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Effects of RNase and RNA on In Vitro Aster Assembly

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RNase alters the in vitro assembly of spindle asters in homogenates of meiotically dividing surf clam (Spisula solidissima) oocytes. Some effects of RNase, such as reduced astral fiber length, appear nonenzymatic and probably result from RNase binding to tubulin. However, RNase-induced changes in the microtubule organizing center are also observed. Since other polycations can mimic RNase effects, the existence of an RNA component of the spindle organizing center remains uncertain.

Effects of RNase and other polycations on astral fiber length can be prevented and reversed by the RNase inhibitor, polyguanylic acid. Polyguanylic acid can also augment astral fiber length in the absence of added RNase or other polycations. Augmentation by polyguanylic acid is favored by high ionic strength, and can be duplicated by polyuridylic acid and, with less efficiency, by polyadenylic acid. Polycytidylic acid and unfractionated yeast RNA, however, are unable to augment aster assembly. Polyguanylic acid can also augment the length of astral fibers on complete spindles isolated under polymerizing conditions. These results demonstrate that specific polyribonucleotides can alter spindle assembly in vitro. The presence of an inhibitor of microtubule assembly in Spisula oocytes, which can be inactivated by specific RNAs, is suggested.

Key words: centrioles, microtubule assembly, microtubule organizing centers, mitotic spindle

INTRODUCTION

The presence of nucleic acid in microtubule-containing organelles has been deduced from cytochemical and analytical studies on the mitotic spindle (1–4) and basal bodies (5–8). Isolation of a specific basal body RNA has been reported (8), and recent electron microscopic studies have revealed specific RNase-sensitive structures in these organelles (9). In addition, the presence of RNA in kinetochores (10), and in the pericentriolar material of spindle microtubule organizing centers (MTOCs) (1, 11–13) has been suggested.

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Although there are many reports suggesting interactions between nucleic acids and tubulin, little is known about the functional significance of such interactions. Spindle microtubules may be closely associated with ribosomes (2–4), and other studies indicate that microtubules may function in the intercellular transport of ribosomes in Hemipteran ovaries (14). Such a process could require specific interactions between microtubules and RNA. Tubulin-ribosome complexes have been reported in chromatoid bodies of Entamoeba invadens cysts (15), and recent evidence for the presence of nucleic acid and tubulin in perinuclear dense bodies of Planarian neoblasts has been obtained (16). It is possible that such complexes consist of inactive storage forms of tubulin in cells where immediate microtubule assembly is not required. The recent demonstration that RNA inhibits the in vitro polymerization of mammalian brain tubulin by binding to factor(s) required for assembly (17) suggests a mechanism by which microtubule assembly could be regulated by RNA.

Much less clear, however, is what role RNA might have in organelles concerned with the initiation of microtubule assembly. According to the exogenous theory of centriole origin proposed by Margulis (18), nucleic acid would be expected in these organelles because they are "autonomously replicating" structures, and, as in mitochondria and chloroplasts, nucleic acid with an informational (i.e., coding) function would not be surprising. Brinkley and Stubblefield on the other hand have proposed that RNA in centrioles and kinetochores may be directly involved in the initiation of spindle microtubule polymerization (11), and there is some support for this view, since ethidium bromide treatment induces both spindle and centriole abnormalities (19).

Since the development in this laboratory of an in vitro system for repolymerization of microtubules onto spindle MTOCs (20), we have sought to determine if RNA has a functional role in spindle assembly. We have found that RNase causes various morphological changes in asters assembled in vitro, including changes in the MTOC, but we are unable to demonstrate that these changes result from RNase interaction with an RNA component of the MTOC. We have also found that certain polyribonucleotides, but not all, augment aster assembly by a mechanism probably unrelated to nonspecific RNA inhibition of brain tubulin polymerization (17). Our results suggest that Spisula oocytes contain an inhibitor of microtubule assembly, with an affinity for specific RNA homopolymers.

METHODS

Spisula solidissima oocytes, obtained from ovaries removed from live animals, were washed in at least three changes of 300 volumes of natural seawater, and parthenogenetically activated by addition of 1 ml of a 0.52 M KCl solution to 14 ml of seawater in which about 1 ml of oocytes (packed volume) were suspended. Oocytes were allowed to develop at room temperature to first meiotic metaphase (13 min), rapidly washed by hand centrifugation in two 15 ml changes of glycerol-phosphate buffer (1 M glycerol, 1 mM potassium phosphate, pH 8.0), and resuspended at 0–4°C in an equal volume of 0.5 M MES buffer [2-(N-morpholino) ethanesulfonic acid], pH 6.6. The temperature of the glycerol-phosphate buffer wash was important for some experiments, but unless otherwise indicated in Results, the wash was performed at room temperature (about 22°C). Resuspended oocytes were homogenized as previously described (20), and the crude homogenate was clarified for 5 min at 5,000 rpm in a Sorvall RC2B centrifuge with an SS-34 rotor, at 4°C.

