Dissociation of Centrosome Replication Events from Cycles of DNA Synthesis and Mitotic Division in Hydroxyurea-arrested Chinese Hamster Ovary Cells

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Abstract. Relatively little is known about the mechanisms used by somatic cells to regulate the replication of the centrosome complex. Centrosome doubling was studied in CHO cells by electron microscopy and immunofluorescence microscopy using human autoimmune anticentrosome antiserum, and by Northern blotting using the cDNA encoding portion of the centrosome autoantigen pericentriolar material (PCM)-1. Centrosome doubling could be dissociated from cycles of DNA synthesis and mitotic division by arresting cells at the G_1/S boundary of the cell cycle using either hydroxyurea or aphidicolin. Immunofluorescence microscopy using SPJ human autoimmune anticentrosome antiserum demonstrated that arrested cells were able to undergo numerous rounds of centrosome replication in the absence of cycles of DNA synthesis and mitosis. Northern blot analysis demonstrated that the synthesis and degradation of the mRNA encoding PCM-1 occurred in a cell cycle–dependent fashion in CHO cells with peak levels of PCM-1 mRNA being present in G_1 and S phase cells before mRNA amounts dropped to undetectable levels in G_2 and M phases. Conversely, cells arrested at the G_1/S boundary of the cell cycle maintained PCM-1 mRNA at artificially elevated levels, providing a possible molecular mechanism for explaining the multiple rounds of centrosome replication that occurred in CHO cells during prolonged hydroxyurea-induced arrest. The capacity to replicate centrosomes could be abolished in hydroxyurea-arrested CHO cells by culturing the cells in dialyzed serum. However, the ability to replicate centrosomes and to synthesize PCM-1 mRNA could be re-initiated by adding EGF to the dialyzed serum. This experimental system should be useful for investigating the positive and negative molecular mechanisms used by somatic cells to regulate the replication of centrosomes and for studying and the methods used by somatic cells for coordinating centrosome duplication with other cell cycle progression events.

THE fidelity of chromosome segregation is dependent upon the regulated replication of the centrosome complex. The centrosome, which nucleates and organizes cytoplasmic microtubules, must be replicated once, and only once, during each cell cycle. At the onset of mitosis, the progeny centrosomes migrate to opposite ends of the cell where the duplicated centrosomes serve as the spindle poles. In this capacity, the mitotic centrosomes nucleate the microtubules that result in both spindle formation and chromosome segregation. Clearly, the integrity of mitotic events depends completely upon the cells' ability to regulate centrosome replication.

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Relatively little is known about the mechanisms used by cells to duplicate centrosomes. Embryonic cells have been used by many investigators as experimental systems for the study of centrosome doubling. These experiments have demonstrated that centrosome replication can occur in the total absence of either DNA or protein synthesis in fertilized eggs (Sluder and Lewis, 1987; Raff and Glover, 1988; Gard et al., 1990; Sluder et al., 1990). Although these investigations have convincingly shown that centrosome duplication can be uncoupled from other cell cycle progression events in embryonic cells, these studies tell us relatively little about the mechanisms of centrosome replication in somatic cells. The reason for this is that embryonic cells usually contain large precursor pools of proteins that can be recruited for the assembly and replication of various cellular structures. Therefore, the multiple rounds of

centrosome replication that have been detected in embryonic cells after various experimental treatments most likely occurred by recruitment of precursor components from the preexisting cytoplasmic pools of proteins in eggs. It seems unlikely that cycling somatic cells have large amounts of stored precursor proteins that are used for centrosome production. Most probably, centrosome components are synthesized and assembled in a regulated fashion during each cell cycle in somatic cells.

Although centrosome replication has been well defined at the morphological level in somatic cells, very little is known about the molecular regulation of centrosome doubling. Ultrastructural studies, which have used centriole doubling as a landmark, have shown that centriole replication begins near the G_1/S boundary of the cell cycle. Centriole doubling then continues through S phase and is completed during G_2 phase of the cell cycle (Robbins et al., 1968; Brinkley, 1985; Vandré and Borisy, 1989). Biochemical studies have demonstrated that centriole doubling in somatic cells will occur in the absence of DNA synthesis (Rattner and Phillips, 1973; Kuriyama and Borisy, 1981; Kuriyama et al., 1986) but requires protein synthesis (Phillips and Rattner, 1976). Moreover, Kochansky and Borisy (1990) have demonstrated that centriole duplication in somatic cells is a semi-conservative process with each progeny cell receiving a parental and daughter centriole at cell division. Finally, studies have shown that unlike unfertilized eggs, in which centriole formation can occur de novo (Palazzo et al., 1992), somatic mammalian cells require the presence of a preexisting centriole to complete centrosome duplication (Kuriyama and Borisy, 1981; Maniotis and Schliwa, 1991). Presumably, the centriole provides a seeding capacity of some form that is required for centriole replication to occur. Still, it is not clear how components of the pericentriolar material are replicated and assembled, nor is it understood how the cell is able to regulate the timing of centrosome duplication each cell cycle.

As stated previously, the centrosome complex must be replicated once, and only once, during each cell cycle. Experimentation has demonstrated that the regulation of centrosome replication is under cytoplasmic control in embryonic cells (Sluder et al., 1986, 1990). However, the cell cycle in somatic cells is much more complex than the embryonic cell cycle and it still is not clear how centrosome replication is controlled during the somatic cell cycle. Two mechanisms have been proposed to explain how a somatic cell is able to coordinate centrosome replication with other cell cycle progression events while limiting centrosome doubling to a single round of replication during each cell cycle. According to one scheme, the somatic cell nucleus could have a direct role in regulating centrosome replication by controlling transcript synthesis each cell cycle thereby directing centrosome duplication events (Sluder et al., 1986). A second possibility is that the cell has an endogenous counting mechanism that both ensures centrosome doubling while at the same time limiting centrosome replication to a single round of doubling during the cell cycle (Rose et al., 1993). An analogous type of system, the so-called re-replication block (Rao and Johnson, 1970; Handeli and Weintraub, 1992), has been demonstrated for nuclear DNA. Although not yet defined, a regulatory mechanism seems to exist that limits DNA replication to a single round during each cell cycle. A similar type of mechanism could be at work to limit the number of centrosomes that are produced during each cell cycle. The experiments reported in this manuscript were designed to address each of these possibilities.

In this manuscript, the dissociation of centrosome replication from the remainder of the somatic cell cycle is reported. CHO cells were arrested at the G_1/S boundary of the cell cycle and then maintained in the blocked state for a period of time that approximated four to five cell cycles. When the arrested cells were examined, it was found that the experimentally treated cells had undergone multiple rounds of centrosome replication. Northern blot analysis using the cDNA encoding portion of the centrosomal autoantigen pericentriolar material (PCM)[1]-1 demonstrated that cells arrested at the G_1/S boundary of the cell cycle maintained centrosomal mRNA at artificially elevated levels when compared to untreated control cells, providing a possible molecular explanation for how the arrested cells were able to perform multiple rounds of centrosome replication in the absence of either S phase or mitosis. These results support a hypothesis by which the somatic cell nucleus, probably under the direction of cell cycle regulatory molecules, directs cycles of centrosome replication by controlling the activation of centrosome genes at the appropriate times during each cell cycle. These results raise interesting questions regarding both the regulatory mechanisms used by cells to direct centrosome replication and the nature of the checkpoint controls in cells that govern centrosome doubling. This unique experimental system should provide an excellent model for investigating the molecular regulation of centrosome replication in somatic cells.

