Development 99, 99–108 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

The effect of the zone of polarizing activity (ZPA) on the anterior half of the chick wing bud

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Summary

Removal of the posterior half of the chick wing bud between stages 17–22 results in failure of the anterior distal tissue to survive and differentiate. This observation has been interpreted in terms of a requirement by the anterior half of a factor supplied by the posterior half of the limb containing the zone of polarizing activity (ZPA). This relationship has been tested by grafting ZPA tissue to the posterior surface of the anterior half after posterior half removal. Grafts made proximally on the cut surface did not significantly improve survival and development, nor did the ZPA prevent the expansion of the cell death in the ANZ beyond its normal boundaries into the distal mesenchyme. However, when grafted distally the ZPA inhibited cell death in the apical mesenchyme and caused the anterior mesenchyme to change its normal prospective fate (radius and digit 2). In all these cases, in addition to digit 2, digit 3 and frequently also digit 4 differentiated. The anterior half went on to develop a full set of digits and zeugopod parts in almost 50 % of cases, although no skeleton resulting from this regulation of the anterior half had totally size regulated. These results demonstrate a developmental 'rescue' effect by the ZPA, and further support the view that the ZPA has a central and unique function in normal limb bud development, controlling survival and differentiation of the mesenchyme along the anteroposterior axis.

Key words: ZPA, wing bud, regulation, cell death, chick.

Introduction

The grafting of the zone of polarizing activity into the chick wing bud and the subsequent duplication of the digital skeleton is a classic experiment in the analysis of control of pattern in development. But the role of the ZPA in normal development remains a subject for debate. According to one group of workers, using barrier and ZPA amputation techniques, the ZPA is indispensible for normal limb bud development (Summerbell, 1979; Summerbell & Honig, 1982; Hinchliffe & Gumpel-Pinot, 1981; Hinchliffe, Gumpel-Pinot, Wilson & Yallup, 1984). By contrast Saunders and Fallon and their co-workers (Fallon & Crosby, 1975; Saunders, 1977; Saunders & Gasseling, 1983) criticize this view, for example Rowe & Fallon (1981) consider that the ZPA has no unique role in normal development or at least ceases to exert any continuing influence after very early stages of limb bud development (stage 17, Hamburger–Hamilton). Another interpretation of wing bud development using the polar coordinate model (French, Bryant & Bryant, 1976) considers that existing positional values in the wing bud are stable (Iten, Murphy & Javois, 1981) and assigns no overriding importance to the ZPA.

Evidence for a role of the ZPA is provided by amputation experiments, which show a relationship between cell death in the subapical mesenchyme and the removal of the ZPA. Amputation of the entire ZPA (for example, by deleting the entire posterior half of the wing bud) results in substantial cell death in the distal mesenchyme of the remaining part of the wing bud. But leaving part of the ZPA *in situ* results in the survival of this distal mesenchyme and the normal development of the wing skeleton (Hinchliffe &

Gumpel-Pinot, 1981; Hinchliffe *et al.* 1984). One way of showing that this effect is due specifically to the ZPA rather than for example to experimental interference with the vascular supply when large slices of limb bud are removed, is to show that the amputation-induced cell death can be inhibited or reversed by ZPA grafting. The experiments reported here examine the developmental fate of the anterior half when the posterior half with the indigenous polarizing region is removed and a ZPA is grafted to the anterior half.

This experiment examines three important aspects of the ZPA control question. The first is the regulatory capacity of the anterior half of the wing bud, and its ability to compensate for the loss with the posterior half of the greater part of the prospective skeleton (Hinchliffe *et al.* 1984) and to give rise to a normal wing from a reduced cell number.

The second aspect examined, by different positioning of the ZPA grafts, is the relationship between the apical ectodermal ridge (AER) and the ZPA. Tickle (1980) showed that, to be effective in limb duplication, a ZPA graft has to be adjacent to the AER. In experiments on reaggregated ZPA cells, she found that the pellets were much more effective if placed directly under the AER rather than using the classical method of placing them in a hole excavated at the preaxial end of the AER. Our own experiments varying the ZPA positions are designed to examine whether close ZPA–AER contact is necessary for expression of polarizing activity, while the quail ZPA grafting experiments are intended to discover whether the ZPA continues to maintain contact with the posterior AER in subsequent wing development.

The third objective is to examine the ZPA role in normal wing bud development. Much of the analysis of ZPA action has been carried out by grafting the tissue in an otherwise intact wing bud to the classical preaxial site. Such preaxial grafting experiments are interpreted in terms of the interaction of the two polarizing regions i.e. those of the host and donor (Tickle, Summerbell & Wolpert, 1975). In our experiments, removal of the host ZPA and the greater part of the prospective wing skeleton in posterior half amputation provides the opportunity to examine the developmental consequences of a grafted ZPA on the anterior half without the complication of the action of the host ZPA, so that this experiment is better able to examine the *normal* action of the ZPA.

In a second series of experiments the effect of grafting a ZPA to the distal tip of a wing bud from which the anterior half had been removed was examined. Previous work has shown that midaxial grafts of ZPA tissue cause duplication of the wing skeleton by initially causing the preaxial AER to thicken, thus expanding the total size of the limb field in which additional skeletal components can then be specified (Saunders & Gasseling, 1968; Summerbell, 1974; Tickle, 1980). In our experiments the grafted ZPA in close proximity to the indigenous host ZPA, and in the absence of the anterior half of the wing, would be expected on the basis of the ZPA–morphogen profile hypothesis to produce a digit **434** pattern from the posterior half in high frequency, and this prediction was tested.

