Time, place and positional value in the chick limb-bud

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SUMMARY

Quantitative experimental evidence is presented for the progress zone theory of limb development. The theory, here formulated mathematically, states that the parts of the limb are specified in proximo-distal succession by an autonomous timing mechanism operating in a 'progress zone' of undifferentiated growing mesenchyme under the influence of the apical ectodermal ridge. By the exchange of distal tips between young and old wing-buds, it is shown that there are no long-range morphogenetic signals from proximal to distal tissue. The width of the progress zone is calculated, and it is found autoradiographically that practically all its cells are dividing.

INTRODUCTION

The vertebrate limb comprises only a few types of tissue, but these are arranged in a precise and intricate pattern. The phenotype of each cell is a function of its position. Two things are needed to bring this about: the cell must get cues from its environment as to its position; and it must interpret those cues appropriately. Specifically, we suggest that the cells of the limb-bud use environmental cues to acquire intrinsic 'positional values' which correspond with their positions, and govern their course of differentiation according to rules embodied in the genome (Britten & Davidson, 1969; Wolpert, 1971; Kauffman, 1973; Wolpert & Lewis, 1975). Like others before us, we take the chick wing as our experimental archetype (Zwilling, 1961; Amprino, 1965; Ede, 1971; Saunders, 1972).

In a previous paper we presented a theory (Summerbell, Lewis & Wolpert, 1973) which explained how cells acquire their positional values along the proximo-distal axis of the chick wing-bud. Here we put that theory in a more quantitative form, and give in detail the crucial experimental evidence against morphogenetic signals from the proximal to the distal part of the limb-bud. We shall discuss only the proximo-distal organization, and not the antero-posterior or dorso-ventral (Saunders, 1972; Caplan & Koutroupas, 1973; Summerbell, 1974a; Wolpert, Lewis & Summerbell, 1974).

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The cells of the limb-bud, we argued, establish their positional values by reference to some sort of internal autonomous 'clock'. They necessarily depend also on an external signal of position, but it is of a very simple kind: it emanates from the apical ectodermal ridge, and marks out a 'progress zone' at the tip of the limb-bud. In this zone, the positional value is allowed to become progressively more distal, in time with the autonomous clock; elsewhere it is held constant. The cells in the progress zone steadily proliferate and overflow from it, becoming fixed in positional value as they cross its proximal boundary. The later a cell leaves, the more distal its positional value at that decisive moment. Thus in the course of time successively more distal positional values are laid down in successively more distal positions. Outside the progress zone, the cells may differentiate autonomously according to their individual positional values. Alternatively, and perhaps more probably, they may interact over short distances even there: the local mean positional value would determine the parameters of these late local interactions so as to give the structures appropriate to the place. The present paper does not decide between these alternatives, though our data set an upper limit to the range of interaction between cells outside the progress zone.

The formal statement of the progress zone theory, which we now give, shows how the eventual pattern should depend on the growth rate, on the width of the progress zone, and on the rate of change of positional value in it.

**Formal model**

To be specific, consider an idealized system growing out along one axis (Fig. 1), and such that at any time the cells are proliferating at the same rate everywhere and maintain a constant packing density (these assumptions are not essential to the theory, but simplify exposition). Let us follow a particular cell lineage, which we label by its initial mean distance \( l \) from the tip. After some time has passed, growth will have carried this lineage to a new distance \( x \) from the tip. If the mean number of mitotic doublings undergone by each cell lineage is \( T \), then this new distance will be \( x = l \cdot 2^T \). One can regard \( T \) as a measure of the time, determined by a clock whose ticks are cell divisions. We shall call \( T \) the age of the outgrowth.

Let \( P(l, T) \) be the positional value, at age \( T \), of the lineage \( l \). Our progress zone hypothesis is that there is a region at the tip where \( P \) changes progressively, while elsewhere \( P \) is fixed so that the pattern can only spread out by interstitial growth. In formal terms

\[
\frac{\partial P(l, T)}{\partial T} = \phi(x) = \phi(l \cdot 2^T),
\]

where \( \phi(x) \) is practically zero except at small values of \( x \), i.e. inside the progress zone. The positional value, \( P \), appearing in this equation is a certain function
Time, place and positional value in the chick limb-bud

Fig. 1. An idealized limb-bud, growing out along the axis $AB$. The progress zone is shaded; $x$ is the distance of a cell lineage $C$ from the tip.

Fig. 2. The pattern of positional values, $P$, as a function of the distance from the tip in an idealized uniformly-growing limb-bud, at three successive ages $\tau$. $C$ marks the successive positions of a typical cell lineage.

(see Appendix) of the eventual level $L$ occupied in the mature structure. We shall later see how to relate $P$ to $L$ experimentally in the chick limb.