In vitro assembly of asters was initiated by incubation of clarified homogenates at either 28°C or at 37°C. As described previously (20), aster assembly in Spisula oocyte homogenates does not require addition of Ca ion chelators or exogenous GTP, and these were routinely omitted in experiments described here. Their presence, however, did not affect results.

Polymerization was terminated and asters fixed for light microscope photography by rapid addition of five volumes of Kane's spindle isolation medium (1 M hexylene glycol, 10 mM potassium phosphate, pH 6.2), at room temperature, followed by immediate chilling to 0°C. Asters were photographed within 1 hr after stabilization using the 40 × objective of a Zeiss Ultraphot microscope with phase contrast optics, on Polaroid 55 PN film.

For electron microscopy, samples were fixed in five volumes of 3% gluteraldehyde in 0.5 M MES buffer added directly to homogenates at the polymerizing temperature, followed by incubation for 30 min at 28°C and collection of asters by centrifugation, or, in some experiments, samples were prestabilized in Kane's medium as described for light microscopy, and asters were collected by centrifugation and fixed for 1 hr at 0°C in Kane's medium containing 3% gluteraldehyde. Asters were postfixed in 1% OsO_4, dehydrated in ethanol, and embedded in epon. Thin sections were stained with aqueous uranyl acetate and lead citrate and photographed in a Philips model 300 electron microscope.

RNase A (protease free) and RNase B as well as other basic proteins and 5'-polynucleotides were obtained from Sigma Chemical Co. Yeast RNA was obtained from Schwartz Bioresearch Co. All other chemicals were reagent grade.

RESULTS

Effects of RNase

Asters formed in the presence of 0.1 mg/ml (8×10^{-6} M) RNase A or RNase B at 28°C exhibit several distinct differences from controls. While there is considerable variability between controls of different preparations, asters assembled in the presence of RNase appear much more symmetrical than controls, and fiber length, compared to controls, is greatly reduced (Figs. 1a,b). Such asters do not exhibit reduction in the number of fibers, however. This is in contrast to the effects of GMP, which also inhibits aster assembly, presumably by competing with GTP binding to tubulin, and results in formation of asters with reduced numbers of fibers. Frequently, asters assembled in RNase appear to actually have more fibers than controls.

Other differences commonly but not always observed in all of the asters formed at these low (0.1 mg/ml) RNase concentrations are changes in the appearance of the organizing center. One such change is the development of a darker, more phase-dense appearance of this structure. A second type of change often observed, which is much more distinctly different from controls than the darkening described above, is the development of a light, "washed out" appearance of the MTOC. This effect becomes even more pronounced when higher RNase concentrations (0.5 mg/ml) at higher incubation temperatures (37°C) are used (Figs. 2a, b). At these higher concentrations, darkening of the centers often occurs initially, followed by development of the light appearance after longer incubation.

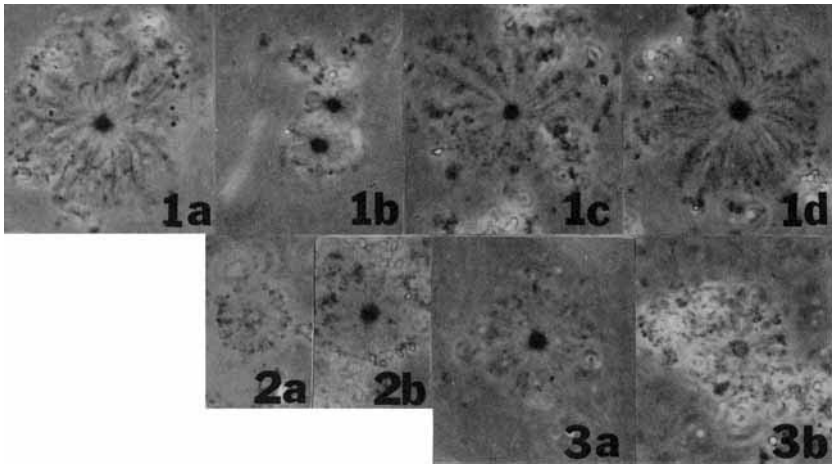

Fig. 1. Reduction of astral fiber length by RNase and reversal by Poly (G). Asters were assembled at 28°C for 14 min without additions (a), with 0.1 mg/ml RNase A (b), or assembled in 0.1 mg/ml RNase A for 14 min and further incubated an additional 6 min after adding 1 mg/ml Poly (G) (c). Figure 1d shows aster from the same preparation formed in the presence of 0.1 mg/ml protamine, 28°C, 14 min incubation. Protamine has no effect under these conditions. × 480.

Fig. 2. Effect of RNase on MTOC appearance. Asters were formed at 37°C, 20 min incubation, with (a) or without (b) 0.5 mg/ml RNase A. Note the pale, "washed out" appearance of the organizing center. × 480.

Fig. 3. Effect of protamine on MTOC appearance. Asters were formed at 28°C, 10 min incubation, without (a) or with (b) 2 mg/ml protamine. × 480.

