Int. J. Dev. Biol. 41: 559-568 (1997)

Original Article

Expression of the transcription factor *slug* correlates with growth of the limb bud and is regulated by FGF-4 and retinoic acid

PAUL G. BUXTON[1], KONSTADINA KOSTAKOPOULOU[2,3], PAUL BRICKELL[2], PETER THOROGOOD[1] and PATRIZIA FERRETTI[1]*

[1]*Developmental Biology Unit,* [2]*Molecular Haematology Unit, Institute of Child Health (University College London), London and* [3]*Department of Anatomy and Developmental Biology, University College London, London, United Kingdom*

ABSTRACT The *slug* gene encodes a zinc finger transcription factor expressed by neural crest cells (Nieto *et al., Science 264:* 835-839, 1994) and by certain non-crest derived mesenchymal cell populations, such as lateral mesoderm and sclerotome (Mayor *et al., Development 121:* 767-777, 1995; Buxton *et al., Dev. Biol. 183:* 150-165, 1997). We report here that *slug* is also expressed in developing chick limbs. The *slug* expression domain in the limb bud expands from posterior to anterior and marks cells that are predominantly destined to become chondrocytes but have not yet differentiated. Its expression is maintained in connective tissue, but is never observed in the premuscle masses. We show that removal of the apical ectodermal ridge results in loss of *slug* expression which can be arrested by the addition of an FGF-4 bead. Retinoic acid bead implants lead to down-regulation of *slug* expression, again accompanied by abolition of limb outgrowth. Dual bead implants demonstrate antagonism between these two factors, suggesting that a localized antagonistic effect between endogenous RA and FGF-4 on *slug* expression underlies the molecular mechanism controlling the transition between undifferentiated and differentiated state during normal limb development. The fact that *slug* expression pattern correlates with areas of growth in the limb, and is maintained by FGF-4 and down-regulated by retinoic acid, indicates that *slug*-expressing cells play a crucial role in growth and patterning of the chick limb. We propose that *slug* expression provides the best correlation to date between a molecular marker and the physical concept of the progress zone, defined as "a labile region where new positional values are successively engendered in the course of growth" (Summerbell *et al., Nature 244:* 492-496, 1973).

KEY WORDS: *slug, limb, progress zone, FGF-4, retinoic acid, development*

Introduction

Slug encodes a zinc finger transcription factor expressed in the dorsal neural tube by cells that will form the neural crest. These cells continue to express *slug* as they adopt a mesenchymal morphology and migrate from the neural tube (Nieto *et al.*, 1994; Buxton *et al.*, 1997). When *slug* expression in the neural tube is knocked out using antisense oligonucleotides, neural crest emigration is inhibited (Nieto *et al.*, 1994). Furthermore, *slug* is re-expressed by regenerated crest cells following ablation of the dorsal hindbrain of the chick embryo (Sechrist *et al.*, 1995; Buxton *et al.*, 1997). These observations suggest that *slug* activity may determine important properties of crest cells, such as adoption of mesenchymal phenotype and migratory behavior. In the course of our studies on *slug* expression in the neural crest, we observed that *slug* is also expressed in the early limb bud, and became interested in establishing whether this gene may be part of the early cascade of events determining limb pattern.

The cell interactions involved in limb patterning have been defined by classical embryological analysis (Wolpert *et al.*, 1975; Saunders, 1977), and recently some of the molecular players in these interactions have been identified (Tickle and Eichele, 1994; Cohn and Tickle, 1996). The emerging limb bud consists of a mass of mesenchymal cells covered by an epithelium which will thicken at the tip of the bud along the anteroposterior axis to form the apical ectodermal ridge (AER), which is initially most prominent posteriorly (Hinchliffe and Johnson, 1980). The zone of mesenchyme immediately below the AER, which is called the progress zone and is

Abbreviations used in this paper: AER, apical ectodermal ridge; FGF, fibroblast growth factor, RA, retinoic acid; *shh, sonic hedgehog*; ZPA, zone of polarizing activity.

***Address for reprints:** Developmental Biology Unit, Institute of Child Health (University College London), 30 Guilford Street, London WC1N 1EH, United Kingdom. FAX: 171-8314366. e-mail: ferretti@ich.ucl.ac.uk

