pictures of the topoisomerase pathway. The gyrase structure shows how the G segment can be held within the head fragments before strand passage, whereas the topo II structure shows how the T segment can pass through the active site. It has been proposed that after passing through the G segment, the T segment exits from the bottom of the molecule via the 'primary' dimer interface (the 'two-gate' model^{12,13}). The structures show how this might be done: the long connecting helices can exist in two distinct conformations, and if we impose the more extended hinge conformation observed in topo II onto the gyrase structure (Fig. 2c), we see that the primary dimer interface opens far enough to allow passage of the T segment.

Methods

Data collection and structure solution. GyrA59 (residues 2 to 523 of *E. coli* GyrA) was expressed and purified as described⁴. Crystals (space group $I4_1$; $a = 119.6 \text{ \AA}$, $c = 95.0 \text{ \AA}$) diffracting to $2.8 \text{ \AA}$ resolution were obtained at 4°C from 8% PEG8K, 20 mM NaCl, 0.1 M Tris-HCl (pH 8.5) after purification by crystallization under the same conditions (see Table 1). The crystals were flash-frozen under a stream of boiled-off nitrogen using 25% ethylene glycol as cryoprotectant. Heavy-atom soak conditions were: 1 mM for 24 h (PtCl_4); 4 mM for 24 h (PIP); and 2 mM for 4 weeks (MMN). The principal heavy-atom binding sites are: Cys 194 (MMN); Met 120 (PtCl_4); Met 101, Met 178, Met 423, Met 499 and Glu 500 (PIP). Data sets were collected with an Raxis-II image plate on a Rigaku RU200HB generator or with a MAR image plate at DESY (Hamburg) and SRS (Daresbury). Data were processed with DENZO, SCALEPACK¹⁴ and the CCP package¹⁵. The initial phases, produced by MLPHARE, were refined by solvent flattening and histogram matching with DM (60% solvent). The structure was solved by rounds of model building with the graphics display program O (ref. 16) (using the model of yeast topo II as a guide) and phase recombination.

Refinement and structural comparisons. Refinement consisted of rounds of simulated annealing and grouped B -factor refinement with bulk-solvent correction in X-PLOR (version 3.8)¹⁷. The present model consists of 477 residues; no convincing density is seen for the N terminus (residues 2–29) or the following surface loops: 251–256; 307–312 and 426–429. The 'tower' domain is especially mobile (average main-chain B value is $56 \text{ \AA}^2$, compared with $27 \text{ \AA}^2$ for the AP-like domain and $34 \text{ \AA}^2$ for the tail and connecting helices), with several loops poorly defined. The stereochemistry is strongly restrained; there is one residue in a disallowed region of the Ramachandran plot; 88.5% lie in the most favourable regions. The head fragments of GyrA59 and topo II A' overlap with an r.m.s. deviation of $1.8 \text{ \AA}$ for 251 homologous $\text{C}\alpha$ atoms; the primary dimer interface overlaps with an r.m.s. deviation of $2.0 \text{ \AA}$ for 37 homologous $\text{C}\alpha$ atoms. Figures are drawn with MOLSCRIPT¹⁸, RASTER-3D¹⁹ and RENDER²⁰.

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erratum

Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb

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In Fig. 7 of this Article, the two arrows indicating signalling by *R-fng* at stage 15 were unclear; the correct figure is shown here.

Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb

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Vertebrate limb outgrowth requires a structure called the apical ectodermal ridge, formation of which follows the previous establishment of the dorsoventral limb axis. *Radical fringe* is expressed in the dorsal ectoderm before the ridge appears, and is repressed by *Engrailed-1*, which is expressed in the ventral ectoderm. Misexpression of these genes indicates that a ridge is formed wherever there is a boundary between cells expressing and not expressing *Radical fringe*. Thus, as in *Drosophila*, *Radical fringe* positions the ridge at the dorsoventral limb boundary.

Regulation of vertebrate development depends on communication between different cells and tissues. Vertebrate limbs arise as a consequence of interactions between mesodermal and ectodermal cells established very early during embryogenesis. These interactions lead to the appearance, some days later, of a limb bud composed of a ball of undifferentiated mesenchymal cells surrounded by an ectodermal jacket. Transplantation experiments have identified three major signalling centres that establish the three major axes of the developing limb: the polarizing region, a group of mesenchymal cells located at the posterior of the limb, mediates patterning along the anteroposterior axis^{1,2}; the ectoderm covering the limb 'instructs' the underlying mesoderm and thereby mediates dorsoventral patterning^{3,4}; and the apical ectodermal ridge (AER), a specialized epithelium that arises at the junction of the future dorsal and ventral ectoderm^{5,6}, integrates some of the information provided by the two other centres and in turn transmits new signals that result in limb outgrowth.

