

## Double anterior chick limb buds and models for cartilage rudiment specification

LEWIS WOLPERT and AMATA HORNBRUCH

*Department of Anatomy and Developmental Biology, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London, W1P 6DB, UK*

### Summary

Most models for the specification of the skeletal elements in the developing limb bud are based on a chemical specification well before overt cartilage differentiation. By contrast, a physico-mechanical model proposes that the process of condensation – an early feature of cartilage differentiation – is itself the basis for patterning the elements. The models thus make quite different predictions as to when the rudiments are specified. Double anterior limb buds have been constructed at stages earlier than condensation, with the expectation that, if specification of the humerus occurs before cartilage condensation, then limbs containing two humeri should develop, since the presumptive humerus lies largely in the anterior region. The development of

anterior and posterior parts, on their own, was in general, consistent with the fate map; both developed a humerus that was thinner than normal. Double anterior limbs developed two humeri in 28 % of cases and a much thicker humerus in 39 %. These results strongly support models based on an early specification of limb rudiments and cannot be accounted for by the physical model. Double anterior limbs in which the two parts were from different stages, developed such that a digit 3 could lie adjacent to the radius, giving further striking evidence for early specification and local autonomy of development.

Key words: cartilage, chick, limb, model, specification.

### Introduction

Most models for the development of the skeletal rudiments in the developing chick limb bud are based on early specification of the cartilage elements by some sort of cell-to-cell interactions involving chemical signals, well before overt differentiation of cartilage. It is assumed that there are signals that specify which cells in the mesenchyme will form cartilage irrespective of whether the mechanism involves a positional signal (Summerbell *et al.* 1973; Tickle *et al.* 1985) or a prepattern (Wilby and Ede, 1975; Newman and Frisch, 1979; Wolpert and Hornbruch, 1987). By contrast, a very different sort of model has recently been proposed which is based on mechanical forces acting at the time of cartilage differentiation (Oster *et al.* 1983, 1985).

One of the earliest indications of cartilage formation in the limb bud is the condensation of the cells in the region of the early cartilaginous element, the cells coming closer together and adopting a different shape (Rooney *et al.* 1984). In the mechanical model, this condensation is seen as the primary event in the formation of the cartilage rudiment; the rudiment forms as a result of the forces arising from degradation of the extracellular matrix together with cell traction forces (Oster *et al.* 1985). An analysis of the physicochemical situation claims to show that a pattern of cartilage

condensations, resembling those in the limb, will develop. A weakness of the model is that it does not specifically deal with the problem of why only the cells that mechanically undergo condensation form cartilage; the implication is that change in cell shape alone is sufficient to specify cartilage. This unlikely hypothesis is further weakened by our recent demonstration that the relationship between cell shape and cartilage differentiation is rather tenuous (Gregg *et al.* 1989). We have thus tried to devise an experiment to test the two classes of model.

The chemical model – whether or not it is based on positional information or prepattern (Wolpert and Stein, 1984) – requires that the cartilage cells are specified *before* condensation and that condensation is merely an early manifestation of cartilage differentiation. By contrast the physical model assumes rudiment specification to result from condensation and thus specification must occur at this time. A test of the different models is to find out when specification occurs. The conventional view, based on apical ridge removal, is that the humerus is laid down at about stage 18 and the radius and ulna at about stage 20 (Summerbell, 1974). However, condensation in the humerus begins at stage 24 and the radius and ulna at stage 25/26; that is some 24 h later. The large interval between the time when specification by a chemical

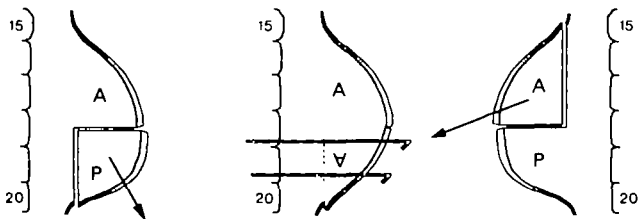
mechanism is assumed to occur and the time at which the physical model operates, offers the possibility of a relatively simple test. If it could be shown that the specification does in fact take place prior to condensation then the physical model can be excluded.

We have constructed double anterior limbs at stages of limb development prior to the condensation. Our expectation was that if specification of the humerus occurs before condensation then such limbs will contain two humeri since the humerus lies largely in the anterior half of the limb.

