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DNA replication and compaction in the cleaving embryo of the mouse

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SUMMARY

The effects of aphidicolin, a reversible inhibitor of DNA polymerase alpha, both on replication and on development of the mouse embryo from the 2- and 4-cell stages to the compacted late 8-cell stage have been assessed. The continuous presence of aphidicolin from G_1 of the 4-cell stage resulted in inhibition of DNA replication and prevention of division from 4 to 8 cells, but was without effect on the timing or incidence of cell flattening, surface polarization and cytoplasmic polarization. The continuous presence of aphidicolin from G_1 of the 2-cell stage resulted in inhibition of DNA replication, division, and polarization. Some slight intercellular flattening in a few embryos did occur. If addition of aphidicolin was delayed by 10 h to early in G_2 of the 2-cell stage, further rounds of replication were blocked and some embryos failed to cleave to 4-cells. Nevertheless, almost all embryos showed evidence of flattening and polarization regardless of cell number. In contrast, if aphidicolin was added in G_1 of the 2-cell stage and removed after 10 h, the cells showed delayed DNA replication, little evidence of division, and no cell flattening or polarization. We conclude that DNA replication at the 2-cell stage may be essential for the components of compaction studied, but that DNA replication at the 4- and 8-cell stages is not.

INTRODUCTION

In many developing systems the expression of new developmental information within a cell is associated with a preceding cycle of replication. Therefore it has been proposed that differentiation may be linked causally to the cell cycle, and that developmental cell cycles might provide a measure of time elapsed since fertilization (Holtzer, Weintraub, Mayne & Mochan, 1972). It is probable that the feature of cell replication most important for developmental timing involves DNA, since inhibition of cytokinesis in most sytems, and of karyokinesis in some, does not influence developmental timing (e.g. Smith & McLaren, 1977; Petzoldt, Burki, Illmensee & Illmensee, 1983; Satoh & Ikegami, 1981*b*). It is not unreasonable to postulate a requirement for DNA replication during developmental change since changing gene expression is associated with local structural changes in the organization and composition of the chromatin, many of which require, or are facilitated by, replication (reviewed Johnson, McConnell & Van Blerkom, 1984).

The earliest phases of development, which involve relatively few cells, are particularly accessible for the study of DNA replication and developmental

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change, and have begun to reveal evidence suggesting a causal association in both *Ascidians* (Meedel & Whittaker, 1979; Satoh & Ikegami, 1981*a*,*b*) and *Xenopus* (Newport & Kirschner, 1982*a*,*b*). In this paper we report on the effect of inhibiting DNA replication on compaction of the mouse 8-cell embryo. Compaction marks a major morphological transition in which cells flatten on each other to maximize cell contact, change their phenotype from radially symmetrical to polar, and engage in formation of specialized intercellular junctions. There is evidence to suggest that these events represent the earliest progress towards the cell diversification observed in the blastocyst (Johnson, 1985).

Using populations of 2- and 4-cell embryos synchronized to the immediately preceding cleavage division, we have determined the effects of continuous or pulsed administration of aphidicolin, a reversible inhibitor of DNA polymerase alpha, on DNA replication, cytokinesis and the events of compaction when added in G_1 of the cell cycle. We conclude that the major features of compaction at the 8-cell stage are not dependent upon proximate DNA replication during the third and fourth cell cycles, but may be dependent upon replication of DNA during the second cell cycle.

MATERIALS AND METHODS

Embryo collection

4- to 6-week-old MF1 strain (Olac) or F_1LAC (C57B1 × CBA/Ca, bred in the laboratory) female mice were superovulated by an intraperitoneal injection of Pregnant Mare's Serum gonadotrophin (PMS Intervet 5–10 i.u. depending on size of animal) followed 48 h later by intraperitoneal injection of the same dose of human Chorionic Gonadotrophin (hCG, Intervet). For *in vivo* fertilization, MF1 females were paired with a stud male (HC-CFLP, Hacking and Churchill, Ltd, Alconbury) overnight. The presence of a vaginal plug was taken as a sign of mating. 48 h after the hCG injection, plug-positive mice were killed by cervical dislocation and the embryos recovered by flushing oviducts with prewarmed (37 °C) Medium 2 (Fulton & Whittingham, 1978) with 4 mg ml^{-1} Bovine Serum Albumen added [M2+BSA]. The numbers of late 2-cell (2c), 3-cell (3c) and early 4-cell (4c) embryos were recorded (dividing cells were recorded as one cell) and all 2-cell embryos were transferred to Medium 16 (Whittingham, 1971) containing 4 mg ml^{-1} BSA [M16+BSA] under oil and pre-equilibrated at 37 °C and 5 % CO_2 in air.

