Patterns of junctional communication during development of the early amphibian embryo

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Summary

Cell-cell communication through gap junctions was examined in *Xenopus laevis* embryos between the 16-cell and early blastula stages using Lucifer Yellow, Fluorescein, lead EDTA and dicyanoargentate as probes of junctional permeability. Injections were made into cells whose position was identified with respect to the primary cleavage axis and the grey crescent. FITC dextrans revealed cytoplasmic bridges between the injected cell and its sister only. In the animal pole at the 16-cell stage at the future dorsal side of the embryo, Lucifer Yellow was frequently and extensively transferred between cells through gap junctions. At the future ventral side gap junctional transfer of Lucifer Yellow was significantly less frequent and less extensive. The asymmetry of transfer between future dorsal and ventral sides of the animal pole was more marked at the 32-cell stage. In the vegetal pole also at the 32-cell stage, a dorsoventral difference in junctional permeability to Lucifer Yellow was observed. At the 64-cell stage the transfer of Lucifer Yellow was relatively frequent between cells lying in the same radial segment in the animal pole; transfer into cells outside each segment was infrequent, except at the grey crescent. At the 128-cell stage, Lucifer transfer between future dorsal or future ventral cells in the equatorial region was infrequent. A high incidence of transfer was restored at the future dorsal side at the 256-cell stage. At the 32-cell stage, fluorescein was infrequently transferred between animal pole cells although lead EDTA moved from cell to cell with high, comparable frequency in future dorsal and ventral regions. Dicyanoargentate always transferred extensively, both at the 32- and 64-cell stages. Treatment of embryos with methylamine raised intracellular pH by 0.15 units, increased the electrical conductance of the gap junction and produced a 10-fold increase in the frequency of Lucifer Yellow transfer through gap junctions in future ventral regions of the animal pole at the 32-cell stage.

Key words: gap junction, *Xenopus*, amphibian embryo, cell-cell communication, Lucifer Yellow, lead EDTA, fluorescein, dicyanoargentate, FITC dextran, ion movement.

Introduction

Cells in early embryos can communicate with one another via gap junction channels, which allow the direct movement of ions and small molecules from cell to cell. Potter et al. (1966) first described ionic coupling, measured electrophysiologically, between cells in the squid embryo, a finding soon extended to the newt (Ito & Hori, 1966), the fish *Fundulus* Bennett & Trinkaus, 1968), the chick (Sheridan, 1968), the starfish (Tupper & Saunders, 1972) and *Xenopus* (Palmer & Slack, 1970). However, the incidence of transfer of small molecules through gap junctions, indicated by the passage of fluorescent dyes between cells that are electrically coupled, varies considerably between embryos of different species. Dye transfer was not observed in the embryo of the starfish at the 32-cell stage (Tupper & Saunders, 1972). The mollusc *Patella* displayed dye coupling at the 32-cell stage (Dorresteijn et al. 1983) although not at earlier stages. In the teleost fish *Fundulus*, dye coupling was reported to be absent between isolated pairs of blastomeres (Bennett et al. 1972) until a reinvestigation yielded the converse result (Bennett
et al. 1978). In the mouse embryo, dye spread through gap junctions occurred from the 8-cell stage onwards (Lo & Gilula, 1979; Goodall & Johnson, 1982). The amphibian embryo showed no transfer of fluorescein from the 2-cell stage to early blastula (Slack & Palmer, 1969). However, another study showed the passage of smaller probes (Turin, 1977) and we have demonstrated that Lucifer Yellow could frequently transfer from cell to cell in the 32-cell Xenopus embryo (Guthrie, 1984).

There are several factors that might contribute to this variability: the position of the injected cell, the developmental stage of the embryo and the probe used to define dye coupling. Many authors used only one probe and did not take account of the position of the injected cell, thus ignoring the possibility that junctional permeability may not be spatially uniform within the embryo. Support for this idea comes from work on the larval epidermis of insects where all cells are ionically coupled (Warner & Lawrence, 1972), but Lucifer Yellow transfers freely from cell to cell only within segments and seldom across the segment border (Warner & Lawrence, 1982; Blennerhassett & Caveney, 1984). In the preimplantation mouse embryo, cell–cell transfer of Lucifer Yellow was restricted at the boundary between the inner cell mass and trophoderm (Lo & Gilula, 1979).

Despite much speculation about a possible role for gap junctions in the transfer of developmentally important signals there have been few systematic studies of the pattern of transfer of small molecules between cells in early embryos. This paper examines the transfer of Lucifer Yellow between positionally identified cells at early stages of the Xenopus laevis embryo. At selected developmental stages we have compared the movement of Lucifer Yellow (M, 457) with that of fluorescein (M, 332), dicyanoargentate (M, 202) and lead EDTA (M, 387). Brief reports of some of these results have already appeared (Turin, 1977, Guthrie, 1984).

Materials and methods

Collection and preparation of embryos

Adult Xenopus laevis frogs were induced to mate and lay eggs by injection of chorionic gonadotrophin (Pregnyl: Organon Ltd; females: 500 i.u.; males: 300 i.u.). Embryos were kept in a large volume of tap water or Holtfreter's solution (60 mM-NaCl, 1.25 mM-KCl, 1 mM-CaCl₂, 5 mM-Tris(hydroxymethyl)aminomethane) pH 7.4 and staged according to the normal Life Table of Xenopus laevis (Nieuwkoop & Faber, 1956). Embryos were selected on the basis of intact appearance and regular cleavage pattern and staged according to the number of cells in the vegetal or the animal pole, depending on which was to be injected. The jelly was usually removed using fine forceps, leaving the vitelline membrane intact. Occasionally embryos were chemically dejellied by soaking in 2% cysteine (Sigma) in Holtfreter's solution at pH 8.0, for 3 to 4 min, followed by extensive washing in Holtfreter's solution.

