Protein Synthesis and the Cell Cycle: Centrosome Reproduction in Sea Urchin Eggs Is Not under Translational Control

Greenfield Sluder, Frederick J. Miller, Richard Cole,* and Conly L. Rieder*

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545; *Wadsworth Center for Laboratories and Research, Albany, New York 12201; and School of Public Health, State University of New York, Albany, New York 12222

Abstract. The reproduction, or duplication, of the centrosome is an important event in a cell's preparation for mitosis. We sought to determine if centrosome reproduction is regulated by the synthesis and accumulation of cyclin proteins and/or the synthesis of centrosome-specific proteins at each cell cycle. We continuously treat sea urchin eggs, starting before fertilization, with a combination of emetine and anisomycin, drugs that have separate targets in the protein synthetic pathway. These drugs inhibit the postfertilization incorporation of [^{35}S]methionine into precipitable material by 97.3–100%. Autoradiography of SDS-PAGE gels of drug-treated zygotes reveals that [^{35}S]methionine incorporates exclusively into material that does not enter the gel and material that runs at the dye front; no other labeled bands are detected. Fertilization events and syngamy are normal in drug-treated zygotes, but the cell cycle arrests before first mitosis. The sperm aster doubles once in all zygotes to yield two asters. In a variable but significant percentage of zygotes, the asters continue to double. This continued doubling is slower than normal, asynchronous between zygotes, and sometimes asynchronous within individual zygotes. High voltage electron microscopy of serial semithick sections from drug-treated zygotes reveals that 90% of the daughter centrosomes contain two centrioles of normal appearance. From these results, we conclude that centrosome reproduction in sea urchin zygotes is not controlled by the accumulation of cyclin proteins or the synthesis of centrosome-specific proteins at each cell cycle. New centrosomes are assembled from preexisting pools of ready-to-use subunits. Furthermore, our results indicate that centrosomal and nuclear events are regulated by separate pathways.

PROTEIN synthesis is required for normal progression through the cell cycle; it not only provides the proteins needed for growth, but also supplies regulatory proteins involved in the pathways that control entry into mitosis. The importance of these regulatory proteins has been most clearly revealed in marine and amphibian embryos, whose cells do not grow between divisions. For such cells, entry into mitosis depends upon the synthesis and accumulation of the cyclin proteins (reviewed in references 1, 32). The cyclins associate with the P34^{cdc2} kinase to form an active complex called maturation or mitosis-promoting factor (MPF)[1] (2, 8, 16). The accumulation of cyclins leads to an increase in MPF activity and subsequent entry into mitosis. At the metaphase–anaphase transition the cyclins are abruptly degraded, MPF activity falls, and the cell finishes mitosis. This cyclical accumulation and degradation of cyclin proteins is thought to drive the cell cycle (1, 32).

The reproduction of the centrosome is a key event in a cell's preparations for mitosis. In animals cells, this consists of the duplication of the centrioles and their associated structures close to the onset of DNA synthesis (24). At a variable time in G_2 the replicated centrosome splits, and the resultant daughter centrosomes migrate to opposite sides of the nucleus. The importance of centrosomes in nucleating spindle microtubules and establishing the spatial organization of the mitotic apparatus (6, 9, 15, 17, 18, 22, 34, 37), mandates that the cell tightly control the number of centrosomes arising from the parent centrosome. If the centrosome fails to double or if it splits into more than two, the ensuing mitosis will inevitably be abnormal. In addition, the cell must coordinate the events of centrosome reproduction with nuclear events.

In principle, the events of centrosome reproduction could be coordinated with the nuclear cycle by the rising level of MPF activity and/or by the timed synthesis of one or more structural components of the centrosome. Moreover, the number of daughter centrosomes formed could be controlled by the synthesis at each cell cycle of a limited number of key centrosome-specific proteins. Indeed, the importance of translational controls in sea urchin zygotes is evident from demonstrations that protein synthesis is developmentally

1. *Abbreviation used in this paper*: MPF, maturation or mitosis-promoting factor.

regulated. Not only are the qualitative mixes of proteins synthesized at the oocyte, early cleavage, and postblastula stages different (10), but also the level of protein synthesis increases markedly at fertilization (4, 39). Specific proteins that are under translational control include the regulatory subunit of ribonucleotide reductase and the cyclins (31, 32).

Whether or not centrosome reproduction is regulated at the translational level has not been studied extensively. For cultured L929 (mouse) cells, at least 4 h of protein synthesis in late G_1 is required for procentriole formation (20). However, this study is subject to the criticism that inhibition of protein synthesis arrests the cell cycle before the point when procentriole assembly is scheduled to occur. By contrast, a recent preliminary report indicates that aster doubling in *Xenopus* embryos may not require translation (Gard, D., S. Hafezi, T. Zhang, and S. Doxsey. 1988. *J. Cell Biol.* 107[6, Pt. 3]:29*a*. [Abstr.]). Cycloheximide treatment that produces a 91–98% inhibition of protein synthesis prevents entry into mitosis and abolishes detectable cycles of mitosis-specific histone kinase activity. After prolonged incubation in the drug, each cell of the embryo contains numerous asters with centrioles. Although this work was carefully executed, its conclusions are subject to the reservation that the 2–9% residual protein synthesis might provide enough new centrosomal components to support aster doubling, even in the absence of a detectable cell cycle.

We designed the study detailed here around a number of observations. First, the sperm contributes the centrosome used in the development of sea urchin zygotes (25, 28). At the time of pronuclear fusion, the sperm aster duplicates to form the two centrosomes used at first mitosis (19, 38). Second, 1×10^{-4} M emetine completely inhibits protein synthesis in the fertilized eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* (5, 35, 36). This inhibition is extremely rapid (within 1 min) and is independent of the phase of the cell cycle at which the drug is applied. Third, when emetine is continuously applied starting shortly after fertilization, the zygotes arrest after syngamy but before first nuclear envelope breakdown. However, the time course and extent of the first round of DNA synthesis, at the time of pronuclear fusion (13), occurs in a normal fashion (35, 36). Therefore, zygotes treated with emetine from the time of fertilization onwards should go through a period in which they are scheduled to duplicate the sperm centrosome. Thus, we should be able to determine if centrosome reproduction requires protein synthesis by following centrosome behavior in such zygotes.

