Nuclear Envelope Breakdown Is under Nuclear Not Cytoplasmic Control in Sea Urchin Zygotes

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Abstract. Nuclear envelope breakdown (NEB) and entry into mitosis are thought to be driven by the activation of the $p34^{cdc2}$–cyclin B kinase complex or mitosis promoting factor (MPF). Checkpoint control mechanisms that monitor essential preparatory events for mitosis, such as DNA replication, are thought to prevent entry into mitosis by downregulating MPF activation until these events are completed. Thus, we were surprised to find that when pronuclear fusion in sea urchin zygotes is blocked with Colcemid, the female pronucleus consistently breaks down before the male pronucleus. This is not due to regional differences in the time of MPF activation, because pronuclei touching each other break down asynchronously to the same extent. To test whether NEB is controlled at the nuclear or cytoplasmic level, we activated the checkpoint for the completion of DNA synthesis separately in female and male pronuclei by treating either eggs or sperm before fertilization with psoralen to covalently cross-link base-paired strands of DNA. When only the maternal DNA is cross-linked, the male pronucleus breaks down first. When the sperm DNA is cross-linked, male pronuclear breakdown is substantially delayed relative to female pronuclear breakdown and sometimes does not occur. Inactivation of the Colcemid after female NEB in such zygotes with touching pronuclei yields a functional spindle composed of maternal chromosomes and paternal centrosomes. The intact male pronucleus remains located at one aster throughout mitosis. In other experiments, when psoralen-treated sperm nuclei are allowed to fuse with normal female pronuclei, over 90% of the zygote nuclei do not break down for at least 2 h after the controls even though H_1 histone kinase activity gradually rises close to, or higher than, control mitotic levels. The same is true for normal zygotes treated with aphidicolin to block DNA synthesis. From these results, we conclude that NEB in sea urchin zygotes is controlled at the nuclear, not cytoplasmic, level, and that mitotic levels of cytoplasmic MPF activity are not sufficient to drive NEB for a nucleus that is under checkpoint control. Our results also demonstrate that the checkpoint for the completion of DNA synthesis inhibits NEB by acting primarily within the nucleus, not by downregulating the activity of cytoplasmic MPF.

ENTRY into mitosis is thought to be driven by the rapid activation of the $p34^{cdc2}$–cyclin B kinase complex, or mitosis promoting factor (MPF),[1] at the end of G_2 (for review see Nurse, 1990; Pines and Hunter, 1990; Maller, 1991; Murray, 1992). On account of its key role in the G_2/M transition, the activity of this kinase complex must be under tight control if the cell is to enter mitosis at the appropriate moment. Although cyclin B accumulates during G_2 and concurrently associates with the $p34^{cdc2}$ kinase, MPF activity does not correspondingly increase because of inhibitory phosphorylations on threonine 14 and tyrosine 15 residues of the $p34^{cdc2}$ kinase molecule (Solomon et al., 1990). Activation of this kinase complex at the time of mitosis appears to involve the dephosphorylation of these residues by the CDC25 phosphatase pathway (Kumagai and Dunphy, 1991; Sebastian et al., 1993; for review see Maller, 1994).

The successful generation of genetically identical daughter cells requires that a sequence of preparative events be completed before nuclear envelope breakdown (NEB), at which point the cell becomes committed to mitosis. Before this time, chromosome replication must have been completed. Although the progression of the cell cycle may normally leave sufficient time for the completion of DNA synthesis before NEB, eukaryotic cells have a feedback control or "checkpoint" mechanism that monitors DNA replication and prevents entry into mitosis until that process is completed (for review see Hartwell and Wienert, 1989; Murray, 1992; Dasso, 1993; Maller, 1994). When

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1. *Abbreviations used in this paper*: MPF, mitosis promoting factor; NEB, nuclear envelope breakdown.

DNA synthesis is experimentally prevented by any of several agents, NEB and entry into mitosis are blocked. That this arrest can be overridden by caffeine treatment (Schlegel and Pardee, 1986; Dasso and Newport, 1990) indicates that all the necessary components for entry into mitosis are present and functional but are downregulated by the checkpoint pathway.

The way in which this DNA synthesis checkpoint mechanism detects incomplete DNA replication and the target of this checkpoint pathway remain important problems in cell cycle regulation. Recent work indicates that this checkpoint monitors the presence of active replication complexes, not the total amount of unreplicated DNA (Kornbluth et al., 1992; Li and Deshaies, 1993; Navas et al., 1995). Work on *Xenopus* egg extracts reveals that the DNA synthesis checkpoint pathway blocks entry into mitosis by maintaining the inhibitory phosphorylations of the threonine 14 and tyrosine 15 residues on the $p34^{cdc2}$ molecule (Kornbluth et al., 1992), perhaps through the upregulation of a tyrosine kinase (Smythe and Newport, 1992), even though cyclin B levels rise to normal levels (Dasso and Newport, 1990). That the checkpoint for the completion of DNA synthesis in *Xenopus* coordinately blocks MPF activation and NEB provides additional evidence that cytoplasmic MPF activity drives NEB.

Despite these observations on *Xenopus* egg extracts, there are indications that the regulation of NEB by the DNA synthesis checkpoint is neither simple, fully understood, or the same for all organisms. For example, mutations in budding yeast that prevent inhibitory phosphorylation of cdc28 (the *Saccharomyces cerevisiae* homolog of cdc2) do not lead to premature entry into mitosis nor do they bypass the checkpoint control for the completion of DNA synthesis (Amon et al., 1992; Sorger and Murray, 1992). Recent work with egg extracts indicates the existence of another level of control through an inhibitor of the cdc2–cyclin B complex that downregulates its kinase activity when DNA replication is blocked (Kumagai and Dunphy, 1995). In addition, for mammalian cells, overexpression of $p50^{wee1}$ kinase, a nuclear protein, ensures completion of DNA synthesis and normal cell division in cells with inappropriately high interphase levels of cytoplasmic MPF activity that otherwise lead to premature mitosis (Heald et al., 1993). This finding indicates that cytoplasmic MPF activity may not be the sole regulator of NEB and raises the important question of how the partitioning of cell cycle regulatory proteins between the cytoplasm and the nucleus, as well as the control of nuclear transport, determine when the nucleus breaks down.

The impetus for the work described in this report was our earlier observation that when pronuclear fusion is prevented in sea urchin zygotes, the female pronuclei always break down earlier than the male pronuclei (Sluder et al., 1994; Sluder and Rieder, 1985). We were surprised by this observation because, with few exceptions, DNA synthesis and NEB are synchronous for naturally occurring and experimentally produced multinucleate cells, even those formed by the fusion of heterophasic cells (Rao and Johnson, 1970; for review see Johnson and Rao, 1971). Also, if the rapid activation of MPF activity alone drives NEB and entry into mitosis in sea urchin zygotes, then both pronuclei should break down at the same time.

We have investigated the basis for asynchronous NEB in sea urchin zygotes and find that it is not due to regional differences in the time of MPF activation. Rather, our data provide a direct functional demonstration that NEB is controlled at the level of each individual nucleus when the checkpoint for the completion of DNA synthesis is activated. A nucleus that does not complete DNA synthesis does not prevent the breakdown of a normal nucleus in the same cytoplasm or the activation of cytoplasmic MPF to mitotic levels. Furthermore, we find that mitotic levels of MPF are not sufficient to drive the breakdown of a nucleus that has not completed DNA synthesis.

Materials and Methods

Living Material

Lytechinus pictus and *L. variegatus* sea urchins were purchased from Marinus Inc. (Long Beach, CA) and Susan Decker (Hollywood, FL), respectively. Eggs and sperm were obtained by intracelomic injection of 0.5 M KCl (Fuseler, 1973). To block pronuclear fusion, unfertilized or just fertilized eggs were treated for 4–8 min with 5×10^6 M Colcemid (Sigma Chemical Co., St. Louis, MO) to prevent future sperm aster assembly. At this dose, Colcemid acts specifically to block microtubule assembly and does not have detectable toxic side effects (for review see Sluder, 1991; Sluder et al., 1994). After Colcemid treatment, the eggs were fertilized and cultured in natural sea water at 16–19°C. Shortly before the expected time of first NEB, the zygotes were mounted in fluorocarbon oil preparations as previously described (Sluder, 1979) or for some experiments in microinjection chambers (Sluder et al., 1986). To promote spindle assembly in the experiments shown in Figs. 5–7, individual zygotes were irradiated on the polarization microscope for 45 s with 366 nm light to photochemically inactivate the Colcemid (for review see Sluder, 1991). Unless specifically noted, all experiments were performed with gametes of *L. pictus*.