The light appearance of the MTOC is always clearly visible, when present, with the 40 × high dry objective using phase contrast, but is usually less apparent when oil immersion is employed. The light appearance of the MTOC is also observed in many asters of some preparations without RNase or other treatments, although such preparations are quite uncommon. It can also be induced in many preparations by prolonged incubation at 37°C, but only after it is already apparent in RNase-treated samples of the same preparation.

The effect of RNase on fiber length is highly specific for the active form of the enzyme. Reduction of fiber length has been observed at concentrations of RNase A as low as 0.075 mg/ml at 28°C. Oxidized RNase A, however, which is enzymatically inactive, can reduce fiber length and cause MTOC lightening as well, but only at concentrations above 6 mg/ml. Protamine and histone, also basic proteins that bind nucleic acids, can induce apparently identical changes in MTOC morphology and also appear to reduce astral fiber length at concentrations of 1.5–2 mg/ml at 28°C (Figs. 3a,b). As is the case with RNase, lower protamine concentrations become more effective in inducing MTOC lightening when longer incubation times at higher temperature (37°C) are used. It should be noted, however, that while protamine is about as effective as RNase in inducing MTOC lightening, only RNase was found to be effective in inducing assembly of small uniform diameter asters at low concentrations (Figs. 1, a,b, and d).

The basic polypeptides spermine and spermidine, at 10 mM, can also induce the light appearance of the organizing center, but effects on fiber length are difficult to determine because of coating of fibers with particulates in the presence of these peptides. DNase I has no visible effects on aster assembly at the highest tested concentrations (4 mg/ml). While RNase T_2 and snake venom phosphodiesterase reduced both size and number of asters in some preparations, consistent results with these enzymes, unlike those with the pancreatic RNases, have not been obtained.

Electron microscope observations of thin sectioned-asters formed in RNase suggest ultrastructural changes in the MTOC, and an apparent change in the association of the centriole with the pericentriolar organizing material. While controls incubated at 28°C are generally observed to contain a distinct centriole (Fig. 8a), many asters assembled in the presence of 0.1 mg/ml RNase A appeared to lack centrioles (Fig. 8c) even though the sections appeared to pass through areas large enough, and thus close enough, to the centers of the asters to reveal centrioles in controls. In addition, the pericentriolar material in these asters appears more condensed than in controls. In the same sections, apparently "free" centrioles, associated with small amounts of organizing material and only a few microtubules, are also observed (Fig. 8d). Asters formed in RNase that do contain centrioles are also found, but in these asters the pericentriolar material still appears more condensed than metaphase controls (Fig. 8b), and such asters resemble those from earlier meiotic stages (see Weisenberg and Rosenfeld, Ref. 20). It should be noted that centrioles are often clearly visible in RNase-treated asters viewed with phase contrast, even when the phase-light appearance of the MTOC is quite pronounced. It is possible, therefore, that loss of the centriole occurs after fixation in RNase-treated asters.

In preliminary experiments attempting to clarify the relationship between the light microscopic image and the ultrastructural appearance of the MTOC, asters were sectioned that had first been assembled at 28°C for 5 min, and then incubated for 16 min at 37°C with either 2 mg/ml RNase A, with 2 mg/ml protamine, or without additions. In these preparations the light appearance of the MTOC was present in nearly all RNase- and protamine-treated asters but not in controls. Sections revealed no centrioles in any of the RNase- or protamine-treated asters observed, while some control asters containing centrioles were found. Many of the controls, however, appeared to lack centrioles, and while asters with highly condensed organizing centers were present in all three samples, only the control sample also contained asters of normal appearance. Our electron microscope results, therefore, though preliminary, do not suggest a strict correlation between the phase contrast and ultrastructural appearance of the organizing center. It should be noted, however, that longer incubation of controls produces lightening of the MTOC, and it is possible that events leading to alterations in ultrastructure occur before light microscopic changes are visible.

While duplication of RNase effects by protamine suggests that RNase does not act on an RNA component of the MTOC, other explanations for this result deserve consideration (see Discussion).

Effects of RNA

The reversibility of RNase effects was investigated using RNase inhibitors. Bentonite, a diatomecious earth that adsorbs RNase (21) can prevent the effects of RNase if added prior to incubation with the enzyme. However, polyguanylic acid [Poly (G)], which is a potent inhibitor of many ribonucleases (22), is able not only to prevent RNase effects, but

is also able to reverse RNase-induced reduction of astral fiber length at 0.5–1 mg/ml (Fig. 1c). Polyadenylic acid [Poly (A)], which also inhibits RNase activity but with less efficiency than Poly (G), also augments fiber length of asters treated with RNase, but to a lesser extent than Poly (G). Poly (G) can also augment fiber length of asters treated with protamine. Attempts to obtain reversal of MTOC changes have led to variable results: In some experiments, both fiber length and MTOC morphology, as viewed by phase contrast, appeared to be restored to normal after addition of Poly (G), but in other preparations only fiber length was restored.

