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The nature of intercellular coupling within the preimplantation mouse embryo

By H. GOODALL AND M. H. JOHNSON[1]
From the Department of Anatomy, University of Cambridge, U.K.

SUMMARY

The changing nature of intercellular coupling during the 4- and 8-cell stages of mouse early development has been investigated by iontophoretic injection of carboxyfluorescein, horseradish peroxidase and current into individual blastomeres in either the intact embryo or after their disaggregation and reaggregation into pairs. Coupling junctions that allowed only molecules of low molecular weight (putative gap junctions) were found not to appear until 2–5 h beyond the 3rd cleavage division (8-cell stage). However, intercellular junctions that were not size selective were detected in intact embryos only throughout the 4- and 8-cell stages. It is proposed that this junctional communication results from the persistence of midbodies through all or part of the two, and in a few cases the three, cell cycles following their formation at the first and second cleavage divisions. We conclude that the cells of the early mouse embryo may be linked in a more extensive syncytial network than was hitherto suspected.

INTRODUCTION

The signalling of relative position within a population of embryonic cells appears to be an important component of the mechanism by which cell diversity is generated (Wolpert, 1978). However, the nature of the developmental signals, and the route by which they are transmitted between cells, are not yet resolved. Gap junctions have been proposed as one possible route by which gradients of low molecular weight, hydrophilic 'informational' molecules might be established across two or more cells during development (Wolpert, 1978; Lo, 1980, 1982; Loewenstein, 1981). Indeed evidence has been presented recently to suggest that the gap junctions within a discrete developmental compartment are qualitatively distinct both from those within other compartments and from those at the boundaries between adjacent compartments (Warner & Lawrence, 1982; Lo, 1982; Weit & Lo, 1982; Schuetze & Goodenough, 1982). These observations have led to the specific suggestion that the nature of the gap junctional contacts might be one feature defining the limits of a developmental field.

Positional signalling and cell interaction are involved both in generating (Ziomek & Johnson, 1980) and in maintaining (Johnson & Ziomek, 1983) the divergent differentiation of cells within the early mouse morula. The first event

[1] *Author's address:* Department of Anatomy, Downing Street, Cambridge, CB2 3DY, U.K.

in the generation of this divergence, namely the cell-contact-mediated induction of polarity in 8-cell blastomeres, occurs during the early period of compaction when gap junctions are first detectable (Calarco & Brown, 1969; Ducibella, Albertini, Anderson & Biggers, 1975; Magnuson, Demsey & Stackpole, 1977; Lo & Gilula, 1979; Johnson & Ziomek, 1981*a*). This coincidence of events raised the possibility that the gap junctions might be involved in the induction process. We reported previously the use of a new technique to measure gap junctional coupling, in which a cell was loaded with the hydrophilic fluorophore carboxyfluorescein (CF) via its nonpolar diacetate derivative (CFDA), aggregated to a non-labelled cell and the transfer of dye monitored. We confirmed the early development of junctional channels during the 8-cell stage and timed their appearance precisely (Goodall & Johnson, 1982). However, we failed to detect any dye transfer when CFDA-labelled 2- or 4-cell blastomeres were aggregated to non-labelled 8-cell blastomeres, under conditions in which the former are known to induce polarity in the latter (Johnson & Ziomek, 1981*a*), a result confirmed by McLachlin, Caveney & Kidder (1983). This result led us to suggest that gap junctional coupling was not important in the induction of polarity.

The use of CFDA was novel and required culture of aggregates for 1–3 h to permit dye transfer to detectable levels. Moreover, using this technique a fixed quantity of dye is loaded initially and cannot be supplemented during the transfer period as can iontophoresed CF. We have therefore applied the more conventional measures of electrical coupling and transfer of iontophoretically injected dye to determine whether or not the results obtained using CFDA could be confirmed and to compare the relative sensitivities of the various techniques. In the process, we have discovered that coupling between early embryonic cells can occur not only via gap junctions but also by the persistence of midbodies for longer than was suspected hitherto.

MATERIALS AND METHODS

Embryos

HC-CFLP (Hacking & Churchill, Alconbury) and MF1 (Olac Ltd) female mice aged 3–5 weeks were superovulated with 5 i.u. pregnant mare's serum gonadotrophin (PMS: Folligon, Intervet) followed after 46–50 h by 5 i.u. human chorionic gonadotrophin (hCG: Chorulon, Intervet). The females were paired with HC-CFLP males. Those HC-CFLP females with copulation plugs were killed at 15, 45, 48 or 68 h post-hCG for recovery of 1-, 2-, 4- or compact 8-cell embryos. Unfertilized eggs were recovered from non-mated females at 12 h post hCG. Eggs and embryos were recovered in phosphate-buffered medium 1 plus 4 mg/ml bovine serum albumin (PB1 + BSA: Whittingham & Wales, 1969) and placed in culture in preequilibrated medium 16 plus 4 mg/ml BSA (M16 + BSA: Whittingham, 1971) at 37 °C and 5 % CO_2 in air. In all cases, removal of the zona

pellucida was effected by brief exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975).

Some 4-cell embryos were recovered at 57 h post hCG, placed in culture in M16 + BSA and observed at 1 h intervals for evidence of division. Any that had achieved five or more cells within 1 h were removed and designated 0 h morulae. These were then cultured for varying periods of time prior to use. Any 4-cell embryos that remained after 20–30 % of their population had commenced the third cleavage division were designated 'late 4-cell embryos'.

Newly formed 8-cell blastomeres were derived by disaggregation of 4-cell embryos to single cells (see below) which were then cultured in M16 + BSA and inspected every hour for evidence of division to form a pair of 8-cell blastomeres. These pairs were then disaggregated to yield two blastomeres designated 0 h, 8-cell blastomeres.

Aggregation of blastomeres

Zona-free embryos were disaggregated to single cells by a 5–20 min incubation in Ca^{2+}-free M16 + 6 mg/ml BSA (Pratt *et al.* 1982) according to techniques described previously (Johnson & Ziomek, 1981*a*). Blastomeres of each stage were treated briefly in a 1/20 dilution of phytohaemagglutinin (PHA: Gibco Biocult) in M16 + BSA prior to aggregation in pairs as indicated in the Results section. Aggregated pairs were then cultured in individual drops of M16 + BSA.

Preparation of couplets and embryos for microinjection

Deep grooves were made with a hot wire on opposite sides of plastic Petri dishes (35 mm Falcon) which were then stored at 37 °C. Five min before use a shallow drop of concanavalin A (Con A: Miles-Yeda Ind, USA: three times crystallized, Lyoph: 100 μg/ml of PB1) was placed in the centre of the dish and left for 5 min at 37 °C. The dish was rinsed three times with phosphate-buffered saline and then filled with PB1 + 4 mg/ml polyvinylpyrrolidone (BDH) (PB1 + PVP). Groups of 6–12 couplets of zona-free embryos were then pipetted in rows of three at the centre of the dish, where they adhered immediately to the Con A-coated surface. The PB1 + PVP was then replaced with medium M2 (derived by replacing most of the bicarbonate of M16 with HEPES: Fulton & Whittingham, 1978) containing 4 mg/ml BSA (M2+BSA), and the dish placed on the heated stage of a Leitz Ortholux Microscope.

