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Control of growth related to pattern specification in chick wing-bud mesenchyme

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SUMMARY

The distribution of raised mitotic index, and the co-incidence of this with lowered cell packing density, has been studied across the anteroposterior dimension of the terminal 500 μm of chick wing buds following various numbers of hours signalling from an anteriorly grafted extra Zone of Polarizing Activity (ZPA). The results show propagation of the situation that causes these correlated phenomena, from graft–host interface essentially right across the limb mesenchyme, frequently within 8 h. This contrasts with the much slower and more local succession of changes in position memory, for differentiation of a duplicated limb pattern, that also occurs in mesenchyme relatively close to the graft after this operation. The results are discussed, in relation to current ideas about the control of pattern during limb development.

INTRODUCTION

In the study of pattern control in the anteroposterior dimension of the chick wing bud, it has been recognized for some time that an enhanced rate of widening during outgrowth of the host rudiment is an early consequence of a successful graft of tissue from the posterior Zone of Polarizing Activity (ZPA, MacCabe, Gasseling & Saunders, 1973) to an anterior site. Such a graft results ultimately in mirror-image duplication of the developed limb pattern around its normal pre-axial border. Several authors (Camosso & Roncali, 1968; Fallon & Crosby, 1975; Calandra & MacCabe, 1978; MacCabe & Parker, 1975; Rowe & Fallon, 1981) have speculated on possible dual roles for the ZPA, including functions in the control of growth. We recently confirmed (Cooke & Summerbell, 1980) that this is correlated with a stimulation of the mean cell cycle rate within host mesenchyme, initiated as an enhanced probability of entry into the 'S' phase of the cycle by cells, and leading to markedly raised mitotic index over the time (12–24 h post-operative) when the elevated widening is morphologically noticeable. When the initial stages of this growth enhancement were studied as a pattern of 'S' phase incidence revealed by brief thymidine labelling before fixation, a surprising feature was the speed and distance of

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its spread posteriorly across the host tissue, from the site of positional disparity among newly joined cells at the host–graft interface. More than the anterior half, and possibly all of the still undetermined mesenchyme of the bud appears to become involved, within hours, in an episode of enhanced cell division. Yet related work indicates that only some 25 % of the bud width, that nearest the graft junction, becomes assigned to produce the final duplicate pattern elements in such cases (Summerbell & Honig, 1981). The reliable change in pattern-forming values appears to require continuous experience of influences from the newly implanted ZPA for 15 h or more (Smith, 1980).

The simplest interpretation of the data so far suggested is perhaps that the growth rate within limb-bud mesenchyme is only loosely tied to the landscape of pattern-specifying signals (e.g. a gradient concentration profile), being largely an expression of how many polarizing regions are in the system rather than of precise position in relation to each ZPA. It is important to ascertain the precise relation between control of the rate of tissue production and that of pattern determination in this system, since it is essentially the first inter-cellular growth control linked to pattern formation *in vivo* that has become accessible to study. More precise knowledge of it should also direct our choice of models (e.g. Summerbell, 1979, 1981*a*, *b*) for the sequence of events in pattern determination.

An additional interest of the system derives from the established fact that, whatever its nature, machinery for pattern specification that is *independent* of tissue dimension or scale over a considerable range does exist in vertebrate embryos (Cooke, 1979, 1981*a*, *b*). This is utilized in control of the primary, or axial pattern in these embryos. Why then, in a secondary pattern-forming field active only a few hours later, should we find that a growth control system has been 'built in' to the machinery? One possible implication is that the spatially repetitive nature of the early patterns of cell differentiation in such organ rudiments as the limb, unlike the unique 'zones' of the primary axial pattern, may involve pre-patterning types of mechanism in addition to monotonic positional gradients (Turing, 1952; Newman & Frisch, 1979; McWilliams & Papageorgiou, 1978; Wolpert, 1969). Such mechanisms may impose constraints on the range of tissue dimensions compatible with production of normal numbers of pattern elements (Murray, 1981), so that a mechanism to adjust scale in relation to the boundaries of pattern (i.e. the position of the ZPA) has evolved.

We report here our attempt to understand further this relationship between growth and pattern by investigating the relative incidence of mitosis across limb-bud sections, 16 h after grafting a ZPA to the anterior margin in a way calculated to cause a profound pattern duplication as in the previous work (Cooke & Summerbell, 1980). In the present experiments however an impermeable, vertical barrier, dividing the bud into anterior and posterior fractions and passing into the body wall, has been interposed between the region of the

graft/host junction and the rest of the responding mesenchyme either 6, 8 or 12 h after grafting and the potential onset of signalling from the new ZPA. We have studied the resulting changes in cell cycle across the anteroposterior dimension of the tissue separated from the new signalling region by the barrier. This tissue has been divided geographically into three equal anterior-most, middle and posterior proximodistal columns, for assessment of mitotic incidence relative to homologous columns of the control (left) limb. The 16 h post-graft time point was chosen because in previous experiments without barrier interposition the 16 h mitotic response was dramatic (up to a doubling of the index), so that we might expect to detect smaller effects with maximum sensitivity. Estimates of the probable length of the (determinate) sequence between 'S' initiation and subsequent mitosis in normal limb-bud cells are around 4–8 h. Therefore, by observing mitotic incidence at 16 h we are probably assessing the experience of the cells, as to signals controlling their mean cycle rate, at times between 8 and 12 h after the implantation of the anterior ZPA graft in the experimental limb of each pair.