Materials and Methods

Cell Culture

CHO cells were cultured in McCoy's 5A medium (Mediatech, Inc., Herndon, VA) containing 0.1 mM minimal essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, and 10% FBS. For some studies, the 10% FBS was replaced by 10% dialyzed FBS. Cultures were maintained in a 5% CO_2 environment.

For centrosome replication studies, cells were arrested at the G_1/S boundary of the cell cycle using methods reported previously (Brinkley et al., 1988; Zinkowski et al., 1989; Zinkowski et al., 1991). Confluent cultures briefly were trypsinized and then plated into complete culture medium that contained 2 mM hydroxyurea (HU). After an appropriate incubation, cells were collected and analyzed. For some immunofluorescence studies, HU-arrested cells were induced to enter mitosis by the addition of 5 mM caffeine for 4 h. Rounded mitotic cells were collected, centrifuged onto coverslips, and then processed for immunofluorescence microscopy. Caffeine addition had no effect on the number of centrosomes produced by HU-arrested cells, but centrosomes were better separated in caffeine-treated mitotic cells which made centrosome counting easier. For some experiments, cells were arrested either at the G_1/S boundary of the cell cycle by substituting 5 μg/ml aphidicolin (Nishioka et al., 1984) for HU in the above procedure or in G_2 by the addition of 10 μg/ml etoposide (Ross et al., 1984).

For studies investigating growth factor control of centrosome doubling, cells were cultured as described above in medium containing 10% dialyzed FBS. For individual experiments, dialyzed serum-containing medium was supplemented with either recombinant human insulin (Boehringer Mannheim Biochemicals, Indianapolis, IN) at a final concentration

1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; HU, hydroxyurea; PCM, pericentriolar material.

of 5 μg/ml, recombinant human epidermal growth factor (Upstate Biotechnology, Inc., Lake Placid, NY) at a final concentration of 10 ng/ml, or both growth factors. The cells then were arrested with HU as outlined above and centrosome replication was assayed.

Indirect Immunofluorescence Microscopy

Mitotic cells were collected, rinsed once with PBS, and then centrifuged onto polylysine-coated coverslips. The cells then were fixed by immersion of the coverslips in −20°C MeOH for 6–8 min and processed for immunofluorescence microscopy using previously published methods (Balczon and Brinkley, 1987; Balczon and West, 1991). Anti-α–tubulin (Sigma Immunochemicals, St. Louis, MO) was used at a 1:200 dilution and SPJ autoimmune human anticentrosome serum (Balczon and West, 1991) was used at a 1:1,000 dilution. FITC-labeled secondary antibodies (Boehringer Mannheim Biochemicals) were used at a 1:20 dilution. After processing, coverslips were mounted in PBS: glycerol (1:1) containing 25 μg/ml HOECHST 33258 dye and then the cells were observed using an Axiovert 35 M microscope (Carl Zeiss, Inc., Thornwood, NY). Images were recorded using T-Max 400 film (Eastman Kodak, Inc., Rochester, NY).

The blockage of cells at the G_1/S boundary of the cell cycle by either HU or aphidicolin treatment was verified by immunofluorescence microscopy. Cell cycle stages were determined by pulsing both treated and control cells on coverslips for 15 min with bromodeoxyuridine (BrdU) and then fixing and processing for immunofluorescence microscopy using monoclonal anti-BrdU antibodies (Sigma Immunochemicals, St. Louis, MO) according to procedures that were published previously (Balczon et al., 1994).

Electron Microscopy

HU-treated cells were fixed and processed for transmission electron microscopy using standard methods (Zinkowski et al., 1989, 1991).

Northern Blotting

Cells were synchronized by serum starvation. Cells then were trypsinized and plated into T-175 flasks containing either complete medium or medium supplemented with 2 mM HU. At appropriate time intervals, one flask was rinsed three times with PBS and then frozen at −80°C until use. To collect total cellular RNA, RNA STAT-60 reagent (Tel-Test "B", Inc., Friendswood, TX) was added to each flask (5 ml per flask) to solubilize cells. The cellular homogenates then were collected and processed according to the manufacturer's instructions. RNA was quantitated by measuring the A_{260} of each RNA sample. RNA samples were glyoxylated (20 μg RNA per sample) using previously published methods (Kovacs and Zimmer, 1993) and then the samples were resolved on 1% agarose gels. The RNAs were transferred to a hybridization membrane and prehybridized using methods that have been published previously (Zimmer et al., 1987). Blots were probed with a ^{32}P-labeled cDNA encoding a 1.6-kb fragment of the cDNA encoding the centrosome autoantigen PCM-1 (Balczon et al., 1994). The 1.6-kb cDNA fragment was labeled with [^{32}P]dCTP using the Prime-a-Gene kit (Promega Corp., Madison, WI). After a 12–16-h incubation in the presence of the ^{32}P-labeled PCM-1 cDNA fragment, the blots were rinsed, dried, and autoradiographed using previously published methods (Zimmer et al., 1987). After development, the autoradiographs were quantitated by scanning with a laser densitometer. The blots then were stripped and probed with control cDNAs using the above procedures. Specific cDNAs that were used as controls were the cDNA encoding calmodulin (Van Eldik et al., 1990) and the cDNA encoding histone H2B (Grandy et al., 1982).

For some experiments, cells were cultured in dialyzed serum that was supplemented with either insulin or EGF as detailed previously. Cells were collected, and PCM-1 mRNA levels were determined using the methods that were outlined in the previous paragraph.

Immunoblot Analysis

Immunoblotting was performed using methods that have been detailed previously (Balczon et al., 1994). Primary antibodies that were used for immunoblotting were either a rabbit polyclonal anti-PCM-1 antibody (Balczon et al., 1994) or sheep anti–human EGF receptor antibody (Upstate Biotechnology, Inc.). Secondary antibodies (Boehringer Mannheim Biochemicals) were either peroxidase-labeled anti–rabbit IgG or peroxidase-labeled anti–sheep IgG.

