

Maps of strength of positional signalling activity in the developing chick wing bud

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SUMMARY

Tissue from the posterior margin of the developing limb bud, when grafted to the anterior margin, evokes the formation of a mirror-image limb duplication from the host tissue. We present maps of the spatial and temporal distribution of this signalling activity in the chick wing bud based on a bioassay that provides a quantitative measure of the completeness of the additional structures (the strength of activity index). Activity is first detected prior to the initial appearance of the limb primordium as early as Hamburger & Hamilton stage 14. It reaches a maximum during early outgrowth of the bud at stages 19 to 25. It then declines as the limb starts to differentiate into its final morphological pattern. The design of the experiment provides serendipitous data showing that two operators can consistently perform grafts with high reproducibility between them while variability between embryos is somewhat higher. The maps of activity are of particular practical value in precisely defining for the experimental embryologist and molecular biologist those positions and stages at which peak signalling activity resides.

INTRODUCTION

In the developing vertebrate limb there is a region at the posterior margin that controls the development of the pattern of tissues across the anteroposterior (AP) axis. If tissue from this region is grafted to an anterior position in a chick host limb it evokes the production by the host of a mirror-image limb duplication. This positional signalling region (Wolpert, 1969) or organizer (Spemann, 1938) was originally discovered by Saunders & Gasseling (1968) and subsequently named the Zone of Polarizing Activity (ZPA). It is a unique region with properties different in kind from any other limb tissue (Honig, 1983*b*, Summerbell & Honig, 1982). The spatial and temporal distribution of polarizing activity in the limb and flank of the developing chick embryo was systematically mapped soon after discovery (A. B. MacCabe, Gasseling & Saunders, 1973) and these maps are still a useful practical guide. However, improvements in the way that we can assay for activity and new ways of quantifying results have led us to suppose for some time that their maps can be misleading and we present here new data.

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Our assay consists of a strength of activity index based on the character or identity of the digit that forms nearest the graft (Honig, Smith, Hornbruch & Wolpert, 1981). Full activity produces extra digits 4 (normally the most posterior digit) while lesser activity causes extra digits 3 or 2, or evokes no duplicated digits. We can therefore ascribe each result in a set of embryos a quantitative score and calculate a mean score expressed as a percentage activity ranging from 0 to 100. The value of this assay has now been proven in a number of experiments in each of which a good relationship has been shown between some quantifiable variable and the strength of activity expressed. These studies include the effects of (a) ionizing radiation (Smith, Tickle & Wolpert, 1978); (b) ultraviolet radiation (Honig, 1982); (c) biochemical inhibitors (Honig *et al.* 1981; Honig & Hornbruch, 1982); (d) number of grafted posterior cells (Tickle, 1981); (e) culture time *in vitro* (Honig, 1983a); (f) exposure to retinoic acid (Summerbell, 1983; Tickle, Alberts, Wolpert & Lee, 1982); and (g) wingless mutant (Wilson & Hinchliffe, 1984).

METHODS

Eggs

Chicken eggs of Little Sussex and Rhode Island Red mixed breed were purchased from Needle Farm (Enfield, U.K.). Eggs were incubated at 38°C in humidified incubators on stationary shelves.

Operative procedure

Grafts were made to the anterior wing margin (opposite somite 16) of stage-19 to -21 host embryos so as to obtain full duplications of the handplate (Summerbell, 1974b; Tickle, Summerbell & Wolpert, 1975). Donors of stage-14 to -16 were prepared by subchorionic injections of Indian ink, as described in Summerbell & Hornbruch (1981), which improves visibility of the somites for counting. Some donor limbs at later stages (25 to 30) were removed from the eggs into Hank's balanced salt solution to prepare the grafts. Graft tissue was excised from the donor embryo using electrolytically sharpened tungsten needles and fixed into an equally sized host site using a 25 µm diameter platinum pin. Operated embryos were sealed with Sellotape and incubated for six to seven days before sacrifice. With the exception of some outlying positions showing little activity, at least six grafts were taken for each donor stage and position.

Particular care was taken to ensure reproducibility and accuracy in staging of donor embryos and in the assessment of position by reference to the somites or to the shape of the limb. Of the 472 surviving embryos, 354 (75 %) were joint experiments involving both authors, using a Wild M8 two-headed discussion stereomicroscope. During joint operations the two investigators agreed upon the stage and somite positions of each of the 66 donor embryos together according to the criteria of Hamburger & Hamilton (1951). From each donor, grafts were removed consecutively in pairs: one operator grafted the piece of tissue from the left wing bud, donor eggs were exchanged, then the other operator grafted the symmetrically corresponding piece from the right wing. Depending on stage, a series of three to seven left-right contiguous pairs of grafts were made from each donor embryo.

