INTRODUCTION

Classical tissue transplantation experiments and recent genetic studies in the limb indicate that ‘signalling centers’ provide the molecular cues necessary for patterning the limb and directing regional outgrowth along the three different axes (Saunders, 1948 and/or Martin, 1995; Johnson et al., 1994; Tickle, 1995; Niswander, 1996; Zeller and Duboule, 1997). One such signalling center is the apical ectodermal ridge (AER), a narrow rim of thickened ectoderm along the limb margin. The AER is critical for normal proximal-distal limb outgrowth (Saunders, 1948) and this function appears to be mediated by members of the fibroblast growth factor family, such as Fgf4 and Fgf8 (Niswander et al., 1993; Ohuchi et al., 1995; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Patterning along the anterior-posterior (A-P) axis is specified by the zone of polarizing activity (ZPA), a cluster of mesenchymal cells in the posterior limb bud (Saunders and Gasseling, 1968). A major component of the ZPA signalling cascade is the protein Sonic hedgehog (Shh) (Riddle et al., 1993). Dorsal-ventral (D-V) axis polarity is influenced by the non-AER ectoderm (Pautou and Keny, 1973; MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987, 1989; Akita, 1996). Wnt7a, a secreted factor expressed by the dorsal limb ectoderm, is required for the differentiation of dorsal mesenchymal structures (Parr et al., 1993; Parr and McMahon, 1995) and mis-expression studies in chick suggest it acts by inducing expression of the LIM homeodomain-containing gene, Lmx1b, in the underlying mesenchyme. We suggest a model whereby, in En1 mutants, ectopic ventral Wnt7a and/or Lmx1b expression leads to the transformation of ventral cells in the broadened AER to a more dorsal phenotype. This leads to induction of a second zone of compaction ventrally, which in some cases goes on to form an autonomous secondary AER.

SUMMARY

The apical ectodermal ridge (AER), a rim of thickened ectodermal cells at the interface between the dorsal and ventral domains of the limb bud, is required for limb outgrowth and patterning. We have previously shown that the limbs of En1 mutant mice display dorsal-ventral and proximal-distal abnormalities, the latter being reflected in the appearance of a broadened AER and formation of ectopic ventral digits. A detailed genetic analysis of wild-type, En1 and Wnt7a mutant limb buds during AER development has delineated a role for En1 in normal AER formation. Our studies support previous suggestions that AER maturation involves the compression of an early broad ventral domain of limb ectoderm into a narrow rim at the tip and further show that En1 plays a critical role in the compaction phase. Loss of En1 leads to a delay in the distal shift and stratification of cells in the ventral half of the AER. At later stages, this often leads to development of a secondary ventral AER, which can promote formation of an ectopic digit. The second AER forms at the juxtaposition of the ventral border of the broadened mutant AER and the distal border of an ectopic Lmx1b expression domain. Analysis of En1/Wnt7a double mutants demonstrates that the dorsalizing gene Wnt7a is required for the formation of the ectopic AERs in En1 mutants and for ectopic expression of Lmx1b in the ventral mesenchyme. We suggest a model whereby, in En1 mutants, ectopic ventral Wnt7a and/or Lmx1b expression leads to the transformation of ventral cells in the broadened AER to a more dorsal phenotype. This leads to induction of a second zone of compaction ventrally, which in some cases goes on to form an autonomous secondary AER.

Key words: Limb development, Engrailed-1, AER, Mouse, Wnt7a, Lmx1b
function in the ventral limb (Loomis et al., 1996; Logan et al., 1997). Recent studies have shown that the function of these different signalling regions is interdependent. Shh signalling by the ZPA is required for maintenance of the AER (Laufier et al., 1994; Niswander et al., 1994), and Fgf4 and Wnt7a expression are required for maintenance of Shh expression (Laufier et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995).

Our studies of mice homozygous for En1 loss-of-function alleles suggested that an additional regulatory relationship exists between D-V limb patterning genes and AER formation. En1 mutant limbs not only demonstrate a partial dorsal transformation of the ventral paw, but they also display a ventroproximal expansion of the AER (Loomis et al., 1996). We suggested that this expansion of the newly formed AER could be responsible for the presence of ectopic ventral transforma
tion on the proximal paws of some mutants. In keeping with our findings, recent misexpression studies in chick have shown that ectopic expression of En1 in the AER and dorsal chick limb either ablates normal AER development or results in ectopic secondary AERs (Laufier et al., 1997; Logan et al., 1997; Rodriguez-Esteban et al., 1997). Recent mutant and misexpression experiments in chick (Laufier et al., 1997; Rodriguez-Esteban et al., 1997), as well as mutant studies in mice (Sidow et al., 1997), indicate that Notch signalling also plays a role in AER development. One of the molecules in this pathway, R-fng, has been shown to be dorsally restricted in chick limbs and appears to be regulated in part by En1 (Laufier et al., 1997; Rodriguez-Esteban et al., 1997).

Correlative molecular data in several chick mutants also suggest a link between D-V patterning and AER development. In the chick limbless mutant in which limb outgrowth does not occur due to failure to form an AER (Prahлад et al., 1979), molecular studies demonstrate that expression of AER marker genes is not initiated and normal D-V axis asymmetries are not established (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). Expression of Wnt7a and Lmx1b is expanded into the ventral limb, whereas expression of the ventralizing gene, En1, is absent in these mutants. On the contrary, chick wingless mutant limb buds initiate Fgf8 expression and display proper D-V patterning early on, but they subsequently fail to form a differentiated AER and molecular D-V asymmetries are lost (Ohuchi et al., 1997).

