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Temporal control of gap junction assembly in preimplantation mouse embryos

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SUMMARY

The de novo assembly of gap junctions during compaction in the 8-cell stage of mouse development is a temporally regulated event. We have performed experiments designed to explore the relationship between this event and DNA replication in the second, third, and fourth cell cycles after fertilization. Inhibition of DNA synthesis by continuous treatment with the DNA synthesis inhibitor, aphidicolin, during the third and fourth cell cycles had no effect on the establishment of gap junctional coupling during compaction. However, a delay of 10 hours in DNA synthesis during the second cell cycle caused by a transient aphidicolin treatment resulted in the failure of gap junctional coupling at the time of compaction. Thus the timing of establishment of gap junctional coupling, like the timing of compaction itself, is linked to DNA replication in the 2-cell stage. Immunofluorescence analysis showed that the failure of gap junctional coupling after aphidicolin treatment in the 2-cell stage is correlated with the failure of nascent connexin43 to be inserted into plasma membranes. We propose that the developmental 'clock' that controls gap junction assembly is set in motion by events surrounding the second cycle of DNA replication, and that this 'clock' ultimately controls the post-translational processing of connexin43.

Key words: dye coupling, connexin43, preimplantation development, compaction, DNA synthesis, protein trafficking

INTRODUCTION

One of the most striking features of embryonic development is the precise timing and orderly sequence of morphological and molecular events. Numerous mechanisms have been suggested to account for temporal regulation, including time elapsed since fertilization, absolute number of cells, number of mitoses, number of DNA replication cycles, critical nucleocytoplasmic ratios, and endogenous cytoplasmic 'clocks'. In a few instances the time-keeping mechanism responsible for the onset of a developmental event has been elucidated. In *Xenopus*, for example, an event termed midblastula transition (MBT) is associated with transcriptional activation of the embryonic genome, the first appearance of G_1 and G_2 phases of the cell cycle, and initiation of both asynchronous cell divisions and cell motility (Kirschner et al., 1985; Satoh, 1985). The timing of the MBT is independent of the number of mitoses, the number of DNA replication cycles, and the time elapsed since fertilization, but is dependent, at least in part, on the nucleocytoplasmic ratio (Kobayakawa and Kubota, 1981; Newport and Kirschner, 1982a,b). It has recently been suggested that the MBT concept is an oversimplification, and that rather than a single transition, several temporally independent transitions take place (Yasuda and Schubiger, 1992). Indeed, more recent experiments with *Xenopus* indicated that the transcriptional activation that accompanies the MBT may not be linked with the N/C ratio but, like gastrulation, may be timed by an autonomous cytoplasmic 'clock' (Lund and Dahlberg, 1992; Kobayakawa and Kubota, 1981; Satoh, 1985; Kirschner et al., 1985; Cooke and Smith, 1990). This illustrates an important point, that all developmental events within an embryo are not necessarily controlled by a single time-keeping mechanism, but rather there may be several mechanisms operating simultaneously and independently. An MBT-like event also takes place during *Drosophila* embryogenesis, the timing of which likewise appears to be controlled by the nucleocytoplasmic ratio (Edgar et al., 1986; Cooke and Smith, 1990). The available evidence on the timing of appearance of three different histospecific enzymes in ascidian embryos indicates that they are all regulated by the absolute number of rounds of DNA replication, but that the actual number of rounds required is specific for each enzyme (Satoh and Ikegami, 1981a,b; Satoh, 1982).

The temporal control of events in preimplantation mouse development has also received considerable attention, although in most cases more is known about mechanisms that are not involved than those that are. For example, transcriptional activation of the zygotic genome in the 2-cell stage is not dependent on cytokinesis or the second round of DNA replication (Bolton et al., 1984), although a dependence on the first round of DNA replication has been documented (Howlett, 1986; Petzoldt, 1984). The timing of the appearance of several

stage-specific proteins has been shown to be independent of both cytokinesis (Petzoldt, 1986) and the nucleocytoplasmic ratio (Petzoldt and Muggleton-Harris, 1987). The timing of compaction has been demonstrated to be independent of immediate transcription or translation (Kidder and McLachlin, 1985; Levy et al., 1986) and of DNA replication in the third and fourth cell cycles after fertilization (Smith and Johnson, 1985). On the other hand, the timing of cavitation does seem to be influenced by the N/C ratio, since lowering it caused a delay in the formation of nascent blastocoels (Evsikov et al., 1990).

The initiation of gap junctional communication in preimplantation mouse embryos is also temporally regulated. It occurs during compaction, shortly after the third cleavage (8-cell stage). The involvement of microtubules and microfilaments, cell flattening, cytokinesis, and immediate gene expression have all been eliminated as participating in the time-keeping mechanism controlling this event (Goodall and Johnson, 1982; McLachlin et al., 1983; McLachlin and Kidder, 1986; Goodall, 1986; Kidder et al., 1987). The question of what does control the timing of gap junction assembly, however, is still unanswered. Here we describe experiments that address this question.

