50 μm serial sections cut using a vibratome. Whole embryos were sectioned transversely, at right angles to the A-P axis of the embryo along the D-V plane. Limb buds were sectioned longitudinally along the P-D axis of the limb, at 90° to the A-P axis and parallel to the D-V axis. Sections were then cleared using 90% glycerol in PBS and mounted under coverslips for photography.

**Probes**

Antisense DIG- or FITC-labelled RNA probes were synthesised according to the manufacturer’s instructions (Boehringer-Mannheim, Indianapolis, IN) using the following appropriately linearised DNA templates.

1. To detect RCAS and RCAN retroviral transcripts containing mouse En-1 cDNA sequences, a 176 bp SstI/ApII fragment from a previously isolated cDNA (Joyner and Martin, 1987) was subcloned into the pBSK Bluescript vector (Stratagene, La Jolla, CA) and the resulting plasmid linearised using SstI. (For Wnt7a, a previously isolated chick cDNA (Dealy et al., 1993) was linearised by cutting internally with XhoI and transcribed using T3 RNA polymerase to yield an approximately 1.4 kb probe covering the 3 untranslated region. (3) Probes for Lmx1 (Riddle et al., 1995), Shh (Riddle et al., 1993), Fgf-4 (Niswander et al., 1994), Fgf-8 (Mahmood et al., 1995) and En-1 (Logan et al., 1996) were made as previously described.

**Scanning electron microscopy**

Embryos infected between stages 4 and 5 were incubated for approximately 72 hours postinfection, prior to their removal from the egg. Once rinsed with Howard’s Ringer and dissected free of embryonic membranes, they were fixed overnight with 4% paraformaldehyde in PBS at 4°C. Following postfixation in 1% osmium in Millonig’s constant osmolarity phosphate buffer (pH 7.3) at 4°C for 90 minutes, embryos were dehydrated in a graded series of acetone. They were then critical point dried, mounted on aluminium stubs and gold sputter
coated. The embryos were observed using a Hitachi S520 scanning electron microscope.

Transplantation of infected limb buds
The viability of embryos infected between stages 4 and 5 was severely compromised and few embryos survived past E8. To obtain E10 limbs for analysis, infected embryos were removed from the egg at stage 20/21 and wing and/or leg buds with visible AER defects were excised at the level of the flank and grafted to the anterior edge of the right wing bud of a non-infected stage 22/23 host embryo, the graft being secured with platinum pins. Orientation was strictly recorded: infected right wing and leg buds were placed with their A-P axis parallel to the host’s wing P-D axis while limb buds from the left-hand side were grafted with their A-P axis parallel to D-P axis of the host’s wing so that the dorsal surfaces of host and graft corresponded. Eggs were sealed with tape and left to heal at room temperature for 20-30 minutes before continued incubation for another 6 days. Embryos were killed at 10 days of total incubation and transplanted limb buds prepared for analysis.

Whole-mount skeletal preparations
Embryos were removed from the egg, dissected free from surrounding membranes and washed in PBS prior to fixing for 3 to 20 hours in 5% trichloroacetic acid. Whole embryos or dissected limb buds were stained overnight in 0.1% Alcan green 2GX in 70% acid alcohol, dehydrated through a graded series of alcohols and cleared in methyl salicylate prior to analysis of their skeletal pattern.

RESULTS

Onset of En-1 mRNA expression in the presumptive ventral limb ectoderm coincides with the acquisition of its ventralising activity
To begin to examine the role En-1 plays in establishment of D-V polarity, we examined the spatial and temporal pattern of En-1 mRNA expression during early chick limb development by in situ hybridisation. En-1 mRNA transcripts were first detected in the flanking ectoderm of the trunk at stage 15, predominantly in the prospective limb fields (data not shown). By stage 16, expression extended throughout the length of the ventral body wall (Fig. 1A). At stage 18, the anterior limit of expression is clearly demarcated at the anterior edge of the wing bud at the level of somite 15. There is no posterior border of expression; rather, it continues under the tail bud through the midline and merges with expression on the contralateral side. During initial stages of limb bud outgrowth, transcripts were uniformly distributed throughout the ventral limb ectoderm and extended into the ventral half of the AER (Fig. 1B,C). Expression persisted up to at least stage 25, the latest stage examined and matches that previously reported for En-1 (Davis et al., 1991; Gardner and Barald, 1992). Thus, both onset and pattern of En-1 expression in the ectoderm coincides with the acquisition of its ventralising activity, as judged by grafting experiments (Geduspan and MacCabe, 1987, 1989).

