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# Nucleo-cytoplasmic interactions that control nuclear envelope breakdown and entry into mitosis in the sea urchin zygote

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## SUMMARY

In sea urchin zygotes and mammalian cells nuclear envelope breakdown (NEB) is not driven simply by a rise in cytoplasmic cyclin dependent kinase 1-cyclin B (Cdk1-B) activity; the checkpoint monitoring DNA synthesis can prevent NEB in the face of mitotic levels of Cdk1-B. Using sea urchin zygotes we investigated whether this checkpoint prevents NEB by restricting import of regulatory proteins into the nucleus. We find that cyclin B1-GFP accumulates in nuclei that cannot complete DNA synthesis and do not break down. Thus, this checkpoint limits NEB downstream of both the cytoplasmic activation and nuclear accumulation of Cdk1-B1. In separate experiments we fertilize sea urchin eggs with sperm whose DNA has been covalently cross-linked to inhibit replication. When the pronuclei fuse, the resulting zygote nucleus does not break down for >180 minutes (equivalent to three cell cycles), even though Cdk1-B activity rises to greater than mitotic

levels. If pronuclear fusion is prevented, then the female pronucleus breaks down at the normal time (average 68 minutes) and the male pronucleus with cross-linked DNA breaks down 16 minutes later. This male pronucleus has a functional checkpoint because it does not break down for >120 minutes if the female pronucleus is removed just prior to NEB. These results reveal the existence of an activity released by the female pronucleus upon its breakdown, that overrides the checkpoint in the male pronucleus and induces NEB. Microinjecting wheat germ agglutinin into binucleate zygotes reveals that this activity involves molecules that must be actively translocated into the male pronucleus.

Key words: Cell Cycle, Checkpoint, Mitosis, Nucleus, Nuclear Envelope

## INTRODUCTION

Entry into mitosis depends upon the rapid activation of the cyclin dependent kinase 1-cyclin B complex (Cdk1-B; historically referred to as p34<sup>cdc2</sup>-cyclin B) at the end of G<sub>2</sub> (reviewed by Nurse, 1990; Pines and Hunter, 1990; Maller, 1994; Murray, 1992). Although cyclin B starts to accumulate and associate with the Cdk1 kinase in S phase, the activity of this complex does not correspondingly increase because of inhibitory phosphorylations on threonine 14 and tyrosine 15 residues of the Cdk1 subunit mediated by the Wee1, Myt1, and related kinases (reviewed by Lew and Kornbluth, 1996; Morgan 1997). Activation of Cdk1-B at the time of mitosis involves the dephosphorylation of these residues by the activation of the CDC25C phosphatase (Kumagai and Dunphy, 1991; Sebastian et al., 1993; reviewed by Maller, 1994; Morgan, 1997).

When DNA synthesis is perturbed, nuclear envelope breakdown (NEB) and the onset of mitosis are blocked by a checkpoint pathway (reviewed by Hartwell and Wienert, 1989; Murray, 1992; Dasso et al., 1992; Maller, 1994; Li and Deshaies, 1993; Elledge, 1996). This checkpoint was initially

thought to inhibit entry into M phase simply by preventing the activation of Cdk1-B activity through the maintenance of the inhibitory phosphorylations on the Cdk1 subunit (Dasso and Newport, 1990; Kornbluth et al., 1992; reviewed by Morgan 1995; Lew and Kornbluth, 1996). However, the notion that this checkpoint operates solely at the level of Cdk1-B activation has been brought into question by studies on a variety of cell types which indicate that a rise in cytoplasmic Cdk1-B activity alone is not sufficient to drive NEB. In sea urchin zygotes this checkpoint slows but does not block the activation of cytoplasmic Cdk1-B activity; nuclei do not break down for at least two hours past the normal time of first NEB even though Cdk1-B activity reaches mitotic or supra-mitotic levels (Sluder et al., 1995). For mammalian somatic cells the microinjection of purified, active Cdk1-B at various points in the cell cycle led to a prophase morphology without breakdown of the nucleus (Lamb et al., 1990). Also, Heald et al. (1993) observed that the overexpression of p50<sup>wee1</sup>, a nuclear kinase, ensures completion of DNA synthesis and normal mitosis in cells with inappropriately high levels of cytoplasmic Cdk1-B activity that would otherwise lead to premature mitosis. Finally, the expression of Cdc2AF (a constitutively active mutant Cdk1

that lacks the threonine 14 and tyrosine 15 inhibitory phosphorylation sites) at normal Cdk1 levels has little effect on the progression of human cells through S phase or mitosis (Jin et al., 1996; Hagting et al., 1998; also see Sorger and Murray, 1992; Amon et al., 1992). Cells with damaged DNA that express Cdc2AF show a reduced but still considerable delay in the onset of mitosis even though they contain high levels of cytoplasmic Cdk1-B activity (Jin et al., 1998).

An explanation for these phenomena has recently been provided by a number of studies indicating that the regulation of NEB in normal and checkpoint arrested cells may be exercised by mechanisms that regulate the partitioning of Cdk1-B between the cytoplasm and the nucleus (Pines and Hunter, 1991; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992; Gallant et al., 1995). Normally, Cdk1-B is localized to the cytoplasm until prophase at which time it accumulates in the nucleus where it may drive NEB (Pines and Hunter 1991). Recent work indicates that Cdk1-B complexes shuttle between the nucleus and the cytoplasm during interphase; the cytoplasmic localization of Cdk1-B during interphase is due to rapid export of Cdk1-B from the nucleus, rather than cytoplasmic retention of the cyclin B subunit as previously proposed (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). That this temporal control of Cdk1-B localization plays a role in checkpoint function was initially suggested by observations that cyclin B remains cytoplasmic in cells arrested in G<sub>2</sub> by DNA damage (Smeets et al., 1994; Jin et al., 1996). More recently Jin et al. (1998) found that expression of a constitutively nuclear cyclin B caused a significant reduction in the G<sub>2</sub> delay due to DNA damage. Coexpression of Cdc2AF and a constitutively nuclear cyclin B in cells with DNA damage led to premature mitosis (also see Hagting et al., 1998).

The studies described in this paper were designed to further investigate the nucleo-cytoplasmic interactions that control NEB using the sea urchin zygote as a model system. First, we directly examine the extent to which the checkpoint for the completion of DNA synthesis effects the import and accumulation of proteins, such as cyclin B1, in the nucleus. Our previous finding that a zygote nucleus unable to complete DNA synthesis does not break down even when cytoplasmic Cdk1-B activity is at higher than mitotic levels (Sluder et al., 1995) could be explained by checkpoint-mediated limitations on the accumulation of cyclin B1 in the nucleus, as is the case for mammalian cells (Hagting et al., 1998). This possibility is supported by the recent finding that functional nuclear pores are required for NEB in sea urchin egg extracts (Collas, 1998). However, cell fractionation and immunofluorescence observations indicate that Cdk1-B activity may be present in sea urchin zygote nuclei from the time of S phase, even though NEB does not occur until much later (Genevriere-Garrigues et al., 1995).

