Interaction between the proximo-distal and antero-posterior co-ordinates of positional value during the specification of positional information in the early development of the chick limb-bud

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SUMMARY

The experiments examine the extent of reduplication of skeletal parts across the antero-posterior axis, following the transplantation of a zone of polarizing activity (ZPA) to the anterior margin of the limb-bud at successively later stages. Previous studies have suggested that the function of the apical ectodermal ridge (AER) is to maintain cells in a special region at the distal tip (the progress zone) labile, with respect to their positional value along the proximo-distal axis. Similarly, the results of these experiments demonstrate that cells in the progress zone are able to change their antero-posterior positional value under the influence of the grafted ZPA, while cells at more proximal levels remain unaffected. In turn, the ZPA may effect the activity of the AER and hence the progress zone.

INTRODUCTION

The early development of the chick limb involves three processes: differentiation, pattern formation and growth. The relationship between the first two of these is clearly very direct, and in fact, changes in the character of a previously undifferentiated cell provide us with our only indication of events occurring during pattern formation. Although a number of useful models exist for pattern formation in non-growing systems – e.g. the insect epidermis (Lawrence, Crick & Munro, 1972) and hydra (Wolpert, Hornbruch & Clarke, 1973) – the relationship between growth and pattern formation has proven more difficult to understand. Recently we have proposed a model in which the process of pattern formation along the proximo-distal axis of the limb is explicitly dependent on growth (Summerbell, Lewis & Wolpert, 1973). Briefly, there is a special region at the distal tip of the limb – the progress zone – in which autonomous changes of positional value occur with time. The appearance and extent of the progress zone are specified by the presence of an apical ectodermal ridge (AER). During outgrowth of the limb, cells leave the progress zone at its proximal boundary,

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and are then fixed at the positional value which they had achieved during their sojourn in the zone. This results in a graded series of positional values along the proximo-distal axis, which may be interpreted by the cells in terms of differentiation into the appropriate tissue types. The model explains only the proximo-distal organization of the limb. If the three-dimensional pattern of differentiated cells is to be determined by positional information, it is necessary to specify positional value along the other two axes: dorso-ventral and antero-posterior. Very little is known about the first of these axes, although recently a mechanism has been proposed by Caplan & Koutroupas (1973). With regard to the antero-posterior axis, much experimental work has been carried out concerning the action of the ZPA (zone of polarizing activity; see review by Saunders & Gasseling, 1968), while Wolpert (1969) reinterpreted the available data in terms of the specification of the antero-posterior co-ordinate of positional information. The ZPA, a specialized region of mesenchyme at the posterior lateral edge of the limb close to the body wall, when grafted (Fig. 1) to a site on the anterior lateral edge of a host limb-bud, causes the limb to develop with duplicated distal parts; the twinned elements are mirror images of one another, with opposite anterior-posterior polarity.

It is important to discover the extent of interaction between the axes. If the AER has the property of conferring lability with respect to proximo-distal positional value on cells within the progress zone, it seems plausible to suggest that it might similarly be responsible for allowing cells to respond to some signal from the ZPA. The simple prediction is that when a ZPA is grafted into a host limb-bud, cells within the progress zone can modify their antero-posterior positional value, while the proximal cells which have left the progress zone remain fixed with respect to both proximo-distal and antero-posterior axes. We should therefore find that when the graft is made to a young host, reduplication occurs from a more proximal level than when it is made to an older host. Evidence supporting this hypothesis would be of particular significance to the whole concept of positional information. It would provide the first described example of what one would expect to be a common feature of developing systems – specification of pattern by a two-dimensional co-ordinate system in a single field. Furthermore, it follows from our model (Summerbell et al. 1973) that the size of skeletal elements is governed by the number of cells in the progress zone. It is therefore of interest to discover whether the secondary axis has its size determined with respect to the host or to the donor norm. This might provide us with information about the relationship between the ZPA and the AER. Does the existing host ridge provide the progress zone for the secondary axis, or does the grafted ZPA cause the host to form a new AER? It is also proposed to examine the consequence of grafting a ZPA to a host limb-bud from which the anterior AER has been removed. It is expected from the model that such operations should not cause the formation of a secondary axis, because of the absence of any structures able to bring about formation of a progress zone.
Fig. 1. The ZPA is excised from a stage 21 or 22 limb-bud, transfixed with a platinum pin, and grafted to a prepared site on the host limb at stages 16–24.
METHODS

Fertilized White Leghorn embryos were incubated at 38 °C and windowed on the third and fourth days of incubation. Host embryos were prepared from stages 16 to 24 (Hamburger & Hamilton, 1951) by excising a small portion of mesenchyme and ectoderm from the anterior lateral edge of the limb-bud near to the body wall (for stages 19–24), or from the presumptive limb mesenchyme level with somite 15 (for stages 16–18) or from the anterior lateral edge near to the distal tip (for stages 22–24). Donor embryos were prepared from stages 21 and 22, when the position of the ZPA is clearly characterized (Saunders, 1972). Part of the ZPA, equivalent in size to the tissue removed from the host, was excised from the posterior lateral edge of the limb-bud, next to the body wall, and transfixed with a platinum pin. The graft was then removed to the host egg and pinned to the previously prepared site (Fig. 1). In a few cases quail embryos were used as donors. Host and donor eggs were then returned to the incubator. In some cases, following the graft, a further operation was carried out. The host egg was removed from the incubator between 1 and 3 h later and the anterior or whole AER surgically excised from the operated limb (Fig. 2). The eggs were then returned to the incubator for a further 7 days, when both host and donor eggs were removed and the embryos sacrificed.

