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Experimental analysis of the role of the ZPA in the development of the wing buds of wingless (*ws*) mutant embryos

D. J. WILSON* AND J. R. HINCHLIFFE

Department of Zoology, University College of Wales, Penglais, Aberystwyth, Dyfed, SY23 3DA, U.K.

SUMMARY

Phenotypically wingless (*ws*) chick embryos have wing buds characterized by the spreading of mesenchymal cell death in an anterior-to-posterior direction beginning at stage 19. It has been argued that this may reflect the absence of a functional polarizing zone (ZPA). When tested by preaxial grafting into normal wing buds (stages 20–21), wingless ZPAs (stage 18–19) had duplicating properties identical with those of normal ZPAs. Equally, normal chick or quail ZPA (stages 20–22) grafted into the posterior margin of wingless wing buds (stages 18–20) failed to inhibit the pattern of cell death or to evoke any improvement in their developmental performance. The wingless (*ws*) condition is not, therefore, due to a ZPA deficiency. Possible explanations are the prior programming for cell death of the wingless mesenchyme, or somitic deficiency, but it appears more likely that the mutant limb mesenchyme fails to transmit or respond to factor(s) produced by the ZPA.

INTRODUCTION

The emphasis of research on the polarizing zone of the avian limb bud has been on the analysis of its ability to produce supernumerary skeletal elements as evidence for a gradient of positional information specifying anteroposterior differentials of the limb (Summerbell, 1974; Tickle, Summerbell & Wolpert, 1975; Summerbell & Honig, 1982). The experimental results of these investigations are indisputable; the ZPA possesses morphogenetic properties capable of establishing a complete new digital field when transplanted preaxially into normal limb bud mesenchyme. Recent experiments have shown that this morphogenetic activity of the ZPA has another facet – that of suppressing limb mesenchymal cell death (MacCabe, Knouse & Richardson, 1981; MacCabe & Richardson, 1982; Hinchliffe & Griffiths, 1984; Wilson, 1984). In their experiments, MacCabe and his colleagues have described an *in vitro* bioassay for polarizing activity by monitoring cell death by estimating the number of macrophages in isolates of anterior tissue when combined with presumed polarizing zone tissue. Cell death in the anterior

tissue was inhibited when combined with ZPA. Hinchliffe & Gumpel-Pinot (1981) observed a similar effect on anterior cell death *in vivo* when they carried out total and partial ZPA amputations at early stages, and they suggested that the increased cell death results either directly or indirectly from the absence of the ZPA. In an attempt to clarify the supposed role of the ZPA in anterior mesenchymal cell death, a wingless chick mutant was subjected to experimental analysis.

Wingless mutants have already proved useful in experiments designed to examine ectodermal/mesenchymal interactions in limb development. In these early studies, Zwilling (1949, 1956) investigated the interaction of the AER and Apical Ectodermal Maintenance Factor (AEMF) by separating the ectoderm and mesoderm components of wingless and normal 3-day embryos and recombining them in various combinations. Zwilling interpreted the results as a reciprocal dependence between the two components – mesodermal outgrowth being evoked by the ectodermal ridge which in turn is maintained by a mesodermal factor. Zwilling considered this mesodermal factor in the wingless mutant to be deficient, causing AER collapse and the cessation of outgrowth and development of the wing. It is possible that Zwilling's experiments reveal more the absence of a polarizing zone than a deficiency of AEMF. In fact, the relationship between the ZPA and maintenance factor still remains to be resolved (Saunders, 1977; Summerbell, 1979). Rubin, MacCabe & MacCabe (reported in Saunders, 1972) examined the possible absence of a ZPA in the American wingless (*wg* – an autosomal recessive gene) and found that the mutant did not possess an area comparable to the polarizing zone of the normal chick wing and concluded that the wingless mutation supresses or eliminates the polarizing zone. The mutant used in the present study was first discovered by Pease (1962), who noted that it differed in its character and mode of inheritance from the *wg* mutant. Winglessness in this mutant resulted from the action of a sex-linked gene (*ws*) (Lancaster, 1968), giving a modal viable type in which the only abnormality was the absence of wings. Further analysis by Hinchliffe & Ede (1973) and Hinchliffe (1977) revealed that the *ws* wingless condition was related to the precocious appearance of cell death in the ANZ in the wing bud at stage 19, followed by progressive distal extension of the necrotic zone beyond its normal boundaries during stages 20 to 23 (Fig. 1). The pattern of cell death in the wingless wing bud closely parallels that obtained by Hinchliffe & Gumpel-Pinot (1981) following experimental amputations. They found that when the posterior ZPA-containing parts of a normal wing are excised between stages 17–22, the remaining anterior parts become necrotic within 18 h of the operation. The cell death is characterized by AER regression and apparent ANZ enlargement with the death of many distal mesenchymal cells which are then engulfed by large macrophages. The subsequent skeletal development of the anterior part is impaired, and in the case of the anterior half it forms less than its prospective fate *viz.* humerus and partial radius rather than humerus, radius and digit 2 (based on the fate map of Hinchliffe, Garcia-Porrero & Gumpel-Pinot, 1981).