We also present data to add to that already in the literature (Smith, 1980; Summerbell, 1973) as to the spectrum of morphological results when limbs are allowed to determine and differentiate pattern after such ZPA grafting operations with subsequent barrier interposition. This is, in effect, an assessment of the timing and spread of acquisition of new 'positional memory' for pattern formation by tissue near the newly implanted ZPA, that parallels our assessment of the timing and spread of initiation of enhanced tissue production in the host. Comparison of the results of these two types shows that conditions stimulating growth rate can spread essentially right across the limb bud in very few hours (less than 10) in a way which contrasts with the slower and relatively localized reorganization of cell position values for pattern formation in the anterior region. The implications of this for overall models of pattern control are discussed.

In addition, we add to the evidence for a geographical correlation between lowered cell packing density and circumstances stimulating the cell cycle in the wing bud (Summerbell, 1977) by presenting the data on cell packing alluded to in Cooke & Summerbell (1980). In cases of experimental pattern duplication, this inverse correlation may be an extension or exaggeration of that seen in the normal limb bud at these stages (Summerbell & Wolpert, 1972).

Neither packing density nor cell-cycle rate were studied in ectodermal limb-bud components. They may be highly relevant to understanding the system, and are the subject of future work.

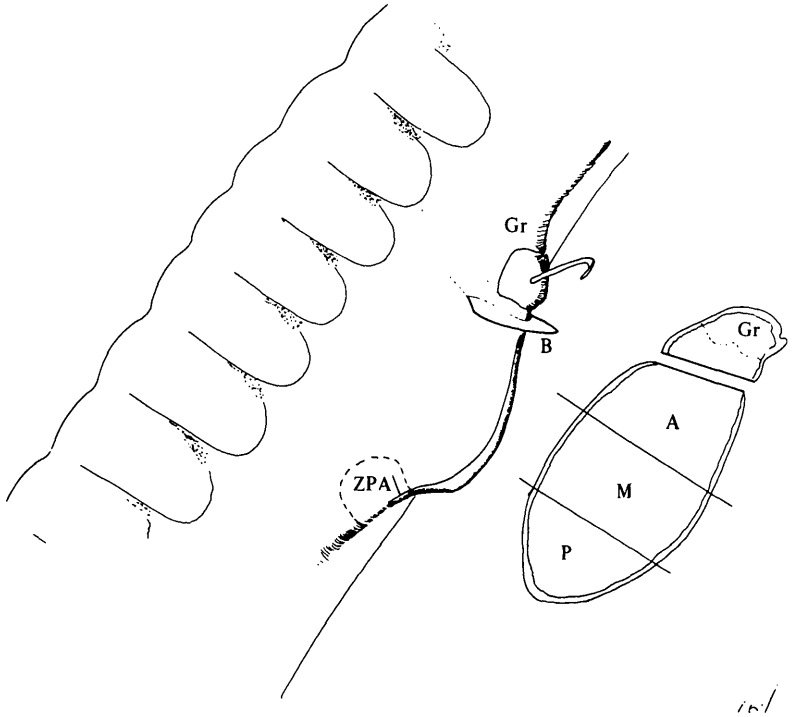


Fig. 1. The ZPA + barrier operation. A right-hand limb bud at stage 18/19 is shown, together with the outlines of the axis (somites), the site of graft implantation (Gr) with the retaining platinum pin, the tantalum foil barrier (B) and the location of the normal Zone of Polarizing Activity (ZPA), the source of grafts. A typical transverse section of the limb bud 16 h later is shown inset, with the graft and barrier sites (apparent in proximal sections of the series only) and the three dimensions of the post-barrier mesenchyme used to compose the Anterior, Middle and Posterior proximodistal columns for recording of mitotic incidence and cell packing density. Control data are from the equivalent cell populations in the left limb buds.

MATERIALS AND METHODS

Details are presented elsewhere of the embryonic operation (Summerbell, 1974) fixation, sectioning and staining, autoradiography and data collection from pre-differentiated wing buds (Cooke & Summerbell, 1980), and the assessment of differentiated skeletal patterns in advanced wing rudiments (Tickle, Summerbell & Wolpert, 1975). Figure 1 shows the version of the ZPA implantation operation used here, followed after 6, 8 or 12 h by interposition of a tantalum foil barrier. Our previous work has established that significant cell-cycle effects observed any distance from the graft/host boundary are seen only following juxtaposition of tissue having widely different position values which will lead to extra pattern formation; in our hands, the relocation of mesenchyme of posterior (ZPA) origin to an anterior site. The operations upon which the present paper is based were accordingly all ZPA grafts to positions opposite somites 15/16 at stages 18 to 19. Without later barrier

placement, this leads in > 90 % of cases to enhanced width increase visible between 12 and 36 h after operation, following ultimately by differentiation of completely duplicated hand, wrist and frequently forearm structures. Embryos were treated in one of two ways after these operations.