Tracers

Lucifer Yellow CH (Stewart, 1978), sodium fluoresceinate and FITC dextrans (Sigma), were made up as a 4% solution in distilled water. The complex of lead (II) and ethylene diamine tetraacetate (PbEDTA) was prepared according to Timm & Arnold (1958). An indication of purity was obtained by paper electrophoresis, followed by hydrogen sulphide precipitation; a single band displaced towards the anode showed that the majority was present as the PbEDTA complex. The apparent overall dissociation constant for PbEDTA is 3 x 10⁻¹⁴ M. No signs of toxicity or failure of cleavage (which would indicate significant quantities of free lead or EDTA) as a result of injection of PbEDTA were observed. The potassium salt of PbEDTA was made up as a 0.5- or 0.1 m-solution in distilled water. Dicyanoargentate, an extremely stable complex of silver (dissociation constant 4 x 10⁻¹⁰, Johnson Matthey) was used as a 0.5 M- or 0.1 M-solution of the potassium salt in distilled water; no sign of toxicity was observed.

Tracer injection

Embryos were placed in a wax bath containing Holtfreter's solution. When appropriate, 5–10% Ficoll was added to the bath solution so that the embryo could be manipulated into any orientation desired (Chung & Malacinski, 1983). The bath solution was connected to ground through an Ag/AgCl Agar–Holtfreter half cell. Electrodes were mounted in similar half cells and connected to FET preamplifiers with current injection facilities (Purves, 1981). Current pulses of known duration and amplitude were delivered by a D100 Digitimer and a Devices Type 2533 isolation unit. The output from the preamplifiers was displayed on a Tektronix 5115 oscilloscope. Electrodes were inserted in the voltage recording mode, and once a membrane potential was obtained, the circuit switched to current injection. For electrophoresis, rectangular, hyperpolarizing 100 nA current pulses of 30 ms duration at a frequency of 10 Hz were used, for about five minutes. The circuit was then returned to voltage recording and the electrode withdrawn.

Micropipettes were made from 1.2 mm outer diameter, fibre-filled glass pulled to a resistance of approximately 20 MΩ and a tip diameter of less than 1 μm. For injection of FITC dextrans (M, 10000), electrodes were broken back to a tip diameter of 1–5 μm. The tip of the electrode was filled with Lucifer Yellow or fluorescein backfilled with 1% lithium chloride. FITC dextrans were injected by applying pressure pulses to the back of the electrode. PbEDTA and dicyanoargentate were injected either by pressure or iontophoresis.

For pressure injections, a half-cell that allowed pressure pulses to be applied at the back of the electrode was used. A Picospritzer II (General Valve Corporation) connected to a 100% nitrogen cylinder (BOC) provided a measured duration pulse of pressure. Generally 40–70 ms pulses were used, at 138 x 10³ N m⁻².
Assessment of tracer distribution

**Fluorescent compounds**

Embryos injected with fluorescent tracers were viewed immediately after injection under ×6-3 or ×10 Planapio objectives on a Zeiss epifluorescence microscope equipped with appropriate filters. ( Lucifer Yellow, Zeiss 10; fluoro-

escin and FITC dextrans, 07). The injected cell was identified, the diaphragm shut down and the embryo scanned, comparing cells near to and distant from the injection site to reduce errors introduced by variations in pigmentation, autofluorescence and scatter from the injected cell. The distribution of dye was classified as no transfer, transfer to the sister cell only, or transfer to one or more cells other than the sister. To check whether the distribution changed with time, some embryos were re-
examined up to 30 min later.

Although the lower limit of detection could not easily be quantified, embryos examined after brief injections (30 s) showed unequivocal fluorescence both in lightly and heavily pigmented regions of the embryo.

**Metal complexes**

After injection, embryos were placed in Holtfreter solution saturated with H2S gas and left until precipitation of the sulphide appeared to be complete (usually 10-15 min). As long as the association constant for precipitation of the metal sulphide is much greater than the association con-

stant between the metal and its complexing anion, a condition met for both complexes used in the present experiments, effectively all the metal will be precipitated. Ammonium sulphide was avoided because preliminary experiments showed that polysulphides, which are fre-

quently present as contaminants (see Schmidt & Sebert, 1973), redissolved the metal sulphide precipitate, generat-

ing an artefactual distribution.

**Histology**

For frozen sectioning, embryos injected with Lucifer Yellow were fixed for 1–3 h in 4% formaldehyde, embed-

ded in OCT, frozen in isopentane in liquid nitrogen and sectioned at 20 μm on a Bright 5030 cryostat. For plastic sectioning, Lucifer-injected embryos were fixed for 5–6 h in 4% formaldehyde, dehydrated in alcohols, followed by overnight infiltration, embedding in hydroxyethyl methacrylate resin (Dupont Sorvall) and sectioning at 10 μm on a Du Pont Sorvall JB4 microtome.