Individual zygotes were followed at 19°C by polarization microscopy with a modified ACM microscope (Carl Zeiss Inc., Thornwood, NY) or BH-2 microscope equipped with differential interference contrast (DIC) optics (Olympus Corporation of America, New Hyde Park, NY). Photographs were recorded on Plus X film developed in Microdol-X (Eastman Kodak Inc., Rochester, NY). Confocal microscopy was conducted on a Diaphot 200 microscope (Nikon Inc., Melville, NY) using a 60X NA 1.40 objective and a MRC 1000 confocal scanner (Bio-Rad Laboratories, Richmond, CA).

Psoralen Treatment of Eggs and Sperm

To form covalent cross-links between base-paired DNA strands in the female pronucleus, unfertilized eggs were treated with 0.5–2 μg/ml 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) (HRI Assoc., Inc. Concord, CA) for 25–30 min and irradiated at 4°C with a long-wave UV box (Blak-Ray Lamp, model UVL-21; Ultra Violet Products, Inc., San Gabriel, CA) for 5 min at 3.6 mW/cm^2. They were then washed with fresh sea water before fertilization. To covalently cross-link paternal DNA, sperm were treated for 15–25 min with 5 μg/ml HMT in sea water and irradiated in the same fashion for 8–10 min at 4°C. Such sperm preparations were then used to fertilize untreated eggs; the final concentration of HMT after sperm dilution and washing of the eggs did not exceed 0.03 μg/ml.

To cross-link DNA in the zygote nucleus after S phase was complete, we treated normal zygotes with 5 μg/ml HMT for 15 min at 16–19°C starting 45 min after fertilization and irradiated them for 5 min immediately thereafter.

Histone H_1 Kinase Assays

Unfertilized eggs were dejellied by suspending them for 3 min in artificial sea water (435 mM NaCl, 40 mM $MgCl_2$, 15 mM $MgSO_4$, 11 mM $CaCl_2$, 10 mM KCl, 5 mM Hepes), pH 5.0. A 1%(v/v) suspension of eggs was then divided into three cultures and kept suspended with stir paddles (60 rpm) in 100-ml beakers. One culture was fertilized with normal sperm and served as the control. The second was also fertilized with normal sperm

and treated continuously starting 5 min after fertilization with 10 μg/ml Aphidicolin (Sigma Chemical Co.). The third culture was fertilized with sperm treated with 5 μg/ml psoralen HMT for 25 min in natural sea water at 16°C and then irradiated at 4°C for 8–10 min with 366 nm light. The control culture was sampled at 6–10-min intervals starting 30 min after fertilization; sampling was terminated when >50% of the zygotes had cleaved. Psoralen- and aphidicolin-treated zygotes were sampled 30 and 60 min after fertilization, and at 60-min intervals thereafter. Percent NEB was determined by observation with DIC optics on a minimum of 100 living zygotes at each sample time.

Samples were prepared and assayed by a slight modification of the methods of Suprynowicz et al. (1994). 3-ml aliquots were pelleted and resuspended in 10 ml of sample buffer at 0°C (250 mM K Gluconate, 250 mM *n*-methyl-D-glucamine, 50 mM Hepes, 10 mM EGTA, 10 mM $MgCl_2$, 1.0 mM DTT, pH 7.2 [adjusted with acetic acid]). Washed samples were then pelleted, resuspended in 470 μl of sample buffer, and lysed by sonication at 0°C by four to six 1-s pulses (Virsonic 3000 with a microprobe tip; Virtis Co., Inc., Gardiner, NY). Lysates were centrifuged for 30 s at 7,500 rpm in microcentrifuge (model 235A; Fisher Scientific Co., Pittsburgh, PA) with a custom-built swing bucket rotor.

To assay histone H_1 kinase activity, 10 μl of lysate was added to 40 μl of sample buffer containing 625 μg/ml histone H_1 (kindly supplied by Dr. James Maller, University of Colorado, Denver, CO; also Ambion Inc., Austin, TX), 125 μCi/ml [^{32}P]ATP (6000 Ci/mmol; Dupont/NEN, Boston, MA), 250 μM Mg ATP, 1.25 μg/ml leupeptin, 1.25 μg/ml pepstatin, 625 μM PMSF, 250 nM cAMP-dependent protein kinase inhibitor, 80 mM β glycerol phosphate at 0°C. Samples were incubated for 10–12 min in a 16°C water bath, and the reactions were terminated with 50 μl of 2× protein buffer at 100°C. Samples were then boiled for 5 min and electrophoresed on 15% polyacrylamide gels. Gels were fixed in 10% acetic acid–45% methanol for 1–2 h, dried, and exposed overnight to a 35 × 43-cm phosphor screen and analyzed with a Phosphorimager:SF (Molecular Dynamics, Sunnyvale, CA). Quantification of counts in H_1 histone bands was performed with Image Quant Software Version 3.3 (Molecular Dynamics). H_1 histone kinase activity is expressed as "volume," which is the sum of pixel values within each H_1 histone band minus background.

Results

Experimental System

Unless specified, all experiments were performed with gametes of *L. pictus*. We prevented pronuclear fusion by treating unfertilized or just fertilized eggs with Colcemid to prevent future microtubule assembly. The doses of Colcemid used were just sufficient to prevent assembly of the sperm aster and were specific for microtubule assembly (for review see Sluder, 1991). Under these conditions, fertilization events were normal with the exceptions that a sperm aster was not assembled and syngamy consequently did not occur. The male pronucleus became visible at a random position at the surface of the zygote ~15 min after fertilization and was always smaller than the female nucleus (Fig. 1 *a*). The female pronucleus broke down before the male pronucleus by an average of 7.5 min (Fig. 1, *b–e*, Table I) for pronuclei that were separate from each other (Sluder et al., 1994).

To determine if this asynchronous NEB was simply due to regional differences in the time of MPF activation, we examined cases in which the pronuclei were fortuitously adjacent or touching each other (Fig. 2). These cases were found in low frequency and were presumably the consequence of the fertilizing sperm entering close to the position of the eccentrically located female pronucleus. In all 42 cases examined, the female pronucleus broke down first, on average 8.3 min before the male pronucleus, even though the nuclei were in a common cytoplasmic environment (Table I). That adjacent nuclei can undergo NEB independently demonstrates that NEB is not a passive response of the nucleus to a global, or even local, activation of MPF activity.

Experimental Manipulation of DNA Synthesis in Male or Female Pronuclei

We next wanted to determine if the delay in male pronuclear breakdown is a property unique to this pronucleus, or perhaps reflects the extra time needed for the decondensation of the highly condensed paternal DNA to allow the onset of chromosome duplication. We used the psoralen HMT to experimentally prevent the completion of DNA synthesis separately in the male or female pronucleus. This psoralen intercalates into DNA and upon activation with 366 nm light, forms covalent cross-links between base-paired DNA strands (for review see Cimino et al., 1985). We reasoned that such covalent cross-links should activate the checkpoint that monitors the completion of DNA synthesis by preventing the propagation of replication complexes thereby preventing or greatly delaying their disassembly. Assembled DNA replication complexes are thought to activate the pathway that prevents entry into mitosis (Kornbluth et al., 1992; Li and Deshaies, 1993; Navas et al., 1995). Since psoralen mediated cross-linking of nuclear DNA after the completion of S phase does not impede entry into mitosis (experiments described below), we assume that we are activating the checkpoint that monitors the completion of DNA synthesis. However, it is conceivable that psoralen cross-links could activate the pathway that detects DNA damage. Bearing this in mind, we will continue to speak of the checkpoint for the completion of DNA synthesis in order to make the presentation of the work less cumbersome.