0214-6282/97/$05.00

Printed in Spain

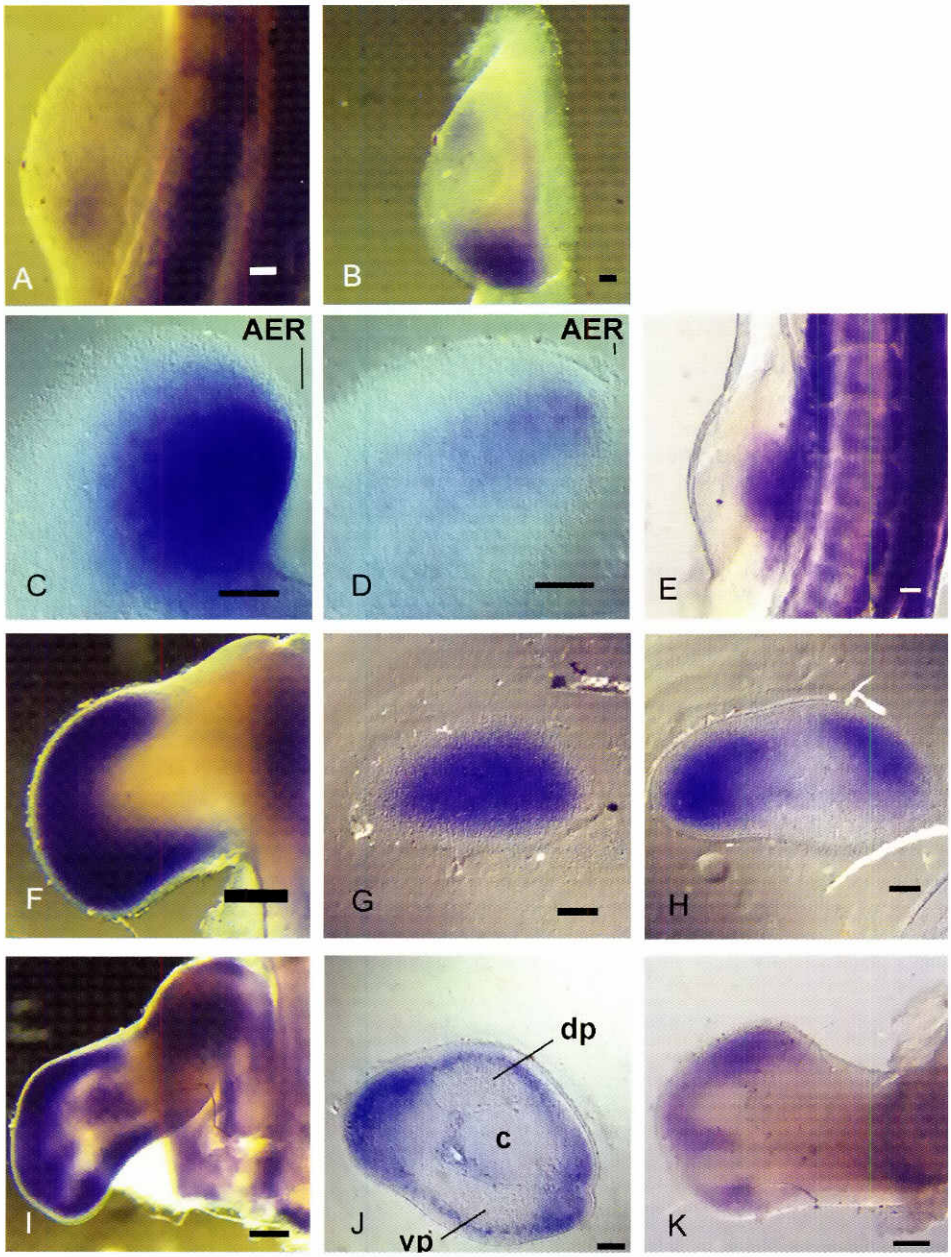

Fig. 1. Normal expression of *slug* (A-D and F-K) and *Pax-3* (E) in the chick forelimb detected by whole-mount *in situ* hybridization. *Distal is to the left and anterior is up in (A,B,E,F,I,K); anterior is to the left and dorsal is up in sections of whole-mount preparations (G,H,J).* **(A)** *In stage 20 limb bud the slug transcript is localized to the posterior region of the mesenchyme.* **(B)** *In slightly older limb (stage 21) the slug domain is expanding as the bud grows out. From coronal sectioning the anterior domain present appears to be continuous with the posterior.* **(C)** *In a section through stage 21 limb bud posterior slug expression is obvious beneath the AER, although a gap of a few cell diameters is present.* **(D)** *In a more anterior section through the same limb bud as (C) it is apparent that slug expression is much lower; note that the AER is still visible.* **(E)** *In stage 20 limb bud Pax-3 expression is observed in the muscle progenitors immigrating from the lateral dermamyotome and is most evident dorsally and medially.* **(F)** *In stage 24 limb bud slug expression has expanded anteriorly and continues to underlie the AER.* **(G)** *In a transverse section through the tip of such a limb, all cells except those immediately below the ectoderm are slug positive.* **(H)** *A more proximal section from the same series shows the down-regulation of slug apparent at the time of pre-chondrogenic condensation; note that slug transcripts are also absent from the premuscle masses located dorsally and ventrally.* **(I)** *In stage 27 forelimb, slug expression is in connective tissue and undifferentiated cells anteriorly and posteriorly, as evident from the transverse section in* **(J)**. **(K)** *In stage 29 limb slug expression is most apparent in the interdigital zones and at the distal tips of the digits. AER, apical ectodermal ridge; dp, dorsal premuscle mass; vp, ventral premuscle mass; c, chondrogenic core. Bars: 100 μm in A,B,C,D,E,G,H,J; 330 μm in F,I,K.*

maintained by signals from the AER, consists of rapidly proliferating, undifferentiated cells (Summerbell *et al.*, 1973). The interaction between apical ridge and mesenchyme is reciprocal, in that the AER is needed to establish a progress zone and the progress zone is needed to maintain the ridge. In addition, maintenance of the progress zone requires a polarizing signal (Niswander *et al.*, 1993) which originates from a group of cells with patterning properties, the zone of polarizing activity (ZPA), located at the posterior margin of the bud (Saunders and Gasseling, 1968). This activity in turn depends on the presence of the ridge, since its removal results in loss of polarizing activity (Vogel and Tickle, 1993). The fine balance of these cell interactions governs growth and patterning of developing limb buds.

A number of molecules which can mimic properties of the AER, such as FGF-2, FGF-4 and FGF-8 (Niswander *et al.*, 1993; Fallon *et al.*, 1994; Cohn *et al.*, 1995; Mahmood *et al.*, 1995; Crossley *et al.*, 1996; Vogel *et al.*, 1996) and of the polarizing region, such as retinoic acid (all-*trans*, RA) and *sonic hedgehog (shh)* have been isolated (Riddle *et al.*, 1993), and the existence of feedback loops involving these molecules proposed. In fact, digit duplications can be produced not only by grafting a ZPA, but also applying either RA or shh to the anterior margin of the limb bud (Niswander *et al.*, 1993; Riddle *et al.*, 1993; Laufer *et al.*, 1994). In addition, RA application to the anterior margin of the bud activates *fgf-4* expression, and it has been suggested that RA and FGF-4 might both be needed to induce *shh*, which would then feed-back to maintain *fgf-4* expres-

sion linking outgrowth and patterning (Laufer *et al.*, 1994; Niswander *et al.*, 1994; Tabin, 1995). It seems currently likely that RA is an endogenous signal in limb patterning (Helms *et al.*, 1996; Stratford *et al.*, 1996). It should also be noted that RA, besides playing a role in patterning, can affect differentiation and/or proliferation of a variety of cell types through its different nuclear receptors (Schilthuis *et al.*, 1993; Mangelsdorf *et al.*, 1994; Pecorino *et al.*, 1994, 1996).

We show here that *slug* is expressed in the undifferentiated loose mesenchyme of the limb, both below the AER and also in more proximal regions where it marks the connective tissue cells. It is not expressed in the prechondrogenic cores or in the premuscle masses. We have manipulated the limb bud by ridge removal and by application of FGF-4 and RA, either alone or in combination. We show that *slug* expression is maintained by FGF-4 but down-regulated by RA, indicating that *slug*-expressing cells play a crucial role in growth and patterning of the chick limb. Furthermore, from our results it is apparent that *slug* expression provides the best correlation to date between a molecular marker and the physical concept of the progress zone, defined as "a labile region where new positional values are successively engendered in the course of growth" (Summerbell *et al.*, 1973).

Results

Correlation of slug expression with undifferentiated cell types in the chick limb

We analyzed the expression of *slug* in the limb between stages 19-29 and found a unique expression pattern. Expression was first found in the posterior mesenchyme of the limb bud at stage 20 (Fig. 1A). By stage 21, expression is also present anteriorly (Fig. 1B). Sections taken through the limb at this stage demonstrated that expression was more intense posteriorly than anteriorly (Fig. 1C) and that the anterior expression domain (Fig. 1D) is continuous with the posterior (not shown). Expression could be seen to extend in an arc both distally and anteriorly to form a continuous band of expression by stage 22 (not shown). *Pax-3* positive muscle progenitors (Fig. 1E) which originate from the dermamyotome of the somites were found within *slug*-negative regions (cells of myogenic lineages never express *slug*). By stage 24/25 (Fig. 1F) the distal limb mesenchyme was all positive for *slug* transcripts, as shown in transverse section (Fig. 1G), with the striking exception of a region immediately below the ectoderm (Fig. 1G). A section 100 μm more proximal showed down-regulation of *slug* in the condensing core region (Fig. 1H). Thus, cells which derive from the progress zone express *slug* for a time, but lose it at the onset of chondrogenic differentiation.

At stage 27 (Fig. 1I) the core (prechondrogenic condensations), and dorsal and ventral premuscle masses (identifiable by cell morphology) remained negative (Fig. 1J) for *slug*. Thus, *slug*-expressing cells occupied those regions which had not yet undergone differentiation (Thorogood and Hinchliffe, 1975), including regions anterior and posterior. Whilst the absolute size of the limb increases from stage 21-27, it was noticeable that the proximodistal extent of *slug* expression appears to remain fairly constant. By stage 29 *slug* expression was confined to the interdigital region (see Fig. 1K), and to the distal tips of the forming digits, where growth is still occurring. A *slug*-negative region was still apparent under the ectoderm.

Expression of *slug* by undifferentiated cells and its exclusion from differentiating cells suggests that *slug* may play a key role in the establishment of the tissue architecture of the developing chick limb.

TABLE 1

EFFECT OF DIFFERENT MANIPULATIONS ON SLUG EXPRESSION IN THE DEVELOPING LIMB

Bead implants or manipulation	Time harvested (h)	Location of bead	Expression of *slug*	Number of limbs	Data shown in Figure number
Ridge removal, stage 21/22	24		none	10	2
	12		posterior only	4	
	5		reduced level	3	
Ridge removal plus FGF4, stage 21/22	24	posterior	Yes	5	2
Ridge removal plus PBS, stage 21/22	24	posterior	No	3	2
FGF4, stage 21/22	24	anterior	none	4	3
		medial	none	5	
		posterior	Yes	4	
Amputation	24		No	2	4
Amputation plus FGF4, stage 24/25	24	posterior	Yes	4	
RA under AER, stage 19/20	24	anterior	slightly reduced	3	5
		medial/posterior	dramatically reduced	3	
RA+FGF4, stage 24		anterior	Yes/No*	8	6
		medial	No	6	
		posterior	Yes/No*	2	

*Expression of *slug* was dependent upon the proximity of the RA and FGF4 beads, see Results for details.

