RIBONUCLEOPROTEIN STAINING OF CENTRIOLES AND KINETOCHORES IN NEWT LUNG CELL SPINDLES

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ABSTRACT

The distribution of ribonucleoprotein (RNP) within the mitotic spindle of newt lung epithelial cells was studied with the high voltage electron microscope (HVEM) using Bernhard's uranyl-EDTA-lead staining of thick sections in conjunction with the ribonuclease digestion of fixed cells. The results indicate that aside from ribosomes, the major RNP-containing components of the spindle are the kinetochores and centrioles, both of which stain electron-opaque after EDTA treatment. In both cases, the electron-opaque material associated with these microtubule organizing centers (MTOCs) can be removed by RNase digestion and cold perchloric acid (PCA) extraction under conditions which leave the spindle microtubules (Mts) centrioles, and kinetochores intact. The staining reaction is not abolished by cold PCA extraction alone or by substituting other positively charged proteins (i.e., cytochrome c or lysozyme) for RNAse. The RNP component of the kinetochore is closely associated with the bases of the kinetochore microtubules. The RNP component of the centriole can be seen to surround the microtubules of the triplet blades. No evidence was found to indicate the presence of RNP in the pericentriolar material. The possible function of both kinetochore and centriolar RNP is discussed.

KEY WORDS mitosis · kinetochores · centrioles · ribonucleoprotein · high voltage electron microscope

At present very little is known concerning the function of the centrioles and kinetochores during mitosis. Although similar with respect to their ability to direct microtubule (Mt) growth in vivo (13, 14, 17, 18) and in vitro (11, 20, 31, 32), these microtubule organizing centers, (MTOC's, see reference 24) are structurally quite different. This problem has prompted investigations into the ultrastructure and chemical composition of these organelles in hopes of understanding their role in the formation and function of the mitotic spindle. An unresolved question has been whether the kinetochores and centrioles contain ribonucleoprotein (RNP). Early preliminary autoradiographic (8) and enzymatic digestion (29) experiments on mitotic cells indicated the possible presence of RNP in both of these MTOCs. More recent in vivo (3, 14, 30) and in vitro (8, 29) experiments lend support to the suggestion that the centriole, its associated pericentriolar material, or both, contain RNP and that this RNP is needed for in vivo function (3, 14). However, the exact location and function(s) of this RNP are unknown.

Few studies have been designed to investigate the presence of RNP at the kinetochore. Braselton (6) applied Bernhard's (2) staining method, which allows for the preferential staining of structures at the kinetochore.
known to contain RNP, to chromosomes of *Allium cepa* root tips. His results suggested the presence of RNP at kinetochores. However, his RNAse digestion destroyed the kinetochore Mts. Thus, he was unable to conclusively demonstrate a kinetochore RNP component since he could find only presumptive kinetochores in his RNAse-digested material.

Recently, this same procedure has been used to localize RNP in the outer plate component of trilaminar kinetochores in HeLa cells arrested during mitosis with colchicine (5). However, colchicine-treated kinetochores are structurally modified when compared to untreated controls; the size of the outer plate is exaggerated while the inner plate is reduced or completely lacking (5, 7, 23, 27).

In the present study, Bernhard's uranyl-EDTA-lead staining method was used in conjunction with the protease-free RNAse digestion of fixed cells to detect and localize RNP components within the astral spindle of the newt. To facilitate the detection of MTOC-associated RNP, thick sections (0.25 μm), which contain more of the structures of interest, were observed and photographed with a high voltage electron microscope (HVEM). The results indicate that both the kinetochores and centrioles in the newt spindle contain tightly associated RNP.

