

The timing of compaction: control of a major developmental transition in mouse early embryogenesis

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SUMMARY

The effect of protein synthesis inhibitors on compaction of the 8-cell mouse embryo has been investigated. The effects observed depended upon the duration and time of drug application and on the features of compaction scored. Continuous application from the late 2-cell or early 4-cell stages allowed cell flattening and surface polarization to occur in most embryos and advanced development of these features in many of them. Cell coupling developed only when drug addition was delayed until the mid 4-cell stage, and cytoplasmic polarization developed only when drug addition was delayed until the late 4-cell stage. We suggest that control over the timing of compaction is achieved at a post-translational level *via* a global permissive change within the blastomeres of the embryo.

INTRODUCTION

It is clear that a committed and differentiated cell expresses, or has available for expression, characteristic subsets of genes that yield the distinctive phenotype of the cell. However, it is not clear whether the selective expression of this discrete genetic subset initiates the processes of commitment and differentiation or is part of the outcome of these events (discussed by Johnson, McConnell & Van Blerkon_L, 1984). We are interested in the process whereby two committed and differentiated cell lineages (the inner cell mass, or ICM, and the trophectoderm) arise in the mouse blastocyst. There is now considerable evidence to suggest that a crucial event in this process is the reordering of cell organization and interaction that occurs at the 8-cell stage. This process is called compaction and involves major changes in the physical relationships between the constituent cells of the embryo with the establishment of gap junctional communication (Lo & Gilula, 1979; Goodall & Johnson, 1982, 1984; McLachlin, Caveney & Kidder, 1983; Goodall, 1986), the appearance of focal tight junctions (Ducibella, Albertini, Anderson & Biggers, 1975; Magnuson, Dempsey & Stackpole, 1977), and the earliest signs of deposition of an extracellular matrix (Leivo, Vaheri, Timpl & Wartiovaara, 1980; Cooper & McQueen, 1983; Wu, Wan, Chung & Damjanov, 1983; Wan, Wu, Chung

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& Damjanov, 1984). Accompanying these changes, the cell membranes become closely apposed resulting in intercellular flattening (Ducibella & Anderson, 1975; Lehtonen, 1980) mediated *via* the Ca^{2+} -dependent adhesion system uvomorulin (also called E-cadherin, L-CAM, cell CAM 120/80 and gp123: Hyafil, Morello, Babinet & Jacob, 1980; Hyafil, Babinet & Jacob, 1981; Ogou, Okada & Takeichi, 1982; Peyrieras *et al.* 1983; Shirayoshi, Okada, & Takeichi, 1983; Damsky *et al.* 1983; Gallin, Edelman & Cunningham, 1983; Vestweber & Kemler, 1984; Johnson, Maro & Takeichi, 1986). This changing pattern of cell associations is believed to be important not only for subsequent morphogenetic events (Lo, 1982; Kimber & Surani, 1982; Kimber, Surani & Barton, 1982; Wiley, 1984) but also for the initiation and regulation of cell diversification (Ziomek & Johnson, 1980, 1981; Johnson & Ziomek, 1983; Fleming, Warren, Chisholm & Johnson, 1984; Johnson *et al.* 1986). The earliest stage at which cell diversity is evident occurs after the transition from the 8- to 16-cell stage, and appears to result from the operation of differential cleavage on the polarized 8-cell blastomeres (Johnson & Ziomek, 1981; Reeve, 1981a; Fleming & Pickering, 1985; Maro, Johnson, Pickering & Louvard, 1985b). The process of polarization occurs during the 8-cell stage, is regulated by cell interaction (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981; Johnson *et al.* 1986), and involves the realignment of many components of the cell both at its surface (Handyside, 1980; Ziomek & Johnson, 1980; Izquierdo, Lopez & Marticorena, 1980; Reeve & Ziomek, 1981; Nuccitelli & Wiley, 1985; Pratt, 1985) and within its cytoplasm and cytoskeleton (Reeve, 1981b; Reeve & Kelly, 1983; Johnson & Maro, 1984; Maro *et al.* 1985b; Fleming & Pickering, 1985). The pervasiveness of the process of compaction, and its significance for subsequent development, make an understanding of its control important.

We now have considerable knowledge about the spatial reordering that occurs at compaction, but we know little about the underlying controls. For example, we wish to know whether the events of compaction are under immediate transcriptional control, and why they first become evident at the 8-cell stage of development. Accordingly, as a first approach, we have analysed the dependence of polarization, cell flattening and gap junction formation on protein synthesis. We find that some elements of compaction can occur even when protein synthesis is inhibited from as early as the late 2-cell or early 4-cell stage. Moreover, some of the events of compaction can be advanced temporally when protein synthesis is inhibited during the early 4-cell stage. We propose a model for the temporal regulation of the process of compaction.

MATERIALS AND METHODS

(A) *Recovery and culture of embryos*

Female MF1 mice (3–5 weeks; OLAC derived, and bred in the laboratory) or F₁LAC mice (C57BL×CBA/Ca, bred in the laboratory) were superovulated by an intraperitoneal injection of 5 i.u. Pregnant Mare's Serum (PMS; Intervet) followed 48 h later by the same dose of human Chorionic Gonadotrophin (hCG; Intervet). For *in vivo* fertilization the females were paired overnight with an HC-CFLP male (Hacking and Churchill) and a vaginal plug taken as an

indication of successful mating. Late 2-cell embryos were flushed from the oviducts 48 h post-hCG with prewarmed (37°C) Medium 2 containing 4 mg ml^{-1} Bovine Serum Albumin (M2+BSA; Fulton & Whittingham, 1978) and cultured in Medium 16+BSA (M16+BSA; Whittingham & Wales, 1969). All incubations were carried out under oil at 37°C in an atmosphere of 5% CO_2 in air.

To obtain early 2-cell embryos, it was necessary to fertilize F_1 eggs *in vitro* in order to overcome the '2-cell block' shown by MF1 embryos (Goddard & Pratt, 1983). Spermatozoa were collected from the epididymides of male HC-CFLP mice and incubated in $500\text{ }\mu\text{l}$ drops of Whittingham's medium pre-equilibrated at 37°C and 5% CO_2 and containing 3% BSA (w/v; W+30; Fraser & Drury, 1975) under oil for 1.5 h to allow for capacitation. Cumulus masses were recovered from the oviducts of F_1 LAC female mice at 12.5 h post-hCG and a maximum of 10 placed in 1 ml drops of pre-equilibrated W+30. Insemination was carried out at 13.5 h post-hCG by mixing a suitable volume of sperm suspension with the cumulus masses to give a final sperm concentration of $(1-4)\times 10^6\text{ ml}^{-1}$. Fertilized eggs were recovered after a 4 h incubation and transferred to M16+BSA for further culture. Removal of the zona pellucida was achieved by brief exposure of the embryos to prewarmed acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975).

(B) Synchronization of embryos

There is considerable developmental heterogeneity amongst embryos, although this is reduced greatly after fertilization *in vitro*. Therefore late 1-cell embryos derived by *in vitro* fertilization and late 2-cell embryos flushed at 48 h post-hCG were examined in culture at hourly intervals; any 2-cell or 4-cell embryos formed were harvested and cultured separately, thereby producing groups of embryos all of which had divided within 1 h of each other. Throughout this paper time is expressed in hours postdivision to 4-cells. Division to 4-cells occurs in most *in vivo* fertilized embryos over the period 48 h to 58 h post-hCG.

(C) Assessment of intercellular flattening

The degree of flattening of embryos was determined by examination using a Wild dissecting microscope. Embryos were compared with the standards illustrated in Fig. 1 and classified as: 'non-flattened' when all cell outlines were still clearly visible (Fig. 1A,C); 'compacting' or 'partially compact' when cell outlines were indistinct but still visible between at least some cells (Fig. 1D – lower embryo); and 'fully compact' when no cell outlines were visible (Fig. 1B,D – upper embryo). To quantify the degree of flattening within a population a scoring system was used, non-compact embryos being assigned a value of 0, compacting embryos a value of 1, and fully compact embryos a score of 2. The extent of flattening for a population was then expressed as the percentage of the maximum possible score, were all the embryos to have been flattened fully.

