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Assembly of microtubules onto kinetochores of isolated mitotic chromosomes of HeLa cells

(electron microscopy/tubulin/phosphotungstic acid staining)

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ABSTRACT **The kinetochores of isolated HeLa cell chromosomes attached to an electron microscope specimen grid, fixed in formaldehyde, and stained with alcoholic phosphotungstic acid are visible as dark, preferentially stained structures distinct from the chromatin with which they are associated. When unfixed chromosomes are immobilized by attachment to grids and incubated with chick brain tubulin, microtubules are observed to assemble onto the kinetochores. This demonstrates the competence of kinetochores in isolated chromosomes to act *in vitro* as microtubule assembly sites and suggests that they also possess this capacity *in vivo*. In addition, the results provide a possible means for isolating and characterizing kinetochores.**

The kinetochore is the site of attachment for the chromosomal microtubules of the mitotic apparatus. While the kinetochore may not be recognizable as a distinct, morphologically identifiable structure in some organisms (1) or may exist as a diffuse region along the chromosome in others (2, 3), in mammalian cells the kinetochore is a localized, well-defined area from which microtubules arise. Considerable detail of the fine structure of the mammalian kinetochore is known from the study of thin sections in the electron microscope (4–6); characteristically, the organelle consists of a tripartite, disc-shaped structure localized at the primary constriction of the chromosome. However, it is only recently that techniques have been defined which permit electron microscopic visualization of the kinetochore in whole mount chromosomes (7). By application of these techniques to surface spreads of the meiotic chromosomes of the locust (7), hamster (8), and man (9) as well as spreads of mitotic chromosomes of HeLa cells (10) the kinetochore has been revealed to be a preferentially stained structure distinct from the chromatin with which it is associated.

There are various sites for the attachment and presumptive assembly of microtubules in eukaryotic cells; among these are the basal bodies of cilia and flagella, centrioles of the mitotic apparatus, and the kinetochores of chromosomes. With the development of methods for the *in vitro* assembly of brain tubulin (11), it has become possible to study the assembly of microtubules onto these sites. Isolated flagellar axonemes of *Chlamydomonas* (12, 13) and sea urchin sperm (12) as well as isolated basal bodies of *Chlamydomonas* (14) will act as initiating sites for the *in vitro* assembly of tubulin. Moreover, it has been demonstrated that brain tubulin will reversibly assemble and disassemble onto the isolated mitotic apparatuses of marine eggs (15) and rat kangaroo cells (16). The poles of marine egg mitotic apparatuses have been separated from the rest of the structure and have been shown to be capable of acting as sites for initiation of microtubule assembly (microtubule organizing centers; 17). However, it has not been demonstrated whether the kinetochores of chromosomes are also capable of initiating microtubule assembly, and an answer to this question would have substantial implications for an understanding of the mechanism of mitosis. Accordingly, studies were initiated to determine if the kinetochores of isolated, intact HeLa chromosomes could act as preferential sites for the *in vitro* initiation and assembly of tubulin subunits into microtubules.

Abbreviations: CIB, chromosome isolation buffer; Pipes, piperazine-*N*-*N*′-bis(ethanesulfonic acid); PTA, phosphotungstic acid.

MATERIALS AND METHODS

HeLa cells were grown as a monolayer in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine and were maintained at 37° in a humid atmosphere with 5% CO_2. The medium was removed and discarded from the culture flasks when the cells attained a density of 2×10^5 cells/cm^2, and new medium was added. Following further incubation for 1 hr, the culture flasks were gently shaken to dislodge mitotic cells, and the medium was decanted. All further operations were performed at 4°, and subsequent centrifugations were carried out in an IEC model PR-6 centrifuge with a no. 253 swinging bucket rotor unless otherwise noted.