One set of embryos was fixed and prepared for Feulgen histology at 16 h after the ZPA graft, but without prior labelling with ^{3}[H]thymidine, as only mitotic incidence was to be studied. Sets of sections at 50 μm intervals for some 500 μm behind the tip of the apical ectodermal ridge (AER) were mounted from right and left limbs to form two proximodistal series of cross sections through the mesenchymes in the parasagittal plane (i.e. parallel to the body axis). That part of each section profile lying posterior to the barrier position, or its equivalent in left control limbs, was treated as three sectors of equal anteroposterior width, that nearest the graft, the middle sector, and that furthest away (being the presumptively posterior zone of the host pattern, nearest to its own ZPA). Density in space of metaphases and anaphases was recorded in the three proximodistal columns of mesenchyme represented by the sectors of sections from each level. Mitotic incidences and ranges of cell density encountered were such that 2–300 mitotic figures and 5–7,000 cells were scanned for each column, the relative areas scanned being determined by planimetry from tracing on to paper at × 165 magnification. Relative cell packing density for each column (in the plane of section concerned) was computed by random 'throwing' of a standard 'quadrat' (i.e. a frame on a television monitor at standard magnification) on to the image of mesenchyme in the appropriate third of each section, summing the nuclei recorded with an electronic colony counter and pen (150–300 per frame) and taking the mean for each column. The *relative* measure of cell density thus obtained for each column within a limb pair was used for computing their relative mitotic incidences on a per cell basis, and also as a biological parameter in its own right to correlate with those corrected mitotic incidences among the columns.

A second set of embryos was allowed to develop to 10 days of incubation after comparable operations, and the limbs prepared for analysis of their skeletal pattern in cleared whole mounts.

Certain limb pairs, after simple ZPA implantation to the right limb without subsequent barrier interposition, were subjected to geographical analysis of cell packing density correlated with the 'S' phase index recorded directly from autoradiographs. In these limb buds, which have been subjected to 1 h [^{3}H]-thymidine incorporation in the embryo immediately before fixation for the study of the 'S' phase stimulation reported elsewhere (Cooke & Summerbell, 1980), the cross sections at each level had been divided into four rather than three sectors of equal width, and these data boxes recorded separately rather than being pooled into mesenchymal columns (see Fig. 3). For the geographical layout of the boxes and thus *distribution* of enhanced labelling, see the previous paper.