Specimen processing

Operated embryos were sacrificed at 9 to 10 days of incubation at stages 34 to 36. The left and right wings were removed and fixed in 5 % trichloroacetic acid for 2 to 3 h, rinsed in 70 % alcohol, stained with 0.1 % Alcian green 2GX in acid-alcohol, differentiated, dehydrated and either cleared in methyl salicylate or embedded in methacrylate resin (Summerbell, 1981).

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Limbs were examined under a Wild M8 stereomicroscope using transmitted illumination and were photographed using an Olympus OM-2 camera back on a trinocular head.

Data analysis

Each limb was drawn and then the drawings and the original specimens were examined by both investigators with particular attention to abnormal digits (8 % of operations, see results). For each set of grafts, percentage activity was calculated (Honig *et al.* 1981). Each operated limb was assigned 3, 2, 1 or 0 points depending on whether the most posterior digit it contained was 4, 3, 2, or whether it was without extra digits (Fig. 1). The number of points so scored was summed and divided by the maximum possible score (number of limbs in set multiplied by 3) to arrive at a percentage activity (100 % if the set contains all digit 4 duplications and 0 % if no duplications). The linearity of this strength of activity percentage depends on the thresholds for digits 2, 3 and 4 as discussed elsewhere (Honig *et al.* 1981). The location of the peak of activity at each stage was clear from inspection of the results but was confirmed together with a standard deviation as a weighted average of positions showing activity, using the percentage activities as weighting factors.

RESULTS

We examined a range of chick embryos from stage-14 to -30 which includes the period (15–29) studied by A. B. MacCabe *et al.* (1973). At stage-14 the lateral plate is forming at wing level and will later give rise to the limb primordium. At stage-30 cytodifferentiation of the digital wing cartilages is nearly complete. A total of 546

Table 1: *Strength of activity and digit next to graft for stages 14 to 19*

Somite position	Stage 14	Stage 15	Stage 16	Stage 17	Stage 18	Stage 19
17					0% 0:0:0:6	
18					8% 0:1:0:7	0% 0:0:0:6
19		25% 2:1:1:8	17% 0:1:2:5	8% 0:0:2:6	70% 4:4:1:1	80% 3:1:1:0
20	30% 1:2:2:5	50% 4:1:1:4	52% 1:2:4:0	52% 2:1:3:1	86% 9:2:0:1	100% 5:0:0:0
21	15% 0:1:2:6	43% 2:3:1:4	75% 3:4:1:0	71% 5:0:0:2	67% 6:0:0:3	78% 4:1:0:1
22	4% 0:0:1:7	17% 1:1:0:8	43% 4:0:1:5	58% 4:0:2:2	13% 0:0:3:5	22% 1:0:1:4
23	0% 0:0:0:1	0% 0:0:0:4	43% 2:1:1:3	33% 0:1:4:1	17% 0:0:1:1	
24		0% 0:0:0:2	27% 0:1:2:2	25% 0:1:1:2		

Each stage/position shows the % strength of activity entered above a set of four values, the number of cases having respectively from left to right duplicate digits 4, 3, 2, or no duplicate digits nearest to the graft.