Chromosomes were prepared by a modification of the method of Wray and Stubblefield (18). Mitotic cells were sedimented at 1000 × *g* (2000 rpm) for 3 min, and the cell pellet was resuspended in 10 ml of chromosome isolation buffer (CIB) containing 1.0 M hexylene glycol (2-methyl-2,4-pentanediol), 0.5 mM $CaCl_2$, and 0.1 mM Pipes [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)], pH 6.9. This suspension was centrifuged at 1000 × *g* as above in 15 ml plastic, conical centrifuge tubes, and the resulting pellet was resuspended in 1 ml of CIB and incubated for 10 min at 37°. The cells were then lysed by passing four times through a 22 gauge needle. The resulting homogenate, containing free chromosomes, was centrifuged at 15 × *g* (250 rpm) for 3 min to sediment any remaining whole cells. The supernatant fluid was carefully removed, and the chromosomes were sedimented by centrifugation at 1400 × *g* (2400 rpm) for 10 min in a 15 ml plastic, conical centrifuge tube. The resulting pellet was gently resuspended in 20–100 μl of CIB, and the chromosomes were attached to grids by floating a carbon-over-Formvar copper grid on 20 μl of the chromosomal suspension for 1–2 min. The grids had been previously made hydrophilic by glow discharging for 2 min in a vacuum evaporator. The grids with the attached chromosomes were rinsed with four drops of polymerization buffer containing 1 mM $MgSO_4$, 1 mM GTP, 0.1 M Pipes, pH 6.9, and then were either floated immediately on 4% para-formaldehyde, 0.1 M sucrose, and 0.1% dimethyl sulfoxide, pH 6.9, for 5

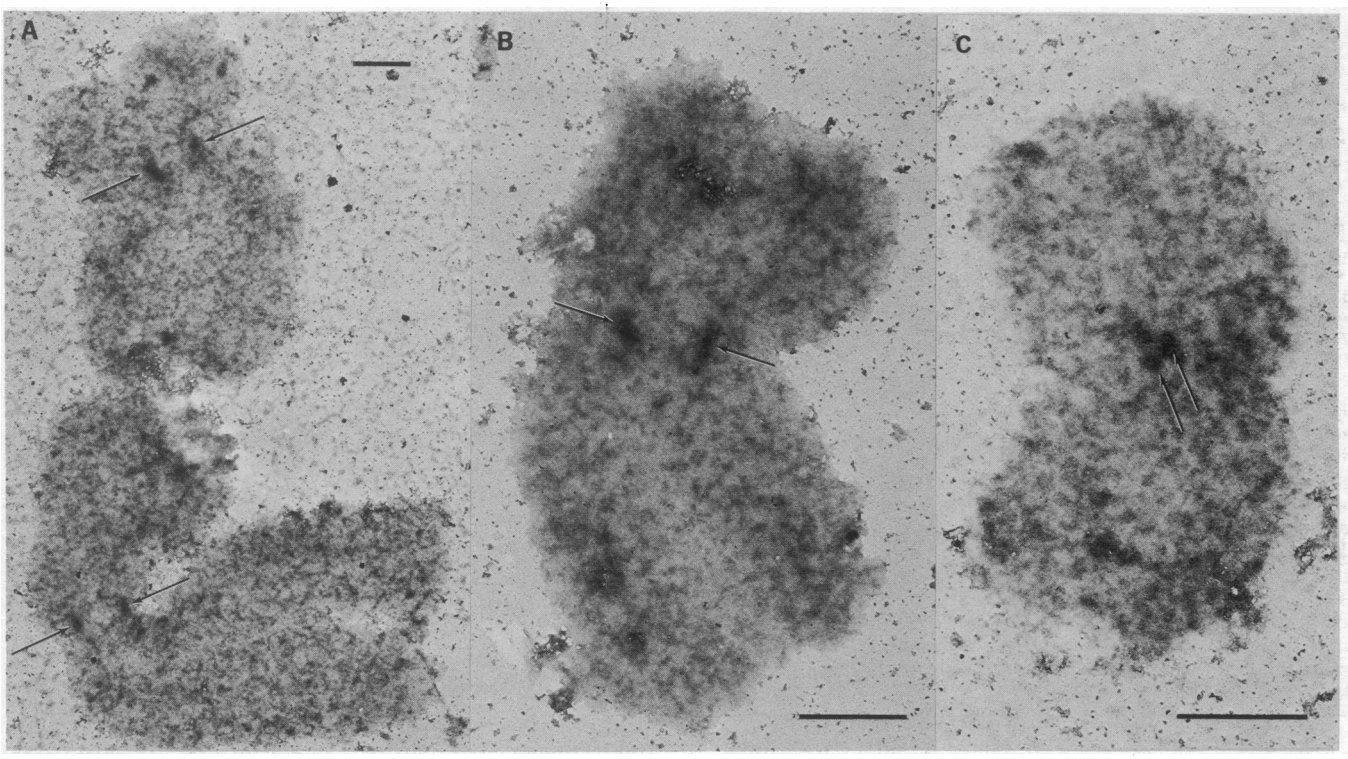

FIG. 1. Isolated HeLa cell chromosomes, immobilized by attachment to grids, rinsed in polymerization buffer, fixed in formaldehyde, and stained with alcoholic PTA. Kinetochores are indicated by arrows. Bar = 1 μm.

min for fixation or first used in the tubulin polymerization procedure prior to fixation.