Mis-expression of mouse En-1 in the developing chick limb
To modify the En expression pattern, a mouse En-1 cDNA was inserted into RCASBP(A) generating RCAS En-1 (Logan et al., 1996). The two mouse En genes have previously been shown to be functionally equivalent to those of other species, including chick (Martinez et al., 1991) and Drosophila (Joyner, 1996), as well as functionally equivalent to each other (Hanks et al., 1995). Concentrated viral supernatant was injected under the vitelline membrane and either layered on top of the blastoderm of stage 4/5 embryos or on top of the ectoderm in the presumptive forelimb and/or hindlimb region of stage 8-10 embryos. Ectopic En-1 expression in developing limbs was subsequently detected by in situ hybridisation (Fig. 1D,E), and distinguished from endogenous En-1 expression using a mouse-specific probe. In embryos analysed between stages 18 and 24, En-1 was typically expressed throughout the mesenchyme and in patches in the ectoderm following infection of stage 4/5 embryos (e.g. Fig. 1D). In contrast, infection of stage 8-10 embryos typically resulted in more complete infection of the ectoderm (e.g. Fig. 1E) and virtual exclusion of transcripts from the mesenchyme. Similar expression patterns were seen using a control vector, RCAN En-1, which lacks the splice acceptor required for proper translation of En protein (data not shown).

En-1 mis-expression represses dorsal-specific gene expression
In embryos infected with RCAS En-1 at stage 4/5 or stage 8-10 and examined between stages 18 and 24, little or no Wnt7a expression was detected in dorsal limb ectoderm (Fig. 1G,I). Expression of Wnt7a in flank ectoderm and spinal cord was also consistently downregulated in embryos infected at stages 8-10 (data not shown) and may reflect the increased extent of En-1 expression in these tissues following infection of stage 8-10 embryos when compared to stage 4/5 embryos. No change in the normal Wnt7a expression pattern was observed in regions of the embryo that did not ectopically express En-1 (data not shown). Furthermore, infection of dorsal limb ectoderm and/or mesenchyme using the control RCAN En-1 vector did not affect endogenous Wnt7a expression (data not shown).

Given that Wnt7a appears to mediate dorsalisation of underlying limb mesenchyme through induction of Lmx1, it was of interest to determine if the observed loss of Wnt7a expression in dorsal limb ectoderm was accompanied by a decrease in Lmx1 expression in the underlying mesenchyme. In some early infected embryos (data not shown) analysed between stages 20 and 22, no Lmx1 mRNA expression was detected in the dorsal limb mesenchyme (Fig. 1L), whereas in the majority (23/38), much reduced expression often persisted in more proximally located dorsal mesenchyme (Fig. 1K). Changes in Lmx1 mRNA expression were less pronounced following infection at later embryonic stages (stage 8-10; data not shown). No change in Lmx1 expression was observed in regions of the embryo that did not ectopically express En-1 (data not shown).

In Drosophila, en is required during a certain developmental period to positively autoregulate its own transcription (Heemskerk et al., 1991). It was therefore of interest to address the question of autoregulation in RCAS En-1-infected limb buds ectopically expressing En-1. The endogenous expression of En-1 mRNA, however, remained unchanged (as detected using a species-specific probe; data not shown) providing further support for the idea that the vertebrate En genes are not autoregulated (Joyner et al., 1991; Logan et al., 1993, 1996). Furthermore, in contrast to the inductive behaviour of grafts in the neuroepithelium (Martinez et al., 1991), ectopic expression
of mouse *En-1* in either limb mesenchyme or dorsal ectoderm did not lead to induction of the endogenous chick *En-1* gene in adjacent cells and/or tissue.