In addition to its ability to reverse RNase effects, Poly (G) was found to augment astral fiber length in the absence of added RNase or other basic proteins. The ability of Poly (G) to augment fiber length varied from preparation to preparation when oocytes were washed in glycerol-phosphate buffer at room temperature. In such preparations, when controls exhibited small asters, augmentation by Poly (G) was pronounced. When controls exhibited asters that were already quite large, little change was induced with Poly (G). In the latter preparations, however, smaller asters could be obtained by either precycling homogenates through assembly-disassembly with alternate warm and cold incubations, or by allowing preparations to stand for several hours at 0°C before adding Poly (G). Poly (G) was able to augment the fiber length of the smaller asters thus obtained (Fig. 5).

These results suggested to us that Poly (G) may act by sequestering an inhibitor of microtubule assembly, which is present and initially inactive in preparations exhibiting large asters, but which could be activated or released from an inactive, complexed form during precycling or aging of the homogenates. Small differences in ionic strength from preparation to preparation, due to the variable degrees of oocyte swelling in the glycerol-phosphate buffer wash, might therefore account for the variable effect of Poly (G) by affecting the release of a complexed inhibitor.

In order to test this idea, we examined the effects of temperature during the wash procedure on Poly (G) augmentation, since we have found that the temperature of the glycerol-phosphate wash changes the degree of oocyte swelling.

When oocytes are washed at 37°C, the swelling that generally occurs at room temperature is greatly reduced. Washing the oocytes at 4°C, on the other hand, results in swelling to over twice their original packed volume. Since the wash buffer contributes very little to the ionic strength but very much to the total volume after resuspension in 0.5 M MES, the differences in swelling will result in a lower final ionic strength in preparations washed at 4°C than those washed at 37°C. We have found that asters prepared from preparations washed at 37°C are very small, and in some experiments, there is no apparent assembly. Preparations washed at 4°C, however, display very large asters. As predicted, the small asters formed at the higher ionic strength (washed at 37°C), were greatly increased in size by 1 mg/ml Poly (G), while those formed at lower ionic strength (washed at 4°C), already large, were not augmented by Poly (G), and inhibition by Poly (G) was sometimes observed.

While the above results demonstrate that differences in oocyte swelling correlate with the variable response to Poly (G), the possibility remained that the temperature of the wash per se, or the reduced concentration of oocyte component(s) in the more diluted 4°C preparations, affected the response to Poly (G). In order to demonstrate that the ionic composition alone (either monovalent ions or the MES zwitterion itself) affects the response to Poly (G), we performed the dilution experiments described below.

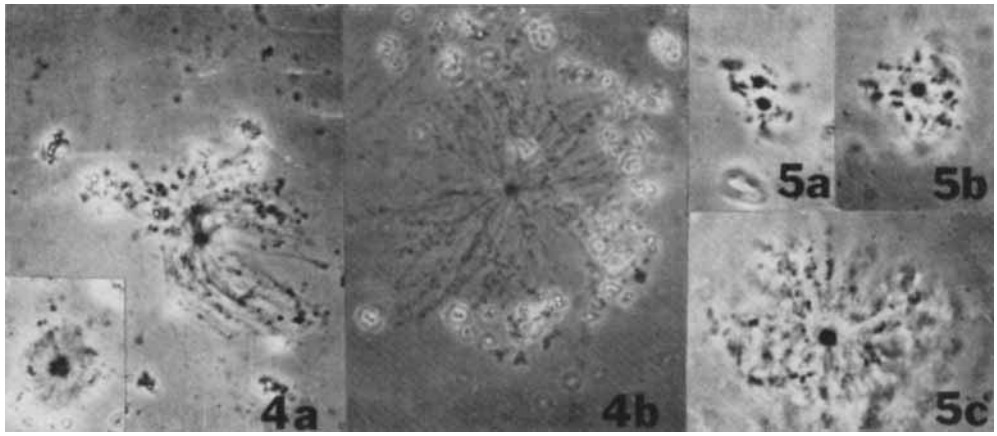

Fig. 4. Effect of RNA homopolymers on astral fiber length. Asters were prepared from homogenates of oocytes washed at 37°C. Homogenates were incubated at 28°C for 10 min with 1 mg/ml Poly (U) (a) or 1 mg/ml Poly (G) (b). Inset in Fig. 4a shows a control aster assembled without additions. Poly (U) is nearly as effective as Poly (G) in augmenting fiber length. × 480.

Fig. 5. Effect of RNA homopolymers on astral fiber length. Asters were prepared from homogenates allowed to stand for several hours at 0°C, resulting in very small asters (a). Figure 5b shows an aster from the same preparation, to which 0.5 mg/ml Poly (A) was added just prior to assembly. Poly (A) results in little augmentation, compared to 0.5 mg/ml Poly (G), shown in Fig. 5c. Incubations were at 28°C for 14 min. × 480.