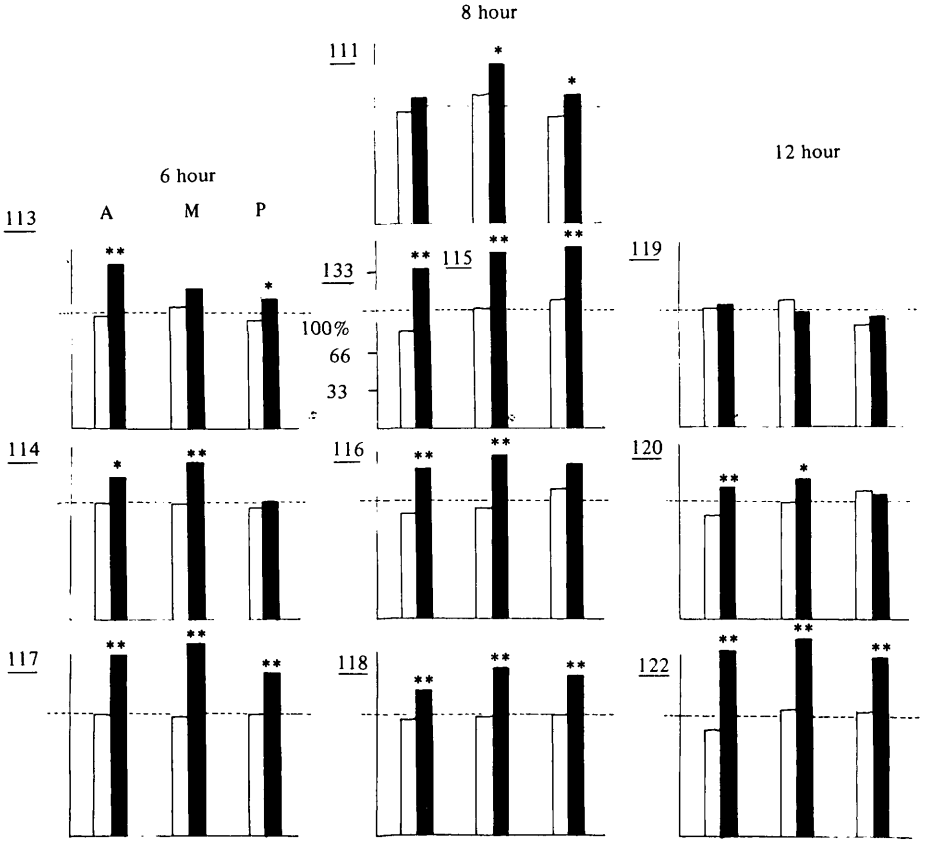


Fig. 2. Mitotic incidence on a per cell basis, 16 h after anterior ZPA implantation. Each histogram refers to a limb pair (underlined numbers), with the control (left) mesenchyme columns open and the experimental (right) columns filled in. Anterior, middle and posterior (i.e. furthest from the implant site) columns read from left to right as column pairs on the histogram. The horizontal dashed line corresponds to the mean mitotic incidence of the control columns in each limb pair, and the heights of columns register deviations from this. Asterisks show significance of *differences* between experimental and control columns at each position, at $P < 0{\cdot}05$* (usually 20% relative incidence or more) or $P < 0{\cdot}01$** (usually 25% relative incidence or more). Number of hours allowed for signalling by the implant before barrier interposition are marked at the top of sets of limb pairs. N.B. Only pair 119 shows a lack of stimulation, presumably because of graft failure. Only 122 and possibly 133, however, show degrees of stimulation comparable with that seen after continuous experience of an extra ZPA for 16–17 h (Cooke & Summerbell, 1980).

RESULTS

The histograms of Fig. 2 give the *relative* incidences of mitoses at grafting + 16 h, in the columns of the three mesenchymal sectors on the right and left (control) sides in limb pairs where communication between grafted ZPA and adjoining tissues had been allowed for the first 6, 8 or 12 h. The relative incidences are on a per cell basis, but are normalized to the mean incidence

Table 1. *Time required for stable change of position values in wing-bud tissue by new ZPA signalling*

No. of hours allowed for signalling before barrier	No. of examples	Mean efficacy score
7–9	9	0
9½–12	9	0·5
13–15	11	0·5
16–18	9	1·4
24	3	2·0

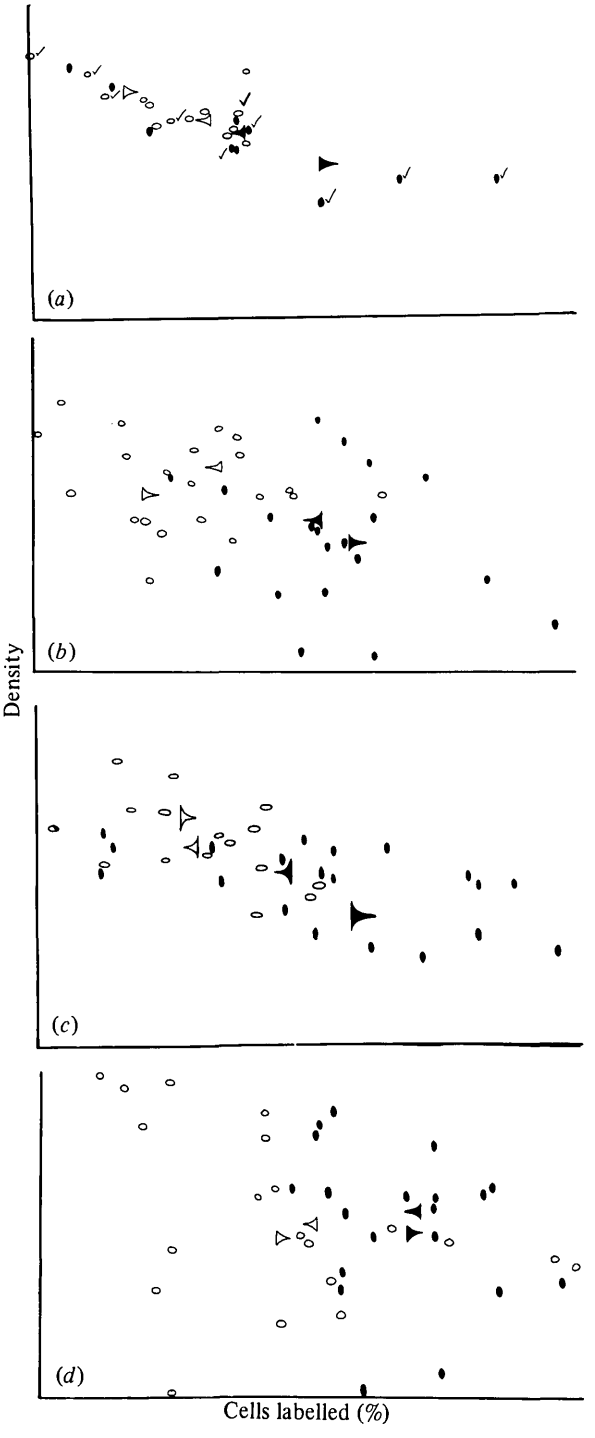
The efficiency of given numbers of hours signalling (i.e. times from graft placement to barrier interposition) was registered by scoring 10-day-incubated, cleared wholemounts for limb skeletal pattern. Normal is: anterior → 2.3.4 → posterior. Efficacy score is → 2.3.4 = 0; → 2.2.3.4 → = 1; → 3.2.2.3.4 → = 2; → 4.3.2.2.3.4 → = 3.