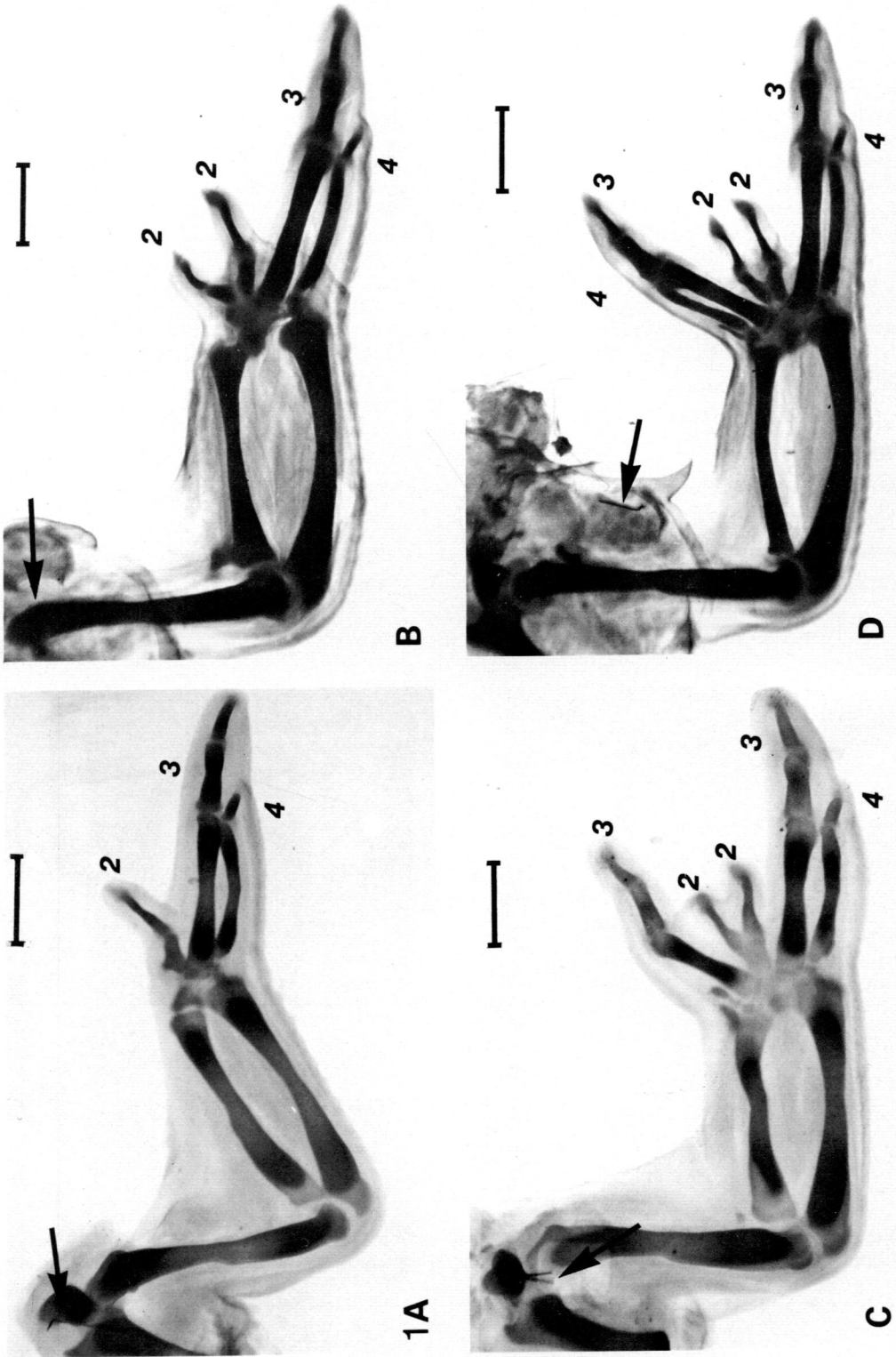


Figure 1. Whole mounts of 10-day embryonic right (operated) wings. (A) No duplicated digits, scores 0 points. (B) Duplicated digit 2 nearest graft (2 2 3 4), scores 1 point. (C) Duplicated digit 3 nearest graft (3 2 2 3 4), scores 2 points. (D) Duplicated digit 4 nearest graft (4 3 2 2 3 4), scores 3 points. Bars are 1 mm; arrows indicate positions of pins used to hold grafts in place.

Table 2: Strength of activity and digit next to graft for stages 21 to 30

Position on donor	Stage 21	Stage 23	Stage 25	Stage 26	Stage 27	Stage 28	Stage 29
distal	50%	15%	50%	0%	0%	0%	0%
	0:3:0:3	0:0:4:5	1:1:1:1	0:0:0:1	0:0:0:2	0:0:0:5	0:0:0:7
middle	95%	94%	100%	57%	42%	38%	27%
	12:2:0:0	9:2:0:0	4:0:0:0	1:5:4:0	0:5:4:2	0:2:4:1	0:2:0:3
proximal	100%	76%	83%	48%	57%	21%	19%
	10:0:0:0	4:2:0:1	3:3:0:0	3:2:0:4	1:6:2:1	0:1:3:4	0:1:2:4
distal	93%	83%	50%	38%	57%	25%	5%
	4:1:0:0	3:3:0:0	1:2:2:1	2:0:3:3	3:4:0:3	0:1:4:3	0:0:1:6
middle	27%	7%	5%	0%	0%		
	0:0:4:1	0:0:1:4	0:0:1:6	0:0:0:1	0:0:0:2		