When homogenates prepared from oocytes washed at 37°C are diluted by 50% with either 0.5 M MES or with distilled water, the MES-diluted samples show some, but very slight, increase in astral fiber length compared to undiluted controls (Fig. 6 a,d). The samples diluted with water, however, show a very pronounced increase in fiber length compared to either undiluted or to MES-diluted samples (Fig. 6g). Poly (G), at 1 mg/ml, results in marked augmentation of undiluted and of MES-diluted samples (Fig. 6 e,f). Little additional augmentation of the already large water-diluted samples is induced by Poly (G) (Fig. 6 g,h). These results demonstrate that monovalent and/or MES ions at higher concentrations result in reduction of astral fiber length, which can be prevented by Poly (G), and suggest the release of an inhibitor that can be sequestered by this polynucleotide.

The ability of other RNAs to augment the length of astral fibers was also investigated. Poly (G), at 1 mg/ml, appeared to be slightly more effective than Poly (U) at this concentration in some preparations, although augmentation by Poly (U) was also pronounced (Figs. 4 a,b). Poly (A) at 1 mg/ml can also clearly augment fiber length but is considerably less effective than Poly (G). At 0.5 mg/ml Poly (A) has little effect in preparations in which augmentation by Poly (G) at this concentration is pronounced (Figs. 5 a–c). Yeast RNA at 1 mg/ml has no effect on aster assembly (Fig. 6 b), and Poly (C), varied over a concentration range of 0.1 to 2 mg/ml, was also without effect (Fig. 6c). Augmentation by Poly (G), however, has been observed at concentrations as low as 0.3 mg/ml, with maximal augmentation occurring at about 1 mg/ml. Concentrations of Poly (G) above 4 to 5 mg/ml appear to inhibit aster assembly.

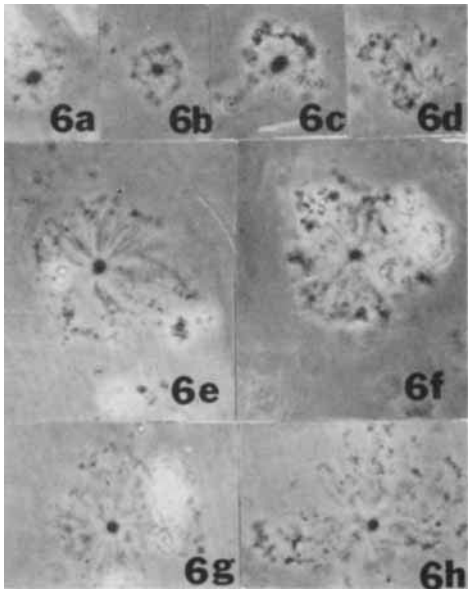

Fig. 6. Effects of polynucleotides and dilution. Asters were assembled at 28°C, 10 min incubation. (a) control without additions; (b) control + 1 mg/ml yeast RNA; (c) control + 1 mg/ml Poly (C); (d) diluted 50% with 0.5 M MES; (e) control + 1 mg/ml Poly (G); (f) diluted 50% with 0.5 M MES, + 1 mg/ml Poly (G); (g) diluted 50% with water; (h) diluted 50% with water, + 1 mg/ml Poly (G). Water dilution (g), but not MES dilution (d), results in pronounced augmentation, although MES-diluted samples (d) can still be augmented by Poly (G) (f). Poly (G) results in little further augmentation of already large water-diluted samples (g and h). Yeast RNA (b) and Poly (G) (c) have no effect. × 480.

The effect of Poly (G) was also tested on intact spindles isolated under polymerizing conditions. When oocytes are washed in glycerol-phosphate buffer at room temperature and resuspended in an equal volume of 0.5 M MES that has been prewarmed to 28°C, intact spindles are liberated after the oocytes have been broken by rapid pipetting. Subsequent incubation of such spindles at 28°C for 10 min results in pronounced elongation of astral fibers in samples containing 1 mg/ml Poly (G) added immediately after oocyte lysis. Elongation of astral fibers also occurs on control spindles incubated in parallel without Poly (G), but asters on Poly (G)-treated spindles are clearly larger than controls. Pole-to-pole distances did not appear different on inspection of the two preparations because of wide variability within each sample. Careful measurement of a large number of spindles, however, showed that the pole-to-pole distance of Poly (G)-treated spindles was slightly greater than controls (average Poly (G)-treated pole-to-pole distance = 27 $\mu \pm 4$, n = 60; average control pole-to-pole distance = 24 $\mu \pm 6$, n = 59; P = 0.0046).