Second, we have pursued a phenomenon that may provide additional information on the control for NEB (Sluder et al., 1995). When normal eggs are fertilized with sperm whose DNA is covalently crosslinked to prevent replication, the resulting zygote nucleus does not break down for at least two hours past the normal time for first mitosis, even though Cdk1-B activity rises to higher than mitotic values. If, however, pronuclear fusion is blocked, then binucleate zygotes are formed, each containing a normal female pronucleus, and a

male pronucleus with cross-linked DNA. The female pronucleus undergoes NEB at the normal time for mitosis, followed shortly thereafter by the male pronucleus. Since the breakdown of the male pronucleus (which presumably is under local checkpoint control) should not be due solely to the rise in cytoplasmic Cdk1-B activity, we have explored the possibility that a normal nucleus has a heretofore unrecognized activity that can override the checkpoint in a nucleus that cannot complete DNA synthesis.

## MATERIALS AND METHODS

### Living material

*Lytechinus pictus* sea urchins were purchased from Marinus Inc. (Long Beach, CA). Eggs and sperm were obtained by intracoelomic injection of 0.5 M KCl (Fuseler, 1973). To block pronuclear fusion unfertilized or just fertilized eggs were treated for 4-8 minutes with  $5 \times 10^{-6}$  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 (Sluder et al., 1994; reviewed by Sluder, 1991). After colcemid treatment, the eggs were fertilized and cultured in natural sea water at 16-19°C. Shortly before the expected time of first nuclear envelope breakdown the zygotes were mounted in fluorocarbon oil preparations or in microinjection chambers as previously described (Sluder et al., 1999). For some experiments fragmentation of unfertilized eggs or just fertilized eggs was conducted as previously described (Sluder et al., 1989). For other experiments the female pronucleus was removed using methodology previously described (Sluder et al., 1986). Wheat germ agglutinin (WGA: Calbiochem, La Jolla, CA) was microinjected at 10 mg/ml in distilled water into the cytoplasm of binucleate zygotes in late prophase (50-60 minutes after fertilization). Our microinjection methods are described by Kiehart (1982) with minor modifications described by Sluder et al. (1999).

Individual zygotes were followed at 19°C by polarization microscopy with a modified Zeiss ACM microscope (Carl Zeiss Inc., Thornwood, NY) or an Olympus BH-2 microscope equipped with differential interference contrast (DIC) optics (Olympus Corporation of America, New Hyde Park, NY). Photographs were recorded on Kodak Plus X film which was developed in Kodak Microdol-X (Eastman Kodak Inc., Rochester, NY). For some figures video images were acquired with a CCD camera (Hammamatsu, Bridgewater, New Jersey), and directly written to a personal computer.

### Assays for protein translocation into the nucleus

Fluorescent bovine serum albumen (BSA) with nuclear localization signals was kindly provided by Dr Bryce Paschal (University of Virginia) This reagent was microinjected into the cytoplasm of normal and aphidicolin treated zygotes in early prophase, approximately 30-40 minutes after fertilization. In these experiments eggs were continuously treated with aphidicolin (Sigma Chemical Co., St Louis MO) from before fertilization as previously described (Sluder and Lewis, 1987). Injected zygotes were observed and photographed on a Zeiss 'Axioscope' fluorescence microscope.

*Xenopus* cyclin B1 cDNA, kindly provided by Dr Carl Smythe (University of Dundee, UK) was amplified by PCR using primers N (5'-GGGGGAATTCAGAAAATGTCGCTACGAGTCACC-3'), and C (5'-GGGGGATCCCATGAGTGGGCGGGCCATTTCAC-3'). To facilitate cloning, primer N was designed to contain *EcoRI* and primer C was designed to contain *BamHI*. The PCR product was digested with *EcoRI* and *BamHI* and cloned into these sites of pGemex-1 vector (Promega, Madison, WI). The green fluorescent protein (GFP:S65T) in pBSKII, kindly provided by Dr Jonathan Pines (Wellcome/CRC Institute, Cambridge, UK), was digested with *NotI* and cloned into the *NotI* site of pGemex-1 to generate the construct

where GFP is fused to the C terminus of cyclin B1. Then the cyclin B1:GFP fusion construct was digested with *EcoRI* and *HindIII* and cloned into the *BglIII* site of pSP64T vector through blunt-end ligation.

mRNA coding for GFP-cyclin B1, containing a 5' 7-methyl guanosine cap, was synthesized from the pSP64T vector using the Ambion mMESSAGE mMACHINE In Vitro Transcription Kit (Ambion Inc., Austin, TX). The mRNA yield from each transcription reaction was determined as per the kit instructions, using percentage incorporation of a trace nucleotide ( $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ ) added to the reaction mixture.

Fertilized eggs were microinjected with GFP-cyclin B1 mRNA (1 mg/ml), and the GFP fluorescence was observed by confocal microscopy on a Nikon Diaphot 200 using a  $\times 60$  NA 1.20 water immersion objective and a Bio-Rad MRC 1024 confocal scanner. Fluorescence intensity in the nuclear and cytoplasmic compartments was quantified with Scion Image Software (Scion Inc., Frederick, MD).

WGA (10 mg/ml), along with GFP-cyclin B1 mRNA was microinjected into the cytoplasm of aphidicolin-treated zygotes 50-60 minutes after fertilization, and fluorescence was observed at 180 minutes.

### Psoralen treatment of eggs and sperm

To form covalent cross-links between basepaired DNA strands in the male pronucleus, live sperm were treated for 15-25 minutes with 5  $\mu\text{g/ml}$  4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) (HRI Assoc., Inc. Concord, CA) in sea water and then irradiated at 4°C with a long-wave ultraviolet box (Blak-Ray Lamp, model UVL-21, Ultra Violet Products, Inc., San Gabriel, CA) for 8-10 minutes at 3.6  $\text{mW/cm}^2$ . Such sperm preparations were then used to fertilize untreated eggs; the final concentration of HMT after sperm dilution did not exceed 0.03  $\mu\text{g/ml}$ .

Histone H<sub>1</sub> kinase assays were performed as described by Sluder et al. (1995). Quantification of counts in H<sub>1</sub> histone bands was performed with Image Quant Software Version 3.3 (Molecular Dynamics, Sunnyvale, CA). H<sub>1</sub> histone kinase activity is expressed as 'volume' which is the sum of pixel values within each H<sub>1</sub> histone band minus background.