For *in vitro* fertilization, spermatozoa were collected from the epididymides of male HC-CFLP mice and incubated in 500 μl drops of Whittingham's medium containing 3 % BSA (w/v) (W+30; Fraser & Drury, 1975) for 1½ h. Cumulus masses were recovered from the oviducts of F_1LAC female mice at 12½ h post-hCG and placed in 1 ml drops of W+30. Insemination was carried out at 13½ h post-hCG to give a final sperm suspension of $1–4 \times 10^6$ ml^{-1}. Fertilized eggs were recovered after 4 h incubation and transferred to M16+BSA for further culture. *In vitro* fertilization using F_1LAC female mice was used in all experiments in which early 2-cell embryos were required, since the MF1 strain of mice shows a developmental block at the 2-cell stage *in vitro* (Goddard & Pratt, 1983).

Manipulation of embryos

During the experiments, culture dishes were removed from the incubator for the minimum possible period and were kept on a hot plate (at 37 °C) or, when viewing, on the heated stage of a Wild binocular dissecting microscope.

Assay for DNA content

Glass microscope slides were scored with a diamond pencil to mark out a grid of small squares. The slides were then cleaned in glacial acetic acid and dried using lint-free paper. Embryos were mounted straight from the culture medium onto the slide grid, one per square, and the position of each embryo noted. Excess medium was removed and the embryos allowed to air dry.

A freshly dissected mouse liver was washed in sterile phosphate-buffered saline (PBS) and smeared onto the same slide that held the embryos. Embryos were fixed by placing the slides in a freshly made solution of 3:1 absolute ethanol: acetic acid for 5 min, transfer to a fresh solution of 85:5:10 absolute ethanol:acetic acid: formaldehyde (40 % w/v) for 1 h, air drying and storage at −12 °C.

Samples were stained by the Feulgen reaction as described in Bolton, Oades & Johnson (1984), and stored at −12°C in the dark for a maximum of one week prior to densitometric quantitation.

Microdensitometry

Embryonic nuclei and liver nuclei were measured for their integrated optical density at 560 nm wavelength, with the ×100 objective of the Vickers M86 microdensitometer.

At each reading session, between 40 and 60 liver nuclei were read in order to obtain 2C and 4C DNA values and also to ensure that the stain had not faded if the readings were taken over more than one day.

For each embryonic nucleus, the average of two readings was taken. Blurred, damaged or obscured nuclei were excluded (5–10 % of the total). Overlapping nuclei were scanned together, and the average of two readings halved to give the value for each nucleus. This procedure was considered to be legitimate as the values obtained were always in the correct range, and the nuclei were from the same time point. For measurements of the third cell cycle, the percentage of overlapping nuclei was 5–10 % while in the fourth cell cycle it was 20–25 %. The number of nuclei in each embryo and the nuclei undergoing mitosis were noted.

When results from different experiments were pooled, the ratio of the 2C DNA values from each experiment was used as a 'standardizing factor' and assigned the value of 100 arbitrary units.

Use of aphidicolin

Aphidicolin (supplied courtesy of ICI Limited) was dissolved in dimethylsulphoxide [DMSO] to give a stock solution of 2 mg ml^{-1}. For embryo culture, the stock solution was diluted to 2 μg ml^{-1} aphidicolin in M16+BSA.

Fluorescent labelling of embryos

Embryos were dezonaed in acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975), washed in M2+BSA and decompacted by incubation in Ca^{2+}-free M2 containing 6 mg ml^{-1} of BSA for 5–10 min. After decompaction embryos were incubated for 5 min at room temperature in tetramethylrhodamine-labelled succinyl concanavalin A (TMRTC-Suc-Con A, 500 μg ml^{-1}; Polysciences). This solution was prepared by dialysing TMRTC-Suc-Con A against two changes of M2 for 8 h at 4 °C, followed by addition of 6 mg ml^{-1} BSA. Embryos were then transferred to PHA-coated coverslips forming the base of a small chamber filled with M2+4 mg ml^{-1} polyvinylpyrrolidone (M2+PVP) (technique described by Maro, Johnson, Pickering & Flach, 1984). The chamber was then sealed with a second coverslip and spun at 500 *g* for 10 min at 20 °C in a Sorvall RC-5 centrifuge using an HB-4 rotor to ensure that the embryos were well attached to the coverslip. The embryos were fixed with 3·7 % formaldehyde in phosphate-buffered saline (PBS) for 30–40 min at 20 °C. They were then washed four times in PBS, permeabilized with detergent (0·25 % Triton X-100 in PBS for 10 min at 20 °C), and finally washed in PBS.

After fixation, the embryos were labelled with affinity-purified antibody against either clathrin or actin as described in Maro, Johnson, Pickering & Louvard (1985) and Johnson & Maro (1984).

Hoechst staining

The chromatin was stained with Hoechst dye 33258 (gift of Dr B. Hogan) by incubation in M2+BSA containing 50 $\mu g\,ml^{-1}$ dye for 1 h at room temperature (Hilwig & Gropp, 1972). They were then rinsed and examined under a fluorescence microscope.

Fluorescence microscopy

Samples were examined using a Leitz Ortholux epifluorescence microscope with the incident source HB200, and equipped with Differential Interference Contrast (DIC) microscopy. Filter set L2 was used for the fluorescein labelling, set N2 for the rhodamine labelling, and set A for the Hoechst staining.