Our interest in this question originated in the finding by Smith and Johnson (1985) that the timing of compaction is linked to DNA synthesis in the second cell cycle. Embryos in which the second round of DNA synthesis was delayed by 10 hours by treatment with aphidicolin, an inhibitor of DNA polymerases α and δ (Sheaff et al., 1991; Sorscher and Cordeiro-Stone, 1991), failed to compact, demonstrating that not only is there a simple requirement for the second DNA replication cycle, but that it must take place within a specific window of time. Given the temporal correspondence between compaction and gap junction assembly, it was of obvious interest to investigate gap junctional coupling in aphidicolin-treated embryos. Experiments were carried out to determine if the onset of gap junctional coupling in mouse preimplantation embryos is dependent on prior DNA replication cycles. The results were correlated with the effects of aphidicolin treatment on the spatial distribution of connexin43 (Cx43), a gap junction protein which contributes to the first gap junctions that form in the preimplantation embryo (Valdimarsson et al., 1991; Nishi et al., 1991; De Sousa et al., 1993).

MATERIALS AND METHODS

Embryo collection and culture

Superovulation and embryo collection were as described previously (Barron et al., 1989). Some of these experiments required that embryos be cultured through the 2-cell stage. The CF1 × CB6F$_1$/J embryos used in these experiments are frequently affected by the '2-cell block' (Goddard and Pratt, 1983). This problem was overcome by using CZB culture medium (Chatot et al., 1989) in all experiments. All culturing was done at 37°C, 5% CO_2 in air, and 100% relative humidity. The culture medium was pre-equilibrated for at least 2 hours prior to embryo addition.

Aphidicolin treatment

Aphidicolin (Sigma) was made up as a 2 mg/ml stock in DMSO and added to the culture medium to a final concentration of 2-4 μg/ml. Control cultures contained DMSO at 0.1-0.2%.

Two distinct experimental protocols were used in this study, both based on that of Smith and Johnson (1985). In the first protocol, 1-cell zygotes were flushed at 29 hours post-hCG and then washed three times with flushing medium I (FM-I; Spindle, 1980) followed by two CZB washes. The embryos were placed in CZB for about 0.5 hour and then transferred, in groups of about 30, to 30 μl drops of CZB under paraffin oil. Observations were made at 1 hour intervals, over a 4- to 5-hour period, and all 2-cell embryos and 1-cell embryos showing signs of cell division were removed. The embryos that were removed at each time point were split equally between CZB drops (30 μl) containing aphidicolin and those with DMSO under paraffin oil, and cultured for 10 hours. At the end of this culture period the embryos were washed separately through five drops of pre-warmed CZB to remove the drug, placed separately in drops of fresh CZB (30 μl) under oil, and cultured until they were either tested for dye coupling (beginning at 80-83 hours post-hCG) or fixed for immunofluorescence (85 hours post-hCG). At 74 hours post-hCG, glucose (2 μl of 100 mg/ml in CZB) was added to each culture drop.

In the second protocol, 2-cell embryos were flushed at 50 hours post-hCG and handled in essentially the same fashion as described above. At hourly intervals 4-cell and 3-cell embryos, and 2-cell embryos showing signs of division to 3 cells, were removed from the CZB drops and transferred to aphidicolin and control drops. The embryos were cultured continously in these drops until testing for dye coupling was begun at 80-81 hours post-hCG. Glucose was added as described above. In some of these experiments the cell number of the embryos was determined by counting DAPI-stained nuclei. The embryos were fixed overnight in 4% paraformaldehyde in calcium- and magnesium-free phosphate buffered saline containing 3 mg/ml polyvinylpyrrolidone (CMF-PBS-PVP), stained for 1 hour in 5 μg/ml DAPI (Polysciences, Warrington, PA) in CMF-PBS-PVP, washed three times in CMF-PBS-PVP, and mounted on a microscope slide in 9:1 (v/v) glycerol:10× Tris-buffered saline. The embryos were examined and the nuclei counted using a Zeiss Photomicroscope I fitted with epifluorescence optics.

Dye injections

Embryos were tested for dye coupling by iontophoretic injection of 10 mM 6-carboxyfluorescein as previously described (De Sousa et al., 1993). Embryos were scored positive for dye coupling if dye could be detected in all the blastomeres within 20 minutes or less.

Immunofluorescence microscopy

The immunofluorescence procedure used was as described by Valdimarsson et al. (1991) and De Sousa et al. (1993). Briefly, embryos were fixed in 1% paraformaldehyde in PHEM buffer (PHEM = 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6.9) for 1 hour at 4°C. All subsequent steps of the procedure were also carried out at 4°C. The embryos were permeabilized with 0.1% Tween-20. After blocking with CMF-PBS-PVP containing 1% bovine serum albumin (BSA) and 0.1% Tween-20 (block solution), the embryos were treated overnight with the primary antiserum (see below) in block solution and then washed extensively with 0.1% Tween-20 in CMF-PBS-PVP. This was followed by a 1 hour incubation with secondary antibody (see below) in block solution, followed by more washes. The immunostained embryos were analyzed on a Bio-Rad MRC-600 Confocal Laser Scanning Microscope. To facilitate comparisons between samples, all parameters were kept constant during image collection (black level = 5; gain = 10; laser neutral density filter = 3; pin hole = 1) except that for embryos treated with the peptide-absorbed antiserum the pin hole setting had to be increased to 4 to make the embryos visible. A series of images was collected by focusing through each embryo and collecting images at 0.5 μm intervals. For presentation, three such images were stacked on top of one another, thus creating an approximately 1 μm optical section, which was then edge enhanced with filter C9A.

