Positional signalling and the development of the humerus in the chick limb bud

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Summary

The positional signal model for specification of the cartilaginous elements in limb development has been tested by examining the effect on the humerus of grafting a polarizing region to different positions along the anteroposterior axis of the limb bud at stage 16. The humerus between the host and grafted polarizing region was largely normal though there were variations in width, particularly the distal epiphysis. The humerus often showed mirror-image symmetry along the anteroposterior axis. When the grafted polarizing region was in a very anterior position, there were a few cases where a second humerus developed. Anterior to the graft an additional humerus often developed. This was associated with the splitting of the bud into two domains. It is suggested that these results are not consistent with a positional signal model and that an additional mechanism involving an isomorphic prepattern may be involved in the specification of the cartilaginous elements.

Key words: positional signalling, chick, limb bud, humerus, cartilage.

Introduction

One model for pattern formation in chick limb development suggests that the limb elements, such as cartilage, arise from the interpretation of positional information. We have proposed that position along the anteroposterior axis is specified by a signal, a gradient in a diffusible morphogen, from the polarizing region at the posterior margin of the bud (Tickle, Summerbell & Wolpert, 1975; Tickle, Lee & Eichele, 1985). The position of the cartilaginous elements would then be determined by thresholds with respect to the concentration of the morphogen. Thus, when an additional polarizing region is grafted to the anterior margin, the pattern of digits is 432234, which is to be compared to the normal pattern of 234 (Fig. 2A,B). Another view is that the cartilaginous elements arise by a mechanism involving an isomorphic prepattern (Wilby & Ede, 1979; Newman & Frisch, 1979); for example, a morphogen may have a spatial distribution similar to that of the observed pattern. There could be a wave-like distribution of a morphogen with one peak for the humerus, two peaks for the radius and ulna and three peaks for the digits. There is as yet no evidence for any sort of a prepattern. However, the observation that when limb bud mesodermal cells are separated, reaggregated and placed in an ectodermal jacket, without a discrete polarizing region, moderately good digits form (Patou, 1973) suggests at least that some other mechanism than positional information is involved. We have thus recently proposed that the cartilaginous elements may be specified by a mechanism involving both a prepattern and positional information (Wolpert & Stein, 1984). In this paper we have examined the effect of a signal from the polarizing region on the formation of the humerus to try and test the predictions from these models.

Our choice of the humerus is dictated by the conditions required to duplicate the humerus if it were specified by a wave-like prepattern or a positional signal. For both mechanisms, the results are dependent on the changes in width of the bud, but the predictions are quite different. For a prepattern mechanism duplication requires fitting in an additional wavelength. For the digits, where the wave length is short, only a modest widening is required. Tickle & Stein (personal communication) have found that a widening of about 150 μm is required for each extra digit. However, for the humerus with only a single wave, the limb width would probably have to almost double to accommodate an additional wave.

Previous studies (Smith & Wolpert, 1981) on the effect of a polarizing region on the widening of the limb suggest that this will not occur early enough for duplication of the humerus to take place. Thus the prepattern model predicts that it will be very difficult to duplicate the humerus with a polarizing region graft and it should be relatively unaffected by such a graft.

It is well recognized that with the positional signal model the concentration of the morphogen following the graft of an additional polarizing region is very dependent on the width of the bud (Smith & Wolpert, 1981; Wolpert & Hornbruch, 1981). If the graft is in an anterior position and the limb bud does not widen then the model predicts that the concentration will increase in the centre of the limb and, typically, the radius and digit 2 will be eliminated (Fig. 1). This has in fact been observed and, when widening is prevented, the digit pattern is **4334** (Smith & Wolpert,
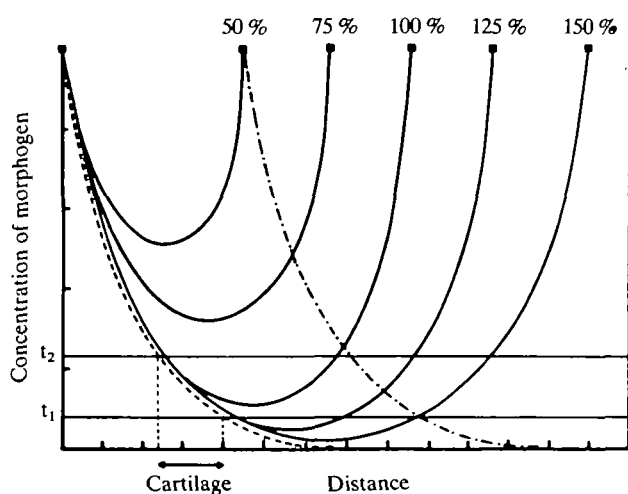