**Materials and methods**

Fertilized eggs from a local breeder were incubated on stationary shelves at 38±1°C in a humidified atmosphere. Operations were performed on stage 19–22 embryos (Hamburger and Hamilton, 1951). Host and donor were always of the same stage, unless specifically mentioned. The graft tissue was the anterior part of a left wing replacing the posterior half of the host right wing, to create a mirror-symmetrical double anterior wing (Fig. 1). To get access to the left wing the embryo was carefully turned over. Grafts were held in place with platinum wire. Three levels were chosen for the plane of symmetry: the junction between somites 17 and 18, the midpoint of somite 18, and the junction between somites 18 and 19. The midpoint of somite 18 corresponds to the midline of the limb bud, so limbs constructed at this level were of normal width whereas those at somite 17/18 were slightly narrower and those at somite 18/19 slightly wider. The apical ridge is much less prominently developed on the anterior half of the limb bud. In a few experiments, very different stages of anterior halves were joined together, for example a stage 24 anterior half was joined to a stage 19 host.

To determine the development of the anterior parts used in the operation, posterior parts were removed at different



**Fig. 1.** Construction of a double anterior limb. The anterior part, A, of a left wing is grafted in place of the previously removed posterior part, P, of a right wing bud.

somite levels and allowed to develop on their own. In addition, posterior parts of the left wing from which the anterior half was taken, were also allowed to develop. Embryos were killed at day 10 of incubation and the wings were fixed in 5% TCA, stained in 0.1% alcian green, dehydrated in alcohol and cleared in methyl salicylate as whole mounts.

**Results**

The development of the anterior part of the limb bud after removal of the posterior part gave quite consistent results (Table 1). Anterior parts from stage 19 failed to develop. Anterior parts prepared from stages 20–22 at somite level 17/18 developed a thinner humerus, which on average measured 65% of the width compared to the contralateral humerus of the same embryo, and the radius was missing or shortened by up to 50%. The average width of the humerus developing from anterior parts from levels 18 and 18/19 for stages 20–22 was 75% and 85%, respectively, and the average length of the radius was 90% and 95%. At stage 22 and somite level 18/19, some anterior parts developed digits. The posterior parts typically developed humerus, ulna and digits 3 and 4, (Table 2) the humerus and ulna being thinner by about 20% and 10% respectively (Fig. 2B).

The 98 double anterior limbs that developed gave quite a range of results but there were some common features. In general, the limbs were truncated at the level of the wrist or more proximally; occasionally a digit 2 developed and more rarely a digit 3. Attention was focused on the development of the humerus (Table 3). 28% of the limbs had two proximal elements which could be up to 40% shorter than normal but could also be of normal length (Fig. 2D,E). Another 39% had a thickened proximal element and in this case the humerus was broader than normal. The epiphysis could be 20% broader, and the diaphysis could have its width increased 50%. In those cases where the humerus was shorter by up to 40%, the diaphysis could have a threefold increase in width (Fig. 2F). In some cases, two humeri were present proximally and fused more distally to give a single thickened element (Fig. 2E). In general, the later the stage of development and the more posterior the somite level, the more often was the humerus either double or thickened. Distal to the

**Table 1.** Development of anterior limb bud parts from which the posterior region has been removed

Level of excision (somite)	Number of limbs containing different elements		
	Stage 20	Stage 21	Stage 22
17/18	2H 1HR 2 no development	5H (incomplete)	2H (incomplete) 3HR (radius short)
18	4HR 1HUR	1H 8HR	3H 5HR
18/19	7HR	5HR 1HR2	7HR2 2HUR2 3

The number preceding each entry is the number of cases. H, humerus; R, radius; 2 and 3, are digits.

**Table 2.** Development of posterior limb bud parts from which the anterior portion has been removed

Level of excision (somite)	Number of limbs containing different elements			
	Stage 19	Stage 20	Stage 21	Stage 22
17		4HU2 3 4	1HU2 3 4	
17/18	2HU3 4 1HU2 3 4			
18	1HU3 4 1HU2 3 4	8HU3 4 5HU2 3 4	9HU3 4 2HU2 3 4	2H 2HU3 4
18/19	2HU3 4 1U4			

The number preceding each entry is the number of cases.  
H, humerus; U, ulna; 2, 3, 4, are digits.

**Table 3.** The development of the humerus in double anterior limbs

Level of junction (somite)	Number of limbs			
	Stage 19	Stage 20	Stage 21	Stage 22
17		3 no development 2H (truncated) 1H	2 no development 2H	
17/18	2H* 13H 2HH	6H 2HH	6H 1HH	1H 6H 1HH
18	6H 1H 1HH	4H 1H 1HH	1H 2H 5HH	1H 5H 3HH
18/19	2H 2H 3HH	1H 2H 4HH	2H 4H 2HH	2H 3HH

The numbers preceding each entry is the number of cases.  
H is a thickened humerus and HH represents two humeri.  
\*These developed as normal limbs.

humerus there could be a single thickened radius, sometimes truncated, or two radii, sometimes thinner than normal (Fig. 2D,C). Otherwise, there were no striking differences between limbs constructed at stage 19 to 22 though there was a tendency for the later stages and limbs constructed at level 18/19 to have more elements.