Immunolabeling was performed with an affinity-purified rabbit

antibody raised against a synthetic peptide corresponding to amino acids 302-319 of Cx43 (a gift from Dr Bruce Nicholson, SUNY, Buffalo; De Sousa et al., 1993). The crude antiserum, absorbed three times with the 302-319 peptide, served as a control. Both the affinity-purified antibody and the peptide-absorbed serum were used at a concentration of 6 μg/ml. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICN Biomedicals Canada Ltd., St. Laurent, Québec) at a dilution of 1:50 was used to localize bound primary antibody.

RESULTS

Effects on dye coupling of inhibiting both the third and fourth rounds of DNA replication

The relation between DNA replication and the onset of gap junctional coupling was investigated using the DNA synthesis inhibitor, aphidicolin. As part of the protocol for these experiments it was necessary to inhibit both the third and fourth rounds of DNA replication. It was therefore important to begin aphidicolin treatment before the initiation of DNA synthesis in the 4-cell stage. Two features of the cell cycles in early mouse embryos made this difficult. First, there is considerable asynchrony in the timing of developmental events within a population of embryos. Second, the G_1-phase of the third cell cycle lasts only about 1 hour (Smith and Johnson, 1986; Pratt, 1987). If drug treatment were initiated too early in an attempt to target this phase, complications could arise because of inhibition of the preceding round of DNA synthesis in some embryos. The experimental protocol was designed to avoid these potential problems. First, by initiating drug treatment only on embryos that had already divided, or were dividing, it was possible to ensure that the preceding S-phase had been complete. Second, by selecting embryos at hourly intervals the possibility that some cells would initiate DNA synthesis before the start of aphidicolin treatment was minimized.

Both aphidicolin-treated and age-matched control embryos were tested for dye coupling at a time when the control embryos had become fully compacted. Since both drug-treated and control embryos were tightly compacted, it was necessary to use DAPI staining to determine the cell number. Counting of DAPI-stained nuclei showed that the DMSO-treated controls were mostly 8-cell embryos (mean number of nuclei=8.7 ± 1.8, n=17), whereas the aphidicolin-treated embryos remained as 4-cell embryos (mean number of nuclei=4.3 ± 0.8, n=28). This is in agreement with the results of Smith and Johnson (1985) and shows that 4-cell embryos cultured continuously in aphidicolin do not undergo further cell divisions, but do become compacted. When the drug-treated, 4-cell compacted embryos were tested for dye