**MATERIALS AND METHODS**

Primary cultures of *Taricha granulosa* lung epithelial cells were grown on 22-mm² carbon-coated cover slips by methods previously described (25, 26). Suitable metaphase or early anaphase cells were photographed with phase optics and immediately fixed for 40 min with 2% glutaraldehyde in 0.05 M Millonig's phosphate buffer. After fixation each culture, containing one to five mitotic cells, was either: (a) postfixed for 1 h in 1% OsO₄ in 0.05 M phosphate buffer before dehydrating and embedding in Epon; (b) washed in 0.05 M phosphate buffer for 1–24 h, dehydrated, and embedded directly without postfixation, or (c) treated with pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) for 1–5 h at 23°C, washed in phosphate buffer, exposed to 5% perchloric acid (PCA) for 1/2 h at 4°C (see reference 1) and washed for 5–10 h in phosphate buffer before embedding in Epon without postfixation. The ribonuclease solution (1.0 mg/ml in distilled H₂O, brought to pH 6.8 with dibasic sodium phosphate; see reference 9) was boiled for 5–10 min before its use to destroy contaminating enzymes. The resulting solution, when assayed for trypsin equivalent units, was found to contain negligible protease activity. In some cases the positively charged proteins cytochrome c and lysozyme, similar in molecular weight to pancreatic ribonuclease, were substituted for RNAse in the solution described above. These control solutions were used exactly as RNAse in the scheme previously outlined.

Mitotic cells were then cut from the embedded cultures, mounted on plastic pegs, and serially sectioned on a Sorvall Mt-2B microtome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). Control cells were sectioned at thickness ranging from 0.1 to 0.5 μm. Those cells that were not postfixed and that were to be stained by Bernhard's (2) method, with or without prior RNAse treatment, were sectioned at 0.25 μm. All sections were picked up on slot grids coated with 0.5% Formvar.

Thin sections were stained for 30 min in 5% uranyl acetate at 23°C followed by 5 min in lead citrate; 0.25-μm sections were stained in 5% uranyl acetate for 90 min at 60°C followed by 20 min in lead citrate at room temperature; and 0.5-μm sections were stained for 4 h in 5% uranyl acetate at 60°C followed by 40 min in lead citrate at room temperature.

Glutaraldehyde-fixed cells with or without RNAse treatment were stained by a modification (28) of Bernhard's RNP-staining procedure. Briefly, 0.25-μm sections were stained in 5% uranyl acetate for 90 min at 60°C followed by a 60°C incubation in either 0.2 M EDTA or dH₂O for 1/2–3 hr. The sections were then washed for 5–10 min and poststained for 15–20 min in lead citrate at room temperature.

All grids were examined and photographed with the AE1-7 HVEM operated at 1,000 kV, using a 35-μm objective aperture.

**RESULTS**

**General**

The structure of *Taricha* metaphase and anaphase spindles prepared by conventional fixation (glutaraldehyde and OsO₄) and staining (uranyl acetate and lead citrate) procedures has been reported elsewhere (25, 26) and will not be described here. Uranyl- and lead-stained spindles fixed only in glutaraldehyde (Fig. 1) are structurally similar to those postfixed in OsO₄. The most notable difference was that those not postfixed in OsO₄ displayed a decrease in the final contrast of the spindle Mts and differential staining of the structural elements of the kinetochore (see below).

The action of EDTA can be seen by comparing Fig. 1 and 2. In general, EDTA destains all components of the spindle, though at different rates, by chelating and removing uranyl ions from the section (2). This destaining is progressive and is influenced by temperature and section thickness. On a 0.25-μm section, a 50- to 60-minute
FIGURE 1 An early anaphase half spindle fixed in glutaraldehyde and stained with uranyl acetate and lead citrate. Notice the staining of the chromosomes, centriole (arrow), spindle microtubules, and background material. Bar. 2 μm. × 8,750. All sections 0.25 μm unless otherwise stated.

FIGURE 2 Same treatment as in Fig. 1, except that this section was destained with EDTA for 100 min at 60°C before poststaining with lead citrate. The early anaphase chromosomes and spindle fiber Mts have lost most of their contrast. The centriole pair (arrow) remains densely stained. No kinetochores are present in this section. Bar, 2 μm. × 8,750.

EDTA treatment at 60°C decreases the contrast of the spindle Mts and of most background components, but fails to destain the chromosomes, kinetochores, centrioles, and background ribosomes. After an 80- to 90-min EDTA treatment, the chromosomes begin to lose their contrast and appear mottled, and by 110-120 min they are uniformly “bleached.” At this time, the most conspicuous components of the spindle are the kinetochores, ribosomes, and centrioles which retain their electron-opaque appearance. Further treatment with EDTA decreases the contrast of kinetochores and ribosomes first, and, after a prolonged treatment (160-200 min), also that of centrioles.