Fig. 1. The concentration of a postulated morphogen produced by the polarizing region, along the anteroposterior axis of the limb (Wolpert & Hornbruch, 1981). The dashed line is assumed to be the distribution in the normal limb. The morphogen is taken to be fixed at a constant concentration in the polarizing region (■) and it diffuses and breaks down. The thresholds for the formation of cartilage are between t_1 and t_2 and the region forming a cartilaginous humerus in the normal limb is shown. The concentration profile when an additional polarizing region is grafted to various positions along the anteroposterior axes on the assumption of differing degrees of widening. The distance between polarizing regions is expressed as a percentage normal bud width. For example, the 150 % curve represents a graft anteriorly followed by a 50 % increase, whereas the 50 % curve is where the graft was central and no widening occurred. In every case the humerus must be eliminated, or duplicated or of mirror-image symmetry of variable width. When the graft is towards the centre of the limb then anterior to the graft a humerus would be expected to develop (—·—·).

1981). In more general terms the positional signal theory requires that, when a polarizing region is placed at different positions along the anteroposterior axis, any element specified by the graded signal will either be duplicated or eliminated in the region between the grafted and the host polarizing regions and, in general, this has been found to be true for the digits (Wolpert & Hornbruch, 1981). This also applies to any part of the element. The reason for this is shown in Fig. 1. Consider first a situation in which the polarizing region is placed in an anterior position. The distribution of the morphogen will depend on the rate of widening and the kinetics of diffusion. It is, however, only necessary to consider three kinds of distribution of the morphogen – when it is all above the thresholds, when part of the distribution is completely below the thresholds and the intermediate case. No other cases are possible. For each of these distributions an altered pattern of humerus development is to be expected. It must be duplicated, or eliminated or be of variable width with mirror-image symmetry. When the polarizing region is grafted near the centre of the limb, the concentration of the morphogen between the polarizing regions would be expected to be substantially higher than when more anterior and the prediction is that the humerus would be eliminated. As the graft is moved more towards the centre an additional humerus would be expected to develop anterior to the grafted polarizing region.

Whilst the position of the presumptive humerus in Fig. 1 has been shown as near the centre of the limb there is evidence that in normal development much of the humerus develops from the anterior half of the limb bud in a position in line with the radius and digit 2 (Hinchliffe & Gumpel-Pinot, 1983). We have very recently confirmed this by constructing double-anterior limbs which develop two humeri lying side by side.

Materials and methods

Fertilized White Leghorn eggs were windowed and staged according to Hamburger & Hamilton (1951). Embryos at stage 21 were taken for donors of the polarizing region which was excised from opposite somite 20. The hosts were stage 16 embryos. It was necessary to graft at this early stage – before even the limb bud is evident – since the humerus is specified at about stage 19 to 20 (Summerbell, 1974). The site for the graft was prepared by removing a cube of tissue of the same size. The graft was kept in place with a platinum wire pin. Grafts were made to different positions along the anteroposterior axis. At about 10 days' incubation the limbs were fixed in 5 % trichloroacetic acid and stained with 0·1 % alcian green in 1 % hydrochloric acid in 70 % alcohol, differentiated in acid alcohol, dehydrated and cleared in methyl salicylate.