For the studies with zona-intact embryos, grooved Petri dishes were coated with poly-L-lysine (Sigma, U.K.), rinsed and treated with Con A as described elsewhere (Goodall, 1983). The Con A was replaced by several rinses of protein-free M2 medium and embryos in their zonas were pipetted onto the dish where upon they adhered rapidly. The medium was then replaced by M2 + BSA.

Measurement of electrical coupling

Hyperpolarising current pulses of 2nA amplitude were applied for 0·5 s every

second via a glass electrode constructed from 1·5 mm 'Kwik-Fil' tubing (Clarke Electromedical Instruments, Pangbourne, U.K.) and pulled to a resistance of 60–80 Megohms when filled with 0·2 M-KCl. The pulse was applied using a Digitimer Neurolog System (Digitimer Ltd, Welwyn Garden City, U.K.) consisting of pulse generator, delay-width and two amplifier modules, the second amplifier being used in conjunction with a similar electrode to record the potential excursion within a coupled cell. The injection and recording circuits were completed with an agar 0·2 M-KCl bridge. The pre-amplifiers holding the electrodes were mounted on Leitz micromanipulators. For dye injection, the electrode tip was backfilled with a solution of 1 mM-carboxyfluorescein (Eastman) that had been dissolved in 0·2 M-KCl at a pH of 10 and then reduced to 7·4 (Socolar & Loewenstein, 1979). The shaft of the electrode was filled with 0·2 M-KCl. Three traces comprising injected current, signal from the injected cell and that from the adjoined cell were displayed on a Tektronix 5111 storage oscilloscope (Tektronix U.K. Ltd, Harpenden, England) and photographed with a Tektronix C5C camera on polaroid 667 film.

Microelectrode entry was induced either by use of the capacitance compensation control or by gentle mechanical vibration. A maintained, stable resting potential of at least −10 mV recorded by each electrode was taken as evidence of entry. It was observed that in some cases impalement of the second cell was accompanied by a small fall in the resting potential of the first cell, and a characteristic brief undulation (max. 5 mV) was observed in both traces when the capacitance compensation control was used to facilitate entry. Invariably where this effect was noted, the cells were found subsequently to be coupled electrically.

The resting potential was monitored constantly to guard against sudden tip blockage since the system measured the injected current with an output monitor. Any microelectrode blockage was observable as an unstable or a greatly offset reading and was confirmed by repeated measurement of the electrode resistance; in such cases the microelectrode and preparation were discarded. Tip potentials were between 0 and −10 mV.

In our system bridge balance cannot be achieved whilst recording from a second electrode, since the bridge balance facility operates by applying a cancellation signal through the ground connection and the second electrode perceives the 'ground' signal. Therefore, the bridge balance control was set to zero. Thus the trace from the injecting electrode (V_1) contains components consisting of the membrane potential excursion and a signal proportional to the membrane input resistance. Measurements of cell coupling ratios, therefore, were not attempted. To ensure that the true potential excursion in the injected cell was not excessive, the input resistance and hence the potential excursion, was estimated for a population of 8-cell blastomeres by dual impalement of single cells.

In addition, the possibility that coupling junctions might be opened artificially by the magnitude of the injected current was investigated by examining couplets of reaggregated blastomeres taken from compact 8-cell embryos 1–3 h after

aggregation. It was found that when current injected into one cell was increased stepwise from zero to 10 nA, the perturbation of potential in a second coupled cell rose approximately linearly until around 5 nA and then plateaued. A stepwise decrease of current from this value resulted in a similar linear reduction in the perturbation of potential in the second cell. Thus, the low resistance junctions (presumed to be gap junctions) appear to be current limiting and furthermore where no coupling was detected at 2 nA, the injection current was increased to 10 nA. In no case did this reveal any coupling. Thus, we may conclude that coupling junctions are not opened artificially by the injected current of the magnitudes used here.

Injection of horseradish peroxidase (HRP)

The procedure for injection of HRP was adapted from those of Lo & Gilula (1979) and Balakier & Pedersen (1982). The single microelectrodes used in this study were pulled from 1·5 mm 'Kwik-Fil' tubing (Clarke Electromedical, Pangbourne, U.K.) to a resistance of 60–80 Megohms when filled with a solution of HRP (Type VI, Sigma, U.K.) at a concentration of 5 mg/ml in 0·2 M-KC1. Zona-enclosed embryos were mounted as described above and injections were performed at 25 °C. Microelectrode entry was achieved either by use of the capacitance compensation control to induce tip oscillation or by use of gentle mechanical vibration. Care in interpretation of the perceived resting potential was exercised, however, since frequently a small resting potential measured after tip oscillation was found to increase after an addition mechanical shock. Although electrical communication between cells was observed after use of either tip entry protocol, HRP ejection appeared to be successful only after the second step. It is possible that tip oscillation leaves the electrode tip just under the surface and physically obstructed in some way and that this may account for failures of injection. The injection system described in the previous section was employed and to guard against any possible susceptibility of midbodies to open in response to the injected current, a group of isolated 4-cell blastomeres were allowed to divide to yield two 8-cell blastomeres and each was impaled by an electrode. Current injected into one cell produced a potential excursion in the linked cell which increased linearly over the whole range of zero to 10 nA. In a few cases, the potential excursion did not increase after the magnitude of the injected current increased beyond approximately 8 nA. Hence it is concluded that, as with gap junctions, mid-body channels do not appear abruptly at high current densities. To ensure sufficient HRP injection 5 s pulses of 5 or 10 nA depolarizing current were used with whole embryos for a total of 2 min, the pulses being separated by intervals of 1 s. This procedure results in faster injection than that described by Lo & Gilula (1979) but the 1 s intervals during the injection period allowed monitoring of membrane potential and electrode integrity which was not possible by the continuous injection procedure of Balakier & Pedersen (1982). After sequential injection of 10–15 embryos over a period of approximately

30 min, the embryos were removed gently from the dish and placed in drops of M16 + BSA at 37 °C for 0 h, 2–3 h or > 3 h, after which they were fixed in a solution of 2·5 % glutaraldehyde in 0·1 M-phosphate buffer, at a pH of 7·2 for 30 min. The embryos were then washed twice in buffer alone.

Visualization of HRP

HRP was visualized as a brown/black reaction product by a modification of the method of Graham & Karnovsky (1966; J. Gearhart, personal communication). The fixed and washed embryos were rinsed in 0·05 M-Tris HC1, pH 7·4 for 5 min and then incubated for 30 min at room temperature in 0·05 M-Tris HC1 containing 1 mg/ml diaminobenzidine tetrahydrochloride (DAB: Sigma, U.K.) and 0·002 % fresh H_2O_2. Since DAB is a suspected carcinogen, great care was exercised in its use. This procedure is similar to that of Bałakier & Pedersen (1982) but differs in that fixation was performed before, rather than after, DAB treatment. Treatment of live embryos with DAB was found to result in a high incidence of peroxidase-positive control embryos (the product tending to occur on the embryo surface). In addition, it was not possible to dissolve DAB in the phosphate buffer of pH 5·5 used by Bałakier & Pedersen and so the more usual pH 7·4 was employed.

Blastomeres were scored for HRP activity by bright-field stereomicroscopy (Wild M5) at a magnification of ×100, the depth of staining tending to fall naturally into dark, light and very light categories. Light and very light staining was assumed to result from passage of HRP into tenuously connected cells. If the sole category scored was 'very light' the embryo was discarded since this would have been the result either of inefficient injection or lysis of the injected cell. Where possible, control embryos were placed in the microinjection dishes and were then subjected to DAB reaction. Also, some embryos were injected for 2 min into the subzonal space between the zona pellucida and the blastomere surfaces to control for ingestion of any HRP that may leak from the injected cell during intra-blastomere injection.