Although it is becoming clear how the anteroposterior axis is established and elaborated^{7–10}, only recently have the mechanisms through which the dorsoventral and proximodistal axes are set up begun to be unravelled. Genes known to specify dorsal cell fate include *Wnt-7a* and *Lmx-1*, which are restricted to the dorsal limb ectoderm and mesoderm, respectively^{11–14}. *En-1*, which is expressed in the ventral ectoderm, has been shown to play a role in establishing ventral cell fate¹⁵. Members of the fibroblast growth factor family, *Fgf-2*, *Fgf-4* and *Fgf-8*, play a key role during limb development as they not only mediate interactions between the mesoderm and ectoderm that allow outgrowth of the limb, but they can also induce formation of the AER and maintain limb outgrowth when the AER is removed^{16–24}.

Here we present the cloning of a chick gene, *Radical fringe* (*R-fng*), that is homologous to the *Drosophila fng* gene²⁵. We show that *R-fng* plays an important role in vertebrate limb outgrowth; in particular, it directs the formation and positioning, at the dorsoventral limb boundary, of one of the key organizer centres of vertebrate limb development, the AER. Further, we have investigated whether *R-fng* expression regulates or is regulated by the genes *Wnt-7a* and *En-1* that establish this boundary. Our results suggest that correct *R-fng* expression requires, but does not influence, the prior establishment of the dorsoventral boundary.

It has been shown previously in *Drosophila* that *fng* is required in an analogous fashion for the formation of the wing margin and hence the wing itself²⁵. The existence of evolutionary parallels

between the vertebrate AER and the *Drosophila* wing margin is supported by our finding that genes which function downstream of *fng* in *Drosophila* (*Serrate* and *Notch*^{26–30}), are also conserved and expressed in the vertebrate AER. Taken together these results suggest that some of the mechanisms leading to limb formation in insects and vertebrates have been preserved. Nevertheless, our data also indicate that not all the molecules involved have been conserved, and this raises questions as to the causes and consequences of changing a subset of players within a specific developmental program.

R-fng* is homologous to *Drosophila fng

Chick genes homologous to the *Drosophila fng* gene were cloned by screening a limb bud cDNA library with a probe complementary to the *Drosophila* gene. One of these genes shows a striking expression pattern in the limb. This clone has particularly high homology to the *Xenopus radical fringe* gene³¹, and hence we call it chick *Radical fringe* (*R-fng*; see Supplementary Information for amino-acid sequence alignment). *R-fng* encodes a protein of 372 amino acids, with a putative amino-terminal signal sequence and a tetrabasic cleavage site, suggesting that it is a secreted signalling molecule like the related *Xenopus* and *Drosophila* proteins. Weak but significant similarity³² of *fng* proteins to the *Lex1* family of biosynthetic enzymes³³ suggests that *R-fng* may function as a glycosyltransferase (see alignment in Supplementary Information). This homology indicates that *R-fng* may operate through the recognition, and perhaps modification, of carbohydrate moieties linked to either extracellular proteins or lipids.

***R-fng* is expressed in dorsal ectoderm and AER**

R-fng mRNA starts to be detected at stage 15 HH³⁴ in the presumptive dorsal-limb ectoderm (Fig. 1a, b), and is absent from the presumptive ventral ectoderm. At stages 16–18, *R-fng* transcripts are clearly restricted to the dorsal side with a higher concentration at the dorsoventral boundary and later in the AER (Fig. 1d, f). Expression remains restricted to the dorsal ectoderm and the AER until stages 22–24 when it starts to fade in the dorsal ectoderm (Fig. 1h–j), but remains in the AER until the latest stage examined (stage 27). The early expression pattern of chick *R-fng* is similar to that of its *Drosophila* homologue, which is restricted to the dorsal region of the wing imaginal disc²⁵.

In comparison with *R-fng*, expression of *Fgf-8*, a gene thought to play a role in formation of the AER^{23,24}, is not detectable at stage 15

(Fig. 1c), but appears a stage later at the dorsoventral boundary and is subsequently restricted to the AER (Fig. 1e, g).

To explore potential parallels between *Drosophila* and vertebrates, we looked for homologues of downstream genes in the *fringe* pathway (*Delta*, *Serrate* and *Notch*^{26,29,30}). Several vertebrate homologues of each gene were cloned. Of these, one *Serrate* clone and one *Notch* clone were found to be expressed in the AER. This is shown in Fig. 2, along with the expression patterns of *Fgf-8*, *Wnt-7a* and *En-1*.

