DEVELOPMENTAL BIOLOGY

We may not have a morphogen

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ATTENTION has centred on retinoic acid (RA) as a potential vertebrate morphogen because of its ability to alter axial specification in avian limb development and urodele limb regeneration (reviewed in refs 1–3). In the chick limb bud the pattern of mesenchymal tissues on the anteroposterior (AP) axis is specified by a mesenchymal cell group on the posterior margin, referred to as the polarizing region or zone of polarizing activity (ZPA). If this cell group is grafted to the anterior margin of a host bud, it establishes a second posterior boundary that results in formation of a mirror-image duplication of digits along the AP axis. The ability of a locally implanted source of RA to mimic such a graft led to suggestions that RA was produced by the ZPA, that its graded distribution across the bud specified the AP axis, and that it was thus the first identified vertebrate morphogen. Reports on pages 81 and 83 of this issue[4,5] provide evidence for a significantly different interpretation — namely that a local source of RA induces neighbouring tissue to become a polarizing region, which then determines the AP axis by some mechanism not involving the graded activity of RA.

It has been proposed for some time that retinoic acid could act by inducing limb tissue to become a ZPA [6]. Wanek *et al.*[4] report that after implantation of an RA-loaded bead in the anterior margin, it is possible to remove a wedge of tissue adjacent to the bead and demonstrate polarizing activity on grafting it to a recipient limb bud. Such wedges show activity after 16 hours of exposure to RA, but not after 12 hours, and induction is essentially complete after 24 hours. The chick-quail marker system has been used to demonstrate that the grafted cells do not make a significant contribution to the pattern duplication, and so are acting as a signalling centre.

Carryover hypothesis

It is important to rule out the possibility that the effect is due to carryover of RA in the grafted tissue. Wanek *et al.* have allowed a clearance interval between removing the bead and excising the wedge. Together with the failure of 12 hours exposure to induce activity and the known kinetics of RA removal and release from the bead, the authors argue that the carryover hypothesis is not valid. It might nonetheless be desirable to obtain direct evidence on this point from studies with labelled RA. Wanek *et al.* suggest that the graded response to exogenous RA may reflect variation in the number of adjacent cells induced to become polarizing cells. They point out that the polarizing activity could be unrelated to RA, or alternatively that RA acts in an autocatalytic relay whereby cells along the axis are signalled to produce more RA, thereby retaining its status as a possible morphogen.

Noji *et al.*[5] provide evidence against this second hypothesis, thus complementing the study of Wanek *et al.* These authors and others[7,8] have identified a ß-type retinoic acid receptor (RAR) expressed in the chick limb bud. The promoter of RARß in human and mouse contains a retinoid response element that mediates a rapid elevation of RARß transcription on application of RA[9,10], and the authors have used this effect as an endogenous reporter of RA activity in the distal bud. When a bead containing RA, its morphogenetically active relative 3,4-didehydro retinoic acid (ddRA)[11] or a synthetic retinoid is implanted on the anterior margin of the bud, the local induction of RARß can be detected by *in situ* hybridization within four hours of implantation. Similar results were obtained after implantation of beads carrying synthetic retinoid into the polarizing region on the posterior margin. Despite the clear response to exogenous retinoid, a graft of ZPA tissue does not provoke any detectable increase in RARß expression, and the authors conclude that RA is unlikely to be released by the ZPA either on the posterior margin or after grafting.

These results are provocative, but it is uncertain that the RARß reponse is a completely satisfactory reporter. Rather little is known about intracellular retinoid traffic, or about the disposition of binding proteins, degradation systems and other components that might modify the responsiveness of the endogenous ß gene. All this might conspire to make a functional distinction between what the authors refer to as "endogenous" retinoid released by the ZPA, and exogenous retinoid applied on a bead. A related possibility is that the ZPA delivers retinoid with a concentration profile that is not detected by the ß response.

These possibilities do not seem particularly likely, but the study by Noji *et al.* will have to be confirmed and extended with other reporter systems before firm conclusions can be drawn from it. The authors have nonetheless established that a bead implant does not precisely mimic a ZPA graft. The morphogen possibility has previously been supported by evidence that graded distributions of exogenous retinoid are the most effective at inducing pattern duplication[11], but this could reflect the time of exposure of neighbouring tissue that is induced to become a ZPA (see page 1919 of ref. 12). The evidence that RA and ddRA are extractable from the limb bud, and that the former at least is present at somewhat higher levels in posterior fragments[13], raises questions about the functional significance of endogenous retinoids, and whether they are responsible for inducing the ZPA *in vivo*.

The other key system for evaluating the effects of retinoic acid on limb morphogenesis is regeneration in urodele amphibians. Amputation of the limb at any level on the proximodistal (PD) axis from shoulder to finger-tip is followed by formation of blastemal (progenitor) tissue at the plane of amputation. Blastemal cells normally give rise only to structures distal to their point of origin, but RA is able to respecify them to more proximal values. Thus a wrist blastema, which normally forms a hand, may regenerate an entire arm[14]. These remarkable effects on the PD axis have recently been extended by Stocum and colleagues[15] to include unidirectional alterations of positional value on the transverse axes in certain experimental contexts. Retinoic acid is therefore able to affect all three axes.

Histological evidence

In view of that, and the lack of evidence for a signalling centre akin to the ZPA, along with the absence of data on endogenous retinoid levels, the case for RA as an endogenous morphogen in limb regeneration has not found strong advocates[2]. The histological evidence suggests that RA treatment of distal regenerates promotes changes in the tissue morphology of the blastema[16], and it is possible that the effects of RA on axial specification are secondary to its ability to influence the extent of the blastema.

In this sense, retinoic acid could be said to establish boundaries, just as local induction of a polarizing region sets the posterior boundary in the chick limb. But what are the molecular bases of these effects, how does the ZPA work and how is position encoded in the blastema and the avian bud? Discussion of these issues has been greatly stimulated by the work on RA, but at present its election to vanguard membership of the club of vertebrate morphogens can be questioned. □

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