Tubulin from chick brain was prepared by a modification of the procedure of Weisenberg (11). Brains of one to 3-day-old chicks were suspended in an equal amount (w/v) of polymerization buffer and homogenized in a glass homogenizer with a motor-driven (10,000 rpm) Teflon pestle. The homogenate was centrifuged at 125,000 × *g* (40,000 rpm, Beckman L2-65B centrifuge, rotor 50Ti) for 1 hr, and the supernatant was removed. This crude supernatant contained the tubulin subunits used in the experiments. The absence of microtubules on a bare, coated grid that had been incubated on this tubulin preparation for 6 min at 37° was taken as an indication that spontaneous microtubule assembly had not occurred.

The grids containing attached chromosomes were floated on 100 μl of the tubulin preparation and incubated at 37° for 2–6 min to permit tubulin assembly to occur. Following this, fixation was performed as described above.

The preparation was preferentially stained according to the procedure of Counce and Meyer (7); see also Miller (19). The grids were first floated on a drop of 0.4% Photo-Flo (Eastman Kodak), pH 8.0, for 15 sec, drained, air dried, and then immersed in freshly prepared alcoholic phosphotungstic acid (1 volume 4% PTA and 3 volumes 95% ethanol) for 1 min. Following a wash in 95% ethanol for 10–15 sec, the grids were drained, air dried, and examined in a Philips EM-201 electron microscope operated at 80 kV.

Alternatively, grids were floated after fixation on drops of distilled water, 0.02% cytochrome *c* in 1% *n*-amyl alcohol, and 2% aqueous uranyl acetate for 10–15 sec on each solution. After immersion for 1 min in each of a series of 50%, 70%, and 95% ethanol solutions, the grids were transferred into 100% ethanol for 10 min and then 100% amyl acetate for 10 min before air drying.

RESULTS

Typical isolated HeLa chromosomes attached to grids, washed in polymerization buffer without tubulin, fixed in formaldehyde, and stained with alcoholic PTA are shown in Fig. 1. The overall morphology of the mitotic chromosome is apparent although details of the chromatin fibrils are obscured as a result of air drying. Each chromosome consists of two parallel chromatids with a pair of kinetochores evident as two dark structures distinct from the surrounding chromatin. The kinetochores present on the two chromosomes shown in Fig. 1A and on the chromosome in Fig. 1B appear as ovoid discs and measure about 150 nm × 250 nm. The kinetochores present within the chromosome in Fig. 1C appear as circular discs more closely spaced; this may be due to the orientation of the chromosome on the grid. Characteristically, each kinetochore is localized at the primary constriction of the chromosome.

Immobilized chromosomes incubated with tubulin and then stained with uranyl acetate are shown in Fig. 2. The majority of microtubules that have assembled onto the metacentric chromosome in Fig. 2A are present as a bundle converging at the primary constriction, the location of the presumptive kinetochore. In contrast, microtubules assembled onto the chromosome shown in Fig. 2B are observed to emanate from the chromosome at several different points. The site of initiation of these microtubules is not visible because of the opacity of the chromosome. However, if the course of each microtubule is projected into the chromosome, the point of convergence is near the constriction.

The results of applying formaldehyde fixation and alcoholic PTA staining to chromosomes onto which microtubules have assembled are shown in Fig. 3. In contrast to the uranyl acetate preparations, the pale staining characteristics of the chromatin in this procedure permit the visualization of mi-