Scanning electron microscopy

The procedure was essentially that described by Johnson & Ziomek (1982). All solutions were Millipore-filtered immediately prior to use. Circular glass coverslips (diameter = 10 mm) notched to specify orientation and cleaned with absolute ethanol and lint-free paper, were air dried for 1 h before immersion in poly-l-lysine solution [1 $mg\,ml^{-1}$, PLL; Sigma type 1B] for 2 h. They were then washed 2–3 times in 0·1 M-cacodylate buffer (pH 7·6) and placed in individual wells of a Linbro 24-well tissue culture dish (Flow Laboratories) containing 0·1 M-cacodylate buffer.

Embryos were dezonaed and decompacted as described earlier and fixed for 30–60 min in 6 % (v/v) glutaraldehyde in 0·1 M-cacodylate buffer. They were then transferred to 1 % glutaraldehyde in 0·1 M-cacodylate buffer before being attached to the coverslips – the fixative ensured firm attachment of the embryos to the PLL-treated coverslips. Samples were then dehydrated through a graded series of alcohols, transferred to the specimen holder for critical-point drying, and dried from alcohol via CO_2 in a Polaron E3000.

The coverslips were then mounted on stubs using a small drop of DAB (silver glue) and stored in a desiccator until examined. Just prior to viewing, samples were coated with a 50 to 60 nm-thick layer of gold in a Polaron E5000 Diode Sputtering system and then examined in a JEOL 35 CF Scanning Electron Microscope.

Photography

Phase photographs were taken on Ilford Pan-F film (exposure time 2–4 s) using a Wild Inverted Phase microscope; for fluorescence photography, the Vario-orthomat photographic system of the Leitz Ortholux epifluorescence microscope was used, the pictures being taken on Kodak Tri-x film.

Statistical analysis of graphs by computer

Curve fitting to graph points was carried out using the Statistical Analysis System – Non-linear Curve Fitting Procedure.

RESULTS

In order to ensure that the round of DNA replication in any given developmental cell cycle is inhibited completely, populations of relatively synchronized cells must be used and the inhibitor must be added during the G_1 phase. G_1 is short (0·5 to 1·5 h) at 2-, 4- and 8-cell stages (Bolton *et al.* 1984; Smith & Johnson, 1985),

and so embryos were examined at hourly intervals for the first signs of division, and any so detected were removed for experimental use.

1. *The effect on compaction of inhibition of DNA replication in the third and fourth cell cycles*

Compaction occurs in the 4th cell cycle at the 8-cell stage. First we examined the effect on compaction of adding aphidicolin in G_1 of the 3rd cell cycle. Late 2-cells were cultured and scored at 1 h intervals. Any embryos in which one or both blastomeres had divided were removed and cultured either in medium containing aphidicolin or in control medium. It was considered legitimate to use both 3- and 4-cell embryos since (a) G_2 of the 2-cell stage is long, being in excess of 8 h (Sawicki, Abramczuk & Blaton, 1978), and (b) 97 % of 2-cell embryos will form 4-cells within 4 h (Smith & Johnson, 1985). Thus, it is unlikely that the slower blastomeres of 3-cell embryos will be in S-phase of the second cell cycle. Both groups of embryos were cultured for up to 25 h during which time they were scored in three ways:–

(i) Embryos were sampled for analysis of DNA content. In the control group many blastomeres were at the 16-cell stage, but the aphidicolin group consisted exclusively of 4-cell embryos containing four nuclei. The values for the DNA content of blastomeres in each group are recorded in Table 1. The values for control blastomeres depend upon whether the cells have completed S phase of the 4th cell cycle, have divided to enter the 5th cell cycle or have entered S phase of that cycle. It is clear, however, that aphidicolin-treated embryos contain approximately 2C levels of DNA, and thus two consecutive rounds of DNA synthesis have been suppressed effectively.

(ii) Embryos were scored for the cell flattening that occurs at compaction. Embryos in both groups showed cell flattening (Fig. 1), and the time course of flattening in the aphidicolin-arrested 4-cell embryos was similar to that of controls (Fig. 2).

(iii) Embryos were examined 23–24 h after division to 4-cells for evidence of the development of polarity. The development of cell surface polarity occurred

Table 1. *Content of DNA in blastomeres from normal embryos and from embryos treated continuously with aphidicolin from the early 4-cell stage*

	Aphidicolin-treated	Controls					
No. of cells per embryo	4c	8 to 9c		10 to 14c		15 to 16c	
Type of blastomere	1/4	1/16	1/8	1/16	1/8	1/16	1/8
Average DNA content	15·33	17·4	32·8	18·2	32·2	19·7	33·3
Standard deviation	1·78	1·6	2·6	2·9	3·6	2·8	–
No. of blastomeres analysed	129	8	56	72	18	141	1