Based on experimental studies of Drosophila limb development and theoretical considerations in mouse, Meinhardt suggested that the juxtaposition of dorsal and ventral limb domains might be a precondition for normal vertebrate AER development (Meinhardt, 1983). In addition to the studies outlined above, further support for Meinhardt’s hypothesis is provided by recent grafting experiments in avian limbs. Chimera studies in which presumptive dorsal or ventral quail limb tissue was inserted into ventral or dorsal limb domains, respectively, resulted in formation of ectopic AERs along the borders between chick and quail tissues (Tanaka et al., 1997). Moreover, the replacement of dorsal chick ectoderm with ventral chick ectoderm at slightly later stages of limb bud development also led to the formation of ectopic AERs (Laufier et al., 1997).

In an effort to better understand the potential relationship between D-V patterning and AER formation, we have used morphological criteria as well as expression analysis of several genetic markers to compare early AER development and subsequent maturation in wild type as well as En1, Wnt7a and En1/Wnt7a double-mutant mice. Our results indicate that early mesenchymal D-V patterning is normal in En1 mutant mice whereas ventral ectoderm specification in En1 mutants is abnormal even at early stages of limb development. At later stages, mesenchymal patterning is abnormal as Lmx1b is reexpressed in the ventral mesenchyme. Moreover, maturation of the AER is aberrant resulting in an expanded ventral AER. With further development, a bifurcated AER is formed following stratification of the ventral border of the mutant AER. Interestingly, ectopic ectodermal stratification occurs at the distal edge of the ventral Lmx1b expression domain. In some cases, the (anatomically) ventral AER rim goes on to form a secondary AER capable of promoting ectopic digit development. Finally, analysis of Wnt7a/En1 double mutants demonstrates that Wnt7a is required for both the ectopic ventral expression of Lmx1b and the development of ectopic secondary AERs and ventral digits in En1 mutant limbs.

MATERIALS AND METHODS

Genotype analysis of En1, Wnt7a and En1/Wnt7a mutant mice

The En1 and Wnt7a mutants used in these studies were previously generated by targeted disruption of the respective genes (Wurst et al., 1994; Hanks et al., 1995; Parr and McMahon, 1995; Matise and Joyner, 1997). In the case of the En1hth allele, the bacterial lacZ coding sequences were targeted to the En1 locus, bringing them under the control of the En1 regulatory sequences and inactivating En1 protein function (Hanks et al., 1995; Matise and Joyner, 1997). En1/Wnt7a double mutants were generated by crossing mice heterozygous for both alleles since homozygosity results in perinatal lethality and/or sterility. DNA was isolated from either yolk sacs or tails and genotyping was performed by either Southern blot analysis as previously described (Hanks et al., 1995) or PCR analysis. Primers used for genotyping the En1 wild-type allele were 5′-GTTCCAGG-CAAAAGCATATC-3′ and for the En1hth allele were 5′-GCCAGCTATCTCCCATC-3′ and 5′-CGGTGTAAGCAG-TTTGCCATG-3′. Primers used to genotype the Wnt7a wild-type allele were 5′-CTCTTGGTTATGCTGCTGG-3′ and 5′-CCCTCCC-GAGACAGTACGC-3′ and for the Wnt7a null allele were 5′-TCAGCTCTGCACGCGAGCTG-3′ and 5′-CCTCCCCGA-AGACAGTACGC-3′ (Cyan et al., 1997).

Histology and in situ hybridizations

Digoxigenin-labelled whole-mount and 35S-labelled section RNA in situ hybridizations were performed essentially as described (Loomis et al., 1996; Matise and Joyner, 1997). The probes used were to En1 (Wurst et al., 1994), Wnt7a (Parr and McMahon, 1995), Fgf8 (Crossley and Martin, 1995), Fgf4 (Niswander and Martin, 1992), Dlx2 (Robinson and Mahon, 1994), Jag1 (Lindsell et al., 1995) and Lmx1b (Cyan et al., 1997). Lmx1b is the mouse homolog of the previously characterized chick Lmx1 gene (Cyan et al., 1997). Embryos for β-galactosidase staining were fixed for 20 minutes to 1 hour, depending on the age, in 4% paraformaldehyde in phosphate-buffered saline on ice and then stained with either X-gal (Sigma) or salmon-gal (Biosynth) substrates diluted in a standard staining buffer containing both 0.02% NP-40 and 0.01% deoxycholate and incubated overnight at room temperature. Some salmon-gal stained embryos were used for double labelling by RNA in situ hybridization. These embryos were postfixed for 24 hours in 4% paraformaldehyde at 4°C and then hybridized with RNA probes as above, omitting the bleaching and methanol dehydration steps.
(Matise and Joyner, 1997). Proteinase K treatments were also reduced so that embryos were incubated in either 2 μg/ml or 0.5 μg/ml for 5 minutes at 37°C for probes to mesenchymal or ectodermal mRNAs, respectively. Cryosections of embryos used in whole-mount RNA in situ hybridizations were prepared by first sinking the embryos in 15% sucrose overnight followed by 30% sucrose for 4 hours. The embryos were then embedded in OCT medium and cut at 10-40 μm.

