The role of gap junctions in patterning of the chick limb bud

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Summary

The role of gap junctional communication during patterning of the chick limb has been investigated. Affinity-purified antibodies raised against rat liver gap junctional proteins were used to block communication between limb mesenchyme cells. Co-injection of the antibodies and Lucifer yellow into mesenchyme cultures demonstrated that communication was inhibited almost immediately. When antibodies were loaded into mesenchyme tissue by DMSO permeabilisation, [3H]nucleotide transfer was prevented for at least 16 h.

Polarising region tissue from the posterior limb bud margin causes digit duplications when grafted to the anterior margin. Quail polarising region cells were loaded with gap junction antibody and grafted into chick wing buds. The antibody had no effect on growth or survival of the grafted cells. As very few polarising region cells are required to initiate duplications, the number of polarising region cells in the grafts was reduced by diluting 1:9 with anterior mesenchyme tissue.

When either polarising region or anterior mesenchyme tissue in the graft was loaded separately with antibody, there was little effect on respecification of the digit pattern. However, loading both tissues in the graft caused a significant decrease in duplications. This indicates that a major role of gap junctions in limb patterning may be to enable polarising region cells to communicate directly with adjacent anterior mesenchyme. A role for gap junctional communication between anterior mesenchyme cells cannot be excluded. The results are discussed in relation to the role of retinoic acid as a putative morphogen.

Abbreviations: gpa, grains per area; AER, apical ectodermal ridge; AM, anterior mesenchyme; PR, polarising region; IR, interquartile range; DMSO, dimethyl sulphoxide.

Key words: gap junctions, chick limb morphogenesis, limb patterning, development.

Introduction

Gap junctions between cells provide channels with the potential to transfer small, regulatory molecules. In embryos, changes in cell-to-cell communication coincide with important developmental events e.g. somite uncoupling (Blackshaw and Warner, 1976). Antibodies raised against gap junction proteins, which are capable of specifically blocking cell communication, have provided direct evidence of the importance of intercellular communication. In amphibian development, blocking gap junctions between the progeny of a future dorsal cell of *Xenopus* from the 8-cell stage resulted in tadpoles with misplaced or even absent structures (e.g. eye or forebrain; Warner *et al.* 1984). In *Hydra*, blocking gap junctions may prevent the passage of a morphogenetic signal. When anterior trunk grafts are made close to the head of the host, a secondary head is rarely formed. This has been interpreted as evidence for a head inhibiting morphogen (MacWilliams, 1983; Berking, 1979). However, when gap junctional communication in the trunk of the animal was prevented by loading gap junction antibodies in bulk, the incidence of secondary head development was significantly increased (Fraser *et al.* 1987). This implies that patent gap junctions are necessary for effective head inhibition and is consistent with direct cell-to-cell transfer of an inhibitory morphogen.

In vertebrates, accessible developmental systems in which to test possible roles of gap junctional communication are few. The development of the chick limb bud is well defined and relatively easy to manipulate *in ovo*. Pattern formation along the anteroposterior axis is profoundly influenced by an area of mesenchyme at the posterior margin of the bud called the polarising region (Saunders and Gasseling, 1968). The polarising region behaves as if it produces a morphogen that diffuses across the limb, specifying digit character by its local concentration (Tickle *et al.* 1975). Retinoic acid is a candidate for the molecule involved in this patterning, as exogenous retinoic acid mimics the effect of polarising region grafts (Tickle *et al.* 1982; Tickle *et al.* 1985),
and retinoic acid is present endogenously in a graded fashion across the limb bud (Thaller and Eichele, 1987). However, it is unlikely that retinoids provide the sole signals necessary for patterning. It is striking that the cells within both the mesenchyme and epithelium of the limb are linked by gap junctions (Kelley and Fallon, 1983). Other signals could require gap junctional communication for their transmission.

We investigated the involvement of gap junctions in the signalling of the polarising region of the chick limb bud by loading gap junction antibodies into polarising region cells before grafting these to the anterior margin of the limb bud. Glyphs of untreated polarising tissue result in mirror-image digit duplications, in a dose-dependent manner, according to the number of polarising region cells in the graft (Tickle, 1981). We used this system to find out how preventing gap junctional communication with antibodies to gap junction protein affects respecification of the limb pattern.

**Materials and methods**

**Materials**

Fertilized White Leghorn chicken eggs (Poyndon Farm, Waltham Cross, Herts, UK) and quail embryos Coturnix coturnix (laboratory breeding stock) were stored at 12°C, until incubation at 38°C. All reagents were obtained from Sigma Ltd (Poole, Dorset, UK) unless otherwise stated.