## RESULTS

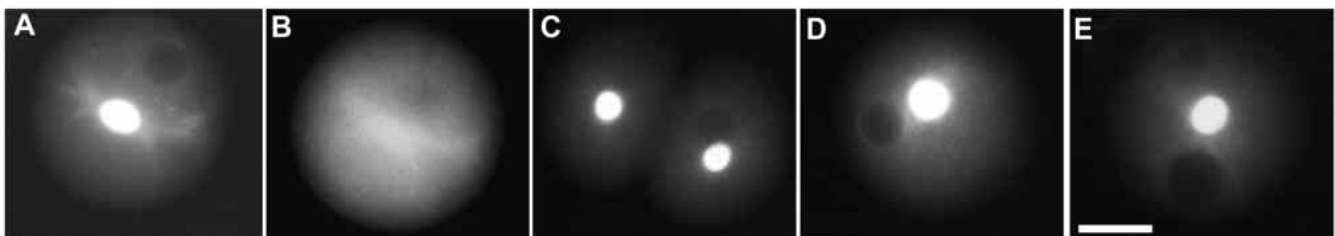
### Protein translocation into the nucleus

Since the checkpoint monitoring DNA synthesis can prevent NEB in the face of mitotic levels of Cdk1-B activity in the cytoplasm, we investigated the possibility that this checkpoint prevents NEB by restricting import of regulatory proteins into

the nucleus. We microinjected fluorescent BSA conjugated with nuclear localization signal sequences into normal zygotes and zygotes prevented from completing DNA synthesis. When injected into normal zygotes in prophase of first mitosis, this protein is efficiently transported and concentrated into the nucleus before onset of mitosis (Fig. 1A). At NEB the fluorescent protein is liberated into the cytoplasm and diffuses throughout the cell (Fig. 1B). When nuclear envelopes reform in telophase, the fluorescent BSA rapidly returns to the daughter nuclei (Fig. 1C).

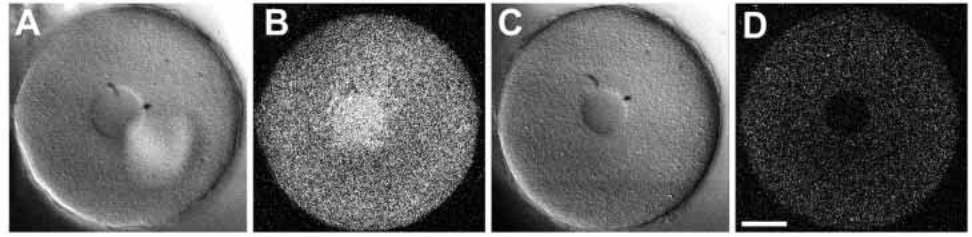
We then injected this protein into zygotes that had been treated from the time of fertilization with aphidicolin, a specific inhibitor of the alpha DNA polymerase (Ikegami et al., 1979). Even though these zygotes were arrested in S phase by the checkpoint for DNA synthesis, the fluorescent BSA was transported and concentrated into the nucleus to the same extent as that in control cells (Fig. 1D,E). This result indicates that the checkpoint monitoring DNA synthesis does not operate by non-specifically inhibiting all protein import into the nucleus; limits, if any, must be substrate specific.

We next sought to determine if this checkpoint blocks NEB by preventing the accumulation of cyclin B1 in the nucleus as has been proposed for mammalian somatic cells and oocytes (Pines and Hunter, 1991; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992; Gallant et al., 1995; Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). We microinjected mRNA encoding green fluorescent protein (GFP) conjugated to the C terminus of cyclin B1 into the cytoplasm of zygotes that were treated with aphidicolin from the time of fertilization. Our rationale was to use the expressed cyclin B-GFP as a tracer to assess the behavior of native cyclin B and Cdk1-cyclin B1 complexes. Cyclin B-GFP binds to Cdk1 and activates its kinase activity; it also shows the same localization in vivo as native cyclin B in fixed cells, and the GFP moiety does not cause cyclin B to localize where the native protein is not present (Hagting et al., 1998). Injected zygotes were then followed for 3 hours using a confocal microscope to record fluorescence intensity in optical sections through the nucleus. Together, the relatively large size of the nucleus in checkpoint arrested zygotes (approximately 23  $\mu\text{m}$ ), the 1.2 NA  $\times 60$  objective, and the confocal optics ensured that cytoplasmic fluorescence above and below the nucleus did not contribute significantly to our observations. To minimize any possible radiation damage



**Fig. 1.** The checkpoint monitoring the completion of DNA synthesis does not block nuclear import of fluorescent bovine serum albumin (BSA) containing nuclear localization signal sequences. (A) Control zygote injected at approximately 30 minutes after fertilization. By early prophase of first mitosis the BSA has translocated into the zygote nucleus. The dark sphere seen in the upper right quadrant of the cell is a drop of the oil used to cap the micropipette. (B) The same cell photographed during mitosis; the fluorescent BSA has dispersed throughout the cytoplasm. (C) The same cell in late telophase of first mitosis. The label has translocated into the blastomere nuclei. The oil drop is visible in the right hand cell. (D,E) Two examples of zygotes treated with aphidicolin from fertilization, injected 120 minutes after fertilization and photographed 60 minutes later. The labeled BSA has translocated into the nucleus even though the cell cycle is arrested by the checkpoint. Oil drops are visible in both cells. Widefield fluorescence microscopy of living zygotes. Bar, 10  $\mu\text{m}$ .

**Fig. 2.** The checkpoint monitoring the completion of DNA synthesis does not prevent cyclin B accumulation in the nucleus. Aphidicolin treated zygotes were injected approximately 50 minutes after fertilization with mRNA for cyclin B1 conjugated to GFP and observed in vivo by confocal microscopy. (A) Injected zygote seen by differential interference contrast optics (DIC) optics 180 minutes after fertilization. (B) Cyclin B1-GFP fluorescence in the same zygote seen by confocal microscopy. (C) Uninjected zygote seen in DIC 180 minutes after fertilization. (D) The same zygote seen by confocal microscopy with the fluorescence channel; the low level of cytoplasmic autofluorescence is not present in the nucleus. The same confocal instrument settings and printer settings were used for all panels. Bar, 20  $\mu$ m.