Results

Studies were performed to investigate the strategies used by somatic cells to regulate centrosome replication. The first experiments performed involved Northern blot analysis to determine the levels of the mRNA encoding the centrosome autoantigen PCM-1 during various stages of the cell cycle. Kuriyama and Borisy (1981) had demonstrated that enucleated cells were unable to replicate centrosomes, and those investigators speculated that synthesis of nuclear transcripts would be required during each cell cycle for centrosome doubling events. Initial Northern blot studies supported the conclusions of Kuriyama and Borisy (1981). For these studies, CHO cells were arrested in early G_1 by serum starvation and then fed complete medium. Cells were collected at 1-h intervals, total RNA was isolated from the cells, the RNAs were resolved on agarose gels, and then the separated RNAs were transferred to a hybridization membrane. The membranes then were probed with a ^{32}P-labeled PCM-1 cDNA fragment. In addition, a parallel coverslip of CHO cells was pulsed with BrdU and then processed for anti-BrdU immunofluorescence to determine the approximate duration of each cell cycle stage. The PCM-1 protein was encoded by an mRNA of ~7.6 kb (Fig. 1), although on longer exposures a second hybridizing transcript could be detected at approximately 9.5 kb (not shown). PCM-1 mRNA could not be detected in serum-starved cells (Fig. 1). However, within 1 h after serum addition the PCM-1 mRNA could be detected in cells, and the mRNA levels peaked within 2 h. PCM-1 mRNA levels remained high through G_1 and S phases before plunging to nearly undetectable levels during G_2 and M phases. The level of PCM-1 mRNA then increased again as cells reentered G_1 phase. A similar pattern of synthesis and degradation was observed in three separate experiments. Attempts to measure PCM-1 mRNA levels for additional cell cycles were difficult due to increasing asynchrony of cell cycle progression in CHO cells. However, the complete degradation of PCM-1 mRNA during the cell cycle demonstrates that at least some of the centrosome mRNAs must be synthesized each cell cycle for centrosome replication to occur in somatic cells. Control blots measuring calmodulin mRNA levels during the cell cycle demonstrated that calmodulin mRNAs followed a similar cycle of synthesis and degradation as the pattern shown by PCM-1 (not shown). As demonstrated by others (Chafouleas et al., 1984), calmodulin mRNA levels increased through G_1 and S phases before peaking late in S phase, and then decreased during G_2 and M phases. The only detectable difference in the patterns of PCM-1 and calmodulin mRNA levels was that the calmodulin mRNA was still detectable in serum-starved cells (not shown). As expected, the levels of histone H2B (Stein et al., 1975) were low in G_1 cells but elevated in S phase cells before decreasing in G_2 and M phases (not shown).

The demonstration of a cyclical synthesis and degradation of PCM-1 mRNA during the cell cycle provided an opportunity for investigating why centrosomes are only replicated once during each cell cycle. Two hypotheses were considered as potential explanations for the tightly regulated centrosome replication that occurs during the somatic cell cycle, and these hypotheses were: (1) cen-

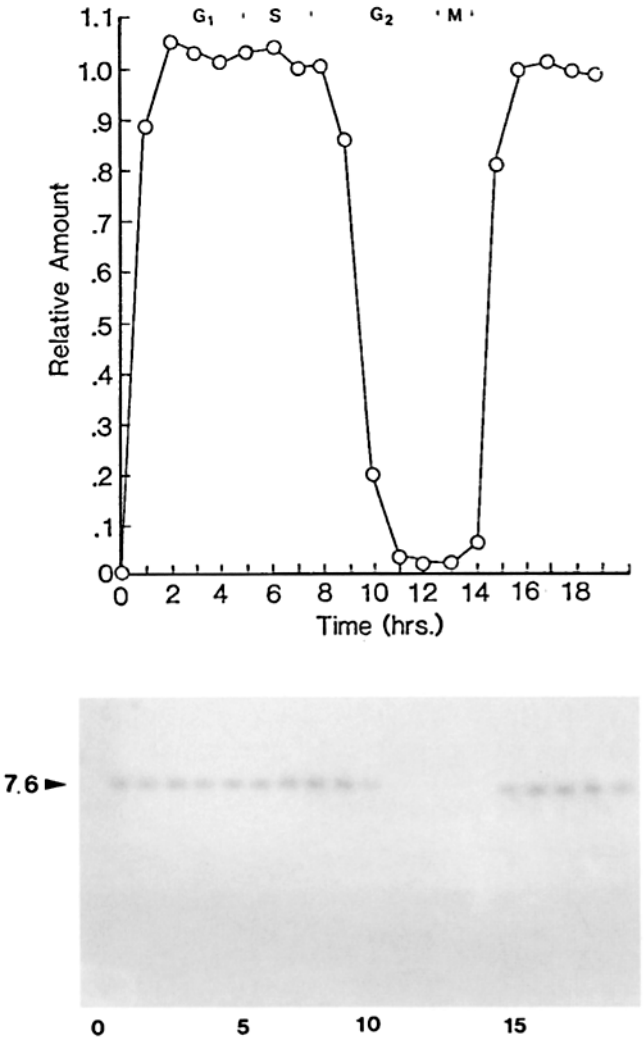

Figure 1. Northern blot analysis of centrosome mRNA levels in cultured CHO cells. CHO cells were arrested in a G_0-like state by serum starvation. Cells were collected at 1-h intervals after feeding with complete medium, total RNA was isolated, the RNA was separated on an agarose gel and then transferred to nylon membrane. The membrane was then prehybridized followed by probing with a 1.6-kb ^{32}P-labeled cDNA fragment encoding portion of the centrosome autoantigen PCM-1. After rinsing, the blot was exposed to x-ray film and the autoradiograph then was scanned using a videodensitometer. The upper portion of this figure shows the densitometric scan and the lower portion shows the actual Northern blot data (time given in hours after serum addition). A corresponding coverslip was incubated with BrdU and then processed for anti-BrdU immunofluorescence to determine the approximate duration of the cell cycle stages (not shown). Cell cycle stages are shown above the graph. A single mRNA at ~7.6 kb can be observed (*arrow*) at this exposure.

trosome replication is limited to one duplication event per cell cycle because of an endogenous counting mechanism that limits centrosome overproduction, and (2) centrosome doubling is controlled by the activation and inactivation of the centrosome replicating machinery at the appropriate times during the cell cycle. If the first hypothesis is correct, then it should not be possible to induce multiple centrosomes in cells; however, if the latter hypothesis is correct then it should be possible to induce the overproduction of centrosomes either by hyperstimulating the centrosome replicating machinery or by arresting cells at a point in the cell cycle when centrosome production is occurring. Various investigators have been able to induce the overproduction of centrosomes in embryonic cells (Sluder et al., 1986; Sluder and Rieder, 1985; Raff et al., 1988), and similar experimental strategies were used in an effort to induce multiple rounds of centrosome replication in CHO cells. For these studies, cultured CHO cells were arrested for extended periods at the G_1/S boundary of the cell cycle by the addition of HU (Brinkley et al., 1988; Zinkowski et al., 1989) and then the cells were processed for immunofluorescence microscopy using SPJ anticentrosome serum. Although centrosome replication could be detected in the HU-arrested cells (Fig. 2 *A*), initial attempts to count centrosomes gave ambiguous results because the centrosomes in the G_1/S-arrested cells were closely packed and it was not possible to make accurate counts using immunofluorescence microscopy. To overcome this problem, HU-treated cells were induced to enter mitosis by the addition of caffeine to a final concentration of 5 mM (Schlegel and Pardee, 1986; Brinkley et al., 1988; Zinkowski et al., 1989). Caffeine drives cells into mitosis, and in mitotic cells centrosomes separate allowing easy counting of spindle poles. Fig. 2 shows results that were obtained when CHO cells were arrested in the presence of HU for increasing lengths of time before processing for immunofluorescence microscopy. Specifically, cells were treated with HU for either 20, 40, or 60 h before being driven into mitosis by the addition of caffeine. The mitotic cells then were collected, centrifuged onto coverslips, and then processed for immunofluorescence microscopy using SPJ serum. As Fig. 2 demonstrates, treatment with HU for progressively longer periods resulted in the generation of increasing numbers of centrosomes in arrested cells. Direct centrosome counts (Fig. 3) demonstrated that cells arrested for either 20, 40, or 60 h in the presence of HU contained 2.45 ± 0.62, 4.16 ± 1.02, and 7.02 ± 1.88 SPJ-reactive foci, respectively. In addition, note that treatment with caffeine induced chromatin fragmentation as reported previously (Brinkley et al., 1988; Zinkowski et al., 1989), and fragmentation of chromatin served as a convenient morphological marker for caffeine-treated cells.