After precipitation of the sulphide, embryos injected with a heavy metal complex were fixed in 5% EM grade glutaraldehyde in 0.1 M-phosphate buffer at pH 7.3. For resin embedding, embryos were fixed for 2 h only, to reduce solution of metal sulphides by the fixative. For frozen sectioning, the fixation time was increased to 24 h to improve the ease of sectioning, despite the possibility of losing some sulphide precipitate.

Embryos that had been injected with PbEDTA were cleared in toluene for 30 min, infiltrated in Araldite overnight at 50°C and embedded in fresh Araldite. This procedure avoided exposure to strong oxidizing agents such as propylene oxide or benzoyl peroxide, both of which were found to bleach lead sulphide, probably converting it to the sulphate. Embryos injected with dicyanoargentate were rapidly dehydrated in alcohols, infiltrated in a mixture of hydroxyethylmethacrylate and butoxyethanol (JB4 Solution A) overnight, followed by one hour in Solution A containing 1% benzoyl peroxide and polymerized by addition of 1 part polyethylene glycol 200 together with N,N'-dimethylaniline (JB4 Solution B). This modified schedule reduced exposure to benzoyl peroxide. Alternatively the embryos were embedded in LR White (Agar Aids).

Sections (7 μm) through metal-injected embryos were intensified using Timm’s method, modified according to Tyrer & Bell (1973). The intensification was carried out in the dark and monitored by examining sections every 5 min until the injected cell became reddish brown and then black. After staining, sections were washed twice in dist-

tilled water, dried and mounted. Alternatively a sodium tungsate developer (Gorgs et al. 1979; see Warner & Bate, 1987) was used. Sections were left to dry overnight, immersed in distilled water for 15 min, followed by 2% sodium tungsate for 10 min. They were then transferred into freshly prepared intensifier containing 8 parts solution A, 1 part solution B and 1 part solution C. (Solution A: 355 ml distilled water, 15 ml 1% Triton X-100, 1.5 g sodium acetate, 30 ml glacial acetic acid, and 0.5 g silver nitrate; solution B: 5% sodium tungsate; solution C: 0.25% ascorbic acid.) Slides were immersed in intensifier for between 2 and 10 min, until the precipitate had turned from orange to dark brown. After rinsing three times with distilled water, slides were dehydrated, cleared in Histo-

clear and mounted.

The sensitivity of the method was determined by processing small blocks of 2% Agar containing known concentrations of cobalt chloride. This gave a lower limit of detection of 10 μM-metal sulphide. Uninjected embryos subjected to the same procedures showed no colouration, indicating that precipitate revealed by the intensification procedure reflected injected metal complex.

**Measurements of intracellular pH**

Microelectrodes were pulled from 1.5 mm outer diameter glass to give relatively blunt (tip diameter approx. 1 μm) electrodes with short shanks. They were silanized in dimethylochlorosilane vapour (Sigma) at 200°C overnight. A small drop of pH-sensitive ion-exchange cocktail (Fluka) was introduced into the back of the pipette and the tip broken to 1–3 μm to aid filling. The electrodes were backfilled with 0.1 M-NaCl and 0.1 M-sodium citrate buffer at pH 6-0 and calibrated with solutions containing 100 mM-

NaCl, 2 mM-KCl, 2 mM-MgCl2, 2 mM-CaCl2 buffered at pH 6.8 (15 mM-Aces), 7.4 (15 mM-Hepes), 8.0 (15 mM-

Hepes) and 8.6 (15 mM-Taps). The electrodes were connec-

ted to high input impedance WPI electrometers designed for use with ion-sensitive electrodes and gave responses close to the Nernstian value of 58 mV per pH unit. Calibration was normally carried out before impalement and repeated at the end of the experiment.

For pH measurements, the embryos were bathed in Hepes-buffered calibrating solution at pH 7.4. The pH-sensitive electrode was inserted into a cell, where it recorded the sum of the potential generated by the pH change and the membrane potential. A second, 0.8 M-
sodium citrate- or 3 M-KCl-filled, electrode was inserted into the same cell to measure the membrane potential. The membrane potential was electronically subtracted from the potential recorded by the pH-sensitive electrode to give a third, direct, reading of intracellular pH. The pH-electrode response, membrane potential and direct reading of pH were displayed on a pen recorder. Impalements were maintained for 15–20 min and the stable value of intracellular pH taken. When the potential did not return to the original baseline on withdrawal of the electrode, the measurements were discarded.

Statistical treatment of data
Comparisons between the frequencies of transfer and failure to transfer between different regions of the embryo or between different probes were normally made using the Chi-squared test using 2×2 contingency tables. When the number of samples in one group was small, comparisons were made using Fisher’s exact test. When appropriate the Mann–Whitney test was employed. P values of less than 0.05 were considered to be statistically significant.

Results
The distribution of cytoplasmic bridges
In rapidly dividing cells cytoplasmic bridges may persist after cytokinesis and provide a direct pathway for tracer movement from cell to cell that can complicate any permeability pattern due to transfer through gap junctions. The extent of cytoplasmic bridges at the 32-cell stage was assessed by injecting FITC dextran, (M₉ 10000), which is too large to move through gap junctions. In 16 injections into one cell of tier 1 or tier 2 or the animal pole, the dye was never seen to pass into more than one other cell, the sister of the previous cleavage. This suggests that by the 32-cell stage cytoplasmic bridges do not persist for longer than a single cleavage cycle. Instances of transfer between sister cells were taken to be occurring via bridges and discounted from assessments of gap junctional communication.