It can be seen that reliable addition even of a digit 2 is not seen until at least 15 h, and that reliable complete new specification may require more than 24 h.

for the entire control limb (i.e. the level marked on the ordinate and called 100 % relative incidence). Within each pair of histogram columns, however, significance of right/left differences marked by asterisks has been assessed on the basis of absolute numbers of mitoses seen and cells scanned in the two populations. Broadly speaking a 20 % relative difference in incidence reaches significance at $P < 0{\cdot}05$, while a 25 % difference reaches significance at $P < 0{\cdot}01$.

Although the picture is variable (as indeed are the morphogenetic results of a series even of simple ZPA implantations) it can be seen that significant and substantial stimulation of cell cycle has frequently spread throughout the presumptive A–P tissue dimension of the buds within the initial 12 h of ZPA signalling. In at least one instance each of the 6 h, 8 h and 12 h barrier versions of the operation, stimulation has had time to reach the posterior third of the host bud in order to cause there a measurably enhanced 16 h mitotic incidence. Examination of the columns, which represent 400–500 mm depth of tissue from the bud top, suggests that the spread of the stimulating 'signal' or situation is by no means only around the periphery underneath the AER in order to reach the back, but also through the core of the undifferentiated mesenchyme. Scanning of the more proximal of the section series, from limbs showing the largest effects, confirms this view.

Data on the 'S' incidence at much earlier times after grafting do indicate that the stimulating conditions are initially concentrated in the pre-axial region near the graft (Cooke & Summerbell, 1980). The present data using the less direct mitotic indicator, some while after a restricted signalling period, show in a more positive way the dramatic rate of spread of stimulation. A concentration of the effect on the pre-axial side is hardly apparent. In the 8 h barrier limbs, however, the 60–100 % relative mitotic enhancement seen after


(a)
(b)
(c)
(d)
Density
Cells labelled (%)

16 h uninterrupted signalling (Cooke & Summerbell, 1980) is not reached. This indicates that the 'lead time' from 'S' phase entry up to mitosis is less than, say, 8 h (i.e. 16 minus 8). One of the 12 h barrier cases is unusual in showing no mitotic (or cell packing) effect, suggesting an unsuccessful graft, but in one of the others the overall response at around +60 % relative mitosis is comparable to that after simple 16 h signalling. The cells might therefore be registering a similar situation experienced at, say 12 h in each case, making the lead time from 'S' initiation to mitosis at least 4 h (i.e. 16 minus 12). Such estimates would fit with our limited knowledge of cycle kinetics in the pre-differentiated mesenchyme.

Table 1 gives our estimate for the mean number of hours normal ZPA signalling necessary to begin the reliable organization of extra pattern elements at the pre-axial side of the future wing, as in the digit pattern 2.2.3.4 compared to the normal 2.3.4. We are in agreement with Summerbell (1973) and Smith (1980) that this is usually 12 h and may be as much as 15, while complete new specification up to 4.3.2.2.3.4 may require more than 24 h. Our method of halting signalling by passing a barrier near the ZPA, like Smith's of re-excising the ZPA plus a little extra tissue, suffers from the criticism that it may ablate the very small territory that is initially instructed to alter its fate where limb patterns like 2.2.3.4 are to be produced. If so, the results only emphasize the relatively confined territory from which tissue for the new pattern is drawn. The data of the present paper should be seen in the light of Honig's (1981) and Summerbell's (1981 *a*) finding and of the discussion in Summerbell & Honig (1981) of their independent experiments, which show that in profound and complete duplications such as we should expect to result from simple ZPA

Fig. 3. Relationships between cell packing density and entry to 'S' phase within limb pairs after simple anterior ZPA grafts. In the scatter diagrams, cell packing densities recorded within all individual data boxes of the host tissues of limb pairs (ordinate) are plotted against the corresponding labelling indices (abscissa). Highest and lowest packings observed (in the transverse plane, not per volume) were 12800 and 6900 cells per mm^2, respectively, while labelling incidence ranges from 23 to 57%.

○, Control limb (left-hand) data boxes; ●, experimental limb (right-hand) data boxes; ◀, mean of (control or experimental) posterior half-bud mesenchyme; ▶, mean of (control or experimental) anterior half-bud mesenchyme. In 4*a*, ticks distinguish the boxes, near graft site, showing a cell cycle effect, together with the homologous control boxes.

(*a*) Pair 623 operation+5 h. (*b*) Pair 619 operation+9 h. (*c*) Pair 617 operation +9 h. (*d*) Pair 634 operation+17 h. A negative correlation between packing density and labelling index is always observed (as it is within the data boxes of control limb pairs), and is statistically significant in the cases (*a*) and (*c*) shown ($P < 0{\cdot}01$). The result of ZPA grafting has been to reverse the positions of posterior and anterior halves of the mesenchyme on the labelling index axis (or, at 17 h, to equalize them) mainly by stimulation of the anterior half. Cell packing density is also decreased. For geographical distribution of the effect see Cooke & Summerbell (1980).

grafts with timing like those in the present work, some 25 % of the host tissue width anteriorly ($\sim$ 300 μm) is probably involved in founding the duplicate pattern 4.3.2.2.3.4, with a time course of at least 24 h. It can readily be appreciated from Fig. 2, Table 1, and these findings on pattern specification, that cellular responses to presence of an implanted ZPA are radically faster and more rapidly spread so far as growth control is concerned than they are in terms of altered position values (Wolpert, Hicklin & Hornbruch, 1971).