Materials and methods

Chick eggs were windowed according to the technique of Summerbell & Hornbruch (1981). Host and donor embryos between stage 20 and 23 were selected for operation (Hamburger & Hamilton, 1951). Grafts of ZPA tissue were prepared as previously described (Wilson & Hinchliffe, 1985). Using the same protocol described by Hinchliffe & Gumpel-Pinot (1981) the posterior half of the host wing buds was removed with iridectomy microscissors (Weiss). To ensure total removal of ZPA tissue with the posterior half, part of the posterior flank tissue was also removed. According to the stage of operation the anteroposterior width of tissue removed varied: at stage 20 tissue equivalent to two somite widths was removed (approximately 700 μm in length), whilst at stage 23 the width of tissue removed was equivalent to one and a half somite widths (approximately 650 μm length). Intersomite 17/18 was used to delineate the midline of the limb buds during the amputations. Polarizing region grafts taken from donor wing buds were trimmed using tungsten needles to a rectangle of 70–100 μm on a side (the grafts include the whole dorsoventral axis), and then grafted either distally (Fig. 1A) or proximally (Fig. 1B) to the cut surface of the anterior half.

The control for the above experiment is the development of the anterior half of the wing bud after posterior half removal at stages 17–22, without ZPA grafting. This control has been carried out and the resulting patterns of cell death and of skeletal development reported in a previous paper (Hinchliffe & Gumpel-Pinot, 1981).

In the second experiment, ZPA grafts were made to the posterior half of wing buds after removal of the anterior half (Fig. 1C). The grafts were performed in the same way as the anterior half plus ZPA operations although only distally positioned grafts were performed.

The operated embryos were then resealed with Sellotape and allowed to develop further. Embryos for skeletal clearance preparations were allowed to develop for a further 5–6 days after operation then fixed in formol alcohol and stained in methylene blue. Embryos examined for cell death were stained *in ovo* between 18–24 h post-operation using neutral red dye (Hinchliffe & Ede, 1973).

To examine the possible contribution of the ZPA to the anterior half when ZPA tissue was grafted to the distal end, quail ZPA tissue was used and the resultant wing examined histologically 3–4 days later. Rather than using the lengthy Feulgen technique, haematoxylin and eosin was found to be perfectly adequate for identifying the grafted quail cells in serial sections in experiments of this type where there is little or no cellular mixing along the host–graft interface.

In all 389 operations were performed of which 296 survived to be examined; the number of limbs examined following the three types of operation is summarized in Table 1.

Results

Skeletal pattern development

Anterior half with ZPA grafted proximally

All 13 wing skeletons obtained following removal of the posterior half and grafting ZPA tissue to the proximal face of the anterior half showed no overall improvement in the pattern of skeletal development as compared with an anterior half without ZPA graft. Typically the anterior half formed a humerus and part radius (Fig. 2A), although the metacarpal of digit **2** was sometimes present.

Anterior half with ZPA grafted distally

Of the 168 preparations obtained following ZPA grafts to the distal end of the anterior half, 75 (44·5 %) showed total pattern regulation, i.e. a complete set of zeugopodal and digital elements were present. The remaining 93 results showed improved skeletal development when compared to anterior half with ZPA grafted proximally, in that a humerus, complete radius and digits **2** and **3** were formed. In the majority of these specimens an ulna was present (90 out of 93 cases), but digit **4** was absent in all 93 cases. When the operation was performed at stage 20 and 22, over half the specimens possessed a full complement of skeletal elements; whilst at stage 21 and 23 slightly less than a third of the results showed total pattern regulation (Table 2). It was noticed that the limb skeletons resulting from these operations exhibited considerable variation in size – for example,