To ensure complete inhibition of protein synthesis, we continuously treated eggs starting before fertilization with emetine and anisomycin, using concentrations at which either drug alone should completely inhibit protein synthesis. By starting the drug treatment before fertilization, when protein synthesis is naturally low (4, 39), we optimized our chances of totally inhibiting translation. In addition, these drugs have different targets in the protein synthetic pathway. Anisomycin binds specifically to the 60S subunit of the ribosome, primarily blocking peptide bond formation and, to a lesser extent, inhibiting substrate interaction with the donor and acceptor sites of the peptidyl-transferase center. Emetine selectively prevents EF-2-dependent translocation in polysomes by possibly binding to the 40S subunit of the ribosome (reviewed in reference 33).

Materials and Methods

Living Material

L. pictus and *S. purpuratus* were purchased from Marinus Inc. (Long Beach, CA). Eggs and sperm were obtained by intracoelomic injection of 0.5 M KCl as described elsewhere (7). 1×10^{-4} M emetine and 1×10^{-5} M anisomycin (both from Sigma Chemical Co., St. Louis, MO) were prepared together in natural sea water immediately before each experiment. Eggs were placed in drug-containing sea water 10–15 min before fertilization. Sperm were then added and the cultures incubated at 18°C for *L. pictus* and 15–18°C for *S. purpuratus*.

Individual zygotes of *L. pictus* were observed and photographed in vivo with a Zeiss ACM microscope modified for polarization microscopy. In some experiments, astral birefringence was augmented by treating the zygotes for 2–5 min with 2% hexylene glycol in drug-containing sea water.

The zygotes of *S. purpuratus* were too refractile to observe asters in vivo. Therefore, they were treated for 2–5 min with hexylene glycol in drug-containing sea water and then extracted in a microtubule stabilizing buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, 0.5% Triton X-100, pH 6.9). Photographs were recorded on Kodak Plus X film which was developed in Kodak Microdol-X.

Measurement of Protein Synthesis

2 ml of pelleted unfertilized *S. purpuratus* or *L. pictus* eggs with jelly coats intact were labeled in 50 ml of sterile sea water, containing 0.18 mCi L-[^{35}S]methionine (97% radiochemical purity; SJ.235; Amersham Corp., Arlington Heights, IL), for 1 h and then washed three times with sterile sea water. The eggs were then split into two 40-ml cultures. One received 10 ml of a 5× drug solution (5×10^{-4} M emetine plus 5×10^{-5} M anisomycin in sterile sea water), and the other 10 ml of sterile sea water. 10–15 min later both cultures were inseminated with 1 ml of diluted sperm.

At 5–10 and 75–85 min postfertilization 10 2-ml aliquots were taken from each culture and each aliquot placed into a 15-ml test tube containing 1 ml of 8% SDS plus 1 mg/ml BSA (both from Sigma Chemical Co.). Immediately thereafter, 3 ml of cold 20% TCA (Mallinckrodt Inc., St. Louis, MO) containing 2 mg/ml unlabeled methionine (Sigma Chemical Co.) were added to each tube. The tubes were then heated for 5 min at 100°C and immediately put on ice. Once cold, they were filled twice with cold 10% TCA containing 1 mg/ml methionine and poured through 2.4 cm filters (GF/B; Whatman International Ltd., Maidstone, Great Britain). The tubes were then filled with 95% ethanol which was poured through each filter. The filters were air dried and then counted for 1 min each in 9 ml of Ecolume (ICN Biomedicals Inc., Costa Mesa, CA). For each time point, the counts from each group of 10 samples were averaged for both control and drug-treated zygotes.

Gel Analysis of Protein Synthesis

3 ml of pelleted, unfertilized, *S. purpuratus* eggs with intact jelly coats were diluted into 80 ml of sterile sea water and split into two 40-ml cultures. One culture was treated with 10 ml of the 5× drug stock solution and the other with 10 ml of sterile sea water. After 10 min, both cultures were labeled with [^{35}S]methionine as described above. 1 h later, both cultures were washed three times with drug-containing sterile sea water and fertilized with 1 ml of diluted sperm. 85 min after fertilization both cultures were pelleted and aspirated to leave 1 ml of packed zygotes. 5 ml of SDS sample buffer were then added to each pellet; the samples were then briefly sonicated to disrupt the fertilization envelopes and heated to 100°C for 15 min. 1-ml aliquots of both samples were centrifuged at 10,000 *g* for 2 min to pellet fragments of the fertilization envelopes. If this step was omitted, labeled particulate material damaged the gels by irregularly forcing its way into their upper portions. 80- and 15-μl aliquots of both samples were then run on 5–15% acrylamide gradient gels as described elsewhere (14). The gels were later stained with Coomassie brilliant blue and autoradiographed on Kodak X Mat XAR-5 film.

Electron Microscopy

Eggs of *S. purpuratus* were continuously treated with 1×10^{-4} M emetine plus 1×10^{-5} M anisomycin starting 15 min before fertilization. At times beginning 90 min after fertilization, aliquots of zygotes were fixed for 90 min in 2% osmium tetroxide in 0.4 M sodium acetate (12, 27). After dehydration and embedment in Epon-Araldite, zygotes were serially semithick

(0.25–0.5 μm) sectioned as previously described (21, 23). After staining with uranyl acetate and lead citrate, the ribbons of sections were screened for content by light microscopy (23). The sections were then viewed and photographed on the Wadsworth Center high voltage electron microscope operated at 800–1,000 kV.

Results

The experiments described in the following sections use two slightly different protocols for the application of drugs and [^{35}S]methionine. These protocols were deliberately used, in lieu of simpler ones, in order to avoid known experimental pitfalls inherent to the measurement of protein synthesis and provide the most stringent tests for the inhibition of translation. To assist the reader, we review the protocol being used for each type of experiment and explain the rationale for its use.

Efficacy of Drug Treatment

To empirically test the effectiveness of our drug treatment in preventing protein synthesis, we preloaded unfertilized eggs of *L. pictus* and *S. purpuratus* for 1 h with [^{35}S]methionine. After the extracellular label was washed out, the culture was split and one batch of eggs was continuously treated with drugs starting 10–15 min before fertilization; the untreated batch of eggs served as the control. Since sea urchin eggs actively sequester and retain amino acids present in sea water (5), preloading labels the intracellular pool of amino acids used in protein synthesis. The eggs were preloaded with [^{35}S]methionine before drug treatment to avoid possible interference emetine might have on amino acid uptake (5). We controlled for this possibility even though the interference should be slight or nonexistent, given the concentration of emetine used and the duration of the preloading (5). Compared to continuous labeling, preloading avoids the possible problem of cell cycle–dependent variations in amino acid uptake after the eggs are fertilized. The incorporation of label into newly synthesized proteins during the preloading period was the same for both drug and control cultures. The drugs were applied well before fertilization to allow more than enough time for them to act and to start the inhibition at a point in development when the rate of protein synthesis is naturally low (4, 39). We quantified the extent of label incorporation only after fertilization to normalize any possible involvement of methionine in the hardening of the fertilization envelope. By averaging the counts from 10 samples taken shortly after fertilization and at the expected time of first mitosis, we sought to minimize the impact of slight but inevitable sampling errors in order to most accurately detect small amounts of label incorporation. For the control cultures, our results consistently showed significant incorporation of label into newly synthesized proteins. In no case did we find <500,000 precipitable cpm in each of the 10 samples taken 75–85 min after fertilization.