RESULTS

D-V and AER marker gene expression in early pre-AER limb buds

Our previous analysis of the En1 mutant AER defect focused on the limb bud stage when a definitive, multilayered AER arises in wild-type mice (10.5 dpc for the forelimb and 11.5 dpc for the hindlimb). To begin to understand the genetic events that trigger and sustain the development of an aberrant AER in En1 mutants, we performed a detailed analysis of the expression patterns of AER and D-V marker genes in wild-type and mutant limb buds. For consistency, we adopted the mouse limb staging system first developed by Wanek et al. (1989) and modified by Bell and Scott (personal communication). At stage 0.25, the limb bud is not yet morphologically discernable along the embryo flank but, by stage 0.5, it is delineated by a slight prominence and its ventral ectoderm is slightly thickened compared to the flattened dorsal ectoderm. At stage 1, the limb is a broad bulge with the distoventral ectoderm thickened relative to the dorsal and more proximal ventral ectoderm (see Fig. 1B,E,H,I). At stage 2, the limb bud looks like a semicircular protrusion emanating from the flank, and a broad crescent or band of bilayered ectoderm is present on the distal third of the ventral limb (Milaire, 1974). At stage 3 (10.5 dpc in the forelimb), the definitive, mature AER is visible along the proximoventral ectoderm and consists of 3-4 layers of ectodermal cells (see Fig. 1C,F,I,L). In mouse, the AER appears to form a true stratified epithelium (Kelley and Fallon, 1983).

Molecular analysis of wild-type limb buds prior to the formation of a morphologically distinct AER demonstrated dynamic shifts in the topological location of both D-V and AER marker gene expression domains. Between stages 0.25 and 0.5, the AER marker gene Dlx2 was expressed throughout the ventral ectoderm (data not shown) but, by stage 1, the Fgf8/Dlx2 expression domain occupied only the distal half of the ventral limb bud (Fig. 1D,E). Over the next few stages, this domain was compressed even further so that Dlx2 and Fgf8 expression was ultimately restricted to the multilayered AER along the D-V interface (Fig. 1F). Over the same stages, the En1 expression domain shifted distally, from a relatively restricted proximoventral position to extend over the entire ventral limb including the ventral AER (Fig. 1A-C). In parallel with these relative distal shifts in the topological location of AER and ventral ectodermal marker genes, the 'dorsal' marker genes, Wnt7a and Lmx1b, were also displaced more dorsally (Fig. 1G-L). Wnt7a, which at stage 1 was expressed in the distal and dorsal ectoderm covering 2/3 of the limb, became restricted to only the dorsal half of the limb ectoderm by stage 3 (Fig. 1H,I). Lmx1b, which was found to be expressed in both the ventral and dorsal limb mesenchyme prior to stage 2, became restricted to the dorsal mesenchyme by stage 3 (Fig. 1K,L).

Differentiation and compression of the ventral AER are aberrant in En1 mutant embryos

To determine if En1 plays a role in setting up the expression domains in the early limb bud, we conducted the same analysis in En1 mutant embryos. D-V ectodermal patterning was abnormal even at the earliest limb stages. Ectopic ventral expression of Wnt7a could be detected as early as stage 0.5 in En1 mutants (data not shown), with intense expression persisting in the ventroproximal ectoderm but clearing from the ventrodorsal ectoderm of older pre-AER and AER stage limbs (Fig. 2 compare A and B, E and F). In contrast, expression of
the mesenchymal gene *Lmx1b* was normal in *En1* mutant limbs at these early stages as it was appropriately expressed in early dorsal and ventral mesenchyme and then repressed despite persistent expression of *Wnt7a* in the adjacent ventral ectoderm (Fig. 2I,J and data not shown).

AER marker gene expression was initiated normally in *En1* mutant limbs, but failed to shift distally as occurs in wild-type limb buds. The *Fgf8/Dlx2* expression domain in stage 1 *En1* mutant limbs was clearly broader than in wild-type limbs (Fig. 2, compare C and D). By stage 3/4, although the proximal boundary of the *Dlx2/Fgf8*-positive domain had shifted distally slightly, the mutant AER remained much broader than its wild-type counterpart (Fig. 2, compare G and H). Expression of *Fgf4*, a late stage AER differentiation marker, was mainly restricted to the dorsal-most cells of the *En1* mutant AER (Fig. 2K,L).

To determine whether the expanded portion of the *En1* mutant AER corresponded primarily to the ventral AER, which normally expresses *En1*, we analyzed mice heterozygous or homozygous for an *En1* targeted null allele, *En1*<sup>kn</sup> (Hanks et al., 1995; Matise and Joyner, 1997), which expresses the lacZ
reporter gene from the En1 locus. Double labelling for β-galactosidase activity (En1-lacZ expression) and Lmx1b mRNA showed that, by stage 3 in both En1+/+ and En1+/− mutants, the size of the dorsal AER, delineated as the ‘negative’ domain between Lmx1b expression dorsally and lacZ ventrally, was very similar (Fig. 2IJ). These observations, together with the Fgf4 expression data and our previous molecular and morphological studies (Loomis et al., 1996), indicate that the dorsal AER is not grossly altered in En1 homozygous mutants; En1 appears to be required primarily for compaction and maturation of the ventral AER.

Development of a second ectodermal thickening along the proximoventral boundary of the expanded En1 mutant AER

Since the ventroproximally expanded AER detected in En1 mutant forelimb buds at stage 3 (Loomis et al., 1996) resembled the thickened ectoderm normally seen in a stage 2 wild-type limb, one question was whether the ventral expansion of the En1 mutant AER at 10.5 dpc simply reflected a delay in the distal shift of cells expressing Dlx2/Fgf8. To address this, we compared AER morphology and marker gene expression patterns in wild-type and mutant limbs at later stages of maturation and found that, over the next several stages, the En1 mutant AER expression domain remained broader than the wild-type AER expression domain (compare Fig. 3A,C with B,D). Surprisingly, whole-mount gene expression analyses revealed that, at limb stages 5/6, the ventral expansion of the En1 mutant AERs exhibited a marked A-P asymmetry (Fig. 3B), which was not prominent at limb stages 3/4 (Fig 2D). The anterior half of the En1 mutant AER at these stages was up to 4-6 times wider than the normal AER, whereas the posterior half was usually only 2-3 times wider than the wild type. This A-P asymmetry was observed in 90% of limbs analyzed (n>40) using a variety of AER-specific probes.