When oocytes were washed at 37°C and the same experiment was repeated, spindles obtained in controls were so small and difficult to find that accurate measurements were rendered impossible. Incubation of these preparations with 1 mg/ml Poly (G), however, resulted in pronounced increase in both astral fiber length and pole-to-pole distance, compared to controls incubated without Poly (G). A typical example of such Poly (G)-augmented spindles and controls is shown in Fig. 7. We have not yet determined if the

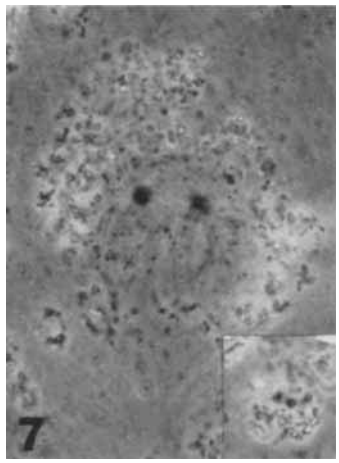

Fig. 7. Effect of Poly (G) on intact spindles. Spindles were obtained under polymerizing conditions as described in the text, from oocytes washed at 37°C, and incubated with or without 1 mg/ml Poly (G), at 28°C for 10 min. The main figure shows a typical spindle obtained after Poly (G) treatment. Inset shows a typical control incubated without Poly (G). Fibers are difficult to distinguish because of the small size of the control, but the two dark centriole-containing regions are clearly visible. Both astral fiber length and pole-to-pole distance are clearly greater in Poly (G)-treated spindles × 480.

apparent increase in pole-to-pole distance in Poly (G)-treated preparations results from actual in vitro growth of pole-to-pole microtubules, effects on possible sliding interactions, or prevention of pole-to-pole microtubule depolymerization in spindles after oocyte lysis.

DISCUSSION

Inhibition of microtubule assembly has been postulated as part of mechanisms by which cells may regulate the precise spatial and temporal distribution of microtubules during the mitotic cycle (23). Recent reports have suggested that inhibitors of microtubule assembly are present in a variety of cell types. Alternate forms of tubulin itself have been reported to inhibit microtubule polymerization (24), and RNA-induced inhibition of assembly has been suggested as the basis for inability to polymerize tubulin in vitro from a variety of cells (17). Our present results suggest that Spisula oocytes contain an inhibitor of microtubule assembly that can be sequestered by specific RNAs. This is suggested by the ability of some but not all RNA homopolymers to augment astral microtubule assembly in vitro, and the inability of RNA from a heterologous source (yeast) to affect aster assembly.

The ionic strength dependence of RNA augmentation further supports the idea that RNA exerts its effects by sequestering an inhibitor. When the ionic strength is increased, astral fiber length is reduced but can be restored by addition of Poly (G). The ability of dilution alone to slightly enhance astral fiber length under conditions where a high ionic strength is maintained may also partially result from the diluting-out of an inhibitor before reduction in tubulin concentration becomes limiting. While reduction of a limiting MTOC concentration could also account for the increase in fiber length upon dilution, it cannot