Photography

Fluorescence photographs, and the corresponding bright-field photographs, were taken on Tri-X film. Fluorescence was excited using a 2 watt Argon-ion laser (Spectra Physics, St Albans, U.K.) at a wavelength of 488 nm. Fluorescence from the specimen passed through a Zeiss no. 50 barrier filter. HRP photography was on Pan-F film.

RESULTS

1. *Resting potentials and input resistance*

The mean value for resting potentials in 8-cell blastomeres was $-27 \pm 7{\cdot}5$ mV (mean ± S.D. n = 40). This is in agreement with previously published values

(Persianov, Limantsev & Leonov, 1973; Biggers, Borland & Powers, 1977). The mean input resistance for a 1 nA injection current was measured as 22 ± 10 MΩ (mean ± S.D., n = 27, range = 10–45 MΩ). Such a low input resistance seems to be characteristic of the earliest stages in mouse development (Powers & Tupper, 1974). Thus a 2 nA injection current produced a mean hyperpolarization of 44 mV beyond the resting potential and in no case was the excursion likely to be more than 90 mV.

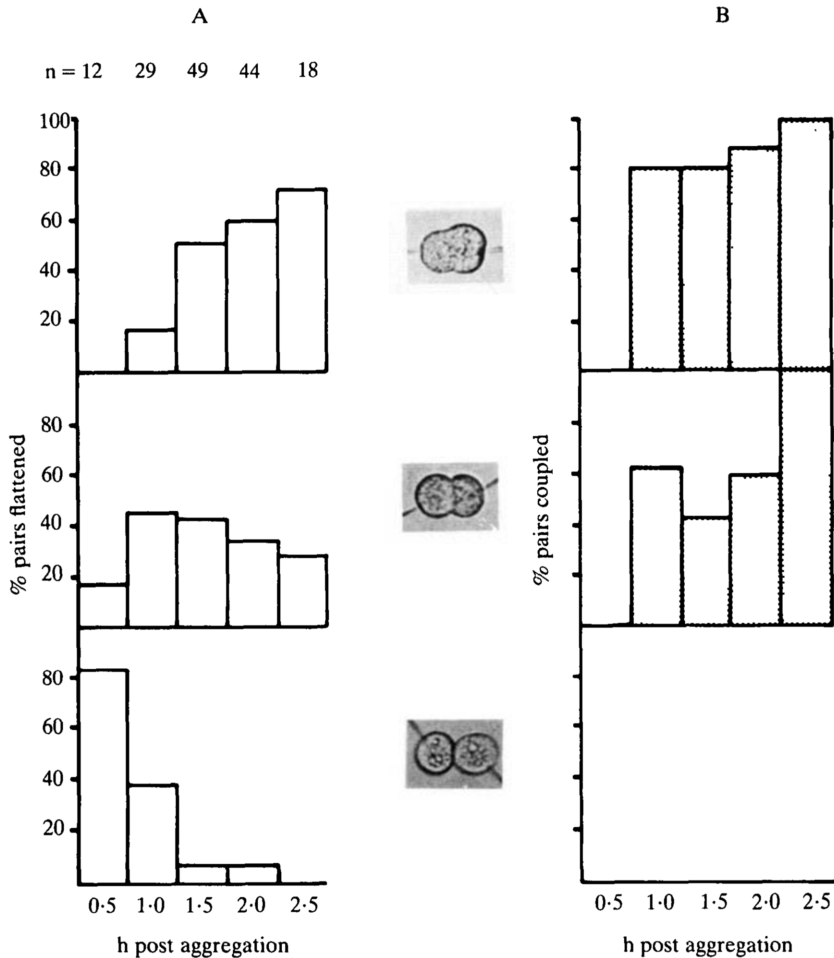

Fig. 1. The onset of electrical coupling in relation to cell flattening between reaggregated blastomeres taken from compacted 8-cell embryos. Flattening is defined as loss of clear cell outlines as cell:cell apposition occurs. (A) The reduction in the proportion of unflattened couplets (lower histogram) correlates with the initial increase and subsequent decrease in moderately flattened couplets (centre) and the steady increase in the proportion of fully flattened couplets (upper). (B) The lack of any electrical coupling within couplets in the non-flattened category compares with the development of complete coupling within the moderately and fully flattened categories over the 1–2 h period postaggregation.

2. *Coupling between cells taken from late 8-cell embryos*

Compact late 8-cell embryos are known to have developed extensive intercellular coupling (Lo & Gilula, 1979; McLachlin, Caveney & Kidder, 1983). We first examined the ability of cells from these embryos to reform such junctions after their isolation as single cells and their reaggregation as pairs. Pairs were cultured for between 0·5 and 2·5 h after aggregation. At intervals over this period, a total of 153 pairs were examined for the degree of cell flattening (Fig. 1A) and for electrical coupling (Fig. 1B). The results were pooled from five separate experiments and reveal rapid flattening of the cells to form a compact mass (Fig. 1A). No electrical coupling was detected prior to any detectable flattening (Fig. 2B), regardless of how long the pairs had been incubated as aggregates. The onset of coupling occurred with the first evidence of flattening and did not require the completion of this process. The data are replotted in Fig. 2, grouped in half hour cohorts for comparison of the percentage of electrically coupled pairs at increasing times after aggregation with the equivalent results gained in experiments using CFDA (Goodall & Johnson, 1982). Coupling between aggregated pairs was also examined simultaneously with electrical recording by iontophoresis of CF. Whilst all aggregates were coupled electrically within $2\frac{1}{2}$ h, variable degrees of dye passage were seen during this time ranging from no detectable passage to full equilibration after 2 min of injection (Fig. 3).

3. *Coupling between newly formed 8-cell blastomeres*

Newly formed 8-cell embryos are known to lack intercellular coupling via gap

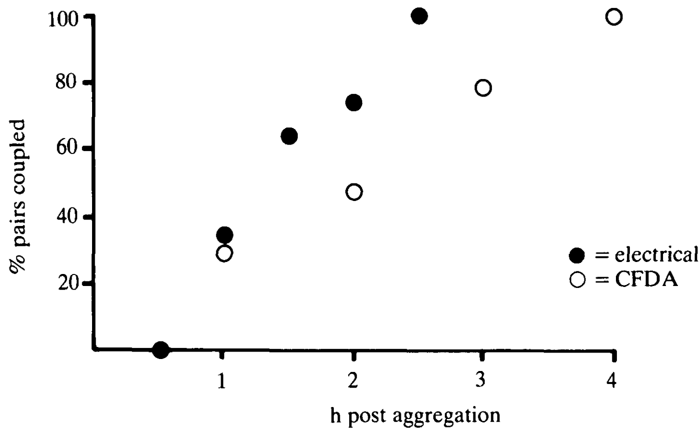

Fig. 2. The onset of cell coupling as measured electrically or by passage of CFDA-loaded CF within reaggregated couplets made with blastomeres taken from compacted 8-cell embryos. The more sensitive index of coupling by electrical continuity shows a steadily increasing proportion of coupled pairs (grouped into $\frac{1}{2}$ h cohorts) until all are coupled within $2\frac{1}{2}$ h. CF passage shows a similar but slightly more prolonged trend over a period of 4 h. CFDA data taken from Goodall & Johnson (1982).

