Centriole Cycle in Chinese Hamster Ovary Cells as Determined by Whole-mount Electron Microscopy

RYOKO KURIYAMA and GARY G. BORISY
Laboratory of Molecular Biology and Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT In interphase Chinese hamster ovary (CHO) cells, the centrosome is attached to the nucleus very firmly. This nuclear-centrosome complex is isolated as a coherent structure by lysis and extraction of cells with Triton X-100 in a low ionic strength medium. Under these conditions, the ultrastructure of the centrioles attached to the nucleus can be discerned by electron microscopy of whole-mount preparations. The structural changes of the centrioles as a function of the cell cycle were monitored by this technique. Specifically, centriolar profiles were placed into six categories according to their orientation and the length ratio of daughter and parent centrioles. The proportion of centrioles in each category was plotted as a frequency histogram. The morphological changes in the centriole cycle were characterized by three distinguishable events: nucleation, elongation, and disorientation. The progress of centrioles through these stages was determined in synchronous populations of cells starting from S or M phase, in cells inhibited in DNA synthesis by addition of thymidine, and in cytoplasts. The results provide a quantitative description of the events of the centriole cycle. They also show that, in complete cells, nucleation, elongation, and disorientation are not dependent upon DNA synthesis. However, in cytoplasts, although elongation and disorientation occur as in normal cells, nucleation is blocked. Procentriole formation appeared to be inhibited by the removal of the nucleus. We suggest that coordination of centriole replication and nuclear replication may depend upon a signal arising from the nucleus.

The centrosome consists of a pair of centrioles and pericentriolar material and serves as a mitotic center in animal cells (21). The reproduction of the mitotic centers is coordinated with other events in the cell cycle and has been dissected functionally into distinct phases of duplication and splitting or separation (12).

With the use of synchronized tissue culture cells and electron microscopy of thin sections, the centriole cycle has been described qualitatively in structural terms (17, 19). Daughter cells formed by a cell division each receive a pair of orthogonally oriented centrioles. The two centrioles become disoriented and lose their orthogonal arrangement in G_1 phase. During late G_1 or S phase, a short daughter centriole, the procentriole, appears at the proximal end oriented orthogonally with respect to the parent. Formation of the procentriole may be considered a nucleation event. The procentriole elongates slowly through S and G_2 phase and attains almost full size at prophase when the two centriolar pairs separate and begin to migrate towards opposite ends of the nucleus. The daughter centriole generally does not attain its full length until early G_1 of the next cell cycle. A pair of orthogonally oriented centrioles is positioned at each spindle pole and is segregated to each daughter cell by mitosis, after which a new centriole cycle is begun. Although different cell types may show slight variations in detail, the fundamental process of the centriole cycle appears to be as described above.

This picture of the centriole cycle is derived largely from electron microscopy of thin sections. A particularly detailed description is that of Rattner and Phillips (16) who investigated the relationship between centriole replication and DNA synthesis. However, with this technique, the precise length and configuration of the centrioles during the cell cycle have to be determined by examining serial sections of individual centrioles. This is obviously a time-consuming procedure that hinders the collection of sufficient data to give more than a qualitative account.

Throughout most of the cell cycle, the nucleus is intact and the centrioles occupy a position adjacent to it. This observation has been confirmed by many techniques including electron (13) and immunofluorescence microscopy (4). The possibility

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 DECEMBER 1981 814-821

that this neighbor relationship arises from a physical connection between the centriole and the nuclear envelope has been suggested by recent studies of Bornens (3) and Nadezhina et al. (14) who have found centrioles in preparations of nuclei isolated from rat liver by homogenization and differential centrifugation.

We have developed a procedure for isolating the nuclear-centrosome complex from tissue culture cells such as Chinese hamster ovary (CHO), Hela, and PtK_1 cells and human neutrophils. After extraction of the isolated nuclei with detergent in a low ionic medium, the ultrastructure of the attached centrioles can be identified easily by electron microscopy of whole-mount preparations. This development has enabled us to reexamine the structural changes occurring in the centrioles throughout the cell cycle with the aim of collecting quantitatively significant data.

In this paper we document the centriole cycle in synchronized CHO cells and study its coordination with nuclear events. We confirm that an inhibitor of DNA synthesis does not prevent centriole nucleation, elongation, or disorientation. In addition, we find that removal of the nucleus by enucleation with cytochalasin B also does not prevent elongation and disorientation but nucleation is blocked. Our results suggest that formation of the procentriole may normally depend on a signal arising from the nucleus.

MATERIALS AND METHODS

Cell Culture

CHO cells were grown as monolayers in Ham's F-10 medium (Gibco Laboratories, Grand Island, N. Y.) supplemented with 10% fetal bovine serum, antibiotics, and 15 mM HEPES, pH 7.2, and were maintained at 37°C in a humid atmosphere with 5% CO_2. Under these conditions, the cells had a mean generation time of 14 h as determined by time-lapse photomicroscopy (P. Kronebusch, unpublished results). HeLa and PtK_1 cells were grown by the same procedure. Human leukocytes were a gift of Mr. B. Soltys.

Cell Synchronization

Arrest of cells in S phase was performed by addition of thymidine to nonconfluent cultures to a final concentration of 2 mM (23). After 12–15 h, all cells were well-spread on the culture dish and no rounded or floating cells were detectable. The monolayers were then washed free of thymidine and returned to culture with fresh medium. It should be noted that the thymidine treatment does not precisely synchronize the cells. Rather, cells in S phase at the beginning of the treatment are inhibited in their rate of DNA synthesis and remain distributed throughout the S phase. Cells in G_1, G_2, and M progress through the cell cycle and tend to accumulate at the G_1/S boundary. After 12- to 15-h exposure, we expect most cells to be either at the G_1/S boundary or in S phase.

