Routine procedures for the in vitro assembly of microtubules from isolated tubulin subunits (12, 17) have provided important new information on the role of microtubule organizing centers (MTOC) in a variety of eukaryotic cells. The assembly of chick brain tubulin onto flagellar axonemes and basal bodies from Chlamydomonas and sea urchin sperm demonstrated that microtubular structures from several species can serve as nucleating sites for exogenous tubulin reassembly (1, 11, 13). More recently, polymerization of microtubule protein from surf clam egg homogenates produced asters containing centrioles with microtubule organizing activity (18). The in vitro assembly of vertebrate brain tubulin into the mitotic spindle of invertebrate marine eggs has also been demonstrated (5, 9). In mammalian cells, rat brain tubulin was shown to stabilize isolated mitotic spindles (4) and increase the number and length of microtubules associated with mitotic poles (14). Evidence for chromosome movement in in vitro reconstructed mitotic spindles has also
FIGURE 1 Light micrographs of mitotic HeLa cells (a) Mechanically collected mitotic cells from monolayer cultures previously treated with Colcemid (0.10 μg/ml) for 2 h. × 1,500. (b) Mitotic cells blocked in Colcemid for 2 h and reincubated in fresh medium without mitotic inhibitor for 45 min. Reversal from C-metaphase block does not occur after removal of Colcemid. × 1,500.

FIGURE 2 Electron micrograph of Colcemid-blocked HeLa cell displaying a typical C-metaphase configuration. Note the frequency of kinetochore profiles (arrows) and absence of associated microtubules. × 16,700.

FIGURE 3 and 4 Sections of cells blocked with 0.06 μg/ml Colcemid. Very few microtubules (mt) were observed. Those present were found to extend between centrioles and kinetochores (arrows) facing centrioles. Fig. 3, × 43,800; Fig. 4, × 14,000.
FIGURE 5  Electron micrograph of a cell treated with 0.10 μg/ml Colcemid showing a kinetochore (arrow) without microtubules located near the periphery of the cell. No microtubules were observed in cells blocked for 2 h at this concentration of colcemid. × 32,800.

FIGURE 6 and 7  Sections of cells blocked in Colcemid and reincubated in fresh medium for 45 min. Fig. 6 represents a cell treated with 0.06 μg/ml Colcemid and reincubated in fresh medium. Some microtubules (mt) within the C-metaphase spindle can be seen. No recovery from C-metaphase block was observed in these cells, and many kinetochores such as the one shown here (arrow) remained free of microtubule
been reported (4, 14). Direct evidence for the nucleation of microtubule assembly from kinetochores on metaphase chromosomes has not been convincingly demonstrated in published reports. In our studies, kinetochores on metaphase chromosomes and centrioles from HeLa cells were demonstrated as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin.

The interesting response of dividing HeLa cells to Colcemid arrest was especially useful for the present study. First, large numbers of mitotic cells can be collected after treatment with Colcemid. In such cells, the spindle fails to form and chromosomes become loosely arranged around two pairs of centrioles. Microtubules are few in number or are completely missing, and those which do form are usually associated specifically with only one member of a pair of daughter kinetochores on each chromosome. The daughter kinetochore facing away from the centriole pair is devoid of microtubule association. Most importantly, HeLa cells are irreversibly arrested with Colcemid, and assembly of spindle microtubules does not occur after removal of the drug (10, 16). In the present study, we utilized chromosomes and centrioles from lysed, Colcemid-arrested HeLa cells to demonstrate the assembly of microtubules at kinetochores and centrioles using bovine brain tubulin and the reassembly system of Weisenberg (17) as modified by Shelanski et al. (12). Our results suggest that centrioles and kinetochores from human cells can serve as nucleating sites for microtubules in vitro and that microtubule assembly under these conditions leads to chromosome and centriole movement in Triton-extracted HeLa cells.

