

# Centrosome Duplication Continues in Cycloheximide-treated *Xenopus* Blastulae in the Absence of a Detectable Cell Cycle

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**Abstract.** Cycloheximide (500  $\mu\text{g/ml}$ ) rapidly arrests cleavage, spindle assembly, and cycles of an M-phase-specific histone kinase in early *Xenopus* blastulae. 2 h after cycloheximide addition, most cells contained two microtubule asters radiating from perinuclear microtubule organizing centers (MTOCs). In contrast, blastomeres treated with cycloheximide for longer periods (3–6 h) contained numerous microtubule asters and MTOCs. Immunofluorescence with an anticentrosome

serum and EM demonstrated that the MTOCs in cycloheximide-treated cells were typical centrosomes, containing centrioles and pericentriolar material. We conclude that centrosome duplication continues in cycloheximide-treated *Xenopus* blastulae in the absence of a detectable cell cycle. In addition, these observations suggest that *Xenopus* embryos contain sufficient material to assemble 1,000–2,000 centrosomes in the absence of normal protein synthesis.

FOR nearly a century it has been recognized that duplication of centrosomes, the major microtubule organizing centers of most animal cells, is tightly synchronized with the cell division cycle (Wilson, 1928; Robbins et al., 1968; Kuriyama and Borisy, 1981; Vorobjev and Chentsov, 1982; Alvey, 1985; for review, see McIntosh, 1983; Brinkley, 1985). However, recent reports have shown that centrosome duplication in embryonic cells can be uncoupled from several key events normally associated with progression through the cell cycle. For example, centrosome duplication in *Drosophila* and echinoderm embryos continues after inhibition of DNA synthesis with aphidicolin (Nagano et al., 1984; Sluder and Lewis, 1987; Raff and Glover, 1988). Furthermore, centrosome duplication continues in echinoderm eggs after enucleation, suggesting that cytoplasmic factors play the major role in regulating this process (Sluder et al., 1986; Picard et al., 1988). However, the mechanisms that coordinate centrosome duplication with other cell cycle functions remain unknown.

The division cycle of early *Xenopus* embryos is governed by a cytoplasmic oscillator that coordinates several nuclear and cytoplasmic events (Newport and Kirschner, 1984; Kirschner et al., 1985). This cytoplasmic cell cycle is not affected by inhibitors of DNA synthesis (Kimelman et al., 1987) or enucleation (Hara et al., 1980; Dabauville et al., 1988). However, inhibition of protein synthesis with cycloheximide arrests all previously examined manifestations of the cell cycle in *Xenopus* embryos, including cycles of an M-phase-promoting factor (MPF; Gerhart et al., 1984), histone kinase activation (Dabauville et al., 1988), surface

contraction waves (Kimelman et al., 1987), nuclear envelope disassembly, and chromatin condensation (Miake-Lye et al., 1983; Newport and Kirschner, 1984). Cycloheximide-arrested embryos thus provide an opportunity to investigate the dependence of centrosome duplication on the cytoplasmic cell cycle.

In this paper we report that cells of *Xenopus* blastulae incubated for extended periods in cycloheximide contained multiple asters, organized by microtubule organizing centers (MTOCs) which are ultrastructurally identical to centrosomes. We conclude that centrosome duplication continues in cycloheximide-treated *Xenopus* blastomeres which lack many features of the cytoplasmic cell cycle. Additionally, our results suggest that *Xenopus* embryos contain a pool of components sufficient to assemble 1,000–2,000 centrosomes.

## Materials and Methods

Cycloheximide and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde was obtained from Ted Pella Co. (Redding, CA). Other reagents for EM were obtained from E. F. Fullham (Latham, NY).

## Immunofluorescence of *Xenopus* Embryos

Adult *Xenopus laevis* were obtained from *Xenopus I* (Ann Arbor, MI). Oviposition, fertilization, and culture of *Xenopus* embryos were as described by Newport and Kirschner (1982).

For most experiments, embryos were allowed to develop for 4 h at 19–20°C, since the resulting cells were the largest that could easily be examined by whole-mount immunofluorescence microscopy. Cycloheximide-arrested embryos were cultured in 5% MR (5 mM NaCl, 0.1 mM KCl, 0.05 mM  $\text{MgSO}_4$ , 0.1 mM  $\text{CaCl}_2$ , 5  $\mu\text{M}$  EDTA, 0.25 mM HEPES, pH 7.8) containing 100–500  $\mu\text{g/ml}$  cycloheximide. Lower concentrations of cycloheximide failed to completely arrest the cell cycle. All experiments reported

1. Abbreviations used in this paper: MPF, M-phase-promoting factor; MTOC, microtubule organizing center.

used 500  $\mu\text{g/ml}$  cycloheximide. Normal embryos were maintained in 5% MR. Where indicated, embryos were dissected by manually removing the vitelline envelope with watchmakers forceps and teasing apart the vegetal pole of the embryo to expose the blastocoel.

Embryos were fixed in 100% methanol for 2–48 h and bleached with 10% hydrogen peroxide in methanol for 8–16 h (Dent and Klymkowsky, 1987). Embryos were rehydrated in TBS (pH 7.4,  $3 \times 30$  min), and were incubated 12–24 h at 4°C in a monoclonal antiserum to  $\alpha$ -tubulin (DM1 $\alpha$ ; 1:250 in TBS plus 2% BSA; ICN Immunobiologicals, Lisle, IL). Embryos were rinsed 24–48 h in several changes of TBS, followed by incubation for 12–24 h in rhodamine-conjugated anti-mouse IgG (1:50 in TBS plus 2% BSA; Organon Teknika-Cappel, Malvern, PA). Embryos were rinsed 24–48 h in TBS, dehydrated in 100% methanol ( $3 \times 30$  min), and cleared in benzyl alcohol/benzyl benzoate clearing solution (1:2; A. Murray, as cited by Dent and Klymkowsky, 1987). Embryos were mounted in 0.5-mm depression slides in clearing solution. Mounting fractured the embryo into small clumps of cells, which aided visualization of microtubules in individual cells.

Embryos were examined on an Ortholux II (E. Leitz, Inc., Rockleigh, NJ) using 10 $\times$  (0.3 NA) and 25 $\times$  (0.6 NA) objectives. Embryos were photographed on Technical pan film (Eastman Kodak Co., Rochester, NY) hypersensitized in a hyper kit (21 d at 30°C; Lumicon Inc., Livermore, CA), using an exposure index of 640–800. Preliminary confocal microscopy was performed on an MRC-600 (Bio-Rad Laboratories, Cambridge, MA) fitted to an optiphot (Nikon Inc., Garden City, NY).

Similar staining patterns were obtained with a monoclonal antiserum to  $\beta$ -tubulin (DM $\beta$ 1, 1:250 in TBS plus BSA; ICN Immunobiologicals). No specific staining was observed with secondary antibody alone. DM $\alpha$ 1 and DM $\beta$ 1 were specific for tubulins in Western immunoblots against total *Xenopus* embryo protein (not shown).

SJ1 anticentrosomal antiserum (a kind gift of Dr. K. Sullivan, Scripps Clinic and Research Foundation, La Jolla, CA) was used at a dilution of 1:500 in TBS plus BSA. This human autoimmune serum was first characterized by staining several mammalian cell lines (BSC-1, 3T3, and N115) and isolated centrosomes from CHO cells (Mitchison and Kirschner, 1984). In all preparation, the pattern of SJ1 staining was indistinguishable from that observed with 5051 antiserum, a well-characterized human autoimmune serum that recognizes centrosomes (Tuffanelli et al., 1983; Calarco-Gillam et al., 1983).