Electron microscopy was performed to verify that multiple rounds of centrosome replication were occurring in HU-arrested cells in the absence of cycles of DNA synthesis and mitotic division. Conceivably, the prolonged treatment of cells with HU may have caused physical disruption of centrosomes in arrested cells which would have been detected as multiple SPJ-reactive foci by immunofluorescence staining. To test this, cells were arrested with HU and then processed for transmission electron microscopy. Fig. 4 shows representative electron micrographs of sections through HU-arrested cells. Multiple intact, mature centriole cylinders were detected supporting the conclusion that centrosome replication, and not fragmentation, was indeed occurring during the HU arrest. Additionally, it should be pointed out that the cells shown in Fig. 4 had only been treated with HU and had not been driven into mitosis by caffeine addition. This reinforces the earlier statement that caffeine does not contribute to

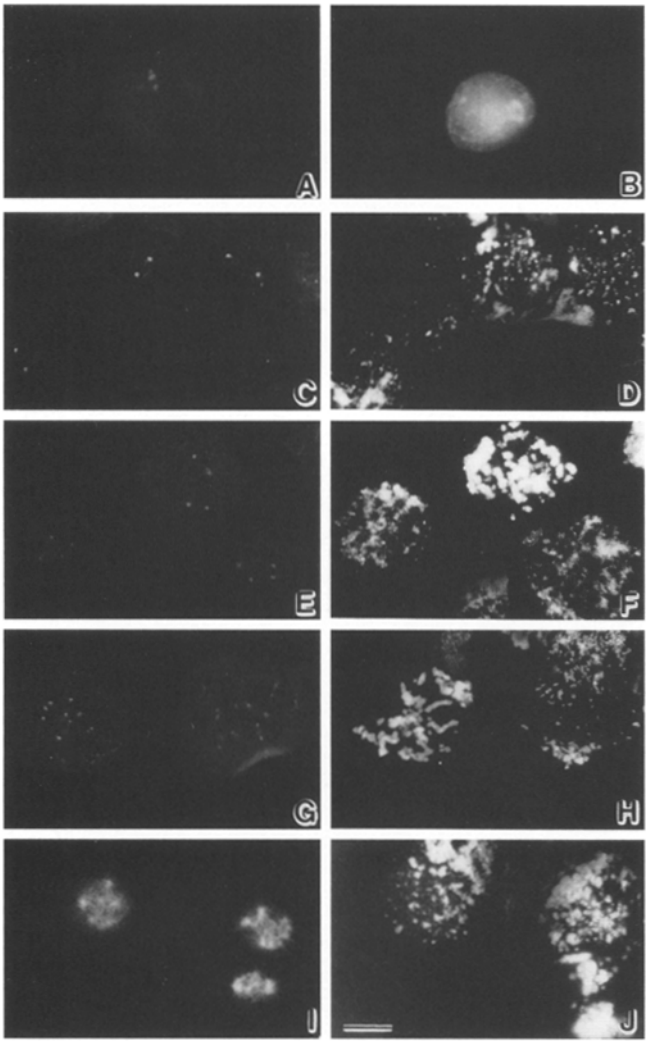

Figure 2. Immunofluorescent staining of HU-arrested CHO cells using SPJ anticentrosome serum. (*A*) A micrograph of a CHO cell that was arrested for 40 h with HU. Note that several closely spaced centrosomes can be observed. (*B*) The corresponding HOECHST-stained cell. *C–H* are micrographs of cells that were arrested with HU for either 20 (*C* and *D*), 40 (*E* and *F*), or 60 h (*G* and *H*) before the addition of caffeine. Caffeine drives cells into mitosis which causes spindle poles to separate allowing easier counting of centrosomes. *C*, *E*, and *G* are SPJ-stained cells, and *D*, *F*, and *H* are the corresponding HOECHST-stained DNA images. Increasing numbers of centrosomes were detected in cells that were arrested for longer periods with HU. *I* shows antitubulin immunofluorescence staining of cells that were treated exactly as the cells shown in *G*, and *J* is the corresponding HOECHST-stained field of *I*. Note the elaborate multipolar spindles that were formed. Bar, 10 μm.

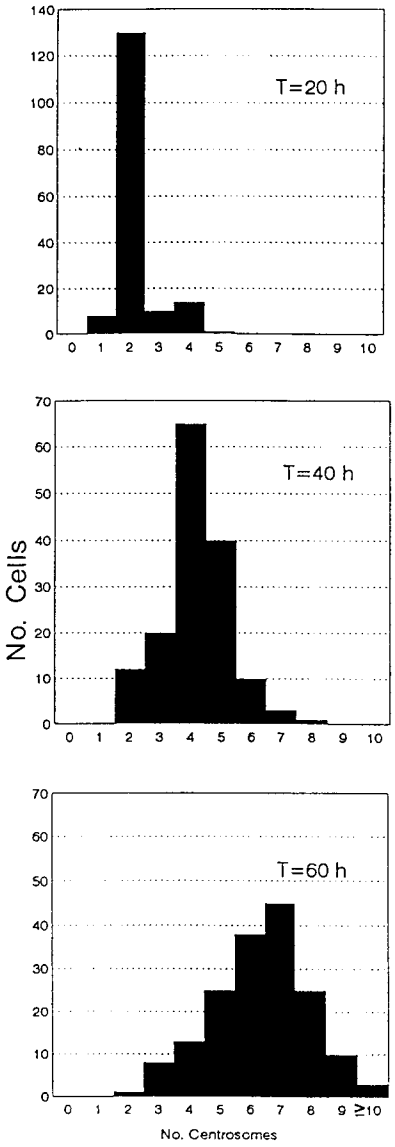

Figure 3. A graph showing the actual number of centrosomes that were counted in cells after incubation in HU for varying lengths of time. (*Upper panel*) Cells that were arrested for 20 h. (*Middle panel*) Cells that were blocked for 40 h. (*Lower panel*) Cells that were treated with HU for 60 h before caffeine addition. The cells then were stained with SPJ antiserum and centrosomes were counted. Between 150 and 160 cells were scored for each time point.

the centrosome doubling events; caffeine only drives cells into mitosis which induces centrosome separation allowing accurate counting of centrosomes by immunofluorescence microscopy.