Kinetochores

In Taricha, conventionally fixed and stained metaphase and early anaphase kinetochores are similar in appearance to the ball and cup structure described for many plants (see references 21 and 22). In this case, the kinetochore Mts penetrate into a lighter staining ball which is surrounded by a dense staining cup indistinguishable from the chromosome (Figs. 3 and 4).

After ~60 min in EDTA, the chromosomes begin to appear bleached while the kinetochores retain much of the uranyl stain (Fig. 5). By 110-120 min in EDTA at 60°C, the kinetochores appear as electron-opaque regions, 0.3-0.5 μm in diameter, and are closely associated on one side with the bleached chromatin (Figs. 6 and 7), and on the other with numerous faintly stained Mts. When viewed from various tilt angles (Fig. 8), these electron-opaque regions can be seen to be associated with the bases of the kinetochore Mts on the kinetochores. This area corresponds closely with the outer region of the lighter staining ball.
structure of conventionally stained kinetochores (cf. Figs. 4 and 8).

The electron-opaque material of the kinetochore is partially removed after a 2-h RNAse digestion and a 30-min cold PCA extraction (Fig. 9 A). This indicates that the 1/2-h cold PCA treatment, which has been shown to differentially extract RNA from tissues without extraction proteins (1), is not sufficiently long enough to remove completely the electron-opaque material of the kinetochore. However, the electron-opaque component of the kinetochore is completely absent if the digestion with RNAse is extended to 4-5 h (Fig. 9 B).

After 4-5 h of RNAse digestion, the kinetochores appear as lightly stained regions on the chromosomes into which numerous Mts insert (Fig. 9 B). They are continuous with and indistinguishable from the chromosome. When compared to nondigested controls, the Mts of RNAse-digested kinetochores are not so smooth in appearance and stain slightly more electron-opaque. The chromosomes remain completely bleached. Substitution of cytochrome c (Fig. 10 A) or lysozyme (Figure 10 B) for RNAse does not block the staining reaction of the kinetochore, even after a 4- to 5-hour treatment and cold PCA extraction.

**Centrioles**

Conventionally fixed and stained centrioles of *Taricha* metaphase and early anaphase spindles appear as paired cylinders approx. 0.2 x 0.5 μm long. They are composed primarily of the nine Mt triplet blades and also of an osmiophilic substance which surrounds the Mts of the triplet blades (Figs. 11 and 12).

After 60 min in EDTA, the centrioles can be recognized as electron-opaque structures in the...
center of a weakly stained centrosome (Fig. 13). At the time when the chromosomes have completely lost their contrast (i.e., 110 min in EDTA at 60°C), the centrioles appear as the most heavily stained component of the spindle (cf. Fig. 2). Cross sections throughout the polar area of such cells when viewed at various angles (Fig. 14) indicate that the areas of high electron opacity appear to be closely associated with the triplet blades of the centrioles. In longitudinal sections (Fig. 15), the electron-opaque material can be seen to run the entire length of the triplet blades of the centriole. If there is an appreciable amount of pericentriolar material in Taricha, then it does not appear electron-opaque after 110 min of EDTA treatment (Figs. 13 and 15). Mts of the aster appear faintly stained.

The electron-opaque material is completely absent in sections of RNase-treated and similarly stained metaphase and early anaphase centrioles (cf. Fig. 16 with Figs. 11 and 14). After RNase digestion and PCA extraction, the centriole appears as a hollow cylinder composed of Mts which have the same electron density as the Mts of the aster. Substitution of cytochrome c or lysozyme for RNase does not block the staining reaction of the centriole (Fig. 17 A and B).

**DISCUSSION**

The observations reported here indicate that kinetochores and centrioles in Taricha lung cells contain a component which can be selectively stained by a method (2) which preferentially stains RNP. This staining reaction is abolished by RNase digestion followed by cold PCA extraction. It is not abolished by cold PCA extraction alone, or by substituting other positively charged proteins (i.e., cytochrome c or lysozyme) of similar molecular weight for RNase. These results support the contention that RNase removes this component from the kinetochores and centrioles and that the extinction of the staining reaction of the kinetochore and centriole

**FIGURE 11** Thin cross section of a metaphase centriole fixed in glutaraldehyde, postfixed in OsO4, and stained with uranyl acetate and lead citrate. Notice the electron-opaque material which surrounds the triplet blades of the centriole and lumen. Bar, 0.5 μm. × 35,000.