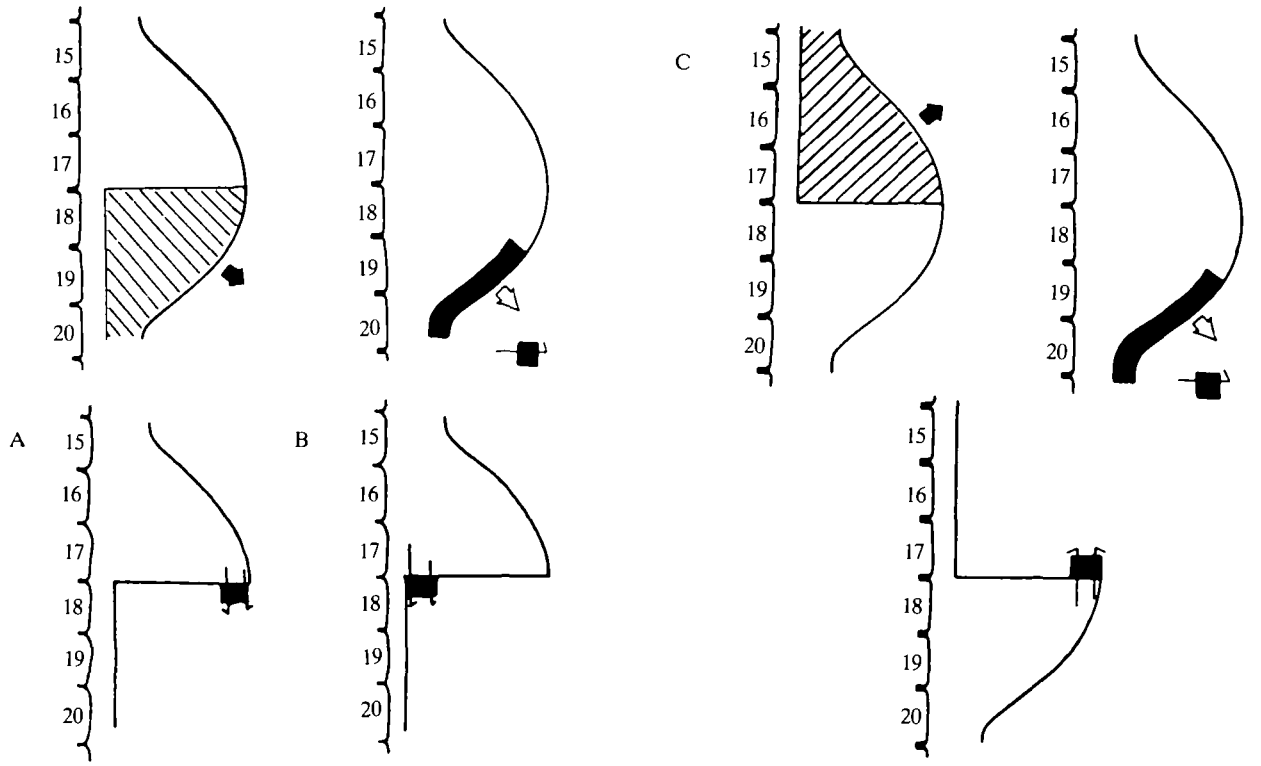

Fig. 1. Grafting protocol for examining the effect of the ZPA on anterior and posterior half wing buds. (A) ZPA grafts from stage 20 wing buds were transplanted to the distal tip of the cut surface of a host wing bud from which the posterior half had been removed. (B) Similar operation except that the ZPA was grafted proximally to the cut surface of the anterior half. (C) ZPA graft to the distal tip of a host wing from which the anterior half had been removed.

Table 1. *Summary of operations*

Type of operation	No. of operations	Survivors (%)	Skeletal preparation	Cell death	Histology
A	258	192 (74)	168	21	3
B	42	30 (71)	13	14	3
C	89	74 (83)	47	23	4

Letters under 'type of operation' refer to the diagrams of operations in Fig. 1.

some skeletons were complete in their skeletal composition but were miniature in comparison to the contralateral control skeleton (Fig. 2B), whilst other skeletons showed only slight reduction in size of the zeugopodal or digital elements (Fig. 2C). In an attempt to quantify this size variation, the elements of the zeugopod and autopod of the operated limbs were measured using an ocular graticule, and were compared with the dimensions of elements in the contralateral limb skeleton. The measurements revealed that size regulation occurred at all the operation stages (i.e. between stages 20–23) although there was no clear reduction in size of the elements on a stage-related basis (Table 3). The variation between the lengths of the elements was considerable, both between control and experimental limbs of the same embryo, between the same elements of the experimental limbs produced by the operation performed at the same stage, and less surprisingly between similar elements in experimental limbs resulting from operations at different stages. However, some overall observations may be made regarding the size of the limb skeleton following this type of operation. None of the 168 experimental limbs achieved a size comparable with its contralateral limb even allowing for the 5 % error of specification variation one would expect from the measurements of Summerbell & Wolpert (1973). In four cases the radii of experimental limbs