Two grafts made at stage 19 at level 18 developed into normal limbs.

Of the 12 limbs constructed between anterior portions of stage 19 and stages 23 to 25, 8 limbs developed with digits distal to a humerus and radius. In four cases, digits developed immediately adjacent to the radius, all contained digit 3 (Fig. 2G) and two had a digit 2 as well.

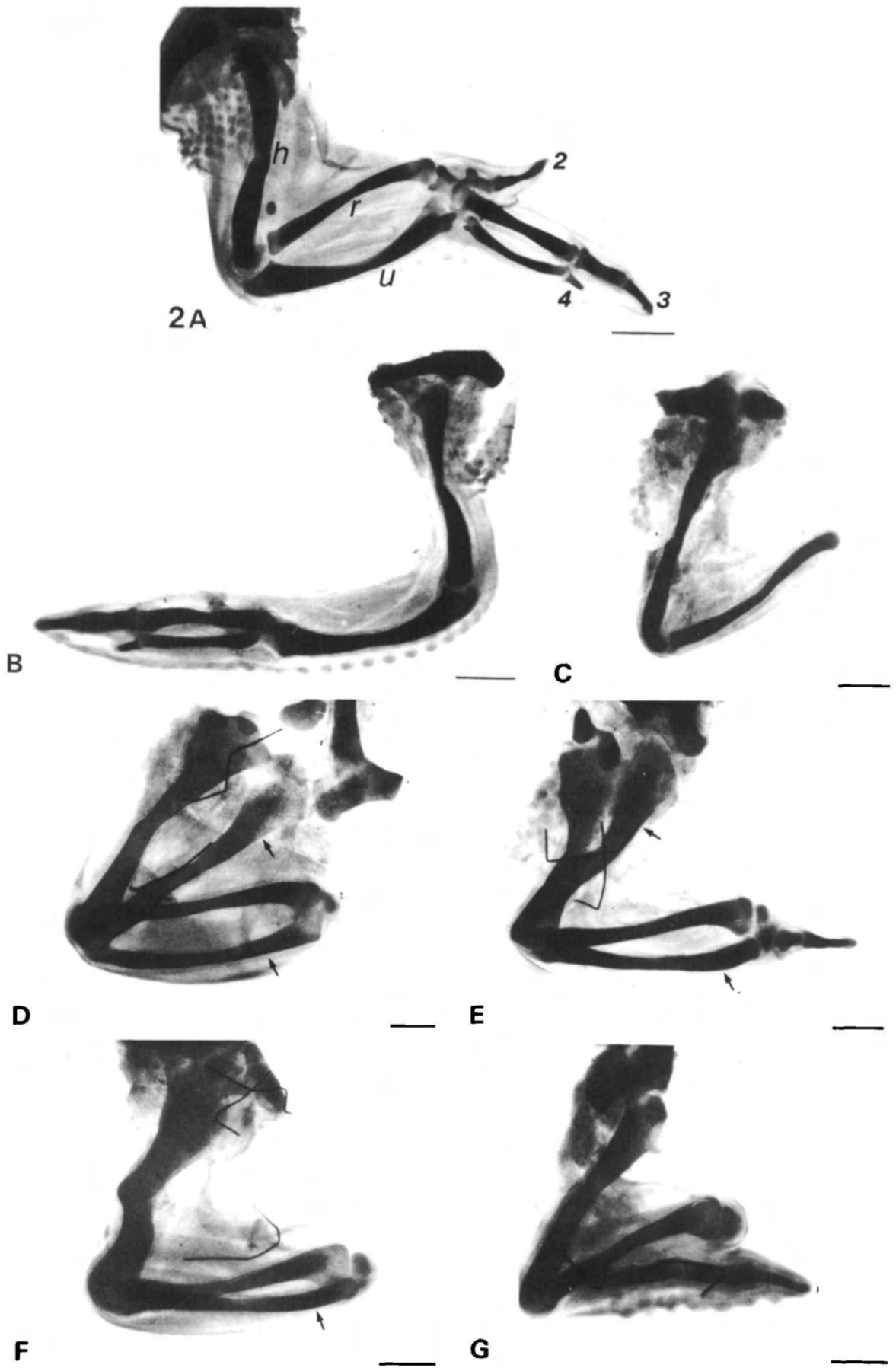
## Discussion

Anterior parts on their own developed a humerus and radius, and the posterior parts on their own developed a humerus, ulna and, unlike the anterior halves, digits. These results are similar to those obtained by Hinchliffe and Gumpel-Pinot (1981), who attributed the failure of

the anterior half to develop distal structures to the role of the polarizing region at the posterior margin in maintaining the apical ridge. The results are consistent with the idea that each part develops more or less autonomously with respect to a fate map in which the radius develops from the anterior part of the wing bud, the ulna from the posterior part, and that both parts contribute to the humerus (Hinchliffe and Gumpel-Pinot, 1981). If that were so and if there were early specification of the humerus and radius, well before cartilage condensation, then it would be expected that double anterior limbs should have a double humerus and radius in mirror image apposition.