It is plausible to argue that the anterior part of a normal chick wing bud (from which the ZPA has been deleted) and the *ws* wingless wing bud like the *wg* wingless wing bud, all lack the same factor required for the survival and subsequent development of the anterior tissue. To investigate this possibility, the *ws* wingless wing bud was examined for the presence of a functional ZPA. The wing bud was tested in two ways: (i) to establish whether normal or quail ZPA (which has been shown to possess a ZPA by Fallon & Crosby, 1977) when grafted into the wingless wing bud, could suppress the mesenchymal cell death and promote normal limb development; and (ii) to test wingless wing buds for areas of posterior mesenchyme with properties comparable to those of the ZPA in normal wing buds.

MATERIALS AND METHODS

Wingless embryos were obtained from Light Sussex descendants of the wingless stock (Pease, 1962). Normal and wingless eggs were incubated for 2·5–3 days and then windowed according to the method of Summerbell & Hornbruch (1981). Phenotypically wingless embryos were
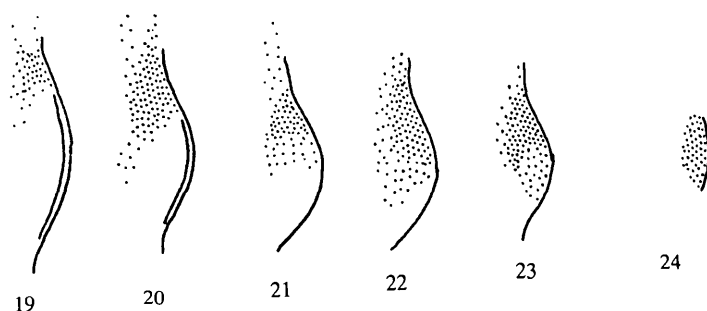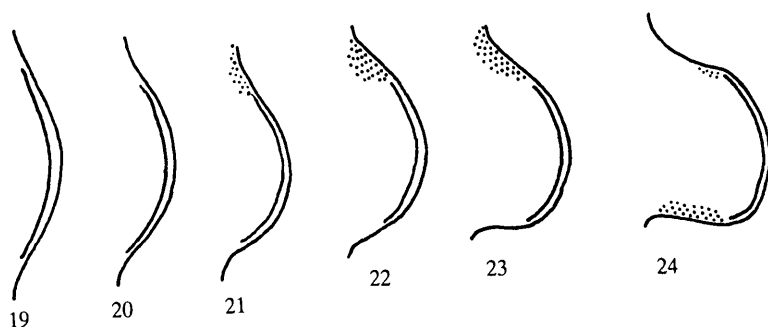

Fig. 1. Pattern of cell death in normal and wingless (*ws*) wing buds. Regions of cell death are indicated by stippling. Bar represents 1mm.