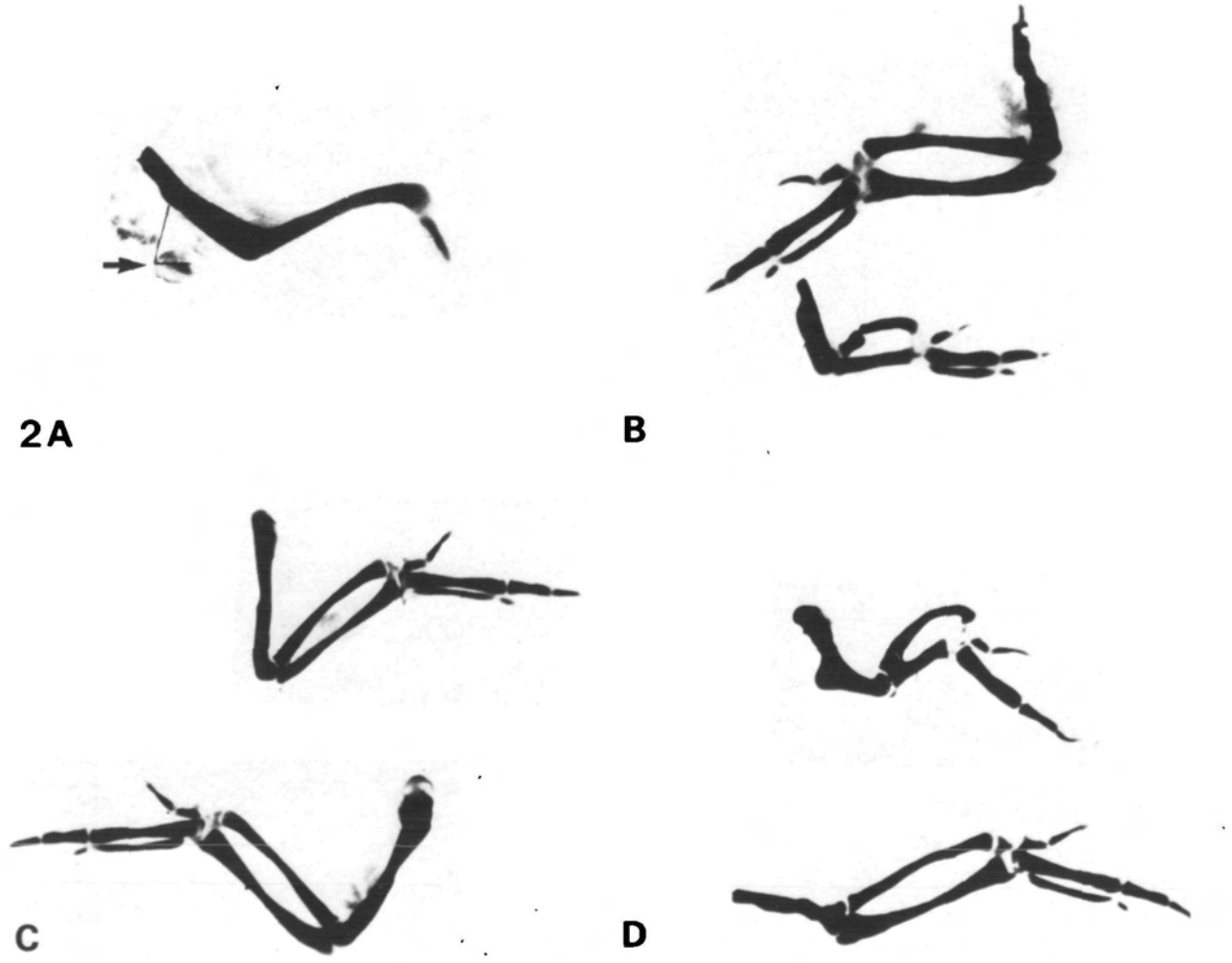

Fig. 2. Skeletal clearance preparations showing the effect of grafting the ZPA to the anterior half. (A) Skeleton resulting from a ZPA grafted to the proximal end of the cut face of the anterior half. There is little skeletal improvement above that which is observed following posterior half removal in that a humerus, partial radius and part digit 2 have been formed (arrow indicates the graft). (B) Skeleton resulting from ZPA distally grafted to the anterior half. The limb has pattern regulated but is considerably smaller than the contralateral limb (top). (C) Skeleton resulting from distally grafted ZPA to the anterior half (top). The limb has pattern regulated and the elements are of very similar proportions to the contralateral control limb skeleton. (D) Skeleton showing shortened ulna that often resulted from distal ZPA grafts. Note the compensatory curvature of the radius allowing articulation at the wrist, and the fusion between the radius and ulna proximally. The absence of digit 4 shows that pattern regulation is incomplete in this skeleton.

Table 2. *Analysis of the number of skeletons showing pattern regulation following ZPA graft to the distal end of the anterior half*

Stage of operation	20	21	22	23
No. showing total pattern regulation	21	19	27	8
No. showing partial pattern regulation	16	37	21	19
% total pattern regulation	57	34	56	30

All the partially regulated skeletal preparations showed digits **2** and **3** but the absence of digit **4**. Only three cases showed the absence of a zeugopodal part, the ulna.

reached the same dimensions as the control radii, and only one ulna and digit **3** (in separate experimental limbs) reached the same size as their contralateral counterparts. In the majority of the specimens (112 out of 168), the ulna was more deficient in length than the radius, and in these cases the short ulna was accompanied by a radius that was bowed anteriorly (Fig. 2D), thus permitting articulation with the wrist elements.

Fusion was frequently observed between the cartilage rudiments of the skeleton following this operation. In 27 cases there was fusion between the distal epiphysis of the humerus and the zeugopod elements. Fusion between the humerus and the ulna occurred in 15 of the specimens and complete fusion of the humerus, radius and ulna occurred in nine cases. Humeral–radial fusion was seen in the remaining three cases. In another seven specimens fusion of the proximal epiphysis of the radius and ulna was seen.

Histological examination of six limbs resulting from the quail grafts to chick anterior half showed that there was no contribution from the donor ZPA cells to the resultant skeletal elements. The quail tissue was confined to the extreme posterior margin in the wing. The quail cells were found as a discrete band in the proximal posterior margin and extended as a tongue of cells distalward almost to the tip of the limb (Fig. 3).