In addition to its marked anterior expansion, the En1 mutant AER also appeared to bifurcate into two distinct ectodermal ridges between stages 5 and 7. In situ staining with Fgf8 or Dlx2 riboprobes outlined two intensely stained rims of ectoderm, which were separated by an intervening region of less intensely stained epithelium (Fig. 3B). The dorsal rim was anchored at the distal limb margin whereas the ventral rim formed along the ventroproximal boundary of the expanded Dlx2/Fgf8 expression domain.

Fig. 3. Loss of En1 function results in reexpression of Lmx1b ventrally and abnormal AER maturation, leading in some cases to formation of ectopic AERs. Whole-mount in situ hybridization and/or β-galactosidase staining of stage 5/6 (A-H,J) and stage 7/8 (I,K,L) wild-type (A,C,E,G), En1 mutant (B,D,F,H,J-L) and Wnt7a mutant limbs (I). Dorsal (d) and ventral (v) are indicated. Dlx2 probe (A,B) illustrating the intense staining of the dorsal and ventral rims of the bifurcated mutant AER and the marked widening anteriorly (arrow, B) relative to posteriorly (arrowhead, B) and compared to the narrow ridge of a wild-type AER (A). β-galactosidase staining (C,D) demonstrating the strong expression of En1-lacZ in the AER and weaker staining in the ventral ectoderm of limbs from (C) heterozygous and (D) homozygous mice. Again note the mutant AER is wider anteriorly (arrow) versus posteriorly (arrowhead). Lmx1b probe (E,F) illustrating the normal dorsal expression (E) and the ectopic reexpression in the ventral mesenchyme of En1 mutant limbs (F). Note that the contours of the distal Lmx1b-negative zone have the same contours of an expanded AER. Jagged-1 expression (G,H), which was restricted to the progress zone of wild-type limb buds (G), extended into these ventral mesenchymal bulges associated with the marked anterior widening of the En1 mutant AERs (H, arrow). Lmx1b expression (purple,K,L) reveals persistent focal expression (arrows) in the ventral ectoderm of some En1 mutant limbs at about the time when Fgf8 transcripts are down-regulated in the primary AER, which is delineated by intense En1-lacZ expression (pink). These secondary AER signalling centers always overlie ectopic mesenchymal outgrowths. Bar: 500 μm.
paralleled by a marked A-P asymmetry in the breadth of the Lmx1b-negative mesenchymal zone. Thus, the ‘footprint’ of the widened mutant AER overlaid the Lmx1b-negative zone and appeared to be outlined by the dorsal and ventral Lmx1b expression boundaries.

**Development of autonomous secondary AERs in En1 mutant limbs**

The AER is a transitory embryonic structure and, by stages 6-8, most AER-specific genes, including Fgf4 and Fgf8, are down-regulated and the AER begins to flatten and regress (Jurand, 1965; Milaire, 1974; Wanek et al., 1989). In some stage 6-8 En1 mutant limbs, however, clusters of cells associated with the ventral AER rim continued to express AER marker genes when they were fading elsewhere (Fig. 3 J-L). These ectopic signalling centers were always associated with underlying mesenchymal outgrowths. As would be predicted if the signalling centers function as AERs, such mesenchymal bulges expressed genes normally restricted to cells of the progress zone that underlie the AERs, such as Evc1 (Niswander and Martin, 1993) and Jagged-1 (Fig. 3G,H and data not shown). Shh, a component of the pathway directing A-P patterning of the distal limb, was rarely detected in the ectopic outgrowths (data not shown). Although almost all En1 mutant limbs demonstrated a marked ventral expansion of the anterior half of the bifurcated AER, only 30-40% (n>40) of mutant limbs gave rise to secondary signalling centers with persistent AER-marker expression. In addition, the formation of such signalling domains occurred only in anterior regions near the widest separation between the dorsal and ventral AER rims.

By limb stages 9-11, the normal regressing AER is difficult to distinguish morphologically from the adjacent ventral and dorsal ectoderm (Wanek et al., 1989). Interestingly, analysis of β-galactosidase activity in limbs of mice carrying the En1imité allele, suggested that a remnant ridge persists along the D-V boundary (Fig. 4A,B) up to at least stage 13 (16.5 dpc) in normal embryos. Relatively intense En1 mRNA expression also delineated a D-V boundary zone up to at least stage 12 (15.5 dpc, data not shown), indicating that the persistent β-galactosidase activity did not solely reflect stability of the β-galactosidase protein.

β-galactosidase activity in En1imité mice was used to follow the evolution of the mutant ectopic AERs. As shown in Fig. 4C, the anterior ventral rim of a stage 8/9 mutant limb was shifted more distally than what was observed at earlier developmental stages, thus positioning the ventral rim in closer proximity to the dorsal rim. In some mutant limbs at this stage (n=5/16 limbs analyzed), the dorsal and ventral rims had become very closely apposed, producing a narrow linear ridge along the D-V interface, which was only slightly wider than the wild-type counterpart (Fig. 4A,B).