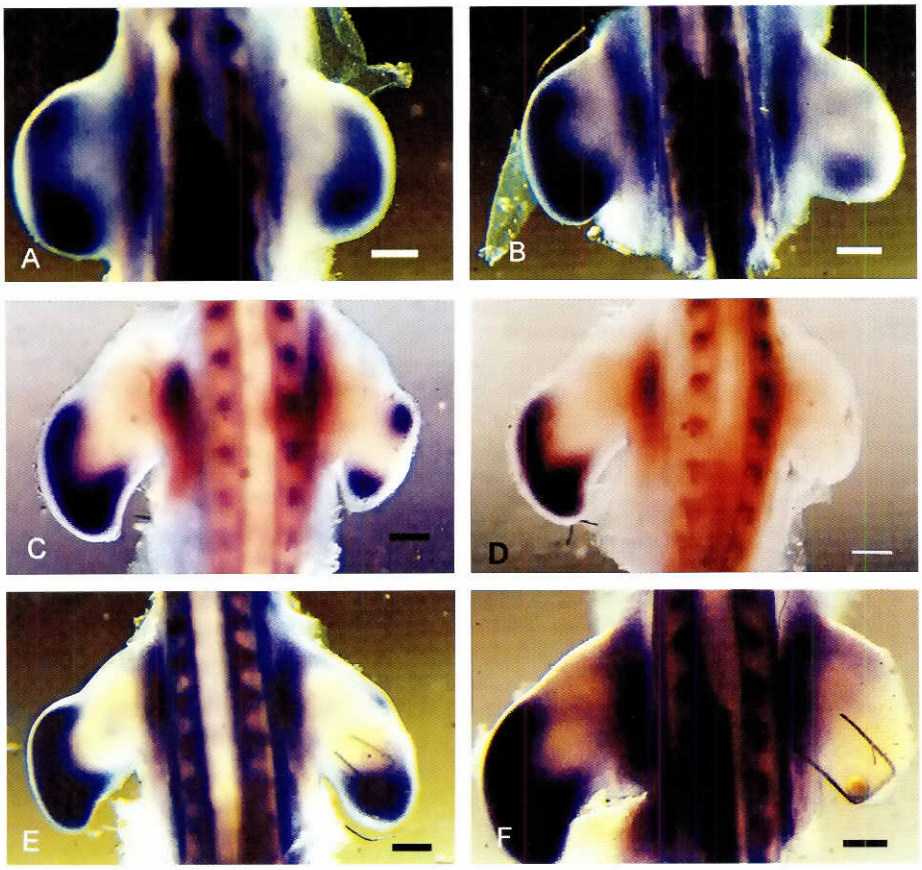

Fig. 2. Response of *slug* expression to ridge removal at stage 21/22. *Manipulated limbs are to the right, anterior is up and all views are dorsal.* **(A)** *5 h after ridge removal expression of slug is evident in a similar pattern to the normal limb but is less intense throughout.* **(B)** *12 h after ridge removal slug expression is still present posteriorly in the manipulated limb although it is absent anteriorly.* **(C)** *24 h after ridge removal the manipulated limb retains slug expression anteriorly and posteriorly in regions where the ridge had not been removed; the limb has broadened (compare to D) but unlike the unoperated controlateral limb has not grown out.* **(D)** *24 h after complete ridge removal there is neither growth nor slug expression in the manipulated limb.* **(E)** *Addition of an FGF-4 soaked bead to the posterior region of the bud immediately following ridge removal results in maintenance of slug expression and growth.* **(F)** *Addition of an PBS-soaked bead following ridge removal does not maintain either growth or slug expression. Bars, 100 μm.*

Apical ectodermal ridge (AER) removal and application of FGF at stage 21/22

To test the significance of this *slug* expression pattern we performed a series of experiments applying molecules, or using manipulations, which affect limb development (summarized in Table 1). Surgical removal of the ridge from wing buds of stage 21/22 embryos abolishes outgrowth of the bud (Saunders, 1948; Summerbell, 1974). Removal of the ridge also abolished *slug* expression within 24 h. After 5 h (Fig. 2A), its expression was less intense, though not dramatically reduced, and the anterior domain had not developed as it had in the control limb. Twelve hours after removal (Fig. 2B) *slug* expression was down-regulated and only remained posteriorly. This correlated with the evident lack of outgrowth of the bud as compared to the control bud. When anterior and posterior margins of the ridge were left intact, *slug* expression was maintained in these regions (Fig. 2C) at the 24 hour time point. When the entire ridge was removed and the embryo allowed to develop for a further 24 h, neither outgrowth nor *slug* expression was evident (Fig. 2D). FGF-4 can substitute for the AER in maintaining limb bud outgrowth. To examine whether FGF-4 was capable of maintaining *slug* expression, we implanted heparin-coated agarose beads, soaked in 0.7 mg/ml FGF-4 protein, at the distal tips of buds from which the ridge had been removed. When the bead was located posteriorly, *slug* expression as well as bud outgrowth was maintained (Fig. 2E). A PBS control bead implanted in an equivalent location had no effect either on *slug* expression or bud outgrowth (Fig. 2F). This suggests that the ability of the FGF-4 to substitute for the AER might involve maintenance of *slug* expression, indicating that this gene plays an important role in the molecular cascade controlling limb outgrowth.

FGF-4 bead implants at stage 21/22

As the bud grows out and chondrogenic differentiation progresses, *slug* expression is switched off. We grafted FGF-4 beads to a number of locations across the anterior/posterior and proximo/distal axes of the wing bud at stage 21-22 to determine whether FGF could induce *slug* expression in regions of the bud where it is not normally expressed. When 3 beads soaked in FGF-4 were implanted at the same proximo-distal level across the anterior-posterior axis, their effects along this axis differed significantly (Fig. 3A). High levels of *slug* were observed around the posterior FGF bead, but no significant changes in *slug* expression were induced by beads located anteriorly or medially in the condensed mesenchyme or in the premuscle mass (Fig. 3A-B).

In 50% of cases (2/4), posteriorly-located beads were surrounded by a halo of *slug* expression, such that the normal expression domain (Fig. 3C) was not continuous with that around the bead (Fig. 3D). Therefore in the posterior margin of the bud FGF may have induced *slug* expression in a region which would have normally been *slug*-negative. However, very proximal bead implants did not induce expression of *slug* in any anteroposterior location (data not shown).

Amputation and addition of FGF-4 beads at stage 24/25

Amputation of the distal limb bud results in truncation (Saunders, 1948) unless FGF-4 is added to the stump (Taylor *et al.*, 1994; Kostakopoulou *et al.*, 1996). This experimental strategy provided another means to test the correlation between differentiation and loss of *slug* expression postulated above. Twenty-four hours after amputation no *slug* expression remained posteriorly, but some anterior expression was still present (Fig. 4A). When an FGF-4 bead was added posteriorly at the time of amputation, *slug* expression was maintained and was accompanied by outgrowth (Fig. 4B).