The results summarized in Figs. 2–4 suggest that our first hypothesis—that either an endogenous counting mechanism or a re-replication block exists that limits centrosome replication to one round of doubling per cell cycle—is incorrect. However, it is conceivable that an endogenous counting mechanism does exist, and that HU is able to inactivate this blocking mechanism. To test this possibility, the centrosome replication experiments were repeated using aphidicolin. Like HU, aphidicolin arrests cells at the G_1/S boundary of the cell cycle, but the two drugs work through completely different molecular mechanisms. Specifically, HU acts by inhibiting the enzyme ribonucleotide reductase while aphidicolin inhibits DNA polymerase (Ikegami et al., 1978; Ikegami et al., 1979; Nishioka et al., 1984). When cells were arrested for extended periods with aphidicolin, results that were identical (not shown) to the results that were shown previously for HU were obtained. Together, these results strongly argue against the hypothe-

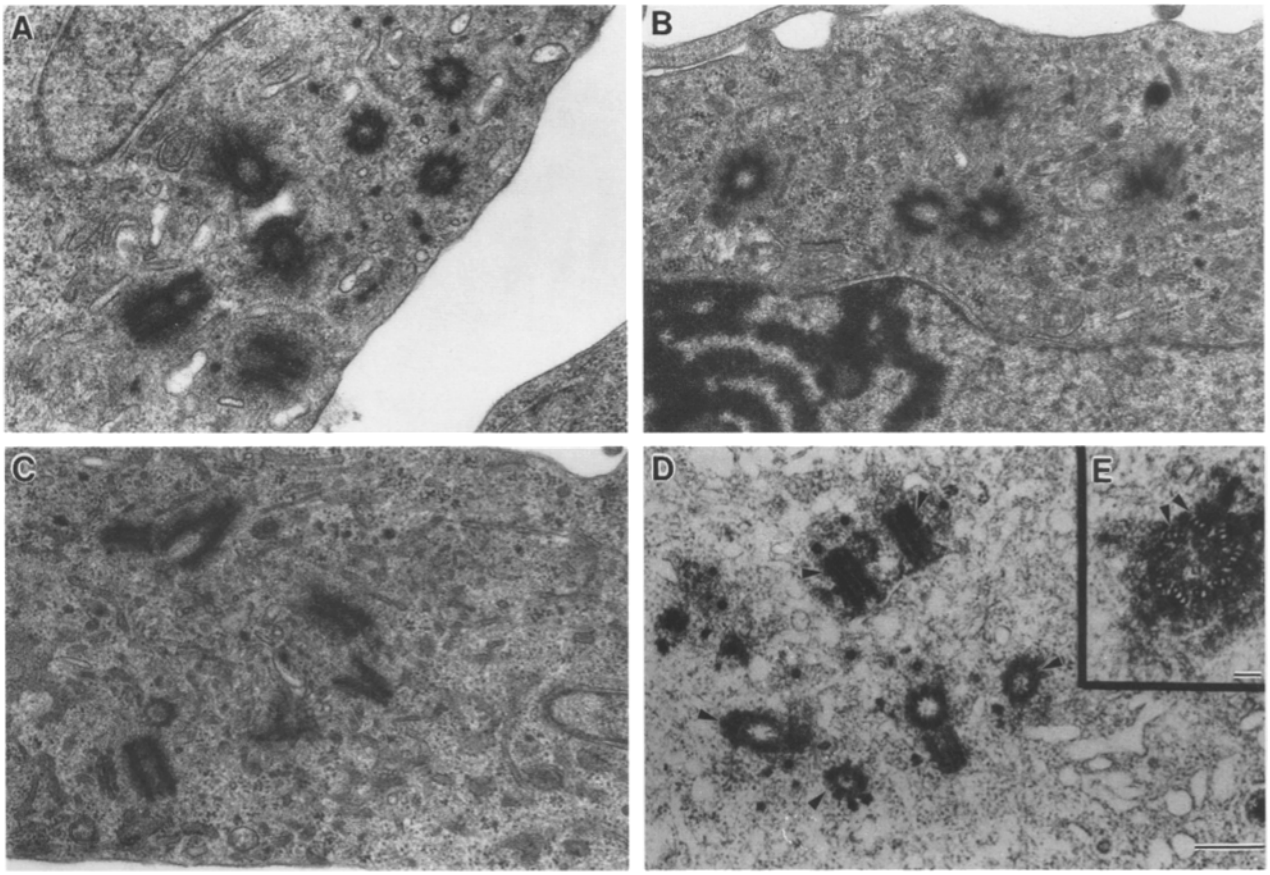

Figure 4. Thin section transmission electron microscopy analysis was performed to determine whether centrosome replication or fragmentation occurred in CHO cells during HU treatment. These sections are from cells that had been arrested for 60 h. (*A–D*) Micrographs showing sections through numerous intact, mature centrosomes in HU-treated CHO cells. Several centrioles can be observed in each field. (*E*) The inset shows a higher power micrograph of a centriole cylinder demonstrating the ultrastructural organization of the nine triplet microtubules (*arrows*) and amorphous pericentriolar material. Bars, (*A–D*) 250 nm; (*E*) 125 nm.

sis that a re-replication block or endogenous counting mechanism exists to limit the number of centrosomes that are produced each cell cycle in somatic mammalian cells.

Additional experiments were performed to investigate the hypothesis that centrosome replication occurs due to the precise activation and inactivation of the centrosome replicating machinery at the appropriate times during the cell cycle. One set of studies involved Northern blot analysis of RNA obtained from HU-arrested cells. From the data shown in Fig. 1, two possible explanations can be made for the multiple rounds of centrosome replication that were observed in HU-arrested CHO cells. In one scenario, the cyclical patterns of synthesis and degradation of centrosomal mRNA could be occurring in the absence of cycles of DNA synthesis and mitotic division in HU-arrested cells. The other possibility is that arrest by HU (and aphidicolin) blocks cells at a point in the cell cycle (G_1/S boundary) when centrosomal mRNA levels normally are elevated allowing excess centrosomal protein to be produced. To test these possibilities, Northern blotting was performed on RNA isolated from HU-arrested cells. As Fig. 5 *A* shows, PCM-1 mRNA levels did not cycle during prolonged HU arrest; instead, the levels of PCM-1 mRNA remained elevated during the arrest period supporting the latter possibility. Control blots of histone H2B and calmodulin mRNA levels in HU-arrested cells (not shown) demonstrated that histone H2B mRNA was not produced in the HU-arrested cells while calmodulin mRNA levels rapidly increased in a manner similar to that shown for PCM-1 mRNA. However, unlike PCM-1 mRNA, calmodulin mRNA levels decreased by ~30% during the prolonged HU arrest period (not shown).