### EM of Cycloheximide-arrested Blastomeres

*Xenopus* blastulae were incubated in cycloheximide for 5.5 h, beginning 3.5 h after fertilization. Arrested embryos were permeabilized with microtubule-stabilizing buffer (80 mM KPipes, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, 30% glycerol, 1  $\mu\text{M}$  Taxol, and 1% TX-100) for 10 min at room temperature, and then fixed in 2% glutaraldehyde (in 50 mM KCacodylate, 100 mM KCl, 10 mM EGTA) for 1 h at room temperature. Fixed embryos were rinsed with cacodylate buffer, and stored overnight at 4°C. Embryos were rinsed with PBS, and incubated in PBS plus 50 mM NaBH<sub>4</sub> overnight at 4°C. After reduction, embryos were rinsed in TBS ( $3 \times 1$  h) and processed for immunofluorescence microscopy with anti- $\alpha$ -tubulin (as described above). Examination of these embryos by fluorescence microscopy revealed that permeabilization had no discernible effect on the number or morphology of microtubule asters.

Arrested embryos fixed in glutaraldehyde and processed for immunofluorescence were dissected into fragments containing three to six blastomeres, with eight or more microtubule asters per cell. Dissected embryos were postfixed in 1% OsO<sub>4</sub> (in cacodylate buffer) for 24 h at 4°C, washed in cacodylate buffer, and dehydrated through a graded series of ethanol (50, 70, 95, 100%;  $3 \times 30$  min each). Samples were incubated in propylene oxide ( $2 \times 30$  min) and infiltrated with propylene oxide:epon (2:1 for 3 h, 1:1 for 3 h) and epon without catalyst (overnight). Samples were embedded in epon, and serial-thin sections were cut and mounted on Formvar-coated slotted grids. Sections were examined on a Phillips EM400 electron microscope.

### Estimation of Protein Synthesis

Normal control and CX-arrested embryos were rinsed three times in 5% MMR and were dissected to expose the blastocoel. Dissected embryos were transferred to 5% MMR containing 1 mCi/ml <sup>35</sup>S-methionine (Amersham Corp., Arlington Heights, IL) for 30 min. Embryos were rinsed in 5% MMR ( $3 \times 1$  ml) to remove extracellular isotope and were lysed in SDS gel sample buffer (100 mM Tris-HCl, 2% SDS, 100 mM DTT, 20%

glycerol, 0.01% bromphenol blue). Duplicate aliquots of 10  $\mu\text{l}$  were batch precipitated with 10% TCA (Gard and Kirschner, 1985) for determination of incorporated <sup>35</sup>S-methionine. Duplicate 5- $\mu\text{l}$  aliquots were counted for determination of soluble isotope. The relative incorporation of radiolabeled methionine into protein was calculated as  $100 \times (\text{incorporated cpm}) / (2 \times \text{soluble cpm})$ . The relative incorporation of <sup>35</sup>S-methionine into protein fell from  $7.5 \pm 1.1\%$  (SD,  $n = 3$ ) in untreated embryos to  $0.15 \pm 0.14\%$  (SD,  $n = 3$ ) within 75 min of cycloheximide addition: a 98% reduction in protein synthesis.

### In Vitro Histone Kinase Assay

Histone kinase activity in embryonic extracts was measured using a modification of the procedure of Dabauville et al. (1988). Synchronized embryos (two/extract) were collected at 5–8-min intervals, rinsed in 500  $\mu\text{l}$  of MPF buffer (80 mM  $\beta$ -glycerophosphate, 15 mM EGTA, 10 mM MgCl<sub>2</sub>, 1 mM DTT, pH 7.3), and lysed in 30  $\mu\text{l}$  of the same buffer by multiple passes through a micropipette tip. Individual extracts were immediately frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until assay. Thawed samples were centrifuged (10 min  $\times$  5,000 g at 4°C) to remove yolk and pigment. 5  $\mu\text{l}$  of lysine-rich histone (1 mg/ml; Sigma Chemical Co.) and 2  $\mu\text{l}$  of 1 mM ATP containing 0.5 Ci/mmol Y-<sup>32</sup>P-ATP (Amersham Corp.) were added to 20  $\mu\text{l}$  of each supernatant. After 10 min incubation at room temperature, 75  $\mu\text{l}$  SDS gel sample buffer was added and samples were heated for 3 min in a boiling water bath. Histones were separated by SDS-PAGE on 15% gels followed by liquid scintillation counting. Results are shown as the percent maximum incorporation.

## Results

### Cycloheximide Arrests the Cell Cycle in *Xenopus* Blastulae

Cycloheximide (at concentrations of 10–200  $\mu\text{g/ml}$ ) has previously been shown to arrest many aspects of the cell cycle in cleaving *Xenopus* embryos (see Introduction for references). We examined several features of cycloheximide-treated blastulae, including cleavage, entry into mitosis, and histone kinase activities, to verify that inhibition of protein synthesis blocked progression of the cell cycle in these later-stage embryos. Preliminary results indicated that cycloheximide concentrations between 100 and 500  $\mu\text{g/ml}$  arrested all detectable features of the cell cycle. The following experiments were performed using 500  $\mu\text{g/ml}$  cycloheximide, a concentration sufficient to reduce protein synthesis to  $\sim 2\%$  of normal levels within 75 min of addition to blastula stage embryos (see Materials and Methods).

Other inhibitors of protein synthesis were also evaluated for their effects on blastulae. Neither puromycin (500  $\mu\text{g/ml}$ ) nor emetine dihydrochloride (up to 10 mg/ml) arrested cell cleavage or mitosis when applied to intact or dissected *Xenopus* embryos (data not shown). Embryos were observed to begin gastrulation despite incubation in 5 mg/ml emetine. These results presumably reflect the impermeability of the *Xenopus* embryos to many compounds.

The first visible effect of cycloheximide on blastulae was the arrest of cell cleavage (Fig. 1). 4 h after fertilization, normal blastulae contained  $86 \pm 7$  cells (SD;  $n = 5$ ) per embryo. Cells on the embryo surface complete one cleavage cycle within 30 min of addition of 500  $\mu\text{g/ml}$  cycloheximide. After 1 h in cycloheximide, the number of cells per embryo had risen to  $162 \pm 22$  ( $n = 5$ ), consistent with previous observations that cycloheximide-treated embryos complete one full cell cycle before arrest (Miake-Lye et al., 1983). After 2 h, the number of cells in cycloheximide-treated embryos had increased to  $203 \pm 19$  ( $n = 5$ ), suggesting that a fraction

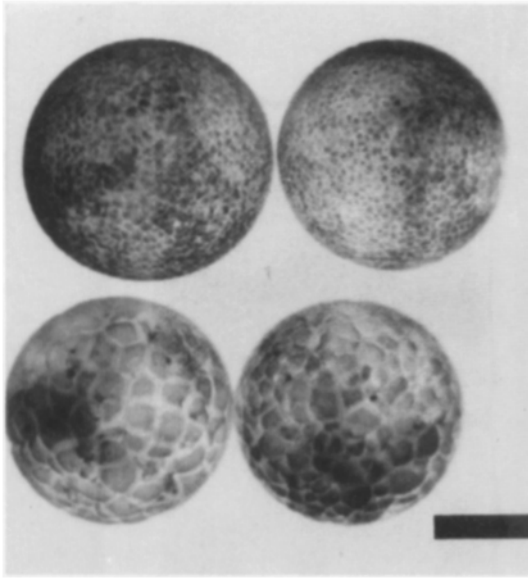


Figure 1. Cycloheximide arrests cleavage of *Xenopus* blastulae. (Above) Normal mid-blastula stage embryos (6.5 h after fertilization) contain 2,000–4,000 cells. (Below) Embryos of identical age incubated in 500 µg/ml cycloheximide beginning 4 h after fertilization contain ~200 cells ( $202 \pm 10$ ; SD,  $n = 5$ ). Bar, 0.5 mm.

of cells on the interior of the embryo had completed one additional division cycle. Cell number then remained constant for at least 5 h of cycloheximide treatment ( $200 \pm 18$ ;  $n = 3$ ).