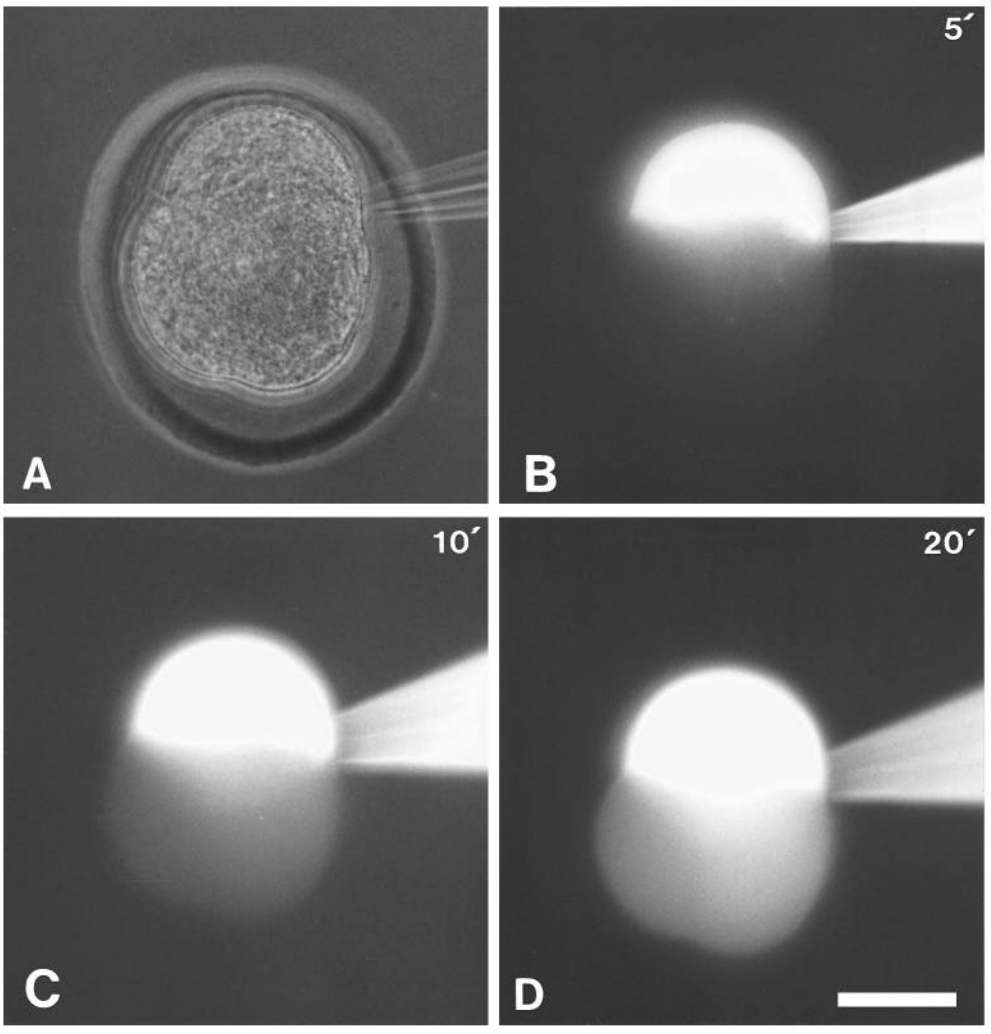

Fig. 1. Inhibition of both the third and fourth rounds of DNA replication does not affect the initiation of gap junction assembly. Embryos were tested for dye coupling after being treated continuously with aphidicolin from the early 4-cell stage (53-56 hours post-hCG) until age-matched controls formed compacted morulae (80-87 hours post-hCG). The treated group remained as 4-cell embryos. These embryos became compacted (as can be seen in the phase-contrast image in A) and they also became dye-coupled, as shown in the fluorescence images in B,C, and D (5, 10, and 20 minutes after the start of dye injection, respectively). Bar, 25 μm.

Table 1. Incidence of dye coupling in embryos cultured continuously in aphidicolin from the early 4-cell stage (53-56 hours post-hCG) to the compacted 8-cell stage (80-87 hours post-hCG)

Treatment	Dye coupled (%)	Not dye coupled (%)
Control (*n*=14)	13 (92.9)	1 (7.1)
Aphidicolin (*n*=16)*	14 (87.5)	2 (12.5)

*Data were collected in three separate test sessions. The percentage of dye-coupled embryos exceeded 83% in each session.

Table 2. Incidence of dye coupling in embryos treated with aphidicolin for 10 hours at the 2-cell stage (beginning at 31-35 hours post-hCG)

Treatment	Dye coupled (%)	Not dye coupled (%)
Control (*n*=28)	27 (96.4)	1 (3.6)
Aphidicolin (*n*=26)*	5 (19.2)	21 (80.8)
Compacted (*n*=12)	5 (41.7)	7 (58.3)
Non-compacted (*n*=14)	0 (0.0)	14 (100.0)

Dye coupling tests were done at 79-88 hours post-hCG.
*Data were collected in five separate test sessions. A single dye-coupled embryo was observed in each session.

coupling, most were found to be fully dye-coupled (Fig. 1; Table 1) and no difference in dye coupling was observed between control and drug-treated embryos (Table 1). Thus, inhibiting both the third and fourth rounds of DNA replication did not prevent gap junctions from being assembled at the normal time.

Effects on dye coupling of delaying the second round of DNA replication by 10 hours

The protocol for the initiation of drug treatment used in this experiment was adopted for essentially the same reasons that were outlined for the previous experiment. The G_1-phase of the second cell cycle is also about 1 hour in duration (Pratt, 1987).

Embryos treated with aphidicolin for 10 hours beginning in the early 2-cell stage remained as 2-cell embryos at the end of the culture period (after about 40 hours in drug-free medium). At this time most control embryos had formed compacted morulae, whereas about half of the aphidicolin-treated 2-cell embryos were compacted (Fig. 2A) and the other half were uncompacted (Fig. 3A).

Dye coupling tests revealed that the spread of dye was severely restricted in aphidicolin-treated 2-cell embryos