(D) Assessment of cell polarity

Surface polarity was assessed immunocytochemically by incubation of zona-free embryos in $700\text{ }\mu\text{g ml}^{-1}$ FITC-labelled Concanavalin A (FITC-ConA; Polysciences) for 5 min at room temperature, followed by three washes in M2+BSA. Labelled embryos were then handled exactly as described in Maro, Johnson, Pickering & Flach (1984) for fixation with 3.7% formaldehyde in phosphate-buffered saline (PBS). Cytoplasmic polarity was assessed by analysis of clathrin distribution immunocytochemically after fixation and detergent extraction of embryos with 0.25% Triton X-100, using an affinity-purified rabbit anti-clathrin antibody followed by rhodamine-labelled anti-rabbit immunoglobulin antibody (see Maro *et al.* 1985b for details). Samples were viewed on a Leitz Ortholux microscope. Filter set L2 was used for the fluorescein staining and set N2 for rhodamine. A Leitz Vario-orthomat photographic system was used with Kodak Tri-X film for immunocytochemically labelled preparations. Ilford Pan-F film was used for photography on the Wild inverted-phase microscope.

Surface polarity and organization were also assessed by scanning electron microscopy (SEM) using the procedure of Johnson & Ziomek (1982) as modified by Maro & Pickering (1984).

Embryos were fixed in 6% glutaraldehyde (Sigma) in 0.1 M-cacodylate buffer pH 7.4, and transferred to freshly prepared poly-L-lysine (PLL, Sigma Type 1B, 1 mg ml⁻¹ fresh solution) coated coverslips. Samples were dehydrated through graded alcohols (30 min each in 20%, 40%, 60%, overnight in 70%, 30 min each in 80%, 90%, 95% and 100%) and then dried from 100% ethanol *via* CO₂ in a polaron E3000 critical-point drying apparatus. Coverslips were mounted on stubs with silver glue and coated with a 50 nm layer of gold in a Polaron E5000 Diode sputtering system. Embryos were examined in an ISM-35CF Jeol microscope under 20 kV.

(E) *Uptake and incorporation of [³⁵S]methionine*

Embryos were incubated for 1 h in a dilution of 2 µl [³⁵S]methionine (1000–1400 Ci mmol⁻¹, Amersham International Ltd) in 40 µl M16+BSA supplemented with 10 µM-methionine (final activity approximately 75 µCi µmol⁻¹), in the presence or absence of 10 µM-anisomyin or

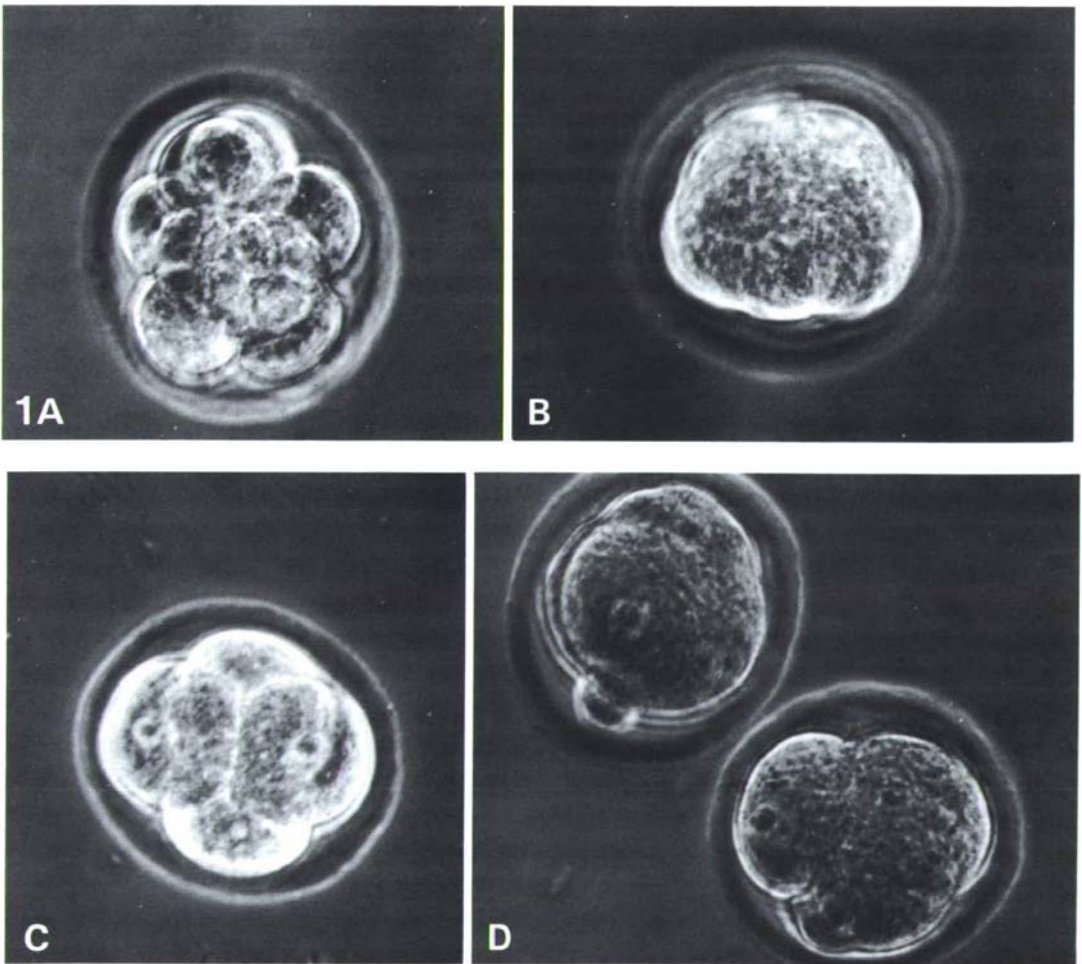


Fig. 1. Photomicrographs of embryos illustrating (A) a non-flattened early 8-cell control embryo – scored as 0. (B) A fully flattened late 8-cell control embryo – scored as 2. (C) A non-flattened 4-cell embryo – scored as 0. (D) Two 4-cell embryos exposed to anisomyin from 0 h; the upper embryo is fully flattened, scored as 2, and the lower is partially flattened, scored as 1. $\times 450$.

10 $\mu\text{g ml}^{-1}$ puromycin. Treated embryos were precultured in medium containing the appropriate drug for 1 h prior to incubation in the radiolabel. After the labelling period embryos were treated as described in Holmberg & Johnson (1979). The drug doses used were adopted after preliminary experiments had shown that the drugs were fully effective within 30 min of addition and yielded incorporation values indistinguishable from background for anisomycin and 12 % of control values for puromycin (0.74 ± 0.27 fmoles embryo $^{-1}$ h $^{-1} \pm$ s.d. in puromycin compared with 6.08 ± 1.28 in controls). The residual incorporation in puromycin probably represents incorporation into prematurely terminated polypeptides. Removal of embryos from anisomycin restored incorporation to about 75 % within 4 h.

(F) Assessment of gap junctional competence

Gap junctional communication was assessed by measurement of both ionic coupling and transmission of carboxyfluorescein between cells (exactly as described in Goodall, 1986), care being taken to avoid the misclassification of residual midbodies as gap junctions (see Goodall & Johnson, 1984, for details). Coupling was measured after culture in anisomycin from the early, mid or late 4-cell stages or from the early 8-cell stage. The mean resting potential and standard deviation of control cells was -16.3 ± 4.3 mV and of anisomycin-treated cells was -16.0 ± 5.4 mV.