Figure 3 gives scatter diagrams of 'S' phase incidence against packing density of mesenchymal cells among the data boxes of certain individual limb-bud pairs fixed 5, 9 or 17 h after simple ZPA grafts to the right-hand limb of each pair. Control side and experimental side data boxes are distinguished, and the pre-axial (anterior) and post-axial (posterior) half mesenchymes' mean values marked out. The general inverse correlation between proportions of cells in 'S' phase during a 1 h labelling period and the mesenchymal packing density is statistically significant in the 5 h post-graft pair, and in one of the two 9 h post-graft pairs. It can be seen from the spread of positions of the right- and left-hand data and the right and left anterior and posterior means, that a decrease of packing density occurs in a coordinate way with early stimulation of the cell cycle. Up to 9 h the effects are sufficiently concentrated towards the pre-axial (graft) side of the host mesenchyme to reverse the trend seen in control limbs for the 'S' index to decline in graded fashion from back to front of the bud. In the 5 h post-graft pair, the marking of the only boxes (adjacent to the graft) which showed the dramatic effects of ZPA signalling, together with their control side partners, shows up the correlation between lowered cell packing and early cycle stimulation in a most striking way. By 17 h after operation the effects have become more generalized geographically and scarcely related to position of the graft within the system, in a way reminiscent of the mitotic response seen in parallel with 'S' enhancement at similar times (Fig. 2). As compared with the earlier limb pairs, the overall relation between packing and cell cycle is largely obscured in this example.

The scatter diagram of Fig. 4 represents the correlation between mitotic incidence per cell and cell packing density within the main series of limb pairs, those providing the geographical data on mitotic stimulation in Fig. 2. Variation in average values characterizing the mesenchymes within different embryos has been dealt with by showing percentage deviation from the average value in the control (left) limb, within each of the six proximodistal columns of each limb pair. The cluster of left limb data points is thus centred, by definition, on the origin. By itself it fails to reveal any systematic relation between the two parameters considered. Signalling by implanted anterior ZPAs for the earlier part of the 16 h period before fixation has however shifted the population of right limb data points, not only in the direction of enhanced and much more variable mitotic indices, but significantly into the lower right-hand quadrant representing diminished cell packing density. The experimental

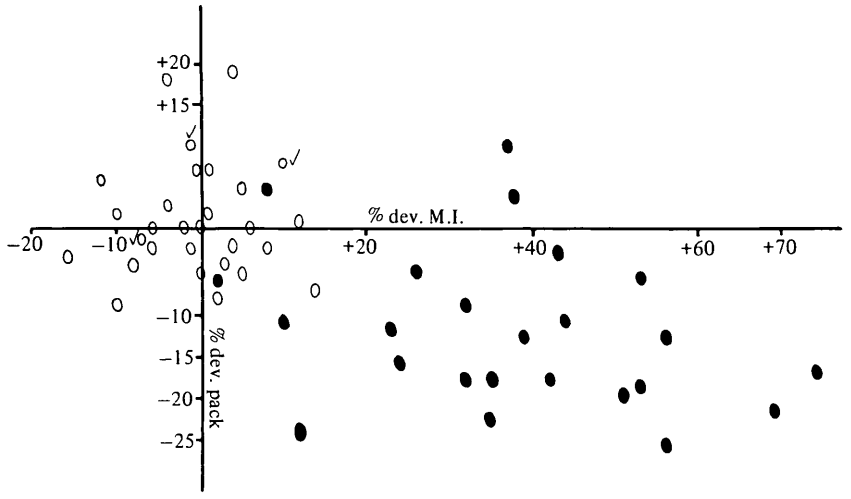


Fig. 4. Relationships between cell packing density and mitotic incidence within limb pairs 16 h after grafting. The *relative* incidences of mitosis per cell, shown in the columns of Fig. 2, are plotted as percentage deviation from their control limb means against the similar deviations for cell packing density. The origin thus represents average cell packing and mitotic incidence in the left limbs within all pairs. Control (left) data points are open circles, with the ticks marking in addition the three *right* data points of pair 119 where no stimulation was seen. Experimental data points are solid. The inverse correlation (−0·35) between packing density and mitosis within the experimental points approaches significance ($p = 0{\cdot}05$), while that for the whole assemblage of data points (−0·61) is highly significant ($P < 0{\cdot}005$). The presence of an extra ZPA anteriorly has diminished packing and raised mitotic index away from normal values in a highly coordinate manner. N.B. the *range* of packing values is less than for Fig. 3. This is largely because the sample for each data point is larger (up to 10 times the cell population of one of the earlier data boxes). But mitosis may be less strongly correlated with cell density than is 'S' entry, because the act of mitosis greatly restores cell numbers even though low density may have stimulated it at an earlier time.

data cluster shows an inverse correlation between packing and mitosis that borders on significance ($P < 0{\cdot}05$), and when the total population of data points is considered a highly significant inverse correlation ($P < 0{\cdot}005$) shows up. The three ticked data points within the control cluster are in fact those of experimental limb 119, the 12 h barrier operation which failed to result in mitotic stimulation. Sitting as they do as typical members of the control cluster, they reinforce the belief that factors diminishing cell packing and enhancing cell division rate away from normal values are (*a*) related to one another and (*b*) a function of successful communication between ZPA and surrounding tissues. The barrier was in normal position at the time of fixation in this limb.