**MATERIALS AND METHODS**

Partially purified tubulin from bovine brain was prepared using the reassembly buffer of Shelanski et al. (12). Fresh bovine brain was homogenized in cold buffer (pH 6.85) containing 0.1 M 2(N-morpholino)ethane sulfonic acid, 1.0 mM EGTA, 0.5 mM MgCl₂, 4.0 M glycerol, and 1.0 mM GTP, and centrifuged for 15 min at 14,000 rpm. The supernate was recentrifuged for 60 min in the cold at 35,000 rpm (100,000 g) and aliquot fractions were frozen at −70°C. Tubulin protein concentrations of our isolations averaged 5.0 mg/ml. Nonionic detergent Triton X-100 (Tx-100) was added to the tubulin-buffer mixture at concentrations ranging from 0.01–0.10% before addition of this reassembly mixture to mitotic collections of HeLa cells. A concentration of 0.10% was found to be most effective.

Mitotic HeLa cells were collected by treating asynchronous, nonconfluent monolayers with 2 mM thymidine for 16 h. Treated monolayers were resuspended in fresh medium for 8 h and exposed to Colcemid (0.06 or 0.10 μg/ml) for 2 h. Mitotic cells were collected by gentle shaking and populations of cells with mitotic indices from 92 to 95% were routinely obtained. The cells were pelleted, resuspended in 5 vol cold tubulin protein in reassembly buffer containing Tx-100 (reassembly mixture) and warmed to 37°C for 45 min with frequent agitation. For controls, cells were collected in Colcemid as described and reincubated at 37°C for 45 min in fresh medium without Colcemid. Other controls were incubated in reassembly mixture without tubulin (reassembly buffer). In order to evaluate the role of endogenous calcium during assembly, some cells were incubated in the reassembly mixture without EGTA.

For electron microscope examination, glutaraldehyde was added to reassembly mixture or reassembly buffer in which HeLa cells were suspended to produce a final fixative concentration of 3%. Control samples reincubated in medium without Colcemid were fixed in 3% glutaraldehyde in Millonig's phosphate buffer. All samples were allowed to fix for 1 h and centrifuged for 20 min at 2,000 rpm. Pellets were postfixed in 1% osmium tetroxide in Millonig's buffer for 1 h, prestained in 2% aqueous uranyl acetate for 30 min, dehydrated in ethanol, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined in a Philips Model 300 electron microscope. For light microscopy, duplicate samples were pelleted, fixed in 45% acetic acid,模特300 electron microscope.
FIGURE 12 A chromosome in longitudinal section showing both daughter kinetochores with microtubules attached at regularly spaced intervals along the face of the kinetochores (arrows). x 42,200.

FIGURE 13 This micrograph shows two kinetochore profiles with microtubules (arrows) attached at various angles to the long axes of the chromosomes. x 57,700.

FIGURE 14 Survey micrograph of microtubule reassembly in an interphase cell. Note the displacement of the centriole (Ct) to the periphery of the cell and the long microtubules which were generated intra- and extracellularly (arrows). x 13,400.

FIGURE 15 A centriole pair located at the periphery of a lysed cell. These centrioles were associated with an abundance of microtubules contiguous with microtubules reassembled from exogenous, extracellular tubulin. x 20,500.
re sus pended in 1% aceto-orcein stain, and pressed between glass slides and coverslips.

RESULTS

The average mitotic index of HeLa cells collected by our technique (see Materials and Methods) was 93% (Fig. 1a). After removal of the drug and reincubation of these cells in fresh medium for 45 min, resumption of normal mitosis failed to occur and mitotic indices remained at 93% (Fig. 1b). Cell cultures from Chinese hamster and mouse cell lines, cultured in our laboratory and blocked with Colcemid as described above, produced mitotic indices as high as 99%. However, removal of Colcemid and reincubation of these cells in fresh medium resulted in rapid recovery from mitotic block and mitotic indices as low as 1% were recorded after only 30-min reincubation.