Each stage/position shows the % strength of activity entered above a set of four values, the number of cases having respectively from left to right duplicate digits 4, 3, 2, or no duplicate digits nearest to the graft. No activity was found at stage 30, when tested at distal positions.

operations were performed of which 472 survived (mortality was 14 %). The results are shown in Tables 1 and 2. Surviving embryos had patterns unequivocally identifiable in 433 cases (92 % of results), but 39 embryos had digits next to the graft that were anatomically atypical. These were identified in 8 cases as digit 4, in 14 cases as digit 3 and in 17 cases as digit 2. In these doubtful cases we ascribed the likely highest value for our analysis. Ascribing the lowest likely value had only a small effect; no peak activity was changed by more than 8 %.

Spatial distribution of activity

The results and corresponding percentage of activity in the posterior half of the chick wing bud are shown in Fig. 2 and Tables 1 and 2. At stages 14 and 15, a low variable level of activity (< 50 %) is observed spread over three to four somites width at the most posterior end of the presumptive wing field. No activity is observed at posterior levels opposite somites 23 and 24 (note that the anterior limit of the leg bud is opposite somite 26). At stages 16 and 17 (at which time the lateral place mesoderm is distinct in the region of the wing field and the wing primordium is just starting to condense) high signalling activity (> 70 %) is observed opposite somite 21: grafts cause duplicated digits 3 and 4. On either side of this peak there is a gentle decline of activity such that activity is spread from somites level 19 to 24, with a "centre-of-gravity" at somite level 21/22, and a standard deviation of 1.5 somite widths. By stages 18 and 19 the peak activity nears 100 % producing always (stage 19) or nearly always (stage 18) additional digits 4. The peak is opposite somite 20, thus slightly more anterior (300 µm) relative to the limb field than at