Operated (right) and control (left) wings of hosts and donors were removed from the embryo, fixed in 5 % trichloracetic acid, stained in 0·1 % alcian green 8GX in 1 % hydrochloric acid in 70 % alcohol, dehydrated, and cleared in methyl salicylate. Operated and control limbs were examined under a Zeiss Stereo IV dissection microscope, photographed and the lengths of the humerus, ulna, radius and digit III measured, according to the method described previously (Summerbell & Wolpert, 1973).

RESULTS

There is a strong correlation between the stage of the host embryo at the time of operation and the most proximal skeletal element reduplicated in the secondary axis. At stage 16 it is possible to produce limbs with a reduplicated humerus; by stage 19 the most proximal reduplicated element is of the level of the ulna/radius, and by stage 23 only elements of the hand are found in the reduplicated axis (Fig. 3, Table 1).

At later stages (22–24), grafts were made at two different sites on the host limb. Those near to the body wall failed to cause the formation of any additional skeletal elements. In contrast, grafts to a more distal position were able to bring about the duplication of distal elements.

In the second series of experiments, grafting of the ZPA was followed by extirpation of the AER (Fig. 2). When the entire ridge was removed, no distal parts were formed. When only the anterior part of the AER was removed, a normal limb resulted—except that occasionally, anterior skeletal elements (radius or digit II) were absent.
Fig. 2. The ZPA is excised from a stage 21 or 22 limb-bud, transfixed with a platinum pin, and grafted to a prepared site on the host limb at stage 18 or 19; 1–3 h later part or all of the apical ectodermal ridge is surgically removed from the host.

Table 1. Reduplication of skeletal parts of chick limb, following transplantation of a ZPA to the anterior margin of the limb-bud at successively later stages

<table>
<thead>
<tr>
<th>Stage</th>
<th>Humerus</th>
<th>Distal humerus</th>
<th>Ulna/ radius</th>
<th>Distal ulna/ radius</th>
<th>Wrist</th>
<th>Hand</th>
<th>Distal hand</th>
<th>Normal</th>
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The lengths of the primary (normal) and secondary (reverse polarity) ulnas and digits III were compared against the lengths of the equivalent host and donor control side limbs (see Summerbell & Wolpert, 1973). The results were very similar for both skeletal elements. At stages 16 and 17 the primary and secondary
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axes both conformed to normal host proportions. The frequency distribution for the percentage difference between host operated and host control limbs peaked strongly in the 'no significant difference' range for both primary and secondary axes (Fig. 4A). At stage 18, elements of the secondary axis tended on average to be fractionally shorter than those for the host control limb, though individual cases were usually not significantly different from the control mean. The primary axis was of normal length. The frequency distributions for both axes are shown in Fig. 4B. By stage 19 the elements of the secondary axis were usually significantly shorter than those of the host control axis. The frequency distributions for both axes are shown in Fig. 4 C. Neither axis of an operated limb was ever longer than that of the control side.

Although the donor embryos were always taken from the same stage and the grafts were transferred into hosts at various different stages, the secondary axes were always more in proportion to those of their host limbs, even in the cases when the ZPA grafts were taken from quail embryos.

DISCUSSION

When the ZPA is grafted at successively later stages to the anterior edge of a host limb, there is a clear proximo-distal sequence of loss of ability to form a secondary axis. This may readily be interpreted by supposing that only tissue in or near the progress zone can respond to the signal from the polarizing region. The later the stage of the host when the polarizing region is grafted, the more distal the level where reduplication starts. In fact, we find that the parts which are reduplicated after the graft are roughly the same as the parts which would have been lost following apical ridge removal at a time about two stages later (D. Summerbell, unpublished observations) and the parts whose antero-posterior organization is unaffected by the graft are the same as the proximal parts which would develop normally despite apical ridge removal at that slightly later stage. Thus, if we suppose that this time lag is accounted for by the time

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**Figure 3**

Photomicrographs of whole limbs fixed on the tenth day of incubation, stained with alcian green 8 GX, and cleared in methyl salicylate.