identified by wing bud form at 2·5–3 days of incubation (in all cases this identification was confirmed by a check on the subsequent lack of development of the left unoperated wing bud). The precise stages of development were determined according to Hamburger & Hamilton (1951), although the stages of winglessness were established using the drawings of Hinchliffe (1977), (Fig. 1). Quail eggs were obtained from a flock (*Coturnix japonica*) maintained in the department and were incubated for 3 days. The eggs were windowed over the airspace, and the embryos staged according to Padgett & Ivey (1960). Stage 20–22 quail donor embryos were selected for operation, removed from the shell, and then placed in a sterile dish containing Biggers BJG (modified) medium supplemented with penicillin and streptomycin. Stage 18–21 wingless and normal host embryos were selected for operation. Following all operations, two or three drops of medium containing antibiotics were placed onto the embryo and the surrounding vitelline circulation to reduce the risk of infection. Eggs were then resealed and returned to the incubator. Operated embryos from which cartilage clearance preparations were to be made were allowed to develop for a further 5–6 days. Experimental and contralateral limbs were removed from the embryos and fixed in formol alcohol and then stained with methylene blue.

The following operations were performed, the number of embryos operated upon in each series being summarized in Table 1.

A1. *Grafts of quail polarizing zone tissue to posterior margin of wingless wing bud*

Quail tissue provides a nucleolar marker by which the contribution of grafted tissue can be assessed by Feulgen staining. The aim here was to establish the relative contribution of grafted ZPA and host tissue to any possible improved development of the wingless wing buds.

ZPA grafts were prepared using microneedles, and were transferred to the host wingless embryo. Each graft was pinned into a site of the wingless wing bud that had been prepared previously (Fig. 2A). At stage 18/19 many of the host wingless wing buds possessed an AER (albeit somewhat lower than that of a comparable staged normal embryo), and the grafted ZPA was pinned in a position slightly posterior to the caudal extremity of the ridge. Control operations were also carried out in which a portion of the normal host ZPA was replaced by a ZPA from another normal chick donor.

A2. *Grafts of normal chick ZPA into the posterior margin of the wingless wing bud*

Normal chick polarizing tissue grafts were made into the posterior margin of the wingless wing bud. Grafts of this type were performed because the quail tissue was found not to have any effect

Table 1. *Wingless and normal ZPA grafts (see Fig. 2)*

Operation	Number of operations	Survivors	Skeleton stained	Cell death preparations
A1: qZPA to ws (postaxial)	21	14	10	4
A2: nZPA to ws (postaxial)	15	9	5	4
B: qZPA to ws (midaxial)	15	12	9	3
nZPA to ws (midaxial)	15	11	5	6
C: wsZPA to normal (preaxial)	32	27	27	–
Controls				
i) Control to A1/A2. nZPA to normal (postaxial)	6	6	6	–
ii) Control to B. nZPA to normal (midaxial)	8	7	7	–
iii) Control to C. nZPA to normal (preaxial)	14	13	13	–