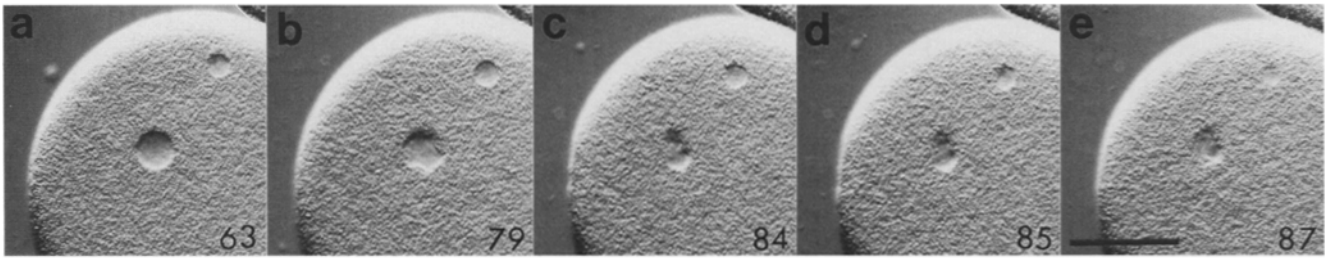

Figure 1. Asynchronous NEB of male and female pronuclei. Normal eggs were fertilized with normal sperm, and pronuclear migration was blocked with Colcemid. The female pronucleus is larger than the male pronucleus. (*a–c*) Breakdown of the female pronucleus; male pronucleus remains intact. (*d* and *e*) Breakdown of the male pronucleus. Female pronucleus always breaks down first. Minutes after fertilization are shown in the lower corner of each frame. DIC optics. Bar, 50 μm.

Table I. Asynchrony of NEB

	Average	*n*	SEM	Range
Minutes between female NEB and male NEB*				
Pronuclei separate‡	7.5	74	0.69	0–33
Pronuclei touching	8.3	42	0.59	2–17
Minutes between male and female NEB, maternal DNA cross-linked§				
1–2 μg/ml psoralen				
Male NEB leads	4.1	11	0.96	0.5–11.0
Female NEB leads	0.5	1		
0.1–0.5 μg/ml psoralen				
Male NEB leads	2.3	7	0.61	1–5
Female NEB leads	2.6	16	0.41	0.5–7.0

The number of cells examined and the SEM in minutes are shown by *n* and SEM, respectively. Ranges are given in minutes. All data are taken from *L. pictus* zygotes followed at 15–19°C.

* Normal eggs were fertilized with normal sperm and treated with Colcemid to prevent pronuclear fusion.

‡ Data from Sluder et al., 1994.

§ Eggs were treated with the indicated concentrations of psoralen and irradiated with 366 nm light before fertilization with normal sperm and treatment with Colcemid to prevent pronuclear fusion.

To demonstrate the efficacy of psoralen treatments in activating the checkpoint for the completion of DNA synthesis, we treated sperm with psoralen, irradiated them with 366 nm light, and then used them to fertilize normal eggs. Fertilization events were normal, and in the absence of Colcemid treatment, the male and female pronuclei fused in a normal fashion. In 17 experiments we monitored percentage of NEB of the zygote nuclei as a function of time using a minimum of 100 zygotes for each time point. As is shown in Fig. 3, we found that <10% of the zygote nuclei had broken down 3 h after fertilization. Thereafter, the percentage of NEB increased to a variable extent in some cultures. This delay of NEB was specific to the psoralen cross-linking of DNA; irradiation or psoralen treatments alone of the sperm did not cause a significant delay in the breakdown of the zygote nuclei. Although the reason why a small percentage of the nuclei break down within 3 h of fertilization is unknown, some NEB occurs when DNA synthesis is completely inhibited by aphidicolin (this report; Sluder and Lewis, 1987).

A characteristic feature of the checkpoint that monitors the completion of DNA synthesis is that it can be overridden by caffeine treatment in both cultured cells and egg extract systems (Schlegel and Pardee, 1986; Dasso and Newport, 1990). To determine if the checkpoint pathway we activated by psoralen-mediated cross-linking of nuclear DNA in sea urchin zygotes has the same functional properties as that found in other cell types, we treated the zygote cultures with 5 and 10 mM caffeine in sea water starting 40 min after fertilization with the psoralen-treated sperm. As illustrated in Fig. 3, for a representative example of four experiments, the zygotes treated with caffeine consistently underwent NEB to completion with good synchrony. Application of 10 mM caffeine led to NEB slightly earlier than 5 mM caffeine.

We also determined whether caffeine could override the checkpoint at later times for zygotes of *L. variegatus*. Normal eggs were fertilized with psoralen-treated sperm, and pronuclear fusion was allowed to occur. Normally, NEB occurs ~1 h after fertilization. At 3 and 4 h after fertilization (percentage of NEB was ≤2%), we continuously applied 10 mM caffeine. For zygotes treated at 3 h, 98% of the nuclei had broken down within 40 min of caffeine application. For zygotes treated at 4 h, 95% of the nuclei had broken down within 40 min of caffeine application. We also conducted this experiment with normal zygotes treated with aphidicolin, a specific inhibitor of the α DNA polymerase. Continuous application of 10 mM caffeine at 3 h after fertilization (percentage of NEB, 4%) led to 84% NEB over a period of 40 min. Thus, nuclei that could not complete DNA synthesis remained competent to undergo NEB well after the normal time for first mitosis.

Additional evidence that we are activating the checkpoint for the completion of DNA synthesis comes from experiments in which we cross-linked nuclear DNA of normal zygotes starting 45 min after fertilization, a time after DNA synthesis is normally completed. We reasoned that under this condition, cross-linking should not activate the checkpoint or impede entry into first mitosis. In three experiments we found that 85–97% of the zygotes treated in this manner entered and proceeded through first mitosis at the same time as the controls (data not shown). Those zygotes that did not enter mitosis at the normal time presumably contained nuclei whose DNA was cross-linked before the completion of DNA synthesis. Importantly, zygotes did not enter second mitosis by 6 h. This is to be expected if the cross-linked DNA of daughter nuclei is incapable of fully replicating. Our finding that psoralen cross-linking of nuclear DNA after the S phase does not block entry into mitosis suggests that we are activating the checkpoint that

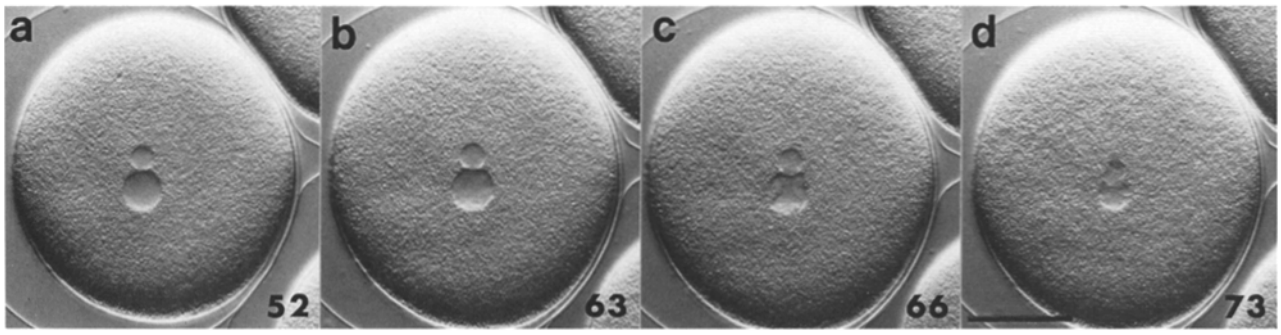

Figure 2. Asynchronous NEB of male and female pronuclei that are touching; the female pronucleus always breaks down first. Normal eggs were fertilized with normal sperm, and pronuclear fusion was blocked with Colcemid. The female pronucleus is the larger nucleus. (*a*) Before NEB. (*b* and *c*) Breakdown of the female pronucleus; male pronucleus remains intact. (*d*) Breakdown of the male pronucleus. Minutes after fertilization are shown in the lower corner of each frame. DIC optics. Bar, 50 μm.

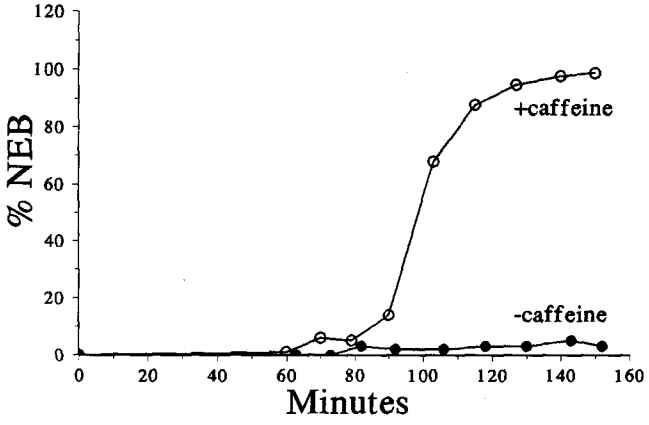

Figure 3. Caffeine relieves the checkpoint for the completion of DNA synthesis in sea urchin zygotes. Normal eggs were fertilized with psoralen-treated, irradiated sperm. Pronuclei were allowed to fuse to form a zygote nucleus. Half of the culture was continuously treated with 10 mM caffeine starting 40 min after fertilization. Plots show the percentage of NEB as a function of time. A minimum of 100 zygotes were scored for each time point.

monitors the completion of DNA synthesis, not the checkpoint that monitors the repair of DNA damage.