(A) Stage 16 host, humerus is reduplicated about its midline.
(B) Stage 17 host, distal epiphysis of humerus reduplicated.
(C) Stage 19 host, twin ulna plus radius.
(D) Stage 21 host, ulna and radius normal, reduplicated hand.
(E) Stage 23 host, reduplicated digit III distal to digit II.
(F) Stage 24/25 host, reduplicated distal digit III.
(G) Normal limb: humerus, ulna, radius, digits II, III and IV.
(H) ZPA graft followed by excision of anterior AER: loss of radius and digit II.
Fig. 4. Frequency distributions showing the number of embryos in which there was a given percentage difference between the lengths of operated (right) and control (left) skeletal elements (ulna or digit III). Each histogram compares elements of the primary host (stipple) against the host control side skeleton; and the secondary, reduplicated, elements (heavy line) also against the host control side. The brackets represent the normal range of variation found between left and right skeletal elements in normal chicks (99% of all cases). (A) Stage 16/17: primary and secondary ulna and digit III are both of normal host length. (B) Stage 18: primary ulna and digit III are both of normal host length; secondary (reduplicate) ulna and digit III are on average slightly shorter than normal host length. (C) Stage 19/20: primary ulna and digit III are both of normal host length; secondary (reduplicate) ulna and digit III are on average markedly shorter than normal host length.
taken for the graft to heal in and for the hypothetical signal to be transmitted to the distal tip, it appears that tissue just beneath the apical ridge, i.e. in the progress zone, is labile with respect to both proximo-distal and antero-posterior character, while more proximal tissues are fixed with respect to both.

When the ZPA is grafted to a proximal level of a late limb, no reduplication occurs. This fact emphasizes that the ZPA affects only the progress zone and not proximal tissues, and that the distance over which it may exert its influence through proximal tissues is limited.

The experiments involving the grafting of a ZPA followed by extirpation of the AER fully support these conclusions. In the absence of the AER, the progress zone loses its special character and cells at the tip are fixed at the level which they had achieved at the time of operating; at the same time they lose their ability to respond to the signal from the ZPA. Neither distal parts nor reduplicated proximal structures are formed.

We must examine the factors governing the lengths of skeletal elements. We make two additional assumptions, both of which we intend to discuss in detail elsewhere. (1) There are no regulatory mechanisms controlling element length at late stages of development. This would imply that the length of an element is determined by the number of cells originally specified with positional values appropriate to the element. (2) The rate of spontaneous change of positional value in the progress zone is proportional to the rate of cell division.

Let us consider specification of an element: cells leave the progress zone with successively more distal values, corresponding to successively more distal levels. The total length of the element will be dependent on the total number of cells crossing the boundary during this period. This, in turn, is governed by the number of cells in the progress zone at that time (J. H. Lewis & D. Summerbell, unpublished observations) and is independent of the rate of cell division, as we have made rate of change of positional value proportional to rate of cell division. So, if we have an abnormally small progress zone, then fewer cells will be specified at a given level, and the size of the element will be shorter. When a ZPA graft has been carried out, the proportions of the secondary elements along the proximo-distal axis are quite independent of the proportions of the donor control limb. If the graft is carried out on a young host (pre-stage 19), there is a marked similarity between the lengths of elements in host control and both primary and secondary axes of the graft. When the graft is carried out at later stages, the secondary axis is reduced in size. This suggests that at later stages there is a smaller progress zone in the anterior half of the limb-bud. However, this poses the question as to how the ZPA graft causes the formation of a normally-sized progress zone in the anterior half, if grafted sufficiently early. There are two possibilities. (1) If the density of cell packing was high centrally and fell off towards the anterior edge of the limb-bud, then fewer cells would leave the progress zone in a given time. If the ZPA graft modified the cell packing so as to raise the density to a level comparable with the central area, then reduplicated
elements would be identical in size to the original host elements. We suggest no mechanism whereby this increase in density could be obtained. (2) If the progress zone is widest centrally and is progressively narrower towards the anterior margin, then again, in the normal limb, for a given time interval, comparatively few cells will leave the anterior portion. One of the effects of a ZPA graft would have to be to increase the width of the anterior part of the progress zone. In this case it is easier to suggest a possible mechanism. It has previously been reported that the AER is most ‘thick and active’ just posterior to the midline (Zwilling, 1955, 1956, 1961) while many workers have reported an increase in the thickness of the anterior AER following a ZPA graft (see Saunders & Gasseling, 1968; Amprino, 1965). We suggest that grafting the ZPA causes the formation of a thick AER, which in turn is able to maintain a wider progress zone and hence to specify larger skeletal elements. We are, however, unable to make any compelling suggestion as to how grafting a ZPA to a young host results in reduplicated elements of normal host length, while a graft to an older stage gives comparatively shorter reduplicated elements.

In conclusion, I should like to emphasize the close interaction between antero-posterior and proximo-distal axes of the developing chick limb-bud. The ZPA appears to have responsibility for the assignation of positional value along the antero-posterior axis of the limb. It also seems to be implicated in the formation of an active AER. The AER first appears at stage 18, just after the first detectable appearance of polarizing activity, and is asymmetrical about the midline in respect of both morphological and functional characteristics. In turn, it seems that the apical ectodermal ridge – and therefore the progress zone – allow changes in positional value along the proximo-distal axis, and also a labile response to the signal from the ZPA.

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REFERENCES


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