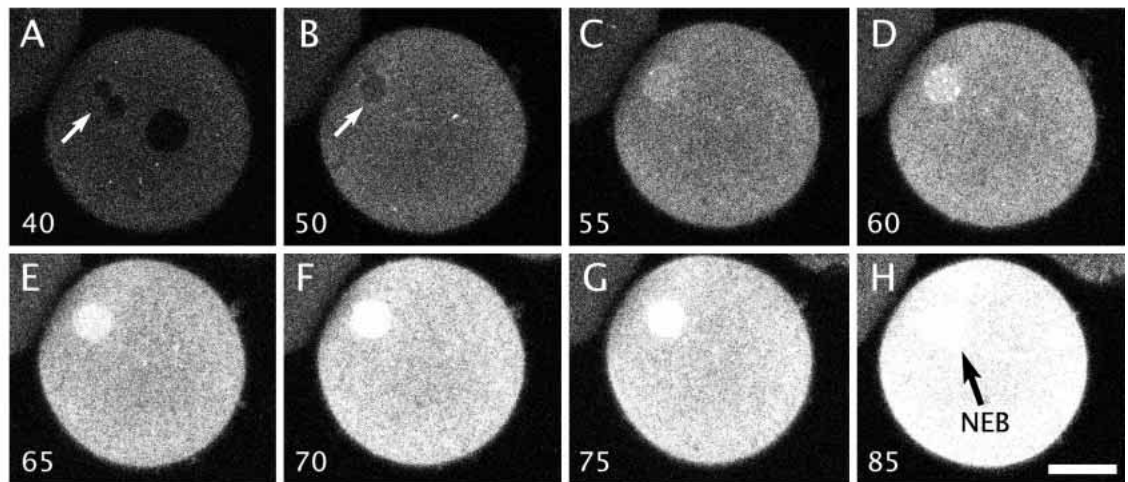


from the blue excitation light, the zygotes were observed intermittently.

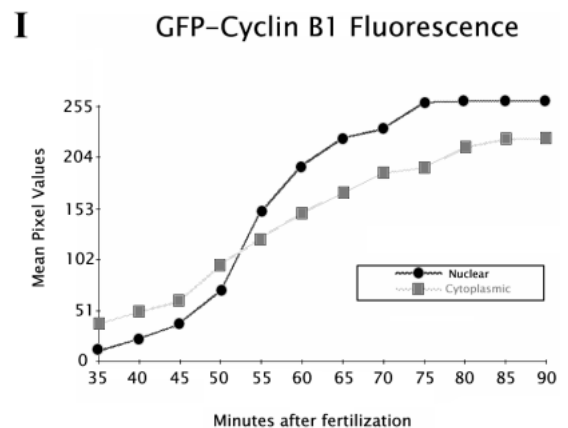
We found that the fluorescence intensity increased with time in both the cytoplasm and the nucleus. Fig. 2A,B shows the fluorescence in the nucleus of a zygote injected with mRNA for cyclin B1-GFP at 50 minutes after fertilization and observed 130 minutes later. Although the fluorescence intensity in the nucleus appears to be greater than that in the cytoplasm, we do not know if this is due to a concentration of cyclin B-GFP in the nucleus or the presence of yolk granules in the cytoplasm that reduce the fluorescence per unit area of the optical section. Nevertheless, cyclin B-GFP, presumably complexed with Cdk1 (Hagting et al., 1998), is clearly present

in a nucleus that cannot complete DNA synthesis and does not break down for the equivalent of three cell cycles. Confocal microscopy of control zygotes treated with aphidicolin but not injected with cyclin B-GFP construct revealed that there is a low level of autofluorescence in the cytoplasm that is not present in the nucleus (Fig. 2C,D). Since the construct we used has the GFP moiety on the COOH end of the cyclin B protein, our results could not reflect the presence of free GFP generated by incomplete translation of the construct.

To determine if this nuclear localization of Cdk1-cyclin B-GFP was peculiar to zygotes that cannot complete DNA synthesis, we examined the distribution of GFP-cyclin B in normal zygotes. The mRNA was injected 20 minutes after



**Fig. 3.** Cyclin B1-GFP accumulates in the cytoplasm and nucleus of normal zygotes during the first cell cycle. Zygotes were injected approximately 20 minutes after fertilization with mRNA for cyclin B1-GFP and observed in vivo by confocal microscopy. (A) The male and female pronuclei (arrow) have migrated together, but are excluded from their normal position at the cell center by the oil drop (large dark sphere) used to cap the microinjection needle. (B) The pronuclei are touching but have not fused (arrow). The oil drop has moved out of the plane of focus. Note that there is little fluorescence in the pronuclei. (C-D) The pronuclei have fused, and there is an increase in both nuclear and cytoplasmic fluorescence. (E-G) Nuclear fluorescence increases before nuclear envelope breakdown. (H) The zygote nucleus (arrow) breaks down. Minutes after fertilization are shown lower left. Bar, 20  $\mu$ m. (I) Quantification of the cyclin B1-GFP fluorescence in the nucleus (circles) and the cytoplasm (squares) of this zygote as a function of time. Ordinate: fluorescence intensity expressed as mean pixel value, which is the mean of the pixel values of a  $6 \mu\text{m} \times 6 \mu\text{m}$  region of the image, minus the field background. Abscissa: minutes after fertilization.



fertilization and the zygotes were followed at 5 minute intervals until the time of NEB. Fig. 3 shows one such zygote from 40 minutes after fertilization when the pronuclei had come together just prior to syngamy (arrows in A and B). We observed that later the fluorescence increased in both the cytoplasm and the zygote nucleus with the nucleus appearing brighter than the cytoplasm after 55 minutes. NEB for this particular cell occurred at 85 minutes after fertilization (Fig. 3H), within the normal range of times for NEB. Quantification of average pixel intensity within the nucleus and cytoplasm for this cell shows that fluorescence increases coordinately in the nucleus and cytoplasm. Other zygotes examined showed qualitatively similar patterns of nuclear and cytoplasmic fluorescence increases, although the rapid increase in nuclear fluorescence between 50 and 65 minutes was not always observed. These results reveal that nuclear localization of Cdk1-B occurs well before NEB during the normal first cell cycle in these zygotes (also see Genevriere-Garrigues et al., 1995).

#### Activity of a normal nucleus that overrides the checkpoint in a separate nucleus

We fertilized eggs from a single female with sperm whose DNA was cross-linked with the psoralen HMT, a compound that intercalates into DNA and upon activation with 366 nm light covalently cross-links basepaired DNA strands (reviewed by Cimino et al., 1985). Such cross-links should inhibit the propagation of replication complexes during S phase 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). Psoralen crosslinking of DNA effectively activates the checkpoint monitoring DNA synthesis (Sluder et al., 1995). Eggs fertilized by sperm treated with 366 nm light or psoralen alone undergo NEB at the normal time (Sluder et al., 1995).