To prevent the completion of DNA synthesis in the female pronucleus only, we treated unfertilized eggs with HMT and irradiated them with 366 nm light. These eggs were then fertilized with normal sperm and treated with Colcemid to prevent pronuclear fusion. We then followed individual zygotes in which the pronuclei were close together or touching each other. For 1–2 μg/ml psoralen treatments of the eggs, we found that in 11 of 12 cases, the male nucleus broke down first, as shown in Fig. 4 (also see Table I). For 0.1–0.5 μg/ml psoralen treatments of eggs, the male pronucleus broke down first in 7 of 23 cases (Table I). This is in sharp contrast to our finding that, in the absence of cross-linking of the maternal DNA, the female pronucleus always broke down first (Table I). These results reveal that, within a common cytoplasmic environment, the checkpoint for the completion of DNA synthesis can be activated in just one of two nuclei, and indicate that the delay in the breakdown of the male pronucleus (Figs. 1 and 2) is not a peculiar property of this pronucleus, such as a slow but otherwise normal response to a rise in cytoplasmic MPF activity.

We next used sperm treated with HMT and irradiated with 366 nm light to fertilize normal eggs. Just after fertilization, we treated the zygotes with Colcemid and followed those individuals in which the nuclei were closely apposed. We found that the breakdown of the male pronucleus was delayed in all cases (on average 13 min), and sometimes did not occur even though the zygote exited mitosis and a female pronucleus reformed. Although the breakdown of the female pronucleus indicates that the zygotes with intact male pronuclei entered mitosis, we confirmed this by functional criteria. Shortly after breakdown of the female pronucleus, we irradiated individual zygotes on the microscope with 366 nm light to inactivate the Colcemid and allow the assembly of spindle microtubules. After irradiation, a functional spindle was assembled from the paternal centrosomes and the maternal chromosomes (Fig. 5, *c* and *d*). For cases in which the male pronucleus did not break down, it remained located in one of the asters throughout mitosis (Fig. 5, *d–f*). In telophase, the male pronucleus was segregated to one of the daughter blastomeres and typically fused with the reforming nucleus containing maternal chromosomes (Fig. 5, *g* and *h*). We did not observe any cases in which the male pronucleus broke down after spindle anaphase onset. In addition to revealing that the DNA synthesis checkpoint operates at the level of the individual nucleus, these observations demonstrate that a checkpoint limited nucleus (here the male) does not prevent the activation of MPF to mitotic levels or the normal progression of mitotic events. Also, mitotic levels of MPF are not sufficient to drive a nucleus into mitosis if it is under checkpoint control.

In these experiments, the male pronucleus appeared to be intact throughout mitosis by visual observation with DIC optics; the margin of the nucleus was sharply defined and spherical. Even so, we felt it necessary to confirm that the male pronuclear envelope was structurally and functionally intact. First, we followed two individual zygotes similar to the one shown in Fig. 5, fixed them, and cut them into serial 0.25-μm sections to systematically examine the ultrastructure of the male pronuclei. Fig. 6 shows

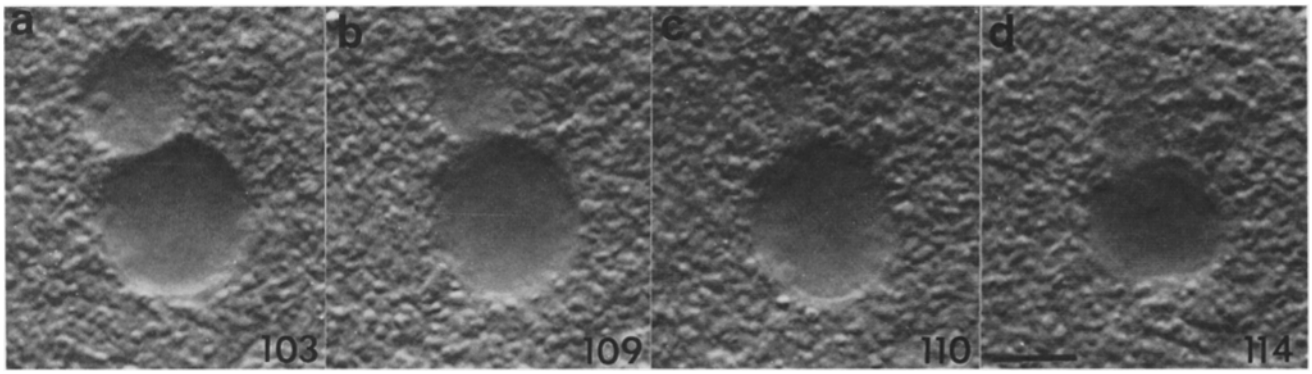

Figure 4. Psoralen-mediated cross-linking of maternal DNA before fertilization causes the female pronucleus to break down later than the male pronucleus. Psoralen-treated, irradiated eggs were fertilized with normal sperm and treated with Colcemid to prevent pronuclear fusion. The pronuclei are touching in the zygote shown here. (*a*) Before NEB. (*b* and *c*) Breakdown of the male pronucleus while the female pronucleus remains intact. (*d*) Start of NEB of the female pronucleus. Minutes after fertilization are shown in the lower corner of each frame. DIC optics. Bar, 10 μm.