DISCUSSION

Summerbell (1981*b*) has reported elsewhere in this symposium volume that by advanced stages of wing development the sizes of the supernumerary structures in the entire or partial extra patterns of reversed polarity, caused by early ZPA grafting, are essentially normal for their stage of differentiation. Thus a completely duplicated pattern of forearm, wrist and hand structures will possess about twice the tissue mass of its single counterpart by 10 days of incubation. Observation of width changes after operations suggests that the doubling of tissue extent relative to incubation data is largely accomplished within 24 or 36 h following operation (Summerbell, 1981*a*). This is reasonably close to the time taken to complete specification of limb pattern in its proximo-distal entirety after such stages ($\sim$ 44 h, Summerbell, 1974). The duplication of pattern and the width increase, after any particular ZPA graft, occur in that mesenchyme which has still to leave the 'tip' zone of 300–400 μm behind the AER during bud elongation. Honig's estimate using orthotopic quail grafts in combination with ZPA grafting (1981), and Summerbell's using the distances between two ZPA grafts necessary to produce two whole sets of structures (1981*a*) agree that the tissue of anterior duplicated patterns is largely descended from a relatively local region embracing much less than half the host bud at time of operation.

Modelling of the control of pattern in the anteroposterior dimension of the bud is currently based on the idea of a monotonically graded positional signal, controlled from normal and implanted ZPA regions, possibly in the form of a diffusion gradient of morphogen (Wolpert, Lewis & Summerbell, 1975). On this basis, two extreme ways of viewing the data on early growth stimulaton accompanying pattern duplication would be as follows.

(1) A small, steep reversed landscape of positional signal is set up anteriorly, embracing tissue which plays little part in building the normal limb structures since the fate map indicates that these are mostly descended from the (normally faster growing) posterior regions of the early bud. The set of position values (Wolpert, 1969) dictating the duplicate pattern is thus present in a much smaller tissue extent than that dictating the normal pattern, whose ZPA has been active for much longer to build up a more extensive gradient landscape. The enhanced growth which then causes abnormal expansion or dilation of the extra fate map at early stages, leading to the differentiation of more normal-sized extra pattern elements, is seen largely as a consequence of small size at foundation, being due to some feedback mechanism that normalizes the sizes of tissue territories relative to the ranges of position value present in them. Early growth rate is considered to be a feedback function of the scale on which pattern determination has occurred. The 'steepness' of a positional gradient at particular times in development could in principle be utilized to set the growth rate. On this view there is no separate control signal from the ZPA

growth, simply the positional signalling system which is used in two ways (see discussion, Summerbell, 1981*a*). Such a view fails to accommodate the data, which show incursion of the growth-stimulating effect into the heart of the presumptive fate map for the original limb pattern. It is true that at very early times after ZPA implantation, and with uninterrupted signalling after 9 or 17 h, the response is concentrated in the anterior half mesenchyme (Cooke & Summerbell, 1980), but it is considerable elsewhere and frequently at the posterior border. The ZPA-plus-barrier results, which leave much stimulation but wipe out the anterior-to-posterior graded pattern otherwise seen, suggest a signal which propagates and equilibrates rapidly over the dimensions of the normal system; one which could scarcely act as the signal for *position* relative to a ZPA (and thus for limb pattern) because gradients in it would be too transient. The results of Smith (1980) and our own, showing that 12 to 15 h is the shortest time for reliable responses of cells to altered signal in terms of position value, are relevant here. They make it impossible to imagine that the system mediating cell cycle control is exclusively tied to pattern-forming values in tissue. On the simple assumptions that absolute incidence of mitotic figures in undifferentiated mesenchyme closely reflects the mean frequency of mitoses, and that all cells are potentially in cycle, the rate of expansion of tissue through most of the limb bud must be dramatically though transiently elevated following implantation of an extra ZPA at an ectopic site.