Table 1. *The effect on the humerus after grafting a polarizing region to different positions along the anteroposterior axis at stage 16. The grafted polarizing region was taken from stage 21 embryonic wings. The limbs were fixed after 10 days of incubation*

Site of graft (Somite number)	Number	Humerus posterior to graft			Humerus anterior to graft	Duplication of digits
		Normal	Mirror image	Duplicated		
14 and 14/15	10	8	—	2	1	3
15	20	10	8	2	4	12
16	13	4	9	—	5	13
17	7	2	5	—	5	7
18	11	—	11	—	10	11

Results

The development of the humerus following the graft of a polarizing region to different positions along the anteroposterior axis of the presumptive limb at stage 16 is shown in Table 1. Posterior to the grafted polarizing region, that is between grafted and host polarizing regions, the humerus took two main forms. It was either normal or it showed mirror-image symmetry along the anteroposterior axis. (Humeri with mirror-image symmetry could be identified mainly by the proximal and distal epiphysial structures as well as by the overall shape.) In each case it was the posterior margin that was duplicated. In no case was the humerus eliminated. By contrast, the radius was absent in most cases, the ulna was usually duplicated and the pattern of digits, though variable, was often **43234**, **4334** (Fig. 2) or **434** depending, as expected, on the position of the graft (Wolpert & Hornbruch, 1981).

In none of the normal or mirror-image humeri was there obvious shortening or proximal broadening, although the distal epiphysis was often much broader where it articulated with three elements as in Fig. 2B. However, when we compared the length and width of the humerus with the contralateral control limb a clear pattern of differences emerged (Table 2). The humerus was usually about 10 % shorter than normal and the proximal epiphysis was in most cases similarly reduced. The middle of the humerus, the diaphysis, and the distal epiphysis showed much greater variation, particularly when the graft was opposite somites 14/15 and 15, ranging from almost half to more than double the normal. There was a clear reduction in the width of the diaphysis as the polarizing region graft was moved to the centre of the limbs; opposite somite 18 it was almost halved. The distal epiphysis was consistently wider than the proximal.

In four cases there was evidence for duplication of the humerus when the graft was in a very anterior position. One case only, gave a completely reduplicated humerus together with radius and ulna and
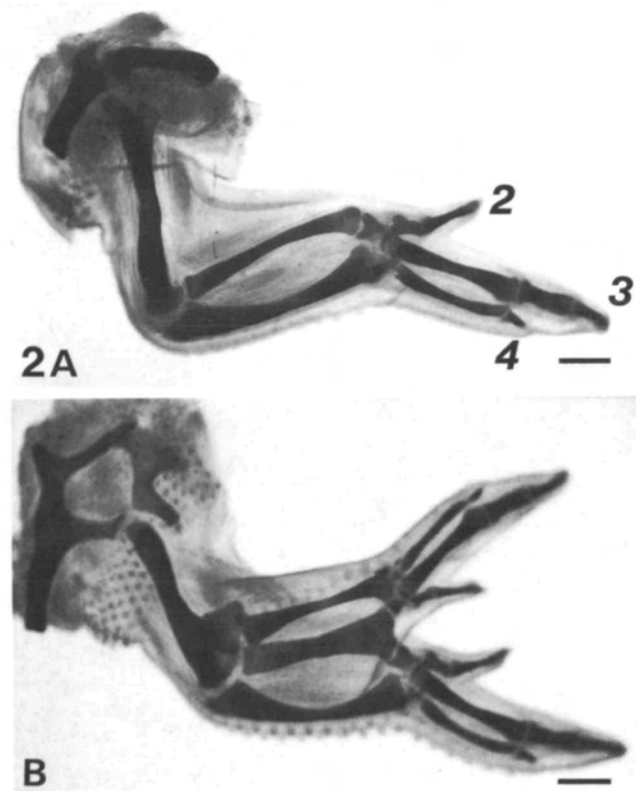

Fig. 2. (A) A normal limb. The anteroposterior pattern of digits is **2 3 4**. (B) A limb that developed after grafting a polarizing region opposite somite 15 at stage 16. The pattern of digits is **432234**. In midarm there is an ulna/mirror-symmetry radius/ulna. Note how the distal end of the single humerus is very broad. Bar, 1 mm.

digits (Fig. 4). In another two, the humerus was only duplicated distally and in one case comprised two humeri lying close together.