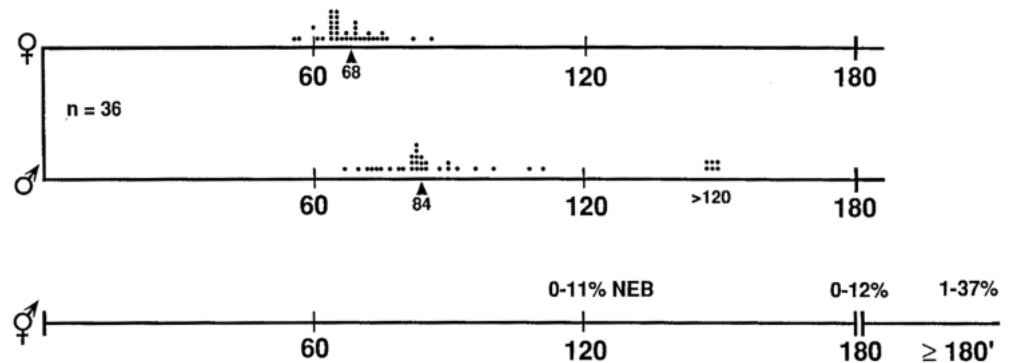
Shortly after fertilization the culture was split. One half was treated with colcemid, at microtubule specific doses, to prevent microtubule assembly and hence pronuclear fusion. In the other half of the culture pronuclear fusion occurred yielding a single zygote nucleus in each cell with half of the genome that could not complete DNA synthesis. In both cultures the source

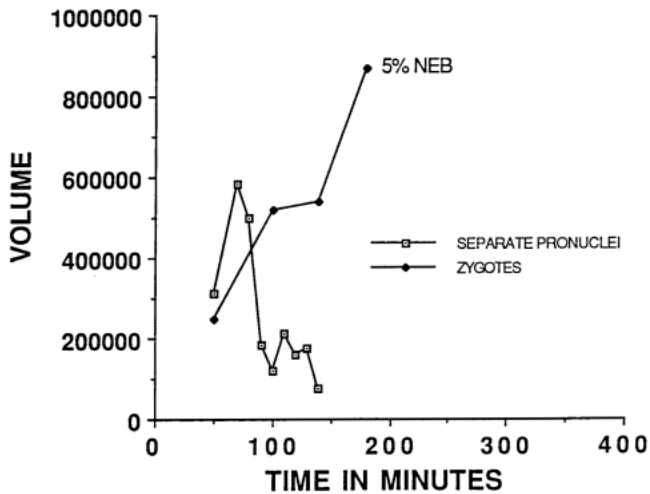
and amount of cross-linked DNA was the same. We then followed both cultures paying particular attention to the time of NEB for the male pronucleus with cross-linked DNA in the binucleate zygotes relative to NEB in cells with a single zygote nucleus. At the outset we note that the time of NEB is not influenced by the presence or absence of astral microtubules (Sluder, 1979).

In the binucleate zygotes the female pronucleus breaks down at the normal time (average 68 minutes post fertilization) (Fig. 4). Here the times of NEB for individual cells are shown by dots above the horizontal line; the numbers under the line represent minutes after fertilization. The smaller male pronucleus breaks down on average at 84 minutes or 16 minutes after the female pronucleus. This average does not include the relatively few cases we find in every experiment in which the male pronucleus does not break down while the zygote completes mitosis with or without microtubule assembly (also see Fig. 5 of Sluder et al., 1995). The delayed NEB of the male pronucleus is not a peculiar characteristic of this nucleus; when the DNA of the female pronucleus is cross-linked with psoralen, the maternal nucleus breaks down after a normal male pronucleus (Sluder et al., 1995). By contrast, NEB in the mononucleated culture does not begin at an appreciable percentage until at least 180 minutes after fertilization (Fig. 4, lowest time axis). Thereafter the rate of NEB is variable in both time course and extent for different batches of eggs (see Sluder and Lewis, 1987; Sluder et al., 1995).

A comparison of the time course of whole cell Cdk1-B activity (measured by histone H1 kinase activity) in paired cultures from single females revealed that Cdk1-B activity rises significantly sooner in cultures with separate pronuclei relative to mononucleated cultures (Fig. 5). For the binucleate culture H1 histone kinase activity peaks at approximately 1 hour post fertilization. In the parallel mononucleate culture H1 activity rises continuously but more slowly and reaches supramitotic values by the end of the experiment at 180 minutes after fertilization even though only 5% of the nuclei have broken down (also see Sluder et al., 1995; Hinchcliffe et al., 1998). For whole sea urchin zygote homogenates, greater than 90% of the H1 histone kinase activity associated with Cdk1 is specific to the kinase bound to cyclin B (Genevriere-Garrigues et al., 1995).

**Fig. 4.** Times of NEB in parallel binucleate and mononucleate cultures. The source and amount of crosslinked DNA is the same in both cultures. The times for male or female pronuclear breakdown in individual binucleate cells are shown by the dots above the time axes, labeled in minutes after fertilization. The mean time of NEB is shown by an arrowhead under the axes and given in minutes after fertilization. Note that in 6 cases the male pronucleus did not break down during mitosis; these were not included in the calculation of the mean. The lowest time axis shows percentage NEB for zygote nuclei (fused male and female pronuclei) for three times after fertilization based on a minimum of 100 cell counts. For four experiments the range of percentage NEB is indicated.





**Fig. 5.** H1 histone kinase activity as a function of minutes after fertilization for zygotes with separate pronuclei (open squares) and zygotes from the parallel culture with single nuclei (filled diamonds). Percentage NEB at 180 minutes for the culture of zygotes with single nuclei is shown next to the last data point. Ordinate: H1 kinase activity expressed as 'volume', which is the sum of the pixel values of the H1 band minus background as determined in the phosphorimager. Abscissa: minutes after fertilization.