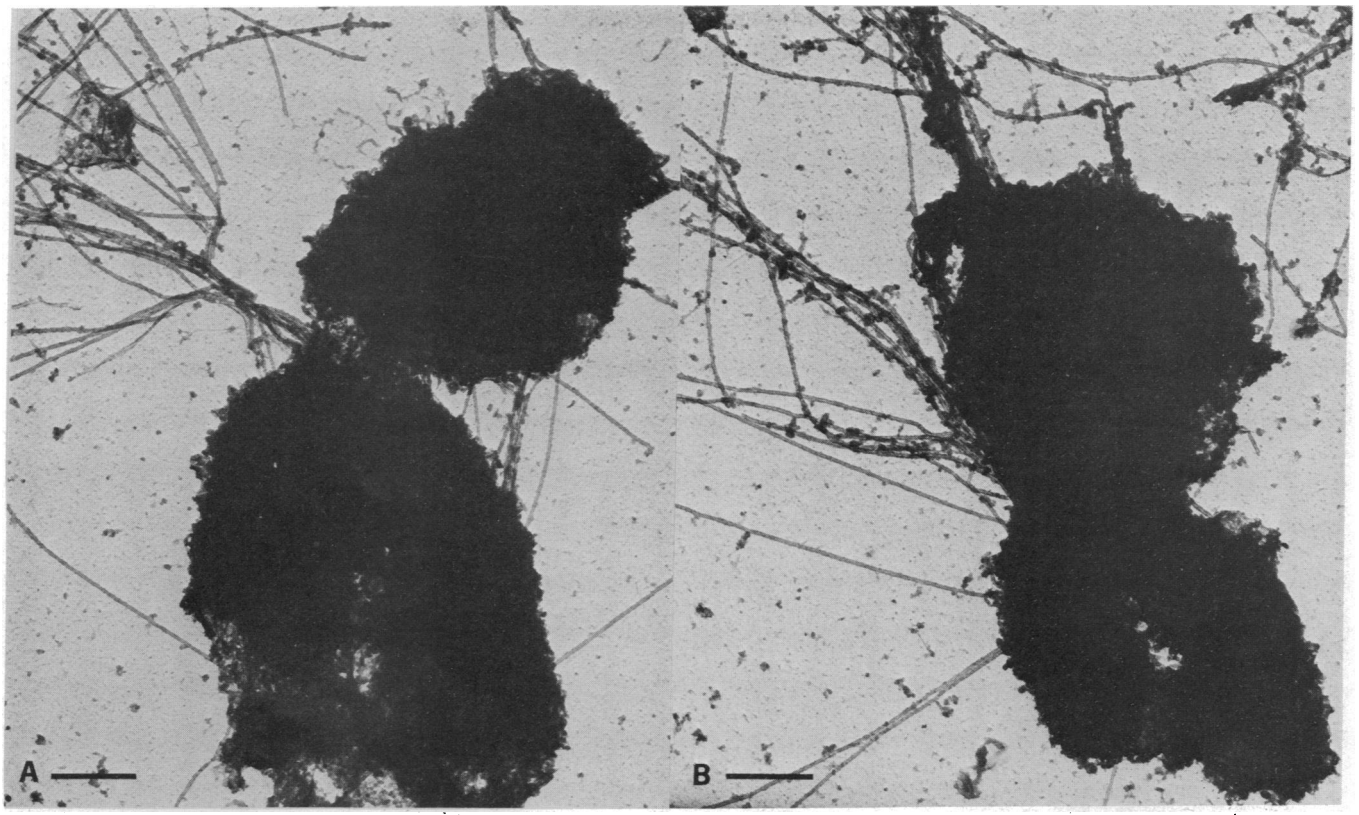

FIG. 2. Isolated HeLa chromosomes, immobilized on grids, incubated in tubulin, and stained with uranyl acetate. Bar = 0.5 μm.

crotubules as they pass over the chromosome. The majority of the microtubules in Fig. 3A emanate from the region of the primary constriction of the chromosome. Microtubules are occasionally bent, some are broken, presumably during handling, and fragments can be seen crossing over the long arm of the chromosome. Such pieces never represent more than a few percent of the total microtubule population; they are not found in the spaces on the grid between chromosomes. In Fig. 3B microtubules are observed to originate from two darkly stained discs, one of which is localized at a position characteristic of the kinetochore while the other is present on the upper half of the left chromatid; this may represent a kinetochore displaced from its normal position.

Further examples of chromosomes prepared by this procedure are shown in Fig. 3C and D. Microtubules radiate from specific regions in these chromosomes which have the staining characteristics of kinetochores.