Mitotic cells were obtained by first treating the cultures with 2 mM thymidine for 12–15 h as before. After washing once with fresh medium to remove the thymidine, they were resuspended in the same culture medium for an additional 7 h until the mitotic index was at a maximum. Rounded cells in mitotic stages were shaken off and collected by centrifugation in a tabletop centrifuge and then plated into new culture dishes. These synchronized cells were cultured for the desired period, then collected by scraping with a rubber policeman, and pelleted in a tabletop centrifuge. In an early series of experiments, Colcemid at a concentration of 0.10 μg/ml in the growth medium was used to gather mitotic cells. After the cells were washed free of Colcemid and resuspended in fresh medium, cell division and attachment of cells to culture dishes seemed to occur normally, although multipolar spindles were observed (19). However, the centriole cycle after cell division was not very regular, and well-ordered progression curves for centrioles were not obtained. Therefore, we abandoned the use of this drug to collect large quantities of mitotic cells and, instead, relied upon the mitotic selection technique described above.

Preparation of Cytoplasts and Karyoplasts

Enucleation was done according to the method developed by Prescott and Kirkpatrick (15), with some modifications. CHO cells were grown on plastic cover slips (3.0 cm in diameter). Cytochalasin B (Sigma Chemical Co., St. Louis, Mo.) medium was prepared by addition of stock 1 mg/ml cytochalasin B in dimethyl sulfoxide to the culture medium to a final concentration of 2.5–5.0 μg/ml. Portions of 4 ml of culture medium containing the cytochalasin B were added to 50-ml Sorvall centrifuge tubes (DuPont Co., Wilmington, Del.). A cover slip was positioned in each tube with the cells facing the bottom. The tubes were spun at 12,000 rpm for 15 min at 37°C in the SS-34 rotor. After centrifugation, the cover slips (with the adherent cytoplasts) were removed, washed once with fresh medium to remove the cytochalasin B, placed cell-side up in a culture dish with fresh medium, and incubated at 37°C for 30–60 min. During this procedure, the total time during which the cells were exposed to cytochalasin was <30 min. For experiments in which the nucleating activity of cytoplast centrosomes was to be compared with that of mitotic centrosomes (see accompanying paper [11]), cytoplasts were cultured for an additional 0.5 h in 0.1 μg/ml Colcemid in medium to depolymerize microtubules *in situ*. The cytoplasts were collected by scraping the cover slips with a rubber policeman. A karyoplast fraction was obtained by transferring the nuclear pellet to fresh medium.

Electron microscopy of embedded material confirmed that in our preparations, as in those reported by others (7, 22), the karyoplast contained the nucleus surrounded by a thin shell of cytoplasm, whereas the cytoplast lacked the nucleus but retained all of the cytoplasmic organelles including the centrosome from which the microtubules radiated (data not shown).

Preparation of Nuclear-Centrosome Complexes from Interphase Cells and Free Centrosomes From Mitotic Cells and Cytoplasts

Interphase or mitotic cells or cytoplasts were scraped off directly from culture dishes with a rubber policeman and were sedimented in a tabletop centrifuge. Samples in 12-ml conical tubes were resuspended for 1 min in 10 vol of distilled water and lysed in a solution of 2% Triton X-100 and 1 mM PIPES by addition of an equal volume of 4% Triton X-100 in 2 mM PIPES at pH 6.7. Under these conditions the nuclei derived from interphase cells became pale with incubation at 30°C. This process was monitored by phase-contrast microscopy and, after 10–60 min, when the nuclei were judged sufficiently pale, 0.5 vol of 3% glutaraldehyde in distilled water was added to stop extraction. The centrosomes remained bound to the nucleus.

In the case of mitotic cells, free centrosomes were produced after lysis and, therefore, glutaraldehyde fixation was done immediately afterwards.

Lysed cytoplasts released large amorphous structures that seemed to be composed of fibrous material in which the centrosome, as well as other cytoplasmic components, was embedded. Since these structures had less electron density than nuclei, it was easy to identify the centrioles in them. In addition, since these structures appeared to dissolve after long incubation with detergent in low ionic strength medium, glutaraldehyde was added to the lysate just after extraction.

Microscopy

For making the whole-mount preparations the methods described by Gould and Borisy (8) were employed with some modifications. For interphase cells and cytoplasts, several drops of a fixed sample were sedimented at 2,000 *g* for 5 min in a tabletop centrifuge onto ionized-Formvar-coated 400-mesh grids that had been heavily coated with carbon. For free centrosomes resulting from lysis of mitotic cells, centrifugation was done at 2,800 *g* for 5–7 min. After washing with distilled water, grids were stained with 2% phosphotungstic acid at pH 6.5 and were examined in a Philips 300 electron microscope operated at 80 kV.

Frequency histogram profiles of centriole configurations were constructed by photographing and measuring the length of the parent and daughter centrioles and then placing them into one of six categories as described in Results. To construct a frequency histogram for one sample, 100–150 photographs of centrioles or centrosomes were taken.

For preparation of thin sections, samples were fixed with 2.5% glutaraldehyde in the medium containing 100 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$ at pH 6.7, postfixed in 1% OsO_4, stained with 0.5% uranyl acetate, dehydrated through an ethanol series, infiltrated, and embedded in an Epon-Araldite mixture according to standard technique. Thin sections were cut on an OmU3 type ultramicrotome (Reichert, Austria), picked up on Formvar- and carbon-coated grids, and stained with uranyl acetate and lead citrate.

RESULTS

The Nuclear-Centrosome Complex in Interphase Cells

When interphase cells were lysed, they released nuclei that

initially appeared as dark spherical or oblong objects and it was difficult to detect any centrosomes attached to them. However, upon continued extraction in a solution of low ionic strength (1 mM PIPES, 2% Triton X-100, pH 6.7), they gradually became more swollen and pale and, as a result, dots on the nucleus became apparent. During the early stages of extraction, many dots were observed on each nucleus which subsequent observations showed not to correspond to the centrosome. These spurious dots were removed from the nuclear surface during the course of extraction and, by 60 min, only one or two pairs of dots remained on the surface. These remaining dots adhered tenaciously and could be freed only by sonication or more drastic extraction procedures which resulted in disruption of the nucleus (11). On the basis of their number (two or four) and their tendency to exist in pairs, we tentatively identified the strongly adherent dots as centrosomes.