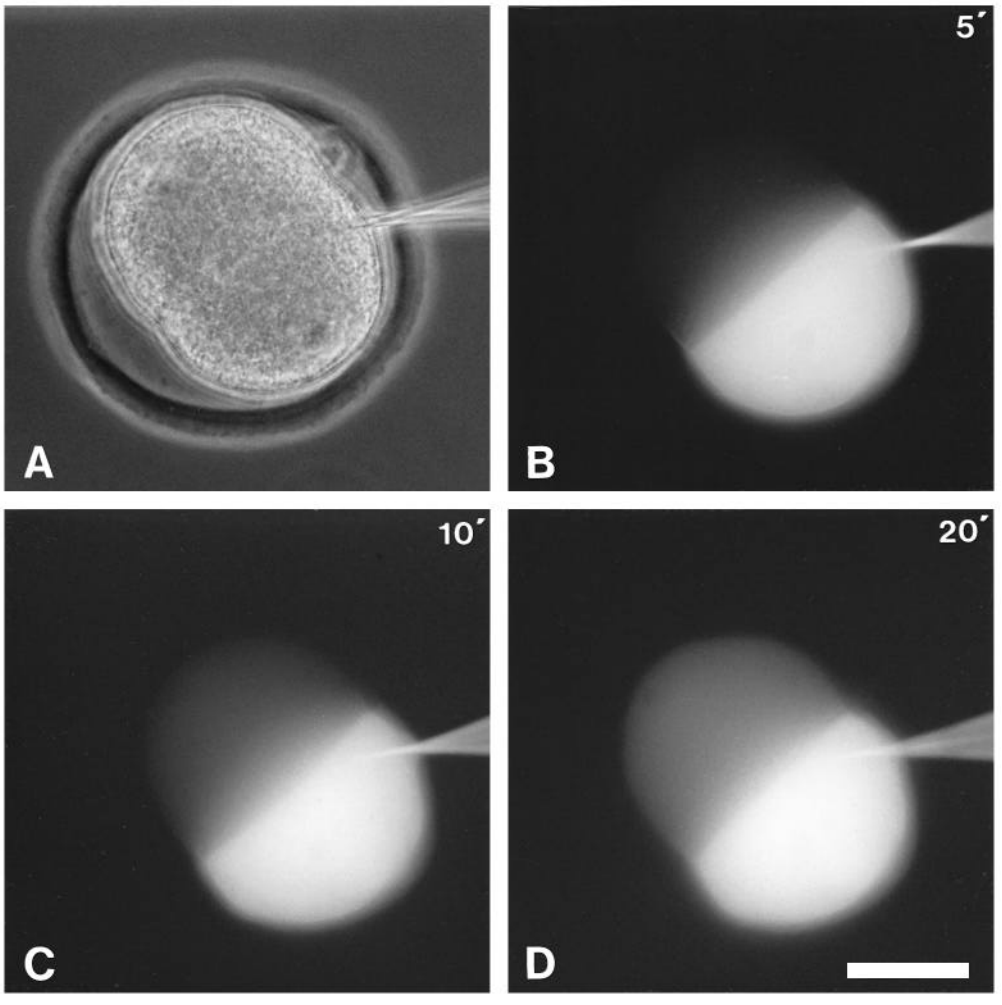

Fig. 2. Delaying the second round of DNA replication does not always inhibit compaction and a few embryos can even become dye-coupled. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 31-35 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age-matched controls formed compacted morulae (79-88 hours post-hCG), at which time both were tested for dye coupling. The treated group remained as 2-cell embryos. About half of the treated embryos compacted, as can be seen in the phase-contrast image in A, and about half of those that compacted were also dye-coupled, as shown in the fluorescence images in B, C, and D (5, 10, and 20 minutes after the start of dye injection, respectively). Bar, 25 μm.