**AER formation is disrupted**

Next, we examined the morphological and molecular effects of ectopic *En-1* expression on AER formation. RCAS *En-1* infection consistently produced limb buds in which the AER was disrupted (Fig. 2) and occasionally displaced or duplicated dorsally (Fig. 10). As shown in scanning electron micrographs of stage 21-22 embryos, the well-defined ridge normally present at the distal margin (Fig. 2G) was discontinuous and completely absent in places (Fig. 2H). Indeed, no morphologically distinct AER is visible in sections through deficient regions (Fig. 1I). Furthermore, *Fgf-8* transcripts, which are normally expressed throughout the AER from very early in limb development (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996), were not detected at stage 21-23 in regions lacking a morphologically defined AER (Fig. 2C,F). Similar results were obtained for *Fgf-4* (Fig. 1M,N), whose transcripts are detected slightly later than *Fgf-8* and confined more posteriorly in the AER (Suzuki et al., 1992; Niswander and Martin, 1992). As might be expected, proximal-to-distal limb outgrowth was decreased in regions lacking an AER, giving the limb buds a scalloped appearance (e.g. Fig. 2B,E).
Limbs infected with the control RCAN En-1 vector developed normally (Fig. 2A,D).

Overall, loss of the AER correlated well with ectopic ectodermal expression of En-1 across the D-V boundary. As shown in Fig. 2L, patches of ectoderm strongly expressing RCAS En-1 following retroviral infection at stage 9 lack a morphologically defined ridge. In contrast, as shown in sections through limbs infected at stage 4/5, a well-defined ridge is present when En-1 is clearly not ectopically expressed in the ectoderm across the D-V boundary (e.g. Fig. 1D,L). On occasions, ectopic En-1 expression coincided with a well-defined ridge (Fig. 1E). Such expression however, may reflect late infection of the ectoderm following viral spread.

Shh expression is reduced

Recent evidence suggests that there is a positive feedback between Shh expression in the ZPA and Fgf-4 expression in the AER (reviewed in Tickle, 1996; Cohn and Tickle, 1996). Furthermore, expression of Wnt7a in the dorsal ectoderm is required to maintain Shh expression. Given that ectopic expression of En-1 in dorsal ectoderm represses Wnt7a expression and disrupts Fgf expression in the AER, we expected that RCAS En-1 infection would also lead to a decrease in expression of Shh in the ZPA. As shown in Fig. 3A-C, Shh expression in posterior mesenchyme of the developing limb of stage 23-24 embryos was decreased and/or undetectable following infection of stage 4/5 embryos. Similar results were obtained following infection of stage 8-10 embryos (data not shown) providing further evidence that signalling along the A-P, D-V and P-D axes of the limb are linked.

Loss of posterior skeletal elements and perturbation of D-V patterning

To determine whether early changes in gene expression induced by ectopic En-1 expression were accompanied by later anatomical changes, we examined skeletal, muscle and tendon patterns in E10 virally infected limbs. Due to poor viability, embryos infected between stages 4 and 5 were killed approximately 72 hours postinfection, their limb buds excised and embryos infected at stages 4 and 5 were killed approximately 4 days following retroviral infection at stage 4/5. Limb buds have been removed from the body and photographed either from the ventral side (A,B) or dorsal side (C). Anterior is toward the top in all panels.

Overall, morphological changes affecting the leg were more frequently observed than those affecting the wing and can be divided into two categories. (1) RCAS En-1 infection resulted in the loss and/or fusion of skeletal elements. As shown in Fig. 4, posterior skeletal elements were preferentially lost following retroviral infection of stage 8-10 embryos. Digit IV was often missing and/or fused (Fig. 4D,F) while the fibula was severely reduced in size (Fig. 4C) or absent (Fig. 4E). Missing or fused digits were also occasionally seen following retroviral infection of stage 4/5 embryos (data not shown), with digits II and III being predominantly affected. The transplantation procedure of the limb buds during their early development also led to most of the structures of the stylopod being lost while the zeugopod was often stunted. (2) RCAS En-1 infection at both stages resulted in changes in the flexion of joints in the leg, affecting primarily the more distal metatarsophalangeal joint and joints of the phalanges (data not shown). In contrast to the feet of control embryos, which normally flex ventrally, the feet of infected embryos were often hyperextended. In severe cases, flexation of the intertarsal joint was also affected (Fig. 4E,F). In addition, some claws did not show the typical ventral curvature but were either straight or dorsally curved.