Fig. 1 illustrates nuclei with adherent dots as revealed by phase-contrast and differential interference contrast microscopy. Panels *A* and *B* both show a pair of putative centrosomes over the nucleus; *C*, *D*, *E*, and *F* show centrosomes at the periphery of the nucleus; and *G* and *H* show two pairs of centrosomes on a nucleus, apparently resulting from replication and separation of the centrosomes.

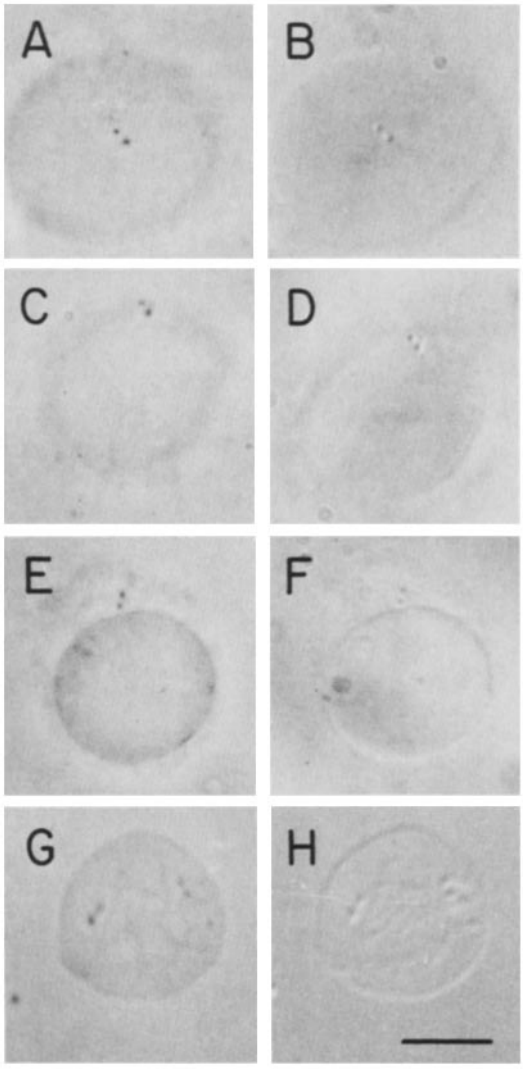

FIGURE 1 Light micrographs of nuclear-centrosome complexes. Micrographs are paired to show the same complex in both phase-contrast and differential-interference microscopy. Pairs of centrioles were detected as pairs of dots. *A, C, E, G*: phase-contrast microscopy. *B, D, F, H*: differential-interference contrast microscopy. Bar, 5 μm.

Proof that the nucleus-associated dots visible in the light microscope were centrosomes was obtained by electron microscopy. Fig. 2 shows an isolated nucleus extracted as described above, fixed, prepared as a whole mount, and stained with phosphotungstic acid. The nucleus appears as a pale grey sphere with two intensely staining cylindrical structures at the periphery corresponding to images seen in Fig. 1 *E* and *F*. Although at this magnification the amount of stain did not permit observation of greater detail in the cylindrical structures, higher magnification and higher beam exposure showed them to be centrioles plus associated pericentriolar material. Similar preparations have been made from HeLa and PtK_1 cells and human leukocytes, which show that the existence of a nuclear-centrosome complex is not a peculiarity of CHO cells. The complex was also stable at 0°C for 30 min or when isolated from cells cultured in the presence of 0.1 μg/ml Colcemid overnight, conditions in which no microtubules were detected.

Fig. 3 shows an electron micrograph of an embedded and thin-sectioned isolated nucleus with its associated centrosome. Observations of numerous micrographs show that the nuclear lamina appeared to remain intact, whereas the lipid bilayers of both membranes of the nuclear envelope were absent as described previously for detergent-treated nuclei (1). The *inset* shows a pair of centrioles in perpendicular orientation to each other and surrounded by a cloud of pericentriolar material, in which are embedded viruslike particles (8, 20). Thin cytoplasmic filaments are generally present, surrounding the centrosome and in association with the nucleus, but few, if any, microtubules survive the isolation procedure.

The Centriole Cycle in CHO Cells

The isolation of nuclear-centrosome complexes permitted an examination of centriole configuration in large numbers of interphase cells. In contrast to interphase cells, lysates of mitotic cells released free centrosomes. Profiles of centriole pairs in both free and nuclear-associated centrosomes were identified,

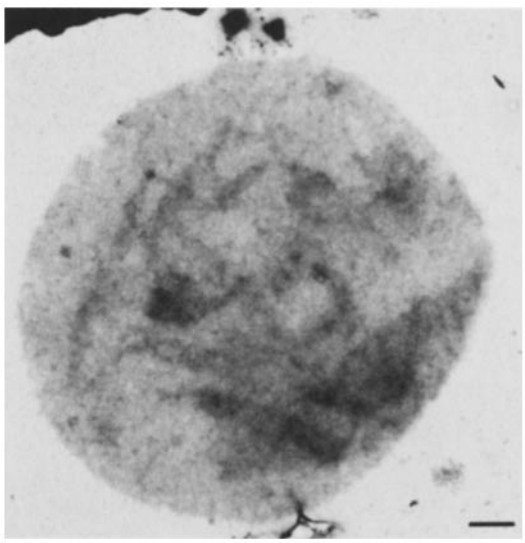

FIGURE 2 Whole-mount electron micrograph of a nuclear-centrosome complex. A pair of centrioles is seen as two dark cylindrical structures at the top of the nucleus. Note that the pericentriolar material appears to contact the nuclear surface. Bar, 1 μm.

measured, and placed into one of six categories according to the relative orientation of daughter and parent centrioles and the ratio of their lengths.