Table 3. *The average lengths (mm) of ulna, radius and digit 3 of control and experimental wing skeletons resulting from ZPA grafts to the distal tip of the anterior half performed at stages 20–23*

	Average length (mm)		Average experimental element length as a % of control length
	Experimental	Control	
Stage 20			
Ulna	2·10	3·45	70·4
Radius	2·38	3·17	78·9
Digit **3**	2·70	3·69	81·2
Stage 21			
Ulna	2·45	3·74	64·4
Radius	2·76	3·38	77·0
Digit **3**	2·79	3·94	70·0
Stage 22			
Ulna	2·04	3·89	54·1
Radius	2·49	3·44	72·3
Digit **3**	2·92	4·06	62·7
Stage 23			
Ulna	2·32	2·72	79·8
Radius	2·52	2·98	81·8
Digit **3**	1·91	3·54	57·0

Stage 20: n = 17, stage 21: n = 19; stage 22: n = 30; stage 23: n = 9. Average experimental element length expressed as a percentage of the contralateral control element is also given. Harvesting limbs at both 5 and 6 days postoperation resulted in large standard deviations from the mean. For example, stage 21 control radii measured 3·38 mm (S.D. 0·89 mm) whilst the experimental average was 2·76 mm (S.D. 0·9 mm).

Control anterior half

As reported previously (Hinchliffe & Gumpel-Pinot, 1981), following amputation of the posterior halves of stage 17–22 wing buds, anterior halves develop poorly, forming in most cases a humerus fused with a shortened radius, with digit **2** usually missing. If the anterior half developed in accordance with its prospective fate it would form the radius and digit **2** (Hinchliffe *et al.* 1984).

Posterior half with ZPA grafted distally

Of the 47 skeletal preparations obtained from these operations, nine produced the normal skeletal pattern that would be expected from an isolated posterior half; a humerus, ulna and digits **3** and **4** – with radius and digit **2** missing and the humerus smaller than normal (see Hinchliffe & Gumpel-Pinot, 1981). The remaining results all showed skeletal abnormalities. Most frequently the zeugopod appeared as a shortened thick element (26 cases, Fig. 4B,C); in seven cases the ulna was comparatively normal in appearance and in five cases the ulna was duplicated (Fig. 4D). The digits showed a range of effects from the grafting procedure. None of the 47 skeletons produced a digit **2**, although 14 produced a small cartilaginous spur on the anterior side of the second phalange of digit **3** (Fig. 4A). In 24 cases digital duplication was observed. The extra elements were present as either a bifurcated digit **3** (six cases), i.e. a **3̲34** pattern (where ⌴ indicates that the digit **3** is a single element proximally but divides into two distally), or a bifurcated digit **3** with complete (seven cases, Fig. 4D) or incomplete (six cases, Fig. 4C) supernumerary digit **4**, i.e. a **43̲34** pattern. The remaining five cases showed a **434** pattern with no bifurcation of digit **3**. No specimen showed duplication of digit **4** alone.

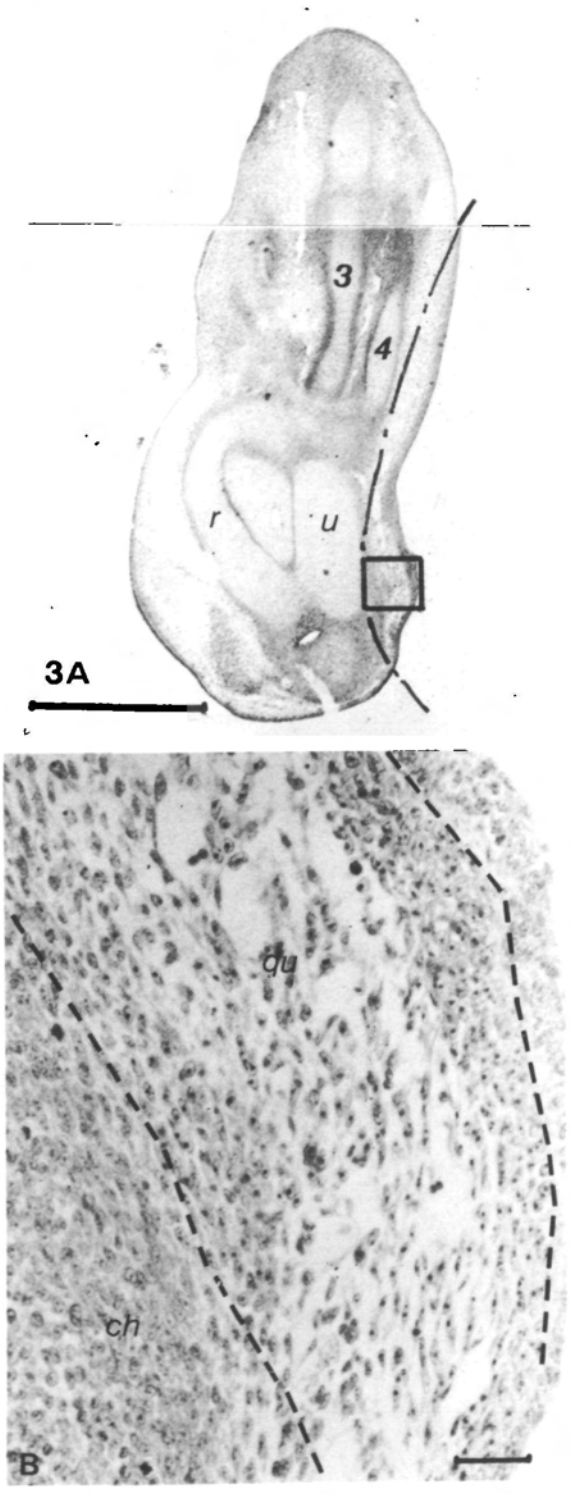

Fig. 3. Histology showing the fate of ZPA cells when grafted to the distal end of the anterior half. (A) Section through a regulated limb showing the distribution of quail cells along the posterior margin (broken line). Bar represents 0·5 mm. (B) Higher magnification of area indicated by box in A. The quail cells (*qu*) were not found in any skeletal component of the regulated limb but were distributed as a narrow band along the posterior border tapering from proximal to distal within the chick tissue (*ch*). Haematoxylin and eosin. Bar, 30 μm.