Histological analysis of muscle and tendon patterns in feet of RCAS En-1-infected embryos proved inconclusive. Overall, there appeared to be a greater abundance of tendons on the dorsal side of the metatarsals compared to controls. However, clear ventralisation of the dorsal mesoderm could not be established. Thus, En-1 expression in the dorsal ectoderm and/or mesoderm perturbs patterning of dorsal mesoderm but does not result in complete transformation of the dorsal mesoderm to a ventral phenotype. No obvious changes were observed in muscle and tendon patterns in the ventral side of the foot. The

![Fig. 3. Expression of Shh is decreased following RCAS En-1 mis-expression. In situ hybridisation of Shh mRNA expression (blue) in limb buds of a control (uninfected stage 23; A) embryo and two infected (B, C) embryos ectopically expressing En-1 (red) 4 days following retroviral infection at stage 4/5. Limb buds have been removed from the body and photographed either from the ventral side (A, B) or dorsal side (C). Anterior is toward the top in all panels.

![Fig. 4. Loss of posterior skeletal elements in whole mounts of E10 legs following RCAS En-1 infection of stage 8-10 embryos. Alcian-green-stained skeletal pattern of a control (uninfected; A, B) embryo and two infected (C, D and E, F) embryos viewed from the ventral side. Zeugopod and autopod of each limb are shown in A, C, E and B, D, F, respectively.](Image 48x626 to 336x718)

differentiation of dorsal scales and ventral foot pads is not sufficiently distinct at half term to draw any conclusions as to phenotypic changes in ectodermal derivatives.

DISCUSSION

Mutation analysis in mice (Wurst et al., 1994; Loomis et al., 1996) has shown that \textit{En-1} is required for ventral limb patterning and suggested that it acts in part by repressing \textit{Wnt7a}-mediated dorsal differentiation. Recent experiments have also shown that expression of \textit{En-1} in ventral ectoderm is essential for proper AER formation (Loomis et al., 1996; Ros et al., 1996; Grieshammer et al., 1996), supporting the idea that the D-V ectodermal interface is preconditional for AER formation (Meinhardt, 1983). The present findings provide direct evidence in support of these two ideas. First, we have shown that ectopic \textit{En-1} expression in dorsal ectoderm is sufficient to repress the endogenous expression of \textit{Wnt7a} in vivo and results in a decrease in \textit{Lmx1} expression in underlying mesenchyme. Second, we have shown that ectopic \textit{En-1} expression across the D-V ectodermal interface results in loss of a morphologically and molecularly defined AER. Furthermore, loss of \textit{Wnt7a}, as well as \textit{Fgf} expression in dorsal ectoderm and AER, respectively, leads to a decrease in \textit{Shh} expression in posterior mesenchyme, lending further support to the idea that molecules involved in signalling along the three limb axes interact with one another.

\textit{En-1} repression of \textit{Wnt7a} mediated dorsalisation

Because the \textit{Drosophila} \textit{en} homeoprotein is known to function as a transcriptional repressor (Jaynes and O’Farrell, 1991; Han and Manley, 1993), it is possible that \textit{En-1} acts directly to repress \textit{Wnt7a} expression. Indeed recent studies have identified two domains in \textit{Drosophila} \textit{en} that are crucial to its transcriptional repression activity in vivo (Smith and Jaynes, 1996) and both are evolutionarily conserved in chick and mouse \textit{En} proteins (Logan et al., 1992). Furthermore, the biological function of \textit{En} proteins has been shown to be evolutionarily conserved (reviewed in Joyner, 1996). In addition, our results, together with mutation analysis in mice, strongly suggest that \textit{Wnt7a} is downstream of \textit{En-1}. \textit{En-1} mRNA transcripts like \textit{Wnt7a} (Riddle et al., 1995) are first detected in the flanking ectoderm of the trunk at stage 15. \textit{Wnt7a} expression, however, is more medial than \textit{En-1} and never extends into the \textit{En-1} expression domain (M. Altabef and C. L., unpublished). In \textit{Wnt7a}−/− mice, \textit{En-1} expression is confined to its normal ventral ectodermal domain, as are other ventral markers such as \textit{Bmp-2} and \textit{Dlx-2} (Parr and McMahon, 1995). Mutant \textit{Wnt7a} transcripts were also restricted to their normal dorsal ectodermal domain suggesting that D-V patterning of the ectoderm is unchanged in these animals. In \textit{En-1}−/− mice, by contrast, the \textit{Wnt7a} expression domain extends into the ventral ectoderm (Loomis et al., 1996). Alternatively, \textit{Wnt7a} repression may be an indirect consequence of \textit{En} function which, by analogy to \textit{Drosophila}, could involve cell-cell communication (Hidalgo, 1994). Interestingly, \textit{Wnt7a} is not expressed in the dorsal half of the AER (Dealy et al., 1993) from which the endogenous \textit{En-1} gene is also excluded. The exact nature of the molecular interaction between \textit{En-1} and \textit{Wnt7a} remains to be elucidated.