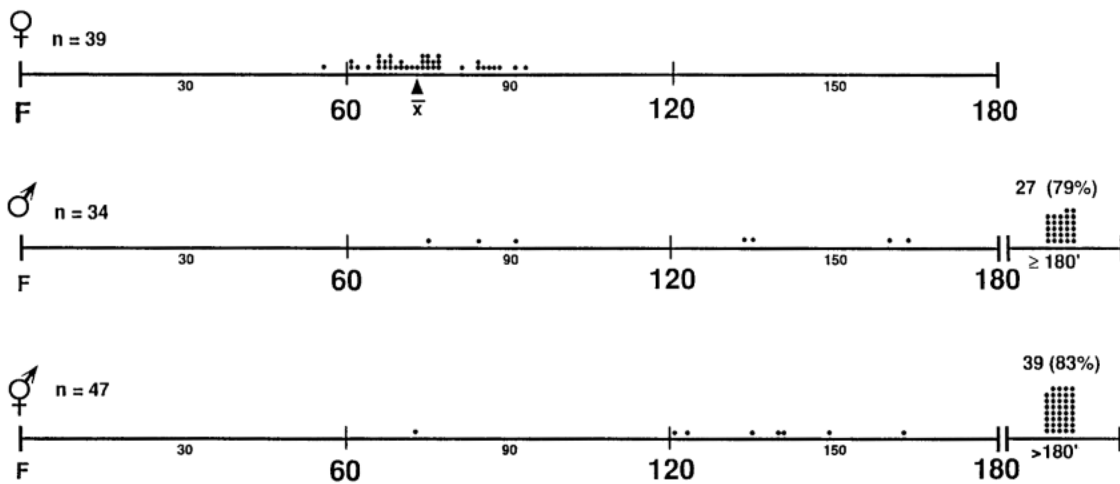
At this point it was important to determine whether or not the breakdown of the male pronucleus in the presence of a normal female pronucleus is due to some peculiar property of the male pronucleus with cross-linked DNA that renders it incapable of arresting the cell cycle before mitosis. To examine this issue we fertilized normal eggs with psoralen treated sperm and then fragmented them with a nylon screen shortly thereafter (Sluder et al., 1989). This yields four classes of fragments: (i) no nuclei, (ii) only a male pronucleus, (iii) only a female pronucleus, and (iv) both pronuclei, which later fuse

to form a zygote nucleus. The various classes of fragments can be easily differentiated shortly after fragmentation by the number of nuclei and by the noticeably smaller relative size of the male pronucleus. The fragmentation procedure does not compromise the viability of the zygote fragments (Sluder et al., 1989).

In this experiment we compared the times of NEB for fragments containing only a female pronucleus, only a male pronucleus, and a zygote nucleus (fused male and female pronuclei). The data for the three classes of fragments are displayed in Fig. 6. For the fragments with only a female pronucleus the mean time of NEB is 74 minutes (range 56-93 minutes) after fertilization. For the fragments containing just a male pronucleus with cross-linked DNA, 79% had not undergone NEB by 180 minutes when the observations were terminated. The fragments with zygote nuclei showed similar behavior with 83% failing to undergo NEB by the end of the experiment at 180 minutes after fertilization. These results reveal that the male pronucleus does not have peculiar properties that preclude it from activating the checkpoint for the completion of DNA synthesis.

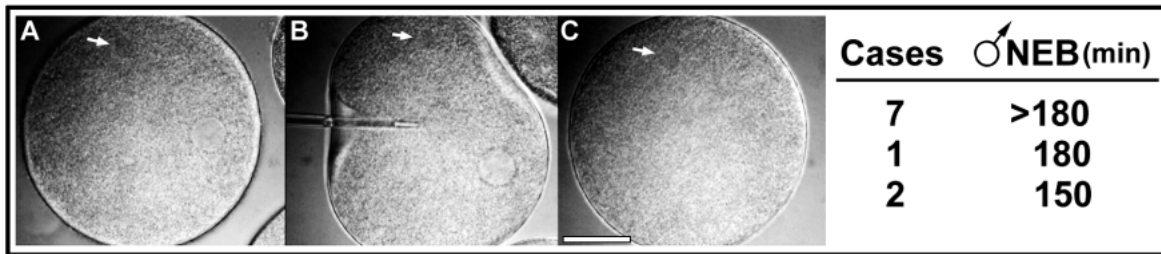
To determine if the checkpoint pathway originating in the male pronucleus with cross-linked DNA was disabled by the presence of a normal female pronucleus, we used a micropipette to remove the female pronucleus at approximately one hour post fertilization, shortly before the expected time of NEB (Fig. 7). In 10 experiments we found that in 7 cells the remaining male pronucleus did not break down by the time the observations were terminated at 3 hours after fertilization. The earliest breakdown of the remaining pronucleus occurred at 2.5 hours (two cells) and one cell underwent NEB at 3 hours after fertilization. Therefore, the checkpoint pathway originating in the male pronucleus is functional, or rapidly becomes so, after the removal of the female pronucleus.

In our final series of experiments we sought to determine if the accelerated breakdown of the male pronucleus requires active nuclear import of molecules presumably originating



**Fig. 6.** Comparison of times of NEB for zygote fragments containing just a female pronucleus (top time axis), just a male pronucleus containing crosslinked DNA (middle axis), and a fused male/female nucleus (bottom axis). Times for NEB of individual cells are shown by dots above the time axes. The mean time of NEB for the fragments with just a female pronucleus is shown by the arrowhead under the time axis. Means were not calculated for the other fragment classes because so many of the cells had not undergone NEB by 180 minutes when the observations were terminated. The number and percentage of zygote fragments that had not undergone NEB by 180 minutes after fertilization are shown above the dots. Axes are labeled in minutes after fertilization.





**Fig. 7.** Removal of the female pronucleus of a binucleate zygote. (A) Before insertion of the micropipette the female pronucleus can be seen in the right side of the zygote; the male pronucleus is indicated by an arrow. (B) Insertion of the micropipette. (C) The same zygote after removal of the female pronucleus leaving the male pronucleus containing crosslinked DNA. For 10 experiments the times for male pronuclear breakdown are shown to the right. Polarization microscopy.

from the female pronucleus. Into binucleate zygotes containing a male pronucleus with crosslinked DNA and a normal female pronucleus we microinjected 10 mg/ml wheat germ agglutinin (WGA) at 50-60 minutes after fertilization, shortly before the expected time of female NEB. WGA is a lectin that has been shown to block active nuclear import in cultured mammalian cells, *Xenopus* egg extracts, and sea urchin lysates by binding to nuclear pores (Finlay et al., 1987; Yoneda et al., 1987; Newmeyer and Forbes, 1988; Collas, 1998). However, WGA does not block passive diffusion of small proteins and other molecules into the nucleus (Finlay et al., 1987; Yoneda et al., 1987; Collas, 1998). The injected zygotes appeared to be healthy for the duration of the observations; there was no sign of granular cytoplasm or surface deformations, the characteristics of compromised viability. We conducted 17 experiments and obtained the following results (summarized in Table 1). In 5 cases the female pronucleus broke down at the normal time and the male pronucleus had not broken down by the time observations were terminated 180 minutes after fertilization. In 5 cases the female pronucleus broke down at the normal time (average 67 minutes) and the male pronucleus broke down late (average 119 minutes); this difference in means of 52 minutes is significantly longer than the 16 minute difference in mean times for NEB in binucleate zygotes that were not injected with WGA. In 4 cases the female pronucleus broke down late (average 115 minutes) and with one exception the male pronucleus had not broken down by the time