The numbers of cells entering mitosis in normal and cycloheximide-treated embryos were compared by examining the number of mitotic spindles visualized by immunofluorescence with antitubulin. Spindles were apparent in normal embryos throughout development from early cleavage to the mid-blastula stage. The mitotic index of normal embryos showed regional variation, ranging from 20% (23:114) to 52% (184:355) during the period from 4–6 h after fertilization. This reflects the occurrence of metachronous waves of mitosis during early amphibian development (Sato, 1977). The mitotic index was lower in embryos fixed after the mid-blastula stage. However, spindles were easily found as late as gastrulation (10–11 h after fertilization).

Within 30 min of cycloheximide addition to 4-h embryos, the mitotic index had decreased to 6% (7:111), and was <1% (2:285) within 2 h. Examination of thousands of cells has failed to reveal any recognizable spindle structures after >2 h in 500 µg/ml cycloheximide.

Previous studies have identified M-phase-specific histone kinase activities in many embryonic cells (Cicirelli et al., 1988; Labbe et al., 1989; Meijer and Pondaven, 1988). We therefore compared histone kinase activities of extracts prepared from normal and cycloheximide-arrested embryos as an indicator of the cytoplasmic cell cycle.

Phosphorylation of exogenous histone was observed in cytoplasmic extracts prepared from normal embryos at all stages of the cell cycle (Fig. 2, A and C). However, a cyclic twofold increase in histone phosphorylation was evident, with a period approximating that of the cell cycle in vivo. This periodic activation of histone kinase was rapidly elimi-

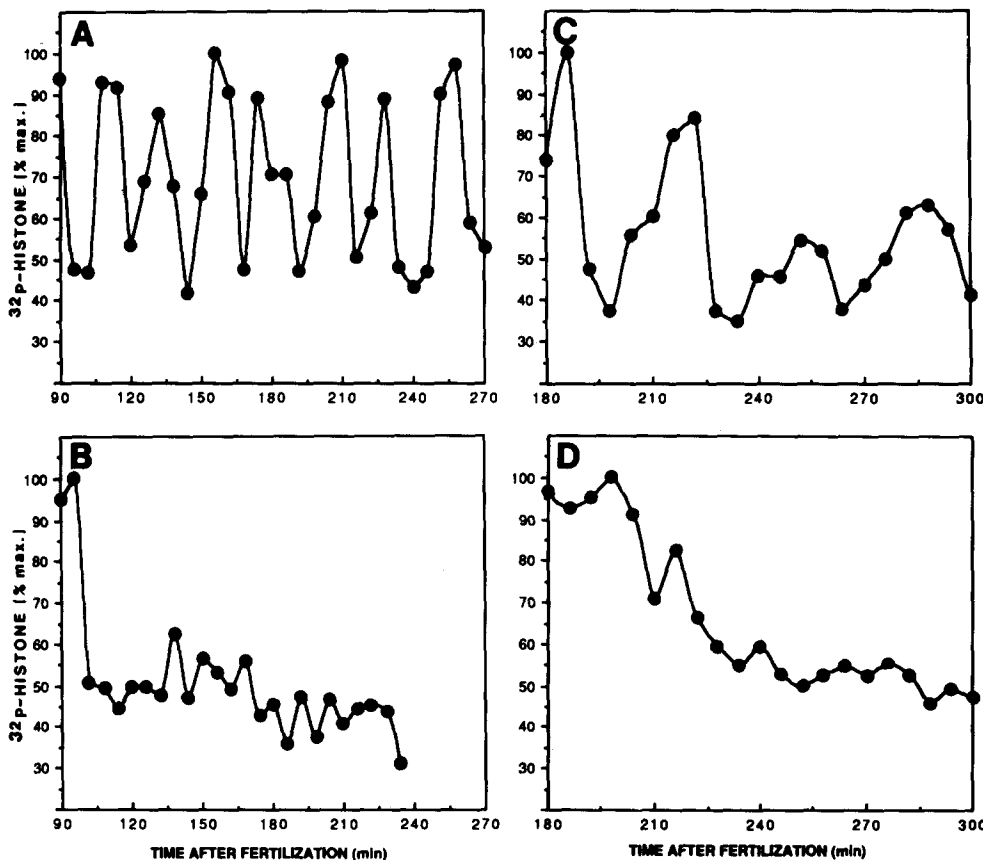
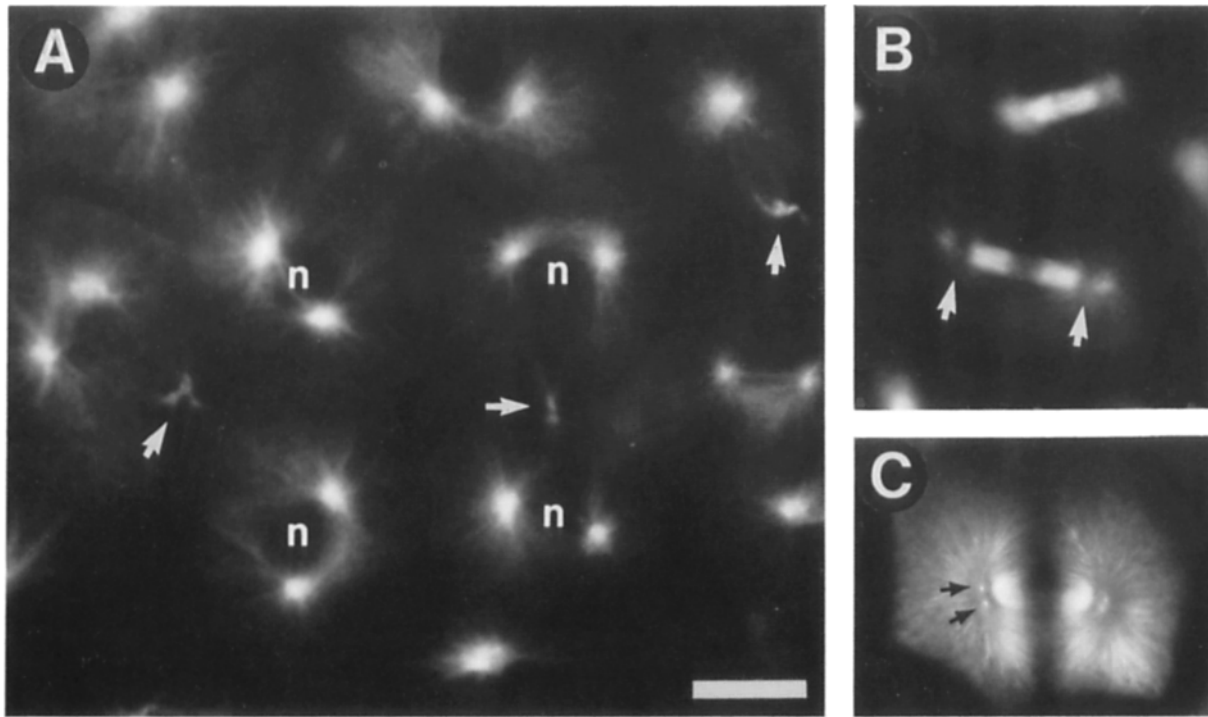


Figure 2. Cycloheximide arrests cycles of M-phase-specific histone kinase. (A) In vitro histone kinase activity in extracts prepared from synchronized normal embryos. Extracts were prepared at the indicated times and assayed for histone kinase activity as described in Materials and Methods. (B) In vitro histone kinase activity in extracts of cycloheximide-treated embryos. Cycloheximide (500 µg/ml) was added 90 min after fertilization, and extracts were prepared at the indicated times. (C and D) In vitro histone kinase activity of normal (C) and cycloheximide-treated blastulae (D). Cycloheximide was applied 180 min after fertilization.