(G) Drugs

Two inhibitors of protein synthesis were used in this study, anisomycin (sample from Pfizer Inc.) and puromycin (Sigma). Both were diluted in M16+BSA from a stock solution to give a final concentration of 10 μM -anisomycin (stock solution, 10 mM in water) and 10 $\mu\text{g ml}^{-1}$ puromycin (stock, 10 mg ml $^{-1}$ water). Nocodazole was dissolved as a stock solution (10 mM) in dimethylsulphoxide (DMSO) and stored at -20°C . Dilutions were made in M16+BSA to give a final concentration of 10 μM . All drugs were equilibrated for at least 12 h in 5 % CO $_2$ in air before being used for culturing embryos.

Monoclonal antibody ECCD-1 directed against E-cadherin, the principal component of the Ca $^{2+}$ -dependent cell-cell adhesion system (CDS) of the early embryo (see Johnson *et al.* 1986, for details of use) was diluted 1/50 in M16+BSA and millipored prior to use.

RESULTS

There is considerable developmental heterogeneity within a population of embryos of the same age in hours postovulation. Therefore, throughout this experimental series embryos were synchronized to the second mitotic cleavage as described previously (Bolton, Oades & Johnson, 1984; Smith & Johnson, 1984). Time axes throughout are expressed in hours postdivision to 4-cells.

(A) Effect of inhibited protein synthesis on cell division

Transition through the cell cycle from interphase to mitosis requires protein synthesis. First, therefore, we determined the period of sensitivity of 2- and 4-cell embryos to anisomycin. Populations of late 2-cell embryos and 4-cell embryos of known age in hours postdivision were placed in anisomycin and then scored at hourly intervals for evidence of cleavage amongst their constituent blastomeres. The results are presented in Table 1, and show that embryos are able to divide normally only if they enter mitosis within 2 h of being placed in the drug. Exposure to anisomycin for a period exceeding the last 2 h of interphase results in the suppression of cleavage in all or most blastomeres of any single embryo. This

Table 1. *Effect of time of application of anisomycin on second and third cleavage divisions*

Time after placing experimental embryos in anisomycin or control embryos in M16+BSA (h) (1)	Percentage of embryos in which one or more blastomeres first divided over the time indicated in column (1)							
	Mixed population of late 2-cells (n = 320) (2)	Control 2-cells (retained in M16+BSA) (n = 644) (3)	Control 4-cells (retained in M16+BSA) (n = 720) (4)	0 h, 3 h and 6 h* 4-cells (n = 115) (5)	8 h* 4-cells (n = 86) (6)	9 h* 4-cells (n = 118) (7)	10 h* 4-cells (n = 109) (8)	11 h* 4-cells (n = 81) (9)
0-1	20	19	9	0	5	19	28	46
1-2	7	16	17	0	6	10	22	38
2-3†	3†	16	19	0	2†	11†	16†	1†
3-4	0	13	14	0	6	8	3	1
4-5	0	11	10	0	7	7	4	1
5-6	0	10	11	0	1	2	5	2
>6	0	10	15	0	12	11	7	4
Undivided residual population	70	5	5	100	61	32	15	7

* Timings indicate the age of the embryos (in hours postdivision to 4-cells) when they were placed in anisomycin.

† Of embryos in anisomycin recorded as undergoing cleavage from 2 h or later, only 2% completed cleavage in all blastomeres and in most only one or two blastomeres cleaved. In contrast, all blastomeres cleaved in control embryos.

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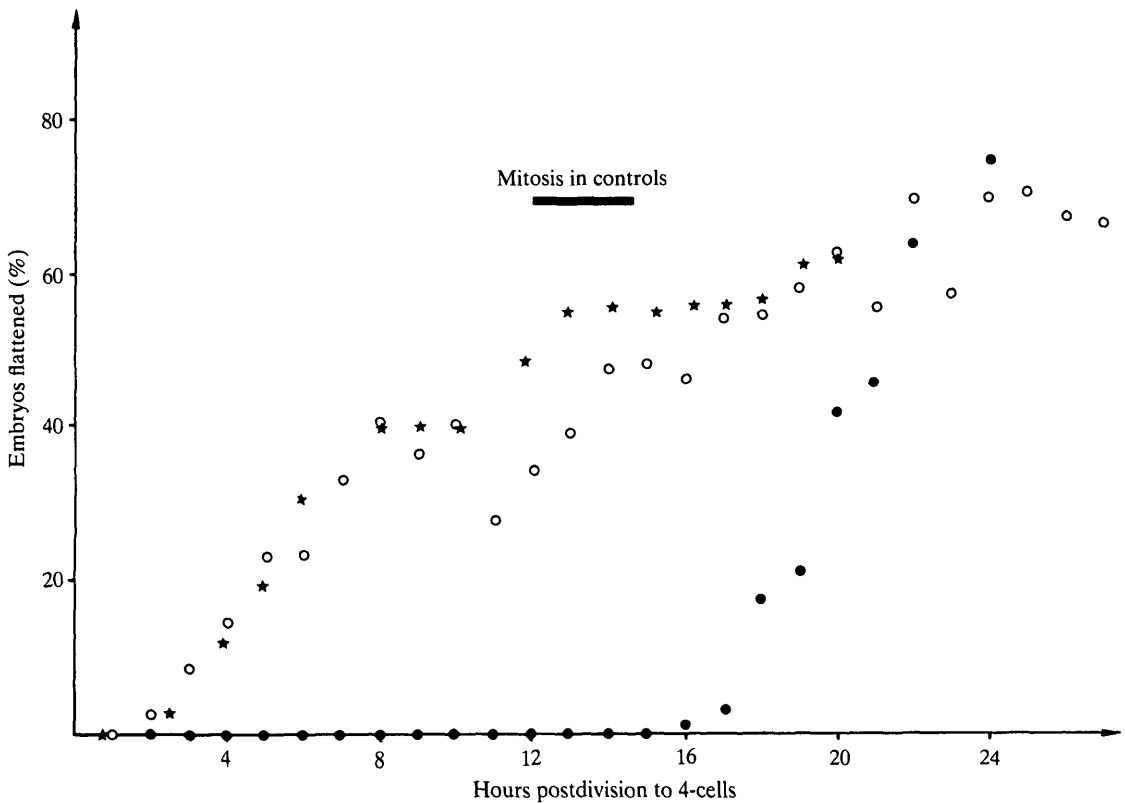


Fig. 2. Plot of the time course of intercellular flattening of 4-cell embryos placed immediately after their formation into (i) control medium: solid dots ($n = 119$), (ii) $10 \mu\text{M}$ -anisomycin: open circles ($n = 186$), or (iii) $10 \mu\text{g ml}^{-1}$ puromycin: stars ($n = 60$). The vertical axis expresses the degree of flattening as a percentage of the maximum score were all embryos to have been fully flattened. Note the premature flattening observed in the presence of protein synthesis inhibitors.

result, which is similar to that obtained for the first cleavage division (Howlett, 1986), suggests that the protein biosynthetic requirement for entry into cleavage is effectively completed 2 h in advance of division. The result does not tell us when this synthetic event is initiated (see below).

(B) *Effect of inhibited protein synthesis on intercellular flattening*

Newly formed 4-cell embryos were placed in anisomycin, puromycin or control medium and scored for intercellular flattening at intervals during the remainder of the third and the ensuing cell cycles. Embryos were compared with the standards shown in Fig. 1 and assigned a score according to the degree of intercellular flattening of 0 (not flattened), 1 (partially flattened) or 2 (fully flattened). The scores for each time point and condition were then expressed as a percentage of the maximum score possible. The results are plotted in Fig. 2. Three features

emerge from these data. First, prolonged exposure to inhibitor does not prevent the occurrence of intercellular flattening. Second, the final level of flattening achieved is always lower in the presence of the drug – a more prolonged incubation does not result in a higher final flattening score. Third, in the presence of the drugs flattening develops prematurely in many embryos, occurring during the third cell cycle. Most embryos incubated in drugs either remained unflattened or flattened fully. Thus the results plotted in Fig. 2 mean that by the end of the fourth cell cycle around 70 % of treated embryos had flattened (this value varied between 50 and 75 % in different experiments). Newly formed 4-cell embryos treated with any concentration of anisomycin ranging from 1 μM to 100 μM behaved in the same way, flattening prematurely to a final flattening score of around 70 %. However, use of 0.1 μM -anisomycin suppressed division in only 30 % of embryos, and 75 % of those dividing to 8-cells flattened at the same time as controls.