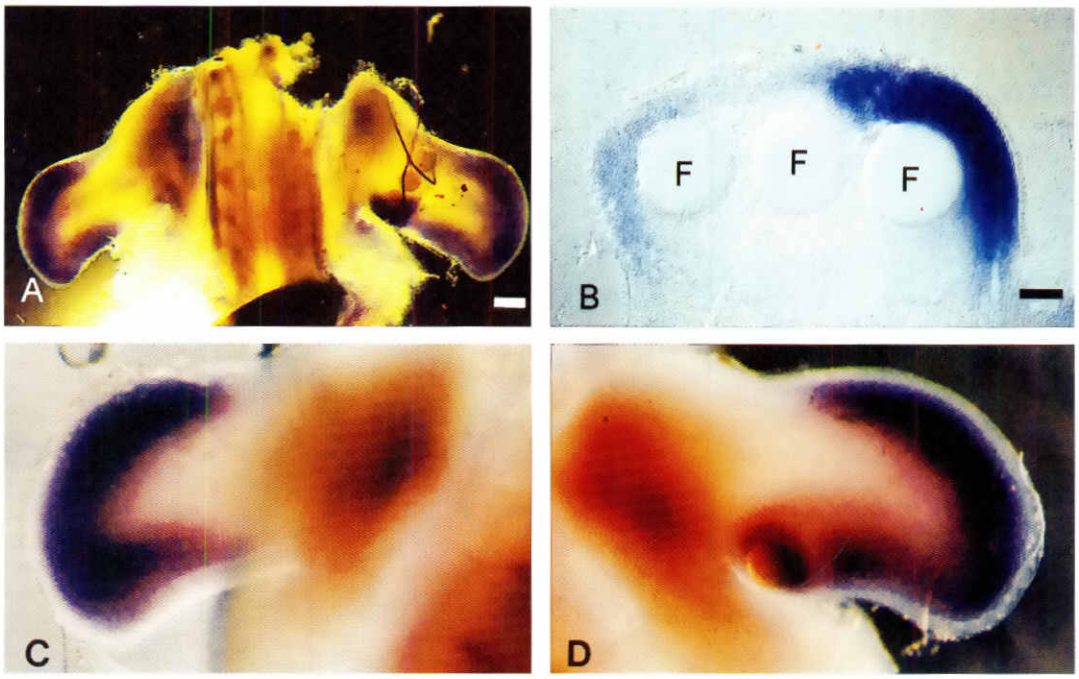

Fig. 3. Response of *slug* expression to beads soaked in 0.7 mg/ml of FGF-4 implanted at stage 21/22 in different locations evaluated by whole-mount *in situ* hybridization; *anterior is up and the views are dorsal.* **(A)** *Effect of 3 FGF-4 beads implanted at the same proximo-distal level across the anterior-posterior axis: 24 h after implantation ectopic slug expression is present only adjacent to the posterior bead; the other beads have no significant effect on slug expression.* **(B)** *Section from the embryo shown in (A) demonstrating that FGF-4 cannot induce slug expression in the condensed mesenchyme of the prechondrogenic core.* **(C)** *Stage 26 control limb.* **(D)** *24 h after a FGF-4 bead posterior implant slug expression is evident in a halo around the bead apparently discontinuous with the normal domain. F, FGF-4 soaked bead. Bars: 330 μm in A,C,D; 100 μm in B.*

Transverse sectioning of such a wing revealed that *slug* expression extended to the surface of the bead but was absent below the ectoderm (Fig. 4C), as in the normal wing. A section 50 μm more proximal showed *slug* expression across the stump of the bud (Fig. 4D), although this domain was reduced in proximal extent when compared to the normal.

Retinoic acid bead implants at stage 19/20

The posterior location of the initial domain of *slug* expression correlated approximately with the zone of polarizing activity. Anterior application of RA induces a new ZPA and should also activate *slug* if this is a component of the ZPA signaling pathway. We therefore implanted into the anterior limb bud mesenchyme beads soaked in RA, using a concentration (0.1 mg/ml) that produces digit duplications in 100% of cases and induces ectopic expression of *shh* (Tickle *et al.*, 1982; Niswander *et al.*, 1994). This did not result in up-regulation of *slug* expression in the vicinity of the bead (Fig. 5A).

Beads soaked in 0.1 mg/ml RA placed in the posterior mesenchyme decrease posterior outgrowth by 24 h, and the apex of the bud is narrower than in controls (Lee and Tickle, 1985). When RA-soaked beads (0.1 mg/ml RA) were placed posteriorly, *slug* expression was down-regulated in the vicinity of the bead, but was maintained in the region of anterior limb bud overgrowth (Fig. 5B).

At doses as low as 0.01 mg/ml, RA beads placed apically affect the shape of the limb bud, and bilobed buds with an AER extending along each lobe can be observed (Lee and Tickle, 1985). When 0.1 mg/ml RA beads were grafted medially, there was a dramatic down-regulation of *slug* expression and inhibition of bud outgrowth. (Fig. 5C). Cells between the ridge and the medially-located bead had reduced *slug* expression (Fig. 5D, compare with normal Fig. 1G), but in more proximal regions, posterior cells adjacent to the bead continued to express *slug* (Fig. 5E), suggesting that retinoic acid does not modulate *slug* expression directly.

Taken together, these experiments demonstrate that *slug* expression is associated with limb bud outgrowth rather than with the ZPA signaling pathway.

Dual implants of FGF-4 and retinoic acid (RA) beads at stage 24

In order to test whether the inhibitory effect of RA on *slug* expression would counter the positive effects of FGF-4 on *slug* maintenance at stage 24, when *slug* is expressed both posteriorly and anteriorly, dual implants were carried out. The beads were implanted in adjacent locations, although growth of the limb during the incubation period led to their dispersal. Beads were implanted as doublets (1xFGF-4 + 1xRA), or triplets (2xFGF-4 + 1xRA, with the RA bead implanted between the FGF-4 beads) at various positions and *slug* expression examined 24 h later. Posterior proximal implants resulted in up-regulation of *slug* around the FGF-4 bead (Fig. 6A,B). Sectioning showed that *slug* transcripts could still be detected adjacent to the RA bead at more distal levels (Fig. 6C), but that they had been completely down-regulated more proximally (Fig. 6D). Since in this embryo the second FGF-4 bead was located within the condensed mesenchyme no up-regulation of *slug* was elicited (Fig. 6E) consistent with the results presented above. There was no *slug* expression around either bead when located medially (data not shown). When the beads were implanted anteriorly the results were similar to those of posterior implants (see Fig. 6F-H). Although they appear to contradict the result of anterior bead implants at earlier stages (Fig. 3A), these results can be explained by the anterior expansion of the domain of *slug* expression at stage 24 (Fig. 1F) and are consistent with a maintenance-only effect of FGF-4 on *slug* expression. When the beads (RA and 2x FGF-4) were in close apposition no ectopic *slug* expression was observed except distal to the more distally located of the FGF-4 beads (Fig. 6H). Therefore RA not only can down-regulate endogenous *slug* expression, but can also antagonize maintenance of *slug* transcription by applied FGF-4.

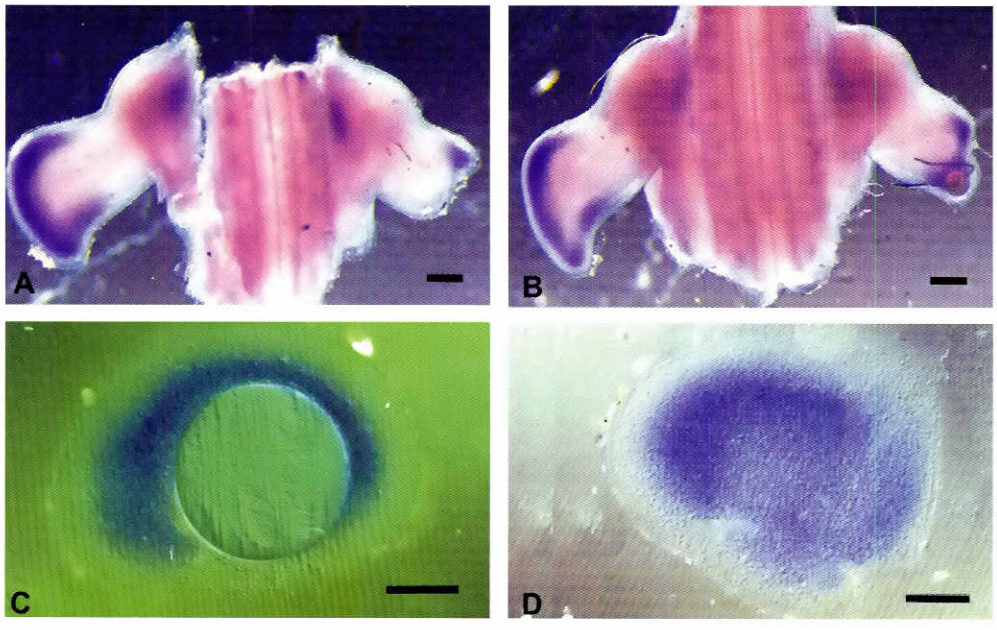

Fig. 4. Maintenance of *slug* expression by FGF-4 following amputation of 400 μm of stage 24/25 limbs. *In whole-mounts anterior is up and views are dorsal; in sections posterior is to the right and dorsal is up.* **(A)** *24 h after amputation slug expression is only maintained by a small region of anterior AER.* **(B)** *An identical amputation followed by addition of FGF-4 to the posterior part of the stump; slug expression is maintained in the region of the bead.* **(C)** *Transverse section through a limb similar to that in (B) showing expression around the posteriorly located bead; note that the sub-ectoderm region is slug-negative as in the normal.* **(D)** *Section immediately more proximal to the bead showing that slug expression is evident across the whole stump, although more prevalent posteriorly. Bars: 330 μm in A-B; 100 μm in C-D.*