**Figure 3.** Immunofluorescence localization of microtubules in normal *Xenopus* blastomeres. (A) Interphase cells, 6 h after fertilization, contain two perinuclear asters. Cells are often observed to be connected by midbodies (arrows; n, nuclei). (B) Mitotic blastomeres from a normal embryo 6 h after fertilization. A zone of decreased microtubule density separates the prominent spindle poles from the central spindle (arrows). (C) A large blastomere in telophase 5 h after fertilization. Spindle poles exhibit two tubulin-containing foci within a microtubule-free region (arrows). Bars: (A and B) 100  $\mu\text{m}$ ; (C) 50  $\mu\text{m}$ .

nated by addition of 500  $\mu\text{g/ml}$  cycloheximide to embryos at 1.5 or 3 h after fertilization (Fig. 2, B and D, respectively). Histone kinase activity then remained low for the duration of the assay (2.5 h). These results suggest that progression of the cytoplasmic cell cycle has been eliminated in cycloheximide-treated blastulae.

#### **Normal Interphase Blastomeres Contain Two Microtubule Asters**

Examination of the microtubule distribution in normal *Xenopus* blastulae by immunofluorescence with antitubulin revealed that all interphase cells contained two prominent microtubule asters (Fig. 3 A). These asters were typically found on opposite sides of the intact cell nucleus. In some cells, asters could be resolved into component microtubules. In others, microtubules could not be resolved and asters appeared as brightly stained perinuclear foci. Pairs of interphase cells joined by midbodies were often observed (Fig. 3 A, arrows). Each cell of such pairs contained two interphase asters, suggesting that centrosome duplication in normal *Xenopus* blastomeres begins in anaphase or telophase and is functionally complete early in the subsequent interphase. This conclusion is supported by changes in spindle pole morphology late in mitosis. In the early stages (metaphase through early anaphase) each spindle pole contained a single prominent focus of microtubules, often separated from the central spindle by a zone of decreased microtubule density (Fig. 3 B). However, in many late anaphase/telophase cells two discrete tubulin-containing foci were apparent (Fig. 3 C), often surrounded by a halo of lesser microtu-

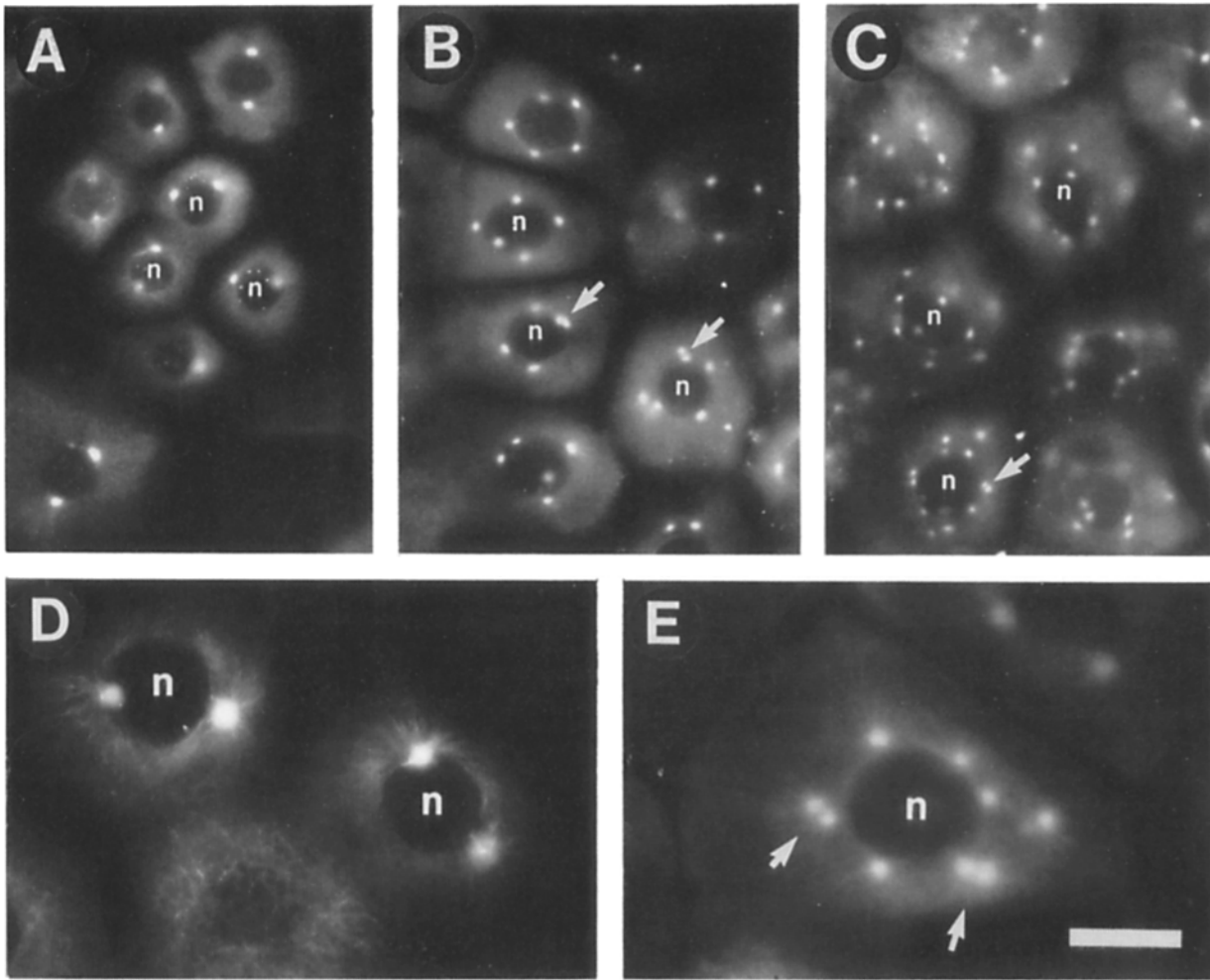
bule density. These foci continue to separate, and become fully functional MTOCs before the completion of cytokinesis (not shown).

#### **Cycloheximide-treated Blastomeres Contain Multiple Asters and MTOCs**

Addition of cycloheximide to 4-h blastulae rapidly arrests both mitosis and cytokinesis (above). After 2 h in cycloheximide, most cells contained two perinuclear microtubule asters (Fig. 4 A). These asters were nearly indistinguishable from those in normal interphase cells (compare Fig. 4 D with Fig. 3 A). Midbodies, remnants of mitotic spindles, were not observed in cycloheximide-treated cells. The lack of midbodies and presence of interphase asters and nuclei are consistent with prolonged arrest of the cell cycle in interphase.

A minority of cells examined after 2 h in cycloheximide contained three or more microtubule asters. With continued incubation in cycloheximide the proportion of cells exhibiting more than two asters and the number of asters per cell increased dramatically (Fig. 4, B and C). Asters were often observed in close proximity to interphase nuclei, a location characteristic of centrosomes, although they were also observed dispersed throughout the cytoplasm. In many instances, asters were found in closely spaced pairs (arrows in Fig. 4, B, C, and E), suggestive of recent duplication or separation of the MTOCs. Often paired asters coexisted with individual asters in cells containing interphase nuclei.