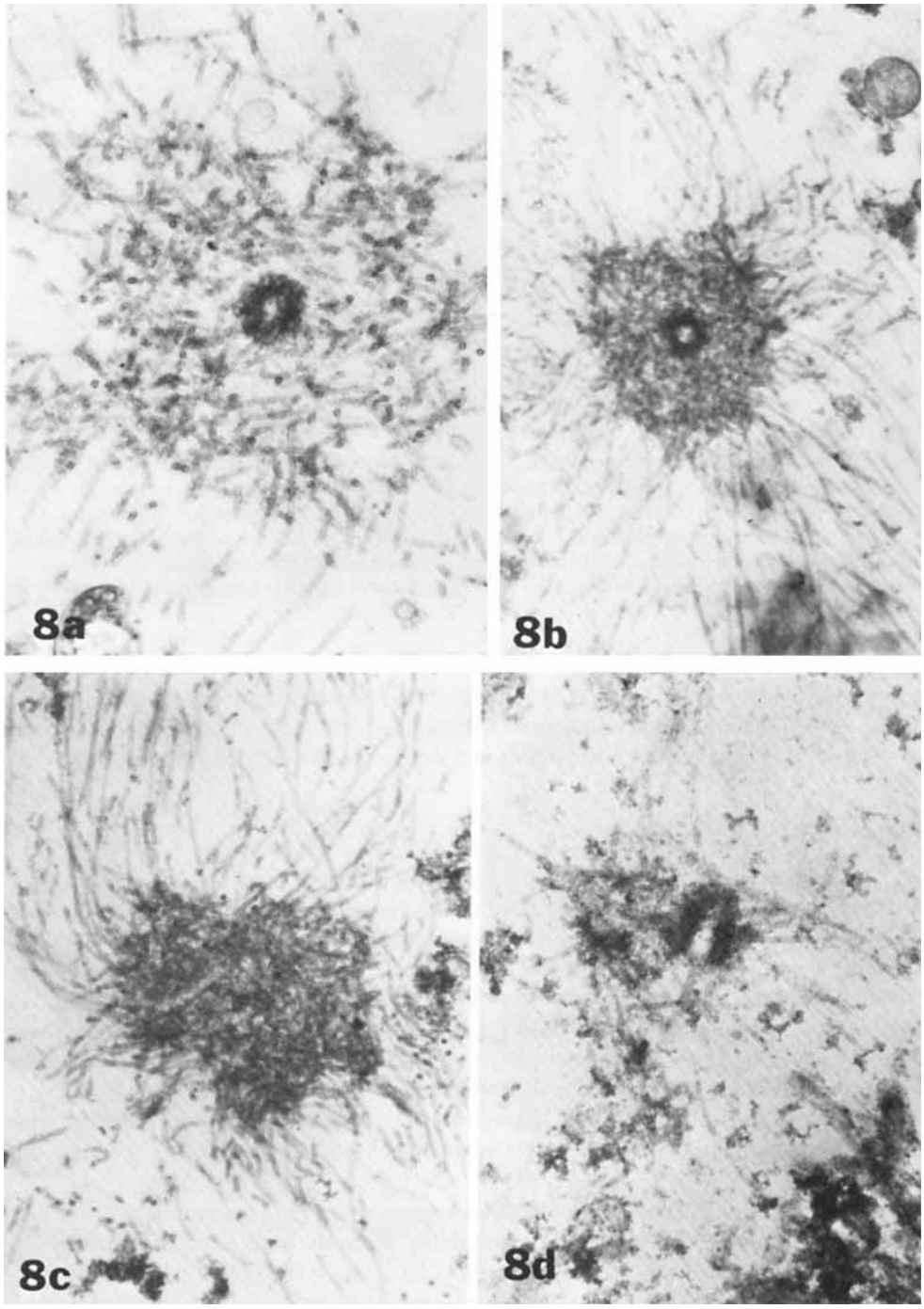

Fig. 8. Ultrastructure of RNase-treated asters. Asters were assembled at 28°C for 15 min without (a) or with (b, c, and d) 0.1 mg/ml RNase A. The MTOC appears more dense and compact in RNase-assembled asters (b, c) than in controls (a). Figure 8c shows an aster formed in RNase that apparently lacks a centriole, while Fig. 8d shows a centriole associated with diffuse granular material and microtubules. (a) × 32,000 (b) × 25,000 (c) × 25,000 (d) × 32,000.

explain the pronounced additional enhancement of astral fiber length that results from dilution and simultaneous lowering of the ionic strength. While it is possible that the higher ionic strength is unfavorable to polymerization, it would be difficult to explain how a polynucleotide at such low concentrations could change the ionic strength dependence of polymerization, unless it were affecting some component present at much lower concentrations than those significantly contributing to the ionic strength, The presence, therefore, of an inhibitor that is released from an inactive complexed form by increasing the ionic strength and is sequestered by some polynucleotides seems likely. Since Poly (G) effects cannot be duplicated by 1 mM GTP or 1 mM EGTA + 0.5 mM Mg^{+2}, either alone or in combination, the possibility that Poly (G) acts by sequestering Ca^{+2} ions or by inhibiting endogenous GTPases is unlikely.

While all of our results are consistent with the idea that Poly (G) and other RNA homopolymers that augment assembly act by sequestering an inhibitor, other explanations cannot yet be ruled out. The possibility, for example, that some RNA homopolymers may duplicate the action of specific cofactors required for assembly should not be overlooked. It is of interest, in this regard, that a transhydrogenase enzyme present in sea urchin eggs with a high specificity for tubulin (26) has been shown to require a specific RNA cofactor for activity (25). Investigation is planned of a possible relationship between these and our own observations.

It has recently been demonstrated that RNA can nonspecifically inhibit the polymerization of mammalian brain tubulin by binding to proteins required for assembly (17). This observation raises the possibility that RNA may enhance astral assembly by preventing the initiation of free microtubules, thereby increasing the number of subunits available for astral fiber elongation. This possibility is unlikely for two reasons. First, Poly (G) can augment astral fiber length when added to preparations after asters have obtained maximal size, and presumably well after initiation events have occurred. For RNA to enhance astral fiber length by binding to a microtubule-associated protein, that promotes, rather than inhibits, assembly, it would have to mobilize such a protein from already formed free microtubules and, in addition, somehow make this protein and/or tubulin subunit available for astral, but not free, microtubule assembly. While such events cannot be completely ruled out, the additional fact that astral fiber elongation is enhanced by specific RNAs, while all RNAs affect brain tubulin assembly (17), makes this mechanism very unlikely. It should also be pointed out that Spisula aster assembly has different ionic strength and buffer dependencies than mammalian brain tubulin. While brain tubulin is routinely repolymerized in a variety of buffers at low (0.1 M) ionic strength, Spisula asters will not repolymerize at ionic strengths much below 0.2 M, and while phosphate buffers allow good polymerization of mammalian brain tubulin, assembly of Spisula asters in phosphate buffer has not been obtained. Preliminary experiments have shown, however, that Spisula nerve tubulin will reassemble at MES concentrations as low as 50 mM, and although assembly of mammalian brain tubulin is nearly completely inhibited at 0.5 M MES, excellent reassembly of clam neurotubulin occurs at this ionic strength. It is likely, therefore, that many of the peculiarities of ionic strength and buffer ion dependence of Spisula oocyte tubulin result from an inhibitor present in the oocytes. This is further supported by the observation that 1 mg/ml Poly (G) inhibits the assembly of clam neurotubulin even at high (0.5 M) ionic strength. It should be noted, however, that our observations also support the idea that proteins required for assembly are present in oocytes as well, as suggested by the inhibitory effect of Poly (G) in dilute, low-ionic-strength preparations, and at higher Poly (G) concentrations.