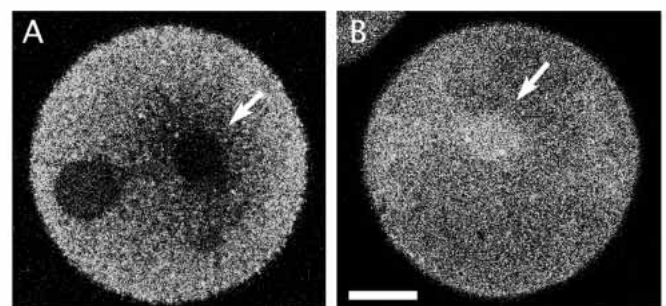
**Table 1. Wheat germ agglutinin blocks or delays male pronuclear breakdown**

Number of cases	Female pronuclear NEB mean + (range)	Male pronuclear NEB mean + (range)
5	69.6 (range 67-74)	>180.0
5	66.8 (range 61-74)	119.4 (range 99-145)
4	115.0 (range 100-120)	>180.0 (one cell 115)
3	None	None

Binucleate zygotes containing a normal female pronucleus and a male pronucleus with crosslinked DNA were injected with WGA just before the expected time of female pronuclear breakdown. The data are broken down into four categories: (i) Female pronucleus breaks down at normal time but male pronucleus does not break down by the termination of the observations; (ii) Female pronucleus breaks down at normal time and male pronucleus breaks down later; (iii) Female NEB is delayed. Female pronucleus does not break down. Values are mean times of NEB in minutes after fertilization. Ranges in parentheses show the highest and lowest individual values in minutes after fertilization.

observations were terminated 180 minutes after fertilization. In the last 3 cases neither the male or female pronucleus broke down by the time observations were terminated 150 minutes after fertilization. To test if the injected WGA, at the concentration we used, is functionally effective in blocking active nuclear import of proteins we coinjected WGA and mRNA for cyclin B-GFP 60 minutes after fertilization into zygotes that had been treated with aphidicolin. In 4 trials we consistently found that cyclin B-GFP was expressed in the cytoplasm but was absent from the nucleus 180 minutes after fertilization (Fig. 8A; compare fluorescence intensity of the nucleus and the oil drop used to cap the micropipette). In contrast, aphidicolin treated zygotes that had not received WGA showed nuclear localization of the GFP signal (Fig. 8B).

To test whether or not the male pronuclei with crosslinked DNA contained all the components necessary for NEB by the time WGA was injected, we conducted two types of experiments. First, we treated binucleate zygotes (not injected with WGA) with 10 mM caffeine starting at 40 minutes after fertilization. Caffeine has been shown to override the checkpoint for the completion of DNA synthesis in mammalian cells (Schlegel and Pardee, 1986) and sea urchin zygotes (Sluder et al., 1995). We found that caffeine treatment reduced the average asynchrony in pronuclear breakdown from 16 minutes to 7.8 minutes ( $n=28$ ). Notably, in one case the male



**Fig. 8.** WGA blocks the import of cyclin B1-GFP into the nucleus. (A) Approximately 50 minutes after fertilization aphidicolin treated zygotes were co-injected with mRNA for cyclin B1-GFP and WGA. At 180 minutes, injected zygotes were observed in vivo by confocal microscopy. There is no GFP fluorescence in the nucleus. (B) Aphidicolin-treated zygote injected with mRNA for cyclin B1-GFP alone, as seen by confocal microscopy at 180 minutes after fertilization. Note the fluorescence within the nucleus. The same confocal instrument settings were used for both panels. Bar, 20  $\mu$ m.

pronucleus broke down 9 minutes before the female pronucleus broke down, something never observed without caffeine treatment. In 4 cases the pronuclei broke down within a minute of each other, which is closer synchrony than ever observed without caffeine treatment. Second, we fragmented eggs and fertilized the fragments with sperm whose DNA had been crosslinked with psoralen. At 40 minutes after fertilization we treated with caffeine and compared the times of first NEB in the fragments containing just a male pronucleus with those containing a zygote nucleus. We found that the fragments with just a male pronucleus underwent NEB on average at 91 minutes after fertilization ( $n=20$ ) and those with a zygote nucleus underwent NEB on average at 89 minutes after fertilization ( $n=13$ ), an insignificant difference. These results reveal that the male pronucleus contains everything necessary for it to break down by the time WGA was injected (including, presumably, cyclin B1), but is prevented from doing so by the regulatory action of the checkpoint pathway.

## DISCUSSION

### Protein translocation into the nucleus

The purpose of this portion of our study was to investigate how the checkpoint monitoring the completion of DNA synthesis can prevent NEB in the face of mitotic levels of cytoplasmic Cdk1-B activity in sea urchin zygotes and mammalian somatic cells (Lamb et al., 1990; Heald et al., 1993; Sluder et al., 1995). We examined protein accumulation in the nuclei of checkpoint arrested sea urchin zygotes because recent work with human cells has revealed that this checkpoint not only slows the activation of cytoplasmic Cdk1-B but also prevents the nuclear accumulation of Cdk1-B which is presumably required for phosphorylation of intranuclear targets involved in NEB (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990; Enoch et al., 1991; Jin et al., 1996, 1998; Hagting et al., 1998).

In principle, limits on the accumulation of proteins in the nucleus can be exercised by modulating nuclear import processes or by controlling the activity of nuclear export of specific proteins such as cyclin B. We first tested whether or not the checkpoint for DNA synthesis operates by shutting down nuclear import pathways by microinjecting fluorescent BSA containing nuclear localization signal sequences into the cytoplasm of zygotes that could not complete DNA synthesis. Our finding that this construct is efficiently translocated and concentrated into the nuclei of these zygotes indicates that the checkpoint does not downregulate nuclear import pathways that depend on the importin  $\alpha/\beta$  sequences. Importantly, cyclin B1 is imported into the nucleus by a direct interaction with importin  $\beta$  (Moore et al., 1999).