The multiple asters observed in cells after prolonged cycloheximide treatment suggested the presence of multiple MTOCs. We therefore examined the number of MTOCs in



**Figure 4.** Immunofluorescence localization of microtubules in embryos treated with 500  $\mu\text{g/ml}$  cycloheximide beginning 4 h after fertilization. (A) After 2 h in cycloheximide, most cells have two perinuclear asters (*n*, nuclei). (B) After 3 h in cycloheximide, cells contain four to seven perinuclear asters. Some asters appear in pairs (*arrows*). (C) After 4 h, cycloheximide-arrested cells contain numerous asters. Arrow denotes a closely spaced pair of asters. Higher magnification of blastomeres after (D) 2 h and (E) 4 h in cycloheximide allows resolution of microtubules. Note the coexistence of paired and single asters surrounding a single cell nucleus in E (*n*, nuclei; *arrows*, paired asters). Bars: (A–C) 100  $\mu\text{m}$ ; (D–E) 50  $\mu\text{m}$ .

normal and cycloheximide-treated cells after microtubule depolymerization, and during subsequent recovery (Spiegelman et al., 1979; Brinkley et al., 1981). Microtubules of control and cycloheximide-treated embryos were completely depolymerized after 30 min incubation at 4°C (Fig. 5, A–C, and E). Antitubulin staining in cooled normal cells was confined to two perinuclear foci in interphase (Fig. 5, A and B) and two pairs of foci in late mitosis (Fig. 5 C). Upon return to room temperature, these cold-stable foci organized either two interphase asters (not shown), or the poles of mitotic spindles (Fig. 5 D).

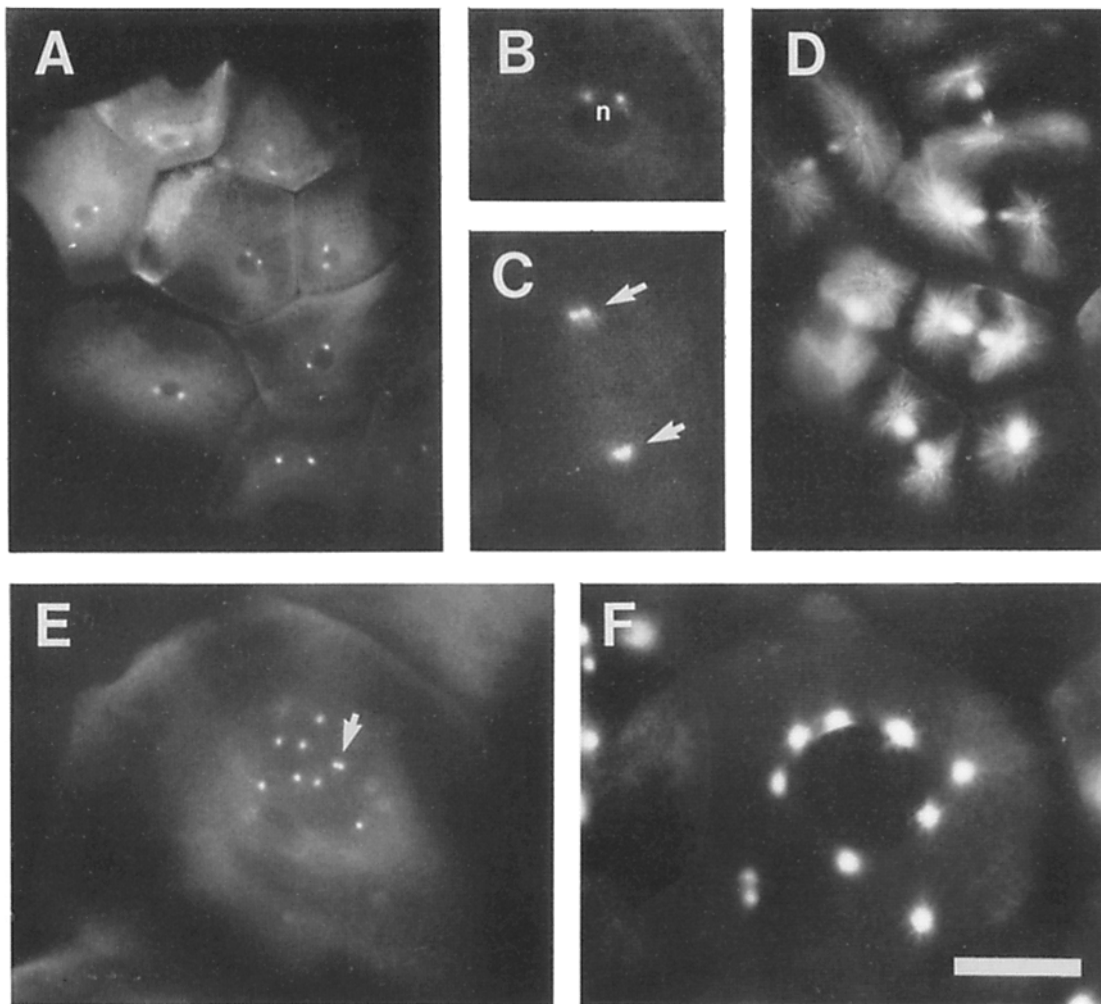
In contrast, numerous cold-stable foci were observed after microtubule depolymerization in cycloheximide-arrested cells (Fig. 5 F). Foci were found in both perinuclear and cytoplasmic locations, and were often observed as closely spaced pairs (*arrows* in Fig. 5 F). The apparent size and intensity of these foci were comparable to those observed in normal embryos after cooling (compare Fig. 5, B and E). The numbers and distributions of cold-stable foci were simi-

lar to those of the microtubule asters in cycloheximide-treated cells.

Numerous centers of microtubule regrowth were apparent in cycloheximide-treated cells within 1 min of return to room temperature (Fig. 5 F). Regrown asters were similar in appearance to interphase asters in normal cells; mitotic spindles were never observed during regrowth in cycloheximide-arrested cells. The number of regrowth centers was comparable to the number of asters in cycloheximide-treated cells before microtubule depolymerization, and the number of tubulin-staining foci in cooled cycloheximide-treated cells. From these results we conclude that most, if not all, asters in cycloheximide-arrested cells are nucleated by functional, cold-stable MTOCs.

#### **MTOCs in CX-arrested Cells Are Centrosomes**

To further investigate the nature of the MTOCs in cycloheximide-treated blastomeres, embryos were examined by immunofluorescence microscopy using an anticentrosome an-



**Figure 5.** Cycloheximide-arrested cells contain multiple MTOCs. (*A* and *B*) Interphase blastomeres from a normal 5-h embryo after cold-induced microtubule depolymerization. Cells contain two perinuclear spots which stain brightly with antitubulin. (*C*) Two pairs of brightly stained spots (arrows) are apparent after depolymerization of microtubules in mitotic cells. (*D*) Extensive regrowth of spindle microtubules is apparent 6.5 min after returning control embryos to room temperature. (*E*) Antitubulin reveals numerous foci in the perinuclear cytoplasm of a cycloheximide-arrested cell (500  $\mu\text{g/ml}$  for 5 h). (*F*) Many foci of microtubule nucleation are apparent 1 min after returning a cycloheximide-arrested cell to room temperature. Bars: (*A* and *D*) 125  $\mu\text{m}$ ; (*B*, *C*, *E*, and *F*) 50  $\mu\text{m}$ .