R-fng regulates AER formation

The role of *R-fng* in vertebrate limb development was studied by inducing its ectopic expression in the ventral portion of the limb. About 300 limb primordia were infected with a replication-competent retroviral vector containing *R-fng*; an abnormal limb phenotype was observed in 16% of the infected embryos.

The limb-bud phenotypes were found to be quite variable, and

can be divided into four classes. In some embryos limb development was arrested after viral infection. These limbs lack a defined AER, which would account for the reduced limb outgrowth (Fig. 3c). In the second phenotype a marked deletion of distal limb tissue results in an AER divided into two or more domains (Fig. 3d, e). A third class of embryos show a phenotype with additional ventral or ventrally expanded AERs giving rise to abnormal outgrowths (Fig. 3f, g); additional or expanded AERs were never observed on the dorsal side of the limb. Finally, some infected embryos exhibited an enlarged limb bud with a lengthwise expansion of the anterior AER (Fig. 3h). All four phenotypes suggest that *R-fng* misexpression perturbs the normal formation of the AER.

When the embryos were allowed to develop further (up to 10 days), the cartilage patterns were found to correlate well with the AER perturbations seen in the early limb buds. In some embryos the limbs were found to be severely truncated, showing a very reduced

Figure 1 Spatiotemporal pattern of expression of *R-fng* and *Fgf-8* during chick limb-bud development. **a, b**, *R-fng* transcripts start to be detected at stage 15 in the presumptive dorsal (D) limb ectoderm. **c**, At the same stage (st) (15), *Fgf-8* transcripts cannot be detected. **d**, At stage 16, higher levels of *R-fng* transcripts begin to accumulate at the dorsoventral boundary, with expression restricted to the dorsal side of the boundary. **e**, *Fgf-8* transcripts appear at stage 16 in a broad ectodermal band at the dorsoventral limb bud boundary. The expression domain

includes both dorsal and ventral (V) cells. **f**, At stage 18, a sharp boundary of *R-fng* expression is seen between the dorsal and ventral limb ectoderm, in addition to expression in the dorsal ectoderm. **g**, At the same stage, *Fgf-8* expression becomes tightly restricted to the AER. **h-j**, *R-fng* expression in the dorsal side of the limb remains until stages 22–24 when it begins to fade. Expression in the AER remains until at least stage 27.

Figure 2 Expression domains of *Wnt-7a*, *En-1*, *R-fng*, *Fgf-8*, *Serrate* and *Notch*. **a**, Scanning electron micrograph of a chicken limb bud (reproduced from ref. 51 (Sinauer) with permission from K. Tosney) illustrating the morphology of the AER and the plane of the sections shown below. **b-g**, Expression domains of *Wnt-7a*, *En-1*, *R-fng*, *Fgf-8*, *Serrate* and *Notch* at stage 20. *Wnt-7a* transcripts are restricted to the dorsal ectoderm. *En-1* expression is restricted to the ventral ectoderm. *R-fng* is expressed in both the dorsal ectoderm and at higher levels in the AER. *Fgf-8* and *Notch* are specifically restricted to the AER; *Serrate* is predominantly restricted to the AER. We have confirmed that the *Notch* clone we show here is identical to that reported previously⁴³ and the *Serrate* clone is the chick homologue of the *Serrate-2* gene reported in rat⁴⁴. D, dorsal; V, ventral.

and deformed humerus or femur with no distal structures (Fig. 3k, l). In other embryos additional distal structures (most frequently extra digits) were observed (Fig. 3m–p). In most of these cases the proximal structures were normal, but in some there was an accompanying reduction of the proximal elements (Fig. 3n). The number and identity of the extra digits was variable, but most commonly digit 2 was duplicated. The extra digits were either in plane or ventral to the other digits (Fig. 3o, p). Additional structures were never observed on the dorsal side of the limb.

The most likely explanation for the variability of the observed phenotypes is that the extent of viral infection varies in the different embryos. Indeed, limb buds that exhibit arrested growth have uniform viral infection (Fig. 4a), and limb buds with expanded or additional AERs have rather patchy viral infection (Fig. 4b–e). In addition to the variability of the extent of viral infection, it is

possible that the dose is also variable, but this is not easily assessed by *in situ* hybridizations. Furthermore, it is difficult to assess the extent of viral infection at the time the phenotype is triggered as the virus will continue to spread through adjacent tissue. Although the inherent variability of viral infection is commonly considered to be a limitation of this technique, in this case the observation of different phenotypes has greatly assisted our interpretation, and seems to indicate that the phenotype is highly dependent on the pattern of *R-fng* misexpression.