Liver standard: 2C DNA value = 17·77
4C DNA value = 35·35

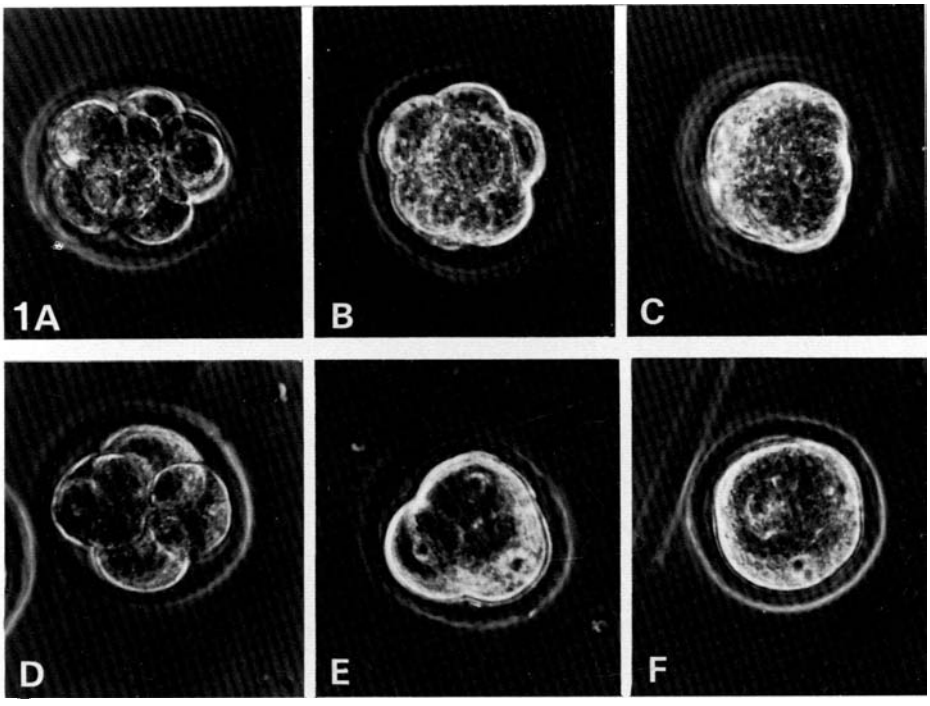

Fig. 1. Phase contrast micrographs illustrating the process of compaction in (i) control embryos: (A) non-compacted early 8-cell embryo; (B) 8-cell embryo undergoing compaction ('partially compacted'); (C) fully compacted late 8-cell embryo. (ii) embryos treated with aphidicolin from G_1 of the 4-cell stage; (D) non-compacted; (E) partially compacted; and (F) fully compacted embryos each containing only 4-cells. Magnification ×300.

normally in aphidicolin-arrested 4-cell embryos, whether assessed by scanning electron microscopy (Fig. 3) or by binding pattern of fluoresceinated concanavalin A (Table 2; Fig. 4A,C,E,G). Similarly, polar redistribution of actin and clathrin was the same in control and aphidicolin-treated embryos (Fig. 4B,D,F,H).

2. *The effect on compaction of inhibition of DNA replication in the second cell cycle*

The lack of effect on compaction of inhibition of the third round of DNA replication led us to investigate the effect of inhibition of DNA replication in the preceding cell cycle. Previous work has shown that addition of aphidicolin to newly formed 2-cell embryos results in inhibition of DNA replication and of cleavage to 4-cells (Bolton *et al.* 1984). Therefore late 1-cell eggs were examined at 1 h intervals, any 2-cell embryos formed during the preceding hour were removed and allocated to one of four groups. Group-1 embryos were placed directly into aphidicolin. Group-2 embryos were placed into aphidicolin but were removed 10 h later after DNA replication was completed in control embryos (Bolton *et al.* 1984); the embryos were then washed thoroughly and transferred to M16+BSA. Group-