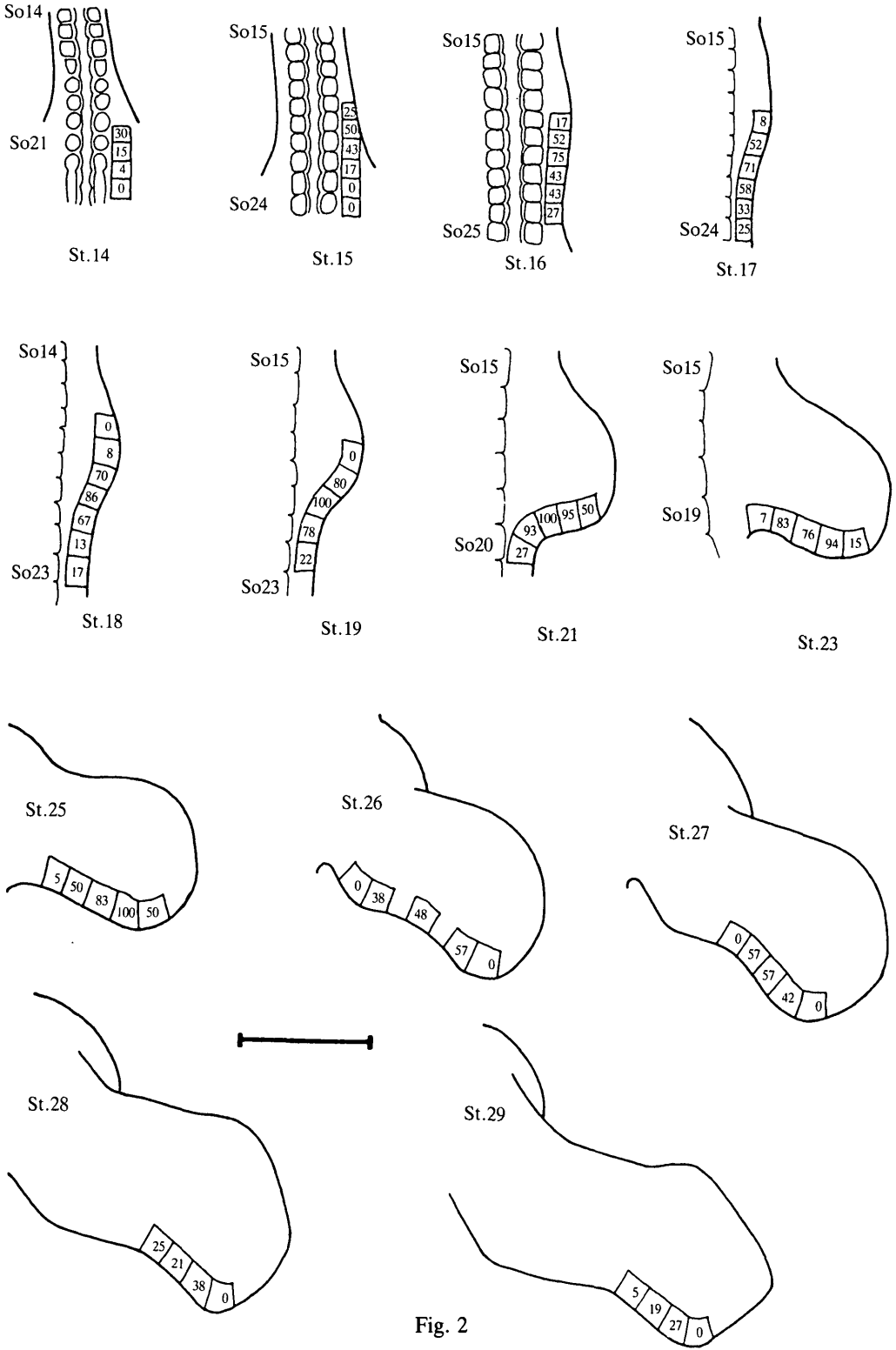


Fig. 2

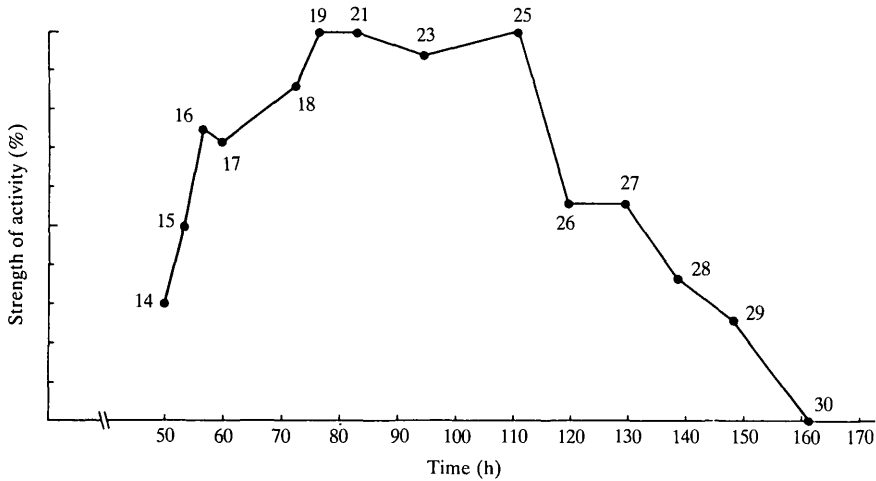


Figure. 3. Strength of activity (%) at its peak location for each limb bud plotted against embryonic incubation time. Stage is printed next to each point.

previous stages. Activity is somewhat less diffuse, with distributions having standard deviations of 1.2 and 0.9 somite widths at stages 18 and 19 respectively. At these stages, and at stage 21, peak activity is at the junction of the limb bud with posterior flank, very high activity (around 80 %) is present both immediately anteriorly on the limb, and posteriorly on the flank. Grafts from anywhere in this locale (which is 700–900 μm in length in the stage-21 embryo) yield predominantly 4-duplications. Grafts from more anterior levels on the bud (into the undifferentiated tip or progress zone area), or from very posterior positions on the flank, less frequently evoke duplications, none of which contain extra digits 4. In stages 23 to 27, activity is present along the posterior margin of the wing bud, with full activity at stages 23 to 25, and reduced levels at stages 26 to 27. Activity of greater than 50 %, with some observed 4-duplications, is spread diffusely over a distance of 900–1200 μm increasing in extent and moving more distal as the wing bud grows to several mm in length. Activity disappears on the flank, at proximal limb locations (e.g. presumptive arm and elbow at stage 27), and also at very distal positions, in the progress zone region.