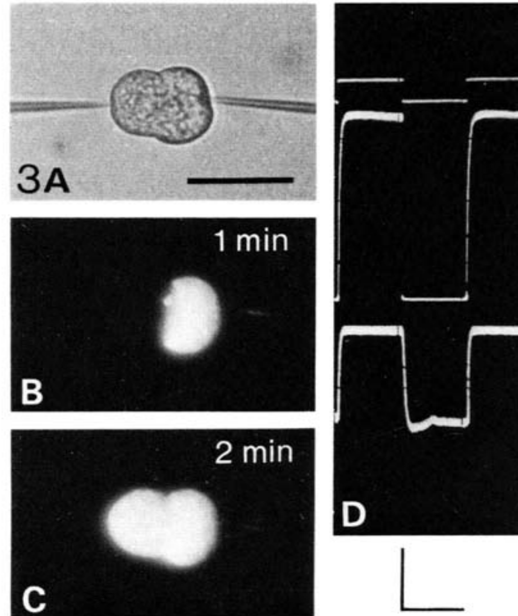

Fig. 3. Electrical coupling and spread of injected CF between reaggregated blastomeres taken from compacted 8-cell embryos. A fully flattened couplet (A) examined 1 h after aggregation showed no CF passage after 1 min of injection (B) but compete passage after 2 min (C). Electrical continuity (D) between the two cells was observed immediately after impalement. In (D) upper trace = injected current; centre trace = signal from injected cell; lower trace = signal from coupled cell. The centre trace (V_I) is included only as evidence of a stable resting potential, the lower edge of its potential excursion was limited by the offset control and is of no quantitative significance. The scale bar in the photographs is 50 μm and in the oscilloscope traces is 0·5 s (horizontal) and 0·5 nA or 20 mV (vertical).

Table 1. *Incidence of cell flattening, ionic coupling and carboxyfluorescein coupling in newly formed 8-cell blastomeres aggregated together in pairs and cultured for up to 7 h*

Time post division (h)	No. of embryos in which intercellular flattening is: zero	intermediate	complete	Proportion showing electrical coupling (%)	Proportion showing dye passage after 10 min of injection (%)
0-1	20	6	0	0/26 (0)	0/26 (0)
1-2	12	13	0	0/25 (0)	0/25 (0)
2-3	11	41	1	13/53 (25)	0/9 (0)
3-4	12	35	24	46/71 (65)	2/14 (14)
4-5	1	26	20	39/47 (83)	3/11 (27)
5-6	1	6	11	16/18 (89)	14/18 (78)
6-7	0	5	14	18/19 (95)	18/19 (95)
>7	0	5	11	16/16 (100)	16/16 (100)

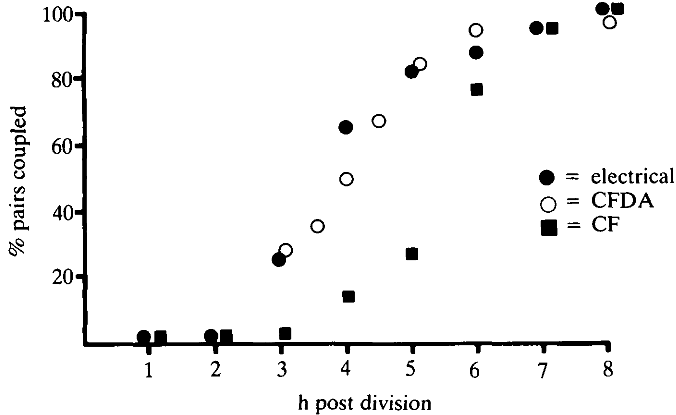

Fig. 4. The incidence of cell coupling with time between 8-cell blastomeres reaggregated immediately after division from 4-cell blastomeres, the coupling being measured electrically, after loading with CFDA (data from Goodall & Johnson, 1982) or after iontophoretic injection of CF.

junctions (Lo & Gilula, 1979). We next examined the time course of development of junctions during the 4th cell cycle by aggregating newly formed 8-cell blastomeres in pairs, culturing them for between 1 and 8 h and then examining them by the approaches validated in section 1.

The results pooled from six such experiments are summarized in Table 1, and compared in Fig. 4 with equivalent data obtained using CFDA (from Goodall & Johnson, 1982). Electrical coupling developed over the period of 2 to 5 h post division, whereas spread of iontophoresed CF into the second blastomere was not detectable prior to 3–4 h, and was only weakly visible up to 5 h post division, even in pairs that were in ionic continuity (Fig. 5A–D). Only 5 h after division was significant or full transfer of dye evident (Fig. 5E,F) comparable to that observed between blastomeres taken from compact embryos (Fig. 3). These results confirm that establishment of junctional communication occurs over the period of induction of polarity, and suggest that use of CFDA is as sensitive as

Fig. 5. The development of communication channels between 8-cell blastomeres reaggregated immediately after division from 4-cell blastomeres and cultured for varying periods of time. (A) Non flattened couplets 25 min after division and reaggregation showed no dye passage between blastomeres (B) after 10 min injection and no electrical coupling (C). 6 h 11 min after division the couplet in (D) showed only faintly observable dye passage (E) after 10 min but electrical continuity (F) was present. 7 h 16 min after division the couplet in (G) showed complete passage of dye (H) after 5 min injection and electrical continuity (I) was present. Electrical traces described in Fig. 3. Note that in (F) the lower limit of excursion in the centre trace (V_1) was limited to avoid collision with the lower trace (V_2). In (I), both were limited. Scale bar 50 μm in photographs. For the oscilloscope traces the horizontal bar is 0·5 s and the central bar is 50 mV (in C) and 20 mV in (F) and (I).

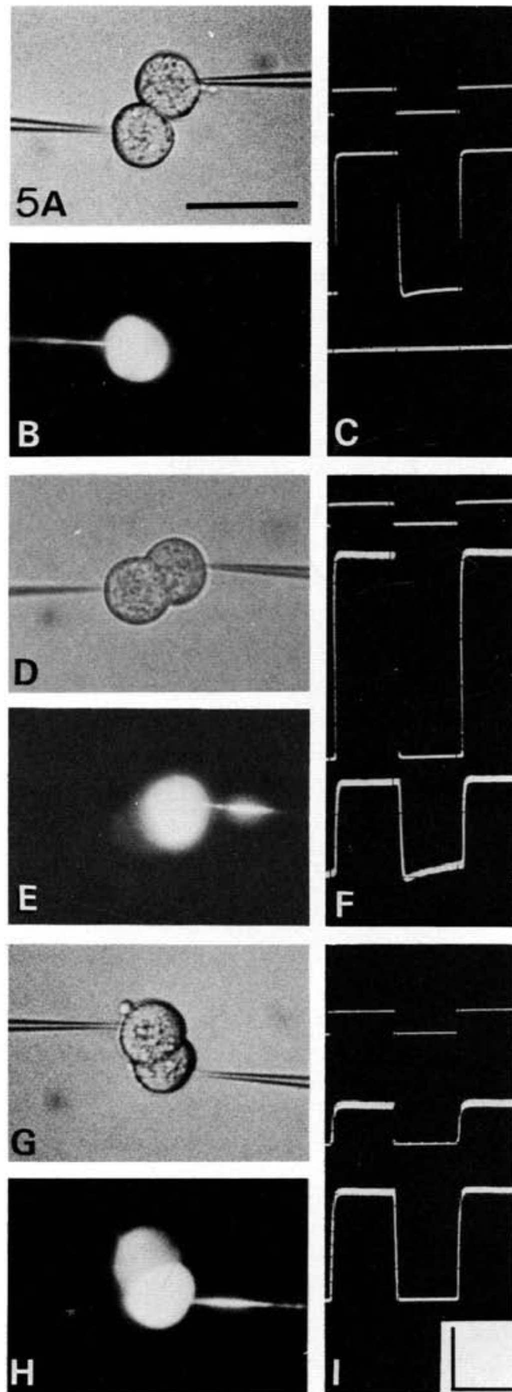

Fig. 5

electrical coupling in this application and both are more sensitive than short term observation of injected CF.