To confirm that the AER-like structures formed as a result of *fng* misexpression were indeed ectopic AERs, and competent to follow normal developmental programs, we characterized the expression of several molecular markers. *Fgf-8* is normally expressed in the AER; several lines of evidence indicate that it is involved in limb initiation as well as maintaining limb outgrowth^{23,24}. Because the

Figure 3 Ectopic *R-fng* expression perturbs normal limb outgrowth. **a, b**, Wild-type limb buds at stage 25. **c–h**, Limb-bud phenotypes are shown at stages 24–25 following injection with virus expressing *R-fng* at stages 8–10. **c**, An extremely reduced wing bud. Note the pointed shape and the absence of a defined AER. **d**, A wing bud showing a marked deletion of tissue (arrow) at its distal end. **e**, A wing bud with a similar, but less severe, deletion of distal tissue leading to an apparent bifurcation of the normal AER (arrows). **f, g**, Limb buds exhibiting additional ventral AERs. The ventral nature of these outgrowths is not easy to visualize (see also **m** and **o**). **h**, Wing bud showing a significant anterior expansion of the AER (broken line). **i, j**, Wild-type, and **k–p**, *R-fng*-infected limbs 8 days after injection at stages 8–10. **k, l**, Severely reduced wing (asterisk) with deformed scapula (S), humerus (H) and coracoid (C), and absence of radius, ulna and digits; also, additional abnormal skeletal elements (&). **m**, Leg with an additional digit (IV) attached ventrally at the junction between femur and tibia. **n**, Leg with an additional digit II (II'), an unidentified structure (Λ) and a deformed digit II (#). The tibia and fibula appear shortened and deformed (\$). **o, p**, Wing with additional digit II (arrow and II') arising from the ventral side of the normal limb, although this is clearer in **o** as the embryo was flattened in **p** to facilitate photography.

Figure 4 Correlation of viral infection with phenotype. **a**, Truncated wing bud 55 h after viral infection. The uniform viral infection correlates with the severe truncation. **b–d**, Ventral views of enlarged limb buds at stages 25–26. The limbs show patchy viral infection. In **b** and **d** these patches correlate with anterior outgrowth (filled arrows). In **d** a marked ventral outgrowth (hollow arrow) also shows a region of viral infection at its tip. **e**, A section from a significantly reduced limb that has a large region of virally infected tissue on the ventral side. The hollow arrow indicates a ventral outgrowth located at a boundary of the viral infection and hence *R-fng* expression.

misexpression of *R-fng* can lead either to the disruption of the AER (hence arrested limb outgrowth) or to the formation of additional or expanded AERs (hence additional limb outgrowth), we would expect to observe perturbations in the *Fgf-8* expression pattern. *In situ* hybridization 55–60 h after viral infection shows that *Fgf-8* expression is absent in the reduced limb buds (Fig. 5a). In limb buds with additional or ventrally expanded AERs, *Fgf-8* transcripts were found to colocalize with the additional AERs (Fig. 5b–e).

The appearance of additional skeletal elements after *R-fng* misexpression would suggest that genes involved in the establishment of the anteroposterior axis are dependent on *R-fng* expression^{7,17,21,39}. Consistent with this, there are enlarged areas of expression of *hoxd-12* and *Fgf-4* in the anterior mesoderm and AER, respectively (Fig. 5f, g). In a few cases, ectopic patches of *Shh* expression were observed at the anterior margin of *R-fng*-infected limb buds (data not shown).

To determine whether *R-fng* influences dorsoventral patterning we examined the expression of *Lmx-1*, an established marker for dorsal mesoderm identity^{13,14}. Because *Lmx-1* expression is absent from both sides of the ventrally derived outgrowths, it seems that these are biventral (data not shown).