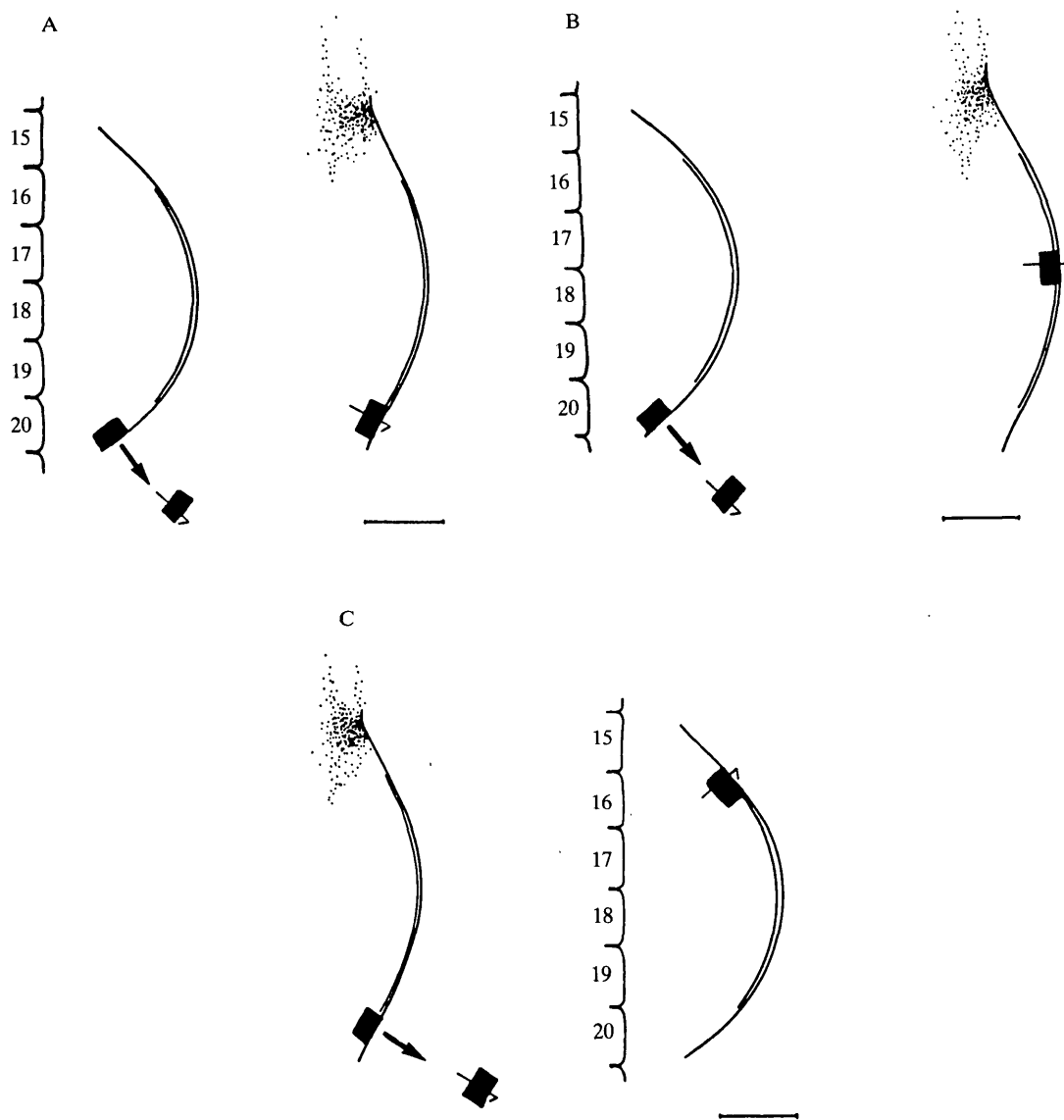n, normal chick; q, quail; ws, wingless.

Fig. 2. Experiments on mutant and normal ZPA duplication. (A) ZPA grafts from normal donor wing buds (stage 20) were transplanted into the posterior margin of wingless host wing buds (stage 19). (B) Graft of chick (or quail ZPA) into the apex of the wingless wing bud. (C) Graft of presumptive wingless wing bud ZPA into pre-axial sites of normal wing buds. Bar represents 0·25mm.

upon the developmental fate of the wingless bud, and to ensure that this failure was not due to some specific feature of the quail ZPA.

B. *Grafts of quail and chick ZPA into the apex of the wingless wing bud*

This series of operations was essentially similar to those of series A1, except that the polarizing graft was pinned in a more anterior position, thereby reducing the distance between the graft and the prospectively necrotic anterior mesenchyme (Fig. 2B). Control operations to the same apical position were again performed to assess the effect of the operation upon normal chick host limb buds.

For the same reason that the A2 series of operations were carried out, a series of grafts using normal chick ZPA was made into midaxial positions of the wingless wing buds.

C. *Grafts of presumptive wingless wing bud polarizing zone to pre-axial sites of normal chick wing buds*

These operations were performed to determine whether the wingless wing bud possessed an area of polarizing tissue comparable to the ZPA of the normal chick wing bud. Stage 18–19 wingless wing buds were selected as donor limbs, and the grafts were taken from sites similarly positioned to those showing ZPA activity in normal embryos (Fig. 2C). It was noticed when cutting out the wingless limb buds, that the mesenchyme was very loose, making the insertion of the platinum pins quite difficult.

The normal chick embryos selected as hosts were usually stage 20 or 21, making the manipulation easier. In control operations, normal ZPAs were grafted into the preaxial position in normal wing buds.