Discussion

We have shown that the transcription factor *slug* is expressed in developing limbs and is first detectable in the posterior region of the limb bud in stage 20 embryos. The expression domain progresses anteriorly following the anterior expansion of the AER and is restricted to a distal and peripheral arc of positive cells around the prechondrogenic condensations. *Slug* expression is maintained in the connective tissue between the premuscle masses and prechondrogenic cores of the growing limb. This pattern of expression is consistent with the idea that *slug* expression marks a population of limb mesenchyme cells that are multipotent, although predominantly destined to contribute to skeletal elements. Recruitment of distal cells by the prechondrogenic condensates (Oster *et al.*, 1988) correlates well with the loss of *slug* expression from this population and the morphological change from loose (*slug*-positive) to tightly packed (*slug*-negative) mesenchyme described earlier (Thorogood and Hinchliffe, 1975).

Slug expression marks progress zone cells

All of the results emerging from the surgical manipulations carried out in this study are consistent with *slug* expression being associated with the progress zone and involved in its maintenance, at least from stage 21 onwards. In addition, its earlier pattern of expression in posterior cells may reflect the bias of this region, from which a larger number of limb structures, as compared to the anterior limb region, originate (Vargesson *et al.*, 1997). Removal of the ridge results in development of limbs truncated at the humerus (Hinchliffe and Johnson, 1980) and leads to the loss of *slug* expression from the underlying mesenchyme, indicating its dependence upon signals that derive from the AER. This loss may be due to the fact that in the absence of the ridge the *slug*-expressing cells stop dividing and are recruited into the prechondrogenic core. This possibility is consistent with the long time required to down-regulate *slug* after ridge removal (between 12 and 24 h) as compared with other genes such as *Cek-8* and *Msx-1*, which are down-regulated within 6 h (Robert *et al.*, 1991; Patel *et al.*, 1996). Therefore there appears to be a link between *slug* expression and undifferentiated state, which is also supported by the fact that at later stages *slug* is maintained in the interdigital regions where no chondrogenic condensation occurs.

The role of *slug* in maintaining a population of cells in an undifferentiated state that can be recruited by the proximal condensations parallels the observations of MacCabe *et al.* (1973) on the work of Rubin and Saunders (1972). These authors showed that when they removed the ectoderm and the most distal mesenchyme, and then replaced the ectoderm, normal limb morphogenesis, rather than the loss of intercalary structures, occurred. Thus, MacCabe *et al.* (1973) concluded that "one aspect of the role of the ridge is to maintain mesodermal cells subjacent to it in a state of developmental plasticity such that they can respond to signals from more proximal structures that communicate the message "make the next most distal part". *Slug* expression correlates with the location and capabilities of these cells, which are retained in an undifferentiated state, can regulate their number and can respond to proximally- and ZPA-derived signals.

It is therefore possible that, as in the head and vertebral column, positional specification is retained and distributed via *slug*-positive cells. Loss of *slug* expression is seemingly irreversible, as FGF-4 beads cannot induce *slug* expression either in condensed mesenchyme or in regions proximal to its endogenous domain. This parallels the permanent fate changes (Vogel and Tickle, 1993) that occur in those cells which we have shown to be initially *slug*-positive, and is also consistent with the finding that condensed thigh mesenchymal cells (*slug*-negative) cannot dedifferentiate and give rise to distal structures (digits) when implanted under the AER (Saunders *et al.*, 1959).

The "irreversible" expression of *slug* is also significant in relation to *Msx-1* expression. *Msx-1* has been considered a marker of the progress zone cells (Hill *et al.*, 1989; Robert *et al.*, 1989), and can be induced in proximal mouse tissue when grafted under the AER

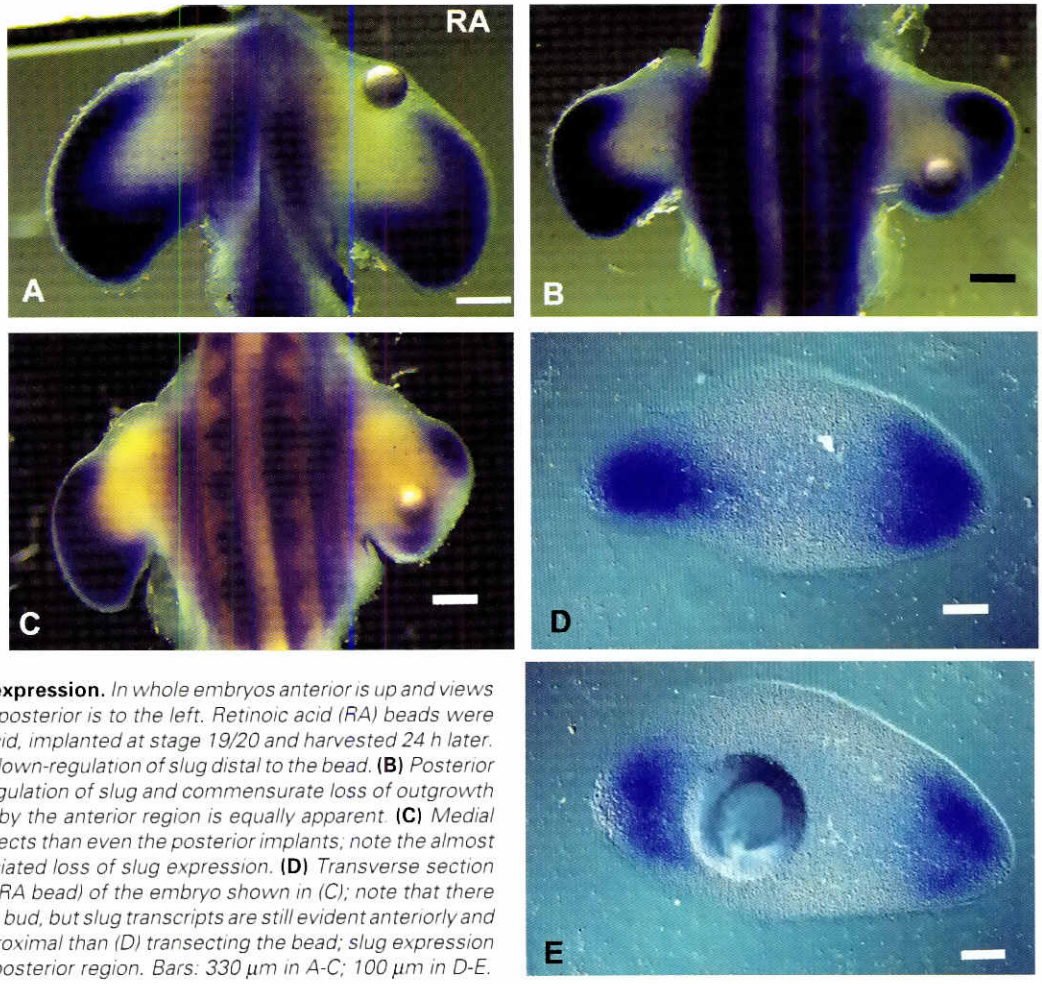

Fig. 5. Effects of retinoic acid on *slug* expression. *In whole embryos anterior is up and views are dorsal; in sections dorsal is up and posterior is to the left. Retinoic acid (RA) beads were soaked in 0.1 mg/ml all trans-retinoic acid, implanted at stage 19/20 and harvested 24 h later.* **(A)** *Anterior beads lead to a detectable down-regulation of slug distal to the bead.* **(B)** *Posterior bead implants cause dramatic down-regulation of slug and commensurate loss of outgrowth from the posterior region; overgrowth by the anterior region is equally apparent.* **(C)** *Medial bead implants cause more profound effects than even the posterior implants; note the almost total cessation of outgrowth and associated loss of slug expression.* **(D)** *Transverse section through the distal tip (also distal to the RA bead) of the embryo shown in (C); note that there is no expression in the medial part of the bud, but slug transcripts are still evident anteriorly and posteriorly.* **(E)** *Section 100 μm more proximal than (D) transecting the bead; slug expression is present adjacent to the bead in the posterior region. Bars: 330 μm in A-C; 100 μm in D-E.*