Figure 5 Misexpression of *R-fng* perturbs the normal expression patterns of *Fgf-8*, *hoxd-12* and *Fgf-4* genes. **a**, *Fgf-8* transcripts are absent from truncated limb buds that result from extensive ectopic *R-fng* expression. **b–e**, Arrows indicate ectopic ventral expression of *Fgf-8* in the additional AERs induced by *R-fng* misexpression. **b**, An ectopic stripe of *Fgf-8* on the ventral side of the *R-fng*-infected limb bud. **c**, *Fgf-8* expression in a section through a ventrally expanded AER. **d**, **e**, Whole-mount *in situ* hybridizations with *Fgf-8* expression in ectopic AERs that are either discrete (**d**) or intersect (**e**) the normal AER. **f**, *Hoxd-12* transcripts, normally restricted to the posterior-distal limb-bud mesoderm, are induced at the anterior-distal mesoderm after *R-fng* viral infection (broken line). **g**, Similarly, *Fgf-4*, restricted to the posterior region of the AER during normal limb development, is induced in the anteriorly expanded AER after *R-fng* viral infection (arrow).

Influence of *Wnt-7a* and *En-1* on *R-fng* expression

Although *R-fng* does not affect dorsoventral identity, several lines of evidence suggest that *R-fng* expression is dependent on previously established dorsoventral cues: (1) *R-fng* expression is restricted to the dorsal ectoderm and directs AER formation at the dorsoventral boundary; (2) correct AER positioning depends on correct *En-1* expression because in mice lacking *En-1*, the AER is significantly expanded on the ventral side of the limb bud¹⁵; (3) in the chick *limbless* mutant, no AER is formed, *En-1* is absent, and *Wnt-7a* is expressed uniformly in the limb bud ectoderm³⁵; and (4) in *Drosophila*, the dorsal selector gene *apterous* regulates *R-fng* expression²⁵. To determine whether either of the ectodermal genes known to specify dorsoventral identity (*Wnt-7a* and *En-1*) influences *R-fng* expression, we used the viral misexpression technology.

As previously reported¹⁴, limbs infected with virus expressing *Wnt-7a* show a bidorsal character, but in no case was any perturbation of the pattern of *R-fng* expression observed (Fig. 6a–c).

Approximately half of the embryos ($n = 58$) infected with virus expressing *En-1* show striking perturbations of limb outgrowth. In some cases a severe truncation of the limb bud was observed, the AER was not well defined, and the limbs exhibited a ‘rippled’

Figure 6 *Wnt-7a* and *En-1* misexpression. **a**, 48 h after *Wnt-7a* viral infection of the limb primordia at stage 12, ectopic patches of *Wnt-7a* are detected in the ventral ectoderm and AER as well as in proximal regions of the limb bud. **b**, Ectopic expression of *Wnt-7a* on the ventral side of the limb induces ectopic ventral expression of *Lmx-1*, but **c**, it does not perturb the expression of *R-fng*. **d–h**, Limb buds fixed 55 h after *En-1* viral infection at stages 8–10. In some cases (**d**, **e**, **h**) the limbs show a rippled or discontinuous AERs; in others (**f**, **g**) ectopic dorsal outgrowths appear (arrows). This correlates with discontinuous (**e**) or ectopic dorsal expression of *Fgf-8* (**f**, **g**). **h–j**, *En-1* misexpression represses *R-fng* in the AER and dorsal ectoderm of infected limbs. Arrows in **h** indicate discontinuities of *R-fng* expression in the AER. **i**, Embryo fixed at stage 17 after *En-1* misexpression. The injected bud (Inj.) shows a clear reduction in *R-fng* expression when compared with the contralateral one (Con.). **j**, The injected *En-1* embryo was fixed at stage 18 and hybridized simultaneously with *R-fng* and *Fgf-8* probes. Although some staining of *Fgf-8/R-fng* is still observed in the AER of the injected limb (Inj.), the dorsal ectoderm expression of *R-fng* is greatly reduced.

appearance with an apparently discontinuous AER (Fig. 6d). In other cases ectopic outgrowths or additional AERs were observed on the dorsal side of the normal limb (Fig. 6f, g). Whole-mount *in situ* hybridization at various stages using probes for *Fgf-8* indicate discontinuous expression in the rippled AERs (Fig. 6e) and ectopic expression in the additional AERs of the dorsal outgrowths (Fig. 6f, g). Hybridization using probes for *Wnt-7a*, *Lmx-1* (data not shown) and *R-fng* show that misexpression of *En-1* strikingly represses expression of these genes. Where viral infection is extensive (data not shown) this correlates with truncated limb buds that exhibit no *R-fng* expression (Fig. 6i, j). Where breaks in the normal AERs are observed, the *R-fng* expression was found to be discontinuous (Fig. 6h).