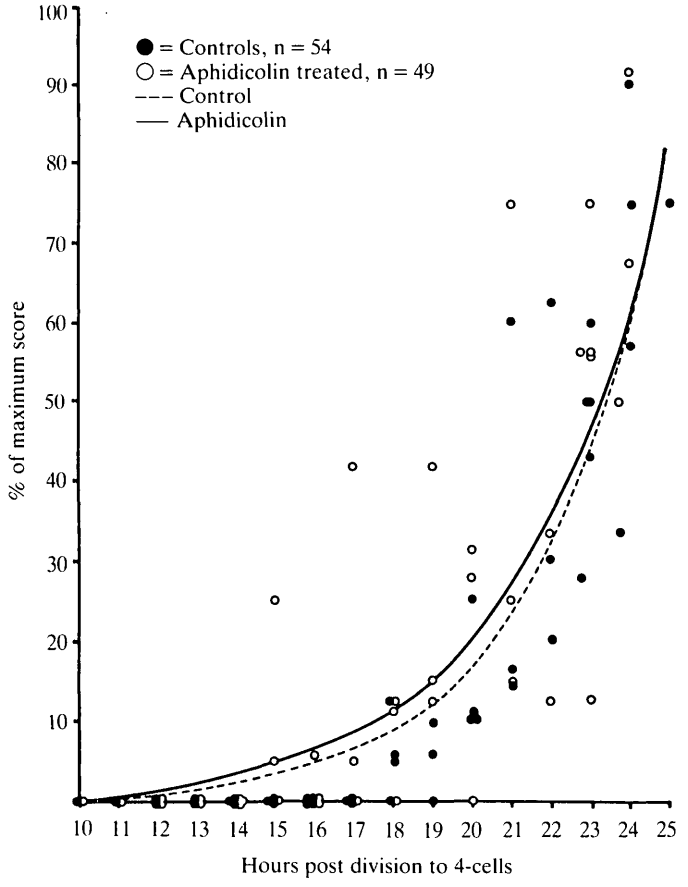

Fig. 2. Timing of compaction in control embryos and embryos exposed to aphidicolin from the early 4-cell stage. Using a scoring system of 1 for a partially compacted embryo and 2 for a fully compacted embryo, points representing each group of embryos were calculated from the total score in each group and expressed as a percentage of the maximum score possible. The curves were calculated by computer from the data points.

Table 2. *Surface phenotype of blastomeres as scored by fluorescent Con A binding in control late 8-cell embryos and in a population of embryos of equivalent age but exposed to aphidicolin from the early 4-cell stage*

Treatment	No. of blastomeres studied	% age of blastomeres		
		Polar	Apolar	Not classified
Controls	159 (8-cells)	72	19	9
Aphidicolin-treated	80 (4-cells)	70	21	9

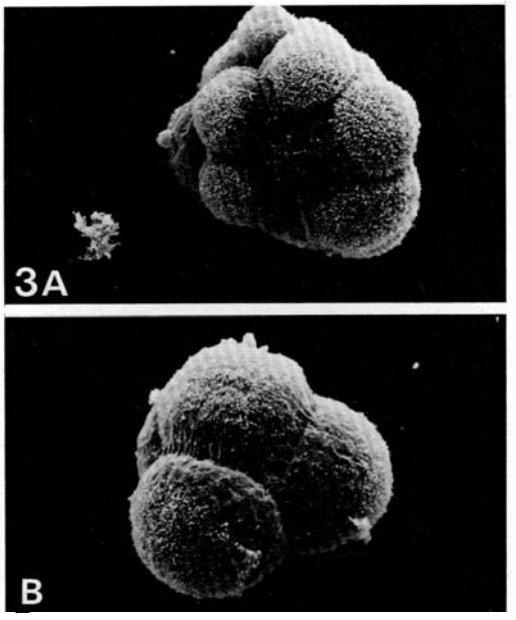

Fig. 3. Polarization in control embryos and in embryos exposed to aphidicolin from the early 4-cell stage as assessed by scanning electron microscopy. (A) Control embryo in transition from 8- to 16-cells and decompacted in medium low in Ca^{2+}. Note the apical pole of microvilli on each blastomere. (B) Embryos treated with aphidicolin also polarize in this manner despite having only four cells. Magnification ×700.

3 embryos were placed in M16+BSA for 10 h and then transferred to aphidicolin. Group-4 embryos were placed in M16+BSA. As expected, 2-cell embryos exposed continuously to aphidicolin did not undergo further cleavage, and each nucleus had a DNA content of only a little above 2C (Table 3, Group 1). Control embryos developed normally (Table 3, Group 4). The majority of embryos in Group 3, which were placed in aphidicolin *after* the period of 2-cell DNA replication divided, and, as expected, all further DNA replication was blocked, the 2-cell nuclei having a 4C and the 4-cell nuclei a 2C DNA content.

Embryos in Group 2, pulsed with aphidicolin over a 10 h period that included the S phase of the second cell cycle, remained as 2-cells but with a DNA content of 4C. Therefore, embryos in this group were examined at 5 h intervals after removal from aphidicolin to determine whether they replicated their DNA immediately after withdrawal from the drug, or whether their DNA replication occurred at the same time as replication was occurring in control embryos at the early 4-cell stage. At the time of withdrawal from aphidicolin, the DNA content of Group-2 embryos was 2C (67 nuclei examined); 5 h and 10 h later the DNA content had doubled to 4C (74 and 73 nuclei examined respectively). Thus, 'catch-up' replication occurred during the late 2-cell stage, but no further rounds of replication occurred, despite the absence of aphidicolin.

When the control embryos had developed to the late 8-cell stage, embryos were taken from each of the four groups and examined at light and electron microscope levels for evidence of flattening and the development of polarity. The results are summarized in Table 4. The control group 4 showed the expected high incidence of flattening and polarization except in three retarded embryos. The embryos exposed to aphidicolin continuously (Group 1) showed no evidence of flattening or polarization (Fig. 5A,C). Group-2 embryos placed in aphidicolin for the first 10 h of the 2-cell stage showed almost complete suppression of flattening (one embryo) and polarization (two blastomeres). Moreover, incubation of these embryos for an extra 10 h or 24 h did not result in any increase in polarization indicating that the drug had exerted an inhibitory rather than a delaying effect. In contrast, Group-3 embryos, exposed to aphidicolin from the mid-2-cell stage onwards, showed a high incidence of polarization and a substantial degree of flattening, whether or not they blocked at 2- or 4-cells (Fig. 5B,D–F). The polarity in this group of embryos differed from controls in that in control embryos, the pole of Con A binding and microvilli is opposite to the point of intercellular contact, whereas in the aphidicolin-treated embryos poles were often eccentrically placed (Fig. 5D,E). Also, in many blastomeres a sharply defined 'ridge' separated the microvillous and non-microvillous domains.