Fig. 4 shows examples in each of these categories. Category *I* represents profiles of two centrioles, the parent being full-sized and the daughter being full-sized or almost full-sized, but not in an orthogonal configuration. This category was always observed as a single pair of centrioles. The centrioles were generally slightly separated from one another and the angle between their axes varied from 0° to 180°. As will be shown subsequently, these profiles were most commonly observed in early G_1 cells. Categories *II-VI* represent stages in the elongation of the daughter centriole. In each of these categories, parent and daughter centrioles were closely juxtaposed and in orthogonal configuration. The ratios of daughter to parent centriole length were 0.0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1.0 in categories *II*, *III*, *IV*, *V*, and *VI*, respectively. Unlike category *I*, centrioles in categories *II-VI* were generally observed as two pairs of centrioles, frequently close together except in mitotic stages when physical separation of the pairs had occurred.

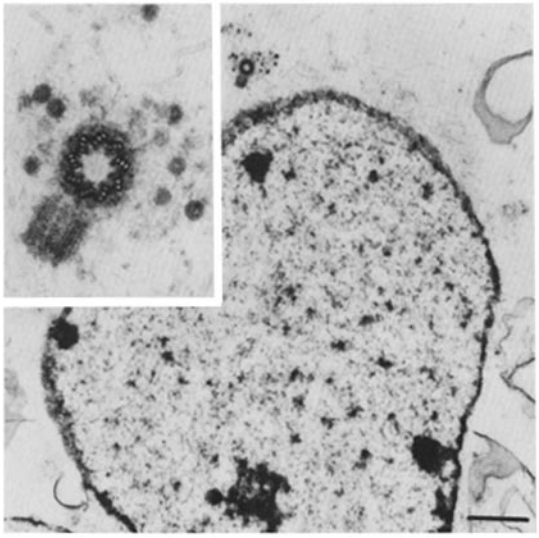

FIGURE 3 Thin-section electron micrograph of a nuclear-centrosome complex. Centrosome region is shown at higher magnification in *inset* (× 42,000) with centrioles displayed in cross and longitudinal section. Note presence of filaments, few microtubules, and viruslike particles embedded in the pericentriolar material. Bar, 1 μm.

The electron micrographs shown in Fig. 4 are examples of centriole pairs that were attached to nuclei (upper row) and centrioles observed free in the lysates (lower row). Most of the centriole pairs observed were attached to nuclei, and, therefore, the micrographs shown in the upper row of Fig. 4 are representative of the majority of our observations. Although the lengths of these centrioles could easily be measured from the negatives, prints of the negatives were not always satisfactory because of the high-contrast levels resulting from the large amount of underlying nuclear material. However, centrioles lying on the periphery of the nucleus could be visualized more clearly. Some centriole pairs in each category were found free in the lysates. Free centriole pairs in categories *I* and *II* were observed only rarely. The other categories were easier to find and were present roughly in proportion to the number of rounded cells in the culture. As will be shown, some of these, especially in categories IV–VI, represent mitotic centrioles, but others presumably resulted from disruption of the nuclear-centrosome complex during lysis and extraction. These relatively rare instances were useful because they better displayed some important structural features of the centriole pairs.

From previous studies (5), it has been established that the daughter centriole arises from the proximal end of the parent, where proximal is defined in relation to the nucleus. The micrographs in the lower row of Fig. 4 show that both parent and daughter centrioles possess a structural polarity that is evident in the negatively stained preparations. The distal end of the parent centriole appears to be more dense or "capped" whereas the proximal end appears open. The daughter centriole displays the same structural differentiation, from which we may conclude that the daughter is oriented with its "capped" end pointing away from the parent. This is the end that will be the distal end in the next cell cycle.

Fig. 5 shows a frequency histogram for categories of centriole pairs in an exponentially growing culture of CHO cells. Category *I* is the most abundant class, which indicates that the centrioles spend a greater proportion of their time in this phase than in any of the other phases. Categories *II-VI* are roughly equally abundant, which indicates that the centrioles elongate in a more or less uniform manner. It should be noted that the frequencies cannot be simply equated with the relative dura-

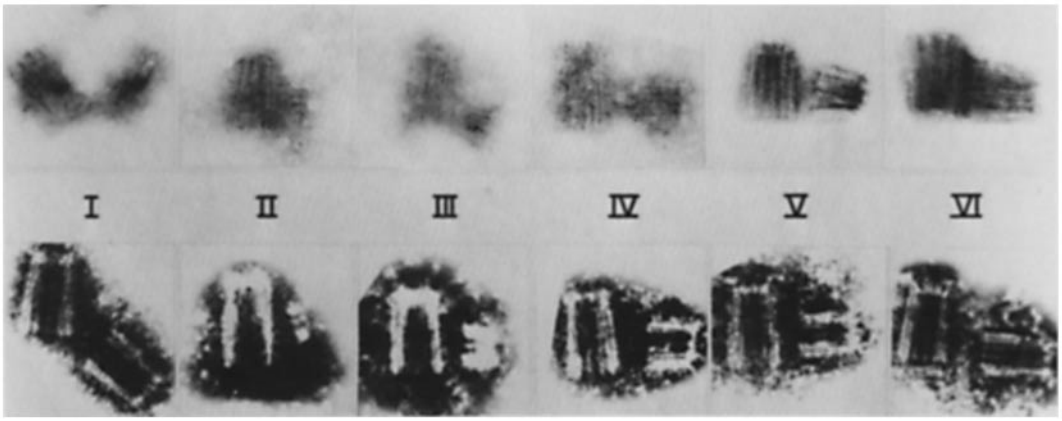

FIGURE 4 Electron micrographs of whole-mount preparations of centrioles, placed into six categories. Upper row: centrioles attached to the nucleus; lower row: centrioles freed from the nucleus. Category *I* represents centriole pairs in late M or G_1. The parent is full-size, and the daughter is also full-size, or nearly so, but not in an orthogonal orientation. Categories *II, III, IV, V,* and *VI* represent stages in the elongation of the daughter centrioles and correspond to ratios of daughter-to-parent centriole length of 0.0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1.0, respectively.