Although the phenotypes of *En-1* misexpression are varied, they are all consistent with the proposal that ectopic *En-1* expression on the dorsal side of the limb-bud represses *r-fng* expression. Thus it seems likely that a deformed structure results when *R-fng* expression is repressed in the presumptive AER, and additional ectopic AERs are induced when *En-1* misexpression causes non-uniform *R-fng* expression in the dorsal ectoderm. These data, taken together with the knockouts of *Wnt-7a* and *En-1*, strongly suggest that a correct pattern of *En-1* expression, but maybe not *Wnt-7a* expression, is necessary for the formation and positioning of the AER.

***fng* activity mediates boundary interactions**

Not all of the genes involved in the control of limb outgrowth and dorsoventral patterning have yet been identified, but we are beginning to understand how the interactions between the known genes combine to regulate limb development. Our experiments suggest that the AER develops at the interface of tissue that is expressing high levels of *R-fng* adjacent to tissue that does not express *R-fng*, that is, at a *R-fng* boundary. Because *R-fng* is not normally expressed on the ventral side of the limb, ectopic expression of *R-fng* here creates new boundaries that result in additional AERs. Expression of *En-1* on the dorsal side represses normal *R-fng* expression and can therefore create new boundaries and ectopic outgrowths. Finally, when *R-fng* is expressed uniformly on both sides of the limb, or when it is absent from both sides of the limb owing to repression by ectopic *En-1* expression, then no *R-fng* boundaries are formed, the AER does not develop, and growth is arrested. It is important to note that in the misexpression experiments the ectopic outgrowths always have a character consistent with their dorsoventral location, indicating that *R-fng* does not influence dorsoventral identity.

The observation that misexpression of *En-1* in the dorsal ectoderm leads to areas without *r-fng* transcripts indicates that *En-1* directly or indirectly represses *R-fng* expression. It is therefore possible that repression by *En-1* defines the dorsal expression domain of *R-fng* when it first appears at stage 15. This suggests that *En-1* may play a critical role in positioning the AER at the dorsoventral boundary. This is supported by the observation that in the early limb buds of the chick *limbless* mutant, *En-1* expression is absent³⁵, *R-fng* is expressed in both the dorsal and ventral ectoderm³⁶, and no AER is formed. The importance of *En-1* in positioning the AER is consistent with the finding that, in mice lacking *En-1*, the AER is mispositioned and expanded ventrally. However, that an AER is formed at all suggests that there is a ventrally displaced *R-fng* boundary, implying that other factors contribute to restricting the domain of *R-fng* expression. It will be important to examine the pattern of *R-fng* expression in mice lacking *En-1*, and also to explain why *R-fng* and *En-1* expression overlap in the ventral side of the mature AER (Fig. 2).

In *Drosophila*, gain- and loss-of-function experiments indicate that wing-margin formation and wing outgrowth occur at the interface of cells expressing *fng* and cells not expressing *fng*²⁵. This is similar to our observation in vertebrates. Together these results indicate the existence of a common signalling process mediated by *fng* in the formation of both the vertebrate AER and the insect wing margin.

We can envisage several mechanisms through which *fng* boundaries might generate an environment that could initiate AER formation. A straightforward model proposed for *Drosophila*²⁵ is that, in those cells expressing *fng*, *fng* receptors are repressed by the ligand itself. Considering that *R-fng* may be a secreted glycosyltransferase, the 'receptor' could be an extracellular carbohydrate substrate, bound to a protein or lipid, whose activity is modulated by *R-fng*. We do not yet know what this target is, but candidates include other extracellular signalling molecules and their receptors. Whatever the nature of the *R-fng* target, it seems either that only ventral cells (lacking *R-fng* expression) are able to respond or that only dorsal cells adjacent to ventral cells can respond. This could be because the *R-fng* target is absent or inactive in *R-fng*-expressing cells on the dorsal side of the limb bud, or because the target is specific to ventral cells, perhaps as a consequence of *En-1* expression. Further insight will require more experiments making use of knockout mice, viral misexpression techniques, biochemical and fate map studies.

A simplified scheme for AER formation is shown in Fig. 7. At stage 14 interaction between the mesoderm and ectoderm establishes the dorsoventral boundary in the ectoderm and the asymmetric pattern of *R-fng* expression. After the *R-fng*-mediated signalling event, cells at the dorsoventral boundary presumably respond and initiate AER formation, perhaps by further signalling to adjacent cells or by establishing new tissue by cell division. Once *R-fng* signalling is established, downstream genes are activated and drive subsequent limb outgrowth. A candidate gene active early in this pathway, or perhaps cooperating with *R-fng*, is *Fgf-8*. We have shown (Fig. 1) that transcripts for *Fgf-8* are detected slightly later than *R-fng* mRNA, just before the formation of a morphologically