**Graft preparation**

The procedure is summarized in Fig. 1. Wings were removed from stage 22 quail embryos or stage 20 chicks (Hamburger and Hamilton, 1955) and maintained in culture medium (MEM, Gibco, Uxbridge, Middlesex, UK, with Hanks' salt solution, 10% FCS, 2mM-L-glutamine, 25mM-Hepes, 100i.u. ml⁻¹ penicillin and streptomycin and 0.25 µg ml⁻¹ fungizone). The wing ectoderm was removed after treating with 2% trypsin (Difco, West Molesey, Surrey, UK, 1:250) in calcium- and magnesium-free Hanks' solution for 1 h at 4°C (Szabó, 1955). 200 µm cubes of mesenchyme were cut either from the anterior margin or from the posterior margin (i.e. the polarising region). The polarising region and/or the anterior mesenchyme tissue was loaded with antibodies against gap junction protein, or preimmune IgGs, using dimethyl sulfoxide (DMSO) permeabilisation (see later).

As further controls the tissue was treated with DMSO alone. After treatment, loaded tissue was washed in culture medium, and used as grafts. For the majority of experiments, mixtures of anterior mesenchyme and polarising region cells were made. To give 10% polarising region cells, 18 cubes of anterior mesenchyme were added to 2 cubes of polarising region cells, mechanically dissociated with a Pasteur pipette and the cell suspension spun at 800 g for 5 min. The pellet was left to consolidate for 1 h at 38°C, 200 µm cubes cut out and grafted into chick limb buds. In general, a pellet yielded 8 to 10 cubes for grafted.

**Experimental procedure**

All grafts were made to the right wing buds of stage 20 chicks. An incision was made under the anterior apical ectodermal ridge and the graft was manoeuvred underneath the resulting ectodermal loop. Host eggs were incubated for a further 6 days at 38°C. The embryos were killed by decapitation and both wings were removed, fixed in 5% TCA (3 h), stained with alcian green and then cleared in methyl salicylate to reveal the cartilage elements. Every experimental limb was photographed and the number and type of digits analysed and expressed as the % respecification that had occurred (see below). When reaggregated cells were grafted, the digits obtained were not always as unambiguous as in limbs which had received non-reaggregated cells. To minimize variations in the assessment from experiment to experiment, the photographs of all the limbs were checked at the end of the series to ensure that the same criteria were applied throughout.

**Calculation of % respecification**

Twice as many polarising region cells are required in the graft to produce an extra digit 3 as a 2, and another doubling of cells is needed to form an extra digit 4 (Tickle, 1981). Accordingly, a limb in which the highest extra digit was a 2 scored 1, an extra 3 scored 2 and an extra 4 scored 4 (Tickle et al. 1985). An extra digit 4 therefore corresponds to 100% respecification of the limb anterior mesenchyme, an extra digit 3 to 50% and a 2 to 25% respecification. All the limbs were scored, and the total score for each treatment divided by the number of limbs, to give an average level of respecification.

Grafts were made with 10% polarising region cells reaggregated with 90% anterior mesenchyme cells. The majority of limbs formed an extra digit 3 (see results), obscuring any subtle effects of communication block. Therefore we also considered the ratio of occurrences of an extra digit 4 to an extra digit 2 (digit 2 includes cases where no digits were formed). This ratio is referred to as the 4:2 ratio, and takes into account only limbs which demonstrate an increase (4s) or
DMSO loading

The technique of loading cells with macromolecules by permeabilising tissue with the detergent DMSO was modified from the previously reported method of Fraser and Bode (Fraser et al. 1987) and will be described in detail elsewhere (Allen, Bode and Fraser, in preparation). DMSO of the highest quality (99.96 % pure, Aldrich Chemical Co., Gillingsham, Dorset, UK) was used and, after opening, stored frozen as 5 µl aliquots. The tissue was washed in serum-free MEM and placed in a 10 µl microwell (Terasaki dish, Nunc, Kamstrup, Denmark) on ice in minimal fluid. 5 µl of DMSO was diluted with 55 µl MEM. 6.5 µl of this solution was added to the microwell to give a final DMSO concentration of 5%. 2 µl of immune reagent in buffer was added and the tissue gently mixed with a needle. After 1 h the tissue was washed 3 times in MEM for 5 min each, gradually warming the tissue to the ambient temperature.

Two polyclonal antibodies, A and B, raised in rabbits against the 32×10^6 M_r rat liver gap junction protein and affinity purified against that protein, were generously provided by N.B. Gilula. A full description of their production has already been published (Warner et al. 1984; Fraser et al. 1987). These antibodies, shown previously to block gap junctional communication in Xenopus embryos (Warner et al. 1984; Warner and Gurdon, 1987), mouse embryos (Lee et al. 1987) and Hydra (Fraser et al. 1987) and immunoblot against chick gap junctional protein (Green, personal communication), were loaded into the cells. The antibodies or preimmune IgGs were used as a stock of 1.5 mg ml^{-1}, based on tests in Xenopus, mouse and Hydra, where higher concentrations did not appear to be more effective.

The antibodies bind specifically to the cytoplasmic surfaces of rat liver gap junctions, both in situ, and to isolated gap junction preparations (Young et al. 1987). Indirect immunofluorescence has demonstrated antibody staining of gap junctions in Hydra (Fraser et al. 1987), rat and mouse livers (Green, personal communication). In EM, protein A gold second layer staining of the antibodies has demonstrated antibody binding exclusively to gap junctions but not to other structures or membranes (Young et al. 1987).