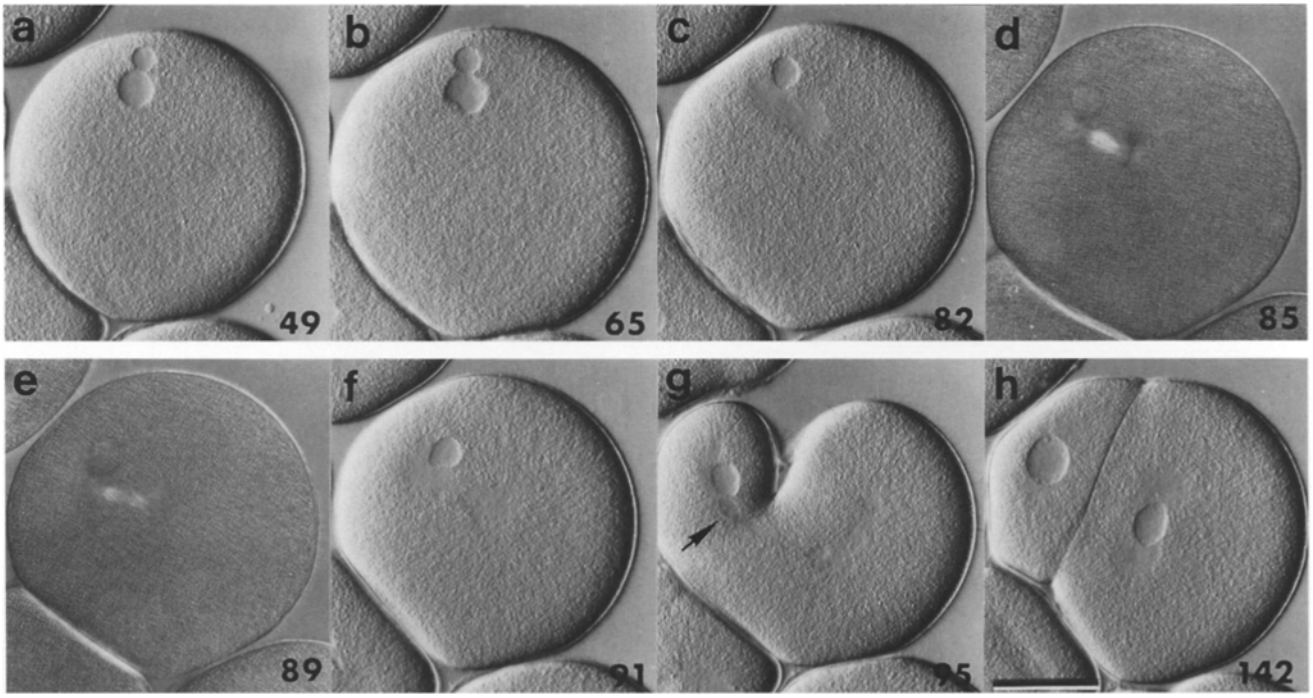

Figure 5. Mitosis in a zygote with a checkpoint-inhibited male pronucleus that does not break down. Normal eggs were fertilized with psoralen-treated, irradiated sperm and treated with Colcemid to prevent pronuclear fusion. This zygote has pronuclei that are touching. (*a*) Before NEB. (*b*) Start of female pronuclear envelope breakdown. (*c*) Zygote after irradiation with 366 nm light to photochemically inactivate the Colcemid. A functional spindle is assembled from the maternal chromosomes and the paternal centrosomes. (*d*) Polarization optics showing assembly of the spindle. The male pronucleus remains closely apposed to one of the spindle poles. (*e*) Early anaphase for the spindle. (*f*) Late anaphase. (*g* and *h*) Cleavage and reformation of daughter maternal nuclei. The male pronucleus fuses with the daughter maternal nucleus (*arrow*) in the lefthand blastomere. Minutes after fertilization are shown in the lower corner of each frame. DIC and polarization optics. Bar, 50 μm.

the nuclear area of one such cell in vivo (*a*), a survey section (*b*), and a higher magnification view of the nuclear envelope of this pronucleus (*c*). Even though the male pronucleus appears crenated in the survey section, the nuclear envelope had the normal double membrane structure (Fig. 6 *c*). Importantly, we could detect no breaks or gaps in the nuclear envelope in any of the serial sections. The crenation of the nucleus is a preparatory artifact because the male and female pronuclei of a zygote fixed 47 min after fertilization, and thus before first mitosis, showed identical crenation of both male and female pronuclear envelopes at the ultrastructural level (data not shown; see McDonald and Morphew, 1993).

Our second assay to determine whether the male pronuclear envelope was intact was to microinject 150,000–mol wt fluorescein-labeled dextran into experimental zygotes before breakdown of the female pronucleus and then follow the zygotes until breakdown of the female pronucleus. After irradiating the zygotes individually with 366 nm light to inactivate the Colcemid and allow spindle microtubule

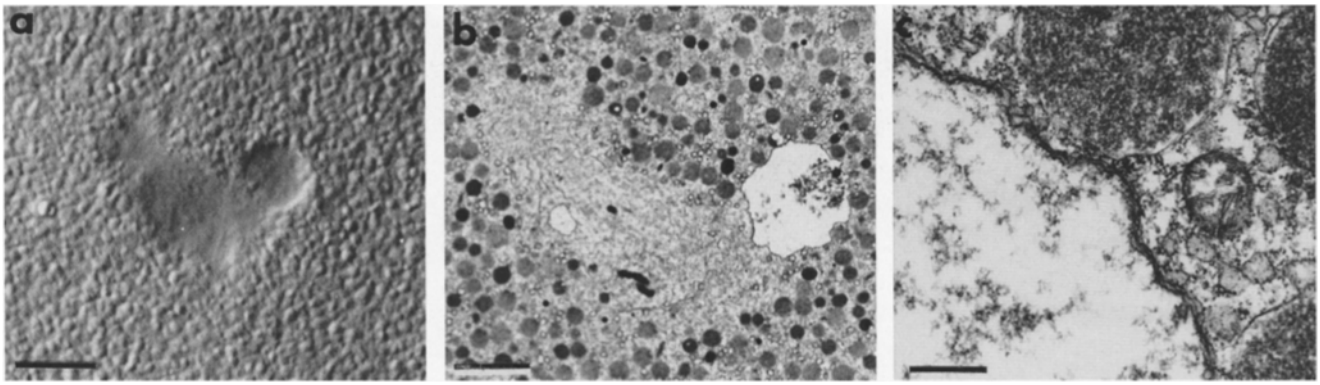

Figure 6. Same-cell correlative light and serial section electron microscopy of the male pronucleus at the pole of the mitotic spindle. The experiment is the same as that shown in Fig. 5 except that the zygote was fixed shortly after the photograph in *a* was taken. (*a*) Spindle and male pronucleus in vivo. (*b*) Survey section of the same spindle and nucleus. Crenation of the nucleus is a preparative artifact. (*c*) Higher magnification view of a portion of the nuclear envelope. The nuclear envelope appears to be intact as determined by complete serial 0.25-μm section reconstruction of this nucleus. *a*, DIC optics. Bars: (*a*) 10 μm; (*b*) 5 μm; (*c*) 0.4 μm.

assembly, we examined them in vivo by confocal fluorescence microscopy to determine if the dextran entered the male pronucleus. Since this dextran does not enter the nucleus of control zygotes, its distribution serves as a functional test for the integrity of the nuclear envelope. In all four cases examined by confocal microscopy, we found that the male pronucleus excluded the dextran during mitosis (Fig. 7, *a–c*).

We also used this same approach to test the functional integrity of zygote (syngamy) nuclei in eggs fertilized with psoralen–cross-linked sperm. 3 h after fertilization, the zygotes were microinjected with the labeled dextran, and 4 h after fertilization, the zygotes were observed by confocal microscopy. In all cases, we found that the dextran was excluded to the same extent in the nucleus and the oil drop used to cap the micropipette (Fig. 7, *d* and *e*).

H_1 Histone Kinase Activities

In *Xenopus* egg extracts and fission yeast, the checkpoint that monitors the completion of DNA synthesis prevents the activation of MPF by maintaining the inhibitory phosphorylations on the $p34^{cdc2}$–cyclin B kinase complex (for reviews see Murray, 1992; Maller, 1994). However, our finding (Fig. 5) that a zygote will enter and complete mitosis with one nucleus under checkpoint control provides a functional demonstration that MPF levels must be rising to mitotic levels in the zygote. This means that either the checkpoint does not downregulate MPF activity in sea urchin zygotes or that some activity of the normal nucleus bypasses the checkpoint pathway that otherwise would prevent the activation of cytoplasmic MPF.

To distinguish between these possibilities, we assayed H_1 histone kinase activity over a period of 3–6 h in embryos with a single zygote nucleus that could not complete DNA synthesis. H_1 histone kinase activity is used as a measure of MPF activity in sea urchin zygotes and other experimental systems (Arion et al., 1988; Meijer et al., 1989; Dasso and Newport, 1990; Solomon et al., 1990; Heald et al., 1993; Kornbluth et al., 1994). From a common pool of unfertilized eggs, we established three cultures. The first was fertilized with normal sperm and served as the control. The second was fertilized with psoralen-treated sperm, allowing pronuclear fusion so that each cell had only one zygote nucleus, thereby removing the possible influence of a separate normal nucleus. The third was fertilized with normal sperm and then continuously treated starting shortly after fertilization with aphidicolin, a specific inhibitor of the α DNA polymerase. This treatment blocks the incorporation of [^{3}H]thymidine by ≥95% in these zygotes (Sluder and Lewis, 1987). The residual incorporation of label is thought to be due to mitochondrial DNA synthesis or repair synthesis of nuclear DNA. For each sample, the percentage of NEB was determined from 100 cell counts at the time of sampling.