(Davidson *et al.*, 1991; Kostakopoulou *et al.*, 1996). Thus whilst the re-expression of this gene occurs, it does not correspond to de-differentiation of the proximal mesoderm. Therefore the domain of *Msx-1* expression does not seem to correlate with the size of the progress zone reported by Summerbell *et al.* (1973) as being approximately 300 μm, since cells outside its domain (*slug*-positive) also have the characteristics of the progress zone. Therefore *slug* expression provides the best correlation yet between a molecular marker and the physical concept of the progress zone (Summerbell *et al.*, 1973).

Antagonistic effects of FGF-4 and RA on slug expression

Application of FGF-4 either after ridge removal or limb amputation consistently results in growth response and *slug* maintenance, whereas down-regulation of *slug* expression in response to RA beads coincides with the cessation of outgrowth.

Anterior and posterior responsiveness to FGF-4 is different depending upon the time of bead implantation. At early stages, an anteriorly located bead cannot induce ectopic expression of *slug*. This may be due to the fact that in addition to ridge/FGF-4 signaling, another signal localized, at least at this stage, in the posterior part of the bud is necessary for *slug* induction. However, at later stages (stage 24), when the *slug* domain is symmetrical, anterior cells can respond to FGF-4 and maintain expression of *slug* independently from the polarizing region.

It is noteworthy that the *slug* expression reported so far is found in migratory cell types (Nieto *et al.*, 1994; Buxton *et al.*, 1997) and sclerotome (Figs. 2D and 6F). This raises the novel possibility that the changing pattern of *slug* expression might reflect, at least in part, a cell migration. In other words, could the anteriorward expansion of the *slug* expression domain during stages 20-21 reflect a directional migration guided by an FGF signal? Such a possibility, although previously discounted, is consistent with several independent observations. Using DiI labeling of mesenchyme in stage 20 limb buds, it has been found that whereas posterior cells (between somite 19 and 20) distribute in a proximodistally linear fashion, less posterior and medial cells fan out towards the anterior tip (Vargesson *et al.*, 1997). DiI tracking has also revealed that FGF-2 can affect movement of posterior (*slug*-positive) but not anterior (*slug*-negative) cells (Li *et al.*, 1996). Moreover, *fgf-4* expression shifts anteriorly with the expansion of the ridge (Duprez *et al.*, 1996), preceding that of the posterior mesenchyme (Vargesson *et al.*, 1997), and the expression domain of *fgf-8* in the AER (Crossley *et al.*, 1996) corresponds to that of *slug* in the underlying mesenchyme. Finally, the loose mesenchyme of the bud is capable of supporting cell motility, as fibroblasts implanted

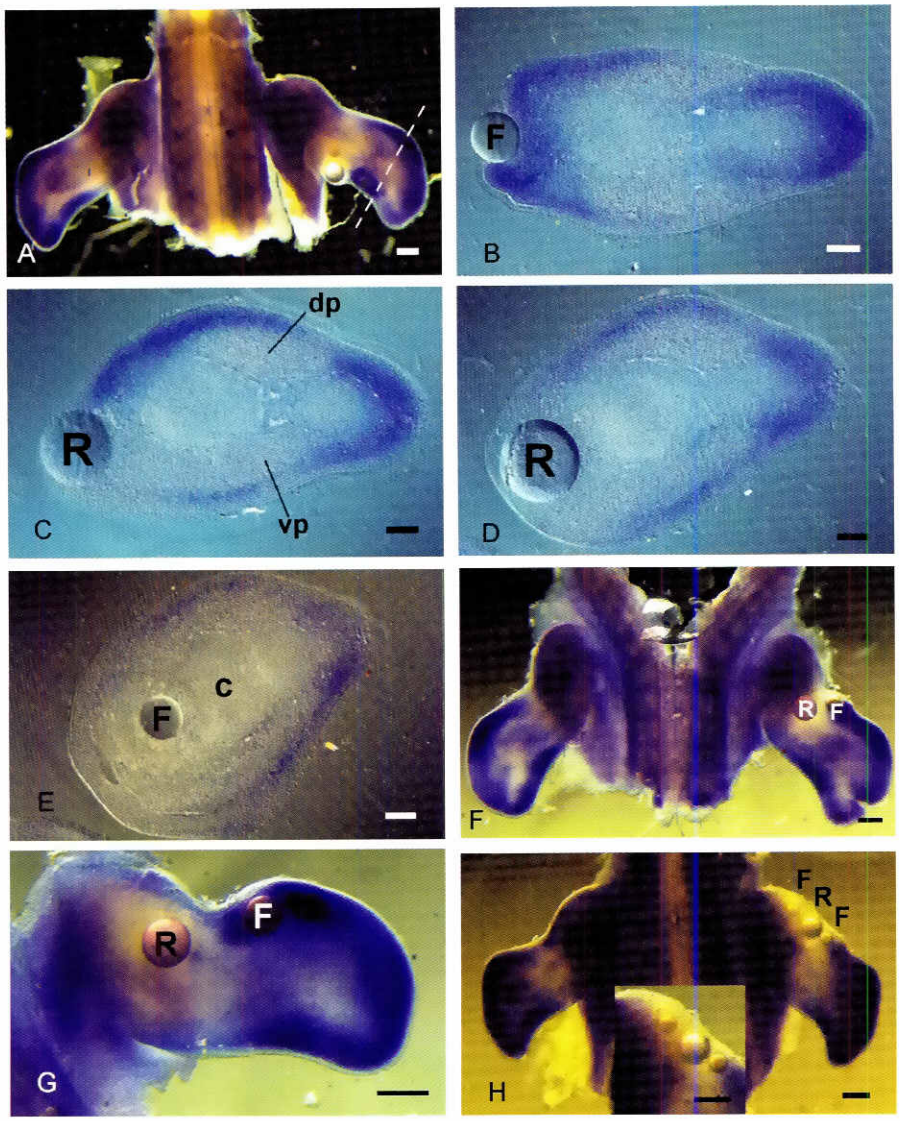

Fig. 6. Antagonism between retinoic acid (RA) and FGF-4 in the maintenance of *slug* positive cells. *Whole embryos are shown in dorsal view and anterior is up; sections are shown with posterior to the left and dorsal is up. Both FGF-4 and RA-soaked beads were implanted at stage 24 (the concentrations were as used previously in this study) and the embryos processed for whole-mount in situ hybridization 24 h later.* **(A)** *Effect of two FGF-4 beads and one RA bead in between them implanted at the posterior margin of the limb: slug expression is evident around the more distal bead, but the endogenous expression present at the proximal dorsoventral boundary is absent. The dashed line indicates plane of section.* **(B)** *Transverse section at the level of the distal FGF-4 bead showing slug expression in the vicinity of the bead.* **(C)** *Section through the distal portion of the RA bead; note that there are slug transcripts close to the bead under the dorsal ectoderm.* **(D)** *Section through the proximal part of the RA bead showing that there are no slug positive cells around the bead.* **(E)** *Section 100 μm more proximal showing the second FGF-4 bead; there is no slug expression around the bead which is located in the condensed mesenchyme undergoing chondrogenesis.* **(F)** *Effect of single RA and single FGF-4 beads implanted close together in a proximal and anterior location; the anterior expansion of the slug domain close to the FGF bead is clear but no slug expression is present between the two beads. The action of the FGF-4 bead also appears to have promoted growth as the anterior flexure is distorted.* **(G)** *Effect of RA bead and FGF-4 bead separated by a greater distance than in the embryo shown in (F); note that here slug expression is evident on both proximal and distal sides of the FGF-4 bead.* **(H)** *Effect of two FGF-4 beads and one RA bead implanted anteriorly: RA has abolished the endogenous expression of slug nearby. Slug expression is maintained in the distal aspect of the more distally located FGF bead, but there is no expression between the FGF-4 and the RA bead. R, retinoic acid bead; F, FGF-4 bead; dp, dorsal premuscle mass; vp, ventral premuscle mass; c, chondrogenic core. Bars: 330 μm in A,F,G,H; 100 μm in B,C,D,E.*

into such an area migrate significant distances (Riddle *et al.*, 1993). Clearly, the possibility of cell motility being an important feature of limb morphogenesis is due for re-assessment.