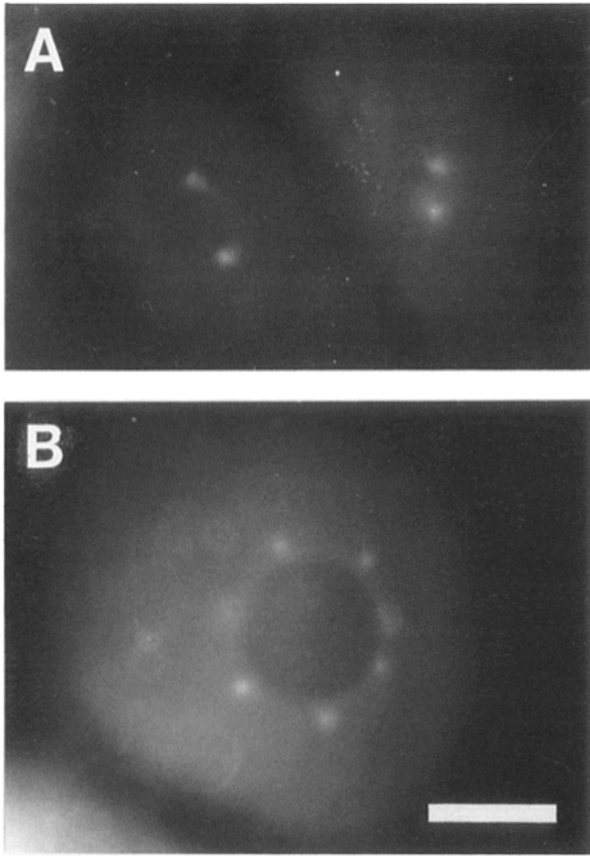
tiserum (SJI). SJI stained two perinuclear foci in interphase cells of normal embryos (Fig. 6 *A*), consistent with the centrosomal pattern of SJI staining observed in mammalian cells (see Materials and Methods). In contrast, SJI stained numerous foci in cycloheximide-treated blastomeres (Fig. 6 *B*). The numbers and distributions of the SJI-stained foci are comparable to the number of asters stained with antitubulin in cycloheximide-arrested cells, suggesting that MTOCs in cycloheximide-treated cells contain the normal centrosomal component recognized by SJI antiserum.

To unambiguously identify these MTOCs, cycloheximide-arrested cells were serial sectioned for EM (see Materials and Methods). Multiple asters were identified in several individual cells. In one cell examined in detail, eight microtubule asters were identified, four of which are shown in Fig. 7. Seven asters were sectioned through most of their diameter. These asters contained typical centrosomes, including a pair of centrioles surrounded by amorphous pericentriolar material. In the eighth aster, which was only partially

sectioned, one centriole was found. Procentrioles, early intermediates in centriole duplication, were not observed, probably due to the limited number of centrosomes that could be examined in detail. Spindles and spindle-like microtubule arrays were never observed in cycloheximide-treated cells, despite the close apposition of some asters (Fig. 7, *A* and *B*). We conclude that the MTOCs that accumulate during cycloheximide treatment of *Xenopus* blastomeres are interphase centrosomes.

#### ***Centrosome Duplication in Cycloheximide-treated Cells Is Asynchronous***

The time course of centrosome accumulation was examined by counting the number of asters in cells (visualized with antitubulin) after various lengths of cycloheximide treatment (Fig. 8). 2 h after cycloheximide addition, most cells contained two centrosomes (mean:  $2.4 \pm 0.9$ ; SEM,  $n = 283$ ; Fig. 8). By 4.5 h after cycloheximide addition, the mean number of centrosomes had increased to eight per cell (8.5



**Figure 6.** MTOCs in cycloheximide-arrested cells stain with anti-centrosome antibody. (A) SJ1 anticentrosomal antiserum stains two perinuclear foci in normal blastomeres. (B) SJ1 antiserum stains numerous perinuclear foci in cycloheximide-arrested cells (5 h arrest beginning 4 h after fertilization). Bar, 50  $\mu$ m.

$\pm 4.3$ ,  $n = 143$ ), corresponding to a doubling time of just over an hour. Thereafter, the mean number of centrosomes remained approximately eight per cell, until cell death.

The distribution of centrosome numbers in individual cells exhibited sequential peaks at two, four, and eight centrosomes per cell (Fig. 8). However, a striking degree of heterogeneity in the number of centrosomes per cell was apparent, which increased with the length of cycloheximide treatment. Cells were often observed with intermediate or odd numbers of centrosomes (see Fig. 3 E, and histograms in Fig. 8). Some heterogeneity undoubtedly results from the difficulty of counting numerous asters in large cells. However, confocal microscopy revealed that many cycloheximide-treated cells unambiguously contained odd numbers of asters (not shown), which could only result if centrosome duplication in cycloheximide-treated cells was asynchronous. Asynchronous duplication is also suggested by the observation of paired and single centrosomes within a single cell (as in Fig. 4, B, C, and E).

Multiple centrosomes were evident in blastomeres when cycloheximide was added to embryos as early as 2.5 h after fertilization (not shown; cells arrested earlier in development were too large for visualization of asters). However, cells in embryos transferred to cycloheximide 5 h after fertilization (or later) arrested in interphase with only two centrosomes

(not shown), suggesting that the capacity for centrosome duplication in cycloheximide-arrested embryos is developmentally limited.