In the electron microscope, Colcemid-blocked HeLa cells displayed typical C-metaphase configurations (3, 6) as illustrated in Fig. 2. Sections of other cells (Figs. 3–7) showed other metaphase chromosomes arranged so that one kinetochore faced toward the center of the cell and one kinetochore faced toward the cell periphery. Figs. 3 and 4 are sections of cells blocked with 0.06 μg/ml Colcemid. With this concentration of mitotic inhibitor, the few microtubules that could be seen were observed to extend between centrioles and kinetochores facing centrioles. With 0.10 μg/ml Colcemid (Fig. 5), no microtubules were seen in cells blocked for 2 h. Most kinetochores remained free of microtubule association in cells reincubated for 45 min after 2-h Colcemid (0.06 μg/ml) treatment (Fig. 6). Cells blocked with 0.10 μg/ml Colcemid produced no kinetochore microtubules for up to 45 min after removal of the drug (Fig. 7). With either drug concentration, C metaphase was maintained after Colcemid removal and no evidence for chromosome movement was observed.

Mitotic HeLa cells reincubated for 45 min in either reassembly mixture or reassembly buffer were lysed and much of their contents solubilized. The cellular remains, predominately chromosomes, centrioles, and membrane, were fixed and processed for electron microscopy. As illustrated in Fig. 8, cells lysed in reassembly buffer displayed no microtubules although centrioles and kinetochores were present. Cells examined after exposure to the reassembly mixture were also lysed and both centrioles and kinetochores were observed. As shown in Figs. 9–13, both centrioles and kinetochores were associated with large numbers of microtubules. Fig. 9 is a low magnification electron micrograph showing chromosomes with distinct kinetochores attached to microtubules. Fig. 10 illustrates the nucleating activity of both kinetochores and centrioles. In most of our sections showing chromosomes with kinetochores attached to microtubules, the chromosomes were arranged into small groups with microtubules extending between kinetochores of different metaphase chromosomes. Fig. 11 is a higher magnification of the chromosome group shown in Fig. 10. Microtubules are clearly observed to extend from the kinetochore of one chromosome to that of an opposing chromosome. Fig. 20 is a low magnification micrograph of a different cell showing three distinct groups of chromosomes seemingly held together by microtubules. In sections of chromosomes revealing the kinetochores of both chromatids (Fig. 12), microtubules were attached to both kinetochores. In Fig. 12, attachment of microtubules to the kinetochore appears to be regularly spaced along the face of the kinetochore. They also appear to associate with the outer layer of the kinetochore face, much as they do in intact cells. More often, however, microtubules were found to be attached to kinetochores from many angles, producing oblique and cross-sections of microtubules in most kinetochore profiles (Fig. 13). The extension of microtubules from kinetochores to centrioles was not observed.

The nucleating activity of centrioles during tubulin reassembly resulted in the formation of vast numbers of microtubules associated with centrioles in interphase and mitotic cells. Fig. 14 illustrates microtubule reassembly in an interphase cell. The centriole in Fig. 14 is located at the extreme periphery of the cell. In five separate profiles of interphase cells examined, the centrioles were characteristically displaced toward the cell periphery. The centriole in Fig. 14 is located at the extreme periphery of the cell. In five separate profiles of interphase cells examined, the centrioles were characteristically displaced toward the cell periphery. Fig. 20 illustrates the usual location of centrioles in mitotic cells following tubulin reassembly. Microtubules associated with the centriole in this section extend beyond the outer periphery of the cell, and the centriole appears not to be associated with metaphase chromosomes. In many cells treated with reassembly mixture, enough membrane remained intact to delineate the outer, circular boundaries of the cell. Centrioles in these cells were regularly located near the broken plasma membrane and were usually associated
with reassembled, extracellular microtubules. These observations suggested the centrioles became relocated from their central position in the cell during microtubule reassembly (compare Figs. 3, 4, and 8 with Figs. 10 and 20).