For simple illustration, suppose that the progress zone has a sharp boundary at $x = w$ such that $\phi(x)$ is a constant distal to that boundary, and zero proximal to it:

$$\phi(x) = \begin{cases} \phi_0 & \text{for } 0 < x < w, \\ 0 & \text{for } x > w. \end{cases} \quad (2)$$

Suppose also that initially the whole outgrowth has the same positional value, which we take as the origin of the positional value scale; then

$$P(l, 0) = 0. \quad (3)$$
Integrating equation (1) we thus find

$$P(l, r) = \int_0^r \phi(l, 2^r) \, dr,'$$

$$= \int_0^{1.2^r} \frac{1}{y \ln 2} \phi(y) \, dy,$$

$$= \begin{cases} \phi_0 r & \text{for } l.2^r < w, \\ \phi_0 \log_2 \frac{w}{r} & \text{for } l.2^r > w. \end{cases} \quad (4)$$

Hence the positional value at the distance $x$ from the tip is $\phi_0 r$ if $x$ lies anywhere inside the progress zone, and is $(r - \log_2 x/w) \phi_0$ if $x$ lies outside the progress zone. This pattern of positional values is shown at three successive ages in Fig. 2.

The size of the rudiment of any structure at a given age depends on $w$ and $\phi_0$. Suppose, for example, that the proximal end of the humerus corresponded to a positional value $P_1$, and the distal end to a value $P_2$. Then at age $\tau$ the rudiment of the humerus, provided it had emerged from the progress zone, would occupy the region from $x_1$ to $x_2$, where

$$\begin{cases} (\tau - \log_2 \frac{x_1}{w}) \phi_0 = P_1, \\ (\tau - \log_2 \frac{x_2}{w}) \phi_0 = P_2. \end{cases} \quad (5)$$

The length of the humerus rudiment would therefore be

$$x_1 - x_2 = w \cdot 2^{\tau - P_1/\phi_0} - w \cdot 2^{\tau - P_2/\phi_0},$$

$$= w(2^{P_1/\phi_0} - 2^{P_2/\phi_0}) \cdot 2^\tau. \quad (6)$$

Thus the size of the primordium at a given age $\tau$ is directly proportional to the size of the progress zone, but decreases with increasing $\phi_0$. Now $\phi_0$ is the rate of change of positional value $P$ with age $\tau$ in the progress zone, and this equals the rate of change of $P$ with time $t$, divided by the rate of change of age $\tau$ with time $t$:

$$\phi_0 = \frac{dP}{d\tau} \bigg|_{x < w} = \frac{dP}{dt} \bigg|_{x < w} (\frac{d\tau}{dt})^{-1}. \quad (7)$$

Since $\tau$ is defined as the mean number of population doublings that have elapsed, $d\tau/dt$ is simply the growth rate. Thus $\phi_0$ is the rate of change of positional value with time $t$, divided by the growth rate. Rapid change of positional value, accompanied by slow growth, would, for example, make for a big $\phi_0$, and hence for small primordia.

If the rate of change of positional value and the growth rate were independently variable, it would be possible to change the relative size of an element, or at least of its primordium, by changing the growth rate, e.g. through heating or
Time, place and positional value in the chick limb-bud

cooling, as it emerged from the progress zone. But if changes of positional value were strictly linked to cell division and proceeded at a directly proportional rate, then \( \phi_t \) would be independent of the growth rate; the relative sizes of primordia would be sensitive to disturbances of the environment only in so far as these affected \( w \), the width of the progress zone. Thus, barring perturbations of \( w \), pattern formation would be reliably coordinated with growth: development would not be distorted, but only hastened or delayed by the variations of temperature to which most embryos are subject. According to our preliminary results, the eventual proportions of chick wings are indeed unaffected by heating and cooling during development. Further experiments to test for a link between change of positional value and cell division are being done, but we shall not try to decide the issue in this paper. Rather, we intend to show how the experimental evidence supports the basic hypothesis that change of positional value is an autonomous process in the progress zone, and is not dependent on any proximal influence.

MATERIALS AND METHODS

Fertilized White Leghorn embryos were incubated at 38 °C and windowed on the third, fourth or fifth day of development. The embryos were staged according to Hamburger & Hamilton (1951), the window sealed over with sellotape and the egg returned to the incubator. A pair of appropriately staged eggs was selected for each operation. The distal part of the right wing was surgically excised from the donor embryo, transfixed with two platinum pins, and transferred to the host egg. The width of the graft from tip to proximal edge was estimated using an eyepiece graticule calibrated at 50 \( \mu \)m per division, in a Zeiss Stereo IV dissection microscope. Although there was some difficulty in measuring the width of the graft (due to the amputation plane being more or less oblique) it was probably correct to \( \pm 50 \mu \)m and certainly to within \( \pm 100 \mu \)m. The distal part of the host right wing was similarly removed and transfixed with pins, and the donor tip was pinned in its place. The second wing tip was then transported in turn to the original embryo and pinned in place on the right wing stump. In all cases, care was taken to ensure that the antero-posterior orientation of the graft was normal with respect to the host. The eggs were then returned to the incubator. In a few cases, embryos were sacrificed between 0 and 24 h after the operation to check that the graft had knitted to the host and that the width of the graft had been estimated correctly. The procedure followed was the same as for preparation of toluidine blue sections in the embryos used for autoradiography (see below). The remaining embryos were left in the incubator until the tenth day of incubation. They were then sacrificed and the wings from operated (right) and control (left) sides fixed in 5 % TCA, stained in 0.1 % Alcian green 8GX in 70 % alcohol with 1 % hydrochloric acid, dehydrated, and cleared in methyl salicylate. Operated and control limbs were examined and photographed using a Zeiss Stereo IV microscope and the lengths of humerus,
ulna, radius and the elements of digit III, the 'middle finger', measured, wherever they were present.