Cell death patterns in wingless wing buds with grafted ZPAs

The cell death pattern following grafts of normal quail and chick ZPA tissue into the host wingless wing buds was examined by vital staining 18–24 h after operation. *In ovo* staining by neutral red via the vitelline circulation enables the areas of individual dying cells and associated macrophages to be mapped out (Hinchliffe *et al.* 1981). The lefthand wing buds of donor wingless wing buds were also stained for cell death. This was done to ensure that (a) the donor embryo used in the operation was, in fact, phenotypically wingless and (b) that the pattern of cell death in the wingless wing bud had not changed since it was last examined by Hinchliffe (1977). (It had been reported (Zwilling, 1974) that in the case of the American wingless mutant, expressivity of the mutant condition had increased and the limbless condition had become more exaggerated between the time of the discovery of the *wg* mutant, and the time the experiments were performed.) Vitally stained specimens were made into permanent preparations by the method of Hinchliffe *et al.* (1981).

RESULTS

In total, 126 embryos were operated upon, of which 101 survived. The number of embryos examined in each series of operations is summarized in Table 1.

A1. *Grafts of quail ZPA into the posterior margin of the wingless wing bud*

From the fourteen operations that survived, ten were examined for skeletal development. In two cases a humerus developed, one of these articulated with a partial ulna. In neither case did any more distal elements develop. The remaining experimental limbs showed no skeletal development beyond the shoulder girdle,

nor did the contralateral control limbs. It was clear from the skeletal preparations that the quail ZPA graft had not improved the developmental capacity of the wingless wing bud; therefore it was considered unnecessary to examine histologically the participation of graft tissue following any of the operations. The occasional development of humerus and ulna elements in wingless forelimbs was reported by Hinchliffe & Ede (1973), and they attributed their formation to a milder expression of the *ws* gene.

A2. *Grafts of normal chick ZPA to the posterior margin of the wingless wing bud*

In all the five cases, the wing skeleton was almost totally absent, with only one embryo possessing even a reduced humerus. The chick ZPA grafts, like the quail, produced no improvement in the development of the wingless wing; and macroscopically the limb was discernible only as a small flange of tissue on the flank of the embryo. The control operations showed that normal ZPA grafts resulted in normal skeletons in all six cases, indicating that the grafting technique had not impaired the skeletal development.

B. *Quail or chick ZPA grafts into the apex of the wingless wing bud*

Of the nine skeletal preparations obtained from quail grafts, only three wingless wings possessed a humerus, in the remaining six all wing elements were absent. The external morphology of the limbs was the same as that in operation A1.

Similarly chick ZPA grafts in the five operations did not produce any improvement in the skeleton of the wing bud, since no limb skeletal elements were found in any case. The control operations of chick ZPA grafted into the apex in normal hosts produced supernumerary skeletal elements in the anterior tissue; the seven cartilage preparations all showed duplications of both zeugopodial elements (e.g. double ulna) and of the digits.

C. *Grafts of presumptive wingless ZPA into the preaxial position in normal chick wing buds*

All 27 operations of this type resulted in duplication of skeletal elements in the normal host wing bud; the extent of the duplication ranged from a single extra digit **2** (minor duplication, Fig. 3) through to complete duplication of all three wing digits (major duplication, Fig. 4). The most frequent result was a skeleton consisting of humerus, radius, digits **2,2,3,4**. In eight cases the ulna was duplicated with the radius either absent or only partially formed, but the duplication of the ulna was not always linked to the formation of supernumerary digits **3** and **4**. The frequency of the duplicated digital sequences resulting from wingless grafts are summarized in Table 2. Digital duplications resulting from control (normal ZPA) preaxial grafts are detailed in Table 3.