Cell death pattern

Anterior half with ZPA grafted proximally

All 14 specimens showed a similar pattern of cell death to that observed following posterior half removal (Hinchliffe & Gumpel-Pinot, 1981). Extensive cell death was found in the anterior margin and the distal mesenchyme and was not confined only to the more distal tissue. In five cases however, cell death in the midproximal mesenchyme corresponding to the opaque patch was absent or reduced in comparison to similarly staged operations without the grafted ZPA.

Anterior half with ZPA grafted distally

The grafting of ZPA tissue produced quite a different effect upon the cell death in the anterior half. In 16 of the 21 specimens examined, regardless of the stage of operation, cell death was absent from the distal mesenchyme (Fig. 5A) and was usually absent in the ANZ or greatly reduced in area. In addition the opaque patch was reduced in area as compared to the contralateral control limb bud. Histologically the distal and central mesenchyme appeared healthy with few or no macrophages present and the extensive cellular debris common in anterior half or anterior half with ZPA grafted proximally, was almost entirely absent. The AER in these wing buds was healthy, and had both lengthened and thickened. The remaining five limb buds did however show limited cell death in the distal mesenchyme although not on the same scale as previously described. Examination of the graft position in all these 21 limb buds showed that it had become slightly displaced proximally in comparison with its original position (Fig. 5A).

Anterior half

The cell death pattern in an anterior half without any ZPA graft has already been described (Hinchliffe & Gumpel-Pinot, 1981). 24 h after removal of the posterior half, there is massive cell death throughout the remaining distal mesenchyme and the AER regresses (Fig. 5B).

Discussion

The experiments reported here show clearly that grafting a ZPA to the distal tip of an anterior half wing bud inhibits the cell death in the apical mesenchyme which would otherwise take place, and enables the anterior mesenchyme to greatly exceed its normal prospective skeletal fate (digit **2**) by forming frequently a full set (digits **2–4**) of wing digits although these are usually reduced in size. Contact between ZPA graft and host AER appears to be an essential condition of this developmental 'rescue'.

These results support the view that the ZPA has a central and unique function in wing bud development