In seven experiments with *S. purpuratus*, emetine in combination with anisomycin inhibited [^{35}S]methionine incorporation into precipitable material by: 99.0, 98.7, 98.1, 98.0, 97.7, 97.4, and 97.3% (average, 98.0%). For the trial in which we observed 99% inhibition, the average increase in precipitable counts (per sample taken) between the two sampling times for the control zygotes was 770,336 counts vs. 7,738 counts for the drug-treated zygotes. In two experiments with *L. pictus*, the drugs inhibited label incorporation by 100 and 99.1% (average, 99.6%).

We were concerned that the slight incorporation of [^{35}S]methionine into precipitable counts for drug-treated zygotes represented residual protein synthesis. Thus, we loaded two identical cultures of *S. purpuratus* eggs before fertilization with [^{35}S]methionine; one in the presence and the other in the absence of drugs. For this experiment, the unfertilized eggs were preloaded with label starting 10 min after the application of the drugs to prevent spurious incorporation of label into proteins during the preloading period. We processed equal aliquots of zygotes from both cultures, 85 min after fertilization, for gel electrophoresis. Fig. 1 *A* shows the electrophoretic pattern of total cell proteins from control and drug-treated zygotes at two loadings of the gel. The autoradiograph of this gel is shown in Fig. 1 *B*. For the control zygotes there is extensive incorporation of label into newly synthesized proteins (Fig. 1 *B*, lanes C_{80} and C_{15}). For the drug-treated zygotes there is label incorporation only into material that does not enter the gel and material that runs at the dye front (Fig. 1 *B*, lanes D_{80} and D_{15}). No other labeled bands were detectable. These gels resolve proteins ranging from at least 500 to ~5 kD (14). The material that does not enter the gel may represent glucosaminoglycans that have incorporated radioactive SO_4; the 3% radiochemical impurities in the [^{35}S]methionine preparations we used include some labeled SO_4. The label running at the dye front may represent unincorporated intracellular [^{35}S]methionine present in these whole cell preparations. Also, some label incorporated into particulate material visible with the light microscope. This material, which included fragments of fertilization envelopes, was centrifuged out of the samples to prevent it from disrupting the upper regions of the gels (data not shown).

Light Microscopy

Eggs of *S. purpuratus* and *L. pictus* were treated continuously with 1×10^{-4} M emetine plus 1×10^{-5} M anisomycin starting at least 15 min before fertilization. The drugs were applied before fertilization to take advantage of the fact that the rate of protein synthesis is naturally low at this stage in development (4, 39) and allow more than enough time for the drugs to act before a centrosome is introduced into each egg by the sperm. At times up to 7 h after fertilization, aliquots of zygotes were directly examined with the polarization microscope (*L. pictus*) or extracted with a microtubule-stabilizing buffer (*L. pictus* and *S. purpuratus*) before examination. In some experiments the zygotes were briefly treated just before extraction with 2% hexylene glycol in sea water to augment astral birefringence. Unlike 10-min treatments with 5% hexylene glycol that produce "miniasters" in some sea urchin eggs (3), the short treatments we used do not induce supernumerary asters. Indeed, control zygotes treated with 2% hexylene glycol at prophase or prometaphase always contained just two asters (data not shown).

For both species the fertilization events, formation of the sperm aster, and syngamy were normal in the presence of the drugs. However, the zygotes arrested before first nuclear envelope breakdown with an enlarged nucleus.