4. *Coupling between heterologous pairs of blastomeres*

Since 2- and 4-cell blastomeres can induce polarity in 8-cell blastomeres (Johnson & Ziomek, 1981*a*), we next examined whether junctional coupling could be detected between heterologous pairs of blastomeres. Blastomeres were taken from 8-cell compact embryos and aggregated with unfertilized eggs (34 couplets), fertilized 1-cell zygotes (22 couplets), 2-cell blastomeres (30 couplets) or 4-cell blastomeres (30 couplets). No electrical or dye coupling was detected regardless of time after aggregation (Fig. 6).

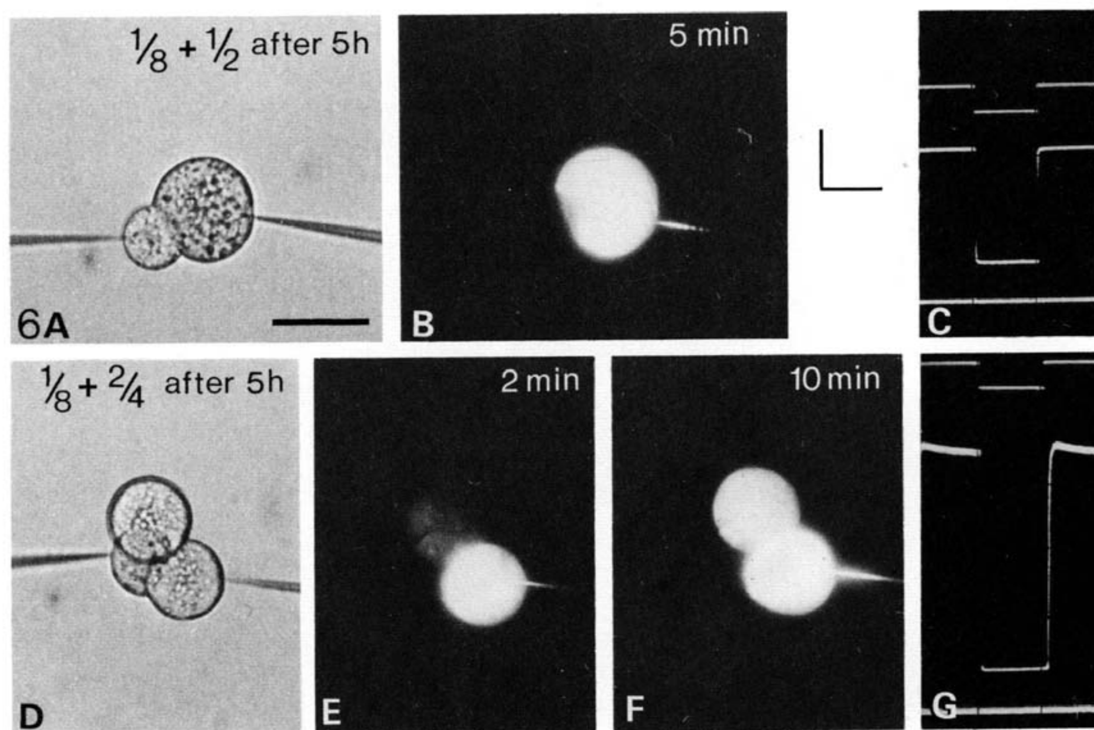

Fig. 6. The inability of 8-cell blastomeres to develop detectable communication channels with blastomeres of earlier stages. A 2-cell blastomere cultured in contact with an 8-cell blastomere for 5 h (A) showed some flattening, but no injected dye passed between them during the 10 min injection period (B), and no electrical continuity had developed (C). A pair of 4-cell blastomeres (D) also showed no detectable dye passage to the 8-cell blastomere over a 10 min injection period (E, F), although dye did pass between the sister 4-cell blastomeres presumably via the midbody connection (Lo & Gilula, 1979). No electrical continuity existed between 4- and 8-cell blastomeres (G). Scale bar is 50 μm in photographs. For the oscilloscope traces, horizontal bar is 0·5 s and vertical bar is 50 mV in (C) and 20 mV in (G). Note that in both (C) and (G) the V_1 trace is limited to avoid collision with the lower trace.

5. *Development of coupling within intact embryos*

The results described above confirm the conclusions of our previous study using CFDA, and suggest that gap junctions develop over a 5 h period after division to 8-cells. We next examined intact embryos culled over this period to determine whether or not a similar time course of junction formation applied. Since individual blastomeres within 4-cell embryos are developmentally asynchronous and therefore divide at different times, it is likely that this approach will provide less precision in timing.

In a preliminary experiment fifteen 8-cell embryos were scored. One 8-cell blastomere was impaled and the recording electrode was inserted serially into two other adjacent 8-cell blastomeres. It was considered necessary to record from more than one other blastomere, since after division the progeny of each 4-cell blastomere retain a tenuous cytoplasmic connection via the residuum of the cleavage furrow, the midbody (Lo & Gilula, 1979; Johnson & Ziomek, 1981*b*). Surprisingly, of the embryos examined in this way the proportion of impaled cells which showed electrical communication with *two* other cells was 1/1 aged 0 h, 4/7 aged 1 h, and 7/7 aged 2 h. In the three exceptional embryos aged 1 h, there was no coupling between the injected cell and either of the two impaled cells. All embryos examined, regardless of whether coupling was detected, showed no sign of intercellular flattening.

There seemed to be two explanations for this preliminary observation. Either electrical communication between blastomeres in intact embryos developed prior to any intercellular flattening and more rapidly than is the case between disaggregated and reaggregated newly formed 8-cell blastomeres, or the midbody that connected the two 4-cell sibling blastomeres, derived by division of one 2-cell blastomere, had persisted not only throughout the 4-cell stage but also during division to the 8-cell stage. We attempted to distinguish between these alternative explanations by undertaking three types of experiment.

(i) Late 4-cell embryos were recovered. One 4-cell blastomere was impaled and recordings made in turn from each of the three remaining 4-cell blastomeres. Of seventeen embryos examined in this way, sixteen showed electrical communication with one other blastomere only, and one with all other blastomeres. In addition, of twelve late 4-cell embryos injected with carboxyfluorescein, eleven showed transmission of the dye to one other blastomere and one showed no transmission to any other cells. This result is consistent with prolonged communication via the midbody throughout the 4-cell stage as has been suggested (Lo & Gilula, 1979). The finding that all four cells were coupled in one case could mean that the midbody from the first cleavage division had also persisted.