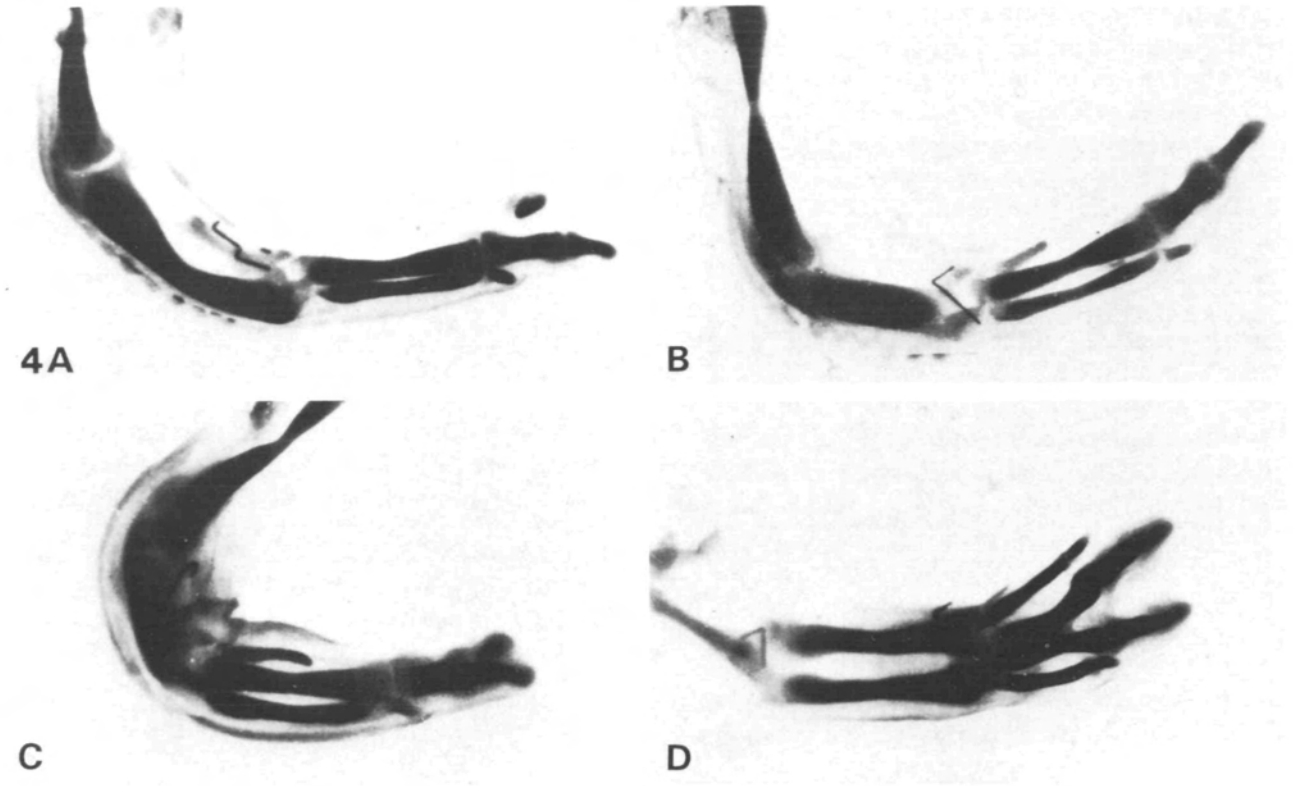

Fig. 4. Skeletal clearance preparations showing the effect of grafting the ZPA to the posterior half. (A) Skeleton showing cartilaginous spur on the anterior side of digit **3**. The skeletal development is similar to that obtained in a posterior half without the ZPA grafted to the distal tip. (B) Skeleton showing incomplete duplication of digit **4**, without bifurcation of digit **3**. (C) Skeleton showing incomplete duplication of digit **4** and bifurcation of the distal phalange of digit **3**. (D) Almost complete duplication of digits **3** and **4** – fusion of only the proximal end of digit **3** is apparent.

(Hinchliffe & Gumpel-Pinot, 1981; Summerbell & Honig, 1982). They do not accord with the view (Rowe & Fallon, 1981) that the ZPA role is limited to influencing the limb field prior to stage 17; nor do they support the view of Smith (1979, 1980) that the effect of a ZPA can be remembered in its absence. Since in the absence of ZPA the distal mesenchyme loses its positional value, with the cells actually dying, while grafting a ZPA to the anterior half creates new positional values in the distal mesenchymal cells adjacent to the graft, raising these from 'anterior' to 'posterior' it is difficult in this case to accept Smith's argument (1979) that 'positional value is a stable cell state that does not depend on the continuing presence of any positional cue'. Instead positional value in the limb mesenchyme seems to remain labile and defined

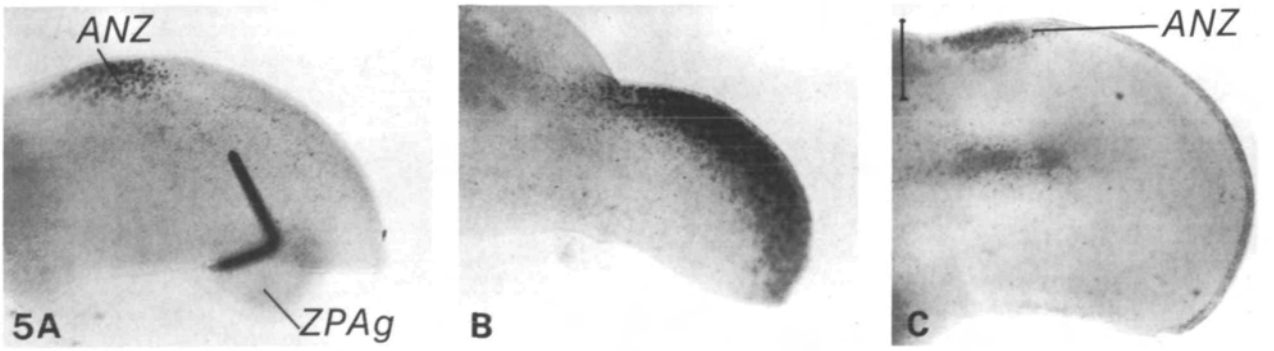

Fig. 5. Effect of the ZPA on cell death in the anterior half. (A) Wing bud following a ZPA graft distally at stage 22, with thick AER overlying viable distal mesenchyme. Note that the ZPA graft (*ZPAg*) is no longer in the extreme distal position. (B) Control wing bud after amputation of posterior half at stage 21. Note massive cell death in anterior and distal mesenchyme accompanied by AER regression. (C) Control unoperated stage 25 wing bud. (A) and (B) are 24 h postoperation and all are stained for cell death with neutral red (*ANZ*, anterior necrotic zone). Bar, 0·25 mm.

only with reference to the ZPA as far as prospective zeugopod and digits are concerned during the stages of operation (20–23) reported here. Our experiments in fact strongly support the conclusion that the ZPA has a continuing role in normal wing development through to stage 22, as suggested by ZPA amputation experiments (Hinchliffe *et al.* 1984). Thus far, the argument for or against a ZPA role has turned on the critical question of whether the whole of the polarizing zone or area of intermediate polarizing activity has been removed in the various amputation experiments (for example, Fallon & Crosby, 1975), since leaving intermediate areas or a small portion of the ZPA itself in position is sufficient to permit normal limb development (Hinchliffe & Gumpel-Pinot, 1981). Grafting the ZPA to the anterior half is a better way of examining the normal ZPA role since it bypasses the question of accuracy of the maps of ZPA activity and intensity in the posterior half (MacCabe, Gasseling & Saunders, 1973; Honig & Summerbell, 1985). None of these maps shows activity in the anterior half.