DISCUSSION

The results in this report show that the kinetochores of isolated HeLa mitotic chromosomes are distinct morphological and functional units that serve as specific sites for the initiation of microtubule assembly. It is significant that the isolated chromosomes alone were never observed to contain microtubule remnants from the mitotic apparatus. Presumably, these microtubules were depolymerized during the isolation procedure at 4° in the presence of calcium, but the possibility cannot be excluded that residual subunits may remain. Initial experiments in which chromosomes and tubulin were used together in an *in vitro* assembly system were hampered by the fact that the optimal conditions for the isolation of chromosomes and for the assembly of tubulin were antagonistic. The presence of high concentrations of hexylene glycol and calcium ions, which were necessary to keep the chromosomes intact, inhibited microtubule assembly; conversely, the low calcium ion concentrations essential for microtubule assembly were detrimental to chromosome integrity. If isolated chromosomes were sedimented by centrifugation, resuspended in polymerization buffer containing tubulin, and then incubated at 37°, they were observed to undergo a rapid loss of form; as a result, microtubule assembly onto recognizable chromosomes could not be observed. To circumvent these problems, the chromosomes were first isolated in solutions containing hexylene glycol and calcium (CIB) and immobilized by attachment to grids to reduce structural deterioration. The grids were then rinsed with tubulin polymerization buffer to remove residual CIB. The kinetochores of these immobilized chromosomes maintained their integrity in the tubulin polymerization solution and served as sites for the assembly of microtubules.

The electron opacity of whole mount chromosomes, either unstained or stained with uranyl acetate, precluded visualization of the kinetochore as in Fig. 2 (also refs. 21 and 22). Observation of the point of origin of the microtubules which had assembled onto uranyl acetate stained chromosomes was not possible because of this opacity. Although the point of convergence of these microtubules appeared near the constriction of the chromosome, it could not be shown that there was a distinct structure serving as a nucleation site at that region (Fig. 2). These problems were overcome by the use of formaldehyde fixation and alcoholic PTA staining in which the kinetochore was preferentially contrasted and appeared as a darkly stained area on a lighter chromatin background. After staining by this procedure, the microtubules could be shown to originate from a specific region on the chromosome (Fig. 3).