We next injected mRNA for cyclin B1-GFP into the cytoplasm of zygotes that could not complete DNA synthesis. This cyclin B-GFP construct complexes with Cdk1 and activates its kinase activity (Hagting et al., 1998), and thus, should act as a faithful marker for the behavior of the endogenous Cdk1-B. We observed the accumulation of fluorescent cyclin B in the nuclei of these zygotes and this accumulation did not compromise the ability of the checkpoint to prevent NEB. The nuclear accumulation of cyclin B-GFP is not peculiar to zygotes that cannot complete DNA synthesis;

we expressed cyclin B-GFP in normal zygotes starting shortly after fertilization and found that it accumulated in the nucleus well before NEB (also see Genevriere-Garrigues et al., 1995). Thus, the checkpoint does not inhibit NEB in sea urchin zygotes by limiting the accumulation of cyclin B in the nucleus, as appears to be the case for mammalian somatic cells (Pines and Hunter 1991; Smeets et al., 1994; Jin et al., 1998; Hagting et al., 1998) or by downregulating cytoplasmic Cdk1-B activity (see Dasso and Newport, 1990); other mechanisms must exist. Certainly the checkpoint could inhibit nuclear Cdk1-B activity by coordinately upregulating Wee1 activity (see Heald et al., 1993) and keeping Cdc25C out of the nucleus by its binding to and subsequent export from the nucleus with 14-3-3 proteins (Lopez-Girona et al., 1999; reviewed by Pines, 1999). However, this may not be the only limit to NEB. We note that a significant percentage of mammalian cells fail to undergo premature NEB when they express both Cdc2AF and an export deficient cyclin B (Jin et al., 1998; Hagting et al., 1998), which should provide a constitutively active nuclear pool of Cdk1-B kinase. These observations raise the possibility that the nucleus contains a cyclin dependent kinase inhibitor that is subject to checkpoint control (see Kumagai and Dunphy, 1995). At a minimum, it appears that this checkpoint can concurrently operate at multiple levels to ensure redundant function.

### Normal nucleus accelerates the breakdown of a nucleus that cannot complete DNA synthesis

We directly compared the times of NEB for male pronuclei with cross-linked DNA in binucleate zygotes with that for cells with single zygote nuclei containing cross-linked paternal DNA. Since the source and amount of cross-linked DNA were identical in the two cultures, the only difference was the presence or absence of a normal nucleus in the same cytoplasm as the nucleus that could not complete DNA synthesis. In the binucleate zygotes the female pronucleus breaks down at the normal time and male pronucleus followed on average only 16 minutes later. In sharp contrast, nuclei that were composed of the fused pronuclei did not undergo NEB at a significant percentage until over two hours later. Also, we found that in Cdk1-B activity rises significantly sooner in cultures with separate pronuclei relative to mononucleated cultures. For the binucleate culture H1 histone kinase activity peaks at approximately 1 hour post fertilization and then drops as the cells exit mitosis; that in the parallel mononucleate culture rises more slowly, but reaches supramitotic values and remains there as long as the zygote nuclei do not break down (also see Sluder et al., 1995; Hinchcliffe et al., 1998). We emphasize that this acceleration in cellular Cdk1-B activation in the presence of a normal female pronucleus should not cause the breakdown of the male pronucleus in binucleate zygotes, because high cytoplasmic Cdk1-B activity per se is not sufficient to drive NEB for a nucleus that cannot complete DNA synthesis (Lamb et al., 1990; Heald et al., 1993; Sluder et al., 1995).

The relatively early breakdown of the male pronucleus in the presence of a normal female pronucleus is not due to the possibility that the psoralen has changed the properties of its nuclear envelope. In sea urchin zygotes the sperm nuclear envelope is disassembled shortly after fertilization and the pronuclear envelope is composed entirely of maternal components (Longo and Anderson, 1968), which in our

experiments were not exposed to psoralen. Nor is this phenomenon due to the inhibition of microtubule assembly by colcemid, because microtubule assembly has no influence on the time of NEB in sea urchin zygotes (Sluder, 1979). In addition, the accelerated breakdown of the sperm pronucleus in binucleate cells is not due to some defect in its ability to activate the checkpoint for the completion of DNA synthesis. When we fragment zygotes just after fertilization by psoralen cross-linked sperm, the fragments with only a male pronucleus (and those with zygote nuclei) do not undergo NEB until at least three hours after fertilization, well after all the fragments containing only female pronuclei have undergone NEB. We also note that the checkpoint pathway originating within the male pronucleus is not permanently disabled in the presence of a normal nucleus because the male pronucleus does not break down for at least another 1.5 hours when the female pronucleus is removed with a micropipet shortly before its expected time of NEB.

Together, our findings provide evidence for the existence of an activity originating from the normal female pronucleus that can override the checkpoint in the male pronucleus thereby accelerating its breakdown. We do not know if this activity is present and functional throughout the cell cycle or alternatively becomes effective only upon the breakdown of the female pronucleus. We favor the latter possibility for two reasons. First, the male pronucleus with crosslinked DNA always breaks down later than the female pronucleus, suggesting the diffusion of some factor and the time required for it to act once it reaches the male pronucleus. Second, accelerated paternal NEB requires active nuclear import starting at least in late prophase, as revealed by the following experiments.

We injected WGA, into binucleate zygotes just before the expected time of female pronuclear breakdown. WGA is a lectin that binds to the cytoplasmic face of nuclear pores and blocks the active translocation of proteins into the nucleus (Finlay et al., 1987) but does not inhibit the passive diffusion of small molecules through nuclear pores (Yoneda et al., 1987). We deliberately injected the WGA in late prophase to give both the male and female pronuclei the greatest amount of time to import enough cyclin B to support NEB. The female pronuclei broke down at the normal time in the majority of the injected binucleate zygotes, which indicates that the WGA per se does not appear to impede NEB if the nucleus is competent to do so. Importantly, we found that the asynchrony in pronuclear breakdown was greatly increased by the introduction of WGA. In over half the cells in which the female pronucleus broke down, the male pronucleus had not undergone NEB by the time observations were terminated three hours after fertilization. In the remaining zygotes male pronuclear breakdown was significantly later than in binucleate zygotes that had not been injected with WGA (mean asynchrony of 52.6 minutes vs 16 minutes, respectively). To empirically test the efficacy of the WGA in vivo, we co-injected this lectin with mRNA for cyclin B-GFP into zygotes that were treated with aphidicolin. We found that the WGA prevented the entry of cyclin B-GFP into the nucleus in all cases.

We do not think that WGA is inhibiting the translocation of some component directly required for male pronuclear NEB, such as cyclin B. Caffeine treatment of binucleate zygotes and egg fragments containing just a male pronucleus with cross-linked DNA reveals that the male pronucleus is competent to

break down at the normal time of mitosis but is prevented from doing so by the action of the checkpoint (also see Sluder et al., 1995). Thus, the action of the activity from the female pronucleus is to override the checkpoint in a regulatory sense rather than just providing something required by the male pronucleus for NEB.

Together, these results provide evidence that the NEB promoting activity of the female pronucleus is composed of macromolecules that must be actively translocated into the male pronucleus for it to break down. The fact that this maternal activity is not functionally downregulated within the male pronucleus suggests that it is either a downstream effector of NEB not subject to checkpoint control or a regulatory factor, such as activated Cdc25C, that is so rapidly imported into the male pronucleus that it overwhelms the checkpoint. This latter possibility is consistent with our finding that cellular Cdk1-B is rapidly activated at the time of female pronuclear breakdown in binucleate zygotes.

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