In order to determine the limits of the developmental period over which drugs advanced flattening, and to determine if and when there was a point in development at which the drugs blocked flattening completely, embryos were placed in the drugs at earlier or later times. 2-cell embryos placed in anisomycin failed to divide and to flatten unless placed in the drug within 1–2 h of division to 4-cells. Of those that did divide to 4-cell embryos, 33 % showed premature flattening and 48 % had flattened when controls were at the mid 8-cell stage ($n = 100$). Thus the flattening process, advanced or otherwise, is sensitive to inhibition of protein synthesis up to late in G_2 of the second cell cycle.

Newly formed 4-cell embryos were cultured in control medium for varying periods of time before transfer to medium containing anisomycin, and then scored for flattening at intervals for comparison with embryos left in control medium throughout. The results are plotted in Fig. 3. Delaying exposure to anisomycin by up to 6 h after division to 4-cells (upper panel, Fig. 3) produced a similar result to the immediate exposure of 4-cell embryos to the drug – the same degree of premature flattening occurring, and a similar final proportion flattened. However, when these newly formed 4-cell embryos were cultured in control medium for 8, 9 or 10 h before transfer to anisomycin, the extent of flattening at the late 8-cell equivalent stage was considerably reduced (Fig. 3, middle panel, data not shown for 9 h but similar to those for 8 h). If these embryos were cultured further, the proportion that had flattened increased a little until by 36 h a maximum of 25 % were flattened. Thus, in contrast to the effects observed with early application of drugs, flattening was impaired and delayed rather than advanced. Placing 11 h 4-cell embryos or embryos transitional between 4- and 8-cell stages in the drugs also resulted in a reduced incidence of flattening, although not so marked as with late 4-cell embryos (Fig. 3, lower panel).

In two sets of experiments, embryos were exposed to anisomycin transiently during the periods from 0 to 3 h (early pulse) or 8 to 12 h (late pulse) postdivision to 4-cells, and, after thorough washing, were then maintained in control medium for the following 13–24 h. The results are included in Fig. 3. Of such embryos, 100 % (early) and 81 % (late) did divide subsequently to 8-cells and continued to

develop apparently normally thereafter. The early pulsed group showed premature flattening and retained the flattened state on removal from the anisomycin, except for a transient period during a delayed third mitosis and cleavage to 8-cells (Fig. 3, middle panel). Of the late pulsed group, over 60% of those that divided flattened, with a similar time course to that of 11 h or transitional embryos treated with anisomycin (Fig. 3, lower panel).

(C) Cell flattening in the absence of protein synthesis resembles flattening in control embryos

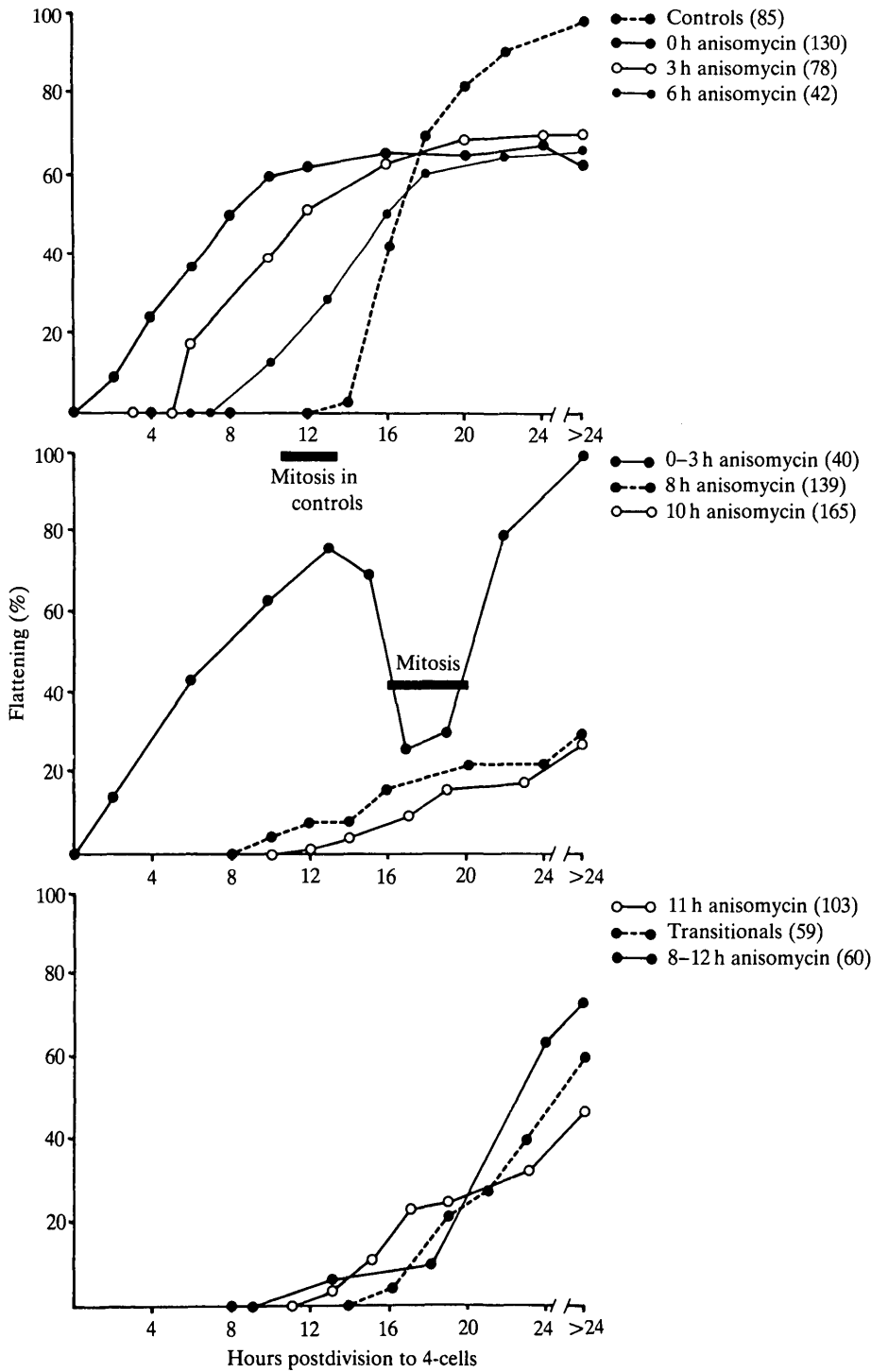
In order to determine whether the premature flattening observed in drug-treated embryos resembled that in controls, we examined the effect of neutralizing the Ca^{2+} -dependent, cell-cell adhesion system (CDS) mediated by E-cadherin and responsible for normal flattening (Hyafil *et al.* 1981; Shirayoshi *et al.* 1983; Johnson *et al.* 1986). Exposure of control embryos to the monoclonal antibody to E-cadherin, ECCD-1, prevents flattening. Embryos were therefore cultured from the beginning of the 4-cell stage in the presence of both anisomycin and ECCD-1. None showed any sign of flattening within the next 24 h. In a similar experiment, early 4-cell embryos were cultured in anisomycin for 6 h before transfer to ECCD-1 in the continued presence of anisomycin. Those embryos that had not flattened in anisomycin remained non-flattened, and those embryos that had flattened prematurely as a result of exposure to the anisomycin reverted to a non-flattened state. Thus, drug-induced flattening appears to involve the CDS used by normal embryos.