For the autoradiography, tritiated thymidine (from The Radiochemicals Centre, Amersham, code No. TRK 61, 22 Ci/mm, 1 mCi/ml, in water) was diluted 1 : 4 with BSS (containing 50 i.u. penicillin, 50 μg streptomycin, and 2.5 μg Nystatin per ml) giving an activity of 200 μCi/ml. Embryos at stages from 20 to 28 were removed from the incubator, the vitelline and amniotic membranes were torn open and 20 μCi in 0.1 ml of BSS was dropped directly on to the embryo. The embryo was then returned to the incubator. (Work in our laboratory by Cheryll Tickle has shown that this simple and convenient method gives the most reliable and consistent uptake of activity.) Four booster doses of 10 μCi were given in the same way at intervals of about 2.5 h thereafter. The wing-buds were cut out 12 h after the first dose (and hence 1 h after the last) and were fixed in half strength Karnovsky's fixative, washed, dehydrated and embedded in Araldite. Sections were cut between 1 and 1.5 μm thick in a plane containing the proximo-distal and dorso-ventral axes of the limb. Some sections were stained with toluidine blue for histological examination and some pre-stained with Feulgen for autoradiography. Autoradiographs were prepared by dipping in Ilford K2 emulsion at a dilution of 1:1 with distilled water, exposed for 2–3 weeks and developed in Kodak D19. The sections were viewed under phase contrast with a ×100 oil immersion objective on a Zeiss Photomicroscope I, and labelled and unlabelled nuclei were counted in a series of non-overlapping rectangular fields. The mean number of grains over a labelled nucleus sectioned through its centre ranged from about 100 at the earlier stages down to about 20 at the later. A nucleus was judged to be labelled if three or more silver grains lay above it. The mean background on every slide was less than, or of the order of, 0.5 grains/nucleus.

RESULTS

We present first some subsidiary findings as to the character and size of the progress zone. We then give the results of our main experiments, the distal tip exchange grafts. We compare these results with the quantitative predictions of our theory.

Cell division in the progress zone

The mesenchyme of the chick wing-bud is roughly homogeneous at first; overt differentiation of muscle and cartilage begins only at about stage 23, in the proximal region (Fig. 3). Distally, i.e. within 300 or 400 μm of the tip, the mesenchyme remains apparently undifferentiated with a comparatively high mitotic index (Hornbruch & Wolpert, 1970) up to about stage 28; by this time almost all the skeletal rudiments are visible (Fig. 4).

We find autoradiographically that, up to stage 28, 97 % or more of the cells sampled within 300 μm of the tip are actively dividing, i.e. become labelled in the course of 12 h continuous exposure to tritiated thymidine. The detailed
Fig. 3. A stage-23 wing-bud sectioned at 1 μm thickness in a plane containing its proximo-distal and dorso-ventral axes, and stained with toluidine blue. AER, apical ectodermal ridge; U, undifferentiated mesenchyme; M, pre-muscle; C, pre-cartilage.

Fig. 4. Photomicrograph of a limb fixed at stage 28, stained with Alcian green 8GX and cleared in methyl salicylate. All of the skeletal elements except for the distal phalanges of the two most anterior digits are already quite distinct.
Table 1. The labelling index, after 12 h continuous exposure to tritiated thymidine, in the mesenchyme within 300 μm from the tip

<table>
<thead>
<tr>
<th>Labelling begun, stage</th>
<th>Fixed, stage</th>
<th>Total cells counted</th>
<th>% cells labelled</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>23</td>
<td>773</td>
<td>98.1</td>
</tr>
<tr>
<td>21</td>
<td>24</td>
<td>702</td>
<td>99.1</td>
</tr>
<tr>
<td>22</td>
<td>25</td>
<td>762</td>
<td>98.6</td>
</tr>
<tr>
<td>23</td>
<td>25</td>
<td>877</td>
<td>97.9</td>
</tr>
<tr>
<td>24</td>
<td>26</td>
<td>963</td>
<td>98.1</td>
</tr>
<tr>
<td>25</td>
<td>27</td>
<td>1112</td>
<td>98.3</td>
</tr>
<tr>
<td>26</td>
<td>27</td>
<td>1157</td>
<td>99.4</td>
</tr>
<tr>
<td>27+</td>
<td>28</td>
<td>1276</td>
<td>97.6</td>
</tr>
<tr>
<td>28</td>
<td>29</td>
<td>1300</td>
<td>75.5</td>
</tr>
</tbody>
</table>

Each line gives measurements on a single chick.
Note the sharp drop in labelling index beyond stage 28; this corresponds to the appearance of differentiating pre-cartilage in the apical mesenchyme.

results are given in Table 1. It should be mentioned that Janners & Searls (1970) have reported a labelling index of only 85% near the tip after 12 h continuous labelling at stage 24; but they did not maintain the level of tritiated thymidine by such frequent booster doses as we did, and they were using 5 μm thick wax sections for their autoradiography — a thickness which may exceed the range of the β-particles from tritium (Rogers, 1967; Simnett, 1968).