Lucifer yellow transfer

Mechanically dissociated limb mesenchyme cells from stage 20 chick or quail embryos, were plated on the lids of 35 mm tissue culture dishes (Nunc, Kamstrup, Denmark) in culture medium and incubated for about 16 h at 38°C, to give a monolayer. The tips of high resistance glass microelectrodes (50-100 MΩ, Corning, Bargaintown, NJ, USA) were filled with 1.7 % Lucifer yellow in distilled H_2O with or without 1.2 mg ml^{-1} gap junction antibody and backfilled with 100 mM LiCl or 100 mM NaCl. Cells in the monolayer were injected by iontophoresis, and the distribution of dye observed under UV illumination during injection. Cells were scored for transfer immediately after injection, and 1.5 and 4 min later, which should be sufficient to allow the antibody to achieve maximal block (see Hertzberg et al. 1985; Lee et al. 1987). Transfer to more than one neighbouring cell was scored as positive, to eliminate transfer between sister cells. Comparisons between control and experimental injections were made blind.

Nucleotide transfer

70 µCi of [3H]uridine was dropped on stage 20 quail or chick embryos in ovo, to label the nucleotide pool of the limb mesenchyme cells. After 1 h the wing buds were removed, polarising region tissue dissected and sampled, immunopurified antibodies or preimmune IgGs as described above. The polarising region tissue was added to an equal amount of untreated and unlabelled chick anterior mesenchyme in culture medium, mechanically dissociated and the cells plated out. After 4, 16 or 24 h the cultures were fixed in half-strength Karnovsky's fixative, dehydrated and air dried for autoradiography to reveal nucleotide incorporated into RNA. The cultures were coated with G5 Ilford Nuclear emulsion (Ilford Ltd, Mobberley, Cheshire, UK), dried, exposed at 4°C for 7 days, and developed at 22°C with Kodak D19 developer. After fixation and washing, the autoradiographs were mounted in Glycerin Jelly (Peacock, 1950) with size 0 glass coverslips (Chance Propper Ltd, Smethwick, Warley, UK).

Autoradiographs were analysed using a ×100 oil immersion objective (Neofluor, Zeiss, West Germany) on a Zeiss, Compound Phase Microscope attached by a Video camera to a Visual Image Processor (V.I.P., Sigh Systems Ltd, Newbury, Berkshire, UK). To generate an image for Computer analysis, two grey images of each field (which included cells and areas of the dish), taken at different focal planes to include silver grains lying at varying levels, were stored, corrected for any unevenness in background light levels and summed by the V.I.P. A binary image (white above threshold, black elsewhere) was superimposed by setting the detection threshold to the range of grey levels corresponding to the silver grains, representing them as white. The grains were counted as the area, in pixels, above threshold with a preset 'box' (total dimensions 6.1 µm×5.8 µm, 35.4 µm^2) which could be manipulated around the field. The pixels were converted into 'grains' per area (gpa) using a calibration curve. Grain densities higher than 50 grains per box (i.e. totally white) could not be resolved and were scored as ≥50 grains.

Cultures were counted blind. Several readings (n) were taken in each field by moving the 'box' to different locations; over cells (often more than one measurement was taken over each cell), and over the dish around the cells (background measurements). Measurements from donor (polarising region) cells, recipient cells (anterior mesenchyme cells in contact with donors), isolated anterior mesenchyme cells and the dish were collected for each culture. Measurements were taken from at least 2 cultures and over an average of 51 cells (range 25–114) for each condition tested. The individual measurements (n) expressed as silver grains per area, were collated for each experiment and plotted as frequency histograms, which approximated to Poisson distributions. Each distribution was described by the median (in gpa) and the interquartile range (IR; 25%–75% of the distribution). Complete distributions were compared using the Median Test.

Freeze fracture replicas of cultured chick mesenchyme cells

Cultures of chick limb mesenchyme cells were set up as described for dye transfer experiments. After 24 h they were fixed (2.5 % glutaraldehyde-buffered in 0.2 m-sodium cacodylate; pH 7.2, 1 h), washed in buffer and treated with 25 % glycerol (in buffer; 1 h). The culture dishes were cut into fragments and each piece frozen in Freon against a polyvinyl-alcohol-coated copper sandwich holder (Balzers) with the
cells in between. The specimens were fractured at $-140^\circ$C in a Balzer BAF400T apparatus by lifting the portion of plastic dish off the copper sandwich. The fractured surface was shadowed with platinum and carbon, cleaned in domestic bleach and the replica viewed in a Philips 301 transmission electron microscope. We are indebted to Dr C. R. Green for assistance with this technique.