The marked decrease and occasional absence of \textit{Lmx1} expression following ectopic \textit{En-1} expression most probably results indirectly from loss of \textit{Wnt7a} expression in the overlying dorsal ectoderm. The complete repression of \textit{Lmx1} shown here, which could not be obtained by ectoderm manipulation (Riddle et al., 1995; Vogel et al., 1995), lends further support to the idea that the proximal expression domain of \textit{Lmx1} in the mesoderm is established too early to be modified after the limb forms. Incomplete \textit{Lmx1} repression probably reflects insufficiently widespread \textit{En-1} mis-expression early in limb development: endogenous \textit{Wnt7a} expression in the dorsal ectoderm may persist for a time and be sufficient to induce and/or maintain expression of \textit{Lmx1} in the underlying mesenchyme. Once induced, its expression may persist proximally despite the subsequent loss of \textit{Wnt7a} due to viral spread.

Given that limb buds deprived of ventral ectoderm fail to express \textit{Lmx1} in ventral mesenchyme cells (Riddle et al., 1995), it is unlikely that ectopic expression of \textit{En-1} in dorsal ectoderm provides instructive signals that act specifically to repress \textit{Lmx1} expression. Nor is the observed disruption of the AER likely to have any effect on \textit{Lmx1} expression as Vogel et al. (1995) have shown that \textit{Lmx1} transcripts were still present 48 hours after complete AER removal. However, it is possible that ectopic expression of \textit{En-1} in dorsal mesenchyme, which is most extensive following infection of stage 4/5 embryos, may interfere with induction and/or maintenance of \textit{Lmx1} expression.

Changes in limb pattern

RCAS \textit{En-1} infection at both stages resulted in loss and/or reduction of skeletal elements as well as changes in the flexion of joints. The loss of posterior skeletal elements (particularly digit IV and the fibula) following later stage infections may be indirectly due to loss of \textit{Wnt7a} and/or \textit{Shh} gene function, as previous experiments have shown that these elements were also preferentially lost following either dorsal ectodermal removal in chick (Yang and Niswander, 1995) or loss of \textit{Wnt7a} gene function in homozygous mutant mice (Parr and McMahon, 1995). Earlier infection also resulted in missing and/or fused digits (particularly II and III). However, the morphological as well as histological (see below) effects of early infection are complicated by the fact that \textit{En-1} becomes ectopically expressed throughout the limb mesenchyme, rather than being largely confined to the ectoderm. Mesenchymal expression may lead to ectopic activation of downstream targets and interfere with normal A-P and/or D-V patterning. Incorrect bending of the digits and/or ankles may have resulted from the development of supernumerary muscles and tendons on the dorsal side of the autopod.

Infected embryos did not show complete ventralisation of the dorsal mesoderm. Other factors in addition to \textit{En} may be required to transform dorsal ectoderm toward a more ventral phenotype, which may then provide instructive signals to the underlying mesoderm. Alternatively, dorsal mesoderm may not be capable of responding to ventralising signals. Interestingly, double ventral phenotypes were rarely observed following D-V rotations of limb ectoderm (MacCabe et al., 1974; Geduspan and MacCabe, 1987, 1989; Akita, 1996). Akita (1996) has recently found that the ventral mesoderm was more readily respecified following rotation of the ectoderm. Along the P-D axis, dorsal ectoderm modified ventral mesoderm before ventral ectoderm affected dorsal mesoderm. In addition, tissue
recombinations made at earlier developmental stages showed a more complete reversal in D-V patterning and distal regions were more affected than proximal ones. In our experiments, distal structures were also more affected than proximal ones and this may reflect an inability to ectopically express \( En-1 \) in a sufficiently widespread manner early in limb development. However, it is interesting to note that loss of \( Wnt7a \) function in homozygous mutant mice also resulted in an incomplete ventralisation of the limb, affecting primarily the distal structures (Parr and McMahon, 1995). The influence of \( En-1 \) mis-expression on ectodermal patterning itself remains to be determined.