The width of the progress zone

It is possible to deduce the width of the progress zone using data on apical ridge removal (Summerbell, 1974b), along with the normal fate maps (Amprino & Camosso, 1958; Lewis, 1975). It is well known (Saunders, 1948) that if the apical ectodermal ridge is excised the distal parts of the limb fail to develop. In the terms of our progress zone model, apical ridge removal halts progress in the zone at the tip. We can, therefore, use it to assay for the positional value at the tip of the bud at any stage. The level of truncation of a de-ridged limb should show us what was the positional value at the tip of the bud at the time when progress stopped, i.e. at or shortly after the moment of ridge removal. The results (Summerbell, 1974b) are summarized in Fig. 5, which indicates for each stage the level of truncation averaged over at least eight limbs from which the apical ridge had been removed.

We define the effective width \( w \) as the width the zone would require in order to generate the observed positional values, if it had the simple step-like characteristic considered in the introduction:

\[
\phi(x) = \begin{cases} 
\phi_0 & \text{for } 0 < x < w, \\
0 & \text{for } x > w.
\end{cases}
\]
In such a case, each cell lineage would normally reach the boundary of the zone, distant \( w \) from the tip, at a clear-cut stage \( S \), and would end its career of change with the positional value \( P_{\text{exit}}(S) \) which it then had. The positional value throughout the progress zone at stage \( S \) would also be \( P_{\text{exit}}(S) \). Thus if apical ridge excision at stage \( S \) stopped all changes of positional value instantaneously, it would cause truncation at the level with positional value \( P_{\text{exit}}(S) \). We could read off that level directly from Fig. 5. From the fate map, we could then locate at stage \( S \) the tissue normally fated to lie at that level. By definition of \( P_{\text{exit}}(S) \), this tissue must normally be leaving the progress zone at stage \( S \). Hence \( w \) is its distance from the tip at stage \( S \). Taking different choices of \( S \), we get a set of different estimates for \( w \), as listed in Table 2. The mean of these estimates is 230 \( \mu \text{m} \) with a variance of \( \pm 70 \mu \text{m} \). In practice, unfortunately, we have to allow for some unknown delay \( \Delta \) between removing the ridge, and stopping the changes of positional value. To find \( w \), we should in principle therefore use in
Table 2. Estimation of \( w \), the effective width of the progress zone

<table>
<thead>
<tr>
<th>Stage, ( S ), at apical ridge excision</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of truncation</td>
<td>0.9H</td>
<td>0.3F</td>
<td>0.4F</td>
<td>W</td>
<td>W</td>
<td>W</td>
<td>0.0M</td>
<td>0.6M</td>
<td>0.8P1</td>
<td></td>
</tr>
<tr>
<td>Estimate for ( w ), from fate map (( \mu \text{m} ))</td>
<td>170</td>
<td>180</td>
<td>250</td>
<td>130–250</td>
<td>100–210</td>
<td>160–310</td>
<td>170–360</td>
<td>210</td>
<td>360*</td>
<td>190</td>
</tr>
</tbody>
</table>

The level of truncation (from Summerbell, 1974) is denoted by a letter to specify a skeletal segment (H = humerus, F = forearm, W = wrist, M = metacarpals, P1 = first phalanges), preceded by a number to specify position within that segment; thus 0.9H means \( \frac{9}{10} \) of the way along the humerus, i.e., just short of the elbow. The estimates for \( w \) are read off from fate maps of Lewis (1975) for stages 18–25, and of Amprino & Camosso (1958) for stages 26–27. The levels of truncation for stages 21–24 are identified only as being somewhere in the wrist (though they seem roughly to represent a progression towards a more complete structure). The mean quoted in the text is taken over stages 18–20 and 25–27, omitting the indeterminate results at stages 21–24.

* The apparent variations of \( w \) from stage to stage are no greater than the experimental errors. If, say, we used Amprino & Camosso’s stage-25 fate map for our stage 26 (as would be reasonable from a comparison with the Hamburger-Hamilton norm), we should estimate \( w = 250 \mu \text{m} \) at that stage, instead of 360 \( \mu \text{m} \).

the above calculation a fate map for a stage later than the stage at apical ridge excision by an amount \( \Delta \). By proceeding as though \( \Delta \) were zero, we underestimate \( w \) by a factor corresponding to the growth of the limb between stage \( S \) and stage \( S + \Delta \). Roughly speaking, the wing-bud elongates uniformly, taking about twice the cell cycle time to double its length (Lewis, 1975). Thus the correction factor to be applied to our estimates for \( w \) is of the order of \( 2^{\Delta/2} \) where \( \Delta \) is measured in cell cycles. Hence our final mean value for \( w \) is

\[
\begin{align*}
  w &= (230 \pm 70) \cdot 2^{\Delta/2} \mu \text{m}.
\end{align*}
\]

If, for example, \( \Delta = \frac{1}{2} \) a cell cycle, or about 5 h, then

\[
\begin{align*}
  w &= 270 \pm 80 \mu \text{m}.
\end{align*}
\]

This estimate of the width of the progress zone tallies well with the histological picture (Fig. 3): we recall that the region of undifferentiated, rapidly proliferating mesenchyme extends inwards from the tip of the wing-bud for about 300 or 400 \( \mu \text{m} \). Since \( w \) was calculated using only fate maps and Fig. 5, without any appeal to histology, this coincidence, rough as it is, constitutes important corroboration for our theory so far.