**Number of polarising region cells in graft**

Quail polarising region cells, loaded with either gap junction antibody or preimmune IgGs, were diluted to 10% with chick anterior mesenchyme cells. 200 $\mu$m cubes of ungrafted, pelleted tissue and limbs containing grafts were fixed in half-strength Karnovsky's, washed in 0.1 m-sodium cacodylate buffer (pH 7.3), dehydrated through alcohol and embedded in methacrylate. 4 $\mu$m serial sections were taken on a Sorvall JB4 microtome and alternate sections viewed. Sections were stained to distinguish the quail cells (Le Douarin, 1973) by a Feulgen technique modified for plastic sections (IN HC1, 60$^\circ$C, 10 min; washed distilled H$_2$O, 5 min; stained in dark, 90 min, room temperature in 2gm pararosanline hydrochloride, 4.4gm K$_2$S$_2$O$_5$, 200 ml 0.15 n-HCl filtered after treatment with activated charcoal; three changes of fresh 5 ml 1 n-HCl, 5 ml 10% aqueous K$_2$S$_2$O$_5$, 90 ml H$_2$O, 5 min each; washed in running H$_2$O, 30 min; washed distilled H$_2$O), air dried and mounted in DPX just prior to viewing. Sections were viewed with a x40 objective on a Zeiss Microscope and the number of quail nuclei on each section counted and corrected for fragmentation by Abercrombie's correction (Abercrombie, 1946).

**Results**

**Gap junction antibodies block communication between limb mesenchyme cells in culture**

Mesenchyme cells in the intact chick limb bud are linked by gap junctions (Kelley and Fallon, 1983). Freeze fracture replicas of mesenchyme cells cultured from stage 20 chick limb buds for 24 h also revealed small, punctate gap junctions linking cells throughout the culture. An example is shown in Fig. 2.

The affinity purified gap junction antibodies to be used in the patterning experiments were tested for the ability to inhibit junctional communication in chick mesenchyme. They were injected together with Lucifer yellow into cultured chick cells. Controls gave efficient transfer (97.6%; n=41; Fig. 3A,B). When gap junction antibody B was injected together with Lucifer, transfer often failed immediately and overall transfer levels fell to 30% (n=23; $P<0.0001$, $X^2$; Fig. 3C,D). Within the limits of experimental variability antibody A was equivalently effective, confirming previous observations (Warner et al. 1984; Lee et al. 1987; Fraser et al. 1987).

The length of time over which antibody A could maintain the block of junctional communication was tested by examining transfer of [3H]nucleotide, recognized by incorporation into RNA, from polarising region to anterior mesenchyme cells after 4, 16 and 24 h of co-culture. Fig. 3E,F illustrates a field from a control culture in which quail donor polarising region cells (heavily labelled) were loaded with preimmune IgGs. Chick anterior mesenchyme cells (outlined by dashes) in contact with the donor cells are more heavily labelled than the isolated anterior mesenchyme cell (right), indicating transfer of nucleotide from donor to recipient through gap junctions. Fig. 4 plots the frequency distributions of the grain count measurements ($n$), taken over a number of cells for each of the different cell populations at one timepoint (16 h; see legend). Table 1 gives the results from all the experiments and details the median and interquartile range (IR) of the grain count over contacting and non-contacting anterior mesenchyme cells after 4, 16 and 24 h in culture.

After 4 h contacting recipients in control cultures contained significantly more grains than non-contacting anterior mesenchyme cells (quail donors: medians of anterior mesenchyme cells in contact (CAM)=4, non-contacting (NAM)=1; chick donors CAM=20, NAM=8; $P<0.0001$; Median Test), indicating transfer of [3H]nucleotide. By contrast, anterior mesenchyme cells in contact with donors loaded with gap junction antibody had levels of label that were insignificantly
Fig. 3. Transfer in cultures of stage 21 chick limb mesenchyme, 24 h after plating. (A, B) Transfer of Lucifer yellow in the control, 4 min after injection. (A) phase-contrast; (B) same field under UV illumination. Dye has spread to several first order neighbour cells of the injected cell and the nuclei of some of the second order neighbours are also dyed.

(C, D) Spread of Lucifer yellow after co-injection with a gap junction antibody. (C) Phase-contrast; (D) same field under UV illumination. Dye has not moved out of the injected cell, even 4 min after injection of dye and antibody mixture.

(E, F) Autoradiographs of mesenchyme cultures demonstrating a field from which measurements were taken. (E) Phase-contrast; (F) bright-field micrograph of the same field as E, showing the silver grains clearly. Quail donor polarising region cells were loaded with preimmune IgGs (heavily labelled) and co-cultured with chick anterior mesenchyme cells, marked by dashes on F. Recipient cells are labelled with silver grains, indicating the previous transfer of $[^3]H$nucleotide. Another uncontacted anterior mesenchyme cell, to the right of the recipients, is not labelled above the dish background level.

Scale bar A–D=27.6 μm; E, F=10 μm.
different from non-contacting cells (CAM versus NAM: quail $P=0.063$; chick $P=0.081$; see Table 1 for medians). At 16 h, contacting and non-contacting anterior mesenchyme cells were still equivalent in antibody-loaded cultures, suggesting that the block of junctional communication persists for at least 16 h (CAM versus NAM: quail $P=0.955$; chick $P=0.882$). After 24 h of co-culture this was no longer the case and antibody-loaded donors had clearly transferred some nucleotide to contacting anterior mesenchyme cells (CAM versus NAM: quail only, $P=0.002$), although transfer was still below the level of control cultures.