The results for eight experiments were qualitatively the same for zygotes unable to complete DNA synthesis, with some variation in the time course of H_1 histone kinase activity increase and in percentage of NEB at later times. The results of three experiments are shown in Fig. 8, and all experiments are summarized in Table II. In Table II, the H_1 histone kinase activity is expressed relative to that experiment's control peak value at first mitosis, normalized to 1.0. For the psoralen and aphidicolin cultures, the indicated time for the kinase activity, in minutes after fertilization, is taken as either the time when the experiment was terminated or as the data point just before the percentage of NEB in that culture exceeded 11%. Although we did not observe a correlation between percentage of NEB and H_1 histone kinase activity in the experimental

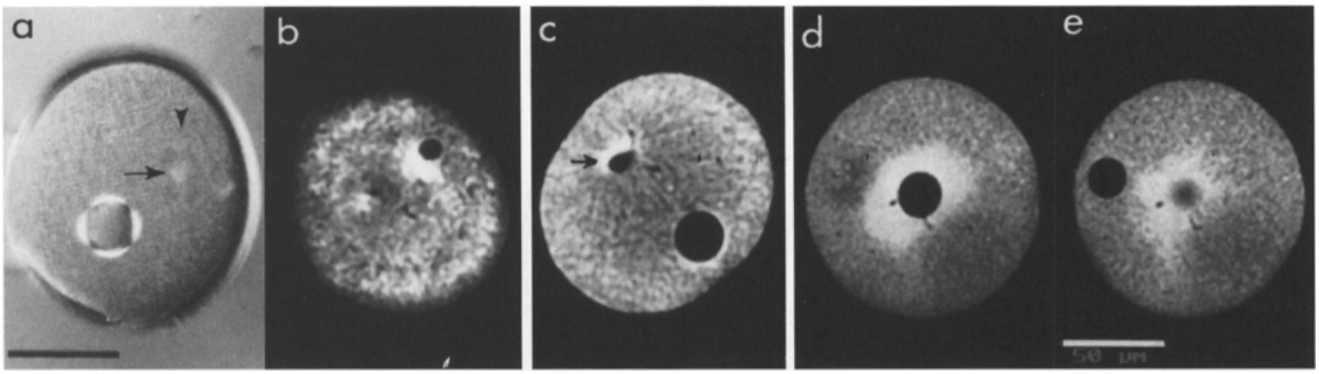

Figure 7. Functional test for the integrity of checkpoint-inhibited nucleus. (*a–c*) The experiment is the same as that shown in Fig. 5 except that the zygotes were injected with fluorescein-labeled dextran (150,000 mol wt) before the expected time of first mitosis and examined by confocal microscopy as the mitotic spindle was being assembled. (*a*) Polarization micrograph showing a zygote shortly after irradiation to inactivate the Colcemid. The birefringent spindle is indicated by the arrow, and the location of the male pronucleus, located at the upper spindle pole, is indicated by the arrowhead. (*b*) Confocal image of the same living zygote. The male pronucleus in the upper right quadrant of the zygote excludes the dextran even though the cell is in mitosis. (*c*) Another zygote experimentally manipulated in the same fashion. The male pronucleus (*arrow*) excludes the dextran even though the zygote is in mitosis. The oil drop used to cap the pipette is seen in the lower right portion of the zygote. (*d* and *e*) Normal eggs were fertilized with psoralen-treated sperm, and pronuclear fusion was allowed to occur. 3 h after fertilization, the zygotes were microinjected in the cytoplasm with labeled dextran. 4 h after fertilization, the zygotes were observed by confocal microscopy. (*d*) Image of a focal plane through the nucleus. (*e*) Same zygote; image of a focal plane through the drop of oil used to cap the micropipette. The label is excluded to the same extent by both the nucleus and the oil drop. Bars, 50 μm.

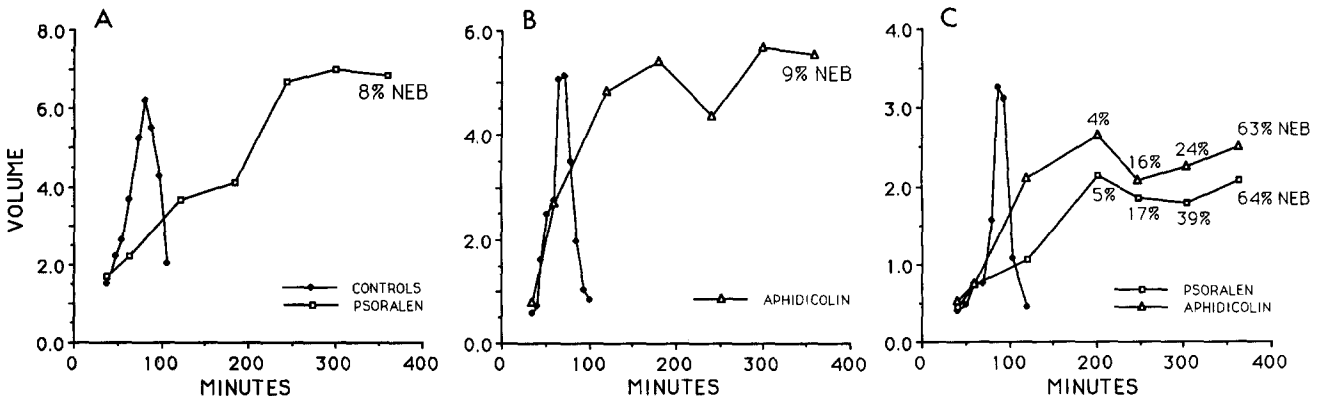

Figure 8. H_1 histone kinase activities in control cultures at first mitosis, zygotes fertilized with psoralen-treated sperm, and zygotes treated with aphidicolin to prevent DNA synthesis. Separate experiments are shown in each panel. For a given experiment, all eggs came from a common pool. Percentage of NEB was determined from 100-cell counts at the time each sample was taken for kinase assay. (*A*) Comparison of control culture and culture fertilized with psoralen-treated sperm. (*B*) Comparison of control culture and culture treated with aphidicolin. (*C*) Comparison of control culture, culture fertilized with psoralen-treated sperm, and culture treated with aphidicolin. This last experiment shows that when the percentage of NEB increases after 200 min, H_1 histone kinase activity does not increase. Ordinate: H_1 histone kinase activity expressed as "volume" $\times 10^6$, which is the sum of phosphorimager-determined pixel values in the H_1 histone band minus background. Abscissa: minutes after fertilization.

cultures (see Fig. 8 *C*), we nevertheless adopted the latter cutoff to minimize the concern that zygotes entering mitosis might accelerate the rate of kinase activation.

The control cultures started entering first mitosis ~1 h after fertilization, and the H_1 histone kinase activity rapidly increased; later when these zygotes finished mitosis, kinase activity precipitously fell (Fig. 8, *A–C*). For first mitosis, H_1 histone kinase activity peaked between 71 and 96 min after fertilization (average 83 min).

For zygotes in which the completion of DNA synthesis had been prevented by either cross-linked paternal DNA or aphidicolin, H_1 histone kinase activity gradually rose for at least the first 3 h from interphase control levels (Fig. 8, *A–C*). Thereafter, depending on the batch of eggs in experiments carried >180 min, H_1 histone kinase activity increased further or reached a plateau. We never observed a precipitous drop in kinase activity at later times. Sometimes, when an increasing percentage of the zygote nuclei broke down 4–6 h after fertilization, H_1 histone kinase activity plateaued or decreased slightly (Fig. 8 *C*). Thus, the increase in H_1 histone kinase activity in the experimental cultures did not appear to be directly correlated with the percentage of the zygotes that had undergone NEB.

In most experimental cultures, the H_1 histone kinase activity rose close to or higher than the peak control levels before the percentage of NEB climbed above 11% (Fig. 8 and Table II). For two of the psoralen cultures and one aphidicolin culture, the percentage of NEB rose above 11% after 60 min, and consequently their kinase activities at this time were relatively low (Table II). When the controls showed peak H_1 histone kinase activity at first mitosis, the kinase activity in the cultures fertilized with psoralen–cross-linked sperm ranged from 20 to 44% of the peak control values (average 34%) and 36 to 61% of peak control values (average 46%) for the aphidicolin-treated cultures.

H_1 kinase activity rose in a similar, but not identical fashion, in zygotes fertilized with psoralen-treated sperm and those treated with aphidicolin. In the majority of the experiments, the H_1 histone kinase activity initially rose slightly faster in the aphidicolin-treated cultures and reached a modestly higher absolute value by the time the experiment was terminated or when the percentage of NEB exceeded 11% (Table II).

Table II. H_1 Histone Kinase Activities in Zygotes Fertilized with Psoralen-treated Sperm and Zygotes Treated with Aphidicolin Relative to Control Zygotes

	Psoralen			Aphidicolin		
Experiment	Value	Time	%NEB	Value	Time	%NEB
1	1.13	300	8	—	—	—
2	—	—	—	1.11	300	5
3	0.65	202	5	0.81	202	4
4	0.75	180	11	0.81	180	3
5	1.12	240	3	0.92	180	5
6	0.20	58	2	1.99	184	1
7	0.30	60	0	0.26	60	4
8	—	—	—	0.76	180	4

Peak kinase activity at first mitosis for the controls is normalized to 1.0 for each experiment. "Value" is the H_1 histone kinase activity relative to that experiment's control peak value at first mitosis. For the psoralen and aphidicolin cultures, the indicated time, in minutes after fertilization, is taken as either the data point when the experiment was terminated or as the data point just before the percentage of NEB in that culture exceeded 11%. For the control cultures, the peak of kinase activity occurred on average 81 min after fertilization. The percentage of NEB (%NEB) was determined from 100-cell counts at the time each sample was taken for processing. For each experiment all cultures were taken from a common pool of eggs. Experiments 1–3 are shown in Fig. 8 *A–C*, respectively.