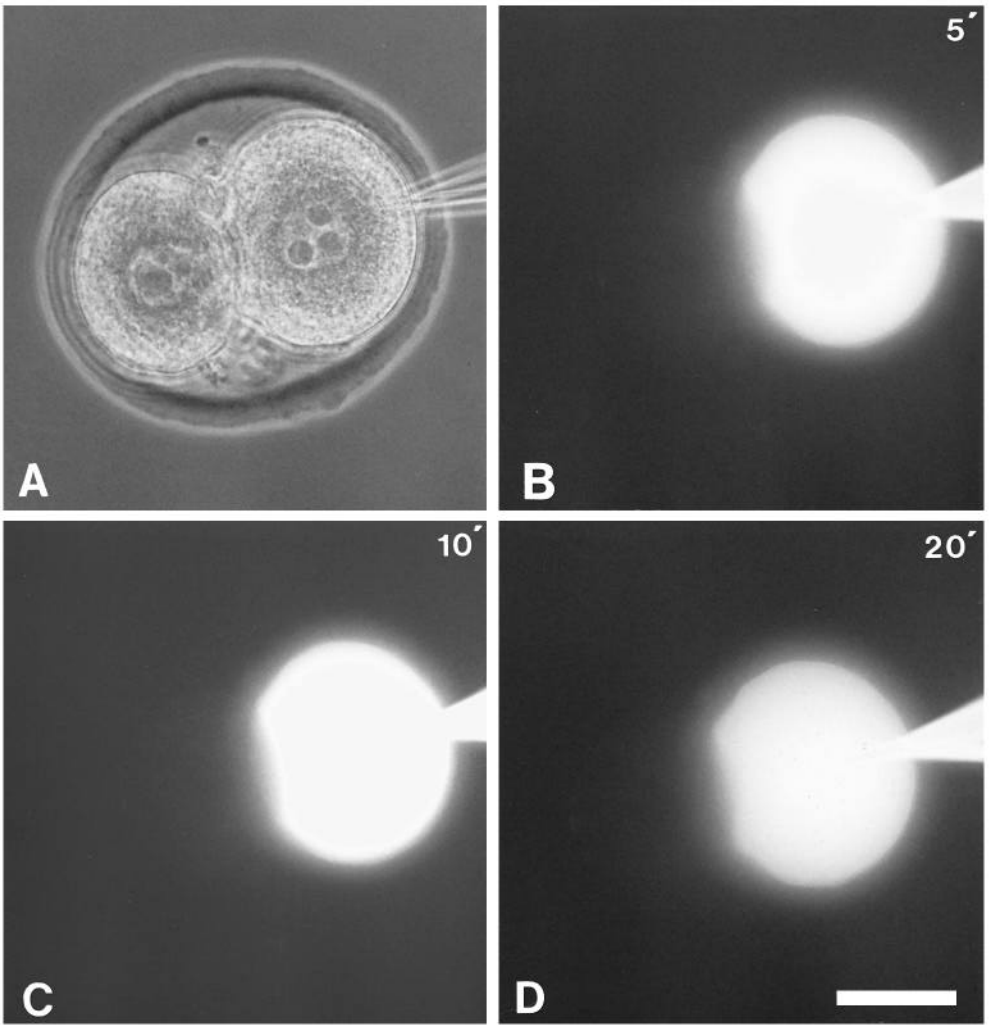

Fig. 3. Delaying the second round of DNA replication inhibits compaction and the initiation of gap junctional communication in most embryos. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 31-35 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age-matched controls formed compacted morulae (79-88 hours post-hCG), at which time both were tested for dye coupling. The treated group remained as 2-cell embryos. Slightly more than half of the treated embryos did not compact, as can be seen in the phase-contrast image in A, and most of them were not dye-coupled, as shown in the fluorescence images in B, C, and D (5, 10, and 20 minutes after the start of dye injection, respectively). Bar, 25 μm.

(approximately 20% coupled) compared to controls (over 90% coupled; Table 2). Delaying the second round of DNA replication by 10 hours is therefore sufficient to inhibit the establishment of gap junctional coupling. More detailed examination of the data (Table 2) revealed that the few aphidicolin-treated embryos that were dye-coupled were also compacted (Fig. 2). However, not all compacted, aphidicolin-treated 2-cell embryos were dye-coupled; in fact, only about one half of them were (Table 2). Conversely, dye coupling was never observed in aphidicolin-treated 2-cell embryos that did not compact (Table 2; Fig. 3).

Effects on Cx43 distribution of delaying the second round of DNA replication by 10 hours

To assess whether delaying the second round of DNA replication by 10 hours had an effect on the spatial distribution of Cx43, aphidicolin-treated 2-cell embryos were immunofluorescently labeled with an antibody specific for Cx43. Embryos were scored for the presence of both cytoplasmic and interblastomeric staining. The staining pattern seen in the control embryos was essentially as described by Nishi et al. (1991), Valdimarsson et al. (1991), and De Sousa et al. (1993), i.e. most embryos displayed both extensive cytoplasmic staining, and punctate interblastomeric staining, which suggested gap junction plaques (Table 3; Fig. 4B). On the other hand, while cytoplasmic staining was detected in the majority of the aphidicolin-treated 2-cell embryos, interblastomeric staining was rare (Table 3; Fig. 4A). No difference was observed in the staining pattern between compacted and non-compacted, aphidicolin-treated 2-cell embryos (Table 3). In many cases the cytoplasmic staining in drug-treated embryos was qualitatively different from that observed in controls. In control embryos, cytoplasmic immunofluorescence was generally localized to

Table 3. Pattern of Cx43 immunoreactivity in embryos treated with aphidicolin for 10 hours at the 2-cell stage (beginning at 31-35 hours post-hCG)

Treatment	Cytoplasmic staining (%)	Intercellular plaque staining (%)
Control (*n*=31)	27 (87.1)	28 (90.3)
Aphidicolin (*n*=27)*	21 (77.8)	3 (11.1)
Compacted (*n*=14)	10 (71.4)	2 (14.3)
Uncompacted (*n*=13)	11 (84.6)	1 (7.7)

The embryos were fixed at 85 hours post-hCG.
*Data were collected in three separate experiments. Two or fewer embryos showed intercellular plaque-like staining in each experiment.