We conducted a total of 38 trials using different female

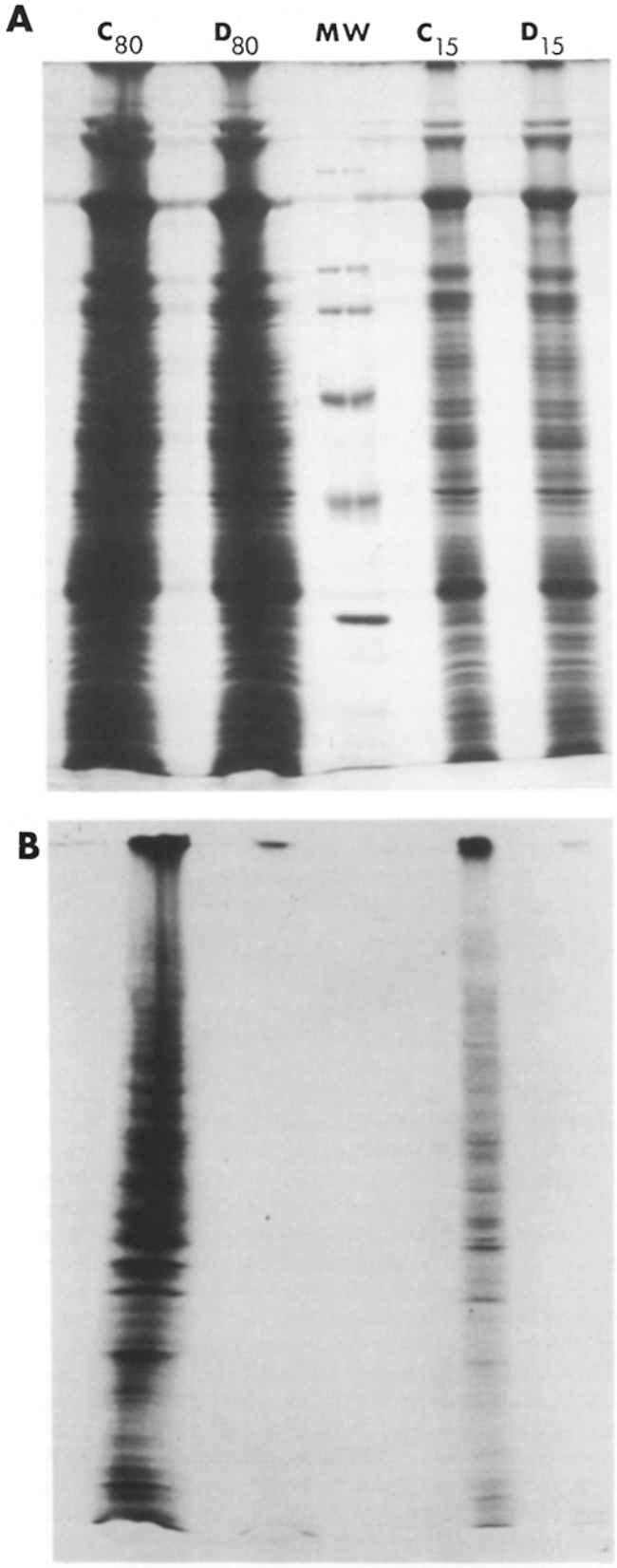

Figure 1. (*A*) SDS-PAGE of total cell protein for control and drug-treated eggs of *S. purpuratus* labeled with [^{35}S]methionine as visualized by Coomassie brilliant blue staining. Lanes C_{80} and D_{80}, 80 μl loadings of control and drug-treated eggs, respectively. Lane *MW*, molecular weight markers. Lanes C_{15} and D_{15}, 15 μl loadings of control and drug-treated eggs, respectively. (*B*) Autoradiogram of the gel above showing the pattern of ^{35}S incorporation. For the control eggs, label extensively incorporates into a wide spectrum of proteins. The drug-treated eggs show label incorporation only into material that does not enter the gel and material at the dye front.

S. purpuratus. In all experiments we observed that the sperm aster in all drug-treated zygotes doubled once by 90 min after fertilization to yield a prophase figure with asters on opposite sides of the enlarged nucleus (Fig. 2 *a*). Later, the average number of asters per zygote progressively increased at a variable rate. After the first doubling of the sperm aster at the time of pronuclear fusion, subsequent doublings were slower than normal and asynchronous between zygotes. We also noted variability in the rate of aster doubling for populations of zygotes from different females. At one extreme, the zygotes from six females all contained two asters 6 h after fertilization. At the other extreme, 70% of the zygotes from another female contained eight asters 6 h after fertilization. For 16 experiments, the average percentages of zygotes having various numbers of asters at 2, 4, and 6 h postfertilization are shown in Table I.

Fig. 2 shows typical examples of eggs with two, four, and greater than four asters extracted 4–7 h after fertilization. The variable number of asters per cell at any given time after fertilization is due to the asynchronous aster doubling within the population. In most cases, the asters were equally spaced around the nuclear envelope, but in some cells they were distributed throughout the cytoplasm (data not shown). Sometimes zygotes contained odd numbers of asters or unexpected even numbers, such as 6 and 10 (Fig. 2, *c–e* and Table I); this indicates that aster doubling is sometimes asynchronous even within individual zygotes. Also, we occasionally observed asters that were in paired configurations (Fig. 2 *d*); these probably represent asters that doubled and were in the early stages of separation when the zygotes were extracted. The astral birefringence shown in Figs. 2 and 3 has been augmented with hexylene glycol to clearly reveal the number of asters. Before augmentation, astral birefringence in drug-treated zygotes is approximately the same as that of normal zygotes in early to mid-prophase.

We conducted eight trials with *L. pictus*. In three experiments, all zygotes arrested for up to 6 h with two asters on opposite sides of the nuclear envelope (Fig. 3, *a* and *b*). In the other five experiments we observed variable percentages of zygotes with between 2 and 16 asters at 5–7 h postfertilization (Fig. 3, *c* and *d*).

In two experiments with *L. pictus*, we observed a few zygotes (< 0.5%) that first underwent nuclear envelope breakdown and divided once. Also, in one experiment with *S. purpuratus* we found a few zygotes (< 0.5%) that had undergone nuclear envelope breakdown 3.5 h after fertilization when they contained four or six asters. The reasons for these exceptions and their significance are not clear. Conceivably, these individual zygotes could have finished their meiotic divisions with small amounts of cyclin B remaining from the premeiotic pool that was not fully degraded at anaphase of meiosis II. If so, residual MPF activity might be sufficient to take these individuals slowly into mitosis.

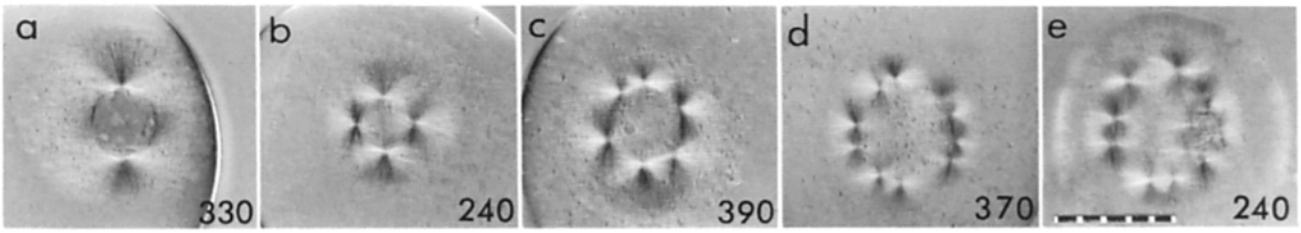

Figure 2. Asters in drug-treated *S. purpuratus* zygotes extracted with microtubule-stabilizing buffer at times after fertilization. Astral birefringence was augmented by hexylene glycol. (*a*) Zygote containing two asters closely associated with the nucleus. (*b*) Zygote containing four asters associated with the nucleus. (*c*) Zygote containing six asters. (*d*) Zygote containing 10 asters closely associated with the nucleus. (*e*) Zygote containing multiple asters, 11 of which are visible in this plane of focus. Minutes after fertilization are shown in the lower corner of each frame. Polarization microscopy; 10 μm per scale division.

With the exception of the few zygotes of *L. pictus* that entered mitosis, none of the zygotes of *L. pictus* or *S. purpuratus* showed overt signs of cleavage, regardless of the number of asters present. This is consistent with the hypothesis that inhibition of protein synthesis causes the cell cycle to arrest at a point before the time when cleavage is scheduled to occur.