(2) The alternative extreme view is that the timecourse of cells' responses to signal, and perhaps the intercellular communication systems themselves, are different as regards control by the ZPA of position value on the one hand and growth rate on the other. Responses to growth control influences occur on a much shorter timescale, and if the signalling systems involved are in fact different, as seems likely from the data in this paper, then the growth control 'signal' is also communicated much faster. It is as if, over the 36 h when growth rate is influenced by implantation of an extra ZPA, the rate of the cycle is largely a function of the number of ZPAs contacting the bud, and only partly of position in relation to any particular ZPA. Typical width changes over this period, together with estimates of the times for determination of pattern in the proximodistal sequence, suggest that by the time duplicated regions of pattern are becoming determined, the pattern elements concerned may each be founded on a scale (i.e. using amounts of tissue) similar to that obtaining in the normal progress of development. Subsequent growth rates within the determined patterns may return to normal because the schedule of growth within established pattern parts is a fixed aspect of their determined character (Wolpert, 1978; Lewis & Wolpert, 1976) and/or because the growth-promoting activities of the ZPAs have re-established the normal amount of territory for each of them, thus returning the growth signal level to normal. If this view holds up under future experimental investigation, the growth-controlling function of ZPA tissue within the limb could be viewed as a

mechanism that ensures that standard amounts of tissue space are available in relation to the numbers of pattern elements that are to be formed. One of us (J.C.) has always experienced difficulty in supposing that biological patterns of the class of the early limb pattern could become organized solely by monotic positional signals of the gradient variety. This is because their deepest feature is their spatially periodic nature, whereby a number of dispersed centres of comparable cellular activity are laid out. The burden placed upon cellular interpretative machinery would seem too large at early stages of formation in such patterns, and the idea of a 'pre-pattern' of morphogen peaks and troughs corresponding to the future pattern of elements, and set up by reaction–diffusion kinetics or allied processes (Turing, 1952; Newman & Frisch, 1979) seems more plausible. The latter classes of process do have the constraint, unless very special accessory mechanisms were built in, that the numbers of elements formed in patterns controlled by them is almost proportional to tissue size at the time of the process. Formation of normal, complete patterns would thus require a degree of control over tissue size that goes quite beyond what is required by the primary embryonic field (e.g. Cooke, 1981*a*, *b*; Dan-Sohkawa & Sato, 1978) which controls a pattern of a different character.

The data now very strongly imply that the cellular mechanisms whereby grafted ZPA tissue controls the sequence of characters in the elements of a new, extra pattern are separate from those whereby it enhances growth, and that these are only coordinated spatially to the extent that both emanate from one site. It has been suggested that a difference in apparent rate of spread between growth stimulation and re-patterning influences could be understood in terms of a 'follow-up servo' mode of positional signalling (Wolpert *et al.* 1971) whereby the dynamic positional variable exists as a relatively transient set of gradient landscapes because of its diffusibility, and cell positional values for pattern formation change by slowly following these gradients. Cell cycle kinetics may be a much more immediate response to the same signal, so that effects appear to spread through more limb tissue per time. It is hard to see how such a 'single signal, dual response' system would stably set up a double, mirror-imaged limb pattern as is observed. The early spread of cycle stimulation and its geographical extent (see Fig. 2, and Cooke & Summerbell, 1980) would imply a 'flooded out' signal landscape. This would lead to obliteration of central pattern parts in a duplicate because of loss of low gradient values, during the time before determination of position values in the anteroposterior dimension is completed.

The rules of continuity and polarity followed by the sequence of element characters (digits, etc.) in relation to grafted and host ZPAs, continue to make a diffusion-controlled positional signal gradient the best concept available for this aspect of pattern. What of the propagated mesenchymal growth control? We are unable to know the causal sequence between the receipt of signal, the decrease of cell packing density and the increase of transition probability to

'S' phase. By analogy with knowledge of growth control in *in vitro* confluent cell systems (see review in Stoker, 1978), the two behavioural responses could be intimately related, with the change in cell packing possibly instrumental in stimulating the cycle. We do not yet understand enough of the mechanism of early morphogenesis in rudiments like the limb bud to know how mesenchymal packing density or shape might be controlled. It seems unlikely that cells can literally push one another apart, even though the appearance of undifferentiated bud mesenchyme from either end of the density range, in toluidine-blue-stained epon sections, shows that low-density cells simply have a more extended surface with less area of mutual contact and more intercellular 'space'. A specific chemical messenger which decreased packing indirectly through causing secretion of colloid from the cells into the space would be surprising in view of the very rapid propagation through tissue and short induction time seen for the effect. Another possibility is that shape and size of the mesenchymal population of the normal bud is constrained by positive elastic pressure and a pattern of deformability in the ectodermal sheath, with the mean cycle rate set well below the maximum possible from the nutritional situation by means of contact density. Signals controlling the growth rates of different parts of the mesenchymal cross-section could then originate as propagated changes of tensile strength within the ectoderm, leading literally to bulges underneath which the amount of space available per mesenchyme cell was increased (Summerbell, 1973). If mesenchyme is organized at all like confluent monolayers or explants in culture, such decrease in cell density would be progressively shared and equilibrated throughout it with an appropriate evening out of the re-setting of mean cycle time (see Folkman & Moscona, 1978). A decrease in constraining forces in ectoderm caused by increased cell division there would accomplish nothing by way of explaining the rapidity of the effect, even though such an increase may finally occur as part of bud widening. If the signal acts initially to decrease the tensile strength of ectoderm, a decrease of nuclear density in the plane of the cell layer, because of a flattening of the cells, might be expected as the earliest sign of the growth-enhancing sequence of events after ZPA implantation. Future studies based on this hypothesis should address the ectoderm and apical ectodermal ridge, as well as questions of the specificity or otherwise to limb mesenchyme of the ZPA's growth-enhancing signal.

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