To verify that the presence of PCM-1 mRNA results in the overproduction of PCM-1 protein, cells that were arrested for either 20 or 60 h in HU were collected and processed for immunoblot analysis. As shown in Fig. 5 *B*, additional PCM-1 protein was detected on blots of 60-h treated cells when compared with 20-h HU-arrested cells. Direct densitometric analysis of the blots determined that the 60-h treated cells contained ~50% more PCM-1 centrosome protein as cells that were arrested for only 20 h. From Fig. 3, one would have expected a 2.5–3.0-fold increase in the amount of PCM-1 protein in cells that were arrested for 60 h in HU if centrosomes were the only cellular components that were being produced during the G_1/S arrest period. A possible explanation for the fact that only 50% more PCM-1 protein was detected in cells that were arrested for 60 h is that numerous cellular proteins, in addition to centrosome proteins, were being produced during the prolonged G_1/S arrest.

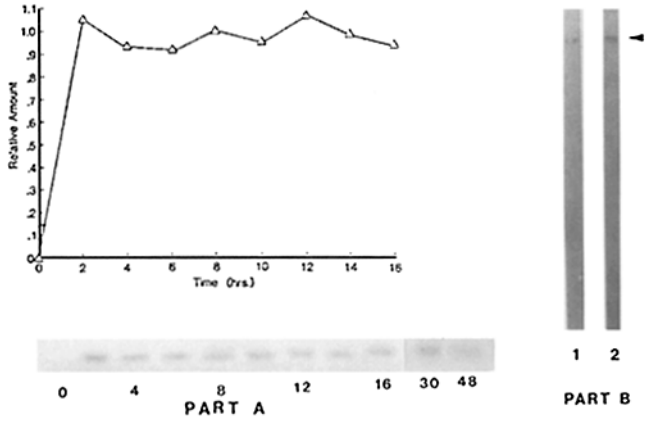

Figure 5. (*A*) Cells were processed exactly as described for Fig. 1, except that cells were plated into medium containing 2 mM HU. One flask of cells was collected at the times shown and the presence of PCM-1 mRNA was assayed by Northern blotting. Recall that the time required for the completion of the first cell cycle under these conditions was 14 h in control CHO cells (Fig. 1). The upper portion of this figure shows the densitometric scan of the Northern blot while the lower portion shows the actual Northern blot data (time given in hours after serum addition). (*B*) Immunoblot analysis of cells that had been arrested for either 20 (lane *1*) or 60 h (lane *2*) with HU. Each gel lane was loaded with 100 μg of protein. After SDS-PAGE and transfer, the blot was probed with antibody against PCM-1. A band corresponding to the 228-kD PCM-1 protein (*arrow*) was detected in each sample.

A final test of the hypothesis that centrosome replication occurs due to the accurate activation and inactivation of the centrosome replicating machinery at the appropriate times during the cell cycle involved arresting cells at a point in the cell cycle when centrosome mRNA levels would be low. From Fig. 1, it can be observed that PCM-1 mRNA levels are nondetectable in late G_2 and M phases of the cell cycle. Therefore, cells were arrested in mid G_2 of the cell cycle using the topoisomerase inhibitor etoposide (Ross et al., 1984). The arrested cells were maintained in etoposide for 50 h before being driven into mitosis by caffeine addition. When etoposide-treated cells were observed after processing for immunofluorescence microscopy, only bipolar spindles were observed (Fig. 6). This result demonstrates that it is not necessarily the prolonged arrest of cultured cells that induces the overproduction of centrosomes in somatic cells, but that the arrest must occur during stages when centrosome replicating machinery is activated for centrosome overproduction to occur. This result further supports the hypothesis that centrosome replication in somatic cells occurs due to the activation and inactivation of the centrosome replicating machinery at the appropriate times during the cell cycle.

A final set of studies was performed to begin to unravel the mechanism used by CHO cells to activate centrosome genes at the appropriate point during the cell cycle. For these experiments, CHO cells were arrested with HU in medium that contained dialyzed FBS. Under these conditions, centrosome replication would not occur (Fig. 7). However, when dialysate was concentrated by lyophilization and added to cells arrested with HU in medium containing dialyzed serum, centrosome replication could be reinitiated (Fig. 7). Moreover, treatment of the dialysate with proteases abolished the capacity of the dialysate to restore centrosome replicating capacity to cells arrested at G_1 (not shown). Together, these results suggest that a low molecular weight protease-sensitive component, such as a growth factor, is responsible for triggering centrosome replication processes. Moreover, the dialysis membrane used for these studies had a molecular mass cut-off of 8 kD suggesting that the regulatory factor involved was smaller than 8 kD.

Various known growth factors have molecular masses below 8 kD (Jenkins, 1991), and studies were performed to identify the growth factor(s) that supplied the signal that drove centrosome replication in CHO cells. As CHO cells have been cultured successfully in defined medium (Hamilton and Ham, 1977; Ham and McKeehan, 1979; Jenkins, 1991), the growth factors responsible for the proliferation of this cell line in vitro are known. The rationale that was used was that if CHO cells can proliferate in minimal medium, then the growth factors in the defined medium must be providing the signal that turns on all cell cycle progression genes, including centrosome genes. It has been reported that CHO cells can be maintained in a defined growth medium that contains insulin ($M_r \approx 5.7$ kD) as the only required growth factor (Hamilton and Ham, 1977; Ham and McKeehan, 1979; Jenkins, 1991), and it was reasoned that either insulin or an insulin-like growth factor would be providing the signal that activates centrosome genes. To test this, recombinant insulin (5 μg/ml) was added to medium containing dialyzed serum and HU. After 40 h, the cells were treated with caffeine and then the cells were collected and processed for immunofluorescence microscopy using SPJ antiserum. As Fig. 8 (Part 1) demonstrates, the addition of exogenous insulin did not reinitiate centrosome replication in treated cells. Moreover, Northern blot analysis determined that insulin did not significantly increase levels of the PCM-1 mRNA in cells (Fig. 8, Part 2). Therefore, other low M_r growth factors were added to the dialyzed serum in an effort to restore centrosome replicating capacity to HU-arrested cells maintained in dialyzed serum. From these studies, it was determined that EGF alone (10 ng/ml) was capable of driving multiple rounds of centrosome replication in cultured CHO cells (Fig. 8, Part 1). In addition, Northern blotting determined that synthesis of PCM-1 mRNA was initiated in CHO cells that were treated with EGF (Fig. 8, Part 2). No stimulation of either centrosome replication or PCM-1 mRNA levels above those initiated by EGF alone was detected when cells were treated with both EGF and insulin suggesting that centrosome replication in CHO cells arrested with HU occurs principally due to the activity of EGF. Finally, because CHO cells can be cultured exclusively in a defined medium that lacks EGF, immunoblot analysis was performed using anti-EGF receptor antibody to verify that CHO cells express receptors for EGF. When whole CHO cell extracts were probed using anti-EGF receptor antibodies, a band corresponding to the 170-kD EGF receptor (Carpenter, 1987) was detected (not shown) suggesting that the effects of EGF on centrosome replication in CHO cells are due to stimulation of EGF receptors.