Exchange of distal tips

Our basic procedure is to exchange the tips of wing-buds of different ages. If both pieces of each composite bud develop autonomously according to their
original presumptive fate, some parts of one resulting limb will be missing whilst corresponding parts of the other will be duplicated. But if the stumps signal positional values to the tips, the presumptive fates of the tips will be radically modified. The interpretation of the experimental results is straightforward if each grafted tip is wider than about 300 \( \mu m \) and so contains the progress zone entire. From fate maps one can then predict directly what the outcome should be if there is no signalling of positional value, and compare this prediction with the observations. It is, however, slightly more complicated to make the corresponding prediction for thinner tip grafts, as in these cases the progress zone due to the apical ridge on the graft may well extend into the host mesenchyme.

We therefore present our findings in two parts—first thick tip grafts, then thin tip grafts. In each case we compare our experimental results with results predicted from fate maps according to the progress zone theory.

**Thick tip grafts**

The experiment is shown schematically in Fig. 6. Embryos were taken in pairs, an early stage with a late. The tip of the late wing-bud was cut off, and the tip of the early wing-bud, measuring about 300 or 400 \( \mu m \) from cut face to
Fig. 7. Photomicrographs of whole limbs fixed on the ninth or tenth day of incubation, stained with Alcian green 8GX, and cleared in methyl salicylate. The figures illustrate results fulfilling the predictions in Fig. 8. The grafted tip in each case is from a stage 19 or 20 wing-bud. (a) Normal limb. (b) Stage-20 host: host gives humerus and parts of ulna and radius. (c) Stage-22 host: note absence of host carpals. (d) Stage-24 host, distal: note presence of host carpals; part of donor girdle has been transplanted with the graft. (e) Stage-24 host, middle: host radius is clearly truncated and distal epiphysis of host ulna is reduced. (f) Stage-24 host, proximal: the large proximal element is the host humerus (it matches the host contralateral control in size). The smaller element parallel with it is the distal part of the donor humerus.
Time, place and positional value in the chick limb-bud

Donor

<table>
<thead>
<tr>
<th>Stage 19/20</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>24</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted composite limb</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>Observed composite limb</td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>No. of cases</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 8. Observed and predicted results of operations in which a stage-19/20 tip, measuring about 300 or 400 μm from cut face to apical ridge, is grafted onto an older stump. The parts that go to make up the composite limb are shown stippled in the top line. The observed results are means, based on the numbers of cases shown in the bottom line. The predictions are based on fate maps (Lewis, 1975; Amprino & Camosso, 1958), assuming no interaction between host and graft.

apical ridge, was pinned onto the late stump in its place. The detached late tip was grafted onto either the early stump or the flank. The resulting wings, together with the contralateral controls, were fixed, stained and measured as whole mounts after a further five or six days of incubation.

The early tips healed well onto the late stumps within 3 or 4 h, and the composite limbs regularly developed in a mosaic fashion, giving reduplications. The detailed results are illustrated by photographs in Fig. 7, and are tabulated in Fig. 8, together with the predictions based on fate maps, assuming that the stump and the grafted tip develop autonomously and independently. The results agree with the predictions to within about half a cartilage element. The variance of the result, for any given combination of stages, is of about the same magnitude. When we compare the dimensions of the experimental wings with those of the contralateral control wings (Summerbell & Wolpert, 1973), we find that the cartilage elements predicted to come from the host have almost exactly the same size as the contralateral control host elements, while the elements predicted to come from the graft have almost exactly the same size as the contralateral control donor elements. That is, the size of each part is determined by its origin, not by its new site. There is a slight discrepancy for late tips grafted onto early hosts: they generally give elements a bit smaller than the contralateral donor controls. But this can be explained as the result of trauma: if a detached late tip is pinned directly back onto its own (late) stump, its development is stunted on average by the same amount. In Table 3, we give the measured ratios of lengths of middle digits, comparing transplanted tips, both young and old, with host
Table 3. The lengths of the middle digits from transplanted tips, compared with host and donor controls, and with controls for the effect of trauma

<table>
<thead>
<tr>
<th></th>
<th>st. 24 sev</th>
<th>st. 24 contr</th>
<th>st. 24 transp</th>
<th>st. 24 donor contr</th>
<th>st. 24 transp</th>
<th>st. 24 host contr</th>
<th>st. 19/20 sev</th>
<th>st. 19/20 contr</th>
<th>st. 19/20 transp</th>
<th>st. 19/20 donor contr</th>
<th>st. 19/20 transp</th>
<th>st. 24 host contr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.86 ± 0.07</td>
<td>0.82 ± 0.10</td>
<td>0.97 ± 0.02</td>
<td>1.29 ± 0.08</td>
<td>1.29 ± 0.08</td>
<td>0.86 ± 0.07</td>
<td>0.97 ± 0.02</td>
<td>1.29 ± 0.08</td>
<td>1.01 ± 0.05</td>
<td>1.01 ± 0.05</td>
<td>0.63 ± 0.04</td>
<td>1.29 ± 0.08</td>
</tr>
</tbody>
</table>

Abbreviations: st., stage; sev, severed from stump, but then pinned back on again, as control for trauma; transp, transplanted to stump of different age; contr, contralateral control.