(D) Effect of inhibited protein synthesis on cell polarization

Newly formed 4-cell embryos were placed immediately in protein synthesis inhibitors and cultured for varying periods of time before sampling for analysis. Constituent cells were scored as polar or apolar by two criteria, namely surface phenotype assessed either by the pattern of FITC-ConA binding or by the distribution of microvilli observed on the scanning electron microscope, and by cytoplasmic phenotype as assessed by the distribution of cytoplasmic clathrin. The results may be summarized as indicating that (i) when embryos flattened (whether prematurely, with a delay or on time compared with controls) their cells also showed an increased incidence of surface polarization, and (ii) when embryos were placed in inhibitor prior to the late 4-cell stage none showed evidence of cytoplasmic polarization.

The results for surface polarity (assessed by FITC-ConA binding) are recorded in Table 2, and show that cells from treated embryos were, as a population, demonstrably polarized in advance of controls. As with the effect on flattening, not all embryos showed advancement of polarization. The phenotype of embryos showing polarization in the presence of anisomycin is shown in Figs 4 and 5, in which it can be seen that poles in some cells appear broader on treated embryos, although this probably reflects the larger size of the individual cells (compare with similar results for polarized, aphidicolin-treated 4-cell embryos in Smith &

Johnson, 1985). In addition, marked retraction fibres are evident in drug-treated, compacted embryos exposed to Ca^{2+} -free medium before fixation. This general organization of the surface pole and the presence of retraction fibres were



observed whether drug-treated embryos were sampled when control embryos were in either the third or the fourth cell cycle. In contrast to the resistance of surface polarity to drug treatment, cytoplasmic polarity was never observed in embryos exposed to anisomycin or puromycin from the beginning of the 4-cell stage, as illustrated in Fig. 6. Of 612 blastomeres examined from treated embryos, none was scored as having a polar clathrin distribution whilst over 50 % showed surface polarity. When 2-cell embryos placed in anisomycin were examined for evidence of surface polarity only a few of those that were within 1 h of division to 4-cells showed evidence of polarity, 16 % of 350 such blastomeres being scored as polar when control embryos were at the mid to late 8-cell stage. All such polar blastomeres were from embryos that had flattened. No 2-cell embryos that failed to divide showed evidence of polarity.

When exposure of embryos to anisomycin was delayed until 10 h into the third cell cycle, only 9 % of 195 blastomeres examined were demonstrably polar for FITC-ConA binding when controls were 80 % polar, and none of the blastomeres showed a polar clathrin distribution (control value 80 %, $n = 81$). Delaying exposure to 11 h (within 1 h of division to 8 cells) yielded values of 44 % and 48 % for surface and cytoplasmic polarity, respectively ($n = 96$). Likewise, 44 % of blastomeres from embryos placed in anisomycin at the 4- to 8-cell transition were scored as having surface poles and 42 % as having cytoplasmic poles ($n = 288$) by the mid to late 8-cell stage when the corresponding values for controls were 80 % and 83 % ($n = 352$).

Embryos pulsed with anisomycin between 8 and 12 h postdivision, and then returned to control medium, were polarized when examined subsequently. Of 527 blastomeres labelled with FITC-ConA after this experimental regimen, more than 50 % were polarized. The same result was obtained with embryos maintained in anisomycin from 8–14 h, 50 % of the blastomeres being demonstrably polar ($n = 190$).

(E) *Effect of inhibited protein synthesis on gap junction formation*

Embryos were placed in anisomycin at various points during the third and fourth cell cycles and analysed for evidence of intercellular coupling when controls were at the mid to late 8-cell stage. The results are summarized in Table 3 and examples

Fig. 3. Time course of intercellular flattening (expressed as in Fig. 2) of embryos exposed to 10 μ M-anisomycin over the various regimes indicated adjacent to each panel. A single time value (e.g. 0, 3, 6, 8, 10 and 11 h) indicates that embryos were at that age in hours postdivision to 4-cells when they were placed in anisomycin; embryos were then maintained in anisomycin for the remainder of the experiment. A range of times (e.g. 0–3 h and 8–12 h) indicates that embryos were exposed to anisomycin only over the specified period. Transitional embryos were those in which one or more blastomeres had undergone division from the 4- to 8-cell stage in the hour preceding placement into the drug. Controls were retained in M16+BSA throughout. The numbers in brackets represent the number of embryos examined under each condition. Individual data points are connected simply to improve visualization of overall effects of each regime. The periods of mitosis are indicated in the central panel for controls and for embryos pulsed from 0–3 h with anisomycin.

Table 2. *Incidence of surface polarity in blastomeres exposed to anisomycin or puromycin from the early 4-cell stage**

Drug used	Total no. of blastomeres scored	% of blastomeres polarized at time of scoring categorized as to the state of flattening of the embryos from which they are derived					
		Late 4-cell (9-11 h)		Early 8-cell (12-17 h)		Mid to late 8-cell (20-24 h)	
		Flattened	Non-flattened	Flattened	Non-flattened	Flattened	Non-flattened
Puromycin 10 µg ml ⁻¹	516	41.2	21.1	40.6	15.9	73.7	42.3
Anisomycin 10 µM	532	48.2	12.4	70.0	66.6	70.2	42.2
Controls	1225	—	0	—	12.3	64.4	46.4

* Surface polarity was assessed by examination of the FITC-ConA binding pattern. Blastomeres with equivocal staining patterns were excluded from the percentage calculations and amount to between 4 and 16 % of cells examined.