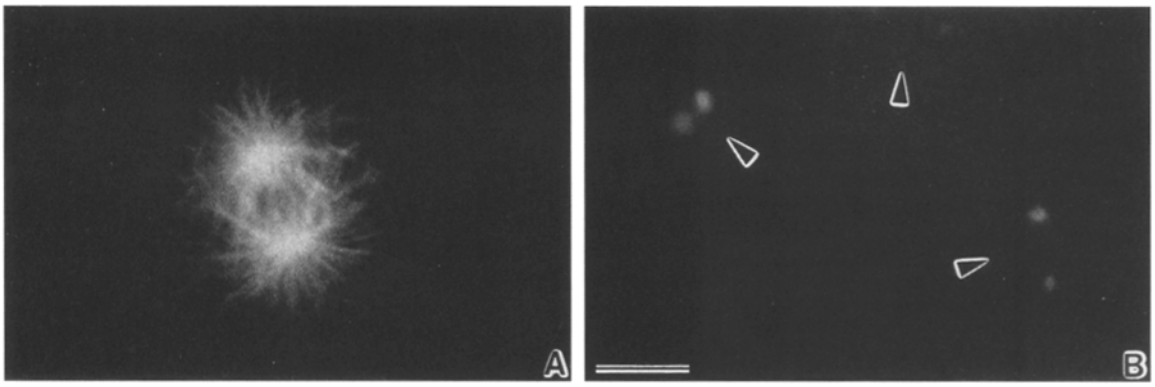

Figure 6. Immunofluorescence staining of CHO cells after a 50-h arrest in the presence of the drug etoposide (VP-16). After the etoposide arrest, cells were treated with caffeine and mitotic cells were collected and processed for immunofluorescence microscopy. (*A*) Antitubulin staining of an etoposide-treated cell showing a bipolar spindle. (*B*) Staining with SPJ serum demonstrating that only two centrosomes were present in each cell. Three cells (*arrows*) can be seen in this field. Bar, 10 μm.

Discussion

Relatively little is known about the mechanisms used by somatic cells to regulate centrosome replication. For cell division to occur properly, the centrosome must be duplicated once, and only once, during each cell cycle. Failure of centrosome replication to occur properly would result either in cell cycle arrest before the onset of mitosis or in the formation of an aberrant monopolar or multipolar spindle (Rose et al., 1993). In this article, experiments are described that investigate whether an endogenous count-

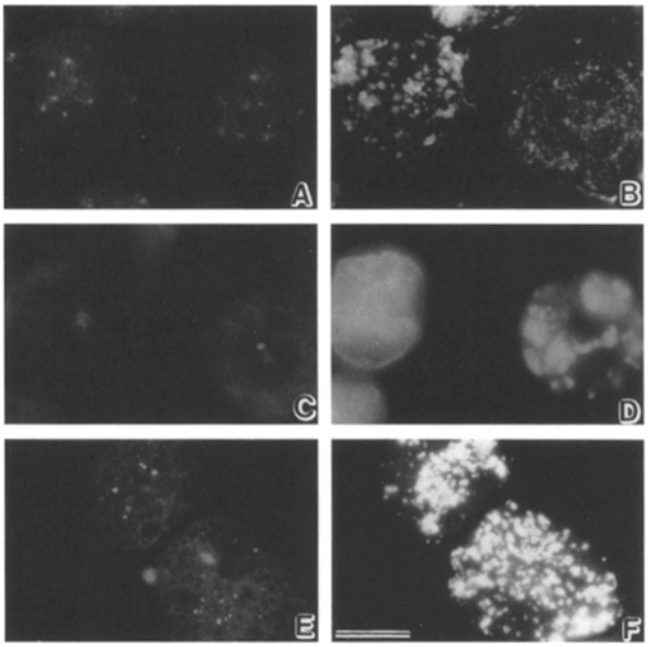

Figure 7. Immunofluorescence staining of HU-arrested CHO cells with SPJ serum after various experimental treatments. *A*, *C*, and *E* are SPJ-stained cells and *B*, *D*, and *F* are the corresponding HOECHST-stained fields of cells. (*A* and *B*) Control cells that were arrested for 60 h in complete medium. (*C* and *D*) Cells that were arrested for the same length of time in medium containing dialyzed serum. Centrosome replication did not occur. Also, note that in dialyzed serum most of the cells could not be driven into mitosis by caffeine addition as demonstrated by the presence of intact nuclei. (*E* and *F*) Cells that were arrested in dialyzed serum-containing medium that was supplemented with dialysate. Centrosome replication was reinitiated. Bar, 10 μm.

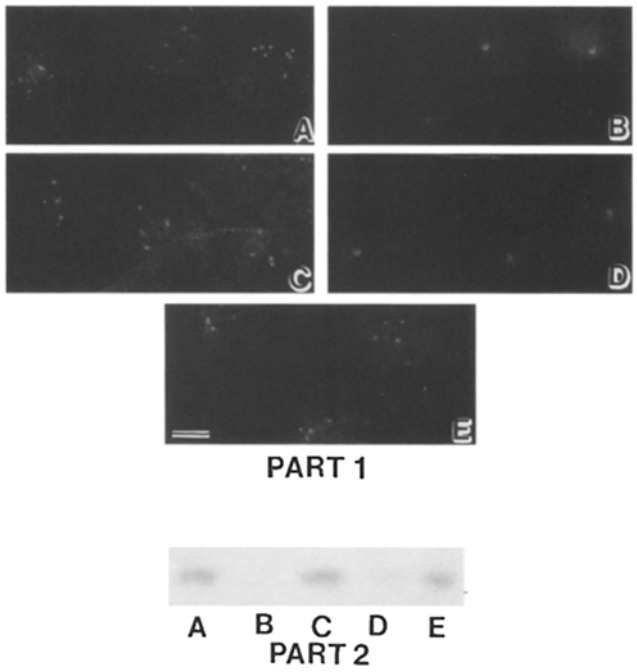

Figure 8. (*Part 1*) Immunofluorescence staining of CHO cells with SPJ serum. Cells were arrested with HU for 48 h in either complete medium (*A*), medium supplemented with dialyzed serum (*B*), or dialyzed serum-containing medium that was supplemented with either EGF (*C*), insulin (*D*), or both (*E*). Cells treated with EGF were able to reinitiate centrosome replication (*C*) while cells treated with insulin (*D*) were not. Bar, 10 μm. (*Part 2*) Northern blot analysis using ^{32}P-labeled PCM-1 cDNA probe. Cells were treated exactly as shown in part 1 and then the cells were collected. Total RNA was isolated from each population of cells, the RNAs were resolved on agarose gels, and then the resolved RNAs were probed with labeled PCM-1 cDNA. Lanes *A–E* correspond to panels *A–E* in part 1. EGF-treated cells contained levels of PCM-1 mRNA that were approximately equal to the levels in control HU-arrested cells.

ing mechanism exists in somatic cells to limit centrosome replication to one round of centrosome doubling per cell cycle or whether centrosome replication is controlled by simply activating and subsequently inactivating the centrosome replicating machinery at the appropriate time during the cell cycle. For these studies, an experimental system was developed that allowed the dissociation of centrosome replication from cycles of DNA synthesis and mitotic division. As demonstrated here, cells arrested at the G_1/S boundary of the cell cycle underwent multiple rounds of centrosome replication in the complete absence of either DNA synthesis or cell division. This conclusion is supported both by immunofluorescence microscopy using human anticentrosome antiserum and by serial section electron microscopy. No evidence of either an endogenous counting mechanism or a re-replication block limiting centrosome replication was obtained (although this possibility can not be discounted totally; see below), and the results reported here support the hypothesis that centrosome replication in somatic cells is controlled by the regulated activation and inactivation of the centrosome replicating machinery during the cell cycle. This has been demonstrated by arresting CHO cells with drugs that block cells at points when centrosome mRNA levels are either elevated or low and then processing the cells for immunofluorescence microscopy to determine the number of centrosomes present in the treated cells.