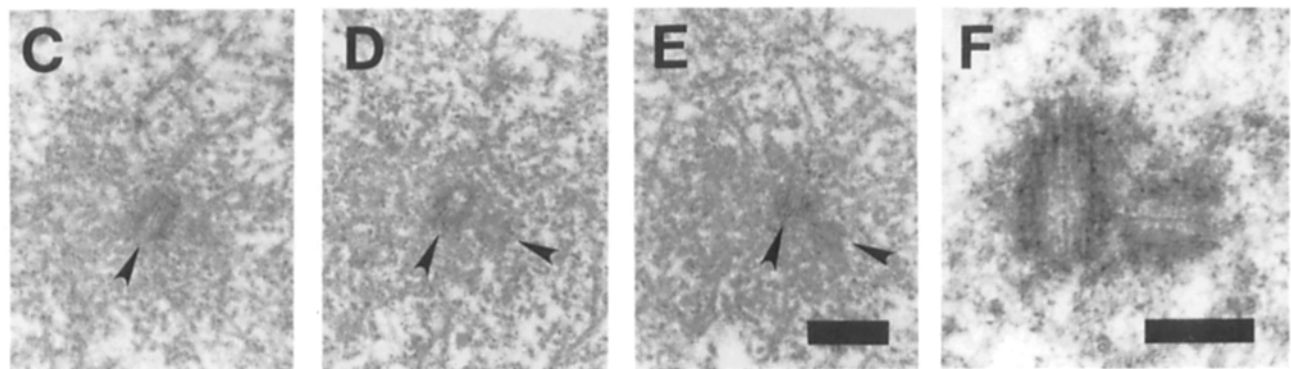
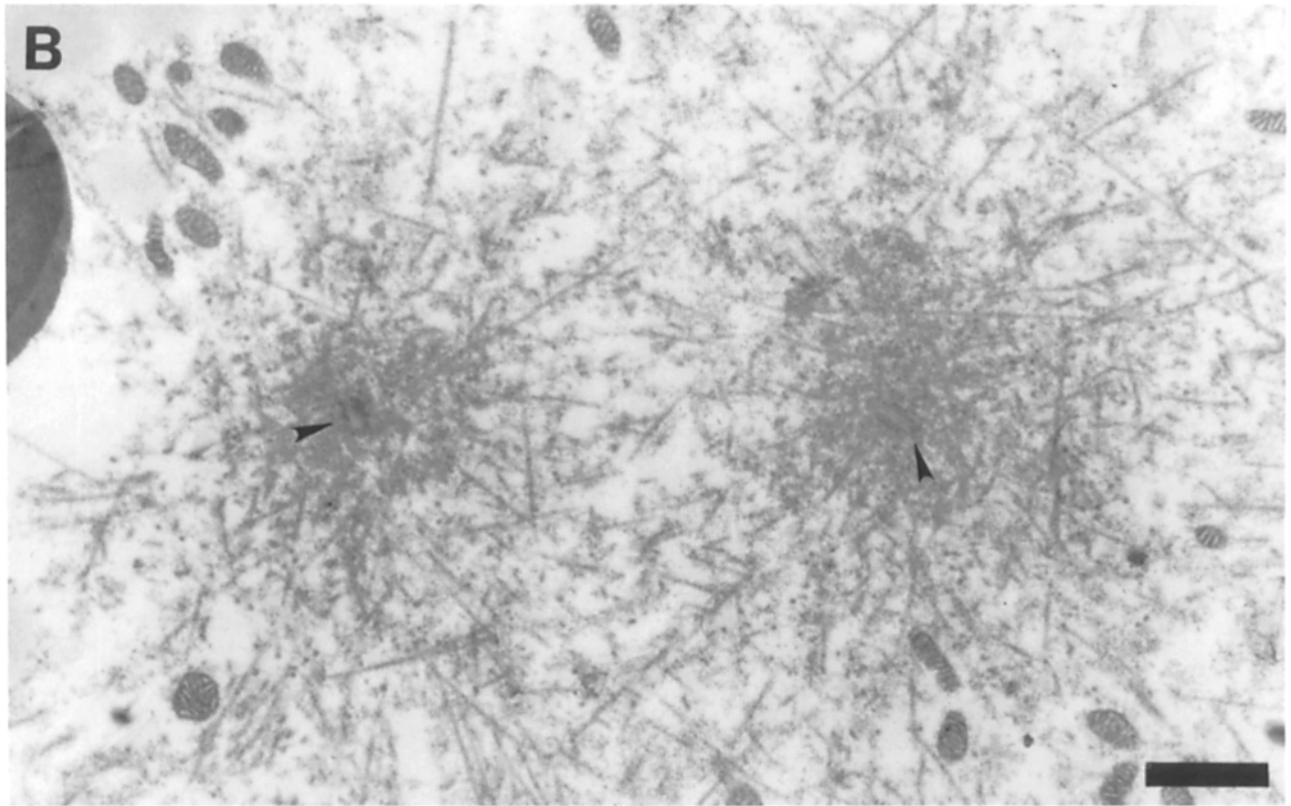
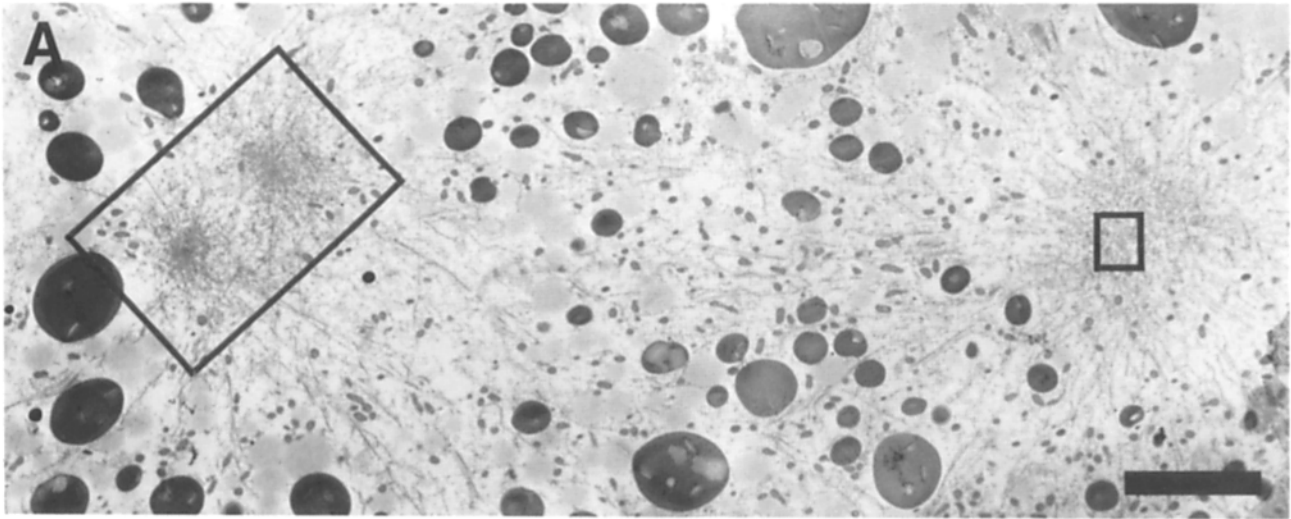
## Discussion

In most cells, the duplication of centrosomes is tightly synchronized to other events of the cell division cycle (for reviews, see Brinkley, 1985; McIntosh, 1983). Recent reports, however, have demonstrated that centrosome duplication can be uncoupled from nuclear events such as DNA replication in embryonic cells (Nagano et al., 1981; Sluder and Lewis, 1987; Raff and Glover, 1988), and can even occur in the complete absence of nuclei (Sluder et al., 1986; Picard et al., 1988). These results have suggested that the process of centrosome duplication is regulated by a cytoplasmic mechanism (Sluder et al., 1986). To further investigate the nature of this regulatory mechanism, we have examined the distribution and number of centrosomes in *Xenopus* blastomeres arrested in interphase with cycloheximide. Under these conditions, centrosomes continued to accumulate despite arrest of many other cell cycle events including cytokinesis, spindle assembly, and cycles of M-phase-specific histone kinase activity.

Several observations suggest that the accumulation of centrosomes in cycloheximide-arrested embryos occurs through a process of binary duplication. For example, accumulation of centrosomes after cycloheximide follows a roughly exponential time course, with peaks in the number distribution corresponding to two, four, and eight centrosomes per cell. Neither fragmentation of preexisting centrosomes (Sellitto and Kuriyama, 1988), nor de novo assembly of centrosomes from preexisting components (Wilson, 1928; Dirksen, 1961; Weisenberg and Rosenfeld, 1975; Miki-Noumura, 1977; Kallenbach, 1982), can readily explain this observation. Fragmentation can also be ruled out by the identification of at least 15 centrioles within a single cycloheximide-arrested cell. The closely spaced pairs of centrosomes observed in cycloheximide-treated cells by immunofluorescence and confocal microscopy are also suggestive of recently completed duplication events. Furthermore, separation of MTOCs has been observed in extracts of cycloheximide-arrested *Xenopus* eggs by video microscopy (Doxsey, S. J., unpublished observation). Taken together, these results suggest that the binary duplication of centrosomes continues in cycloheximide-arrested *Xenopus* blastomeres.