Table 1. Medians of Ag grains over cells and dish in mesenchyme cultures

<table>
<thead>
<tr>
<th>Area</th>
<th>4 hours</th>
<th></th>
<th>16 hours</th>
<th></th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prel IgGs</td>
<td>Gap Jn Ab</td>
<td>Prel IgGs</td>
<td>Gap Jn Ab</td>
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<td>gpa</td>
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<tr>
<td>(A) Quail donor cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Background over dish</td>
<td>1 (0-1)*</td>
<td>1 (0-1)</td>
<td>1 (1-1)</td>
<td>1 (0-1)</td>
<td>1 (1-1)</td>
</tr>
<tr>
<td>Uncontacted AM cells</td>
<td>5 (1-2)</td>
<td>3 (2-5)</td>
<td>3 (3-8)</td>
<td>3 (3-8)</td>
<td>3 (3-8)</td>
</tr>
<tr>
<td>Contacted AM cells</td>
<td>14 (2-7)</td>
<td>14 (2-7)</td>
<td>14 (2-7)</td>
<td>14 (2-7)</td>
<td>14 (2-7)</td>
</tr>
<tr>
<td>Donor Quail PR cells</td>
<td>43 (36-49)</td>
<td>51 (33-48)</td>
<td>47 (46-51)</td>
<td>47 (46-51)</td>
<td>47 (46-51)</td>
</tr>
<tr>
<td>(B) Chick donor cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background over dish</td>
<td>4 (2-7)</td>
<td>2 (1-7)</td>
<td>4 (1-6)</td>
<td>4 (1-6)</td>
<td>4 (1-6)</td>
</tr>
<tr>
<td>Uncontacted AM cells</td>
<td>8 (3-12)</td>
<td>6 (3-8)</td>
<td>6 (3-8)</td>
<td>6 (3-8)</td>
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<tr>
<td>Contacted AM cells</td>
<td>21 (5-12)</td>
<td>8 (3-12)</td>
<td>8 (3-12)</td>
<td>8 (3-12)</td>
<td>8 (3-12)</td>
</tr>
<tr>
<td>Donor Chick PR cells</td>
<td>51 (15-30)</td>
<td>51 (15-30)</td>
<td>51 (15-30)</td>
<td>51 (15-30)</td>
<td>51 (15-30)</td>
</tr>
</tbody>
</table>

$gpa$="grains per area" (for calculation see Methods). AM=anterior mesenchyme; PR=polarising region.

*Interquartile range=25-75% of the distribution.

*All donor chick PR cells were densely labelled, with $>51$ gpa. Each value represents measurements taken over between 25 and 114 cells.
The signalling of the polarising region can be demonstrated by grafting it to the anterior margin of a wing to-cell communication. A blocks intercellular communication between polarising cases an additional digit developed (equivalent to with gap junction antibody A, or treated with DMSO consisting entirely of polarising region cells were loaded bud. This operation almost invariably leads to a mirrorimage symmetrical limb along the anteroposterior axis. With either quail or chick donors at both 4 and 10% of antibody-loaded cells might still be gap junction antibody was present. By the same reckoning, after 24 h up to 40% of the polarising region cells could have escaped the antibody block.

It is common practice to use non-contacting recipient cells as a background in nucleotide transfer experiments despite the possibility of transient contact during culture. When donors contain gap junction antibodies this should not be a complication. The finding that there was no systematic difference in the label over isolated anterior mesenchyme cells between control and antibody loaded cultures, confirms that readings over these cells provide a realistic background.

These experiments show that gap junction antibody A blocks intercellular communication between polarising region and anterior mesenchyme cells for at least 16 h, and still has some effect after 24 h. A polarising region graft is required in the limb for at least 12 and 18 h to give respecification of a digit 3 (Smith, 1980). Experiments using implants of beads soaked in retinoic acid indicated that if there is a delay of 8 h before a polarising signal is introduced, no duplications are obtained (Eichele et al. 1985). Thus, the antibody block of cell-to-cell communication should last sufficiently long to reveal any effect on polarising region signalling.

The consequences for limb patterning of inhibiting cell-to-cell communication

The signalling of the polarising region can be demonstrated by grafting it to the anterior margin of a wing bud. This operation almost invariably leads to a mirror-image symmetrical limb along the anteroposterior axis (e.g. see Fig. 6D).

In a first series of experiments, cubes of tissue consisting entirely of polarising region cells were loaded with gap junction antibody A, or treated with DMSO alone, and grafted into host wing buds. In virtually all cases an additional digit developed (equivalent to 100% respecification; data not shown), whether or not gap junction antibody was present.

The nucleotide transfer experiments indicated that potentially 10% of antibody-loaded cells might still be able to communicate even during maximal block. Since very low numbers of polarising region cells in mixed reaggregates can lead to the specification of additional digits (Tickle, 1981), only tests with small numbers of polarising region cells are likely to be valid. The appropriate number of polarising region cells was determined by constructing a dose–response curve using quail polarising region cells, treated with DMSO, and diluted with untreated anterior mesenchyme tissue. A steep non-linear relationship between the number of polarising region cells and respecification was obtained (see Fig. 5; see also data from Tickle, 1981, dashed curve). Examples of the range of digit patterns obtained with different proportions of polarising region tissue in the graft are shown in Fig. 6; limb scoring was applied as described in the Methods.