Electron Microscopy

To determine if centrioles duplicate in a normal fashion when asters double, we examined with the high voltage electron microscope serial semithick sections of drug-treated *S. purpuratus* zygotes that contained two or more asters. We completely reconstructed four zygotes fixed 90 min after fertilization, when each cell contained two asters. We found two centrioles of normal appearance in both asters of each zygote (Fig. 4, Table II). The particular cell shown here was chosen because the two centrioles in both centrosomes appeared in adjacent sections. When a pair of centrioles appeared in a single 0.5-μ section, a tilt series was required to clearly visualize them as paired organelles. The fact that astral microtubules are not visible in these micrographs is due to the thickness of the sections. Although present, microtubules in longitudinal view have little contrast due to electron scattering by the plastic. Partial sectioning of five additional zygotes revealed two centrioles in each of the five asters completely reconstructed (Table II).

We also serial sectioned zygotes containing multiple asters. Over 70% of the cells in the culture from which these zygotes were taken contained eight asters when fixed 6 h after fertilization (see lowest row of Table I). Of the total of 20 asters completely reconstructed, 17 contained two centrioles and 3 contained 0 centrioles (Table II). Fig. 5 shows a portion of a complete serial 0.25-μm section series through two closely adjacent asters that may have been in the process of separating. Both asters contain two centrioles.

Table I. Asters per Zygote after Fertilization

	Asters per zygote								
Hours	2	3	4	5	6	7	8	8+	Total zygotes counted
2	59.8	1.1	25.5	1.7	1.8	<1	8.3	1.1	1,678
4	45.0	1.5	28.6	1.2	3.9	<1	16.6	1.3	1,601
6	37.9	8.3	26.7	1.7	3.7	<1	18.8	1.4	1,395
6	2	4	17.8	0	6	0	70	–	101

The average percentages of drug-treated zygotes (*S. purpuratus*) with various numbers of asters at 2, 4, and 6 h after fertilization (16 experiments with separate females). A minimum of 100 zygotes were scored at each time for each experiment. The culture showing the highest percentage of zygotes with multiple asters at 6 h is shown on the bottom row.

Discussion

The goal of this study was to determine if the reproduction of centrosomes in sea urchin zygotes is controlled by the required synthesis of key centrosomal components at each cell cycle or by the accumulation of regulatory molecules, such as the cyclin proteins. Since sea urchin zygotes do not grow between divisions, we can examine the role of protein synthesis as a fundamental control mechanism for centrosome reproduction without the concern for cell growth limitations on the centrosomes or nuclear cycles.

We had to be certain that protein synthesis was completely blocked in the zygotes we used. Our results show that 1×10^{-4} M emetine in combination with 1×10^{-5} M anisomycin inhibits the incorporation of [^{35}S]methionine into cold TCA precipitable counts by an average of 98% for *S. purpuratus* and 99.6% for *L. pictus*. Furthermore, autoradiography of SDS-PAGE gels shows that the minor incorporation of label into precipitates of drug-treated zygotes is exclusively in material that does not enter the gel and into low molecular weight species that run at the dye front. The material that does not enter the gels may be glucosaminoglycans that presumably acquire label from radioactive SO_4 present in the preparations of [^{35}S]methionine used. The material at the dye front includes unincorporated intracellular [^{35}S]methionine. Unless awkward assumptions are made concerning the selective synthesis of proteins that do not enter the gel and peptides that run at the dye front, we completely inhibited protein synthesis. In addition, the fact that zygotes of both species are arrested before first mitosis for at least 7 h (five to seven normal cell cycle durations depending upon the species) provides functional evidence that cyclin synthesis is completely inhibited.

One of the primary observations of this study is that asters can repeatedly double in the absence of protein synthesis. The sperm aster always doubles once, and in many zygotes up to three times more in 7 h, even though the nuclear cell cycle is completely arrested. However, the period of aster

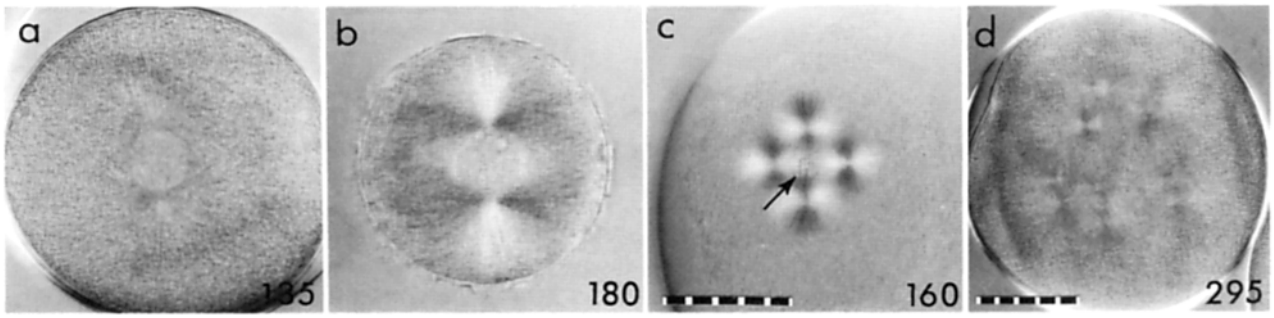

Figure 3. Asters in drug-treated zygotes of *L. pictus* at times after fertilization. Astral birefringence was augmented by hexylene glycol. (*a* and *b*) Zygotes containing two asters photographed in vivo and after extraction with microtubule-stabilizing buffer, respectively. (*c*) Extracted zygote containing four asters; the nucleus (*arrow*) has collapsed. (*d*) Living zygote with many asters; the nucleus is out of the plane of focus. Minutes after fertilization are shown in the lower corner of each frame. Polarization microscopy; 10 μm per scale division.

doubling is slower and more variable than normal. Also, asters may double asynchronously within individual zygotes as indicated by cases of odd numbers of asters or unexpected even numbers of asters, such as 6 or 10.