The development of the double anterior limbs was found to be variable and 28% of limbs had two proximal elements, and these were often thinner than normal. In 39% of cases, the humerus was significantly thicker than the normal humerus (Table 3).

These results strongly support the idea that specification of the humerus occurs many hours before cartilage condensation presumably by chemical signals involved in cell-to-cell interactions. The development of two proximal elements is inexplicable in terms of the physical model. The shape of the limb bud is essentially



**Fig. 2.** (A) Whole mount of a normal 10-day-old embryonic chick wing stained with alcian green. h, humerus; u, ulna; r, radius. Digits 2, 3, 4. (B) Development of the posterior half of a left wing after the removal of the anterior part for grafting at level mid somite 18 at stage 21. The humerus is thinner and radius and digit 2 are absent. (C) Development of the anterior part of a right wing after the removal of the posterior part of the wing bud at somite level mid 18 at stage 21. Only humerus and radius develop both of which are thinner. (D) Double anterior wing constructed from two stage 22 wing buds at somite level 17/18. There are clearly two separate humeri and two radii. Arrows show the host's skeletal elements. (E) Double anterior wing constructed from two stage 22 wing buds at somite interface 18/19. Arrows indicate host's skeletal elements. Note the two humeri proximally which fuse just distal to their diaphyseal region; there are two separate radii and a common digit 2. (F) Double anterior wing constructed from two stage 21 wing buds at somite level 18/19. Here we see a complete fusion of both humeri into one element and two separate radii. Arrows indicate the host radius. (G) Double anterior wing constructed by grafting a stage 24 anterior half tip to a stage 19 anterior right half. The host part has developed a humerus and a radius (compare with Fig. 2C) while the graft has developed autonomously into a digit 3. Scale bars are 1 mm.

unaltered though it may be broader or narrower, by no more than 20%, and yet two elements develop rather than one at the time of cartilage condensation. Increasing or decreasing the width of the early limb bud along the anteroposterior axis has no effect on the pattern of the limb provided the polarizing region is present (Yallup and Hinchliffe, 1983). The physical model similarly cannot account for the large number of cases in which the humerus is thicker than normal. However, a thicker humerus would be the expected consequence if the presumptive humeri from each half were already specified and fused in the region where the graft was made. If the humerus is specified at an early stage and the specification is stable and has some capacity for regulation (Hornbruch, 1980), a satisfactory explanation of the results can be provided. From Table 1, it is seen that a humerus will develop in an anterior fragment terminating at level 17/18; that is inside the anterior half if somite 18 is regarded as the middle of the limb bud. This would account for the small number of double humeri found in double anterior limbs constructed at this level which is only 16% compared to 32% and 44% at levels 18 and 18/19 respectively. With these larger double anterior limbs there is less chance of the two presumptive humeri fusing to give a single humerus.

The development of a digit 3 in direct contact with the radius (Fig. 2G) is a remarkable demonstration of local autonomy that persists when anterior parts at different developmental stages are joined together. It is remarkable that their development is quite unaffected by the abnormal adjacent element. However, the development of a digit 3 and not a digit 2, as would be expected from the fate map of an anterior part may be the result of the influence of latent polarizing activity as

discussed below. In the majority of cases, the older part, which formed digits, took up the more distal position.

The absence of distal elements in both anterior wings and the double anterior limbs is most likely due to the lack of a prominent apical ectodermal ridge: the ridge is naturally less developed in anterior parts and, in addition, anterior parts lack a polarizing region which probably maintains the ridge (Hinchliffe and Gumpel-Pinot, 1981). The development of two normal limbs from double anterior parts, constructed at stage 19, may be due to the presence of polarizing activity in the flank at the posterior margin of the limb bud (Hornbruch and Wolpert, 1990 in preparation).

We conclude that cartilage specification occurs well before condensation and once specified is quite stable with respect to removal of adjacent regions. Condensation itself is not involved in any way in the specification of the elements themselves and may merely represent an early phase of cartilage differentiation in which hyaluronic acid surrounding the cells is degraded and cartilage matrix starts being produced. Physical forces very likely do play a role in the further development of the rudiments particularly in relation to cell orientation and the development of the perichondrium (Archer *et al.* 1983).

The early and stable specification of the cartilage elements still requires an explanation of the considerable capacity of the limb bud to regulate these patterns when parts of the early limb bud are rotated or removed, provided there is a continuous set of proximo-distal positional values (Wolpert *et al.* 1975).

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