Lucifer Yellow transfer between identified cells in the animal pole at early stages
Cell position was noted with respect to the grey crescent and the primary cleavage axis. The grey crescent is characterized by a lightening of the dark pigment of the animal pole and appears at the 2- or 4-cell stage opposite the sperm entry point. It marks the future dorsal side of the embryo and is bisected by the primary cleavage axis (see Fig. 1A).

The 16-cell stage
The 8 radial cells in the animal pole were labelled a–h, beginning at the primary cleavage axis on the future ventral side of the embryo (see Fig. 1B). Fig. 2A scores the incidence of dye transfer, based on 8 injections into each cell, monitored by visual inspection after injection. Injection began once the cleavage furrows were superficially complete and monitoring continued for up to 30 min (approximately the beginning of the next cleavage cycle). Cells at the future dorsal side (c, d and e) transferred dye in a majority of cases while there was a low frequency of transfer from cells at the future ventral side (a, b, g and h). Thus the frequency of transfer was highly asymmetric. The amount of dye in adjacent cells increased with time, but there was seldom any alteration in the pattern of dye transfer. It is very unlikely that this pattern arises by chance. The incidence of dye transfer from cells c, d, e and f, in the future dorsal half of the embryo was significantly greater than that from cells a, b, g and h, in the
The 32-cell stage

The incidence of dye coupling between cells of the animal pole at this stage has already been briefly described (Guthrie, 1984); some of that data are represented here for direct comparison with results obtained at other stages. The 16 cells in the animal pole generated by tangential cleavage were labelled al-hl (tier 1) and a2-h2 (tier 2) (Fig. 1C). The literature shows some divergence in the nomenclature used to identify particular blastomeres. Table 1 equates our nomenclature at the 32-cell stage with that of Dale & Slack (1987). Fig. 3A,B plots the frequency of transfer of Lucifer Yellow for each of these cells, provided by 16 injections into each cell, based on visual inspection of intact embryos. Transfer to other cells was most frequent in the region of the grey crescent, cells c1, d1 (tier 1) and c2, d2, e2, f2 (tier 2). Comparison of the incidence of transfer from cells c, d, e and f with that from a, b, g and h showed a highly significant difference between dorsal and ventral halves of the embryo ($\chi^2$ test, tier 1: $P<0.0001$; tier 2: $P<0.001$).

22 embryos, most of which had been assessed as failing to transfer, were subsequently frozen sectioned. 20 gave results which completely confirmed the previous visual assessment. 36 embryos were sectioned after methacrylate embedding. 18 of these gave the same result as visual observation of the intact embryo; the remainder gave lower estimates of transfer than originally noted, probably because of loss of Lucifer during histological processing. Fig. 4 shows examples of whole embryos assessed as no transfer (A), transfer to the sister cell (C), and transfer to one or more other cells (E), together with frozen or plastic sections through sibling embryos. Frozen sectioning gives an unequivocal confirmation of no transfer (B), while plastic sectioning better preserves cell morphology (D and F).

The pattern of transfer within the animal pole is illustrated in Fig. 3C,D and shows that cells c1, d1 and e1 of tier 1 regularly transferred dye laterally into nearly half the embryo. Cells that transferred dye well also received dye well, e.g. cells c1 and d2, while cells that transferred dye poorly also received dye.

Table 1. Comparison of nomenclature for labelling blastomeres at the 32-cell stage with that of Dale & Slack (1987)

<table>
<thead>
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<th></th>
<th>Present nomenclature</th>
<th>Dale &amp; Slack (1987)</th>
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<tr>
<td><strong>Tiers</strong></td>
<td>1 (animal)–4 (vegetal)</td>
<td>A (animal)–D (vegetal)</td>
</tr>
<tr>
<td><strong>Dorsal cells</strong></td>
<td>d1–d4 (rt) el–e4 (left)</td>
<td>A1–D1 (no rt/left distinction)</td>
</tr>
<tr>
<td><strong>Ventral cells</strong></td>
<td>a1–a4 (rt) h1–h4 (left)</td>
<td>A1–D4 (no rt/left distinction)</td>
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The future ventral half of the embryo ($P<0.0001$; Fisher's exact test).

Fig. 2B identifies the cells that received dye from each injected cell, excluding transfer into the sister cell of the previous cleavage. It shows that cells that transferred dye frequently also transferred dye into a number of other cells. There is considerable symmetry of transfer between cells at equivalent positions on either side of the primary cleavage axis.
poorly, e.g. cells al and h1. The position of maximal transfer is biased towards the future right-hand side of the embryo (compare cells el and f1). At the future ventral side of the embryo transfer across the primary cleavage axis was not observed.

Dye transfer into tier 3 was only seen along with transfer into cells of tier 1 or 2. It was infrequent after injections into cells of tier 1 (al–hl), although injections into tier 2 (a2–h2) often lead to dye movement into tier 3, both in future dorsal regions (50–70% of injections showing transfer) and in future ventral regions (35% of injections showing transfer).

