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, KONSTADINA KOSTAKOPOULOU, PAUL BRICKELL, PETER THOROGOOD and PATRIZIA FERRETTI

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 pre-muscle 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.
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 gfg-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 gfg-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 downregulated 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., 1975).

**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**

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<th>Bead implants or manipulation</th>
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<th>Expression of slug</th>
<th>Number of limbs</th>
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*Expression of slug was dependent upon the proximity of the RA and FGF4 beads, see Results for details.
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 medi ally 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).

**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

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 contralateral 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 a PBS-soaked bead following ridge removal does not maintain either growth or slug expression. Bars, 100 μm.

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 outgrowth (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 implantation 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 2xFGF-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.
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 pre-chondrogenic cores of the growing limb. This pattern of expression is consistent with the idea that slug expression marks a population of limb mesenchymal 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 Mxs-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 mesenchymal 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 de-differentiate 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 Mxs-1 expression. Mxs-1 has been considered a marker of the progress zone cells (Hill et al., 1983; 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 posteroirly. (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 dedifferentiation 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 Dil 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). Dil 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, tgf-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 embryos 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.

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 patterning. 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-acryl beads (HS263, 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 mm², 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 bud 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 FGF-4 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 and Hill (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 10 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 Axioshot. Images were either recorded photographically or captured electronically using a Kontron ProRes 3012 digital camera. Figure montages were compiled using Adobe Photoshop.

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References


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