In other mutant limbs (n=8/16), however, a region of the ventral ridge remained associated with proximal mesenchymal outgrowths and, as a result, formed a small deflection or bow in the otherwise linear contour of the ventral rim (Fig 4D). Over the next few developmental stages, such bows were completely replaced by linear ridges (n=3/16 at stages 8-9 and n=6/12 at stages 10-12) which intersected the primary ridge at a 90° angle (Fig. 4E,F). In some cases, a proximal segment of the ventral AER rim became discontinuous with the primary rim and was at an approximately 90° angle to the primary rim (5/30, stages 8-12). These more proximal, autonomous ridges always overlaid elongated outgrowths, which resembled developing digits (Fig 4G,H,K). Intermediate stages could...
occasionally be detected in which such autonomous En1-lacZ-positive ridges could be shown to be indirectly connected to the primary ridge through a ventral mesenchymal thickening (Fig. 4H).

**Lmx1b expression boundaries are associated with ectopic secondary AERs**

Lmx1b limb expression, like En1, persisted long after most AER-specific marker genes and Wnt7a expression had faded. By stage 9, Lmx1b expression was closely associated with the developing dorsal skeletal elements of the foot and had cleared from the interdigital webs of the dorsal domains of both wild-type and mutant limbs (Fig. 4I).

The ventral Lmx1b expression pattern in En1 mutant limb buds at these later stages demonstrated several striking features. First, the pattern of interdigital web clearing of Lmx1b was more irregular on the ventral limb than on the dorsal limb. Second, Lmx1b expression was not maintained in the posterior ventral footplate (Fig. 4J). This observation was consistent with our earlier observation that the posterior-most digits of En1 mutant limbs were less dorsalized than the more anterior digits (Loomis et al., 1996). Second, there was dramatic clearing of Lmx1b expression below the ectopic AERs (Fig. 4I). This was similar to the striking Lmx1b-negative footprint produced by the widened primary AER at earlier stages in the En1 mutant limbs. Finally, the ectopic ventral outgrowths expressed Lmx1b on both sides of the Lmx1b-negative zone beneath the ectopic AERs, suggesting they had a double-dorsal identity (Fig. 4I). Interestingly, the two Lmx1b-positive dorsal domains were on the anterior and posterior sides of the ectopic digit relative to the rest of the limb, reflecting the 90° rotation of the ectopic ridge. In rare En1 homozygotes that survive past birth, it was clear that morphologically, the well-differentiated ventral digits had cylindrical nails similar to the En1 double-dorsal digits (Fig. 4L).

**Wnt7a is required for ectopic ventral digit formation in En1 mutants**

Our expression studies implicated the ectopic expression of Wnt7a and Lmx1b in the formation of ectopic AERs and digit formation. To further investigate this association, En1/Wnt7a double homozygous mutants were constructed by interbreeding En1 and Wnt7a heterozygotes (Wurst et al., 1994; Hanks et al., 1995; Parr and McMahon, 1995; Matise and Joyner, 1997). In En1/Wnt7a double mutants, as in the Wnt7a mutants, the AERs were found to be slightly (1.5-2 fold) wider than wild-type AERs (Fig. 5A,C,D), and staining for En1-lacZ expression suggested that the dorsal, rather than the ventral, portion of the AER was primarily expanded in these mutants (data not shown). None of the stage 3/4 En1/Wnt7a double mutant AERs (n=12) displayed the dramatic ventral expansion characteristic of En1 mutant AERs (Fig. 5B). Also, none of the AERs of stage 5-8 En1/Wnt7a double mutants developed a second ventral rim or appeared bifurcated (n=16). This is in contrast to the >90% of En1 mutant limbs which display these features. Finally, none of the stage 7-10 En1/Wnt7a double-mutant embryonic limbs displayed ventral mesenchymal outgrowths associated with functional ectopic AERs (n=8) nor did postnatal double mutants have ectopic ventral digits (n=30). Thus, ectopic Wnt7a signalling in the ventral ectoderm appears to be essential for the delay in compaction of the ventral En1 mutant AER and for subsequent ectopic AER and digit formation.

The embryonic and postnatal D-V patterning defects require Wnt7a expression. (A-D) Whole-mount RNA in situ hybridizations of stage 4 wild-type (A), En1 mutant (B), Wnt7a mutant (C) and En1/Wnt7a double-mutant (D) limb buds probed with Fgf8 (purple). Some of the embryos were also stained for En1-lacZ (pink). Note, that the Wnt7a and En1/Wnt7a mutant AERs are slightly wider than the wild-type AER, but much narrower than the En1 mutant AER. Whole-mount dorsal view (E-G) and high power ventral view (H-J) of young adult wild-type (E,H), En1 mutant (I), Wnt7a mutant (F) and En1/Wnt7a double-mutant (G,J) paws. The dorsal surface of a wild-type paw is covered with hair, but lacks dermal pads (E). In contrast, the dorsal surface of Wnt7a and En1/Wnt7a double-mutant paws (F,G) show variable loss of hair follicles distally and develop ectopic pigmented pads, which grow hard, nail-like structures (arrowheads). In contrast to the ectopic dorsal pads, wild-type ventral pads (H) are soft, compressible bulges with a non-shiny epidermal surface which lacks pigmentation except at the opening of sweat gland ducts. The ventral pads as well as the transverse digit ridges of En1 mutant (I) and En1/Wnt7a (J) double-mutant paws, however, are firm, shiny bulges with marked pigmentation throughout the surface ectoderm. As described previously (Loomis et al., 1997) these go on to form nail-like structures in older mice similar to the ectopic dorsal pads described above. Bar: 500 μm.