The 64-cell stage
Injections were made into the outer ring of 8 cells, produced when tier 1 of the 32-cell stage divides, and labelled al.2–hl.2 (see Fig. 1C). The average frequency with which dye transferred beyond the sister cell was 46.8% (Fig. 5A) and there was no significant difference between dorsal and ventral halves of the embryo. However, the pattern of transfer (Fig. 5B) showed strong positional dependence. Injection of cells other than cell d1.2 seldom showed transfer laterally outside the radial segment originating at the 16-cell stage, now consisting of 4 cells. Bilateral transfer was observed, on average, in only 10% of injections. By contrast, injections into cell d1.2 (on the future right-hand side) frequently led to substantial bilateral transfer (50% of injections). This cannot be due simply to changes in cell numbers or in the geometry of the embryo, since all cells have the opportunity to transfer laterally in two directions.

Lucifer Yellow transfer between cells in the vegetal pole at early stages
A diagram of the vegetal pole view of an embryo at the 16-cell stage is shown in Fig. 6A. Cells were labelled av–hv so that ventral cell av lies directly below ventral cell a in the animal pole. The incidence of dye transfer, based on 8 injections per cell, is shown in Fig. 6B. Most cells transferred dye with a frequency of about 50%, apart from cell cv (87.5%) and hv (25%); the dorsoventral difference was thus less marked than in the animal pole at the 16-cell stage (compare Fig. 2A). Sister cell transfer constituted a more substantial component of the total cases of no transfer than observed in the animal pole,

Fig. 3. The transfer of Lucifer Yellow in the animal pole at the 32-cell stage. (A,C). The incidence of transfer of Lucifer Yellow out of identified cells after injection into cells of tier 1 (A) and tier 2 (C) based on 16 injections into each cell. Ordinates: Number of observations. Abscissae: Cell label. In each case, transfer into the sister cell of the previous cleavage only has been classed as failure to transfer. Note the asymmetry of the two distributions, with dye transfer through gap junctions occurring most frequently after injections into cells in the region of the grey crescent. (B,D). The pattern of dye transfer after injection into each cell of tier 1 (B) and tier 2 (D) of the animal pole. In each case, the injected cell is blocked in, the sister cell is shaded and the future dorsal side is downmost. The numbers indicate the frequency with which the labelled cell received dye. Note that dye spreads most widely after injection into cells cl, dl and el in tier 1 and cells c2, d2, e2 and f2 in tier 2. Instances of transfer into tier 3 are not included.
Fig. 4. Examples of the outcome of Lucifer Yellow injections at the 32-cell stage. (A,C,E) Photographs of whole embryos taken shortly after the end of the injection. (A) Injection into cell al. Lucifer is restricted to the injected cell. (C) Sister cell transfer only; example taken from an injection into cell dl. (E) Transfer into several cells after injection into cell dl. (B,D,F) Sections taken through embryos to confirm assessments made in the intact embryo. (B) Frozen section after an injection into cell al, which had been assessed as complete failure to transfer. The frozen section confirms this assessment. (D) Plastic section through embryo after injection into cell dl, previously assessed as transfer to the sister cell only. The plastic section completely confirms this assessment. (E) Plastic section taken after injection into cell dl showing transfer to a number of cells other than the sister. Section confirms previous assessment of intact embryo. For A, C and E, bar, 200 μm; for B, D and F, bar, 100 μm.
Fig. 5. The transfer of Lucifer Yellow after injection into cells al.2–hl.2 in the animal pole at the 64-cell stage. (A) The incidence of transfer of Lucifer Yellow to cells other than the sister of the injected cell based on 8 injections into each cell. Ordinate: no. of cells. Abscissa: cell label. Although the highest frequency of transfer occurs after injection into cell dl.2 there is no significant difference between future dorsal and future ventral halves of the animal pole. (B) The pattern of transfer of Lucifer Yellow. Each diagram shows the distribution of Lucifer Yellow after injection into each cell. Injected cell filled in. The number of occasions on which dye was observed in cells other than the sister is also indicated. Note that transfer into cells lying in the same radial segment as the injected cell occurs throughout the animal pole. Transfer outside each radial segment is rare, except after injection into cell dl.2.

implying that cleavage bridges may persist for longer in vegetal regions.

At the 32-cell stage, 8 injections were made into each cell in tier 3 (see Fig. 6C for nomenclature) and the results are summarized in Fig. 6D. Cells in the dorsal region transferred dye most frequently, i.e. cells c3, d3, e3 and f3, showing a close positional correspondence with those displaying most transfer in the animal pole. The incidence of transfer in future dorsal and future ventral regions is significantly different ($P < 0.01$). The pattern of dye transfer from every injection was not recorded systematically, but was usually lateral within the vegetal pole. Dye was seldom seen to move into the animal pole. This contrasts with the pattern of transfer after injection into cells of tier 2 in the animal pole, where transfer into the cells of tier 3 was relatively frequent.

Fig. 6. The transfer of Lucifer Yellow in the vegetal pole. (A) Diagram to show cell labels at the 16-cell stage. The vegetal pole lies in the centre of the diagram. Cell av lies immediately below cell a of the animal pole. (B) The incidence of dye transfer after injection in cells av–hv in the vegetal pole at the 16-cell stage based on 8 injections into each cell. Ordinate: number of observations. Abscissa: cell label. There is no dorsoventral asymmetry in the frequency of transfer. (C) Diagram to show cell labels in the vegetal pole at the 32-cell stage viewed with the vegetal pole in the centre. Cells of tier 3 were labelled a3–h3, with cell a3 lying immediately below cell a2 of the animal pole. (D) The incidence of transfer of Lucifer Yellow, based on 8 injections into each cell. Ordinate: number of observations. Abscissa: cell label. Note asymmetry in frequency of transfer, with cells d, e, f at the future dorsal side showing the highest frequencies.