(ii) Embryos were recovered on detection of the earliest blastomere to divide to 8-cells, designated 0 h old, and then cultured for up to 8 h. One 8-cell (or, in some transitional embryos, 4-cell) blastomere was impaled for injection of current. The recording electrode was then placed in turn into as many of the

Table 2. *Extent of electrical coupling among cells of individual embryos examined by multiple impalement at different times after the initiation of the 4- to 8-cell transition. Those embryos marked * show communication among more than 4 × 1/8 blastomeres*

Age of embryos (h after first 1/4 divided)	No. of cells in embryo	Cell type injected	No. of cells recorded as being				Number of cells in continuity of those measured (expressed as 1/8, where 1/4 is taken as being equivalent to 2/8)
			in communication with injected cell		not in communication with injected cell		
			1/4	1/8	1/4	1/8	
0-1	5	1/4	–	2	1	–	4/6
			2	2	–	–	6/8*
	6	1/4	–	–	–	1	2/3
			–	–	1	3	2/7
			–	3	–	–	5/5*
	6	1/8	–	3	2	–	4/8
			–	3	1	–	4/6
	7	1/4	–	–	1	2	2/6
			–	–	–	1	2/3
	8	1/8	–	4	–	–	5/5*
			–	3	–	2	4/6
1-2	6	1/4	–	1	–	1	3/4
			1	–	–	3	4/7
			1	–	–	4	4/8
			–	1	–	–	3/3
	6	1/8	–	3	1	–	4/6
			–	1	–	2	2/4
	8	1/8	–	1	–	3	2/5
			–	1	–	2	2/4
			–	–	–	5	1/6
			–	7	–	–	8/8*
			–	1	–	4	2/6
2-3	6	1/4	1	–	–	3	4/7
			–	–	–	1	2/3
	8	1/8	–	4	–	1	5/6*
			–	1	–	3	2/5
			–	3	–	2	4/6
			–	2	–	3	3/6
			–	1	–	3	2/5
			–	2	–	5	3/8
3-4	6	1/4	1	–	–	4	4/8
			–	1	–	2	3/5
	6	1/8	–	3	2	–	4/8
	8	1/8	–	1	–	6	2/8
			–	2	–	–	3/3
			–	2	–	2	3/5
			–	2	–	1	3/4
			–	3	–	4	4/8
			–	2	–	2	3/5
			–	3	–	3	4/7
			–	2	–	2	3/5

Table 2. *cont.*

Age of embryos (h after first ¼ divided)	No. of cells in embryo	Cell type injected	No. of cells recorded as being: in communication with injected cell ¼	in communication with injected cell ⅛	not in communication with injected cell ¼	not in communication with injected cell ⅛	Number of cells in continuity of those measured (expressed as ⅛, where ¼ is taken as being equivalent to 2/8)
4-5	8	⅛	–	2	–	4	3/7
			–	2	–	4	3/7
			–	7	–	–	8/8*
			–	7	–	–	8/8*
			–	7	–	–	8/8*
			–	3	–	2	4/6
			–	2	–	4	3/7
			–	1	–	5	2/7
			–	3	–	3	4/7
			–	7	–	–	8/8*
5-6	8	⅛	–	3	–	3	4/7
			–	5	–	–	6/6*
			–	6	–	–	7/7*
			–	4	–	1	5/6*
			–	7	–	–	8/8*
			–	5	–	–	6/6*
			–	5	–	–	6/6*
			–	4	–	–	5/5*
			–	6	–	–	7/7*
			–	3	–	2	4/6
			–	3	–	4	4/8
6	8	⅛	–	7	–	–	8/8*
			–	6	–	–	7/7*
			–	–	–	3	1/4
			–	4	–	–	5/5*
			–	7	–	–	8/8*
			–	7	–	–	8/8*
			–	7	–	–	8/8*
			–	5	–	–	6/6*
			–	7	–	–	8/8*
			–	3	–	2	4/6

remaining blastomeres as possible and in each case the size of the group of electrically connected cells was estimated. The results of five such experiments are summarized in Table 2. It is clear that in many cases of transitional or early 8-cell embryos (up to 4 h), there is extensive electrical coupling within the embryo, in most cases between two, three or four blastomeres. In a few embryos aged up to 4 h (asterisked), the communicating cluster was larger than four cells. This result could reflect either the early formation of gap junctions or the persistence of the midbody from the first cleavage division, in addition to that from the second. Persistence of the midbody from the second cleavage division at least is suggested by the observation of continuity between 4-cell blastomeres and 8-cell

Table 3. *Extent of spread of carboxyfluorescein over a 10 min. period among cells of embryos at various times after the initiation of the transition from the 4- to the 8-cell stage*

Age of embryo (h after division of the first ¼ cell)	No. of cells labelled fluorescently in individual embryos	Nos. of embryos classified as		
		precompact	semicompact	compact
0-2	2,2,2,2,2,2,2,2,4	9	0	0
2-4	2,2,2,2,3,2,8, 4,2,2,2,2,2,4	14	0	0
4-6	2,8,4,8,4,4,2,4, 2,4,4,4,5,2,2,8	13	3	0
6-8	4,4,2,2,4,8,4,2, 3,4,4,4,4,4,8,4	6	6	4
>8	2,2,8,2,8,8,8, 8,8,8,8,8,8,8	2	5	7

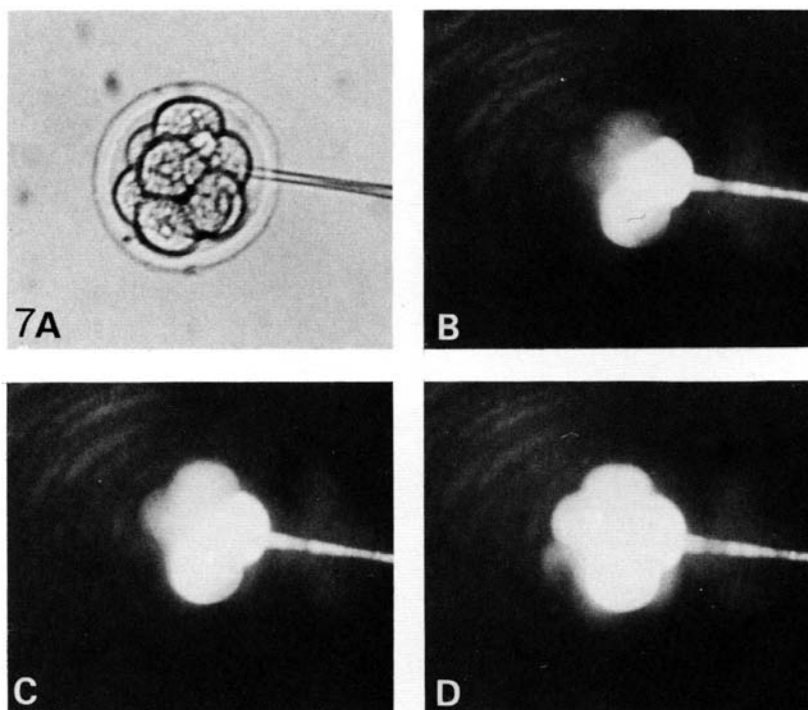

Fig. 7. The spread of injected CF through a mid 8-cell embryo (A) 4 h 16 min after division of the first blastomere from the 4-cell stage. Dye injected into one blastomere spread within 3 min to an adjacent cell (B) presumably via a mid-body connection (Lo & Gilula, 1979), and thence into two other cells by 5 min (C). Equilibration of dye between these four cells was followed within 10 min by spread to the remaining cells (D).

blastomeres. The incidence of embryos in which more than four blastomeres are interconnected becomes greatly elevated after 4 h, a time at which our couplet data would suggest corresponds to the period of gap junction formation.