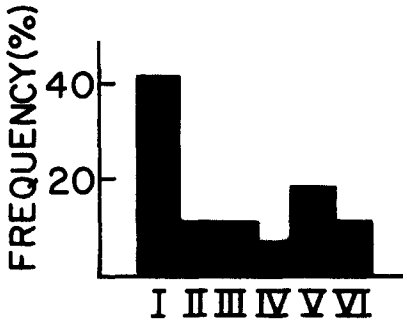

FIGURE 5 Frequency histogram of centriole profiles in exponentially growing cells. Ordinate: frequency in percentage; abscissa: category of centriole profile. Centriole profiles are categorized according to their orientation and the ratio of daughter and parent centriole lengths.

tions of each phase since the data must be corrected for the age distribution of the population. In exponentially growing cultures, there are always twice as many cells that have just divided as there are about to divide. When the necessary corrections are made, the effect is to reduce the apparent predominance of category *I*, but otherwise the conclusions are only slightly changed.

Fig. 6 shows the centriole cycle in CHO cells that were treated by addition of thymidine. As indicated in Materials and Methods, under these conditions most of the cells will be distributed through S phase or accumulate at the G_1/S boundary. After blocking the cells for 12 h (Fig. 6*a*), the most abundant categories were *I-III*; that is, centriole pairs before formation of the procentriole and centriole pairs in which the daughter had reached a maximum of 40% of full length. When the cells were washed free from thymidine (Fig. 6*b-f*), category *I* declined, indicating procentriole formation (nucleation), and the peak in the frequency histogram shifted to later categories, indicating elongation of the daughter centriole. By 5 h of culture (Fig. 6*c*), the peak in the histogram had moved to a daughter/parent ratio of 0.4–0.6. The maximum mitotic index was obtained at 7 h after removal of the thymidine (Fig. 6*d*). At this time, almost all of the cells had long daughter centrioles (ratio: 0.6–1.0) and at the same time category *I* began to increase. This can be easily understood as arising from the short duration of M, <1 h, and the subsequent reentry of cells into the G_1 phase. However, this experiment does not permit us to determine precisely the time of transition from category *VI* to category *I*. The data permit this to occur at the end of M or in early G_1. Cells went into G_1 phase after mitosis, and the number of category *I* profiles increased significantly. At 13.5 h (Fig. 6*f*), daughter centrioles 20% of full length began to appear again.

When cells were synchronized and started from M phase, essentially the same centriole cycle was observed (Fig. 7). As shown in panel *a*, mitotic cells contained centriole profiles primarily in categories *V* and *VI* but also in category I. Since panel *a* was obtained 0.5 h after the cells were replated the category *I* profiles probably came from round cells that had reentered G_1 phase. Panels *b-d* show the progress of disorientation, nucleation, and elongation of the daughter centrioles. By 13 h (Fig. 7*d*), almost one full cycle has occurred.

The Effect of Thymidine on the Formation and Elongation of the Daughter Centriole

Since formation of the daughter centriole begins at around

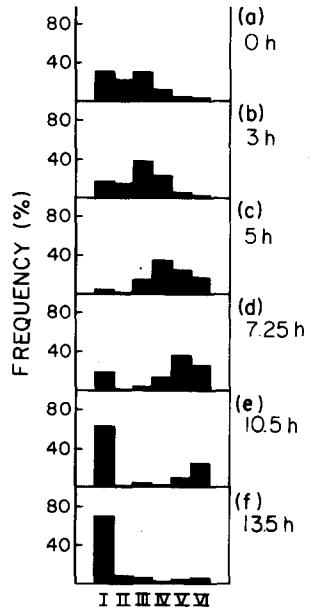

FIGURE 6 Centriole cycle in cells arrested in S phase. After treatment with 2 mM thymidine for 12 h, cells were washed and then continued in culture with fresh medium for (*a*) 0, (*b*) 3, (*c*) 5, (*d*) 7.25, (*e*) 10.5, and (*f*) 13.5 h. Ordinate: frequency in percentage; abscissa: category of centriole profile.

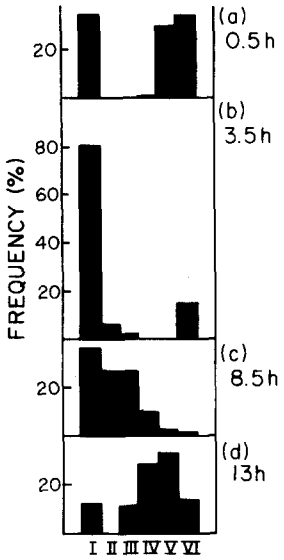

FIGURE 7 Centriole cycle in cells synchronized at M phase. Isolated mitotic cells were plated in culture dishes and continued in culture with fresh medium for (*a*) 0.5, (*b*) 3.5, (*c*) 8.5, and (*d*) 13.0 h.

S phase, the possibility that centriolar replication is closely linked to DNA synthesis should be considered. Rattner and Phillips (16) have reported that in cells of the mouse line, L929, the course of procentriole formation was not contingent upon nuclear DNA synthesis. Although their results were clear, they relied upon electron microscopy of thin sections to assay the centrioles and so their sample size was small and they were not able to provide quantitative data on the course of centriole elongation. Therefore, we thought it useful to reinvestigate this question using our new techniques applied to CHO cells.