To compare the duplicative capacity of wingless ZPA with that of normal ZPA grafts, a scoring system was employed (taken from Honig, Smith, Hornbruch &
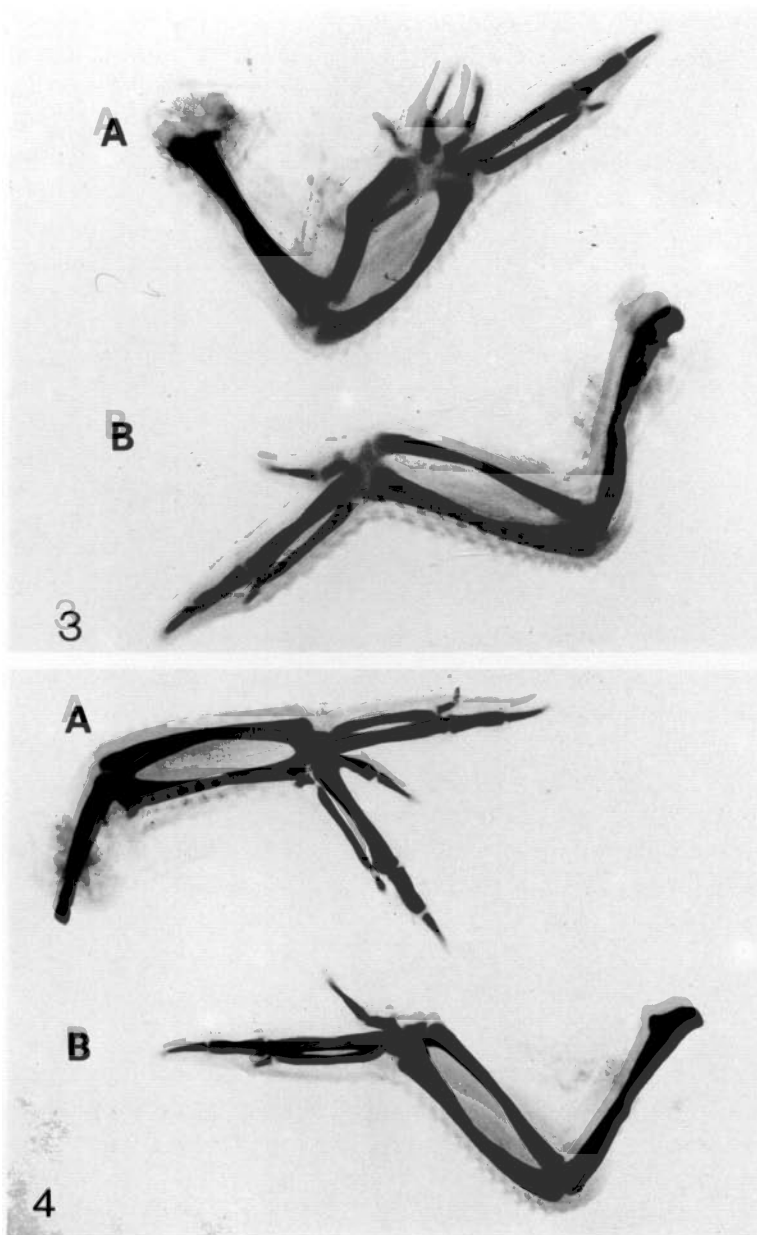
Fig. 3. (A) Example of minor duplication resulting from wingless graft into the apex of a normal host wing bud: digit 2 has been duplicated, and the radius has been affected. (B) Contralateral control wing bud.

Fig. 4. (A) Wing skeleton showing major digital duplication following wingless ZPA graft into pre-axial tissue of normal host wing bud. Note the duplicated ulna. (B) Contralateral control wing bud.

Wolpert, 1981). Grafts that resulted in the duplication of all three digits scored the maximum of 6; 3 for a digit **4**, 2 for a digit **3** and 1 for a digit **2**. The aggregate score for each duplication type was totalled and an average was taken. In the case of wingless ZPA tissue the average result was 3·5, whilst control operations produced an average of 3·1. These averages indicate that grafts of both normal and wingless ZPA tissue produced comparable duplications (equivalent to a duplication of 32234).

Cell death in the wingless wing bud following grafts of normal chick or quail ZPA

In all, eight embryos were stained with Neutral red 18 h after ZPA grafts had been made to the posterior margin of the wingless wing bud. None of the experimental limbs showed any reduction or deviation from the normal cell death pattern of the wingless wing; the anterior mesenchyme had become highly necrotic and numerous macrophages were observed throughout the entire anterior half of the wing bud and the AER overlying the dying anterior mesenchyme was also necrotic (Fig. 5). Grafts of polarizing tissue to the apex of the wingless wing bud similarly had no effect upon the cell death of the anterior tissue, the pattern