Postnatally, the proximal paws combined the phenotypic characteristics of both the Wnt7a and En1 single mutants, whereas the digit tips resembled those of the Wnt7a mutants. The nails of the double mutants, like those of the Wnt7a mutants, were shortened cones which were overgrown by...
et al., 1995; Vogel et al., 1995), the dorsalizing gene observed that, in mouse, unlike that reported for chick (Riddle to form a morphologically visible ridge. Second, we have restricted to the tip, and this change in the expression domain of ectoderm covering the ventral limb and then to become

AER marker genes was found to be induced in a broad region important in these processes. First, our results reinforce and

En1 appears to coincide with compaction of the ventral ectoderm and then becomes dorsally restricted just prior to AER formation. In Wnt7a mutants, we observed that this early expression of Lmx1b is independent of Wnt7a. Third, our mutant analysis indicates that En1 is required during AER formation specifically for maturation of the ventral AER, where it is normally expressed. In En1 mutants, the dorsal AER appears to form normally, whereas the ventral AER does not compact resulting in an abnormally broadened AER in the ventroproximal dimension. The resulting expanded AER later bifurcates and, in some cases, forms ectopic AERs that can promote digit formation ventrally. Finally, our analysis of Wnt7a/En1 double-mutant limb buds demonstrates that Wnt7a is required for formation of ectopic AERs in En1 mutants. We suggest that this is because ectopic ventral Wnt7a and/or Lmx1b expression in En1 mutants induces ventral AER cells to take on dorsal characteristics and undergo a second compaction process ventrally.

Role of ectodermal morphogenetic movements in early AER formation
The data to date do not distinguish between whether the initial induction of the AER is coincident with formation of a morphologically thickened ridge or begins at an earlier stage. Milaire (1974) proposed that many of the important morphogenetic functions of the definitive AER are present in the ventral ectoderm of the pre-AER limb bud, based on histochemical and morphological studies of early limb buds. He suggested that mammalian AER development involves progressive thickening of the ventral ectoderm and then a convergence of these cells to the limb margin. Consistent with this, recent chick-quail chimera studies and cell-labelling experiments in chick indicate that a lineage relationship exists between cells that initially overlie a broad region of presumptive limb mesoderm and cells that later reside along the ventrodorsal surface of the pre-AER limb bud and finally localize to the mature AER (Altabef et al., 1997; Michaud et al., 1997).

Our gene expression studies and morphological data provide further evidence that early morphogenetic movements are critical for normal mouse AER development (Fig. 6A). We showed that, prior to formation of a morphologically distinct AER, the AER marker genes Dlx2 and Fgf8 are expressed in a broad region of ventral ectoderm, consistent with prior observations (Lyons et al., 1990; Bulfone et al., 1993; Parr et al., 1993; Crossley and Martin, 1995; Morasso et al., 1995). We went on to further show that the ventro-proximal border of this expression domain shifts distally with increasing limb maturity and that this shift correlates temporally and spatially with the progressive morphological thickening of the ventrodorsal limb ectoderm observed by Milaire (1974) (also see Fig. 1). Additionally, the En1 expression domain initially occupies only the proximal region of the early ventral limb but later extends to the distal margin. It is not clear from our studies if all the Dlx2/Fgf8-expressing cells in the ventral ectoderm of the pre-AER limb bud ultimately come to reside in the definitive AER. Indeed, recent cell-labelling studies in chick suggest that this might not be the case (Altabef et al., 1997). Nevertheless the cumulative molecular and morphological data indicate that multiple waves of ectodermal morphogenetic movements are probably critical for normal mouse AER development (Fig. 6A).

Wnt7a and En1 are required only for late regulation of Lmx1b
D-V patterning in the early limb involves complex interactions between mesoderm and ectoderm. Tissue rotation experiments in chick suggested that D-V polarity of the early limb field is initially specified by mesoderm and, just prior to limb outgrowth, control is transferred to the overlying ectoderm (Geduspan and MacCabe, 1989). Given these results, we were somewhat surprised to find that expression of the dorsalizing gene Lmx1b is not restricted to early dorsal limb mesenchyme, but expression extends into the ventral mesenchyme. However, by stage 2, Lmx1b expression has receded from the ventral mesenchyme, resulting in distinct dorsal and ventral molecular identities of the limb mesoderm.

Analysis of En1 and Wnt7a mutants demonstrated that late, but not early, Lmx1b regulation in mouse is dependent on En1 and Wnt7a. In pre-AER En1 mutant limbs, the lack of En1 expression and persistent Wnt7a expression ventrally has little apparent effect on initiation of Lmx1b expression and its subsequent dorsal restriction. However, ventral reexpression of Lmx1b does occur in En1 mutants, at stages 5-6, and this is

DISCUSSION
Detailed analysis of D-V patterning and AER formation in wild-type and En1 and Wnt7a mutant limbs has provided further insights into the genetic and cellular pathways important in these processes. First, our results reinforce previous histological studies suggesting that mouse AER development occurs in multiple stages. Expression of early AER marker genes was found to be induced in a broad region of ectoderm covering the ventral limb and then to become restricted to the tip, and this change in the expression domain appears to coincide with compaction of the ventral ectoderm to form a morphologically visible ridge. Second, we have observed that, in mouse, unlike that reported for chick (Riddle et al., 1995; Vogel et al., 1995), the dorsalizing gene Lmx1b is initially expressed in both dorsal and ventral limb mesenchyme and then becomes dorsally restricted just prior to AER formation. In Wnt7a mutants, we observed that this early expression of Lmx1b is independent of Wnt7a. Third, our mutant analysis indicates that En1 is required during AER formation specifically for maturation of the ventral AER, where it is normally expressed. In En1 mutants, the dorsal AER appears to form normally, whereas the ventral AER does not compact resulting in an abnormally broadened AER in the ventroproximal dimension. The resulting expanded AER later bifurcates and, in some cases, forms ectopic AERs that can promote digit formation ventrally. Finally, our analysis of Wnt7a/En1 double-mutant limb buds demonstrates that Wnt7a is required for formation of ectopic AERs in En1 mutants. We suggest that this is because ectopic ventral Wnt7a and/or Lmx1b expression in En1 mutants induces ventral AER cells to take on dorsal characteristics and undergo a second compaction process ventrally.