Strength of peak activity during development

The strength of activity for the peak position of each donor stage is shown in Fig. 3. It reaches a maximum at stages 19 to 25, with almost full activity: virtually every

Figure. 2. Maps of positional signalling activity at stages 14 to 29. The areas assayed are shown on one side of the embryo. Drawings are to scale (bar is 1 mm) number on graft shows % strength of activity. The size of each graft piece as drawn does not indicate an identical graft size in that position for each embryo. But the variation in drawing freehand approximately equal size squares is similar to the variation in excision of approximately equal size graft tissue pieces.

graft from the peak position evokes a digit 4. And every tested embryo between these stages has at least one graft area on each side that gives a 4-duplication. Reliable but distinctly attenuated activity is shown by stages 26 to 27; the predominant results are 3- and 2-duplications. For the most part, by stage 28, the only duplications caused by graft tissue are extra digits 2; still fewer duplications occur at stages 29 and 30. The decline in peak activity from stage 25 onwards occurs despite the slightly larger volume of graft pieces, due to increased dorsoventral limb bud thickness.

At stages of development before stage 19, prior to the 35 h period of 100 % peak activity, there were fewer duplications. Strength of activity at its peak location was only about 70 % at stages 16 to 17, 50 % at stage 15 and 30 % at stage 14. In the earlier embryos, the distribution of results at any position did not suggest the presence of a weak or attenuated signalling region since rather than getting predominantly 2- or 3-duplications there was an all-or-nothing pattern with mostly 4- and 0-duplications (e.g. stage 15 opposite somite 20 and stage 17 opposite somite 21).

DISCUSSIONS

The results presented here show the regional localization of chick wing polarizing activity over a range of 17 stages. These stages extend over 4.5 days (110 h) during which time the wing anlagen arises and develops through early limb primordium and limb bud to a stage at which digits have begun to appear. Signalling activity is present for the entire period but reaches the maximum over a period of duration 35 h from stages 19 to 25. At each stage there is a single peak location of activity. Signalling activity decreases in neighbouring regions to negligible levels anterodistally in the presumptive digit field, and posteriorly in the flank. A similar profile is seen in the leg bud at stage 21–22 (Hinchliffe & Sansom, 1984). Hornbruch & Wolpert (personal communication) have carried out a series of experiments at still earlier chick stages starting at stage 9, when Hensen's node is regressing past the limb field, and overlapping the earliest stages reported here. Their findings open the possibility of a relationship between the node and the posterior limb organizing region. The maps of MacCabe *et al.* (1973) are significantly different from ours. The results presented here show more extensive, wider areas of signalling activity in both early, stage-14 to -16, and late, stage-27 to -29, limbs; importantly we show strong, albeit submaximal activity at the earliest stages. However these studies cannot be exactly compared because MacCabe *et al.* (1973) used a host site at the midline of the recipient wing, which grafting position frequently yields limbs that are more difficult to interpret than those resulting from grafts to the anterior margin. Furthermore, the grey and black shading on their maps may be misleading as they are not based on a simple quantitative assay: indeed their darkened areas do not necessarily represent signalling activity. For example, their criteria consisted of 'major' duplications which included 'forearm structures without corresponding

duplication of the digits', and 'minor' duplications which included 'feather germs without the corresponding duplication of underlying cartilaginous structures'. Both of these classes of limbs are likely to be the result of self-differentiation of the graft and/or disturbance of the host pattern, when graft emplacement is in the midline. Our mapping experiments do not have this difficulty, and furthermore are based on a clearly defined and simple quantitative assay.

The new maps (Fig. 2) showing locations of signalling activity will be of great practical help to experimental embryologists by precisely delimiting the stages and locations in the wing bud at which peak signalling activity resides. This is useful data for the design and interpretation of experiments in which this posterior tissue is used as either donor graft or recipient site (Iten & Murphy, 1980; Summerbell & Honig, 1982). Furthermore it will greatly facilitate experiments at the cellular or biochemical level which require as pure as possible populations of signalling cells, often in large quantities.