Breakdown of Separate Male and Female Pronuclei in the Presence of Caffeine

These observations indicate that NEB is not simply driven by a rise in cytoplasmic MPF activity. This could be due to a functional separation of the nuclear compartment from the cytoplasm, which could allow for the independent regulation of a possibly distinct nuclear pool of MPF responsible for NEB. Alternatively, a nucleus under checkpoint control may not be able to respond, as it normally would, to a rise in nuclear or cytoplasmic MPF activity. To ad-

dress this issue, we fertilized normal eggs (*L. variegatus*) with psoralen-treated sperm and prevented pronuclear fusion with Colcemid. For each experiment, half of the culture was treated with 10 mM caffeine starting 35 min after fertilization, and the other half served as the control. We then followed individual zygotes of both populations to determine the times of male and female pronuclear envelope breakdown.

For the control populations (not treated with caffeine) the female pronuclei always broke down first (by an average of 13.1 min, $n = 19$, range 5–40 min), and in three additional cases, the male pronucleus did not break down during first mitosis. In the populations treated with caffeine, both pronuclei broke down synchronously in 13 cases, the female pronuclei broke down first in 20 cases (by an average of 6 min, range 1–36 min), and the male pronuclei broke down first in 13 cases (by an average of 17.5 min, range 4–36 min). Although we do not know why the two pronuclei did not always break down synchronously or in a consistent order, these data support the possibilities that the checkpoint for the completion of DNA synthesis operates either by limiting the transport of cell cycle regulatory proteins through the nuclear envelope or by downregulating a distinct pool of nuclear MPF. However, the mechanism by which caffeine overrides the checkpoint is not known. Since we cannot rule out the possibility that this agent modifies the ability of the nucleus to respond to nuclear or cytoplasmic MPF, drawing firm conclusions from these experiments may be premature.

Discussion

Our initial finding that separate pronuclei in sea urchin zygotes break down asynchronously was surprising given that NEB and entry into mitosis are thought to be driven by the rapid activation of MPF (for review see Nurse, 1990; Pines and Hunter, 1990; Maller, 1991; Murray, 1992), and that, with few exceptions, entry into mitosis is synchronous for nuclei in naturally occurring or experimentally produced multinucleate cells (for review see Johnson and Rao, 1971). We have explored the basis for this asynchronous NEB, and our results address two interrelated issues: first, whether the control NEB is exercised at the nuclear or cytoplasmic level, and second, the location of the target of the checkpoint pathway that monitors the completion of DNA synthesis (or possibly the repair of DNA damage).

Nuclear Envelope Breakdown Is Controlled at the Nuclear Not Cytoplasmic Level

We initially demonstrated that asynchronous NEB is not simply due to regional differences in the time of MPF activation in these relatively large cells. When the pronuclei are touching, the female pronucleus consistently breaks down first despite what should be a common cytoplasmic environment. This result suggests that NEB is regulated at the level of the individual nucleus and is not simply the result of global changes in the cytoplasmic environment. That the asynchrony of NEB is essentially the same whether the pronuclei are touching or separate argues against the possibility that the breakdown of one nucleus liberates activities that immediately drive the breakdown of the other nucleus.

We next wanted to evaluate the possibility that this asynchrony in NEB reflects a peculiarity of the male pronucleus, such as a slow but otherwise normal response to the rise in cytoplasmic MPF activity. Thus, we sought to determine if the order of male and female pronuclear breakdown can be manipulated by activating the checkpoint that monitors the completion of DNA synthesis selectively in one of the two pronuclei. If NEB is under nuclear control, then we should be able to experimentally control which nucleus breaks down first. For this purpose, we used psoralen-mediated cross-linking of base-paired DNA strands in either the male or female pronuclei as a nondiffusible inhibitor of DNA synthesis. We reasoned that such covalent cross-links should stall the propagation of replication complexes, thereby preventing or greatly delaying their disassembly. It has been proposed that assembled DNA replication complexes produce an inhibitory signal that activates the pathway that prevents entry into mitosis (Kornbluth et al., 1992; Li and Deshaies, 1993; Navas et al., 1995).

Before conducting this experiment, we wanted to first demonstrate that psoralen-mediated cross-linking of nuclear DNA will activate a checkpoint that blocks NEB in sea urchin zygotes. When we fertilized normal eggs with psoralen-treated sperm and allowed pronuclear fusion, NEB and entry into mitosis did not occur in the vast majority of zygotes for at least 3 h—well past the normal time of NEB starting ~1 h after fertilization. The psoralen-mediated cross-linking of the paternal genome appears to be as effective in maintaining the checkpoint activity as the complete inhibition of nuclear DNA synthesis by aphidicolin (also see Sluder and Lewis, 1987). That the psoralen- or aphidicolin-induced block to NEB can be overridden by millimolar concentrations of caffeine reveals that this checkpoint pathway in sea urchin zygotes has the same functional properties as the checkpoint described for mammalian cells (Schlegel and Pardee, 1986) and *Xenopus* egg extracts (Dasso and Newport, 1990). Additional evidence that we are activating this checkpoint, and not looking at an artifact, comes from experiments in which we cross-linked nuclear DNA in zygote (syngamy) nuclei after completion of S phase. The zygotes entered and proceeded through mitosis in synchrony with the controls but did not enter second mitosis. This would be expected if the DNA of the daughter nuclei is cross-linked and thus incapable of completely replicating after first mitosis.

In discussing our results, we assume that psoralen cross-linking of nuclear DNA activates the checkpoint that monitors the completion of DNA synthesis. However, it is possible that psoralen activates the checkpoint that monitors the repair of DNA damage. We suggest that this is a less likely possibility because cross-linking nuclear DNA after the completion of S phase does not impede entry into mitosis. In any case, our analysis does not depend on which checkpoint is activated, and the two checkpoints may share a portion of a signal transduction pathway that leads to cell cycle arrest (see Navas et al., 1995).

Our observations do not appear to be an artifact of the psoralen treatments or 366-nm light irradiations since nei-

ther psoralen or 366-nm light irradiations administered alone significantly delay NEB of the zygote nuclei. The fact that caffeine will allow NEB to proceed to completion, even 4 h after fertilization, indicates that the psoralen/366 nm light treatments do not inactivate the nucleus. Furthermore, these treatments cannot be cross-linking proteins in the nuclear envelope in a way that could prevent NEB, because the sperm nucleus loses its envelope shortly after fertilization (Longo and Anderson, 1968). Thus, the male pronuclear envelope must be composed entirely of maternal subunits that have not been exposed to psoralen/366 nm light. Why a small percentage of the zygotes fertilized with psoralen-treated sperm (and zygotes treated with aphidicolin) undergo NEB within 3 h of fertilization is not understood but may indicate that this checkpoint may be leaky to a variable extent in these zygotes (see Sluder and Lewis, 1987).

When only the maternal DNA is cross-linked, leaving a separate but normal male pronucleus in the zygote, the female pronucleus breaks down after the male pronucleus, especially for eggs treated with higher doses of psoralen. Conversely, cross-linking of just the paternal DNA does not delay the breakdown of the female pronucleus but always causes the male pronucleus to break down substantially later in mitosis than it normally would have, whether or not the pronuclei are touching. Thus, the delayed breakdown of the male pronucleus we originally observed (Sluder et al., 1994) is not some peculiarity of the male pronucleus but rather reflects the fundamental properties of the mechanisms that control NEB. In the absence of experimental intervention, delays in the breakdown of the male pronucleus may be due to the extra time required to reorganize the highly condensed sperm chromatin, which could delay paternal chromosome replication relative to that in the female pronucleus.