Lucifer Yellow transfer between cells at the early blastula stage

At the 128-cell stage it is difficult to identify cells individually. Cells injected at the 128- and 256-cell stages were therefore located by their position relative to the grey crescent and injections made within an equatorial ring, running midway between the animal and the vegetal pole. Visual assessments were made as described previously and the data divided into the same categories.

Table 2 shows that at the 128-cell stage very little
dye transfer occurred in either the future ventral or dorsal aspects of the embryo, 10% and 20%, respectively (n = 10 for each). At the 256-cell stage, transfer increased slightly at the future ventral side (25%), and decisively at the future dorsal side, 65% (n = 20). Transfer at the future dorsal side was significantly different from transfer between cells in the ventral region (P < 0.01).

At these stages there are 4 or 5 cell layers between the surface cells and the blastocoel cavity. Fig. 7A illustrates a section from an embryo injected at the 256-cell stage, in the ventral equatorial region, which had been scored initially as failing to transfer. The section shows transfer to 4 or 5 deep cells. Fig. 7B shows an embryo injected into a dorsal cell, where dye transfer has occurred extensively into about 13 cells, clearly more than with the ventral cell in 7A. Sections through other embryos confirmed that assessments of dye transfer based on visual inspection of surface cells underestimated the overall transfer, although a difference in transfer between dorsal and ventral regions was always present.

Fluorescein transfer between cells in the animal pole
Injections of fluorescein (M, 332) were made into cells al–h1 in the animal pole at the 32-cell stage and dye transfer was assessed visually since fluorescein is not immobilized by fixation. In contrast to observations of Slack & Palmer (1969), fluorescein was found to transfer between cells. However, the incidence of transfer was considerably lower than observed with Lucifer Yellow; out of 70 injections only 14 (20%) showed clear transfer of fluorescein. Examples of transfer and no transfer are illustrated in Fig. 8A, B and the collated results are shown in Fig. 9. Cells in the region of the grey crescent transferred dye most frequently. The difference between dorsal and ventral halves of the animal pole is just statistically significant (P < 0.04; Fisher’s exact test).

The transfer of heavy metal complexes between cells in the animal pole
The range of molecules tested for the ability to move between cells in the amphibian embryo was extended by injecting two heavy metal complexes: PbEDTA and dicyanoargentate.

Dicyanoargentate always transferred to cells other than the sister of the injected cell, regardless of position, both at the 32-cell and 64-cell stages (n = 20). Transfer occurred to at least five other cells, and usually to at least ten cells, with transfer occasionally extending throughout the embryo. Injections into the blastocoel did not provoke uptake by the cells from the blastocoel fluid. A section showing transfer of dicyanoargentate is shown in Fig. 10C.

At the 32-cell stage, PbEDTA was injected into cells al (at the future ventral side) and dl (at the future dorsal side) of tier 1 in the animal pole (see Fig. 1B). For injections into future ventral cell al there were five instances of transfer into 3 or more cells (56%; n = 9), implying transfer beyond the sister cell. For injections into future dorsal cell dl, eight injections (57%; n = 14) yielded transfer into 3 or more cells. Thus dorsal and ventral cells showed equal tendencies to transfer PbEDTA. Fig. 10A shows a frozen section through a 32-cell embryo exhibiting transfer.

Twelve injections of PbEDTA were made at the 64-cell stage, six into cells of the animal pole which had not been identified positionally and six into cells dl.2. The tracer was never found in more than 2 cells, indicating complete failure to transfer. An Araldite section through one of these embryos is illustrated in Fig. 10B. Calculations using the binomial theorem show that this result makes it unlikely (P < 0.035) that the average incidence of transfer of PbEDTA in the animal pole at the 64-cell stage is more than 25% and very unlikely (P < 0.00025) that the average frequency of transfer is as high as that observed for Lucifer Yellow (46.8%).

The effect of raising intracellular pH on the transfer of Lucifer Yellow between future ventral cells
Gap junctions in the amphibian embryo are highly pH-sensitive (Turin & Warner, 1977; 1980). We therefore tested whether the frequency of dye transfer out of cells in the future ventral region could be increased by raising the intracellular pH in the 32-cell embryo, when the dorsoventral difference is most marked.
Methylamine raised the intracellular pH

An increase in intracellular pH can be brought about by soaking in a solution of weak base, which enters the cells in the associated form and then dissociates, liberating free base (see Thomas, 1984). The speed and magnitude of the rise in intracellular pH are controlled by the rate of entry of the undissociated salt, its pK and the resting intracellular pH. The weak base methylamine has previously been shown to raise intracellular pH in snail neurones (Thomas, 1984) and was used for the present experiments.

The ability of methylamine to raise intracellular pH in the Xenopus embryo was tested in six batches of eggs. Measurements with pH-sensitive microelectrodes in control embryos gave a mean intracellular pH of 7.81 ± 0.018 (±1 standard error; n = 55),
Gap junctions in amphibian embryos

Fig. 8. The transfer of fluorescein in the animal pole at the 32-cell stage. (A) Photograph of whole embryo after injection into cell al. Dye is restricted to the injected cell, indicating failure to transfer. (B) Photograph of whole embryo after injection into cell dl showing an example of fluorescein transfer. 4 cells are fluorescent. Bar, 100 μm.