**FIGURE 12** (A and B) Serial longitudinal sections through the centrioles of a metaphase spindle. This cell was fixed only in glutaraldehyde and stained with uranyl acetate and lead citrate. Note that the electron-opaque material is present along the entire length of the centriole Mts. Bars (A and B), 0.5 μm. × 36,000.

**FIGURE 13** Low power micrograph of the centrosome of a metaphase cell fixed and stained as in Fig. 12, except that it was destained in EDTA for 60 min at 60°C before poststaining in lead. The centrioles appear more electron-opaque than the astral Mts. If there is an appreciable amount of pericentriolar material in Taricha centrosomes, then it does not stain electron-opaque. Bar, 1.0 μm. × 18,000.

**FIGURE 14** Centriole pair of an early anaphase cell fixed in glutaraldehyde, stained in uranyl acetate for 90 min at 60°C, destained in EDTA for 110 min at 60°C, and poststained in lead citrate. Fig. 14 B is tilted 25° with respect to Fig. 14 A in the horizontal axis. An electron-opaque material can be seen to surround the triplet blades (B) of the centriole. Mts of the centrosome appear faintly stained. Compare with Figs. 11 and 16. Bar, 0.5 μm. × 36,000.

**FIGURE 15** (A–C) Serial sections through the centrosome of a metaphase cell fixed and stained as in Fig. 14. Electron-opaque material can be seen to be closely associated with the Mts of the centrosome. Bars (A–C), 0.25 μm. × 44,000.

**FIGURE 16** (A and B) Early anaphase centrioles digested with RNase for 5 h and PCA for 30 min before embedding and staining as in Figs. 14 and 15. The electron-opaque material seen to surround the Mts of the triplet blades is removed by this treatment. Notice that the centriolar Mts are now as electron-opaque as those of the centrosome. Bars (A and B), 0.5 μm. × 36,000.

**FIGURE 17** (A) A metaphase centriole stained as in Figs. 14–16. This cell was treated with cytochrome c for 5 h and cold PCA for 30 min before embedding. The electron-opaque material associated with the centriolar Mts is still present. Bar, 0.5 μm. × 36,000 (B) Similar to Fig. 17 A, except that lysozyme was substituted for cytochrome c. Bar, 0.25 μm. × 36,000.
is not due to the nonspecific binding of RNase. They strongly suggest that the kinetochore and centriole contain an RNP component.

That the kinetochore contains RNP was first suggested by Brinkley and Stubblefield (8) who viewed it as a specialized gene. Preliminary high resolution autoradiographic evidence suggested to them that kinetochores actively incorporate radioactive RNA precursors. Since then, Braselton (7, see also reference 5) has shown that kinetochores in *Allium cepa* stain electron-opaque with Bernhard's (2) staining procedure, a method which preferentially stains cell structures known to contain RNP. This staining procedure is abolished by RNase treatment, suggesting that the kinetochore does indeed contain RNP. However, Braselton was unable to definitely demonstrate that the stained regions were in fact kinetochores in RNase-digested material since his digestion destroyed the kinetochore Mts. The present results, in which the kinetochore Mts were preserved during protease-free RNase digestion, confirm Braselton's expectations and further indicate that in *Taricha* the RNP component is associated with the bases of the kinetochore Mts.

Howell (15) has recently used a Giemsa-silver staining procedure to visualize the RNP associated with the nucleolar-organizing region in cricket oocyte chromosomes. This staining procedure has also been used to visualize centrioles and the nucleolar organizer region in squashes of human metaphase chromosomes (16). In addition, careful scrutiny of Figs. 1 b and 3 b from the work of Howell et al. (16) shows that the kinetochores are also stained, an observation not mentioned by these authors (see also reference 19). Thus, at the light microscope level, silver stain, which has been shown to preferentially stain RNP (15), stains both centrioles and kinetochores. These observations provide further evidence for the existence of RNP in these structures. Thus, besides the protein tubulin (23), there is at least one other type of macromolecule that is common to both of these MTOC's. Whether other types of MTOC's (i.e., spindle polar bodies, blepharoplasts, persistent polar complexes, etc.) also contain RNP is currently being investigated.