Role of ectodermal morphogenetic movements in early AER formation
The data to date do not distinguish between whether the initial induction of the AER is coincident with formation of a morphologically thickened ridge or begins at an earlier stage. Milaire (1974) proposed that many of the important morphogenetic functions of the definitive AER are present in the ventral ectoderm of the pre-AER limb bud, based on histochemical and morphological studies of early limb buds. He suggested that mammalian AER development involves progressive thickening of the ventral ectoderm and then a convergence of these cells to the limb margin. Consistent with this, recent chick-quail chimera studies and cell-labelling experiments in chick indicate that a lineage relationship exists between cells that initially overlie a broad region of presumptive limb mesoderm and cells that later reside along the ventrodorsal surface of the pre-AER limb bud and finally localize to the mature AER (Altabef et al., 1997; Michaud et al., 1997).

Our gene expression studies and morphological data provide further evidence that early morphogenetic movements are critical for normal mouse AER development (Fig. 6A). We showed that, prior to formation of a morphologically distinct AER, the AER marker genes Dlx2 and Fgf8 are expressed in a broad region of ventral ectoderm, consistent with prior observations (Lyons et al., 1990; Bulfone et al., 1993; Parr et al., 1993; Crossley and Martin, 1995; Morasso et al., 1995). We went on to further show that the ventro-proximal border of this expression domain shifts distally with increasing limb maturity and that this shift correlates temporally and spatially with the progressive morphological thickening of the ventrodorsal limb ectoderm observed by Milaire (1974) (also see Fig. 1). Additionally, the En1 expression domain initially occupies only the proximal region of the early ventral limb but later extends to the distal margin. It is not clear from our studies if all the Dlx2/Fgf8-expressing cells in the ventral ectoderm of the pre-AER limb bud ultimately come to reside in the definitive AER. Indeed, recent cell-labelling studies in chick suggest that this might not be the case (Altabef et al., 1997). Nevertheless the cumulative molecular and morphological data indicate that multiple waves of ectodermal morphogenetic movements are probably critical for normal mouse AER development (Fig. 6A).

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Ectopic AER development in En1 mouse mutants

dependent on Wnt7a expression as it does not occur in En1/Wnt7a double mutants. Furthermore, in Wnt7a mutant limbs, Lmx1b expression is normal until stages 5-6, when it is lost dorsally, but only in distal regions, consistent with a study in chick demonstrating selective distal loss of Lmx1b expression after removal of the Wnt7a expressing dorsal ectoderm (Riddle et al., 1995).

**Loss of En1 leads to aberrant ventral AER maturation and the formation of ectopic AERs**

Our mutant studies have demonstrated that En1 is not required for the process of AER induction or the initial stages of AER formation. Loss of En1 function has no apparent effect on the early thickening of the pre-AER ventral ectoderm (stage 0.5-1) or on the initial induction of AER marker genes, such as Dlx2 and Fgf8. Furthermore, at stage 3, the cells of the dorsal AER of En1 mutant limbs, like those of wild-type limbs, do not express Wnt7a and they stratify and initiate Fgf4 expression, an AER differentiation marker (Niswander et al., 1994; Chan et al., 1995; Haramis et al., 1995).

In contrast, differentiation of the ventral portion of the AER, where En1 is normally expressed, appears to be delayed and abnormal in En1 mutants. At the time when a mature AER is apparent in wild-type limbs, the ventral portion of the En1 mutant AER remains extended with the anterior region being much broader than the posterior. This difference in the En1 mutant AER phenotype along the A-P axis likely reflects an exaggeration of the inherent A-P asymmetry characteristic of

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**Fig. 6.** Schematic drawing illustrating a model for the morphogenetic movements important in mouse AER development. Based on our results, prior histochemical studies in mouse and lineage data in chick (Milaire, 1974; Michaud et al., 1997; Altobef et al., 1997), we propose that cells which give rise to the mouse AER initially overlie much of the presumptive limb mesoderm and that sequential, convergent morphogenetic movements are required for normal ridge formation (A, left). A first wave of lateral morphogenetic movements results in the compaction of the AER precursor cells onto the ventral surface of the early limb bud and early ventral ectodermal thickening. A second wave compresses this domain to the distal 1/3 of the ventral limb, and a final wave constricts the cells into the densely packed AER. In En1 mutants (A, right), the first morphogenetic waves occur normally. The final wave, however, is markedly inhibited and a secondary compaction process is initiated at the ventroproximal border of the widened mutant AER (A, bottom right). We further suggest that this final wave of ectodermal movements resembles the closing of a zipper (B, ventral view of limb). This process initiates posteriorly and proceeds anteriorly, bringing the ventral domain of the wild-type AER into close proximity to the dorsal domain, which is anchored at the D-V interface (top). In En1 mutant limbs (middle and bottom), the anatomically ventral AER cells take on dorsal characteristics, causing them to become partially anchored and to recruit cells into an ectopic proximoventral rim. The two rims of the bifurcated mutant AER ultimately converge, although in some cases the ventral rim also begins to zip up on itself (middle). If this self-zipping begins at late stages of limb development, the ectopic secondary AER remains contiguous with the primary AER and intersects the latter at a 90° angle (middle, far right); if it begins earlier, the ectopic secondary AER becomes discontinuous with the primary AER and goes on to promote the outgrowth of distinct ventral digits (bottom).
normal ridge constriction, which begins posteriorly and proceeds anteriorly (see Fig. 2G; Milaire, 1974). The ventral AER cells of stage 3/4 En1 mutants remain relatively flat and, while they express Dlx2 and Fgf8, they do not express Fgf4 (Fig. 2L) and do not repress expression of Wnt7a until late (Loomis et al., 1996 and Fig. 2B,F). Nevertheless, the ventral AERs of En1 mutants appear to have some characteristics of a functional AER, since the underlying mesenchyme proliferates. In fact, the expanded En1 mutant ventral AER at these stages appears to secrete more growth factors than normal, since the anterior ventrodorsal mesenchyme is visibly wider than normal and it expresses genes usually restricted to AER-associated distal mesenchyme, such as Msx2 (data not shown).