The results are also more easily interpreted by the ZPA control theory than by the polar coordinate interpretation (Iten & Murphy, 1980; Iten *et al.* 1981; Javois, 1984) since it suggests that the distal mesenchyme does not possess fixed positional values that are only impressed on it with reference to the polarizing zone. The polar coordinate theory can explain the effect of ZPA grafts to the anterior half as an infilling of missing positional values at the graft–host interface. But since it argues (Iten *et al.* 1981) that existing positional values are stable, it cannot explain the loss of positional value represented by the distal mesenchyme cell death in the absence of a ZPA.

The regulative capacity of the anterior mesenchyme is also emphasized by these experiments. From approximately half the normal mesenchymal cell number an attempt is made, frequently successful, to form a complete wing skeleton. The regulative property is not restricted to the distal mesenchyme or the progress zone (Summerbell, Lewis & Wolpert, 1973), since the proximal mesenchyme frequently forms both zeugopod elements even though prospective ulna tissue has been removed. How are the additional mesenchyme cells generated? There are two possibilities, which are not mutually exclusive: increased cell division or decreased cell death. Though not yet investigated, increased cell division is a likely contributor, since Cooke & Summerbell (1980) have demonstrated stimulation of cell division throughout the limb mesenchyme especially between 4 and 17 h after a preaxial ZPA graft. The possibility that the ZPA acts as a mitogenic stimulator has been discussed by Bell & McLachlan (1985) and Caplan (1985).

Decreased cell death of two types makes a contribution to the regulation. There is inhibition of first the apical cell death following ZPA amputation and frequently also of the normally occurring anterior necrotic zone (ANZ). The apical cell death may well represent an extension in a posterior direction of the ANZ. Participation of the ANZ in regulation is demonstrated in other experiments (Yallup & Hinchliffe, 1983; Yallup, 1984). If excesses or deficiencies of tissue along the anteroposterior axis are created experimentally up to stage 23 there is respectively inhibition or extension of the ANZ detectable by 6 h during the regulation process. A morphogen profile model (Hinchliffe, 1980) for control of cell death in the ANZ has been proposed which fits the experimental data. Tickle *et al.* (1975) had proposed that the ZPA is the source of a morphogen profile declining along the anteroposterior axis and is responsible for the specification of digits. If the morphogen maintains the distal mesenchyme above a certain low threshold, then removal of the ZPA will result in a lower concentration across the whole limb field, resulting in the extension of the ANZ posteriorly. But if the ZPA is grafted to the anterior half, then all the remaining distal mesenchyme will be brought above the threshold level, and cell death will be inhibited.

In the second experimental series ZPA tissue was grafted to the distal tip of the posterior half of the wing bud. These experiments also provide clear evidence of ZPA control of anteroposterior polarity, since a **434** or **4334** digital pattern was produced in 38 % of cases. What still has to be explained is the relatively low incidence of skeletal duplication in posterior halves. One possible explanation is that there is attenuation of ZPA influence if the graft heals poorly or subsequently becomes displaced proximally (see Fig. 5A). In terms of the morphogen profile hypothesis, a higher level of morphogen is required posteriorly than anteriorly to produce a detectable change in the normal pattern of skeletal differentiation. This interpretation of the posterior half experiments is in accord with data obtained from experiments using retinoic acid (Tickle, Lee & Eichele, 1985). These authors have shown that retinoic acid closely mimics the putative ZPA morphogen, and calculations of the total amount of retinoic acid needed in the wing bud to obtain each additional digit showed that there is a tenfold difference between the amount required to specify a digit **3** and that required to specify a digit **4**. If these levels reflect that of the putative ZPA morphogen in the wing bud, then we can interpret ZPA grafts to the distal tip of the posterior half as sometimes sufficiently raising the

morphogen level to generate **4334** and **434** duplication patterns, but in other cases insufficiently to respecify the adjacent host tissue as digit **4**.

The emerging picture of normal wing development is consistent with the morphogen profile hypothesis and depicts the ZPA in control of cell division and maintenance and also of pattern formation in the otherwise labile distal mesenchyme. The AER possibly in association with the subridge mesenchyme appears to mediate transmission of the ZPA-derived morphogen or other control signal. It is now becoming important to discover the cellular basis of such an interpretation. Kelly & Fallon (1983) have directed attention to the importance of intercellular communication *via* gap junctions at the AER–mesenchyme interface, and Fallon and Sheridan (unpublished data) claim that the cells in an active AER are physiologically coupled as a channel of communication. Preliminary results (Hinchliffe and Griffiths) at the electron microscope level suggest that both the apical mesenchyme and AER cell profile and the organization of the extracellular matrix (ECM) under the proximal surface of the AER change drastically once ZPA influence is excluded from anterior parts of the wing bud. Distal mesenchyme cell death following ZPA exclusion is likely to be a secondary effect of other, earlier changes, possibly involving loss of organization at the AER–mesenchyme interface and its associated ECM. More detailed work on this interface, as influenced by the presence or absence of the ZPA, in terms of gap junctions and the precise composition of ECM and the AER basal lamina is currently planned.

This work was supported by a grant from the SERC. Our thanks go to Dennis Summerbell for his comments on the manuscript.

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(Accepted 8 September 1986)