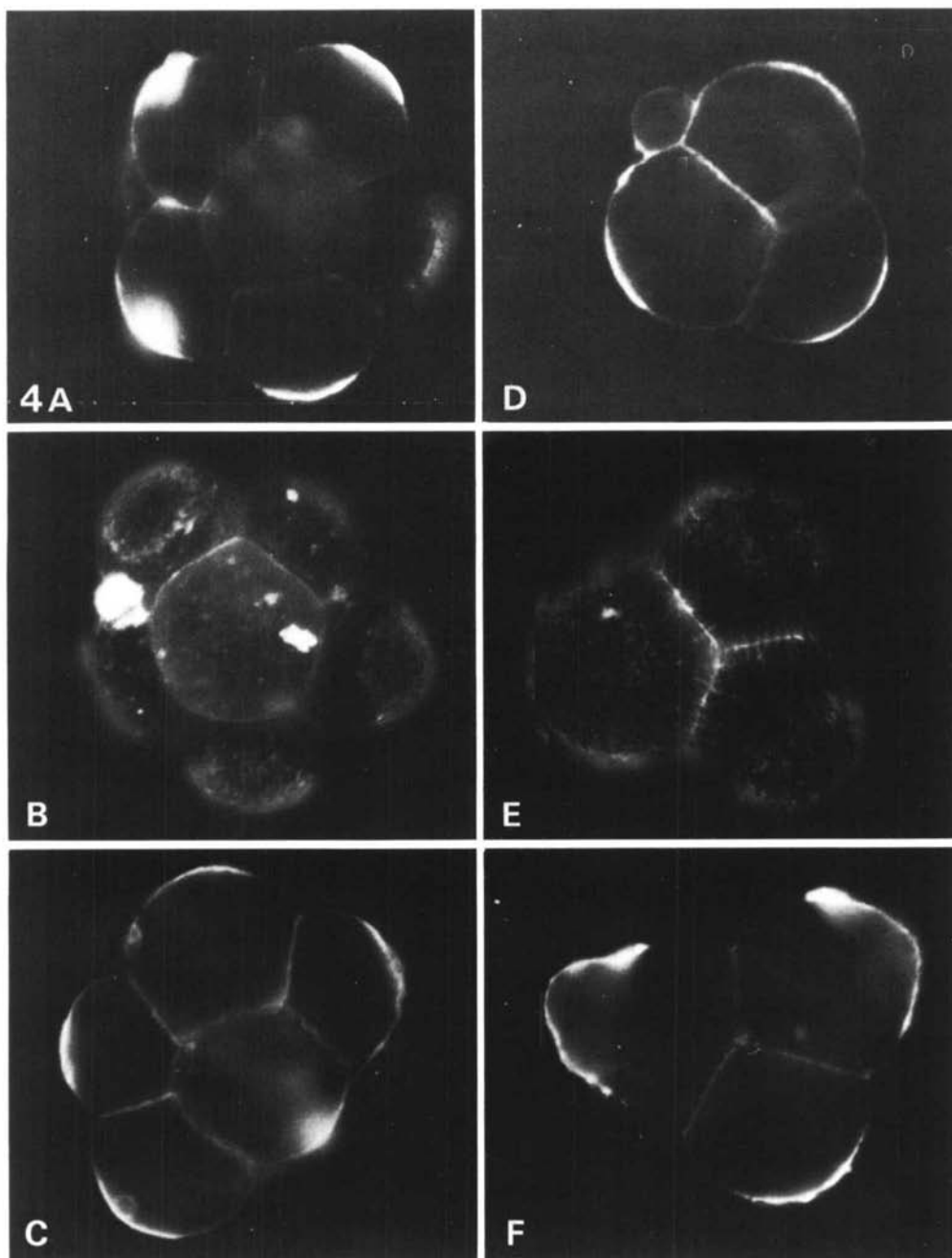


Fig. 4. Examples of control embryos (A,B,C) and embryos treated with protein synthesis inhibitors from the early 4-cell stage (D,E,F) after exposure to FITC-ConA. The embryos in B,E are viewed in a focal plane across their surfaces, the others in planes through the embryo. Note the distinct polar staining patterns in each embryo. $\times 500$.

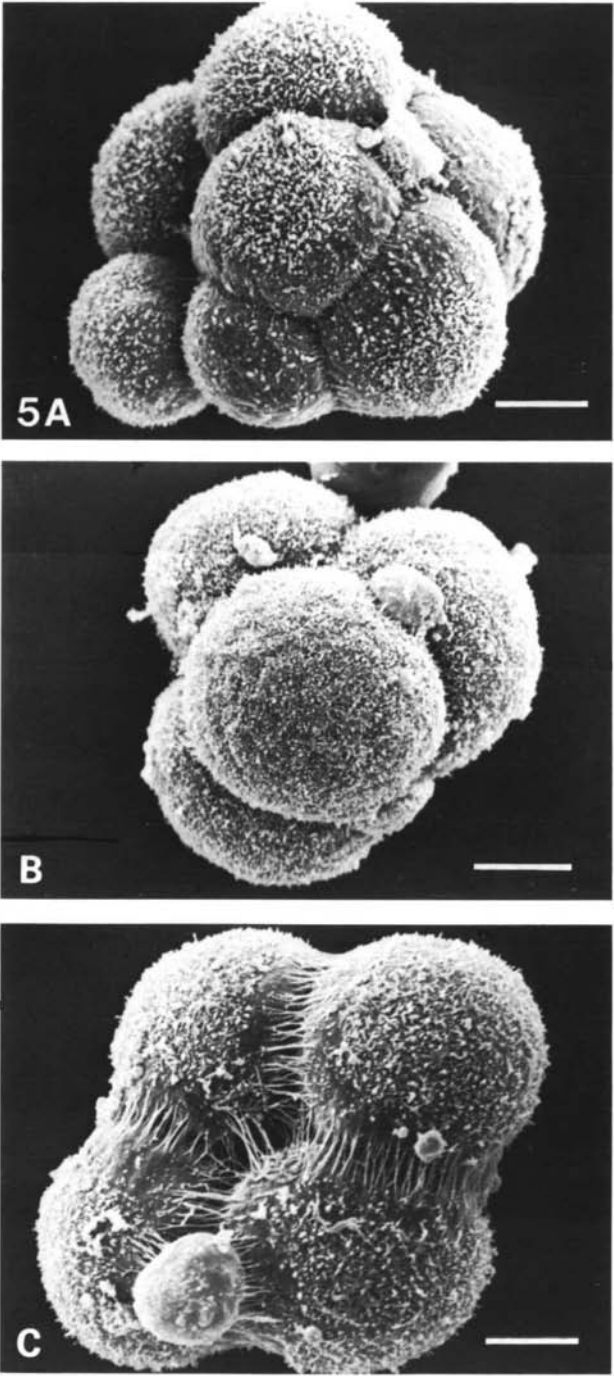


Fig. 5. Scanning electron micrographs of (A) control, and (B,C) anisomycin-exposed embryos. Clear evidence of polar regions of microvilli in A and C but not in B. Note the retraction fibres that persist between blastomeres of the flattened embryo exposed to Ca^{2+} -free medium to separate the cells in C. Bar, $10\mu\text{m}$.

are shown in Fig. 7. Coupling was never detected in embryos placed in anisomycin at the early 4-cell stage. However, when mid or late 4-cell embryos or early 8-cell embryos were placed in anisomycin, coupling could be detected, whether or not division and/or intercellular flattening had occurred. The independence of coupling from flattening has been reported previously (Goodall, 1986).

DISCUSSION

Compaction appears to occupy a central role in the generation of cell diversity during blastocyst formation, and an understanding of its control is therefore important. One approach to such an analysis is to inhibit a particular biosynthetic activity and to observe the effect on compaction. Such an approach does not

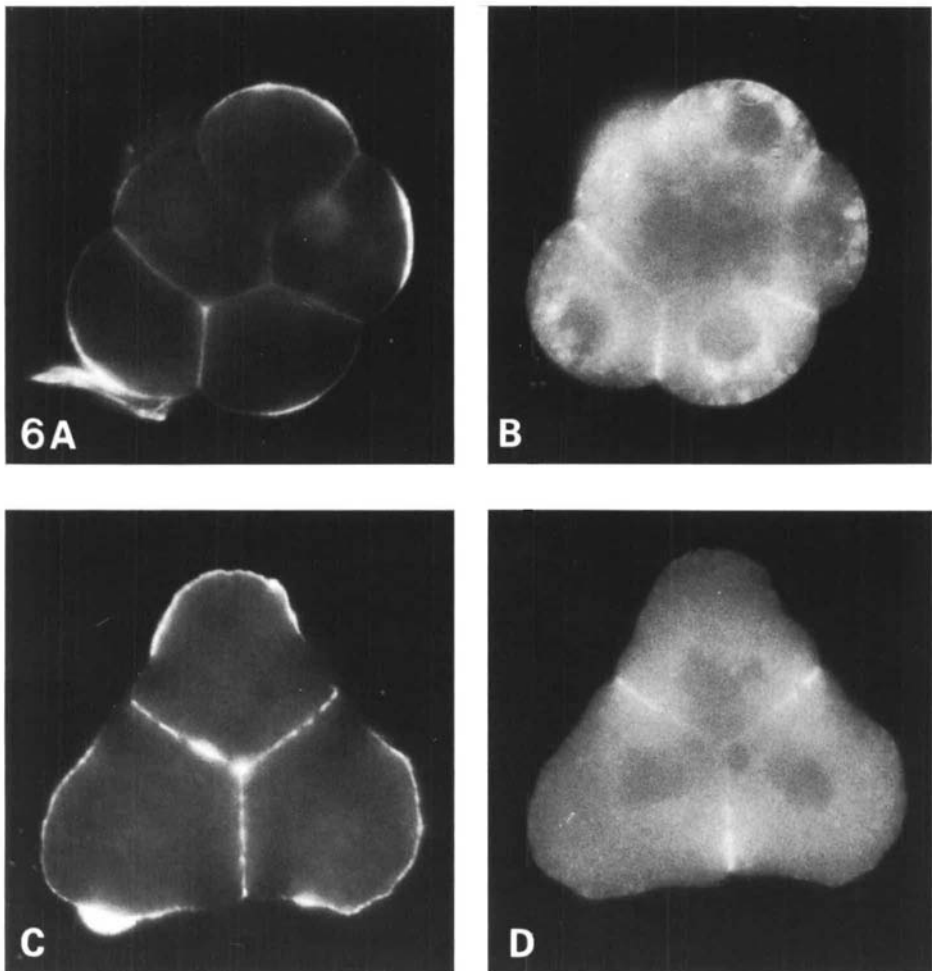


Fig. 6. (A,B) Control embryos and (C,D) embryos treated with anisomycin from the early 4-cell stage, and then stained with either (A,C) FITC-ConA or (B,D) antiserum to clathrin. Note focal concentration of clathrin apically in control, but uniform distribution in experimental embryo. $\times 500$.