**En-1 may act to define ventral ectodermal identity and be indirectly involved in AER formation**

In *Drosophila*, *engrailed* acts as a selector gene to confer polarity to (e.g.) the wing disc, where it is expressed in the posterior compartment and plays an important role in establishment and maintenance of the A-P compartment boundary (reviewed in Lawrence and Struhl, 1996). By analogy, *En-1* in vertebrates may act as a selector gene defining ventral ectodermal identity and be involved in the cell-cell communication which establishes and/or maintains a boundary between dorsal and ventral ectoderm; it may thus be involved in defining distinct D-V ectodermal compartments. In addition, *En-1* may be indirectly required for proper formation of the AER by virtue of its role in establishment of a D-V boundary. However, proper formation and maintenance of the A-P boundary in *Drosophila* does not depend exclusively on *engrailed* (Hidalgo, 1994). Interestingly, the loss of *En-1* function in homozygous mutant mice (Loomis et al., 1996) leads to disruption rather than complete loss of the AER.

Transplantation experiments have shown that flank ectoderm also has the ability to form an AER when in contact with limb field mesoderm (Saunders and Ruess, 1974; Carrington and Fallon, 1984). Its competence to respond is maximal at stage 16 and lost by stage 17. Furthermore, application of exogenous Fgps to the flank region of stage 13-17 chick embryos induces ectopic limb buds between the normal wing and leg buds, which are capped with a well-developed AER (Cohn et al., 1995; Vogel et al., 1996). Intriguingly, although these additional limb buds have reversed A-P polarity, they arise on the same D-V plane as normal limb buds, regardless of the position of the bead (M. Altabef, personal communication). Hence, normal expression of \( En-1 \) in flank ectoderm may function to establish a D-V interface along which an ectopic AER may be induced. These results are consistent with Meinhardt’s (1983) boundary model.

In our experiments, ectopic expression of \( En-1 \) in dorsal limb bud ectoderm disrupted the AER. Here, the normal interface between \( En-1 \) expressing and non-expressing cells was effectively abolished and infected cells at the distal margin are no longer responsive to induction by the underlying mesoderm. Mosaic ectodermal expression of \( En-1 \) across the D-V boundary at early developmental stages (stage 15-18) during the time at which ectoderm is competent to respond to induction, may account for the observed partial as opposed to complete disruption of the AER. Interestingly, embryos (7/152; <5%) were recovered following infection at stage 8-10 which completely lacked or had severely reduced forelimb and/or hindlimb buds (data not shown). In situ hybridisation analysis showed that these embryos were extremely heavily infected. Conversely, ectopic *En-1* expression in stage 20-24 embryos across the D-V boundary in regions where the AER is not disrupted may reflect late infection of the ectoderm following viral spread.

In conclusion, we have shown that *En-1* plays an important role in both the ectodermal regulation of D-V patterning and in proper formation of the AER. Our results provide additional support for the idea that, in its normal domain of expression, *En-1* acts to repress *Wnt7a*-mediated dorsal differentiation by limiting expression of *Wnt7a* to the dorsal ectoderm. Elucidation of the molecular nature of this interaction should provide valuable insight into the specification of cell position along the D-V axis. In addition, our results are consistent with the idea that the D-V ectodermal interface is preconditional for apical ridge formation. Furthermore, the recent proposal that D-V patterning and apical ridge formation are intimately linked (Grieshammer et al., 1996) is supported by our data.

This work was supported by the Welcome Trust and Howard Hughes Medical Institute, of which A. L. is an International Research Scholar. C. L. was supported by the MRC (Canada) and by HFSP. We thank K. Brady for excellent assistance with SEM and C. Tabin, A. Brown, H. Roelink, L. Niswander and I. Mason for providing some of the probes. We are grateful to A. Joyner for many helpful discussions and for providing the mouse *En-1* cDNA. We also thank C. Tickle and I. Mason for commenting on an earlier version of this manuscript.

**REFERENCES**


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


(Accepted 15 April 1997)