A smaller number of observations was made on intact embryos using injection of CF (Table 3 & Fig. 7). These data, in contrast to the results with multiple impalement, indicate that initially (up to 4 h) only two cells are coupled in most embryos, that between 4 h and 8 h four cells are coupled in most embryos and only after 8 h is more extensive coupling encountered. CF, like ionic current, can pass through both gap junctions and midbodies but its passage is a much less-sensitive indicator of coupling channels than is electrical coupling. The early coupling of only two cells could therefore represent passage via the residual midbody of the third cleavage division, that remaining from the second cleavage division being of too low a capacity for detectable transfer to occur. The coupling of four cells over 4 h to 8 h could represent the appearance of gap junctions among one quartet of blastomeres and full coupling between both quartets (as detectable by CF) may not be evident till 8 h or more. Indeed, in Fig. 7 it can be seen that first one quartet and then the second quartet label in sequence.

(iii) A third way of discriminating between the existence of persistent midbodies and gap junctions is to inject horseradish peroxidase (HRP) into one cell. This molecule will not pass through the gap junctions but can pass through midbodies (Lo & Gilula, 1979; Bałakier & Pedersen, 1982). Therefore, we injected HRP into one blastomere of zona-intact embryos at the 4-cell and 8-cell stages and analysed the distribution of the enzyme, either immediately or after a period of further incubation to allow equilibration of the HRP by available midbody routes. The distribution of the HRP injected into cells of experimental embryos is summarized in Tables 4 & 5 (Fig. 8). Of 24 embryos that had four to seven cells at the time of injection (Table 4) and were cultured for 2 h prior to

Table 4. *Extent of HRP distribution within embryos after injection into one blastomere and culture of embryos for 2 h before fixation and visualisation with DAB*

No. of embryos	No. of cells at injection	Injected cell type	No. of cells at fixation	Distribution of HRP + ve cells	
				¼	⅛
14*	4	¼	4	2	0
2	4	¼	4	1	0
1*	4	¼	4	2	0
1	4	¼	6	0	2
1*	4	¼	8	0	4
1*	5	⅛	6	1	2
1	6	¼	8	0	2
1*	6	⅛	8	0	4
2	7	⅛	8	0	2

* indicates embryos in which cells constituting half the total embryo are HRP positive.

Table 5. *Distribution of HRP among blastomeres of 8-cell embryos after injection into one blastomere*

Current magnitude nA	Age of embryo (h after division of first 1/4 cell)	Time of analysis (h after injection)					
		0–1 h Proportion of embryos (%) in which number of HRP +ve cells was		2–3 h Proportion of embryos (%) in which number of HRP +ve cells was		3 h Proportion of embryos (%) in which number of HRP +ve cells was	
		>1	>2	>1	>2	>1	>2
5	5–10	N.D.	N.D.	14/17 (82)	6/17 (35)	N.D.	N.D.
10	0–4	19/20 (95)	2/20 (10)	38/39 (97)	13/39 (33)	25/26 (96)	9/26 (35)
	5–7	N.D.	N.D.	27/30 (90)	12/30 (40)*	N.D.	N.D.
	>8	N.D.	N.D.	26/31 (84)	8/31 (26)	N.D.	N.D.

* In 5 out of the 50 positive embryos examined in this category, 5, 6 or 8 cells were HRP +ve. In the remainder only 3 or 4 were HRP +ve.

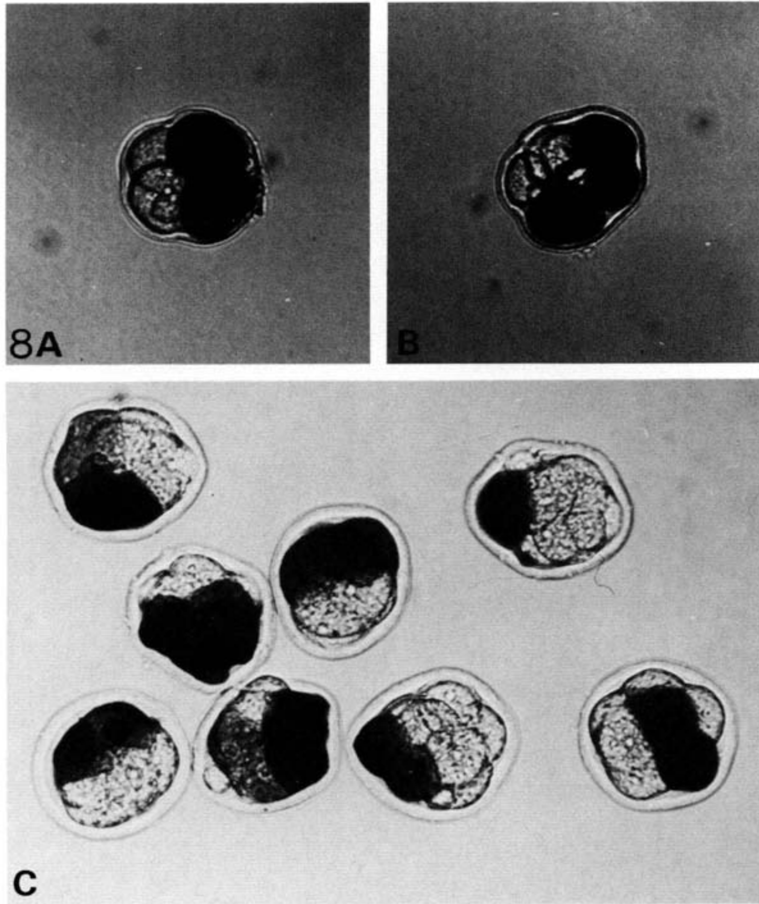

Fig. 8. Horseradish peroxidase was injected into a single blastomere of (A) a 4-cell embryo, and (B) a 1/4 blastomere of a 6-cell embryo, and (C) one blastomere of an 8-cell embryo. The embryos were then cultured for 3 h (A, C) or 0 h (B) prior to visualization of the enzyme. In (A), HRP is seen to have passed between the injected cell and one other cell. In (B) the 1/4 cell is shown in continuity with the 2/8 cells. In (C) HRP is localised to one, two or four cells showing evidence of persistent midbodies from the 2nd and 3rd cleavage divisions.

fixation, 18 showed clear evidence of transmission of the HRP through half of the embryo. Of particular note are those embryos in which a 1/4 blastomere was in communication with 2/8 blastomeres. Of embryos having 8-cells at the time of injection, 135/146 (93 %) showed communication with one other blastomere (Table 5), regardless of the time of analysis, indicating free and immediate passage of HRP through the midbody remaining from the 3rd cleavage division. HRP transmission through more than two blastomeres occurred in only 2/20 (10 %) of newly formed embryos analysed immediately after injection but in 33–35 % when several hours elapsed between injection and fixation, suggesting that with time, detectable levels of HRP could also pass through the midbody

remaining from the 2nd cleavage division. The observation that the third midbody is more permissive to transport than the second midbody is consistent with the observations on CF transfer reported in (ii) above.