At this time, the function and composition (i.e., whether a heterogeneous or homogeneous RNP population is present) of the kinetochore RNP are unknown. An appealing idea is that this kinetochore RNP is a gene product which has a role in regulating either the function of the kinetochore (see reference 8) or the timing of the kinetochore cycle (i.e., its maturation) during mitosis, or both.

It is presently unknown when, in the cell cycle, kinetochore RNA is synthesized. However, Gonzalez-Fernandez et al. (10) have shown that *Allium cepa* root tip cells treated with inhibitors of RNA synthesis or with RNase during prophase fail to undergo normal prometaphase chromosome movements and are thus arrested in prophase. These authors conclude that a specific RNA is synthesized during prophase which is necessary for the prophase-metaphase transition. EM autoradiographic studies are currently being planned to determine whether the RNA described by these authors is the RNA component of the kinetochores.

The present study also reports the direct visualization of the tightly bound RNP component of the mitotic centriole. This observation is in agreement with early enzymatic digestion experiments of glutaraldehyde-fixed spindles (29) which indicate the presence of RNA on the inner surface of the triplet blades and at the foot of the A tubule. Other recent investigations (14) have shown that isolated basal bodies from *Tetrahymena* and *Chlamydomonas* contain a tightly bound RNase-sensitive component which remains associated with the basal body through both velocity and density gradient sedimentation. These results corroborate those published on the ultrastrucural effects of nucleases which indicate the presence of RNase-sensitive material within the lumen of *Paramecium* basal bodies (9).

Thus, there is ample evidence to suggest that the mitotic centriole and basal bodies contain tightly bound RNP. However, other experiments with enzymatic digestion (8) and laser irradiation of acridine orange-sensitized mitotic cells (3, 4) suggest that the pericentriolar material, which has been shown to nucleate the growth of Mts in vitro (11), may also contain RNP. The results presented here indicate that, with the methods used in this study, the pericentriolar material in the newt does not contain a detectable amount of RNP. However, newt lung cells, unlike newt fibroblasts (21, 22), contain very little pericentriolar material. Further studies are presently being conducted by the author to determine whether the large amount of pericentriolar material in certain mammalian cells (i.e., PtK1,
Chinese hamster ovary) contains RNP.

The function(s) of centriolar RNP is presently unknown. In *Taricha*, the RNP component, which extends the full length of the centriole, surrounds the Mts of the triplet blades. This location suggests that it may play a structural role in forming or maintaining the arrangement of the Mt blades of the centriole (see Fig. 14). The tightly bound RNP component has recently been implicated in the Mt generating and organizing capacity of the centriole. For example, Heidemann et al. (14) have shown that a tightly bound RNase-sensitive centriolar RNP component is responsible for the initiation of aster formation when injected into eggs of *Xenopus laevis*.

Experiments with actinomycin (30) indicate that RNA synthesis is necessary for procentriole formation, suggesting that RNA plays a role in nucleation of new centrioles. This theme was later expanded by Hartman (12) who believes that the centriolar RNP, like the ribosome, is morphic and can localize itself at the site of the old centriole before nucleating a new one. Went (33) theorizes that the RNP of the centriole represents the primary genome of the organelle which is replicated via a DNA intermediate with the aid of a reverse transcriptase. These results suggest that there may be more than one type of centriolar RNA: one responsible for the nucleation of new centrioles and another for initiating the growth of Mts.

The author would like to thank Dr. H. Ris, Dr. J. Lilien, Mr. J. Grunwald, and Mr. C. Cyper for their comments and support during the writing stages of this investigation. The author is also indebted to Mr. H. Howard (University of Oregon, Eugene) for his generous photographic assistance.

This work was supported by National Institutes of Health research grant RR 00570 from the Biotechnology Resources Branch, Division of Research Resources to the AEI-EM7 HVEM, University of Wisconsin at Madison.

Received for publication 12 May 1978, and in revised form 21 August 1978.

REFERENCES