DISCUSSION

In early embryos of most species studied, a close relationship exists between the occurrence of identifiable developmental events and the number of developmental cell cycles elapsed since fertilization. In the mouse embryo, for example, the first expression of the embryonic genome, the changing morphology at compaction and the beginnings of blastocoel expansion each occur at characteristic times, namely in the second, fourth and sixth developmental cell cycles respectively (Flach *et al.* 1982; Lehtonen, 1981; Smith & McLaren, 1977). A number of studies have investigated the extent to which this association is incidental rather than causal, and, should the latter be true, the nature of the underlying time-keeping mechanism. In this paper, we report on our examination of the relationship between DNA replication in the second, third and fourth cell cycles and compaction in the fourth cell cycle.

The major developmental event of compaction was found to be remarkably independent of proximate replication. Thus, complete suppression of DNA replication in the third and fourth cell cycles is without detectable effect on compaction. This observation makes it unlikely that 'developmental' time is measured solely by replication cycles. Indeed, we noticed that when embryos in the control population were cleaving from 4- to 8-cells, the aphidicolin-treated embryos remained as 4-cells but showed surface deformation as though attempting unsuccessful division. Similar observations have been made on enucleated *Xenopus*, sea-urchin, newt and mouse eggs (Yoneda, Ikeda & Washitani, 1978;

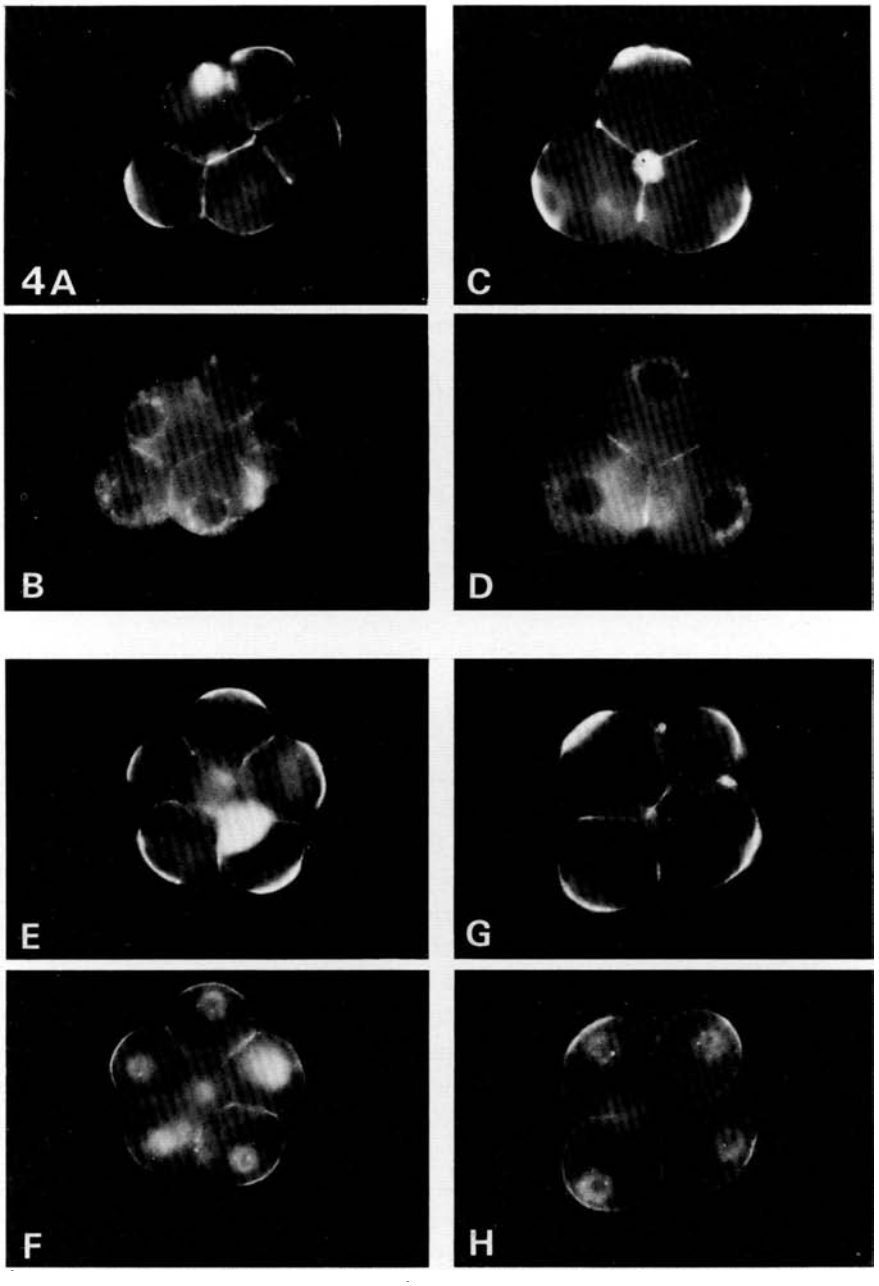
4A
C
B
D
E
G
F
H

Table 3. *Cell number and blastomere DNA content of embryos incubated from the very early 2-cell stage to the late 8-cell stage under various conditions*