Previous studies have shown that thymidine reduces the rate, and is therefore an effective inhibitor, of DNA synthesis in cultured cells (2). To investigate the question of whether the nucleation and elongation of daughter centrioles is dependent

upon DNA synthesis, we kept cells in culture in medium containing thymidine. Cells were synchronized in M phase as described in Materials and Methods, were immediately plated into fresh medium containing 2 mM thymidine, and were maintained in the same medium until they were lysed. Fig. 8 *a* shows the frequency histogram of centriole profiles for mitotic cells just before treatment with thymidine. As before, the profiles were clustered in categories *V*, *VI*, and *I*. After 11 h of blocking in thymidine (Fig. 8 *b*), categories *V* and *VI* declined dramatically and >70% of the profiles contained daughter centrioles <40% of full length. This distribution should be compared to the one shown in panel *c*, in which the cells were cultured for 11 h in fresh medium that did not contain thymidine. These two histograms are very similar although a slight delay in the progression of the centriole cycle can be detected in the thymidine-treated cells. Further culturing of the cells in thymidine permitted continued elongation of the daughter centrioles (Fig. 8 *d*). This, it may be concluded that both the nucleation and elongation of daughter centrioles do not depend upon DNA synthesis, and that these processes occurred at almost the same rate as in control cells. Also, since centrioles could pass into category *I* from category *VI*, disorientation does not depend upon DNA synthesis. Similar results were obtained when the cells were started from S phase and when they were blocked using 2 or 10 mM thymidine (data not shown).

However, we should note that completion of the centriole cycle was inhibited by thymidine. Cultures maintained in media containing thymidine for up to 46 h showed no change in the frequency distribution of profiles over that obtained at 17.5 h, and, under these conditions, the most abundant categories were *IV* and *V* (data not shown). It seems that cells blocked with thymidine cease their centriole cycle sometime before the configuration corresponding to M phase.

The Effect of Enucleation on the Formation and Elongation of the Daughter Centriole

It was clear from the results of the previous section that DNA synthesis was not required for both the formation and elongation of daughter centrioles. Thus, we next asked whether these events were dependent upon the presence of the nucleus. We prepared cytoplasts from the cells by enucleation with cytochalasin B. We first examined cells synchronized in S phase.

Cells grown on plastic dishes were treated with 2 mM thymidine for 12 h. They were then centrifuged at 37°C for 15 min in culture medium containing cytochalasin as described in Materials and Methods. After washing out the remaining cytochalasin B, the cytoplasts were placed in fresh medium until they were lysed.

Frequency histograms of centriolar profiles just before and after enucleation of S phase cells are shown in Fig. 9 *a* and *b*, respectively. The profile distributions for the two cases are essentially identical; therefore the surgical process of enucleation resulted in no perturbation of the centriolar structures. After 8 h in fresh medium, centrioles harvested from the cytoplasts had the frequency histogram shown in panel *d*. This distribution should be compared to the one shown in panel *c*, in which the profiles obtained from whole cells cultured for an equivalent period of time are shown. The centrioles in category *I* probably derived from cells that reentered G_1 phase (see Fig. 6). The two distributions are similar, although elongation of centrioles in the cytoplasts is evidently a little delayed as

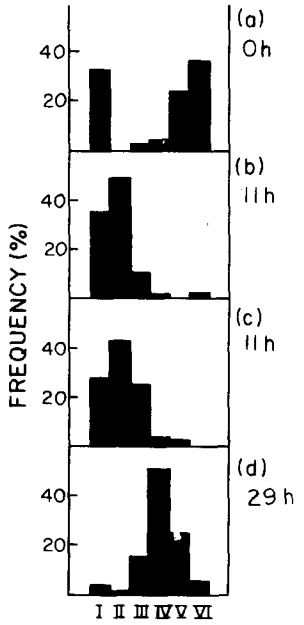

FIGURE 8 Effect of thymidine on nucleation and elongation of the daughter centriole. Isolated mitotic cells were plated into dishes containing 10 mM thymidine and cultured for (*a*) 0, (*b*) 11, and (*d*) 29 h. In c, cells were cultured for 11 h in fresh medium that did not contain thymidine.

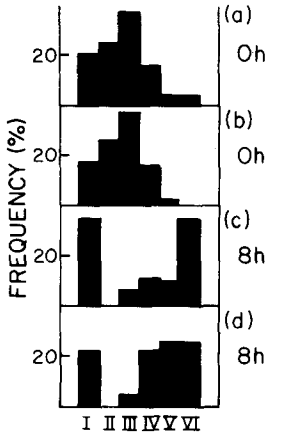

FIGURE 9 Effect of enucleation on elongation of the daughter centriole. After treatment with 2 mM thymidine for 12 h, cells were enucleated and continued in culture for 8 h. Centriole profiles are shown for (*a*) just before removal of the nucleus, (*b*) just after removal of the nucleus, (*c*) intact cells cultured for 8 h, and (*d*) cytoplasts cultured for 8 h.

compared with the controls. As shown by Prescott and Kirkpatrick (15), cytoplasts incorporate ^{3}H-labeled amino acids at a high rate for the first several hours after removal of the nucleus, but the rate of incorporation becomes severely depressed by 12 h. In another experiment, we found that the distribution of centriole profiles obtained from cytoplasts cultured for 23 h was essentially unchanged from that at 11 h. Therefore, the difference between panels *c* and *d* in Fig. 9 may be attributable, in part, to the reduced viability of the cytoplasts. Nevertheless, it is clear that, at least up to 8 h of culture, the centriole cycle progressed almost normally in the cytoplasts. The results show that centriole elongation and disorientation can occur in the absence of the nucleus. However, we could not draw any conclusions concerning the dependence of nucleation of centrioles since insufficient time elapsed for procen-

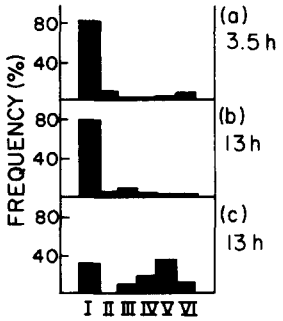

FIGURE 10 Effect of enucleation on nucleation of the daughter centriole. Isolated mitotic cells were plated into dishes and continued in culture for 3.5 h. After enucleation at 3.5 h, the cytoplasts were continued in culture in fresh medium. Centriole profiles are shown for (*a*) just before removal of the nucleus, (*b*) cytoplasts cultured for 10 h, and (*c*) intact cells cultured for 10 h.

triole formation to occur in the control cells, and, as mentioned above, it was not practical to carry out longer-term incubations of the cytoplasts because of their reduced viability. Therefore, we conducted a similar experiment but with cells synchronized in G_1 phase.