Analysis of operator and embryo variability

Since the large majority of the experimental data in Tables 1 and 2 are the result of coordinated joint experiments an analysis can be made comparing *inter*-embryo variability (which depends on embryo heterochronicity, heterogeneity, staging discrepancies, somite arrangements) with *intra*-embryo variability (which, considering that the embryo can reasonably be taken as bilaterally symmetric (Summerbell & Wolpert, 1973), refers to the inter-operator variability). The general impression from the data is that inter-embryo variability is greater than inter-operator variability and is substantiated by computing and comparing correlation coefficients (r) of paired results. Overall, the correlation between the results of the two operators, $r = 0.53$ ($n = 124$, $P < 0.01$) was higher than that between embryos for the two operators each individually: 0.36 and 0.45 (both $n = 62$, $P < 0.01$). However use of the whole range of results is somewhat artificial because of the heavy weighting of all 4 and all 0 duplications towards a high correlation. The measure becomes much more meaningful and sensitive if only data points in the range 20–80 % activity are included. By this measure inter-operator correlation has $r = 0.30$ ($n = 68$, $P < 0.01$) while inter-embryo values for the two operators are $r = 0.09$ and 0.14 (both $n = 34$, $P > 0.10$). On detailed analysis it is found that inter-operator reproducibility is equally high at all stages by this measure ($r = 0.3$), while inter-embryo correlation is close to this value only at stages 21–30; at stages 14–19, it is much lower. The above analysis shows that two operators can consistently perform grafts with high reproducibility between them, while variability between embryos is somewhat higher particularly at early stages.

Positional signalling activity at different stages

All of our grafts were made to anterior positions in stage-19 to -21 hosts. During this host stage period (14 h) the recipient wing responds well to classical polarizing region (ZPA) grafts, producing reliable duplications from about the level of the

wrist; older hosts show increasingly poor duplications from grafts of similar stage and position (Summerbell, 1974*b*). For donors of stages later than 22, this experimentally labile recipient stage should yield the maximal estimate of activity. However for donors of earlier stages that we have used, stages 14 to 17, one might argue that younger, isochronic hosts would show higher activity. It is important however that we use the standard older hosts (stage 19–22) because we are interested in activity at the time of making the graft and not in activity as it might develop if the graft tissue is left to mature. We therefore deliberately limit the stage of host embryos.

This leads us to consider how the same group of cells at early stages yields between 20 and 80 % activity as shown here but will later give us 100 % activity. There are several possible interpretations

(1) It is possible that signalling activity is not yet fully functional (there is no limb field yet). Increased activity at a later stage could arise through (a) increased synthesis (Summerbell, 1979), (b) a diffusely distributed low activity becoming concentrated at a single position by a mechanism involving positive feedback, such as the reaction diffusion model (Meinhardt, 1982), or (c) the proliferation of signalling cells so as to produce larger numbers (Tickle, 1981).

(2) Alternatively it is possible that conditions may be sub-optimal for these early stage grafts. There is evidence that intact apical ectodermal ridge is important to ensure good interaction between graft and host and early grafts may be deficient in this respect (Honig, 1982; Rowe & Fallon, 1981; Tickle, 1981), or may simply be smaller than the grafts made from later stage donor embryos. All these considerations are however not relevant to the main point of this paper, which is to consider operationally how well these tissue grafts signal positional information.

CONCLUSION

These new maps of positional signalling activity update those of MacCabe *et al.* (1973). Particularly they show significant levels of activity in an area near the posterior margin of the limb rudiment as early as stage 14. This result was specifically predicted in a computer simulation of pattern control across the anteroposterior axis using a diffusible morphogen produced at the posterior margin (Summerbell, 1979). However the observation of erratic position and level of activity at these early stages is typical of what one would expect if the positional signalling region was produced by the reaction–diffusion mechanism (Meinhardt, 1982) and more detailed observations on these early stages would be of considerable interest. The results also show that at late stages the level of activity gradually declines even though the distal parts of the limb are still undifferentiated and in the process of specification (Summerbell, 1974; Summerbell, Lewis & Wolpert, 1973). This is again a prediction of the diffusible morphogen model. Maximal activity is still found in the grafts from some embryos up until stage 27, well after the time that the limb can develop normal morphology following removal of the posterior margin. (Fallon

& Crosby, 1975; Hinchliffe, Gumpel-Pinot, Wilson & Yallup, 1984; Summerbell, 1979). The overall pattern is the gradual formation of a discrete focus of activity at limb primordium stages. This zone persists as a relatively localized region during early limb bud stages. Later as determination and differentiation start the activity spreads out along the posterior margin and dissipates. Perhaps there is a fixed population of non-dividing cells that either (a) exhibits a finite functional signalling lifetime, or (b) is gradually diluted by cell division of other mesenchyme cells to number densities which cannot effectively signal.

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