Roughly coincident with the up-regulation of the dorsalizing gene Lmx1b in the ventral mesenchyme of stage 5/6 En1 mutant limbs, the cells along the ventral border of the expanded AER begin to thicken (Fig. 6A) and occasionally initiate Fgf4 expression. Morphologically, the AER of En1 mutants appears bifurcated with the two rims being markedly further apart anteriorly than posteriorly. In some cases (~30%), independent ectopic AERs form, which display prolonged expression of AER marker genes and go on to promote either independent digit outgrowths or partial distal digit duplications on the ventral surface of En1 mutants limbs.

Based on these observations, we propose a model that likens the final phase of AER constriction to the closing of a zipper (Fig. 6B). In wild-type limb bud形态s, the two halves of the zipper are the dorsal and ventral AER domains. The zipper occurs between stages 1 and 3 with the dorsal half remaining relatively fixed and the ventral half being pulled towards it in a posterior-to-anterior direction. In En1 mutants, differentiation of the ventral half of the AER is markedly delayed and the cells eventually acquire characteristics of dorsal AER cells. Although a zipper process occurs in the normal posterior-to-anterior direction in En1 mutants, the cells in the middle of the broadened AER are pulled toward both the dorsal and ventral AER borders due to the transformation of the ventral half of the AER into a functionally ‘dorsal’ AER. In some cases, distinct secondary AERs form, at right angles to the normal AER axis, due to self-zipping of the ectopic rim (Figs 2B, 4D-H, 6B).

Role of Wnt7a in ectopic AER formation in En1 mutants

The finding that, in En1/Wnt7a double mutants, bifurcated AERs do not form, nor do later ectopic AERs or ventral digits, demonstrates that Wnt7a is required for initiation of abnormal secondary AER formation in En1 mutants. In addition, the finding supports our suggestion that the early abnormal AER structures seen in En1 mutants later produce ectopic ventral digits. Since the AER of En1/Wnt7a double mutants is only slightly broader than normal, this indicates that the early ventral expression of Wnt7a in En1 mutants plays a role in the delay in compaction of the ventral AER cells. This could be due to early expression of Wnt7a in the ventral AER; for example, if Wnt7a prevents necessary cell-shape changes. Alternatively, if during normal limb development Wnt7a secreted from the dorsal non-AER ectoderm is involved in specifying the adjacent AER cells to take on a dorsal phenotype then, in En1 mutants, the ectopic signalling of Wnt7a ventrally would lead to specification of the ventral AER cells as dorsal. Since one apparent characteristic of dorsal AER cells is to stay anchored and pull or recruit cells toward them, this could account for the initial delay in compaction of the En1 mutant ventral AER cells and the later formation of a bifurcated AER.

With respect to the question of the genetic mechanism by which Wnt7a expression leads to formation of secondary AERs in En1 mutants, it is interesting to note that the position of the secondary AER thickening roughly coincides with both the timing of, and the contour of, the ectopic ventral Lmx1b expression domain. Furthermore, in the En1 mutants, ectopic ventral digits and AERs never develop in the posterior autopod where ventral Lmx1b expression is not maintained (Fig. 4J). Since in En1/Wnt7a double mutants Lmx1b is not reexpressed in the ventral mesenchyme, it is possible that Lmx1b also plays a role in the stratification of the ventroproximal AER border and formation of a bifurcated AER.

In Wnt7a mutants, the dorsal AER is only slightly broader than normal, indicating that Wnt7a alone does not normally play a major role in AER formation. Since, Lmx1b is expressed in the dorsal mesenchyme of Wnt7a mutant limbs at the time of AER formation, it is possible that Lmx1b might compensate for the lack of Wnt7a expression in the dorsal ectoderm. Alternatively, or in addition, another Wnt gene might partially compensate for loss of Wnt7a dorsally. It will be interesting to determine whether the Notch pathway also regulates the later process of AER maturation and/or earlier process of AER induction.

In summary, En1 is essential for the normal maturation of the ventral AER, as well as ventral limb patterning. The proper compaction and stratification to form a mature AER requires En1. Our studies indicate that the process of forming ectopic AERs in En1 mutants does not involve a recapitulation of the entire process of AER formation, but instead involves duplication of part of the later process of AER differentiation. We suggest that this formation of a second dorsal AER domain and focal ectodermal thickening requires the juxtaposition of presumptive AER ectoderm and functionally dorsal non-AER ectoderm or mesoderm. These studies have provided a unique example of a genetic pathway impacting on both cell fate and morphogenetic movements, which combine to generate form and pattern.

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