Synchronized cells in M phase were placed into culture for 3.5 h to allow them to enter G_1 phase. The cells were then enucleated according to the cytochalasin B procedure, and the cytoplasts were returned to culture. Fig. 10*a* shows the frequency distribution of centriole profiles in the G_1 cells just before enucleation. As is evident from the panel, >80% of the profiles were in category *I*, that is, disoriented and without procentrioles. After the cytoplasts were cultured in fresh medium for 10 h, essentially the same frequency histogram was obtained (panel *b*). On the other hand, during the same time interval, intact cells (panel *c*) advanced normally through their centriole cycle and showed a peak in the frequency histogram in category *V*, corresponding to a daughter/parent ratio of 0.6–0.8. Control cells centrifuged according to the standard enucleation procedure but against culture medium lacking cytochalasin B gave essentially the same distribution of centriole profiles as shown in Fig. 10*c*. In addition, treatment of whole cells with 3 μg/ml cytochalasin B at 37°C for 20 min with no centrifugation also resulted in the same distribution as shown in Fig. 10*c*. Thus, neither centrifugation alone nor cytochalasin B treatment alone prevented formation of procentrioles. From these results taken together with the previous experiments on cells blocked in S phase, we conclude that removal of the nucleus from the cell resulted in an inhibitory effect on daughter centriole nucleation but not on elongation or disorientation.

DISCUSSION

The Nuclear-Centrosome Complex

Our results indicate that the centrosome and nucleus may be isolated together as a coherent complex. The mechanism of this association is not clear but some comments may be made. The association clearly does not require the lipid bilayers of the nuclear envelope, since detergent treatment removes these layers but the complex persists. The association also does not depend upon the presence of microtubules since (*a*) most of them do not survive the isolation procedure, and (*b*) the remaining ones can be depolymerized at low temperature or with Colcemid without affecting the stability of the complex. The association may be mediated by the pericentriolar material since the centrioles are always separated from the nucleus by a small distance, whereas the pericentriolar material sometimes is seen in apparent contact with the nuclear envelope. Microfilaments and/or 100-Å filaments may play some role since they are frequently observed in the region of the centrosome and the nuclear envelope both in our isolated preparations and *in situ* (16, 18); however, this possibility requires further investigation. Another question concerns the elements within the nucleus to which the centrosome is attached. Possible candidates are the nuclear pores and the nuclear lamina (1). Again, these represent problems for future study.

Why should the centrosome be situated next to the nucleus? One answer might be that a juxtanuclear position would facilitate the formation of the mitotic spindle. If the centrosomes were remote in the cytoplasm, how would they come to arrive at the poles of the spindle? Another possibility is that the cytoplasmic microtubules that seem to radiate from the centrosome might serve to anchor the interphase nucleus within the network of intracellular filaments.

The salient point for this study, however, is that the combination of whole-mount electron microscopy and isolation of the nuclear-centrosome complex provides a new technique for studying the activity of the centrosome throughout the interphase portion of the cell cycle. The results obtained with the new method fully confirm the pattern of events in the centriole cycle as determined by electron microscopy of thin sections (16, 17, 19). In addition, the method has the important advantage that, with it, the collection of quantitatively significant data becomes a feasible undertaking. Future applications of the method may include determination of the effects of various drugs on centriolar events and the coupling of centriole cycle with mitosis.

Since the centriole cycle is normally tightly coordinated with other cellular events, the technique permits one to assay the cycle position of an individual cell from inspection of its centriole configuration. As an example, when centrioles of human neutropils were observed with this technique, they were all found to have the structure designated as category *I* (unpublished results).

The Centriole Cycle

We have used the method to document the events of the centriole cycle. We distinguish three morphological events, which we have designated as nucleation, elongation, and disorientation. Nucleation, which has been referred to in the literature (16, 17, 19) as formation of the procentriole, occurs in late G_1 or early S phase. Elongation occurs throughout S and G_2 and does not generally attain completion until early G_1 of the next cell cycle. Disorientation occurs in late M or early G_1 and is an event that has not previously received attention in the literature. These three events refer to the relationship of the two centrioles within a given pair. A fourth event is the separation of pairs of centrioles at the onset of M phase.

Mazia et al. (12) have studied the reproduction of mitotic centers in fertilized sea urchin eggs and identified on functional considerations three distinct events, which they referred to as duplication, splitting, and separation. An important question concerns the relationship between the reproduction events defined in functional terms and the events defined in morphological terms. It is clear that the separation event of Mazia et al. (12) is equivalent with the physical separation at the onset of M phase of centrosomes each containing a pair of centrioles as we observed in CHO cells. However, identification of the morphological counterparts of the duplication and splitting

steps is more problematic. Is duplication represented by the formation of the procentriole (category *II*), or by the daughter centriole attaining maturity at some later stage? Can maturity be equated with the daughter achieving its full length (category *VI*), or is maturity not dependent upon complete elongation? Is splitting to be correlated with the event we have designated as disorientation (category *I*), or does it simply represent the onset of separation? The answers to these questions will require correlated functional and ultrastructural analyses.