Various configurations of reassembled tubules at centrioles are shown in Figs. 15-18. The centrioles in Fig. 15 were located near the lysed plasma membrane and were associated with an abundance of microtubules extending outside the cell. Microtubule reassembly at centrioles also included extension of tubules in the triplet blades. The centrioles illustrated in Figs. 15 and 16, and sections of many not shown, indicated that microtubules can be assembled at either end of centrioles of HeLa cells but preferentially appeared at the distal end. The centriole cross-sectioned in Fig. 17 is associated with a large number of microtubules including a group of three passing through the lumen. As indicated in Fig. 18, both parent and daughter centrioles can act as nucleating sites for tubulin assembly under the conditions of our experiment. The family of centrioles shown in Fig. 19 was from a cell treated with reassembly mixture lacking EGTA. No microtubule reassembly occurred in cells suspended in reassembly mixture without EGTA; but after such treatment, centrioles were frequently found to be embedded in an electron-dense, fibrous material.

**DISCUSSION**

The system described in this report permits a direct evaluation of partially isolated human chromosomes and centrioles as microtubule assembly sites in vitro. Two lines of evidence support our view that microtubules which become associated with centrioles and kinetochores are assembled largely from exogenous bovine brain tubulin. First, intact HeLa cells blocked with Colcemid at concentrations of 0.06–0.10 μg/ml fail to recover and do not progress through mitosis when placed into fresh media without the drug. Thus, it is unlikely that HeLa tubulin would be available for reassembly in lysed cells. In this regard, it should be pointed out that tubulin from sources other than brain has been difficult, it not impossible, to assemble in vitro. Secondly, reassembly of tubules fails to occur in lysed cells when either reassembly buffer alone (minus tubulin) or the reassembly mixture minus EGTA is used in the incubation. The latter observation suggests that Ca++ is antagonistic to in vitro reassembly of microtubules in our system just as it has been reported by others (17). Evidence that centrioles and kinetochores are functioning as microtubule nucleating sites is supported by electron micrographs which show a dramatic increase in the number of microtubules associated with these structures within a brief time following incubation of lysed cells in the reassembly system. Not all of the available bovine brain tubulin is assembled by kinetochores or centrioles as evidenced by the fact that many random tubule profiles were observed outside the cell boundaries. It is interesting to note, however, that random microtubules were seldom seen within the cell mass and that most microtubules were associated with either kinetochores or centrioles. Thus, HeLa centrioles and kinetochores appear to be serving as microtubule organizing centers in vitro.

Our observation also suggests that centrioles and chromosomes become redistributed within the extracted cells during microtubule reassembly. In

**FIGURE 16** This centriole exhibits microtubule reassembly from a single tubule in a triplet blade resulting in extension of the single tubule (arrows). Micrographs of many other such centrioles indicate that single tubule extension occurs preferentially from distal ends. × 66,000.

**FIGURE 17** The increased nucleating activity of centrioles in our system is demonstrated by this cross-section of a centriole showing numerous microtubule attachments at the cylinder wall and three microtubules passing through the centriole lumen (arrow). × 60,000.

**FIGURE 18** A centriole pair, parent and daughter, acting as nucleating sites for microtubule reassembly. Note the arm-like projections on the microtubules. × 50,800.

**FIGURE 19** This family of centrioles (C) was observed in a cell lysed in reassembly mixture lacking EGTA. No microtubules were observed in these cells, and centrioles were found most often in cell centers. Metaphase chromosome at (C). × 34,000.

**FIGURE 20** Low magnification micrograph of a cell lysed in reassembly mixture showing three chromosome groups associated with numerous kinetochore microtubules and a centriole (arrow) located at the cell periphery. Note that the centriole microtubules extend to the extracellular space and are not associated with kinetochores. × 10,200.

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intact Colcemid-blocked metaphase cells, the centrioles are positioned near the cell center; and in interphase cells, the centrioles are usually closely juxtaposed to the nucleus. In sections of lysed cells exposed to the reassembly system, the centrioles were most often located near the outer periphery of the cell. Thus, it would appear that microtubule assembly at the centrioles leads to their redistribution. The possibility that centriole redistribution is due to lysis alone seems unlikely in view of the fact that in lysed mitotic cells exposed to reassembly buffer minus tubulin or reassembly system minus EGTA, no microtubules formed and centrioles remained near the cell centers.