The figures for the transplanted tips are means and standard deviations for a set of 4 complementary pairs, in which stage-24 and stage-19/20 tips were exchanged. The figures for tips severed but replaced are means and standard deviations for six cases for each stage.

and donor contralateral controls, and with controls for trauma, i.e. tips which have been severed from their stumps, but then pinned back on again. We conclude that both pattern-formation and growth proceed autonomously in these 300 μm-wide tip grafts, and are not significantly affected by any long-range interactions between tip and stump.

There may be some sign of short-range interactions, however, at the boundary between the host and the graft. It is usually quite easy to see where the boundary lies in the whole mounts. The cartilaginous elements formed from the host are bigger and more mature than those from the graft (Fig. 7d, f), and though they may sometimes fuse with them (Fig. 7b), the junction in such cases is generally marked by some sort of irregularity. Elements both from host and from graft may often be cut short somewhere along their diaphysis. Elements truncated in this way do not regulate so as to reconstruct the lost epiphysis: the diaphysis may just end abruptly, or it may taper to a spike, or peter out in some other fashion (Fig. 7b, c, e, f). Fragmentary elements of this sort from host and graft may come close without fusing (Fig. 7e). Elements from the graft that are entire may form peculiar articulations with inappropriate elements from the host (Fig. 7c). Setting aside these phenomena in the immediate neighbourhood of the boundary, the most striking feature of the composite limbs is their mosaic character: host tissue and graft tissue develop autonomously according to their origins.

In the complementary cases in which thick tips cut from late buds were grafted onto early stumps, we again saw mosaic behaviour, giving rise to composite limbs with medial deficiencies as predicted. We have, however, only a small number of instances in which such grafts healed well to the host: the parts that developed from large tip fragments cut from late (e.g. stage-24) buds tended to be stunted and deformed; they often consisted mainly of a big haemorrhagic vesicle, and their bad development was probably the result of a disrupted blood
Fig. 9. Photomicrographs of whole limbs fixed on the ninth or tenth day of incubation, stained with Alcian green 8GX, and cleared in methyl salicylate. The figures illustrate the results obtained in Fig. 10. The grafted tip is from a stage-19 or 20 wing-bud in 10 d, e and f, and is to a stage-19 or 20 host in 10 a, b and c. (a) Stage-22 donor: no wrist and short ulna and radius. (b) Stage-24 donor: no wrist. (c) Stage-26 donor: no wrist and proximal parts of metacarpals missing. (d) Stage-22 host: the forearm region is partly reduplicated, the radius more obviously than the ulna. (e) Stage-24 host: again the forearm is partly reduplicated but host wrist parts are present between the two regions. (f) Stage-26 host: even more of the host is present, all of digit II and parts of the metacarpals of digits III and IV.
supply. In the cases where a late tip was grafted not to the wing-bud stump but to the flank of an early embryo, there seemed to be less trauma of this sort. Again, the transplanted late tip and the naked early stump developed, separately, according to their original presumptive fates. One of us (D. S.) has recently done a more extensive set of experiments to test for regulation of medial deficiencies by cutting out transverse slices from wing-buds. The results, which again show that there is very little interaction or regulation along the proximo-distal axis, have been described briefly elsewhere (Wolpert et al. 1974). A detailed account of them is in preparation (D. Summerbell, unpublished observations).

Thin tip grafts

An early and a late wing-bud are each cut through at the level of the marginal vein, i.e. about 150 μm from the apical ridge, and the detached tip slivers are exchanged. As before, the composite wings are fixed, stained and measured, together with the contralateral controls, after a further 5 or 6 days of incubation. The results are illustrated by photographs in Fig. 9, and tabulated in Fig. 10. In each case, some deficiency or reduplication occurs, but it is rather slight. This is to be expected. For since the grafted tip is narrow, it cannot contain a great excess of presumptive territories; and for the same reason, according to our theory, the progress zone due to its apical ridge can extend into the host, and there maintain development of more distal parts, without any signalling of positional value. Because the discordance between host and graft tissue is not great, the boundary between them is not so clearly marked as in the thick tip transplants, though in most cases it can still be distinguished without much difficulty. At first glance, and without measurement, however, it is easy not to notice significant abnormalities that are present — for example, the abnormally short metacarpals in Fig. 9c, or the loss of carpals in Fig. 9b. In general, elements in the immediate neighbourhood of the boundary behave in much the same way as for the thick tip grafts.