A 10% graft, which gives approximately 50% respecification of the limb pattern, was chosen for experiments to test signalling by the polarising region and the response of surrounding anterior mesenchyme cells. With this number of polarising region cells (538±148, mean±s.d.; see Table 4), even if the level of escape from the block was substantially greater than the upper limit of 10% estimated from [3H]nucleotide transfer experiments, any change in signalling should not be masked. More effective signalling would be detected by an increase in the number of limbs with extra digit 4s than in the controls, while a weaker signal would result in more limbs with extra digit 2s. Quail polarising region tissue was diluted with quail anterior mesenchyme tissue, and grafted into host chick limbs. The effects of loading gap junction antibody into polarising region cells only, diluting anterior mesenchyme cells only, or both tissues in the graft was tested by comparison with the appropriate preimmune or DMSO controls.

Consequences of blocking gap junctional communication between the polarising region cells in the graft (experiment A)

Table 2A shows the digit patterns obtained after loading polarising region cells in the graft with gap junction antibody and Table 3A summarizes these results. Antibody-loaded polarising region cells gave an overall respecification of 54% (No. of limbs (n)=47) compared with 63% (n=42) for the DMSO controls, and 56% (n=8) for the preimmune IgG controls. The ratios of occurrence of an extra digit 4 to an extra digit 2 (the 4:2 ratio) are shown at the bottom of Table 3. For controls (including preimmune IgGs) the 4:2 ratio is 1.29, and for grafts containing antibody-treated polarising region

![Fig. 5. The relationship between the % of the graft consisting of quail polarising region cells and the % respecification of the digit pattern obtained. The upper curve (●) illustrates data obtained from experiments when quail polarising region tissue was pretreated with DMSO prior to mixing with the anterior mesenchyme in the graft, while the lower curve (○) was data obtained previously using chick polarising region cells without any DMSO treatment (Tickle, 1981). Respecification of 100% is obtained when an extra digit 4 is formed at the anterior edge, 50% with mainly extra digit 3s, and 25% with mainly extra digit 2s.](image-url)
F. Allen, C. Tickle and A. Warner

Fig. 6. Host chick wings showing examples of duplicated digits 6 days after receiving a mesenchyme graft to the anterior edge. (A) Graft of anterior mesenchyme tissue treated with DMSO. Digit pattern is normal, 234 (0% respecification). (B) Graft of 10% polarising region tissue, with both the polarising region and the anterior mesenchyme tissue in the graft treated with gap junction antibody. Digit pattern is 2234 (25% respecification). (C) Graft of 10% polarising region tissue, previously loaded with gap junction antibody. Digit pattern is 3234 (50% respecification). (D) Wing after graft of 10% polarising region treated with DMSO. A full mirror-image duplication has occurred, with digit pattern 432234 (100% respecification).

from the level of respecification for the DMSO controls. Clearly, uncoupling polarising region cells does not abolish their ability to signal.

Consequences of blocking gap junctional communication between the diluting anterior mesenchyme cells in the graft (experiment B)

In reciprocal experiments, antibody or preimmune IgGs were loaded into the anterior mesenchyme cells of pellets containing 10% polarising region tissue. In this series of experiments, both gap junction antibodies were used, and the results were indistinguishable (see Tables 2B and 3B).

In these experiments, the overall respecification is 54% in the antibody grafts (n=28) and 63% in the preimmune controls (n=15). The 4:2 ratio shows a difference between the experimental and control treatments, with a value of 5.0 in the controls and 0.83 in the antibody grafts, but this is not significant (4s: P=0.126; 2s: P=0.106). The antibody block of communication has little effect on the ability of anterior mesenchyme cells to respond to a polarising signal.

Consequences of blocking gap junctional communication in both the anterior mesenchyme and polarising region cells in the graft (experiment C)

When cell communication is reduced throughout the graft (Tables 2C and 3C), the overall respecification is 42% in the antibody-treated grafts (n=17) compared with 64% in the preimmune control (n=22). This decrease to 42% respecification is equivalent, from the dilution curve, to a graft containing only 4% polarising region cells, effectively less than half the expected strength of the polarising region introduced into the limb. Furthermore, the 4:2 ratio is 2.25 for the control grafts but only 0.38 for the antibody grafts. The proportional test indicates that in antibody-treated grafts, fewer digit 4s (P=0.059) and significantly more digit 2s (P=0.026) are formed than in controls. When both tissues are prevented from communicating via gap junctions, the effect of the graft on limb respecification is significantly reduced.

Comparison of control treatments

The control grafts were not equivalent (see Table 3), probably because, in each experiment, tissue that was not to be loaded was not treated with DMSO. When
polarising region tissue alone was treated with DMSO (A) there were fewer digit 4s and more digit 2s than when the anterior mesenchyme alone was treated (B). When both tissues were treated (C), an intermediate 4:2 ratio was obtained. DMSO treatment may slightly decrease polarising signal production and increase the ability of the anterior mesenchyme in the graft to respond to the signal. However, these effects are relatively small and insufficient to disguise any effect of the gap junction antibodies.