An RA bead implanted at the anterior margin of the bud at stage 20 does not induce up-regulation, but rather down-regulation of *slug* in the region surrounding it. This indicates that *slug* expression and polarizing activity are not equivalent. RA application at the apex (medial region) of the ridge at the same stage leads to profound down-regulation of *slug* by 24 h. Thus it may be that addition of RA beads to the apex of the bud, which have been shown to abolish the ridge (Tickle *et al.*, 1985), causes subsequent down-regulation of *slug* (as following ridge removal). However, two factors weigh against this interpretation. Firstly, RA effects on the AER have been shown to be mediated by the mesenchyme (Tickle *et al.*, 1989). Secondly, RA was effective at down-regulating *slug* in more proximal regions, in the absence of any ridge. Thus, the *slug* down-regulation that results from apical application of RA may actually cause the decay of the AER. This view is consistent with the fact that progress zone cells are essential for ridge maintenance, loss of *slug* expression indicating loss of the progress zone.

Dual implants of RA and FGF-4 have demonstrated that RA is capable of antagonizing the *slug*-maintaining effect of FGF-4. RA-induced down-regulation of *slug* seems to be a short range effect, since it occurs only in proximity of the bead. It is conceivable therefore that a localized antagonistic effect between endogenous RA and FGF-4 on *slug* expression underlies the molecular mechanism controlling the transition between undifferentiated and differentiated state during normal limb development.

Conclusion

In the limb *slug* expression marks a population of undifferentiated mesenchymal cells, mainly prechondrogenic and possibly motile, with the characteristic of the progress zone. From the nature of the cells known to express *slug* and the likelihood that some at least of their properties derive from the action of this gene, it is probable that the phenotype and behavior of the cells in the *slug* zone are critical for limb patterning. However, cells expressing *slug* are not specifically cells with polarizing activity; rather they provide the link between maintenance of the AER and the propagation of polarizing activity. Finally, as *slug* marks progress zone cells, the antagonistic effects of RA and FGF on its expression provide a unique insight into the properties of

this cell population and into the signalling cascades whereby growth is translated into form and pattern. The precise role of *slug* remains to be elucidated but it should now be included among the set of genes having a key role in vertebrate limb morphogenesis.

Materials and Methods

Experimental manipulations of chick embryos

White Leghorn chicken eggs were incubated at 38°C until they reached the appropriate stages (Hamburger and Hamilton, 1951). The eggs were windowed and the membranes covering the embryo were slit and pulled back to reveal the wing bud. For AER removal, a fine tungsten needle was employed. For amputations, an anterior-posterior incision through the limb bud with fine needles was made and then the tissue cut off was removed with fine forceps. The level of amputation was measured using an eyepiece graticule calibrated at 20 μm per division; half of the limb (approximately 400 μm) was removed. For FGF-4 application, heparin acrylic beads (H5263, Sigma) of size of 200-250 μm were soaked in 2 ml of 700 mg/ml (kind gift from John Heath) for at least 1 h at room temperature before application to the mesenchyme of the limb. To keep the beads in place, we used staples made out of platinum wire (0.025 mm^2, Goodfellow Metals). In another series of experiments, a small slit was made at a measured position in the proximal posterior, proximal central, or proximal anterior part of the wing bud with a needle and FGF-4 bead(s) was inserted into the mesenchyme. In order to ensure that the bead was held in place, a staple was used. For retinoic acid application AG1-X2 beads were soaked in all-trans-retinoic acid (Sigma) as described (Tickle *et al.*, 1985). To examine gene expression, embryos were removed from eggs between 5 and 24 h after the operation and fixed overnight in 4% (w/v) paraformaldehyde at 4°C. The embryos were then washed twice in PBS, dehydrated and stored in absolute methanol at -20°C before being used for whole-mount *in situ* hybridization.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out essentially as described (Wilkinson, 1992). The *slug* probe used was as described (Nieto *et al.*, 1994) and was hybridized at 65°C. The *Pax-3* probe was derived from that cloned by Goulding *et al.* (1993) and comprised the *EcoRV/HindIII* interval; it was hybridized at 70°C. Embryos to be sectioned were embedded in gelatin/albumin solution (1:2) and 50 μm sections cut with a Pelco 101 Vibratome (St Louis, USA). Embryos and sections were viewed with Nomarski, differential interference contrast, optics using a Zeiss Axiophot. Images were either recorded photographically or captured electronically using a Kontron ProgRes 3012 digital camera. Figure montages were compiled using Adobe Photoshop.

Acknowledgments

We would like to thank Cheryll Tickle and Paul Hunt for critical reading of this manuscript, and the Child Health Research Appeal Trust and Action Research for financial support. Clones were kindly provided by M. Sargent (slug), and A. Lumsden (Pax-3). FGF-4 protein was the kind gift of John Heath.

References

BUXTON, P.G., HUNT, P., FERRETTI, P. and THOROGOOD, P. (1997). A role for midline closure in the reestablishment of dorsoventral pattern following dorsal hindbrain ablation. *Dev. Biol. 183:* 150-165.

COHN, M.J. and TICKLE, C. (1996). Limbs: a model for pattern formation within the vertebrate body plan. *Trends Genet. 12:* 253-257.

COHN, M.J., IZPISUA-BELMONTE, J.C., ABUD, H., HEATH, J.K. and TICKLE, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell 80:* 739-746.

CROSSLEY, P.H., MINOWADA, G., MACARTHUR, C.A. and MARTIN, G.R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell 84:* 127-136.

DAVIDSON, D.R., CRAWLEY, A., HILL, R.E. and TICKLE, C. (1991). Position-dependent expression of two related homeobox genes in developing vertebrate limbs. *Nature 352:* 429-31.

DUPREZ, D.M., KOSTAKOPOULOU, K., FRANCIS-WEST, P.H., TICKLE, C. and BRICKELL, P.M. (1996). Activation of *Fgf-4* and *HoxD* gene expression by BMP-2 expressing cells in the developing chick limb. *Development 122:* 1821-1828.

FALLON, J.F., LÓPEZ, A., ROS, M.A., SAVAGE, M.P., OLWIN, B.B. and SIMANDL, B.K. (1994). FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science 264:* 104-107.

GOULDING, M.D., LUMSDEN, A. and GRUSS, P. (1993). Signals from the notochord and floorplate regulate the region-specific of two *Pax* genes in the developing spinal cord. *Development 117:* 1001-1016.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HELMS, J.A., KIM, C.H., EICHELE, G. and THALLER, C. (1996). Retinoic acid signalling is required during early chick limb development. *Development 122:* 1385-1394.

HILL, R.E., JONES, P.F., REES, A.R., SIME, C.M., JUSTICE, M.J., COPELAND, N.G., JENKINS, N.A., GRAHAM, E. and DAVIDSON, D.R. (1989). A new family of mouse homeobox containing genes: molecular structure, chromosomal location and developmental expression of Hox-7.1. *Genes Dev. 3:* 26-37.