Our demonstration that DNA cross-linking can be used to selectively delay NEB in one pronucleus or the other shows that NEB is controlled at the level of the individual nucleus, not by the cytoplasmic environment when the checkpoint for the completion of DNA synthesis is activated. This is perhaps most graphically illustrated in those experiments in which we fertilized normal eggs with psoralen–cross-linked sperm, treated them with Colcemid, and followed zygotes in which the pronuclei were touching. In these cases, a short irradiation with 366 nm light to photochemically inactivate the Colcemid just after female pronuclear breakdown allowed the cell to assemble a functional bipolar spindle from the paternal centrosomes and the maternal chromosomes. However, in some of these zygotes, the male pronucleus remained structurally and functionally intact throughout mitosis as determined by same-cell correlative light and serial section electron microscopy as well as fluorescent dextran exclusion studies. Since the male pronucleus is located within one of the asters throughout mitosis, it is in the cytoplasmic environment of the spindle and is exposed to MPF activity sufficient to support normal mitosis. Thus, NEB cannot simply be a passive response of the nucleus to the activation of cytoplasmic MPF in sea urchin zygotes. This conclusion is supported by studies on other experimental systems, which indicate that the nucleus and cytoplasm are functionally distinct compartments from the standpoint of cell cycle regulation. In human cells, cyclin B1 has a cytoplasmic location, and its entry into the nucleus just precedes NEB (Pines and Hunter, 1991); in mammalian cells, inappropriately high levels of cytoplasmic MPF activity during interphase will not induce premature NEB if wee1, a nuclear protein, is overexpressed (Heald et al., 1993); and in budding yeast, the constitutive activation of the cdc28 (budding yeast homolog of cdc2) does not lead to precocious entry into mitosis or bypass the checkpoint for the completion of DNA synthesis (Sorger and Murray, 1992; Amon et al., 1992).

Target of the Checkpoint Monitoring the Completion of DNA Synthesis in Sea Urchin Zygotes

Studies on *Xenopus* egg extracts containing >300 nuclei/μl (Dasso and Newport, 1990) and fission yeast (Enoch and Nurse, 1990) provide strong evidence that the checkpoint for the completion of DNA synthesis prevents entry into mitosis by downregulating cytoplasmic MPF activity (measured as H_1 histone kinase activity) through the maintenance of the inhibitory phosphorylations on $p34^{cdc2}$ by a tyrosine kinase (Smythe and Newport, 1992). Additionally, egg extracts appear to have a titratable inhibitor of the cdc2–cyclin B complex that downregulates MPF activity in response to unreplicated DNA (Kumagai and Dunphy, 1995).

Our results, however, reveal that the checkpoint monitoring DNA replication must operate differently in sea urchin zygotes. The fact that spindle assembly and progression through mitosis can occur in the immediate presence of a male pronucleus with cross-linked DNA provides functional evidence that the checkpoint acts primarily at the nuclear level and is not downregulating cytoplasmic MPF activity. Nevertheless, this result could also be interpreted to indicate that the normal female pronucleus exerts a dominant positive effect on MPF activation that somehow bypasses the checkpoint pathway. To evaluate this possibility, we compared H_1 histone kinase activities as a function of time for control zygotes, eggs fertilized with psoralen-treated sperm where pronuclear fusion occurs, and normal zygotes treated with aphidicolin at doses sufficient to prevent DNA synthesis. For both eggs fertilized with psoralen-treated sperm and zygotes treated with aphidicolin, H_1 histone kinase activity progressively rose over 3–6 h to values close to or higher than the peak values for the controls at first mitosis, even though the vast majority of the zygotes had not undergone NEB. This result clearly reveals that sea urchin zygotes behave differently than aphidicolin-treated frog egg extracts in which H_1 histone kinase activity reaches a plateau of one third of the peak activity found in the controls at M phase (Dasso and Newport, 1990). Since the rise in H_1 histone kinase activity is not correlated with corresponding increases in the percentage of NEB, it is unlikely that the rise in kinase activity we observed was due to that small percentage of zygotes that had entered mitosis. Also, our results are not due to the possibility that we are working with an embryo system that has a rigidly programmed cell cycle, as may be the case for *Xenopus* egg extracts with <100 nuclei/μl (see Dasso and Newport, 1990; Murray, 1992). The ratio of nuclear number to total cytoplasmic volume for first-division sea urchin zygotes is ~2,000/μl.

It is important to note, however, that our results indicate that the checkpoint does in fact downregulate the rate of MPF activation to some extent, but its action is not absolute. H_1 histone kinase activity rises to mitotic levels only over a period of hours in checkpoint-inhibited sea urchin zygotes with a single nucleus and is 20–44% of peak control values in cultures fertilized with psoralen-treated sperm and 36–61% of peak control values for aphidicolin-treated cultures when the controls show peak kinase activity at first mitosis. Nevertheless, the fact that the vast majority of zygote nuclei at ≥3 h do not break down in the presence of mitotic or supramitotic levels of MPF activity indicates that the checkpoint must act also at the level of the nucleus to prevent NEB, regardless of its effect on the time course of MPF activation.

It is noteworthy that when the cross-linked male nucleus is prevented from fusing with the normal female pronucleus (Fig. 5), MPF activity appears by functional criteria to reach mitotic levels sooner than in zygotes where the pronuclei have fused to form a single checkpoint-limited nucleus. This suggests that the normal nucleus has activities that promote the activation of cytoplasmic MPF. The liberation of activated cdc25 phosphatase from the female pronucleus at its breakdown could, for example, accelerate the activation of cytoplasmic MPF.

It is currently unknown how the checkpoint for the completion of DNA synthesis acts to limit NEB at the nuclear level in sea urchin zygotes. In principle, the checkpoint could directly regulate the activation of a distinct intranuclear pool of MPF by shifting the cdc25 phosphatase–wee1 kinase activity balance, modulate the activity of an inhibitor (see Heald et al., 1993; Maller, 1994; Kumagai and Dunphy, 1995), or it could indirectly control intranuclear MPF activity by modulating transport of cyclin B (or other proteins) into the nucleus (Pines and Hunter, 1991). Support for the possibility that the checkpoint may operate by regulating the transport of cell cycle regulatory proteins into the nucleus comes from the finding that RCC1, an abundant chromatin protein that is part of the checkpoint pathway, is a guanine nucleotide release factor for ran/TC4 (reviewed in Dasso, 1993). Ran/TC4 is not only involved in nuclear import (Melchior et al., 1993), but also appears to play a direct role in the activation of cytoplasmic MPF in nucleus-free *Xenopus* egg extracts (Kornbluth et al., 1994). The importance of nuclear transport in the checkpoint that monitors DNA synthesis, even in *Xenopus*, is also suggested by the demonstration that wheat germ agglutinin, an inhibitor of transport through the nuclear pores, blocks the activation of the $p34^{cdc2}$–cyclin B complex (Kumagai and Dunphy, 1991).

Species-specific Differences in the Control of NEB?

That NEB is controlled at the nuclear level in sea urchin zygotes and at the cytoplasmic level in other experimental systems challenges only the notion that all cells control their cell cycle in the same way (discussed in Murray, 1992). Indeed, there may well be species-specific differences in the extent to which cell cycle regulatory proteins are compartmentalized and how the checkpoint for the completion of DNA synthesis modulates nuclear transport. In the words of Mazia (1961), "variability would be most perplexing if we insisted on endowing the ideas of 'control mechanisms' nuclear or cytoplasmic, with some absolute cybernetic significance. But if we look upon nuclear division as a problem of nucleus–cytoplasm interaction in which either partner may be the limiting one in a given case, the problem is not so serious from a theoretical standpoint and much more serious—because it calls for work—from a practical standpoint."

We thank Dr. James Maller for giving us purified histone H_1 for our kinase activity assays, and Dr. Earl Baril and Sandy Mayrand for kindly giving us labeled ATP for some experiments. We also thank R. Cole and G. Osorio for serial sectioning zygotes and the high-voltage electron microscopy; Drs. Fatima Gebauer and Hideake Kuge for helping us learn to make and run gels; Dr. Barbara Stebbins-Boaz for starting us on the phosphorimager; and Christine McKinnon for expert help with graphics software. The help of Steven Norman and Andrea Werme in printing the manuscript is most appreciated.

Support for this research was provided by National Institutes of Health grants GM-30758 to G. Sluder, GM-40198 to C. L. Rieder, and PHS-01219 awarded by the Department of Health and Human Services/Public Health Service to support the Wadsworth Center Biological Microscopy and Image Reconstruction Facility as a National Biotechnological Resource.

Received for publication 11 November 1994 and in revised form 29 March 1995.

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