Do gap junction antibodies influence proliferation of cells in the grafts? The number of quail cells in grafts containing 10% quail polarising region cells and 90% chick anterior
mesenchyme cells was compared after gap junction antibody treatment and control treatments (preimmune IgGs or DMSO). Control or antibody-treated cubes from a pellet were fixed, sectioned and stained to provide an initial time point. The number of quail polarising region cells was counted in wings fixed 4, 24 and 48 h after grafts had been made. Some grafted limbs were left for 6 days to confirm that the respecification was within the range of the other experiments.

In host limbs, the polarising region cells tended to form irregularly shaped aggregates \((n=10)\). Fig. 7 shows a section from a 24 h limb with a 'whorl' of quail polarising region cells. The number of quail cells at different time points are given in Table 4.

In 200 \(\mu\)m cubes cut from a mixed control pellet, the total number of cells ranged from 4319 to 8953 (mean±s.d. 6117±1841), and there were 538±148 (mean±s.d.) polarising region cells (range 6.9–10.1%). The dilution curve (Fig. 5), subject to the same degree of inherent variability, shows that 6.9–10.1% polarising region cells would give respecification in a narrow window between 52–59%. This illustrates that, despite differences in total cell number, pellet variation would not affect the outcome of the experiments. Cubes cut from a pellet containing antibody-treated polarising region cells had an average of 683 quail cells (10.9%; see top part of Table 4).

By 4 h after grafting, 601 cells were found in a control limb, showing little cell division had yet occurred. After 24 h, the number of quail cells in all the grafts was comparable (controls average = 1464; antibody average = 1515). The range of quail cells at 24 h is well within the variation found in the cubes from the original pellet (see above), and the 48 h quail numbers generally confirm this finding. There is no evidence that antibody treatment either causes massive cell death or stimulates growth of the polarising region cells \textit{in vivo}. This finding is likely to hold also for the anterior mesenchyme cells. In contrast, previous experiments on cells in culture suggested a link between cell-to-cell communication and the rate of growth (Mehta \textit{et al.} 1986; 1989).

An estimate of the cell cycle time of the polarising

\textbf{Table 4.} The assessment of cell growth in control and antibody-treated cells

<table>
<thead>
<tr>
<th>Time point</th>
<th>Quail PR</th>
<th>Total Cells</th>
<th>% PR</th>
<th>Quail PR</th>
<th>Total Cells</th>
<th>% PR</th>
<th>Quail PR</th>
<th>Total Cells</th>
<th>% PR</th>
<th>DMSO control Quail PR cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>644</td>
<td>6021</td>
<td>(10.7)</td>
<td>437</td>
<td>4319</td>
<td>(10.1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(Cubes of Pellet)</td>
<td>723</td>
<td>6618</td>
<td>(10.9)</td>
<td>468</td>
<td>4843</td>
<td>(9.7)</td>
<td>469</td>
<td>6797</td>
<td>(6.9)</td>
<td>–</td>
</tr>
<tr>
<td>Mean</td>
<td>683</td>
<td>6319</td>
<td>s.d.</td>
<td>521</td>
<td>5675</td>
<td>(9.2)</td>
<td>797</td>
<td>8953</td>
<td>(8.9)</td>
<td>538</td>
</tr>
<tr>
<td>4 hours</td>
<td>674</td>
<td></td>
<td></td>
<td>601</td>
<td></td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24 hours</td>
<td>1248</td>
<td>1622</td>
<td></td>
<td>2131</td>
<td>1095</td>
<td></td>
<td>1166</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean</td>
<td>1515</td>
<td></td>
<td></td>
<td>All Controls 1464</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>48 hours</td>
<td>7601</td>
<td>9917</td>
<td></td>
<td>12161</td>
<td>3078</td>
<td></td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean</td>
<td>8759</td>
<td></td>
<td></td>
<td>All Controls 7619</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6 day digit patterns</td>
<td>432234</td>
<td>2334</td>
<td></td>
<td>23234</td>
<td>23/334</td>
<td>23234</td>
<td>23/334</td>
<td>6234</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
region cells in the grafts, obtained from their doubling time, gives a median and mode of 11 h, and a mean of 12.7 h. This is close to the estimate of 13.2 h (stage 24), calculated from the mitotic index (Janners and Searls, 1970).

**Discussion**

The main conclusion from these results is that, when both polarising region and anterior mesenchyme cells are prevented from taking part in gap junctional communication, the ability of a graft to respecify the limb was substantially reduced. However, uncoupling either the polarising region cells or the anterior mesenchyme cells alone in the grafts had little effect. Gap junctional communication was inhibited by loading polyclonal antibodies, raised and affinity purified against the \( 32 \times 10^9 M \), rat liver gap junction protein, by DMSO permeabilisation. These antibodies blocked cell-to-cell communication in cultures of chick limb mesenchyme, both immediately and in the long term.