Table 2. *Patterns of digital duplications obtained following grafts of presumptive wingless ZPA into pre-axial sites of the normal chick wing bud (Operation type C).* (*Numerals underlined indicates supernumerary digits*)

Pattern of digits	(Score)	Number of cases	(Aggregate score)
2 2 3 4	(1)	8	(8)
3 2 3 4	(2)	3	(6)
3 2 2 3 4	(3)	4	(12)
4 3 3 4	(5)	4	(20)
4 3 2 3 4	(5)	3	(15)
4 3 2 2 3 4	(6)	5	(30)

Score obtained as percentage of possible score $\frac{91}{162}$ = 56 %.

Table 3. *Patterns of digital duplications obtained following grafts of normal ZPA into pre-axial sites of normal chick wing buds*

Pattern of digits	(Score)	Number of cases	(Aggregate score)
2 2 3 4	(1)	6	(6)
3 2 3 4	(2)	0	(0)
3 2 2 3 4	(3)	1	(3)
4 3 3 4	(5)	3	(15)
4 3 2 3 4	(5)	2	(10)
4 3 2 2 3 4	(6)	1	(6)

Score obtained as a percentage of possible score $\frac{40}{78}$ = 51·3 %.

produced in the nine embryos being similar to that in the maps of Hinchliffe (1977) for stage 20–21 wingless wing buds.

DISCUSSION

The analysis of the American wingless (*wg*) mutant led to the suggestion that the wingless condition is due to the absence of a functional ZPA (Saunders, 1972). The results of the experiments described here on the *ws* wingless mutant, have clearly demonstrated that the *ws* mutant possesses a ZPA which, when grafted into pre-axial tissue of a normal host wing bud is capable of producing the same duplication of skeletal wing parts as control normal ZPA grafts. In addition, it was shown that grafts of normal ZPA into the wingless wing bud did not improve the skeletal development in the mutant limb bud. These results suggest that the mechanisms involved in the genesis of winglessness in the sex-linked mutant and the autosomal *wg* mutant are different.

The cell death studies revealed that the enlargement of the ANZ in the wingless wing bud was unaffected by the presence of normal polarizing tissue in the posterior margin. Midaxial grafts of normal ZPA – performed to examine the
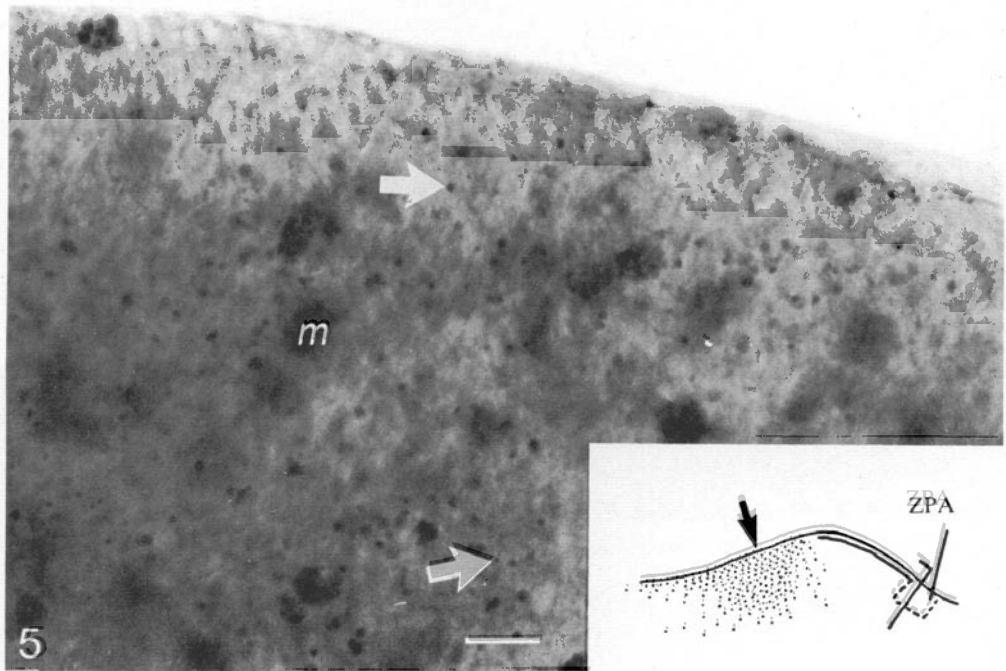