Anterior to the grafted polarizing region, an additional humerus often developed (Fig. 3 and Table 1). For example, when the polarizing region was opposite somite 18, i.e. towards the centre of the limb, a humerus developed anterior to the graft in ten out of eleven cases. This humerus formed part of a supernumerary limb, which was usually incomplete,

Table 2. *Mean dimensions of the humerus as percentage (standard deviation) of that on the contralateral side. The normal 14 and 15 refer to limbs without alteration in digit pattern. The limbs measured were wholemounts fixed after 10 days of incubation*

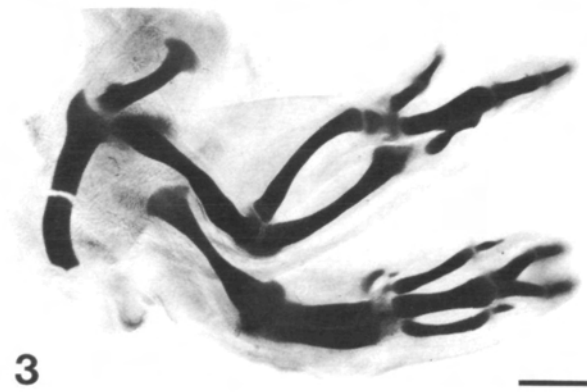| Site of graft (Somite number)	Length	Width		
		Proximal	Middle (Diaphysis)	Distal
14 and 15 (Normal)	99 (2)	95 (7)	105 (11)	99 (7)
14/15	77 (20)	95 (53)	114 (70)	168 (109)
15	81 (12)	122 (45)	121 (37)	197 (53)
16	89 (7)	100 (17)	100 (30)	180 (28)
17	88 (10)	80 (13)	69 (20)	123 (21)
18	89 (8)	89 (17)	62 (13)	130 (21)
All humeri anterior to graft	83 (11)	78 (22)	58 (21)	82 (22)

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Fig. 3. Two limbs that developed following the grafts of a polarizing region opposite somite 18 at stage 16. The anterior one is more or less normal. The posterior limb has a mirror-symmetrical posterior humerus. The digit pattern is **4334** with digit **3** fused in the metacarpal region. The ulna is also duplicated and partly fused. Bar, 1 mm.

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Fig. 4. Two almost complete limbs in mirror symmetry that developed after grafting a polarizing region opposite somite 14/15 at stage 16. The anterior limb lacks only an anterior digit **4**. The humerus is duplicated along almost the whole of its length. Bar, 1 mm.
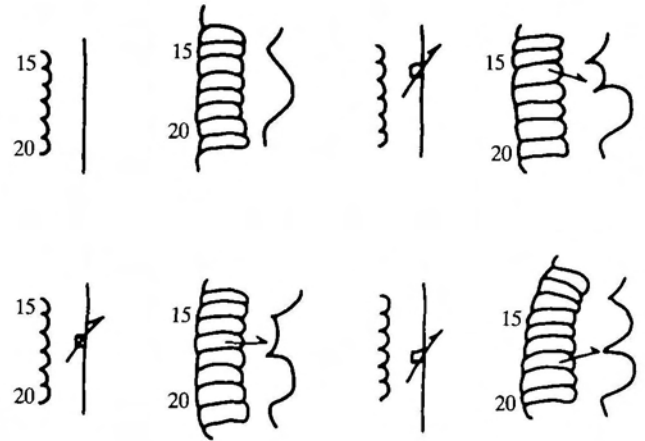

Fig. 5. Camera lucida drawings of typical effects of a polarizing region graft on the shape of the limb bud 24 h after grafting, i.e. at stage 20–21. When the graft is in an anterior position opposite somite 16 little widening occurs, whereas opposite somite 18 the graft splits the bud and there is an overall widening of about 40 %. Only when the graft split the bud did a humerus develop anterior to the graft.

but could be normal. The development of this anterior humerus was related to the splitting of the bud into two regions.