It was occasionally observed that some chromosomes incu-

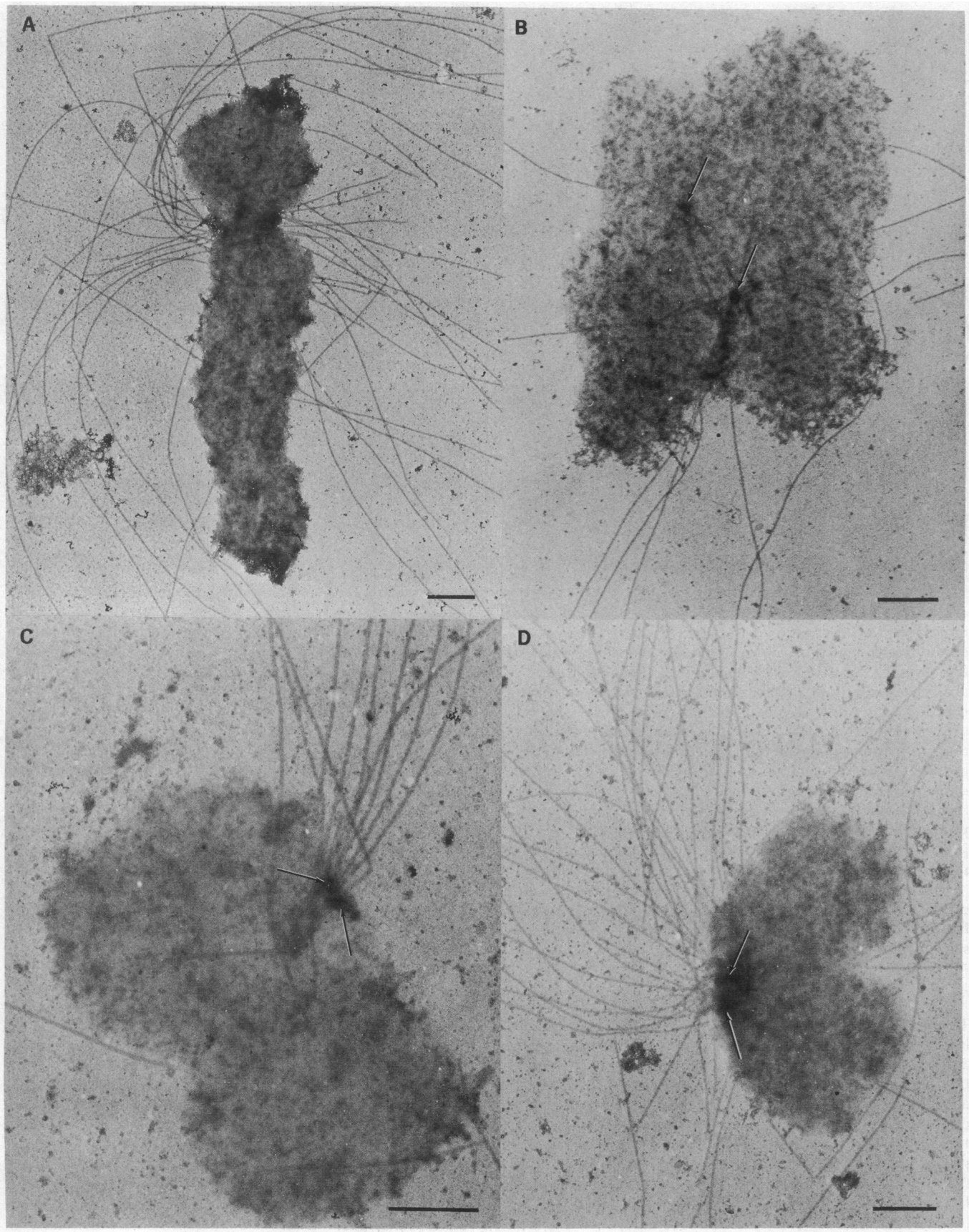

FIG. 3. Isolated HeLa chromosomes, immobilized on grids, incubated in tubulin, and then fixed in formaldehyde and stained with alcoholic PTA. Kinetochores are indicated by the arrows. Bar = 1 μm.

bated with tubulin failed to serve as assembly sites for microtubules although other chromosomes on the same grid did so. These chromosomes without microtubules resembled those in controls, as in Fig. 1; they possessed well-defined kinetochores at the primary constriction and maintained their morphology. The nature of the attachment of the chromosomes to the grid may have precluded assembly of tubulin onto a kinetochore if it were located between the chromosome and the grid. Only those kinetochores situated on the outer surface of the chromosome presumably would be free to initiate microtubule assembly.

Chromosomes onto which microtubules had polymerized were often observed to have undergone a change in morphology; the kinetochores from which the microtubules arose were sometimes displaced from their normal positions (Fig. 3B, C, and D). That the initiating sites are, in fact, displaced kinetochores is suggested by their size, shape, staining intensity, and the fact that no other kinetochore-like structure is present.

Such distortions of the kinetochores were not seen either in controls which had been treated in polymerization buffer alone or in tubulin-treated chromosomes upon which no microtubules had assembled. A reasonable explanation is that the microtubules attached to the kinetochore had caused it to become dislocated from the rest of the chromosome, possibly when the grid was transferred between solutions or when it was dried. However, the possibility cannot be excluded that the change in morphology may also reflect an alteration necessary for, or associated with microtubule assembly itself.

Chromosomes with apparently only one kinetochore also were observed but only in association with formed microtubules. These cases represent either the fusion of two kinetochores or the loss of one of them from the grid; anaphase chromosomes were rarely observed in controls. The second possibility, when taken together with the observed dislocation of the kinetochores, is a reasonable interpretation if it is considered (*a*) that the unfixed chromosome upon which polymerization occurs is a nonrigid structure, (*b*) that the attachment of microtubules to the initiating site is a firm and stable one, and (*c*) that the mass of microtubules may create an appreciable drag force which could dislodge and move the kinetochore from its normal place or even detach it completely from the chromosome.

From the present observations *in vitro*, the kinetochore appears to be the only chromosomal component that can initiate microtubule assembly. Establishment of this fact now makes possible the isolation and characterization of presumptive kinetochores from fractionated chromosomes by assessing their ability to serve as sites for microtubule assembly. The isolated kinetochores then may be analyzed for their macrocmolecular composition.

The mechanism by which chromosomes become associated with the microtubules of the mitotic apparatus *in vivo* is not known. The kinetochore could associate with preformed microtubules, or it could serve as an initiation site for the assembly of the chromosomal microtubules. In this report it is shown that in the absence of formed microtubules, the kinetochore itself has the capacity to act as an initiation site for the assembly of microtubules *in vitro*, and it may do so as well *in vivo*.

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