Fig. 5. Cell death in the anterior tissue of wingless wing bud revealed by vital staining. Numerous macrophages (*m*) can be seen, while the small points of staining (arrows) probably represent lysosomes in individual dying cells cellular debris. The AER has flattened and a macrophage can be seen within the ridge. Inset shows position of photograph (arrow) of wingless wing bud 18 h after transplantation of a normal ZPA posteriorly. Bar represents 30 μm.

effect of a reduction in distance between the polarizing zone and the anterior prospectively necrotic tissue – also failed to inhibit the increased cell death. It has been suggested by Hinchliffe (1980) that the similar cell death patterns and subsequent developmental failure in both the ZPA-deleted normal wing bud and the *ws* wing bud might be due to the absence in the mutant of the ZPA or to it having less 'potency' than normal. In view of the finding that the *ws* wingless wing bud possesses a ZPA of normal duplicative strength, this hypothesis is clearly incorrect. It is possible that the anterior cell death in wingless mesenchyme is programmed from an early stage of development, and this cell death programme may render the anterior cell incompetent to respond to a signal from the ZPA. For example, another area of cell death in the limb, the posterior necrotic zone (PNZ), has been shown to have a cell death programme (Fallon & Saunders, 1968) – the cells possess an intrinsic commitment to die at a particular developmental time. Perhaps a similar 'death clock' exists in the mutant anterior mesenchyme which may already be programmed and thus unable to respond to the ZPA signal. But there is reason to believe that prior ANZ programming is not the explanation, since Yallup (1984) has found that the ANZ is respectively increased or decreased within 6 h of making experimental excesses or deficiencies along the anteroposterior axis of the normal wing bud.

Another alternative explanation for the wingless condition comes from the work on limblessness in reptiles (Raynaud, 1977) from which it has been suggested that the somites exert a stimulatory effect upon the pre-limb bud somatopleure. Raynaud suggests that in the limbless reptiles the absence of the somitic stimulation results in a mesodermal deficiency of the limb rudiment and causes AER involution. Experimental work on the chick wing bud supports this interpretation of the stimulatory role of the somites (Pinot, 1970).

If the development of winglessness proves not to be a question of either programmed cell death or a failure of somitic contribution to the limb bud, then a further possibility remains. Earlier it was pointed out that excision of ZPA tissue from wingless wing buds and the insertion of pins into the graft tissue were difficult to perform. There was a looseness of the tissue not encountered in normal posterior wing bud tissue, and frequently pieces of mesenchyme fell away from the ectoderm of the graft. The wingless mesenchyme appeared to be less cohesive than normal and this observation raised the question – is the wingless mesenchyme capable of transmitting or reacting to a signal from the ZPA? This question is of particular interest because Tickle (1980) has proposed that the supposed ZPA morphogen may be transmitted through gap junctions between mesenchyme cells. It is possible that the junctional contacts in the wingless mesenchyme are defective, and might therefore impair the diffusion of the supposed morphogen across the limb bud and prevent mesenchymal response to the signal. It is of interest to note that Sawyer (1982), working on the American wingless mutant (*wg*), has shown that in this mutant the mesenchyme cells are more compact and rounded than normal, with shorter filopodial processes. In view of these findings, a

similar electron microscopical analysis of the wingless (*ws*) wing bud mesenchyme (prior to the onset of cell death) might provide further insight into the development of winglessness in the sex-linked wingless mutant.

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NOTE ADDED IN PROOF

Since this paper went to press a further account of winglessness in the American wingless (*wg*) chick mutant has been published (Carrington, J. L. & Fallon, J. F., *J. exp. Zool.*, **232**, 1984). This study shows that the ectoderm is affected by the wingless gene, and that there may be a pre-limb bud stage interaction between the wingless ectoderm and mesoderm causing a defect in the mesoderm at a later stage. In addition, the study shows that the *wg* wingless mutant possesses a polarizing zone, but its duplicative activity is lower than in normal ZPA tissue.