Figure 7 *R-fng* signalling and AER formation. A schematic illustration of the expression patterns of genes present in the ectoderm at the dorsoventral boundary (broken line) during AER formation. At stage 14, expression of *Wnt-7a* and *En-1* is restricted to the dorsal and ventral sides of the presumptive limb ectoderm, respectively. At stage 15, *R-fng* expression appears in the dorsal side of the ectoderm and forms a *R-fng*/no-*R-fng* boundary. It is likely that *R-fng* signals (arrows) to cells immediately at the dorsoventral boundary. (Note that this model suggests that the primary role of *R-fng* in AER formation occurs before stage 17.) These cells then give rise to the AER, either as a consequence of further signalling to adjacent cells or by cell division. In the mature AER, two cell types can be distinguished characterized by *R-fng*/*Fgf-8* expression with no *En-1* or *Wnt-7a*; *R-fng*/*Fgf-8*/*En-1* expression. The layout of the figure suggests that the dorsal half of the AER derives from dorsal cells, and the ventral half from ventral cells, but this need not be the case and requires further investigation. Arrows beneath the ectoderm indicate that there is an interplay with signals from the underlying mesoderm (progress zone and polarizing region, ZPA).

distinguishable AER, in a symmetrical stripe of cells encompassing the dorsoventral ectodermal boundary. If *Fgf-8* is indeed involved at an early stage in the *fng/no-fng* effector pathway then we should expect that its misexpression would have a similar phenotype to creating ectopic *fng/no-fng* boundaries. Indeed, application of *Fgf-8* to the early limb bud of the *limbless* mutant can rescue the phenotype and give rise to limb outgrowth³⁵.

Evolutionary conservation of developmental programs

The observation that *fng* homologues in vertebrates seem to operate in essentially the same way as their counterparts in *Drosophila* raises the general question of the similarity of limb development in these distant organisms. The insect and vertebrate limbs are morphologically quite distinct, and so it is difficult to correlate particular tissues and structures in the two species. However, it is becoming clear that both limbs develop through the integration of different signals from the dorsoventral and anteroposterior axes that allow further elaboration of the proximodistal axis and outgrowth of the limb^{11,12,17,21,27,28,37}. Remarkably, some of the molecules involved in this process seem to be conserved. In particular, *hedgehog*, *patched* and *decapentaplegic* in *Drosophila* and their counterparts in vertebrates (*Sonic hedgehog*, *patched* and the *Bone morphogenetic* proteins) establish the anteroposterior axis in both species^{21,38–41}. In contrast to the similarities along the anteroposterior limb axis in insects and vertebrates, conservation of the molecules that govern dorsoventral identity is less apparent. *Lmx-1* has been suggested to be a vertebrate counterpart of *apterous*, but here we show that, unlike *apterous*, it is not involved in *R-fng* expression. *Wnt-7a* does not regulate *R-fng* expression, and does not seem to have a functional counterpart in the dorsal compartment of the *Drosophila* wing, and the homologue of *En-1* in *Drosophila* is involved in establishing the anteroposterior axis⁴². However, with the characterization of chick *fng*, we have shown that a key player in the transduction of dorsoventral signalling to proximodistal signalling is highly conserved between insects and vertebrates. Because chick homologues of the genes *Serrate* and *Notch*^{43,44} are also expressed in the AER, it seems that in general, the genes responsible for transducing the dorsoventral information at the boundary, and hence establishing the proximodistal pathway, are conserved, although functional equivalence has yet to be demonstrated. Interestingly, counterparts of other genes known to be involved in the formation of AER and wing margin (the *Fgf* genes in vertebrates and *vestigial* in *Drosophila*) have not yet been identified.

The apparent conservation of *R-fng* and downstream molecules in the proximodistal pathway suggests that these comprise a conserved developmental unit, or syntagma⁴⁵. Some of the genes that direct the formation of the anteroposterior axis form a similarly conserved syntagma. The conservation of these two developmental subroutines highlights the apparent lack of conservation in the molecules that establish the dorsoventral axis. It will be important to establish whether these apparent differences are real differences responsible for establishing evolutionary diversity between insect and vertebrate limbs, or whether homologous counterparts will be discovered. In conclusion, our results suggest that, in making a limb, a similar combination of genetic building blocks or syntagmata have been retained in flies and vertebrates, although the identity of certain 'selector' genes may have changed. These changes may in turn subtly alter the interaction between the different syntagmata accounting for differences in the morphology of insect and vertebrate limbs. Nevertheless, the basic functional homologies, such as the vertebrate AER and the *Drosophila* wing margin, have been preserved. □

Methods

Identification of *R-fng*. The probe used for screening covered the entire open reading frame of *Drosophila fng* and was provided by K. Irvine and E. Wieschaus. Approximately 2×10^6 phage from a chick limb cDNA library

(stages 20–23; ref. 34) were screened under low-stringency conditions⁴⁶. Three of these were found to encode *R-fng*. Several related clones were also obtained, and will be published elsewhere. Positive clones were excised and sequenced according to manufacturer's instructions (Stratagene, Qiagen and USB).