**Gap junction antibody blocks communication between chick and quail limb mesenchyme cells**

These antibodies recognize gap junction proteins extracted from a wide range of tissues and species (Warner et al. 1984; Fraser et al. 1987; Lee et al. 1987; Zervos et al. 1985). They have previously been shown to inhibit gap junctional communication in Xenopus (Warner et al. 1984; Warner and Gurdon, 1987) and mouse embryos (Lee et al. 1987), and Hydra (Fraser et al. 1987), a finding which can now be extended to chick and quail limb mesenchyme. Escape from functional block did not begin for a minimum of 16 h and was only partial at 24 h. The antibodies may become less effective because of dilution through successive cell division; chick limb mesenchyme cells have a cell cycle time of 12–13 h (present results; Janners and Searls, 1970). There was no evidence to suggest that gap junction antibodies influenced cell growth or cell death. The block of junctional communication lasted sufficiently long to allow detection of any contribution by gap junctions to the respecification of anterior mesenchyme brought about by grafted polarising region cells (cf. Smith, 1980).

**Gap junctions between polarising region and anterior mesenchyme cells may be important**

The effective strength of the polarising signal was halved when gap junction antibodies were loaded into both polarising region and anterior mesenchyme cells in a graft. Preventing polarising region or anterior mesenchyme cells separately from communicating through gap junctions had little influence on their ability to send, or respond to, the polarising signal. This suggests that signalling through gap junctions formed between the polarising region and the anterior mesenchyme cells is important. The pathway might only be revealed when both tissues are communication incompetent because of the greatly reduced probability of both cell types escaping antibody block simultaneously; even if a 10% escape level were operating within the population as a whole, there would be only a 1% chance that adjacent polarising region and anterior mesenchyme cells would both be competent to communicate through gap junctions.

The results of blocking junctional communication cannot be explained by the inability of non-communicating cells to take part in digit formation, since the outcome of loading antibody into polarising region alone or anterior mesenchyme alone is the same despite the tenfold difference in cell numbers. The results also argue against a substantial contribution by gap junctional communication between anterior mesenchyme cells, although a minor role cannot be excluded.

If an important interaction between polarising region and anterior mesenchyme is mediated by cell-to-cell communication, why does blocking gap junctions between polarising region and anterior mesenchyme cells not inhibit respecification completely? It is difficult to prevent limb respecification by a polarising region graft for two reasons; the polarising region cells are remarkably potent and anterior mesenchyme cells can remain responsive over a long time. Transfer of \([3H]\) nucleotide was blocked by the antibodies for 16 h, but we cannot be confident that this communication block was absolute. Even minimal signalling between polarising region and anterior mesenchyme cells in the first 8 h could be sufficient to ensure that anterior mesenchyme cells become primed for respecification. Only complete absence of a polarising signal over the first 8 h prevents the generation of additional digits (Eichele et al. 1985). A weak initial signal extends the time for which anterior mesenchyme cells are competent to respond (Eichele et al. 1985). Failure to prevent completely respecification by blocking gap junctional transfer is not surprising. Rather, the arguments favour an important role for direct cell-to-cell communication, since despite the resilience of the respecification process, signalling is reduced effectively by a factor of two.

**Cell-to-cell communication and retinoic acid in limb patterning**

The need for polarising region cells apparently can be abolished if a source of retinoic acid is provided, which seems to conflict with the evidence suggesting that gap junctions between polarising region and anterior mesenchyme cells are important. A signal passing through heterologous gap junctions may initiate the generation of a morphogen, which does not itself need to be transferred through gap junctions. On this hypothesis, retinoic acid application simply bypasses an earlier, gap junction dependent, step in the process of limb patterning. Alternatively retinoic acid could induce some mesenchyme cells to take on the properties of polarising cells, so reconstituting the normal situation in which heterologous junctions between polarising region and anterior mesenchyme cells can be formed.

It is not known how retinoic acid influences communication through gap junctions in chick limb mesenchyme. In mouse C3H cells (Mehta et al. 1989), very
low concentrations (below $10^{-10}$ M) inhibit junctional communication, while intermediate levels ($10^{-9}$–$10^{-8}$ M), in the range found endogenously in chick limbs (Thaller and Eichele, 1987), enhance communication in the long term. If chick mesenchyme cells behave similarly, then the endogenous retinoic acid gradient (Thaller and Eichele, 1987) may lead to enhanced junctional communication close to the polarising region and reduced coupling further away. The generation of retinoic acid by chick limb cells might speed the escape of mesenchyme cells from the block achieved by gap junction antibodies. Clearly experiments to test directly the effects of retinoic acid and its derivatives on cell-to-cell communication in chick limb bud mesenchyme would be illuminating.

The present results focus attention on an interaction between polarising cells and directly contacting, responding mesenchyme cells and suggest that this interaction may be mediated by gap junctions. Any signalling molecule involved therefore would have to be small and hydrophilic. It also appears to be exceedingly potent. The timing of this interaction in normal limb development is hard to pin-point. It could initiate the production of (say) retinoic acid by polarising region cells, or it could be an essential consequence of the action of a primary morphogen. The identity of the signals that pass between polarising region and anterior mesenchyme and the precise part played by this gap junctional interaction in the overall process of limb patterning will not be simple to resolve.

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References


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