To assess our observations, we compare them with quantitative predictions based on the hypothesis that there is no signalling of positional values to one region from another. Consider first the young host bud with the old grafted tip. At the time of operation, let the age of the former be \( r_h \) and the age of the latter \( \tau_g \); we define the age, on the same lines as before, as the mean number of division cycles that have elapsed in the tip mesenchyme since the wing began to grow out (Lewis, 1975). The progress zone set up by the grafted tip will initially extend into the host. Let \( \theta_g \) be the number of division cycles required by the grafted tip mesenchyme to grow to fill the progress zone to the exclusion of host tissue. Let \( \theta_h \) be the number of division cycles occurring meanwhile in the distal host tissue. (The young cells divide faster than the old.) Then the last of the host tissue will emerge from the progress zone aged \( \tau_h + \theta_h \). Immediately after this, the first of the grafted tissue will emerge, aged \( \tau_g + \theta_g \). According to our theory, in normal development the age of emergence from the progress zone
Fig. 10. Observed and predicted results of operations in which thin tip slivers, measuring about 150 μm from cut surface to apical ridge, are exchanged between young and old wing-buds. The parts that go to make up the composite limb are shown stippled in the top lines. The observed results are means, based on the numbers of cases shown in the bottom lines. The predictions are according to the calculations in Table 4.
Table 4. Predicted outcome of thin tip exchange grafts

| Donor stage | 19/20  | 19/20  | 19/20  | 22    | 24    | 26    |
| Host stage  | 22/23  | 24     | 26     | 19/20 | 19/20 | 19/20 |
| Donor age, $\tau_g$ | 1     | 1      | 1      | 2.7   | 4     | 5.3   |
| Host age, $\tau_h$ | 3     | 4      | 5.3    | 1     | 1     | 1     |
| $\tau_g + \theta_g = \tau_g + 1$ | 2     | 2      | 2      | 3.7   | 5     | 6.3   |
| $\tau_h + \theta_h = \tau_h + \mu_h/\mu_g$ | 3.7   | 4.7    | 5.9    | 2.4   | 2.5   | 2.6   |
| Most prox. level from donor = $L_T(\tau_g + \theta_g - \Delta)$ | 0.6F  | 0.6F   | 0.6F   | 0.5W  | 1.0W  | 0.2P1 |
| Most distal level from host = $L_T(\tau_h + \theta_h - \Delta)$ | 0.5W  | 1.0W   | 0.7M   | 0.0W  | 0.0W  | 0.0W  |

$L_T(\tau)$ = level of truncation after apical ridge excision at age $\tau$

writes = level of truncation if change of positional value is stopped at age $\tau + \Delta$. Notation for level as in Table 2.

Determines the ultimate positional value. Thus if there is no local signalling or averaging of positional value in the composite wing, it will eventually lack the structures whose normal age upon emergence from the progress zone lies between $\tau_h + \theta_h$ and $\tau_g + \theta_g$. We can identify these structures by referring to the apical ridge removal results shown in Fig. 5. (Again the identification depends on the delay $\Delta$ between excising the ridge and freezing positional values. Uncertainty about $\Delta$, however, will not here affect the width of defect predicted, but only its level.)

An exactly similar argument applies to the reciprocal graft of a young tip on to an old host. In this case, if there is no local averaging of positional value, we should predict duplication of certain levels, which we can calculate analogously.

In Fig. 10, we compare the predictions with the experimental results. The predictions are based on the plausible guesses

$$\theta_g = 1 \text{ cell cycle}, \quad \Delta = \frac{1}{2} \text{ a cell cycle}.$$  

(An error of $\frac{1}{2}$ a cell cycle in either of these estimates would not in fact make very much difference to the deductions.) We derive $\theta_h/\theta_g$ from the known ratio of mitotic indices $\mu_h/\mu_g$ for young and old tissues (Hornbruch & Wolpert, 1970). The steps of the calculation are set out in Table 4. We see that the observations agree with the predictions to within about half a cartilage element. We conclude that the positional value of the cells in the progress zone is not specified by a signal from the proximal stump, either in the thick tip grafts, or in the thin.

In particular, our results seem to exclude mechanisms by which the labile apical mesenchyme is assigned a positional value by reference to the positional value of determined mesenchyme immediately proximal to it (Rubin & Saunders, 1972). For according to such a mechanism, normal limbs should result from the grafting of the thin tip slivers in which (as we have argued above) the final positional value has not yet been assigned at the time of grafting.
We cannot, however, rule out the possibility that there may be some short-range communication between mesenchyme cells, tending to smooth out local discontinuities to the extent of, say, half a cartilage element; though equally our grafting experiments provide no firm evidence that this sort of minor local regulation along the proximo-distal axis occurs (Summerbell, in preparation). The rules governing regulation along the other two axes may, however, be very different.

**DISCUSSION**

The main proposition of our theory is that the mesenchyme cells under the apical ectodermal ridge autonomously change their positional value, becoming progressively more distal in their intrinsic character as they proliferate. This statement rests heavily on the evidence from two investigations. One, as described in detail in this paper, shows that proximal tissue cannot modify the fate of a mismatched distal tip. The other is the crucial experiment of Rubin & Saunders (1972), in which they demonstrated the indifference of distal mesenchyme to the age of the apical ridge with which it was covered. We must now consider other related evidence as reported elsewhere.

**Regulation**

Results similar to our own have been reported by Amprino & Camosso (1959, 1965). In contrast, Hampe (1959) and Kieny (1964a, b; 1967) have stressed the regulative capacity of chick limb-buds following operations of roughly the same type. Since their results might be supposed to contradict ours, we must discuss them in some detail. Unfortunately, their findings are not set out in a quantitative form that would make the comparison easy, and many of their experiments involve the leg-bud, while ours concern the wing only. We do not, however, believe that there is any serious conflict between their very interesting observations and our own.