Cycloheximide arrests many features of the cell cycle of early *Xenopus* embryos in interphase, including cycles of MPF (Gerhart et al., 1984), a highly conserved cell cycle regulator (Dunphy et al., 1988; Gautier et al., 1988). Recently it has been reported that the periodic activation of MPF, which is a key event of the cytoplasmic cell cycle in *Xenopus* embryos, is dependent upon the synthesis of cyclins (Minshull et al., 1989; Murray and Kirschner, 1989): a family of cell cycle-dependent proteins originally identified in echinoderm embryos (Evans et al., 1983). In our experiments, cycloheximide (at 500  $\mu$ g/ml) inhibited protein synthesis by 98%, blocking cytokinesis, spindle assembly, and cycles of histone kinase activation. The major mitosis-specific H1 kinase has been found to be a component of MPF (Arion et al., 1988; Labbe et al., 1989), indicating that cytoplasmic oscillations of MPF have also been eliminated in





**Figure 7.** Cycloheximide-arrested cells contain multiple centrosomes. (A) Three cytoplasmic asters are apparent within the same micrograph of a cycloheximide-arrested blastomere (5.5 h, beginning 3.5 h after fertilization). The left box in A encloses two asters, shown in higher magnification in B. These asters originate from characteristic centrosomes containing centrioles (*arrowheads*) and pericentriolar material. (C-E) Serial sections of the centrosomal region in the right box in A. Two centrioles (*arrowheads*) were identified. (F) A section through a fourth centrosome from the same cell shows two centrioles in an orthogonal orientation. Bars: (A) 5  $\mu\text{m}$ ; (B) 1  $\mu\text{m}$ ; (C-E) 0.5  $\mu\text{m}$ ; (F) 0.25  $\mu\text{m}$ .



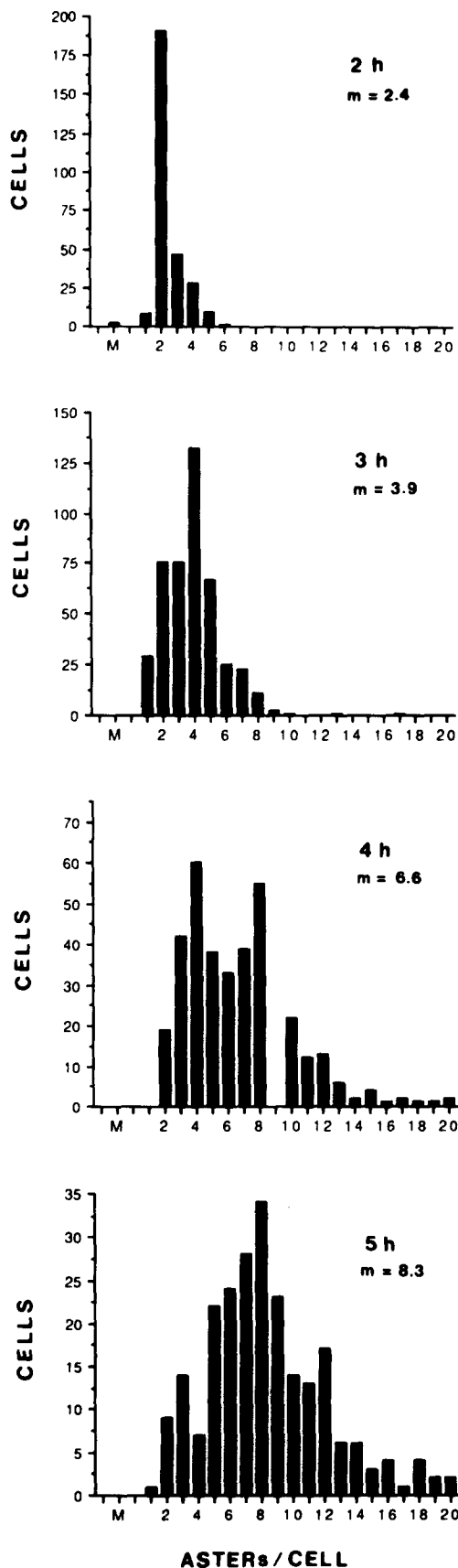


Figure 8. Cycloheximide (500  $\mu\text{g}/\text{ml}$ ) was added to embryos 4 h after fertilization. After cycloheximide treatment for the indicated time, embryos were fixed and processed for immunofluorescence with antitubulin. The number of centrosomes per cell was esti-

cycloheximide-arrested *Xenopus* blastomeres. The continued duplication of centrosomes under these conditions suggests that centrosome duplication can be experimentally uncoupled from the synthesis of cyclins and cycles of MPF activation, and that cycloheximide arrests cells at a stage conducive to multiple rounds of centrosome duplication. While we cannot exclude the possibility that the residual levels of protein synthesis support an undetectable cytoplasmic cell cycle, we think it unlikely since it has recently been shown that low levels of cyclin synthesis are insufficient to drive several cell cycle features, including nuclear envelope breakdown, MPF activation, and histone kinase activation, in extracts of *Xenopus* eggs (Murray and Kirschner, 1989).

Centrosome duplication in normal embryonic cells is highly synchronous. This synchrony is not disrupted by enucleation of echinoderm embryos (Sluder et al., 1986; Picard et al., 1988), nor by inhibition of cytokinesis in cytochalasin-treated *Xenopus* blastomeres (Gard, D. L., unpublished observations), manipulations which leave the cytoplasmic cell cycle intact (Picard et al., 1988; Newport and Kirschner, 1984). In cycloheximide-arrested embryos, however, many cells were observed with odd or intermediate numbers of centrosomes. In addition, single and closely paired centrosomes were observed to coexist in the same cells. Loss of synchrony in cycloheximide-treated cells would suggest that the cytoplasmic cell cycle, though not necessary for centrosome duplication and assembly, provides cues which coordinate centrosome duplication with other cell cycle events. Although the nature of these cues remains undefined, recent reports suggest that cell cycle-dependent phosphorylation of centrosomal components might modulate centrosome function or assembly in normal cells (Vandre et al., 1984; Riabowol et al., 1989).

Continued duplication of centrosomes in cycloheximide-treated embryos suggests that early *Xenopus* blastulae contain extensive pools of centrosomal components. From the average number of centrosomes in cycloheximide-arrested cells and the number of cells in arrested embryos, we estimate that early blastulae contain sufficient component pools to assemble at least 1,000–2,000 centrosomes. This conclusion is consistent with reports showing that *Xenopus* embryos contain extensive stores of many cell components including lamins (Forbes et al., 1984), histones (Woodland and Adamson, 1977), and tubulin (Gard and Kirschner, 1987). Echinoderm eggs have also been found to contain maternally-derived pools of proteins or mRNA required for centrosome duplication in the absence of nuclear function (Sluder et al., 1986; Picard et al., 1988).

Cycloheximide has been reported to arrest the centriole/centrosome cycle in cultured mammalian cells (Phillips and Rattner, 1976) and early *Drosophila* embryos (Raff and Glover, 1988). However, a recent report indicates that centrosome duplication continues after inhibition of protein synthesis in embryos of several sea urchin species (Sluder, G., F. J. Miller, S. King, and C. L. Rieder. 1989. *J. Cell Biol.*

ated by counting microtubule asters in individual cells. The mean number of asters per cell at each time is indicated (excluding mitotic cells). Successive peaks in aster number correspond to two, four, and eight centrosomes per cell, though the broad distributions suggest that duplication is asynchronous.

109:95a). This suggests that the uncoupling of centrosome replication from the cell cycle and protein synthesis is not unique to *Xenopus* blastomeres.

In summary, we report that multiple rounds of asynchronous centrosome duplication proceed in the absence of a cytokinesis, spindle assembly, or a measurable cytoplasmic cell cycle in cycloheximide-treated *Xenopus* embryos. In addition, we suggest that *Xenopus* embryos contain a pool of components sufficient to assemble at least 1,000–2,000 centrosomes. Thus, cycloheximide-treated *Xenopus* embryos may provide a useful system for studying the regulation of centrosome assembly and maturation into functional MTOCs.

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