Our serial semithick section characterization of zygotes containing two or more asters shows that the doubling of asters seen at the light microscope level represents the reproduction of complete centrosomes in the great majority of cases. Of the 33 asters completely reconstructed, 30 contained just 2 centrioles. The pair of centrioles introduced by the sperm at fertilization must have duplicated and must have been distributed in a normal fashion with the splitting of the centrosome as a whole. This rules out the possibility that the repeated doubling of the original sperm aster is due simply

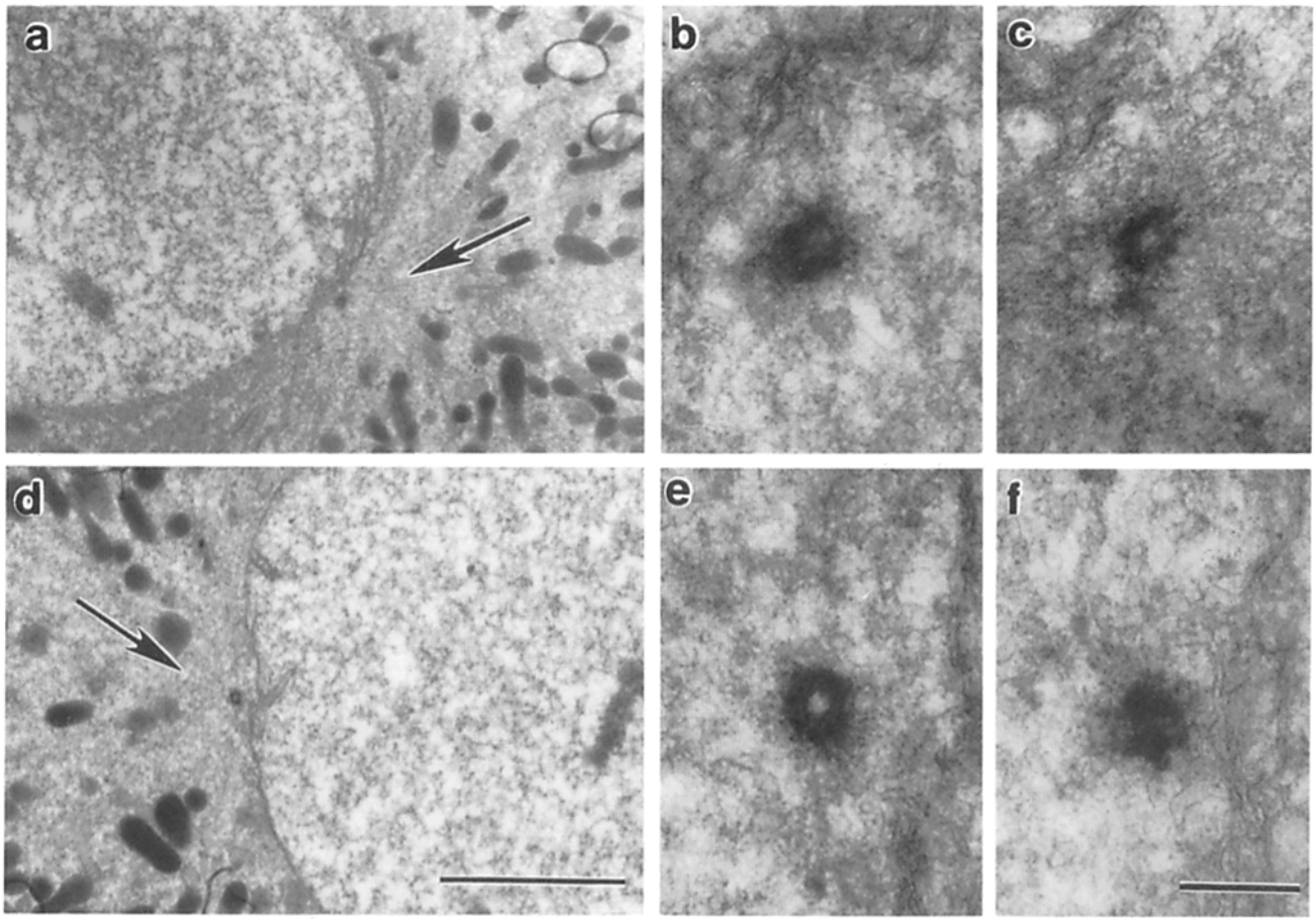

Figure 4. Ultrastructural analysis of a drug-treated zygote fixed 100 min after fertilization when it contained two asters. Shown here are sections from a complete serial 0.5-μm section series of the whole zygote. (*a*) Survey view of one centrosome (*arrow*) next to the nucleus. (*b* and *c*) Two sequential sections showing the two centrioles in this centrosome. (*d*) Survey view of the other centrosome (*arrow*) in the same zygote. (*e* and *f*) Two sequential sections showing the two centrioles in this centrosome. Although present, astral microtubules in longitudinal view produce little contrast in these semithick sections. Bars: (*d*) 5 μm; (*f*) 0.5 μm.

Table II. Centrioles per Aster

	Asters/ zygote	Zygotes examined	Total asters reconstructed	Centrioles/aster
		n	*n*	
Whole zygote reconstructions	2	4	8	8/8: 2 centrioles
	6	1	6	4/6: 2 centrioles
				2/6: 0 centrioles
Partial zygote reconstructions	2	5	5	5/5: 2 centrioles
	>4	6	10	10/10: 2 centrioles
	5	1	4	3/4: 2 centrioles
				1/4: 0 centrioles
Totals		17	33	30/33: 2 centrioles
				3/33: 0 centrioles

Summary of serial semithick section reconstructions of drug-treated zygotes. Whole zygote reconstructions lists cells that were completely sectioned and all asters serially sectioned with no loss of sections. Partial zygote reconstructions lists cells that were partially sectioned; only those asters completely reconstructed with no missing sections are tabulated. The fractions under the centrioles/aster heading indicate the number of asters containing 2 or 0 centrioles over the total number of asters reconstructed for that category.

to the fragmentation or subdivision of its microtubule organizing center.

The finding of three asters containing no centrioles may be explained by the occasional splitting of the centrosomal microtubule-organizing center before the centrioles duplicate. This possibility is consistent with our complete reconstruction of a whole zygote containing six asters; four asters contained two centrioles and two were acentriolar. None of the asters in this cell contained four centrioles. Perhaps the occasional generation of acentriolar asters may contribute to the apparent asynchronous duplication of asters in some individual zygotes. We have previously shown, for sea urchin zygotes, that the reproductive capacity of a centrosome is correlated with the number of centrioles it contains (27). Furthermore, sea urchin centrosomes without centrioles do not double between mitoses (30). If only the centriole-containing asters continue to double in drug-treated zygotes, the formation of acentriolar asters should eventually lead to individual cells with unexpected numbers of asters.

From our results we conclude that the sea urchin zygote does not control the reproduction of centrosomes by requiring the synthesis of key structural components of the centrosome at each cell cycle. Even before fertilization the egg must contain pools of all the necessary components, in ready-to-use forms, for the assembly of at least 16 centrosomes (also see reference 29). Furthermore, our results demonstrate that the proteins that control the recruitment of these components and their assembly into centrosomes are not themselves under translational control. Unless low levels of MPF are present before fertilization, the cyclin proteins and the $P34^{cdc2}$ kinase are not directly involved in the assembly of new centrosomes.