HINCHLIFFE, J.R. and JOHNSON, D.R. (1980). *The Development of the Vertebrate Limb.* Oxford Science Publications, Clarendon Press, Oxford.

KOSTAKOPOULOU, K., VOGEL, A., BRICKELL, P. and TICKLE, C. (1996). 'Regeneration' of wing bud stumps of chick embryos and reactivation of *Msx*-1 and *Shh* expression in response to FGF-4 and ridge signals. *Mech. Dev. 55:* 119-131.

LAUFER, E., NELSON, C.E., JOHNSON, R.L., MORGAN, B.A. and TABIN, C. (1994). Sonic hedgehog and FGF-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell 79:* 993-1003.

LEE, J. and TICKLE, C. (1985). Retinoic acid and pattern formation in the developing chick wing: SEM and quantitative studies of early effects on the apical ectodermal ridge and bud outgrowth. *J. Embryol. Exp. Morphol. 90:* 139-169.

LI, S., ANDERSON, R., REGINELLI, A.D. and MUNEOKA, K. (1996). FGF-2 influences cell movement and gene expression during limb development. *J. Exp. Zool. 274:* 234-247.

MacCABE, J.A., SAUNDERS, J.W. and PICKETT, M. (1973). The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm. *Dev. Biol. 31:* 323-335.

MAHMOOD, R., BRESNICK, J., HORNBRUCH, A., MAHONY, C., MORTON, N., COLQUHOUN, K., MARTIN, P., LUMSDEN, A., DICKSON, C. and MASON, I. (1995). A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol. 5:* 797-806.

MANGELSDORF, D.J., UMESONO, K. and EVANS, R.M. (1994). The retinoid receptors. In *The Retinoids. Biology, Chemistry and Medicine* (Eds. M.B. Sporn, A.B. Roberts and D.S. Goodman). Raven Press, New York, pp. 319-349.

MAYOR, R., MORGAN, R. and SARGENT, M.G. (1995). Induction of the prospective neural crest of *Xenopus*. *Development 121:* 767-777.

NIETO, M.A., SARGENT, M.G., WILKINSON, D.G. and COOKE, J. (1994). Control of cell behaviour during vertebrate development by Slug, a zinc finger gene. *Science 264:* 835-839.

NISWANDER, L., JEFFREY, S., MARTIN, G. and TICKLE, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature 371:* 609-612.

NISWANDER, L., TICKLE, C., VOGEL, A., BOOTH, I. and MARTIN, G.R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell 75:* 579-87.

OSTER, G.F., SHUBIN, N., MURRAY, J.D. and ALBRECH, P. (1988). Evolution and morphogenetic rules: the shape of the vertebrate limb in ontogeny and phylogeny. *Evolution 42:* 862-884.

PATEL, K., NITTENBERG, R., DSOUZA, D., IRVING, C., BURT, D., WILKINSON, D.G. and TICKLE, C. (1996). Expression and regulation of Cek-8, a cell to cell signalling receptor in developing chick limb buds. *Development 122:* 1147-1155.

PECORINO, L.T., ENTWISTLE, A. and BROCKES, J.P. (1996). Activation of a single retinoic acid receptor isoform mediates proximodistal respecification. *Curr. Biol. 6:* 563-569.

PECORINO, L.T., LO, D.C. and BROCKES, J.P. (1994). Isoform-specific induction of a retinoid-responsive antigen after biolistic transfection of chimaeric retinoic acid/thyroid hormone receptors into a regenerating limb. *Development 120:* 325-333.

RIDDLE, R., JOHNSON, R., LAUFER, E. and TABIN, C. (1993). *Sonic Hedgehog* mediates the polarizing activity of the ZPA. *Cell 75:* 1401-1416.

ROBERT, B., LYONS, G., SIMANDL, B.K., KUROIWA, A. and BUCKINGHAM, M. (1991). The apical ectodermal ridge regulates Hox-7 and Hox-8 gene expression in developing chick limb buds. *Genes Dev 5:* 2363-74.

ROBERT, B., SASSOON, D., JACQ, B., GEHRING, W. and BUCKINGHAM, M. (1989). *Hox-7*, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J. 8:* 91-100.

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

SAUNDERS Jr, J.W. (1948). The proximo-distal sequence of the origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool. 108:* 363-404.

SAUNDERS Jr, J.W. and GASSELING, M.T. (1968). Ectodermal-mesodermal interactions in the origin of limb symmetry. In *Epithelial-mesenchymal Interactions* (Eds. R. Fleishmajer and R.E. Billingham). Williams and Wilkins, Baltimore, pp. 78-97.

SAUNDERS Jr, J.W., GASSELING, M.W. and CAIRNS, J.M. (1959). The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. *Dev. Biol. 1:* 281-301.

SAUNDERS, J.W.J. (1977). The experimental analysis of chick limb bud development. In *Vertebrate Limb and Somite Morphogenesis* (Eds. D.A. Ede, J.R. Hinchliffe and M. Balls). University Press, Cambridge, pp. 1-24.

SCHILTHUIS, J.G., GANN, A.A. and BROCKES, J.P. (1993). Chimeric retinoic acid/thyroid hormone receptors implicate RAR-alpha 1 as mediating growth inhibition by retinoic acid. *EMBO J. 12:* 3459-66.

SECHRIST, J., NIETO, M.A., ZAMANIAN, R.T. and BRONNER-FRASER, M. (1995). Regulative response of the cranial neural tube after neural fold ablation: spatiotemporal nature of neural crest regeneration and up-regulation of *slug*. *Development 121:* 4103-4115.

STRATFORD, T., HORTON, C. and MADEN, M. (1996). Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Curr. Biol. 6:* 1124-1133.

SUMMERBELL, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb bud. *J. Embryol. Exp. Morphol. 35:* 241-260.

SUMMERBELL, D., LEWIS, J. and WOLPERT, L. (1973). Positional information in chick limb morphogenesis. *Nature 244:* 492-496.

TABIN, C. (1995). The initiation of the limb bud: growth factors, Hox genes, and retinoids. *Cell 80:* 671-4.

TAYLOR, G.P. ANDERSON, R., REGINELLI, A.D. and MUNEOKA, K. (1994). FGF-2 induces regeneration of the chick limb bud. *Dev. Biol. 163:* 282-4.

THOROGOOD, P.V. and HINCHLIFFE, J.R. (1975). An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. *J. Embryol. Exp. Morphol. 33:* 581-606.

TICKLE, C. and EICHELE, G. (1994). Vertebrate limb development. *Annu. Rev. Cell Biol. 10:* 121-152.

TICKLE, C., ALBERTS, B.M., WOLPERT, L. and LEE, J. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature 296:* 564-565.

TICKLE, C., CRAWLEY, A. and FARRAR, J. (1989). Retinoid acid application to the chick wing buds lead to a dose-dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. *Development 106:* 691-705.

TICKLE, C., LEE, J. and EICHELE, G. (1985). A quantitative analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. *Dev. Biol. 109:* 82-95.

VARGESSON, N., CLARKE, J.D.W., VINCENT, K., COLES, C., WOLPERT, L. and TICKLE, C. (1997). Cell fate in the chick limb bud and relationship to gene expression. *Development 124:* 1909-1918

VOGEL, A. and TICKLE, C. (1993). FGF-4 maintains polarizing activity of posterior limb bud cells *in vivo* and *in vitro*. *Development 119:* 199-206.

VOGEL, A., RODRIGUEZ, C. and IZPISUA-BELMONTE, J.C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development 122:* 1737-1750.

WILKINSON, D. G. (1992). Whole mount *in situ* hybridization of vertebrate embryos. In *In Situ Hybridization: A Practical Approach* (Ed. D.G. Wilkinson). IRL Press, Oxford, pp. 75-83.

WOLPERT, L., LEWIS, J. and SUMMERBELL, D. (1975). Morphogenesis of the vertebrate limb. In *Cell Patterning*, Vol. 29, new series. Associated Scientific Publishers, Amsterdam, pp. 95-119.

Received: April 1997
Accepted for publication: May 1997