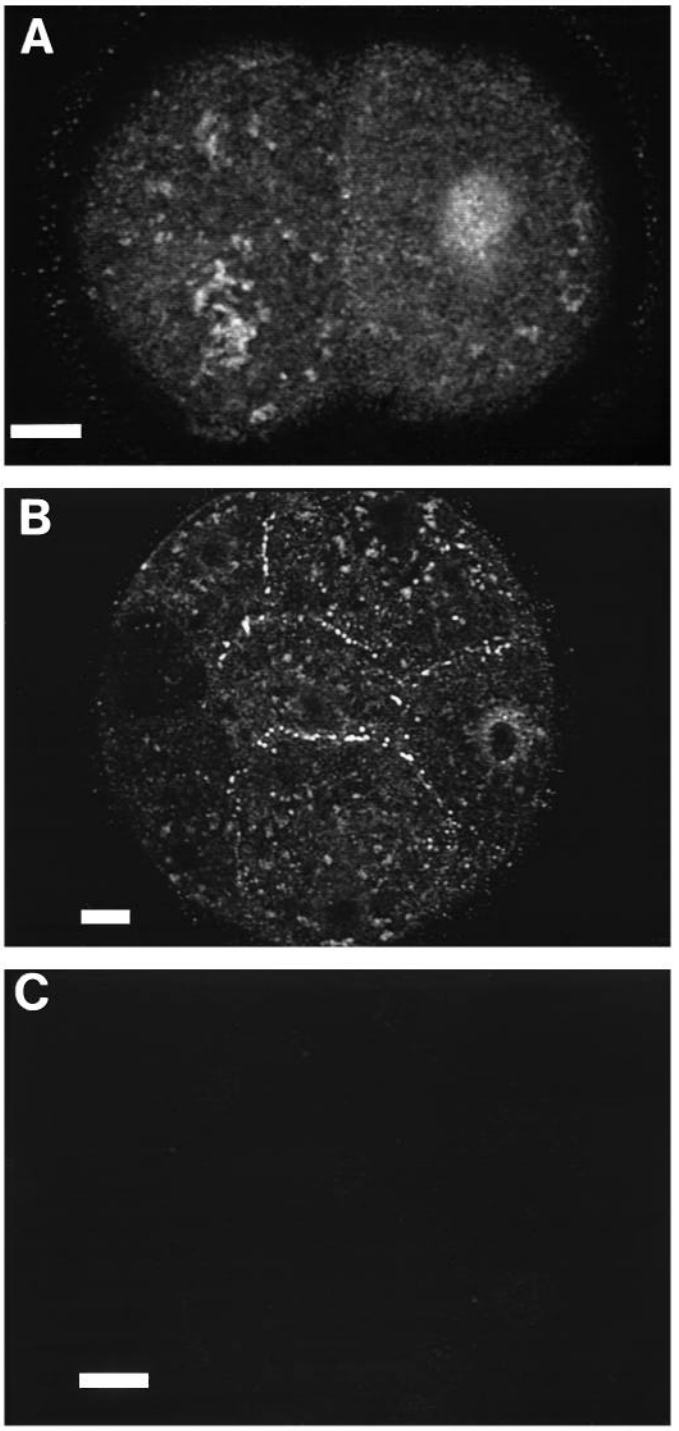

Fig. 4. Assembly of Cx43 into gap junctions is blocked by delaying the second round of DNA replication. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 31-35 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age-matched controls formed compacted morulae (85 hours post-hCG), at which time both groups were fixed, labeled with a Cx43-specific antibody, and viewed with the confocal microscope. The treated group remained as 2-cell embryos which contained considerable cytoplasmic Cx43 immunoreactivity (A). Punctate interblastomeric staining indicative of gap junction plaques was rarely seen. Control (DMSO-treated) embryos displayed both extensive cytoplasmic and punctate interblastomeric staining (B). No staining was observed in control embryos stained with antibody absorbed three times with the synthetic peptide against which it was raised (C). Bar, 10 μm.

numerous small, diffuse foci (Fig. 4B), whereas in many of the aphidicolin-treated embryos a portion of the immunoreactivity was seen in large, reticulated clouds (Fig. 4A). This array of cytoplasmic immunoreactivity is similar to that seen in compacting embryos after treatment with the protein trafficking inhibitor, brefeldin-A, and interpreted to be nascent Cx43 interrupted in its journey to the plasma membrane (De Sousa et al., 1993). None of the staining patterns described was ever seen when peptide-absorbed antiserum was used in place of the affinity-purified antibody (Fig. 4C).

DISCUSSION

The present results demonstrate that the third and fourth rounds of DNA replication are not required for the initiation of gap junctional communication in 8-cell embryos. Conversely, a 10 hour delay in DNA replication in the 2-cell stage leads to a failure to initiate gap junctional communication when these embryos reach the chronological age of 8-cell compacted embryos. Because the immunofluorescence results indicated that there were substantial levels of Cx43 in the drug-treated embryos, the failure to initiate gap junctional communication must arise at a post-translational level. Furthermore, the lack of punctate interblastomeric staining suggests that the underlying cause of the communication failure is an inability of Cx43 to assemble into gap junctions because it is not transported to the plasma membrane (De Sousa et al., 1993).

These results parallel the findings of Smith and Johnson (1985) on the effects of DNA replication on compaction. They found that inhibition of the third and fourth rounds of DNA synthesis had no effects on cell flattening but that a 10 hour delay of the second round inhibited this process. It thus appears that it is critical that the second round of DNA replication takes place within a specified temporal window for both cell flattening and gap junction assembly (and perhaps other developmentally significant events) to be initiated two cell cycles later. This type of time-keeping mechanism may not be restricted to mouse embryos because a similar mechanism has been shown to operate in the nematode *Caenorhabditis elegans*. In *C. elegans* embryos, the development of two biochemical markers (carboxylesterase and gut granules) in cells belonging to the gut cell lineage is dependent on DNA synthesis during the first cell cycle after the gut cell lineage is clonally established (in the 8-cell stage; Edgar and McGhee, 1988). The transcription necessary for the development of these markers takes place one to three cell cycles after the critical DNA replication cycle, and the markers are not expressed until the embryos contain 50-150 cells.