Under normal circumstances, centrosome reproduction is tightly coordinated with the progression of nuclear events during the cell cycle. An important finding of our study is that the centrosome cycle can proceed even though the cell cycle, as defined by cycles of MPF activity or nuclear events, is arrested. This indicates that centrosomal and nuclear events are controlled by different metabolic pathways. Thus, the reproduction of centrosomes is not dependent on the accumulation of the cyclin proteins, as appears to be the case for entry into mitosis. Even though the centrosome cycle can run independently of the cycle of nuclear events, the normal precise coordination between these cycles indicates that they are probably linked in some way. Perhaps the cycle of MPF activity drives the timing of the centrosome cycle, thereby providing the essential coordination between centrosomal and nuclear events during the cell cycle.

The report that protein synthesis in late G_1 is required for daughter centriole formation in cultured cells (20), may raise questions about the applicability of our results to somatic cells. In this regard, the important finding of our study is that the synthesis of new proteins, such as structural components or regulatory enzymes, at each cell cycle is not a strategy

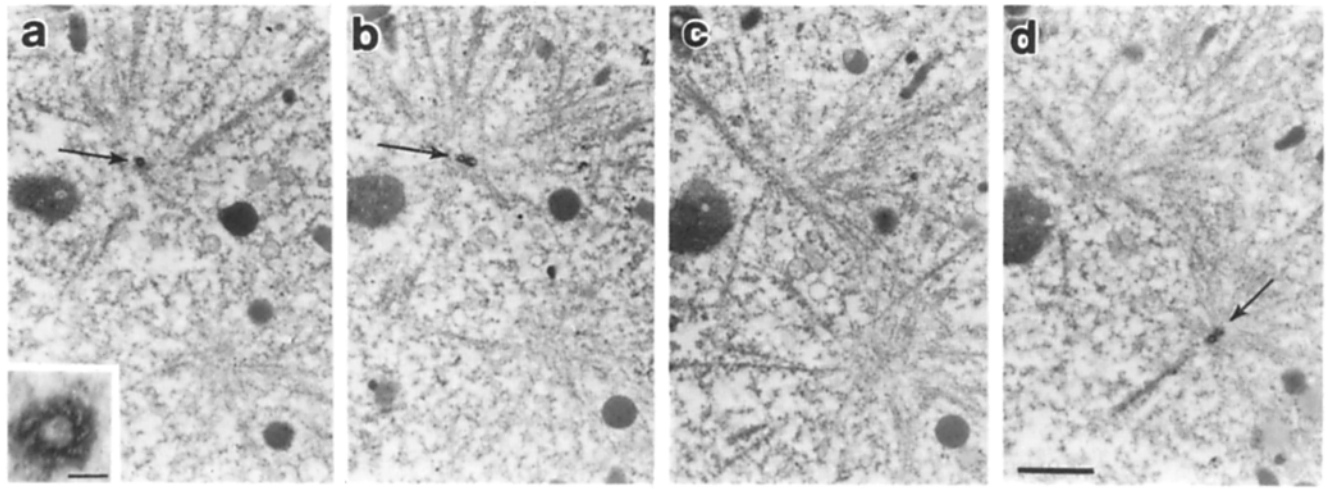

Figure 5. Serial 0.25-μm sections through two closely associated asters in a drug-treated zygote containing multiple asters fixed 6 h after fertilization. (*a*) One centriole (*arrow*) of the upper aster seen in cross section. (*Inset*) Higher magnification view of this centriole. (*b*) Both centrioles (*arrow*) of the upper aster are visible in this section. (*c*) No centrioles are included in this section. (*d*) Both centrioles (*arrow*) of the lower aster are seen in this section. Subsequent sections showed no additional centrioles in either aster. Bars: (*inset*) 0.12 μm; (*d*) 2 μm.

used by all cells to control centrosome reproduction. It is, however, possible that the pools of centrosomal components in cultured cells need to be replenished during the cell cycle in which they are used. If so, this limitation operates in addition to the nontranslational control mechanisms for centrosome reproduction that are revealed only in cells that do not need to grow before they can divide.

Hall et al. (11) recently presented evidence that the basal bodies of *Chlamydomonas* contain DNA which encodes genes for basal body formation. If sea urchin centrioles contain such DNA, the observations that centrioles can duplicate repeatedly in the absence of a nuclear cell cycle (the present study) and that centrosomes can reproduce when nuclear DNA synthesis is inhibited by the drug aphidicolin (26) suggest that the replication of this putative DNA must use different regulatory pathways and replicative enzymes than the nuclear genome.

We thank Dr. Stephen King for running the gels shown in this paper; his help was invaluable. We are also pleased to acknowledge the valuable consultations provided by Drs. David Epel, Charles Glabe, Beth Luna, and Matthew Winkler. We are most appreciative of the help provided by Ms. Rebecca Bell, Ms. Sandy Johnson, and Ms. Ann Moss in the preparation of the manuscript. We also thank Dr. Joel Richter for loaning us the apparatus to conduct the label uptake experiments.

This work was supported by National Institutes of Health (NIH) grant GM-30758 to G. Sluder, NCI grant PO 30-12708 to the Worcester Foundation for Experimental Biology, NIH grant GM-40198 to C. L. Rieder, and NIH grant PHS-01219 awarded by 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 24 October 1989 and in revised form 20 February 1990.