Fig. 9. The incidence of transfer of fluorescein in the animal pole based on 8 injections per cell except for cell dl (12 injections) and al (10 injections). Ordinate: number of observations. Abscissa: cell label. Note the frequency of transfer is low throughout the animal pole, although it is significantly higher in the future dorsal half.

The finding that ventral cells can, under the appropriate conditions, transfer Lucifer Yellow with high frequency makes it extremely unlikely that the low with highest control). Thus the weak base methylamine consistently increased the intracellular pH by about 0.15 pH units.

Methylamine had no obvious effect on cell membrane potential, cleavage or development up to stage 37/38 (the latest time examined) even when treatment continued for many hours.

Methylamine treatment improves dye transfer between future ventral cells

The transfer of Lucifer Yellow injected into cell al, which lies at the future ventral side of the 32-cell embryo, was compared in control embryos and embryos that had been treated with 15 mM-methylamine for 15 min. Fig. 11 compares the incidence of transfer in the absence (A) and presence (B) of methylamine. In control embryos, Lucifer Yellow transferred out of cell al only once (n = 16). Treatment with methylamine significantly (P < 0.001, Fisher's exact test) increased the incidence of transfer, bringing it up to 67% (n = 17). The rise in frequency of transfer in methylamine was not the consequence of opening of cytoplasmic bridges; injections of FITC dextrans showed that cytoplasmic bridges were only present between sister cells of the previous cleavage, as found in controls (see earlier).

The finding that ventral cells can, under the appropriate conditions, transfer Lucifer Yellow with high frequency makes it extremely unlikely that the low
level of transfer observed in controls is a consequence of the high level of pigmentation.

A limited study of the effects of raising intracellular pH on the electrical conductance of the gap junction was made on isolated pairs of blastomeres. Each cell was impaled with two microelectrodes, one of which voltage clamped each cell at the resting membrane potential, while the other recorded the current flowing into one cell after application of a 5 mV voltage step to its neighbour, so measuring junctional current. In seven experiments, the weak base propylamine induced an increase in junctional current ranging from 30–100%. Application of 100% CO₂ to acidify the cytoplasm, completely abolished current flow through the junction.

Discussion

Cells of the early amphibian embryo can exchange a variety of molecules larger than small ions, through gap junctions. However, the ability of molecules to move through gap junctions depends on the position of the injected cell relative to the future dorsoventral axis, the stage of the embryo and the properties of the probe used to assess junctional permeability.

The pattern of transfer of Lucifer Yellow was explored most extensively. At early cleavage stages both the frequency and the extent to which Lucifer moved from cell to cell was determined by the position of the injected cell with respect to the future dorsoventral axis of the embryo. Fig. 12 shows the major movements of Lucifer at the 16- (A), 32- (B) and 64- (C) cell stages, based on the radial segments formed at cleavage to the 16-cell stage. The most striking feature is the gradual focussing of the pattern. At the 16-cell stage, dye transfer is most frequent and extensive in the region of the grey crescent, but dye can move between all segments. The symmetry found at the 32-cell stage has disappeared, and there is marked directionality in the pattern.

There was considerable variation in the frequency
Gap junctions in amphibian embryos

Fig. 11. Methylamine increases the frequency of dye transfer out of future ventral cell al at the 32-cell stage. Ordinates: percentage of embryos. Abscissae: clear bars give the percentage of embryos in which Lucifer was completely restricted to the injected cell. Solid bars give the percentage in which dye moved into the sister of the injected cell only. Hatched bars give the percentage showing transfer of Lucifer Yellow to one or more cells excluding the sister cell. Results based on 16 injections for controls and 17 injections for embryos treated with 15 mM-methylamine for 15 min. Note that in methylamine-treated embryos transfer through gap junctions is frequent compared with controls. The percentage of cells showing transfer to the sister cell only is slightly less in the methylamine-treated group.

Fig. 12. Diagrams illustrating the major movements of Lucifer Yellow after injections in the animal pole at the 16-cell stage (A), 32-cell stage (B), and 64-cell stage (C) derived from observations of the pattern of dye spread after each injection. In each case, the future ventral side of the animal pole lies at the top of the diagram, which is bisected by the primary cleavage axis. The diagram is drawn in terms of the radial segments formed at the 16-cell stage. Segments a–h are labelled clockwise, starting at the primary cleavage axis on the future ventral side. The numbers give the frequency with which dye movement in the direction of the arrows was observed. (A) (16-cell stage) shows that dye transfer is greatest at the future dorsal side, but shows no directionality. (B) (32-cell stage) shows that dye movement was never observed across the primary cleavage axis at the future ventral side; elsewhere dye transfer occurs reciprocally between each radial segment. (C) (64-cell stage) shows how the pattern focusses: dye transfer does not occur out of segments a, h, c, and e. Segments c and e receive dye only. Frequent transfer into neighbouring segments only occurs after injection into segment d, which donates to, but does not receive dye from, its neighbours.

with which Lucifer moved out of individual cells, which undoubtedly arises in part from the relatively small samples. Additionally, the position of maximal and minimal transfer of Lucifer may vary from embryo to embryo. The dorsoventral axis was defined by the pattern of pigmentation and cleavage, which does not give an invariable indication of polarity and the ultimate fate map (Dale & Slack, 1987; Moody, 1987). Failure of Lucifer transfer in dorsal regions could imply a major shift in the pattern of transfer relative to the perceived dorsoventral axis. Alternatively, the absence of Lucifer transfer in dorsal regions could indicate individuals where cell-to-cell communication was poor throughout the embryo. This might reflect poor viability or a disturbance of patterning.