DISCUSSION

Studies on reaggregated pairs

The measurements of electrical coupling between pairs of reaggregated cells from the early mouse embryo supports the interpretation drawn from our results obtained previously with the new technique involving the use of CFDA (Goodall & Johnson, 1982). Thus, the pattern of development of communicating channels after division from the 4-cell stage, the pattern of reestablishment of channels after reaggregation of cells taken from compact 8-cell embryos and the failure to detect channels in heterologous pairings all confirm our earlier observations. Moreover, the two techniques seem to be of similar sensitivity (see Fig. 4), both being more sensitive than that of acutely injected CF, as may also be concluded from the results of earlier work (Loewenstein, 1981).

Junctional channels first form over the period 2 to 5 h post division, as assessed from our studies on pairs of blastomeres. This range of time may reflect a real heterogeneity amongst the couplets of cells that has also been reported with respect to similar studies on cell flattening (Lehtonen, 1980; Ziomek & Johnson, 1980), induction of a stable axis of polarity (Johnson & Ziomek, 1981*a*) or development of a polarized phenotype (Ziomek & Johnson, 1980). The last feature has been shown to depend on cell size, a smaller cell polarizing earlier than a larger cell (Ziomek & Johnson, 1980). However, it is also probable that some additional heterogeneity is introduced experimentally, since populations of newly-formed 8-cell blastomeres are recovered from hourly culling of 4-cell blastomere cultures. This means that each population of newly formed 8-cell blastomeres will have a 0–1 h age range. Additionally, the manipulations involved in disaggregation and reaggregation are likely to introduce delays in development that may vary slightly for different cells.

The development of electrical continuity correlates in time with the close apposition of cell membranes that occurs as the cells flatten together and with the induction of polarity. In addition to their temporal correlation, all three events are insensitive to α-amanitin applied during the 4- and 8-cell stages and thus all appear to show an independence of proximate transcriptional activity (McLachlin *et al.* 1983; M. H. Johnson & H. P. M. Pratt, unpublished data). However, it is not clear whether the three are linked causally. Thus, although extensive contact undoubtedly facilitates the establishment of functional gap junctional channels, it is found not to be an absolute requirement (Goodall, 1984). Moreover, our results provide further evidence against the idea that development of gap junctions is involved in inducing polarity. Thus, 2- and 4-cell blastomeres are able to induce polarity in 8-cell blastomeres (Johnson & Ziomek, 1981*a*) yet are

unable to develop electrical coupling with them. Since polarity also develops under conditions in which gap junction development is delayed or prevented (Pratt, Ziomek, Reeve & Johnson, 1982; Goodall, 1984), a developmental role for the junctions, if one exists, must lie in the subsequent interactions that characterise either the continuing divergence of the lineages or their ultimate commitment (Johnson & Ziomek, 1983).

Studies on intact embryos

In order to determine whether the development of junctional coupling in reaggregated pairs of cells provides a real comparison for the situation in the intact embryo, we examined newly formed 8-cell embryos for the earliest signs of coupling. Our results were unexpected. We detected extensive electrical coupling between impaled 4-cell blastomeres and other 4- and 8-cell blastomeres. Moreover, the coupling among newly formed 8-cell blastomeres was also extensive. Electrical measurement of coupling is sensitive. It may detect coupling mediated by small numbers of channels regardless of size selectivity, or selective for low molecular weight molecules regardless of channel number. To discriminate between these alternatives we used a number of approaches.

Electrical coupling within 8-cell embryos aged 0 to 4 h old is limited in most cases examined to four cells or less (5 exceptions out of 41 embryos or 12 %, Table 2). Embryos that were 4–5 h old or greater than 5 h old showed more extensive coupling among 5–8 cells (40 % and 76 % respectively). However, most embryos aged upto 4 h showed coupling between only two blastomeres (78 % of embryos) as judged by injection of CF; only by 4–8 h were 81 % of embryos communicating via more than two cells but in most cases (67 % of total) the coupling was among three or four cells only. Beyond, 8 h, most embryos (80 %) showed dye communication among all eight cells. We know (see earlier) that CF is a less sensitive indicator of coupling than that provided by electrical measurements. The simplest interpretation of these observations therefore is as follows. In all embryos midbodies from the third cleavage division persist into the 8-cell stage and account for early coupling between pairs of blastomeres as revealed by CF. In addition, in most embryos midbodies from the second cleavage division also persist. These are readily detected by electrical techniques but more rarely (22 % of cases only) by CF injection. We conclude that these persistent midbodies have become in some way more constricted or obstructed and have a lower transmission capability. Very few embryos (12 % by electrical detection, 4 % as detected by CF) may also retain the midbody from the first cleavage division. Superimposed upon this extended midbody continuity is the development of gap junctions. Our electrical measurements would place this event at around 4–5 h after division of the first ¼ blastomere, and our CF injection experiments would place it around 4–6 h after such a division. Both times are in reasonable agreement with the 2–5 h estimated from our use of reaggregated couplets.

If this interpretation is correct, we should be able to test it by use of HRP which will pass through midbodies but not through gap junctions. Data in Tables 4 and 5 show clearly that extended continuity between 1/4 and 2/8 blastomeres and among quartets of 1/8 blastomeres can be demonstrated, if 2–3 h is allowed for passage of the enzyme among cells. Complete equilibration among all four blastomeres in all embryos might not be expected if indeed we are correct in interpreting the CF data to show the midbody from the second cleavage division to be of lower transmission capability.

It is possible that previous workers failed to detect extended survival of midbodies by not giving sufficient time for equilibration after injection of HRP at the 8-cell stage (Lo & Gilula, 1979; Bałakier & Pedersen, 1982). The latter authors do however report continuity among more than two blastomeres in a subpopulation of 16-cell embryos, suggesting that the phenomenon may not be restricted to the 4- and 8-cell stages. In addition J. Gearhart (personal communication) has detected alterations in the effective continuity between 2-cell blastomeres via the midbody from the first cleavage division after experimental manipulation of the embryo.

Implications

Our results have a number of implications for studies on early embryos. First, our results here together with those reported previously (Goodall & Johnson, 1982; Goodall, 1984; McLachlin *et al.* 1983) render any critical *causal* role for gap junctions in inducing polarity highly improbable; whether they are a consequence of polarity development remains to be established.

Second, if most embryos are composed of two clusters of syncytially-linked blastomeres, then passage of information among them could lead to synchronisation of cells within each group. We note in this paper that gap junctions, as assessed by CF passage, appear first in one quartet. In addition, one quartet often appears to flatten, and its cells to polarize, ahead of the other quartet (unpublished observations). Graham and his colleagues have indeed shown that the progeny of each cell at the 2-cell stage tend to develop more synchronously (Kelly, Mulnard & Graham, 1978). It will be of interest to determine whether this synchrony is dependent upon continuing cytoplasmic continuity or is inherent to the constituent cells. However, syncytial linkage cannot be a requirement for a cell to contribute successfully to either tissue of the blastocyst, as the many studies using reaggregated blastomeres indicate. Whether the balance of contribution to each tissue might be influenced is not, however, clear.

Finally, HRP has been used as a lineage marker in early mouse development with rather contradictory results. Use of iontophoresed HRP suggested that extensive mixing across putative cell lineages within the embryo occurred (Bałakier & Pedersen, 1982) whereas use of pressure injected HRP confirmed the proposed lineages (Gearheart, Shaffer, Mussen & Oster-Granite, 1982). At least part of this contradiction may be explained by our observations here. We

find that HRP will label two blastomeres in most cases and four blastomeres in many cases when injected iontophoretically. Such a labelling pattern could result in an appearance of lineage-crossing as concluded by Balakier & Pedersen (1982) from their own observations.

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