Our results with thymidine have shown that inhibition of DNA synthesis affected neither the formation of the procentriole (nucleation) nor its elongation and subsequent disorientation from its parent. However, long-term incubation in thymidine caused the centriole cycle to become arrested sometime before M. In all these respects, our data confirm the results of Rattner and Phillips (16). These findings might have suggested that the centriole cycle was completely independent of nuclear events, but subsequent results indicated that this was not the case.

Removal of the nucleus of the cell by treatment with cytochalasin B and centrifugation prevented formation of new centrioles, whereas elongation and disorientation proceeded normally as in control cells. This suggests that the presence of the nucleus but not DNA synthesis is necessary for the induction of new centrioles. Possibly the requirement for the nucleus results from a need for an RNA component, but if this is true we infer that the RNA species is not necessary for elongation.

An additional explanation for this result is that the formation of the procentriole is facilitated by a physical association between the centrosome and the nucleus. We have already demonstrated that in interphase cells the centrosome is firmly bound to the nucleus and that a complex of the two may be isolated as a coherent unit. It is clear that centriole reproduction is normally strictly coordinated with nuclear division. Centriole production does not get out of phase with nuclear division and neither too many nor too few centrioles are formed. Our results suggest that some nuclear factor is involved in centriole formation.

However, it is also clear that centriole production is not absolutely dependent upon the nucleus. It is known that large numbers of cytasters, each containing one or more centrioles, may be induced in unfertilized sea urchin eggs by a variety of treatments (6, 10). Most of these cytasters form with no apparent connection to the nucleus. Moreover, it is possible to observe *de novo* formation of cytasters and centrioles even in enucleated fragments of eggs (9). Thus, one must conclude that centrioles can be formed without the nucleus. However, to control the precise number of centrioles within the cell, the nucleus may be required. Perhaps under normal conditions, parent centrioles are stimulated to nucleate their daughters only when they receive the proper signal from the nucleus.

This work was supported by National Institutes of Health research grant GM25062 to G. G. Borisy.

Received for publication 26 April 1981, and in revised form 6 July 1981.

REFERENCES

1. Aaronson, R. P., and G. Blobel. 1974. On the attachment of the nuclear pore complex. *J. Cell Biol.* 62:746–754.
2. Bootsma, D., L. Budke, and O. Vos. 1964. Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res.* 33:301–309.
3. Bornens, M. 1977. Is the centriole bound to the nuclear membrane? *Nature* (*Lond.*). 270: 80–82.
4. Connolly, J. A., and V. I. Kalnins. 1978. Visualization of centrioles and basal bodies by fluorescent staining with nonimmune rabbit sera. *J. Cell Biol.* 79:526–532.
5. DeFoor, P. H., and E. Stubblefield. 1974. Effects of actinomycin D, amethopterin, and 5-fluoro-2′-deoxyuridine on procentriole formation in Chinese hamster fibroblasts in culture. *Exp. Cell Res.* 85:136–142.
6. Dirksen, E. R. 1961. The presence of centrioles in artificially activated sea urchin eggs. *J. Biophys. Biochem. Cytol.* 11:244–247.
7. Goldman, R. D., R. Pollack, C. M. Chang, and A. Bushnell. 1975. properties of enucleated cells. III. Changes in cytoplasmic architecture of enucleated BHK21 cells following trypsinization and replating. *Exp. Cell Res.* 93:175–183.
8. 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.
9. Kato, K. H., and M. Sugiyama. 1971. On the *de novo* formation at the centriole in the activated sea urchin egg. *Dev. Growth Differ.* 13:359–366.
10. Kuriyama, R., and G. G. Borisy. 1978. Cytasters induced within unfertilized sea urchin eggs. *J. Cell Biol.* 79(2, Pt. 2):294*a* (Abstr.).
11. Kuriyama, R., and G. G. Borisy. 1981. Microtubule-nucleating activity of centrosomes in Chinese hamster ovary cells is independent of the centriole cycle but coupled to the mitotic cycle. *J. Cell Biol.* 91:822–826.
12. Mazia, D., P. J. Harris, and T. Bibring. 1960. The multiplicity of the mitotic centers and the time-course of their duplication and separation. *J. Biophys. Biochem. Cytol.* 7:1–20.
13. Murray, R. G., A. S. Murray, and A. Pizzo. 1965. The fine structure of mitosis in rat thymic lymphocytes. *J. Cell Biol.* 26:601–619.
14. Nadezhina, E. S., D. Gais, and Y. S. Chentsov, 1979. On the association of centrioles with the interphase nucleus. *Eur. J. Cell Biol.* 19:109–115.
15. Prescott, D. M., and J. B. Kirkpatrick. 1973. Mass enucleation of cultured animal cells. *Methods Cell Biol.* 7:189–202.
16. Rattner, J. B., and S. G. Phillips. 1973. Independence of centriole formation and DNA synthesis. *J. Cell Biol.* 57:359–372.
17. Robbins, E., G. Jentzsch, and A. Micali. 1968. The centriole cycle in synchronized HeLa cells. *J. Cell Biol.* 36:329–339.
18. Starger, J. M., W. E. Brown, A. E. Goldman, and R. D. Goldman. 1978. Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells. *J. Cell Biol.* 78:93–109.
19. Stubblefield, E. 1968. Centriole replication in a mammalian cell. *In* The Proliferation and Spread of Neoplastic Cells. 21st Annual Symposium on Fundamental Cancer Research. The Williams and Wilkins Co., Baltimore.
20. Wheatley, D. N. 1974. Pericentriolar virus-like particles in Chinese hamster ovary cells. *J. Gen. Virol.* 24:395–399.
21. Wilson, E. B. 1928. The Cell in Development and Heredity. MacMillan, Inc., New York. 3rd Edition.
22. Wise, G. E., and D. M. Prescott. 1973. Ultrastructure of enucleated mammalian cells in culture. *Exp. Cell Res.* 81:63–72.
23. Xeros, N. 1962. Deoxyriboside control and synchronization of mitosis. *Nature* (*Lond.*). 194:682–683.