Microtubule assembly at centrioles included the generation of individual microtubules from proximal and distal ends of triplet blade tubules and assembly of microtubules at both parent and daughter centrioles. The microtubule extensions of single tubules in triplet blades were observed more frequently at distal ends of centrioles. This observation is consistent with the studies of Snell et al. (13), demonstrating single tubule extensions from isolated basal bodies treated with chick brain tubulin. In mammalian cells, formation of normal, bipolar metaphase spindles occurs as pole-to-pole microtubules are generated between separating centriole pairs. Each centriole pair is composed of one parent and one daughter centriole, but usually only the parent is associated with spindle microtubules (8, 15). In our lysates, it was interesting to observe that both parent and daughter centrioles served as nucleating sites for microtubule assembly. Tubule assembly on both proximal and distal ends of centrioles, as well as at both parent and daughter components, may be due to higher concentrations of tubulin in our system. Binder et al. (1) observed in vitro microtubule assembly on both proximal and distal ends of axonemes of flagella from Chlamydomonas and sea urchin in higher concentrations of tubulin (1.7–5.0 mg/ml), but, at concentrations of less than 1 mg/ml, tubules were only assembled at the distal end.

In many sections of cells treated with the reassembly system, kinetochore-to-kinetochore microtubules were seen in association with small groups of metaphase chromosomes. The clustering of chromosomes into random groups following microtubule reassembly may be facilitated by kinetochore-to-kinetochore microtubule attachments. The fact that chromosomes are not clustered into small groups in intact C-metaphase cells suggests that chromosome redistribution must have occurred during in vitro reassembly of microtubules. Moreover, in cells treated with reassembly buffer alone or reassembly mixture without EGTA, microtubule polymerization failed to occur and no chromosome rearrangements were observed. Thus, in our system, reassembly of microtubules appears to induce a type of chromosome movement which leads to the formation of small chromosome clusters. Clearly, the attraction of one chromosome to another seems to coincide with microtubule assembly between the chromosomes. It is possible that tubulin subunits are attracted to regions which are acting as MTOCs. Thus, when polymerization is initiated in our system, some random kinetochore-to-kinetochore attachments are formed which hold chromosomes together. Alternatively, one or two kinetochores may become preferentially active as nucleating sites during in vitro microtubule reassembly which, in turn, “attracts” kinetochores of other chromosomes. However, the mechanism of how microtubule assembly can lead to chromosome redistribution in our system remains unclear.

The haphazard orientation of reassembled microtubules at the outer face of kinetochores of most chromosomes suggests that some controlling factor (or factors) which normally leads to parallel orientation of kinetochore microtubules is missing in our in vitro system. Thus, the precise lateral association between kinetochore-to-pole microtubules and interpolar microtubules fails to occur. It is interesting to note that we frequently see short “arms” extending from reassembled microtubules but typical cross-arms between adjacent microtubules were rarely seen. Cross-arms between parallel bundles of microtubules are normally seen in spindle tubules of mammalian cells (2, 7), and their absence in our in vitro system may explain the loss of parallel orientation of microtubules.

In summary, we have described a new system, whereby metaphase chromosomes and centrioles from Colcemid-arrested HeLa cells can serve as nucleating centers for microtubule assembly in vitro. Thus, kinetochores of mammalian chromosomes and centrioles can be added to the growing list of MTOCs which function in vitro to initiate microtubule assembly from brain tubulin. The present system offers several distinct advantages for studies of kinetochore and centriole function. As a result of Colcemid synchronization, millions
of cells at precisely the same stage of mitosis can be studied and extraction of cells with Tx-100 does not alter the C-metaphase configuration appreciably. Since HeLa cells are irreversibly arrested in Colcemid, most, if not all, of the microtubules which appear after incubation are derived from the exogenous tubulin source. In our system, chromosomes and centrioles become redistributed after microtubule assembly, allowing direct investigations of this form of microtubule-mediated movement. Hopefully, this system will also allow identification of the factors which control metaphase spindle formation and chromosome movement in mammalian cells.

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