Treatment	Mean of microdensitonometric values for DNA content ±S.E. (No. of blastomeres)		
	2-cell blastomeres	4-cell blastomeres	8-cell blastomeres
Group 1. Continuous aphidicolin	19·49 ± 2·24 (70)	–	–
Group 2. Aphidicolin pulse for first 10 h of 2-cell stage	34·66 ± 3·5 (64)	–	–
Group 3. Aphidicolin added 10 h into 2-cell stage	35·31 ± 2·19 (35)	18·30 ± 2·28 (60)	–
Group 4. Control	–	–	32·75 ± 3·6 (40)
Liver standard: 2C DNA value = 17·77 4C DNA value = 35·35			

Sawai, 1979; Hara, Tydeman & Kirschner, 1980; Waksmundzka *et al.* 1984), leading to the suggestion that a 'cytoplasmic clock' can run for a few cell cycles in the absence of a nucleus. Whether the run down of this putative clock, that occurs several cell cycles after enucleation, is due simply to the lack of adequate metabolic support in the absence of a nucleus, or whether the nucleus gives a short-term periodicity to the cytoplasm that can then run for a while after enucleation is not clear (Satoh, 1982).

In contrast to the resistance of compaction to aphidicolin applied early in the third cycle, suppression of the second round of DNA replication prevented compaction. This inhibition was not due to the inability of embryos containing only two cells to undergo compaction, as revealed by the few arrested, but nonetheless compacted 2-cell embryos observed when aphidicolin was added early in G_2 of the second cell cycle (see also Goddard & Pratt, 1983). Neither was the inhibitory effect of aphidicolin likely to be due merely to its prolonged presence

Fig. 4. Polarization in control embryos and in embryos treated with aphidicolin from the early 4-cell stage. Control 8-cell embryos are shown in the left-hand series of photographs (A,B,E,F). The aphidicolin-treated 4-cell embryos, on the right (C,D,G,H), express an identical polarization pattern to the controls. (A,C,E,G) the apical surface poles on the blastomeres are visualized by the binding of Con A. (B,D) double staining of embryos shown in A and C – antibody to clathrin reveals clustering of clathrin-coated endocytotic vesicles between the nucleus and the overlying membrane. The position of these aggregates correlates with the position of the apical surface pole visualized by Con A binding (A,C). (F,H) staining with anti-actin antibodies. The polar localization of actin both cortically (in the region of the membrane) and subcortically (between the nucleus and the overlying membrane) follows the same orientation as the Con A pole (E,G).

Table 4. *Incidence of intercellular flattening and polarity in blastomeres of embryos exposed to various treatments and examined when control embryos were at the late 8-cell stage*

Treatment	Total No. of embryos analysed	No. as 2-cells	% of these flattened	% of their blastomeres polarized	No. as 4-cells	% of these flattened	% of their blastomeres polarized	No. as 8-cells	% of these flattened	% of their blastomeres polarized
Group 1. Continuous Aphidicolin	185	185	0	0	0	–	–	0	–	–
Group 2. Aphidicolin for first 10 h of 2-cell stage	70	67	2	1	3	0	12	0	–	–
Group 3. Aphidicolin added 10 h into 2-cell stage	175	71	46	70	104	51	93	0	–	–
Group 4. Controls	78	2	0	0	1	0	0	75	91	91

from early in G_1 of the 2-cell stage, since the inhibitory effect was *not observed* when the drug was added only 10 h later (*i.e.* was present for about 32 h instead of 42 h) but *was observed* when the drug was present for a total of only 10 h during the first half of the long 2-cell stage. Strikingly, in this latter group of embryos, DNA replication occurred over the 5 h period immediately following withdrawal of the drug. Thus, the inhibition of compaction was only observed under conditions in which the second round of DNA replication did not take place at the appropriate time in the second cell cycle. It is possible that a mismatch between cytoplasm and replicating DNA results in a failure of some modifying action of the cytoplasm on