In situ hybridization. Whole-mount and sectional *in situ* hybridizations using digoxigenin and ³⁵S-labelled probes were performed as described previously⁴⁷. The *r-fng* digoxigenin and ³⁵S-labelled antisense probes span the entire open reading frame. The *Serrate-2* and *Notch* antisense probes span the intracellular domain. The details of the remaining probes have been reported elsewhere: virus (ref. 21); *Fgf-8* (ref. 24); *Hoxd-12* (ref. 48); *Fgf-4* (ref. 16); *Lmx-1* and *Wnt-7a* (ref. 14).

Retroviral infection. Chicken embryos (either MacIntyre Poultry (San Diego, CA) or SPAFAS (Norwich, CT)) were infected with virus in the wing or leg primordia at stages 8–10 as previously described⁴⁹. The replication-competent retroviral vector used, RCAS(BP)A⁵⁰, contained cDNAs spanning the entire coding region of the *Wnt-7a* (provided by A. MacMahon and L. Niswander), *R-fng*, or *En-1* genes, respectively. Virus preparation was carried out as described previously¹⁴. After different incubation time points, embryos were fixed in 4% paraformaldehyde overnight and dehydrated in methanol. After evaluation under a dissecting microscope for possible alterations, embryos were stored at –20 °C until processed for *in situ* hybridization. Visualization of cartilage structure was as previously described¹⁴.

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Expression of *Radical fringe* in limb-bud ectoderm regulates apical ectodermal ridge formation

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The apical ectodermal ridge of the vertebrate limb bud lies at the junction of the dorsal and ventral ectoderm and directs patterning of the growing limb. Its formation is directed by the boundary between cells that do and cells that do not express the gene *Radical fringe*. This is similar to the establishment of the margin cells at the *Drosophila* wing dorsoventral border by *fringe*. *Radical fringe* expression in chick-limb dorsal ectoderm is established in part through repression by *Engrailed-1* in the ventral ectoderm.

Inductive signals from discrete signalling centres regulate much of embryonic development. The apical ectodermal ridge (AER) is an inductive centre that runs anteroposteriorly along the distal margin of the limb-bud ectoderm, and has several distinct roles during limb ontogeny¹. It maintains developmental plasticity within the underlying limb mesenchyme; maintains expression of the signalling molecule *Sonic hedgehog* in the posterior mesenchyme; synergizes with other factors to pattern the limb mesenchyme; and controls the anteroposterior width of the developing limb bud. The signals produced by the AER that mediate each of these activities are members of the fibroblast growth factor family, notably *Fgf-2*, *Fgf-4* and *Fgf-8*.

The correct spatial and temporal control of the formation of signalling centres such as the AER is critical for normal embryogenesis. However, as with most embryonic signalling centres, its induction is only partly understood. Expression of molecular markers, notably *Fgf-8*, indicates that AER induction begins by stage 16 (refs 2–4). Tissue grafting experiments have shown that

signals from the limb mesoderm induce AER formation in the overlying ectoderm^{5–7}, and that the capacities of both the mesenchyme to signal and the ectoderm to respond diminish over time, being lost from both tissues by stage 20 (ref. 8). Although mesodermal signals trigger AER formation, the information specifying both the position and number of AERs is encoded within the ectoderm^{9,10}.

The AER is positioned at the junction of the dorsal and ventral limb ectoderm. Several genes are expressed in dorsoventral restricted domains within the limb bud before AER formation, and are therefore candidates for regulating AER formation. *Wnt-7a* and *Lmx-1* are expressed in dorsal ectoderm and mesoderm, respectively^{11–13}. They specify dorsal-limb mesodermal fates, but do not seem to be directly involved in AER formation, as neither loss of *Wnt-7a* function¹⁴ nor gain of *Wnt-7a* or *Lmx-1* function^{12,13} alters normal AER formation. *Engrailed-1* (*En-1*) is a ventral limb ectodermal marker that is also expressed in the ventral half of the AER¹⁵. It has been directly implicated in regulating AER formation