The comparison between the ability of different probes to permeate the gap junction was most complete at the 32-cell stage. In contrast to Slack & Palmer (1969), who failed to see fluorescein transfer from cleavage stages to the blastula, fluorescein moved through gap junctions. But the incidence was low, although more substantial in dorsal than in ventral regions. Since random sampling with respect to position and a low average incidence together make the likelihood of failing to observe fluorescein transfer high, our findings and those of Slack & Palmer can be simply reconciled. The dicyanoargentate anion, which has a similar dimension to the chloride ion across its shortest axis, always moved through gap junctions. PbEDTA transferred with equivalent, relatively high frequency in both dorsal and ventral regions.

Taking all four probes together, for future ventral cell al, dicyanoargentate was the most permeant. PbEDTA transferred significantly more frequently than either Lucifer Yellow or fluorescein (PbEDTA versus Lucifer: \( P < 0.01 \); PbEDTA versus fluorescein: \( P < 0.03 \)), with Lucifer and fluorescein being poorly permeant and approximately equivalent (\( P > 0.2 \)). This gives a permeability series of dicyanoargentate > PbEDTA > Lucifer = Fluorescein. CPK models suggest that this can be explained on the basis of size, since dicyanoargentate is the smallest, followed by PbEDTA, the Lucifer Yellow, with fluorescein being the largest. Warner & Lawrence (1982) attributed the transfer of PbEDTA, but not Lucifer Yellow, between epidermal cells lying on either side of the segment border in Calliphora to the slightly smaller size of PbEDTA.
For future dorsal cell $d_{1}$, dicyanoargentate was again the most permeant. Lucifer and PbEDTA were relatively permeant and not significantly different ($P > 0.1$), but both probes were significantly more permeant than fluorescein ($P < 0.01$ for both). The permeability series for gap junctions between cells in the future dorsal region is not the same as for future ventral regions, being dicyanoargentate > Lucifer Yellow = PbEDTA > fluorescein. This cannot be explained simply on the basis of more gap junctions between dorsal cells or a higher proportion of open gap junctional channels in dorsal regions. Some difference in selectivity is required to account for the observations.

At the 64-cell stage, dicyanoargentate remained the most permeant, Lucifer Yellow displayed marked positional dependence in its permeability properties while PbEDTA was never seen to transfer. The permeability of PbEDTA must, therefore, fall between the 32- and 64-cell stages. Whether more subtle differences in the permeation of these probes through gap junctions exist at this stage remains to be resolved.

The weak base methylamine raised the intracellular pH by about 0.15 units and significantly increased the frequency with which ventral cells exchanged Lucifer Yellow. Gap junctions in the early *Xenopus* embryo are highly sensitive to alterations in cytoplasmic pH (see Turin & Warner, 1980; Bennett & Spray, 1985). Previous estimates of the pH–gap junctional conductance relation (Turin & Warner, 1980; Spray et al. 1981), made electrophysiologically, have suggested that the junctional channels are fully open by pH 7.6. The present results show that the permeability to both small ions and larger molecules can be significantly increased by a relatively small rise in intracellular pH.

The use of metal complexes to determine the permeability of gap junctions deserves comment. The major disadvantage is the processing required before the distribution can be assessed, which leaves open several steps at which the concentration of the metal sulphide can be reduced by bleaching or redissolution. The cumulative effect is to reduce the threshold for detection of transfer. It follows that observations of transfer are unlikely to be the consequence of artifact, although some underestimation of absolute junctional permeability to such molecules is probably inevitable. Nevertheless judicious choice of metal complex should generate probes of variable size and charge to complement the currently restricted set of fluorescent dyes. Dicyanoargentate may, because of its small size, prove to be most useful as a tracer for ionic coupling, allowing a permanent record of the movement of small ions in a population of electrically coupled cells.

We have no evidence to suggest that the early amphibian embryo contains reproducible, restricted communication domains analogous to the compartments found in insect epidermis (see Warner & Lawrence, 1982; Blennerhassett & Caveney, 1984). However, the correlation between the pattern of molecular transfer through gap junctions and the future dorsoventral axis could be related to patterning of the embryo. There is already evidence that important developmental events, related to embryonic patterning, occur between the 16-cell and early blastula stages. Pattern deficiencies induced by lithium treatment are more pronounced when lithium is applied before the 64-cell stage (Breckenridge et al. 1987). Furthermore, lithium treatment of the *Xenopus* embryo at the 32- to 64-cell stages rescues embryos from axis deficiency induced by previous u.v. irradiation (Kao et al. 1986). U.v.-induced axis deficiencies may be overcome also by transplantation of dorsal vegetal pole cells at the 32- and 64-cell stages (Gimlich & Gerhart, 1984; Gimlich, 1985). The sensitivity of gap junctions to alterations in intracellular pH increases as the embryo progresses from early cleavage to the 64-cell stage (Turin & Warner, 1980). Such correlations suggest that it would be worthwhile testing whether agents that interfere with patterning of the embryonic axis, such as u.v.-irradiation and lithium treatment, also disturb the pattern of dye transfer through gap junctions. Such experiments are now in progress (D. Nagajski, S. C. Guthrie, C. Ford and A. E. Warner, manuscript in preparation).

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