References

1. Cross, F., J. Roberts, and H. Weintraub. 1989. Simple and complex cell cycles. *Annu. Rev. Cell Biol.* 5:341–395.
2. Draetta, G., F. Luca, J. Westendorf, L. Brizuela, J. Ruderman, and D. Beach. 1989. Cdc 2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell.* 56:829–838.
3. Endo, S., M. Toriyama, K. Ohta, and H. Sakai. 1990. Formation of miniasters in the cytoplasm of hexyleneglycol-treated sea urchin eggs. *Cell Motil. Cytoskeleton.* 15:23–33.
4. Epel, D. 1967. Protein synthesis in sea urchin eggs: a late response to fertilization. *Proc. Natl. Acad. Sci. USA.* 57:899–906.
5. Epel, D. 1972. Activation of Na^+-dependent amino acid transport system upon fertilization of sea urchin eggs. *Exp. Cell Res.* 72:74–89.
6. Euteneuer, U., and J. R. McIntosh. 1981. Structural polarity of kinetochore microtubules in PtK-1 cells. *J. Cell Biol.* 89:338–345.
7. Fuseler, J. W. 1973. Repetitive procurement of mature gametes from individual sea stars and sea urchins. *J. Cell Biol.* 57:879–881.
8. Gautier, J., J. Minshull, M. J. Lohka, M. Glotzner, T. Hunt, and J. L. Maller. 1990. Cyclin is a component of MPF from *Xenopus*. *Cell.* 60: 487–494.
9. Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* 73:601–615.
10. Grainger, J. L., A. Van Brunn, and M. M. Winkler. 1986. Transient synthesis of a specific set of proteins during the rapid cleavage phase of sea urchin development. *Dev. Biol.* 114:403–415.
11. Hall, J. L., Z. Ramanis, and D. J. L. Luck. 1989. Basal body/centriolar DNA: molecular genetic studies in *Chlamydomonas*. *Cell.* 59:121–132.
12. Harris, P. 1962. Some structural and functional aspects of the mitotic apparatus in sea urchin embryos. *J. Cell Biol.* 14:475–487.
13. Hinegardner, R. T., B. Rao, and D. E. Feldman. 1964. The DNA synthetic period during early development of the sea urchin egg. *Exp. Cell Res.* 36:53–61.
14. King, S. M., T. Otter, and G. B. Witman. 1986. Purification and characterization of *Chlamydomonas* flagellar dyneins. *Methods Enzymol.* 134: 291–306.
15. Kuriyama, R. 1984. Activity and stability of centrosomes in Chinese hamster ovary cells in nucleation of microtubules *in-vitro*. *J. Cell Sci.* 66: 277–296.
16. Labbe, J.-C., J.-P. Capony, D. Caput, J.-C. Cavadore, J. Derancourt, M. Kaghad, J.-M. Lelias, A. Picard, and M. Doree. 1989. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3053–3058.
17. Mazia, D. 1961. Mitosis and the physiology of cell division. *In* The Cell, Biochemistry, Physiology, Morphology. J. Brachet and A. Mirsky, editors. Academic Press, Inc., New York. Vol. 3. 77–412.
18. Nicklas, R. B. 1971. Mitosis. *In* Advances in Cell Biology. D. M. Prescott, L. Goldstein, and J. E. McConkey editors, Vol 2. Appleton-Century-Crofts, New York. 225–297.
19. Paweletz, N., D. Mazia, and E. M. Finze. 1987. Fine structural studies of the bipolarization of the mitotic apparatus in the fertilized sea urchin egg. I. The structure and behavior of centrosomes before fusion of the pronuclei. *Eur. J. Cell Biol.* 44:195–204.
20. Phillips, S. G., and J. B. Rattner. 1976. Dependence of centriole formation on protein synthesis. *J. Cell Biol.* 70:9–19.
21. Rieder, C. L. 1981. Thick and thin serial sectioning for the three-dimensional reconstruction of biological ultrastructure. *Methods Cell Biol.* 22:215–249.
22. Rieder, C. L., and G. G. Borisy. 1982. The centrosome cycle in PtK_2 cells: asymmetric distribution and structural changes in the pericentriolar material. *Biol. Cell.* 44:117–132.
23. Rieder, C. L., G. Rupp, and S. Bowser. 1985. Electron microscopy of semi-thick sections: advantages for biomedical research. *J. Electron Microsc.* 2:11–28.
24. Robbins, E., G. Jentzsch, and A. Micali. 1968. The centriole cycle in synchronized HeLa cells. *J. Cell Biol.* 36:329–339.
25. Schatten, H., G. Schatten, D. Mazia, R. Balczon, and C. Simerly. 1986. Behavior of centrosomes during fertilization and cell division in mouse oocytes and in sea urchin eggs. *Proc. Natl. Acad. Sci. USA.* 83:105–109.
26. Sluder, G., and K. Lewis. 1987. Relationship between nuclear DNA synthesis and centrosome reproduction in sea urchin eggs. *J. Exp. Zool.* 244:89–100.
27. Sluder, G., and C. L. Rieder. 1985. Centriole number and the reproductive capacity of spindle poles. *J. Cell Biol.* 100:887–896.
28. Sluder, G., and C. L. Rieder. 1985. Experimental separation of pronuclei in fertilized sea-urchin eggs: chromosomes do not organize a spindle in the absence of centrosomes. *J. Cell Biol.* 100:897–903.
29. Sluder, G., F. J. Miller, and C. L. Rieder. 1986. The reproduction of centrosomes: nuclear versus cytoplasmic controls. *J. Cell Biol.* 103:1873–1882.
30. Sluder, G., F. J. Miller, and C. L. Rieder. 1989. Reproductive capacity of sea urchin centrosomes without centrioles. *Cell Motil. Cytoskeleton.* 13:264–273.
31. Standart, N. M., S. J. Bray, E. L. George, T. Hunt, and J. V. Ruderman. 1985. The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal RNA species in clam and sea urchin eggs. *J. Cell. Biol.* 100:1968–1976.
32. Swenson, K., J. Westendorf, T. Hunt, and J. Ruderman. 1989. Cyclins and regulation of the cell cycle in early embryos. *In* The Molecular Biology of Fertilization. H. Schatten and G. Schatten, editors. Academic Press, Inc., New York. 211–232.
33. Vazquez, D. 1979. Inhibitors of protein synthesis. *Mol. Biol. Biochem. Biophys.* 30:136–139 and 162–163.
34. Vorobjev, I. A., and E. S. Nadezhdina. 1987. The centrosome and its role in the organization of microtubules. *Int. Rev. Cytol.* 106:227–293.
35. Wagenaar, E. B. 1983. The timing of synthesis of proteins required for mitosis in the cell cycle of the sea urchin embryo. *Exp. Cell Res.* 144:393–403.
36. Wagenaar, E. B., and D. Mazia. 1978. The effect of emetine on first cleavage division in the sea urchin, *Strongylocentrotus purpuratus*. *In* Cell Reproduction. E. R. Dirsken, D. M. Prescott, and C. F. Fox, editors. Academic Press, Inc., New York. 539–545.
37. Wheatley, D. N. 1982. The Centriole: A Central Enigma of Cell Biology. Elsevier Biomedical Press, New York.
38. Wilson, E. B. 1925. The Cell in Development and Heredity. MacMillan Publishing Co., New York. pp. 400.
39. Winkler, M. M., R. A. Steinhardt, J. L. Grainger, and L. Minning. 1980. Dual ionic controls for the activation of protein synthesis at fertilization. *Nature (Lond.).* 287:558–560.