Sluder and co-workers (Sluder et al., 1986, 1990; Sluder and Lewis, 1987) have demonstrated that the replication of centrosomes in embryonic cells is completely under cytoplasmic control with no contribution at all from the nucleus. However, our demonstration that PCM-1 mRNA must be synthesized each cell cycle, as well as work from Kuriyama and co-workers (1981, 1986) and Maniotis and Schliwa (1991) demonstrating that centrosome assembly would not occur in enucleated somatic cells, argues for a role for the nucleus in centrosome assembly in somatic cells. Although the results obtained using embryonic and somatic cells differed, the basic ideas of Sluder and coworkers can be applied to somatic cells if one assumes that cytoplasmic molecules may be controlling the activity of centrosome genes thereby regulating centrosome replication processes. The data presented here are consistent with a model in which a cytoplasmic signal is generated in CHO cells due to the influence of EGF and then maintained in an active state during the HU arrest period. Such an active signal conceivably could play a role in driving the synthesis of the centrosome components required for the multiple rounds of centrosome production that were detected in these studies by regulating the activity of factors responsible for either the synthesis or degradation of centrosome mRNA and protein. In this manner, one can incorporate the essential nature of the nucleus for centrosome replication in somatic cells to the observations made using embryonic cells that the cytoplasm controls centrosome replication. Clearly, our understanding of the integration of centrosome duplication with nuclear and cell cycle progression processes is limited. The experimental system described here provides a promising approach for investigating how stimulatory and inhibitory growth signals are transmitted from the cell surface to the nucleus to regulate the activity of genes whose transcription is essential for centrosome replication and how these events are coordinated with other cell cycle processes.

The results presented here are inconsistent with the hypothesis that either an endogenous counting mechanism or a re-replication block exists within cells to limit the number of centrosome doublings to a single round of replication during each cell cycle. However, the idea of a centrosome re-replication block can not be discounted completely. Although the data as presented are more consistent with a model in which centrosome replication occurs as a result of the simple activation and inactivation of centrosome replicating machinery at the appropriate times during the cell cycle, the timing of centrosome replication in HU-arrested cells lagged considerably behind the cell cycle time of control CHO cells. The duration of the cell cycle in the CHO cells used for these studies was 11–12 h. As shown in Fig. 3, cells that were arrested for 40 h in HU contained 4.16 ± 1.02 centrosomes while cells arrested with HU for 60 h contained 7.02 ± 1.88 centrosomes. As the cells that were arrested for 40 h were arrested for a period that covered over three cell cycles for CHO cells, those cells should have contained ~8 centrosomes. Likewise, cells arrested for 60 h were blocked for a period that corresponded to five cell cycles, and those cells should have contained as many as 32 centrosomes if centrosomes were doubled every 11–12 h. Similar observations have been made by Sluder and Lewis (1987) who observed centrosome doubling in fertilized eggs only when the eggs were treated for extended periods in the presence of aphidicolin. A simple explanation for the difference in centrosome number observed versus the number that might have been expected based on the duration of the cell cycle is that not all of the components of the centrosome replicating machinery are maximally activated at the G_1/S boundary of the cell cycle. Conceivably, other cellular factors involved in centrosome doubling and maturation may be maximally activated later during S phase. However, a second possible explanation is that a re-replication block does exist within cells to inhibit multiple rounds of centrosome replication in cells. If this putative block does exist, it is conceivable that it has a finite half-life. If such a counting mechanism is present, this could explain the difference in the number of centrosomes that were actually detected versus the number that might have been expected. According to this scheme, centrosomes would be replicated but additional rounds of centrosome doubling would be delayed until the re-replication block degenerates or is inactivated, perhaps by protein degradation. Then an additional round of centrosome replication would ensue. Therefore, although the data obtained were consistent with the hypothesis that centrosome doubling occurs due to the cell cycle stage–specific activation of centrosome replicating machinery, the possibility that an endogenous counting mechanism exists can not be discounted completely.

The finding that EGF triggered PCM-1 synthesis and replication of centrosomes in CHO cells was unexpected (Fig. 8). CHO cells have been cultured in a defined medium that contained insulin as the only growth factor (Hamilton and Ham, 1977; Ham and McKeehan, 1979; Jenkins, 1991). Therefore, it was reasoned that insulin would induce centrosome doubling in CHO cells. How-

ever, insulin was unable to drive cycles of centrosome replication in HU-arrested CHO cells. The reason for the discrepancy between the results reported here and those reported for the growth of CHO cells in minimal defined medium is not clear. One possible explanation is that different sublines of CHO cells have separate growth factor requirements. Another possibility is that CHO cells undergo some sort of adaptation or change in growth factor requirement during the transition into defined medium (Jenkins, 1991). Cells are slowly weaned from serum-containing to serum-free medium during the transfer to a defined medium, and it is conceivable that CHO cells undergo biochemical changes during the transition period. Alternatively, it is possible that a small subpopulation of CHO cells is selected for that exhibits insulin-dependent growth during the lengthy transition from serum-containing to serum-free medium.

The results presented here suggest that EGF plays a key role in activating centrosome doubling in CHO cells. It is worth mentioning that an effect of EGF on centrosomes has been reported previously. Sherline and Mascardo (1982*a*, *b*; 1984) reported that EGF induced the rapid separation of centrosomes in cultured HeLa cells. As it has been reported that centriole separation is the first detectable morphological event in the doubling of centrosomes (Brinkley, 1985; Vandre and Borisy, 1989), it is conceivable that Sherline and Mascardo actually detected early stages of centrosome replication in their studies. The significance of EGF regulation of centrosome activity in CHO cells is not clear at this time. However, the fact that this single growth factor can induce the formation of multiple functional centrosomes suggests that all of the genes encoding centrosome proteins may be regulated in a similar manner. Experiments are in progress to determine how positive and negative signals regulate centrosome doubling.

In summary, the strategies used by somatic cells to coordinate centrosome replication with other cell cycle progression events have been investigated. No evidence of an endogenous counting mechanism or re-replication block limiting centrosome doubling to one duplication event per cell cycle could be detected. Instead, the data support a hypothesis that centrosome replication in somatic cells is controlled by the activation and subsequent inactivation of the centrosome replicating machinery at the appropriate times during the cell cycle.

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