Hampe and Kieny both present their results under two headings: regulation to accommodate excess tissue, and regulation to make up for a deficiency of tissue. To test the former, they cut the extreme tip off a limb-bud (typically at stage 20) and pin on in its place another almost entire limb-bud. To test the latter, they make a cut roughly at the level of the prospective elbow or knee, and pin a narrow sliver cut from the tip of a limb-bud on to the remaining proximal stump, omitting what they judge to be the primordium of the forearm or lower leg. The left-over fragments of limb-buds are kept as controls. Both workers define regulation by reference to normal presumptive fate maps, showing what parts would have developed from the cells making up the experimental limb, if they had been left undisturbed in their natural situations. Skeletal regulation is said to occur if the experimental limb develops a set of cartilage elements different from the sum of those appearing in the normal fate maps for the constituent tissues in their original sites. The degree of regulation is assessed
quantitatively by counting the number of limb segments (stylopod, zeugopod and autopod), but without measuring their lengths.

The observed degree of regulation of excess tissue is rather slight: taking an average over Kieny's (1964a) four different graft combinations of leg and wing, we find the mean number of excess segments developed in the experimental limb to be 1.3. If there were no regulation, according to Kieny, the corresponding figure would be 2.0; if there were perfect regulation, it would be 0.

The regulation of deficiencies does not lend itself so easily to this kind of quantitation and calls more for analysis by measurement of lengths. Without measurement, one may, for example, obtain a half-length forearm in the experimental limb, plus a half-length forearm in the control fragment, and by scoring these simply as two forearms, conclude wrongly that regulation has doubled the total amount of forearm tissue. The general impression, however, from Hampé's and Kieny's results is that their experimental limbs with an initial deficiency end up more nearly normal than those with an initial excess. Kieny (1964b, 1967) reports also that the distal fragment of the experimental limb-bud often becomes more proximal in character to supply the medial deficiency. This judgement is based partly on the morphology of the medial parts in leg/wing combinations, and partly on the final position of carbon particles put at the interface at the time of the operation; it depends also on the fate maps used.

Our comments are as follows:

1. Regulation in the sense of Hampé and Kieny does not necessarily imply signalling of positional value within the mesenchyme. Tissue can be diverted from its normal presumptive fate by disturbing its normal relation to the apical ectodermal ridge, so that it spends either less or more time than usual in the progress zone. In the experiments on regulation of excesses, the most distal part of the host contribution to the experimental limb-bud is prematurely deprived of the influence from the ridge; so it may fail to make distal parts that it should normally have made. In the experiment on regulation of deficiencies, the progress zone due to the ectodermal ridge on the grafted apical sliver can extend into the host; there it can bring about regulation by regeneration from proximal tissue, by the same basic mechanism that operates when an ectodermal ridge on its own is applied to a cut face.

2. Even by the rather loose criterion of the number of limb segments that develop, the skeletal regulation observed by Hampé and Kieny is far from complete.

3. Differential growth of the limb-bud can give misleading impressions of the degree of regulation. In particular, the early primordium of the wrist is disproportionately large, so that even without changes of presumptive fate, quite big early excesses or deficiencies of tissue in this region may produce barely perceptible abnormalities (cf Fig. 9b).

4. As for the contention that distal tissue often becomes more proximal in
character to compensate for a medial deficiency, the evidence is not at all conclusive. It depends on carbon particle marking, both to derive the fate maps used, and to indicate the boundary between host and graft. We have argued elsewhere (Lewis, 1975) that this technique is unreliable. To check the fate maps by reference to the control fragments, it would be necessary to measure the lengths of the elements that develop, and this was not done.

(5) We admit that there might be some local short-range interactions tending to smooth out discontinuities of positional character, over distances of not more than about half a prospective cartilage element. Our own experiments provide no strong evidence for or against this possibility (see also Summerbell, in preparation). The experiments of Hampé and of Kieny might perhaps be construed as evidence for it. But to make a convincing case it would be necessary to confirm the fate maps used by some technique other than carbon particle marking, and to measure the sizes of the elements and bits of elements that develop, rather than rely on subjective impressions.

APPENDIX

The numerical scale on which one specifies positional value is largely arbitrary: if \( P \) is one satisfactory measure, then any monotonic function \( \hat{P}(P) \) of \( P \) will also serve. But \( \hat{P}(P) \) will satisfy a different progress zone equation:

\[
\frac{\partial P}{\partial \tau} = \hat{P}(P) \frac{\partial P}{\partial \tau} = \hat{P}' \phi(x).
\]

Conversely, we may start with some arbitrary scale of positional value, \( \hat{P} \), and hypothesize that it satisfies the more general progress zone equation

\[
\frac{\partial \hat{P}}{\partial \tau} = f(\hat{P}) \phi(x).
\]

Provided \( f(\hat{P}) \neq 0 \), we can then always define a new scale

\[
P = \int_0^\hat{P} \frac{1}{f(y)} dy
\]

such that

\[
\frac{\partial P}{\partial \tau} = \phi(x)
\]

as before. In short, our progress zone equation (1) implies a special choice of scale for the positional value \( P \). It is in fact the scale such that the positional value at the tip changes linearly with age. The relationship between the special scale \( P \) and other scales such as \( \hat{P} \) is rather like the relationship between absolute and empirical scales of temperature.

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