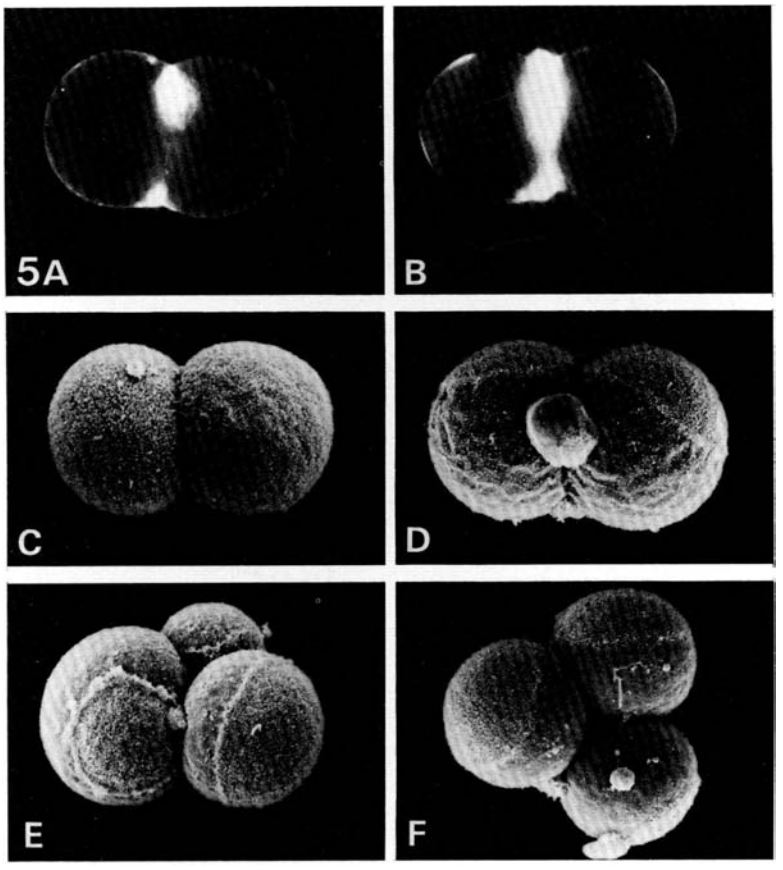

Fig. 5. Fluorescence photomicrographs (A,B) and scanning electron micrographs (C–F) of embryos placed in aphidicolin during the 2-cell stage and cultured until control embryos were compacted late 8-cells or early 16-cells. All embryos were then dezonaed and exposed to Ca^{2+}-free medium. (A,C) embryos cultured continuously in aphidicolin from G_1 of the 2-cell stage. Note absence of surface polarity. (B,D–F) embryos cultured continuously in aphidicolin from early G_2 of the 2-cell stage. Note that polarity develops whether or not division from 2- to 3- or 4-cells has taken place. However, frequently the polarity is not opposite to the point of intercellular contact, e.g. (D,E) as in controls (see 3A,B). Also, a marked ridge defines the polar and unpolar regions in some blastomeres.

the DNA and thereby prevents the events of compaction at the 8-cell stage from occurring. However, interpretation of an inhibitory action of a drug beyond its immediate target, in this case DNA replication, is difficult. For example, mismatched replication also resulted in a failure to divide to 4-cells, and whilst aphidicolin does not directly inhibit division (e.g. see Group-3 embryos in Table 3), it does not necessarily follow that the failure of Group-2 embryos to divide resulted from a direct effect of the drug on DNA replication. The only conclusion that can be drawn with confidence from these results is that the third and fourth rounds of DNA replication are irrelevant to compaction.

This independence from proximate replication of a key developmental event in early mouse embryogenesis has also been reported with respect to the major activation of many embryonic genes that occurs at the mid-2-cell stage in the absence of the preceding second round of DNA replication (Bolton *et al.* 1984). Moreover, when the fourth round of DNA replication was delayed by 8 h, blastocoel formation occurred, but did so slightly earlier, and with half the number of cells, and therefore presumably after one less round of DNA replication (although this was not tested directly: Dean & Rossant, 1984). These results on the mouse are reminiscent of those reported for Ascidian embryos, in which nuclear replication cycles appear to be critical for developmental events observed two or more cell cycles later (Meedel & Whittaker, 1979; Satoh & Ikegami, 1981*a*,*b*).

There are two conclusions that might be drawn from these results. The conservative conclusion is that when drugs such as aphidicolin only have developmental effects after relatively long exposure periods, the results are artefactual and should be questioned seriously. Despite the controls for long exposure to the drug described above, this view cannot be denied. A less-conservative conclusion is also possible, and its value lies in directing attention to more definitive experimental approaches. There is evidence to support the notion that changes in the organization of chromatin may be required if the potential for subsequent transcriptional activity of genes is to be achieved (e.g. Groudine & Weintraub, 1982; Burch & Weintraub, 1983). The actual expression of the genes in the form of utilizable transcripts, and the use of these transcripts, may occur later in response to temporal or spatial signals. Many of the relevant changes to chromatin organization that yield the potential for expression require, or are facilitated by, replication (e.g. Razin & Riggs, 1980; Leffak, 1984; Poccia *et al.* 1984). In some types of early embryo, there is evidence that the reduction in nucleocytoplasmic ratio that occurs during cleavage without growth can 'dilute out' maternally derived cytoplasmic components and thereby permit nuclear changes (Newport & Kirschner, 1982*a*,*b*). Such a mechanism appears to be less likely in the mammalian embryo (reviewed Johnson *et al.* 1984), and perhaps more likely is the temporally programmed synthesis of a cytoplasmic factor that could interact with replicating chromatin at the 2-cell stage (e.g. see Bolton *et al.* 1984; Howlett & Bolton, 1985). It should be possible to test directly these alternative mechanisms and this we are now attempting.

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