permit conclusions to be drawn about the control of features that fail to develop, but can be revealing about those that are resistant to inhibitor action (for discussion of this point see Bolton *et al.* 1984; Smith & Johnson, 1985). Both anisomycin and puromycin arrested cleavage in most embryos, a result also observed previously when inhibitors of protein synthesis were applied to cleaving embryos (Thomson & Biggers, 1966; Tasca & Hillman, 1970; Monesi, Molinaro, Spalletta & Davoli, 1970; Kidder & McLachlin, 1985). Almost all of those embryos that escaped complete or partial cleavage arrest were within 2 h of entering mitotic cleavage, reflecting the known requirement for a burst of protein synthesis if the transition from interphase to the mitotic phase is to be achieved successfully in cleaving blastomeres (Howlett, 1986; Miake-Lye, Newport & Kirschner, 1983; Gerhart, Wu & Kirschner, 1984). Otherwise, embryos incubated in inhibitors showed evidence of remarkable health and resilience over a prolonged period, as judged, for example, by their membrane resting potentials. In particular, exposure to drugs of most embryos from the early 4-cell stage and of many from the very late 2-cell stage, did not prevent either cell flattening or surface polarization. Moreover, the pattern and properties of cell flattening and surface polarity in drug-exposed embryos resembled those observed in control embryos. In addition, gap junctional communication developed in most embryos placed in the drug from the mid 4-cell stage onwards, a result which confirms and extends a previous observation (McLachlin *et al.* 1983). Cytoplasmic polarity was the feature of compaction most sensitive to the action of the drugs, and was inhibited unless their addition was delayed to the late 4-cell or early 8-cell stages. We know from previous work that cytoplasmic features of polarity are particularly labile and

Table 3. *Effect of anisomycin on development of gap junctional coupling**

Time of transfer to anisomycin (h postdivision to 4-cells)	No. of cells per embryo	No. of embryos analysed	% coupled†	% of coupled embryos that had flattened	% of non- coupled embryos that had flattened
Early 4-cell (0–6)	4	14	0	—	79
Mid 4-cell (6–8)	4	16	31	100	82
Late 4-cell (9–11)	4–8	16	50‡	71	67
Early 8-cell (12–14)	8	18	78	92	60
Controls	8	24	88	90	0

* All analyses undertaken at mid to late 8-cell equivalent stage.

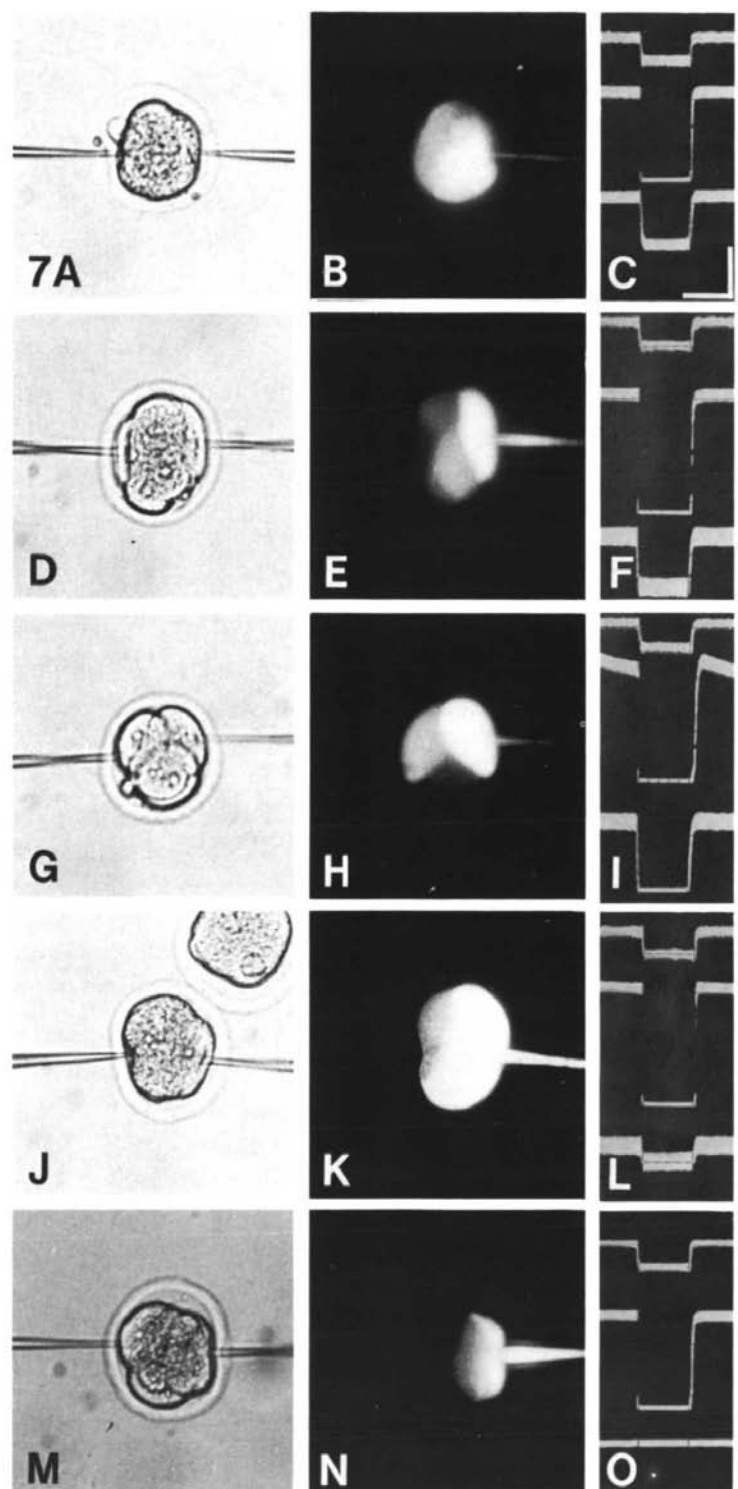
† Coupling was scored as present if more than $4 \times 1/8$ or more than $2 \times 1/4$ blastomeres were in communication.

‡ Of these embryos, two had 4 cells, two had 5–7 cells and four had 8 cells. Non-coupled embryos consisted of four with 4 cells and four with 8 cells.

sensitive to disruption (Johnson & Maro, 1985; Fleming, Cannon & Pickering, 1986).

The simplest conclusion to be drawn from these results is that most embryos possess sufficient of the required proteins to achieve much of the process of compaction well in advance of the normal occurrence of the event. Such a conclusion does not exclude the possibility that in normal development continuing synthesis of these proteins occurs up to and during compaction. Of those proteins known to have a role in compaction, most appear to be present in the embryo prior to the event itself, e.g. uvomorulin is present from as early as the 1-cell stage (Hyafil *et al.* 1981; Ogou *et al.* 1982; Johnson *et al.* 1986), as are actin and tubulin (Abreu & Brinster, 1978). Moreover, the relative constancy of the qualitative pattern of protein biosynthesis between the late 2-cell and 8-cell stages has been known for some time (Van Blerkom & Brockway, 1976), and the half lives of the bulk of protein species are known to be many hours (Merz, Brinster, Brunner & Chen, 1981; Pratt, Bolton & Gudgeon, 1983; Howlett, 1986). Thus, we may conclude that most if not all of the major physical rearrangement that occurs at compaction can function with proteins made on templates present as early as the late 2-cell stage. It is of interest that shortly before this time, the embryonic genome first becomes active in the production of many new transcripts; these transcripts are translated into protein, and maternal mRNA is destroyed (Flach *et al.* 1982; Piko & Clegg, 1982; Giebelhaus, Heikkila & Schultz, 1983; Bolton *et al.* 1984).