One discrepancy between the current results and those reported by Smith and Johnson (1985) is that, while they observed a nearly complete inhibition of cell flattening in embryos in which the second round of DNA replication had been delayed by 10 hours, about half of the embryos in this group compacted in our experiments. One possible reason for this discrepancy is that some of our embryos managed to escape the inhibitory effect of aphidicolin, at least to a degree

sufficient for them to become compacted and for dye coupling to develop in some of them. How this escape might have occurred is difficult to say, but it is conceivable that the G_1-phase of the second cell cycle is shorter in the mouse strain used in the present study ($CF_1 \times CB6F_1/J$) compared to the strain ($F_1LAC \times$ HC-CFLP) used by Smith and Johnson (1985). If this were the case then it is possible that some of the embryos had already entered S-phase when aphidicolin treatment commenced, and partial DNA replication could have taken place to an extent that was sufficient for compaction to develop. It is known that there are strain differences in the length of cell cycle-related events (Molls et al., 1983; Smith and Johnson, 1986); for example, relative to hCG injection, first cleavage occurs about 1 hour earlier in $CD_1 \times CB6F_1/J$ embryos than in $CF_1 \times CB6F_1/J$ embryos (G. Valdimarsson, unpublished observations). Moreover, the fact that the drug did inhibit cleavage in all of the embryos demonstrates that the failure to inhibit compaction is not simply due to inactivation of the aphidicolin.

The results presented here make it possible to exclude two more potential time-keeping mechanisms from being responsible for the timing of gap junction assembly. Embryos treated with aphidicolin during the first 10 hours of the second cell cycle undergo a catch-up round of DNA synthesis when they are released from the drug and then arrest DNA synthesis permanently (Smith and Johnson, 1985). These embryos have therefore undergone the same absolute number of DNA replication cycles (2) and have the same nucleocytoplasmic ratio as have the embryos that were treated continuously with the drug from the early 4-cell stage. Since this latter group of embryos initiated gap junctional communication normally, one can conclude that neither the absolute number of DNA replication cycles nor the nucleocytoplasmic ratio is an important determinant of the timing of gap junction assembly.

The inhibition of gap junction assembly observed in the aphidicolin-treated 2-cell embryos could be due to such non-specific effects as: an inability of 2-cell or non-compacted embryos to assemble gap junctions; inhibition of the activation of the zygotic genome; or non-specific toxicity of the drug. These non-specific effects are unlikely, however, since: (1) inhibition of compaction has been shown not to interfere with the initiation of gap junction assembly (Goodall, 1986; Kidder et al., 1987); (2) experiments with cytochalasin show that inhibition of cytokinesis from the 1-cell stage onward does not alter the timing of gap junction assembly (Kidder et al., 1987); (3) aphidicolin inhibition of DNA synthesis in the 2-cell stage does not affect the activation of the embryonic genome (Bolton et al., 1984); and (4) 4-cell embryos treated continuously for about 25 hours with aphidicolin initiated gap junctional communication at the normal time, whereas 10 hour treatment of 2-cell embryos led to inhibition of same. It is thus clear that the initiation of gap junction assembly in the 8-cell stage is dependent on the timing of the second round of DNA replication, in a specific manner.

How does the timing of the second round of DNA replication control the initiation of gap junction assembly, two cell cycles later? Previous work from this laboratory (De Sousa et al., 1993) demonstrated that the assembly of nascent Cx43 into gap junctions during compaction is dependent on a post-translational event that is sensitive to inhibitors of intracellular protein trafficking. Our present finding, that Cx43 remains in a cytoplasmic compartment after aphidicolin treatment in the 2-cell stage, implies that it is this trafficking step that is blocked when the timing mechanism is disrupted. An interaction of some kind between the cytoplasm and the DNA must take place in the 2-cell stage, and this interaction requires that DNA replication takes place at the normal time. Perhaps, a cytoplasmic 'clock' that is already running interacts with the replicating DNA in such a way as to set in motion a regulatory cascade that does not require further DNA synthesis to operate, and that takes two cell cycles to run down, culminating in the assembly of gap junctions. Indeed, an autonomous cytoplasmic or zygotic 'clock' has been reported to operate in 1- and 2-cell mouse embryos (Waksmundzka et al., 1984; Poueymirou and Schultz, 1987, 1989; Manejwala et al., 1991; Wiekowski et al., 1991). The nature of the interaction between this putative cytoplasmic 'clock' and the DNA might involve some sort of gene-modifying activity that can only occur during the major chromatin reorganization that takes place in the 2-cell stage (Wiekowski et al., 1991), in preparation for activation of the embryonic genome. The steps in the proposed regulatory cascade are unclear but presumably the last step would be the release of nascent Cx43 from its state of suspended trafficking so that its insertion into plasma membranes and into gap junctions can begin. It is interesting to note in this regard that the antibody used in the present study does not normally detect cytoplasmic Cx43 in pre-compaction embryos, presumably because the epitope it recognizes is unavailable for binding (De Sousa et al., 1993). The 'maturation' of Cx43 that normally takes place at compaction is therefore not dependent on the second round of DNA synthesis and is not sufficient for gap junction assembly to occur.

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