Observations on the limb bud 24 h after the operation showed that the graft of a polarizing region often caused the limb bud to be split into two domains particularly when it was grafted towards the centre of the limb opposite somite 18 (Fig. 5). At the site of the graft the bud was prevented from growing out, which resulted in the formation of a twinned bud. In some cases, the effect was temporary and the two buds fused; in others, it was permanent. There was a very strong correlation between the development of a humerus anterior to the grafted polarizing region and the splitting of the bud into two domains. Only if the

bud was still split into two domains after 48 h did an additional humerus develop anteriorly to the graft. Measurements on these split buds revealed a total widening by about 40 % after 24 h compared to the contralateral control bud. By contrast when the graft was in a more anterior position opposite somite 16 and no splitting occurred the bud only widened about 15 %.

Discussion

The result of grafting an additional polarizing region to different positions along the anteroposterior axis of the limb bud at stage 16 has remarkably little effect on the humerus that lies between the two polarizing regions and thus presents severe difficulties for the positional signal model. The results do not conform to the predictions of Fig. 1. Out of 61 grafts to various positions along the anteroposterior axis none eliminated the humerus and only 4 gave some sort of complete duplication to give a second humerus, even though the pattern of digits varied as expected. 24 results gave a normal humerus and 33 a mirror-image duplication of a single humerus. The mirror-image duplication was predicted by the positional signal model and was associated with the expected variation in width, but the positional model cannot account for the presence of the humerus when the polarizing region was near the centre of the limb and the distance between polarizing regions was substantially reduced (Fig. 5). In this case, the humerus should have been eliminated (Fig. 1). It is striking how little the width of the proximal end is affected. The model also has difficulty in accounting for the proximal end of the humerus being of normal width and the distal end being almost doubled. It cannot be argued that the signal is ineffective at the stage of grafting, as 25 grafts gave an additional humerus in an anterior position.

The results can, however, to quite a large extent, be interpreted if the formation of cartilaginous elements involves an isomorphic prepattern, which interacts by the positional signal. The prepattern mechanism could be generated by a reaction diffusion mechanism (Murray, 1981), which would explain why it is difficult for two humeri to develop within the same domain whereas it is possible to get an additional humerus when the domain is split into two. The reason for this is that to accommodate another wavelength in the region of the humerus requires doubling of the width. A doubling was presumably achieved in the case shown in Fig. 4 where the graft was anterior to somite 15 and the bud was thus sufficiently wide for an additional humerus to form between the graft and host polarizing region. This could also explain the considerable variations in

width of the humerus when the graft was in a very anterior position. However, if the domain is divided into two and each widens then it is still possible to set up a single wavelength in each. For the more distal regions where there are several and shorter wavelengths, much less widening of the bud is required in order for one element to be duplicated. Widening of the bud would also account for the increase in thickness observed at the distal end of the humerus compared to the proximal end. This explanation immediately accounts for the puzzle posed by Slack (1977) in relation to the duplications he found in axolotl limb following what are effectively polarizing region grafts. Cases of total distal duplication occurred where the humerus was not even branched.

If our interpretation is correct, the specification of pattern in the limb bud must be considered in terms of mechanisms involving both prepatterning and positional information, and this may also apply to other systems, particularly where there are a number of similar units in the pattern. For example, in the early development of the insect embryo division into segments appears to involve mechanisms somewhat different to the morphogen gradient that gives the segment its positional character (Russell, 1985; Meinhardt, 1986). Again, the feather patterns in birds require the buds to be both appropriately spaced and named.

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