If the protein biosynthetic requirements for compaction are fulfilled from as early as the late 2-cell stage, what does this tell us about the regulatory mechanisms at work? It seems reasonable to conclude that many of the changes in cell organization that occur at compaction are regulated at a post-translational level by a change in the structure, stability, conformation and/or activity of the previously synthesized proteins. A similar conclusion concerning cell flattening has been reached recently by Kidder & McLachlin (1985). How might such a control be exercised? Some light on this question comes from examination of the other principal result to arise from the experiments reported here. Previously, we have encountered no condition in which elements of compaction have been advanced to a stage of development earlier than the 8-cell embryo. However, in these experiments we have observed that many late 2-cell and early 4-cell embryos placed in anisomycin or puromycin not only flatten and polarize at the surface but do so prematurely. It is not surprising, given both the complexity of the developmental process under consideration and the pervasive effects of the drugs used, that in our experiments not all embryos showed premature flattening and surface polarity. What is surprising is that this result occurred regularly in all experiments in such a substantial proportion of embryos. Moreover, when the period of exposure to inhibitor was limited to the first 3 h of the third cell cycle, 100 % of embryos flattened. Taken together, these results appear to indicate that some of the proteins synthesized during the 4-cell stage may in fact be involved in *suppressing* compaction.



Inhibition of protein synthesis is likely to have wide-ranging effects within the cell, not only selectively depleting proteins that turn over rapidly, amongst which are included putative regulatory proteins (Oren, Maltzman & Levine, 1981; Croy & Pardee, 1982), but also elevating the levels of damaged or incomplete proteins with consequential changes in the balance of ubiquitination activity within the cell that may itself lead to the selective alteration of regulatory molecules (Munro & Pelham, 1985). Alternatively, the drugs may elicit less-specific, secondary effects within the cells, such as a global change in calcium levels or in pH. Whichever feature of the response to the drugs might be responsible for initiating the signal to compact, once this signal has been given the restoration of protein synthesis does not reverse the process. Indeed, restoration of protein synthesis permits the progress of compaction to go to completion in most or all embryos. Presumably, in normal development a similar signal is not given until the early 8-cell stage, where it could be controlled either negatively by removal of a prior restraint or positively by supply or activation of a previously limiting component. The precise form that this putative permissive signal takes may perhaps be determined from the analysis of other agents that cause premature compaction. The nature of the post-translationally mediated processes by which the signal achieves the events of compaction will come from an analysis of the changes undergone by the structural proteins involved.

If the proposed model is correct, it must take account of the paradoxical observation that maintained arrest of protein synthesis at the late 4-cell stage (from 8 h postdivision onwards) tends to *reduce and delay* compaction considerably (a finding also observed for flattening by Kidder & McLachlin, 1985). In contrast, pulsing late 4-cell embryos with anisomycin, or delaying addition of anisomycin until cleavage is imminent or under way, permits compaction to occur, albeit with a delay and less completely than in controls. There appears to be a window, spanning the latter part of the third cell cycle, during which sustained inhibition of protein synthesis delays compaction. Does this paradoxical result really mean that the putative permissive state is no longer being induced in the presence of

Fig. 7. Junctional coupling in anisomycin-treated embryos. Left column, bright field. Middle column, carboxyfluorescein distribution 10 min after start of ionophoresis into a single blastomere. Right column, oscilloscope traces of ionic coupling between two cells within an embryo: top trace, injected current; centre trace, signal from injected cell; lower trace, signal from second cell. The trace from the injected cell is barred, and thus not proportional to the current injected, but is included as evidence of a stable resting potential. (A–C) Fully flattened control 8-cell embryo with all cells in communication. (D–F) Partially flattened embryo that had been cultured in anisomycin (10 μ M) from the early 8-cell stage and shows dye spread among 4-cells and ionic coupling between two opposite cells. (G–I) Embryo treated with anisomycin from the late 4-cell stage that had not divided or flattened fully, and in which coupling was evident between two cells by dye, although three cells were impaled successfully and found to be coupled to each other ionically. (J–L) Fully flattened embryo treated with anisomycin from the mid 4-cell stage, and in which all cells are coupled. (M–O) Partially flattened embryo treated with anisomycin from the early 4-cell stage, and revealing no coupling that could not be explained by transmission *via* residual mid-body of sister blastomeres (see Goodall & Johnson, 1984).

anisomycin in the latter half of the cycle? Or could the result mean that the induction is still occurring in these embryos, but that its effects are being obscured by the known deleterious effects of inhibited protein biosynthesis on mitotic cleavage (see Table 1)? This second explanation would not be unreasonable, since the features by which compaction is assessed are either modified, or lost transitorily, at mitotic cleavage, e.g. cell flattening (Lehtonen, 1980; Maro & Pickering, 1984; Goodall & Maro, 1986), gap junctional coupling (Goodall & Maro, 1986), cytoplasmic polarity (Reeve, 1981; Fleming & Pickering, 1985; Maro *et al.* 1985*b*) and surface polarity (Johnson & Ziomek, 1981). Hourly examination of individual embryos exposed to inhibitors from the late 4-cell stage onwards (8, 9, 10 or 11 h) supports such an explanation. Thus, those embryos that divide to 8-cells within 1 h of being placed in anisomycin proceed to compact at about the same time as controls, whereas of those embryos containing blastomeres that enter cleavage at later times after being placed in the drug, many do not complete cleavage in all blastomeres, others do so over a very protracted period, and many of those that do complete cleavage do not flatten subsequently or do so only after a delay. Thus, the depressed compaction rate in these groups of embryos appears to be an artefact arising from an abnormal prolongation of the mitotic state. As this depressant effect is not observed when anisomycin is added at 6 h postdivision to 4-cells but is observed with addition at 8 h, we can conclude that the synthesis of proteins that initiate entry into mitosis may begin between 6 and 8 h, even though levels adequate for completion of mitosis are not reached until 2 to 4 h later.

The hypothesis, that a key developmental event such as compaction might be regulated in a *permissive* way by setting up the required structural components and then modulating the level of a regulatory factor or cell state, is plausible, since there is good evidence that a similar type of system operates to regulate the activation of development at fertilization. Thus, the postfertilization development of the mouse embryo, as well as various other types of embryo, is (i) dependent upon presynthesized proteins, (ii) activated artificially by the addition of protein synthesis inhibitors (Masui & Markert, 1971; Zampetti-Bosseler, Huez & Brachet, 1973; Siracusa, Whittingham, Molinaro & Vivarelli, 1978; S. K. Howlett and B. Maro, personal communication), (iii) arrested by the presence of putative restraining (cytostatic) factors that hold the cell at a restricted phase of the cell cycle (Masui & Markert, 1971; Masui, 1982), and (iv) released naturally from restraint by destruction of the restraining factor, possibly as a result of changing levels of Ca^{2+} (Meyerhof & Masui, 1977; Newport & Kirschner, 1984). Moreover, grey crescent formation and the laying down of the dorsoventral axis in amphibia are also induced by inhibitors of protein synthesis (Gautier & Beetschen, 1985), and both early cleavage in the sea urchin (Evans *et al.* 1983) and proliferation of mouse cell lines in culture (Mita *et al.* 1980; Cienchanover, Finley & Varshavsky, 1984) are associated with the periodic destruction of putative regulatory proteins.

The use of a 'permissive' signal to initiate a post-translationally mediated programme of development has a number of advantages, a principal one being the relative ease with which 'instructive' positional signals could be integrated into the

developmental process. Such instructive signals do indeed operate at compaction, cell interaction regulating both the synchronization and spatial organization of the process (Ziomek & Johnson, 1980; Johnson *et al.* 1986; Johnson & Maro, 1986). We are now examining how the integration of a permissive temporal signal with an instructive positional signal might be achieved.

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