Reduction of astral fiber length by RNase and other basic proteins appears to occur through interaction of these polycations with tubulin, which is highly negatively charged. It has been demonstrated that polycations can induce abnormal assembly of brain tubulin (27). It has also been shown that polycations can mimic in vitro the "decorations" seen on isolated microtubules formed in vivo (28), and they can also substitute for microtubule-associated proteins (MAPS) required for the assembly of purified mammalian brain tubulin (29). The apparent inhibitory effect of RNase and other basic proteins on aster assembly may result from nonspecific interactions. It should be noted, however, that changes in polycation levels occur in sea urchin eggs during the division cycle (30), and spindle poisons have been shown to inhibit key enzymes in polycation metabolism (31). Tubulin-polycation interactions, therefore, may be important in the cell.

The nature of RNase effects on the MTOC remains uncertain. While other polycations can mimic these effects, this does not rule out direct enzymatic interaction of RNase with an RNA component of the organizing center. Such polycations would also be expected to interact with RNA, or with tubulin, either or both of which may be present in the MTOC. Alternatively, other polycations may exert their effects through liberation of endogenously complexed and inactive RNase in our crude homogenates. Reversal of RNase as well as protamine-induced reduction of astral fiber length by Poly (G), a potent inhibitor of many RNases (22), and the ability of Poly (G) to augment assembly in the absence of added polycations, suggest that the inhibitor we have postulated may itself be RNase. This is further suggested by the observation that Poly (A), a less potent RNase inhibitor than Poly (G) (22), is also less efficient than Poly (G) in augmenting astral fiber length in the absence of added proteins. It should be noted that polycation-induced reduction of astral fiber length appears, at low polycation concentrations, to be highly specific for the enzymatically active form of RNase. While the effect of RNase on fiber length might result from subtle interactions with the MTOC, it appears likely that this effect results from RNase binding to tubulin, and is independent of MTOC alterations. It has recently been demonstrated that G-actin inhibits the activity of DNase I (32) and that DNase I can depolymerize F-actin (33, 34). The possibility of fine control mechanisms coupling nucleic acid metabolism and filamentous protein polymerization through these or other such interactions should not be overlooked.

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NOTE ADDED IN PROOF

After completion of this manuscript a paper appeared by M. W. Berns, K. Leonardson and M. Witter [J. Morph. 149: 327-338 (1976)] which also reports both phase lightening and phase darkening of the centriole region after laser microbeam irradiation of cells treated with ethidium bromide to selectively sensitize RNA.

REFERENCES

1. Shimamura, T., and Ota, T.: Exptl. Cell Res. 11:346–361 (1956).
2. Goldman, R. D. and Rebhun, L. I.: J. Cell Sci. 4:179–209 (1973).

3. Zimmerman, A.: Exp. Cell Res. 20:529–547 (1960).
4. Hartman, J. F., and Zimmerman, A. M.: Exp. Cell Res. 50:403–417 (1968).
5. Randall, J., and Disbrey, C.: Proc. R. Soc. B 162:473–491 (1965).
6. Smith-Sonneborn, J., and Plaut, W.: J. Cell Sci. 2:225–232 (1967).
7. Smith-Sonneborn, J., and Plaut, W.: J. Cell Sci. 5:365–372 (1969).
8. Hartman, H., Puma, J. P., and Gurney, T.: J. Cell Sci. 16:241–259 (1974).
9. Dippell, R. V.: J. Cell Biol. 69:622–637 (1976).
10. Braselton, J. P.: Cytobiologie 12:148–151 (1975).
11. Brinkley, B. R., and Stubblefield, E.: In D. M. Prescott, L. Goldstein, and E. M. McConkey, (Eds.), "Advances in Cell Biology" (Vol. 1). New York: Appleton-Century-Crofts, pp. 119–186 (1970).
12. Stubblefield, E., and Brinkley, B. R.: In K. B. Warren (Ed.), "Formation and Fate of Cell Organelles" (Vol. 6). New York and London: Academic Press, pp. 175–218 (1967).
13. Berns, M. W., and Rattner, J. B.: J. Cell Biol. 67:30a (1975).
14. Macgregor, H. C., and Stebbings, H.: J. Cell Sci. 6:431–449 (1970).
15. Keller, P. M., Morgan, N. H., Morgan, R. S., and Czeto, A. R.: J. Cell Biol. 59:165a (1973).
16. Betchaku, T.: J. Cell Biol. 67:31a (1975).
17. Bryan, J., Nagle, B. W., and Doenges, K. H.: Proc. Nat. Acad. Sci. USA 72:3570–3574 (1975).
18. Margulis, L.: In M. Borgers and M. De Brabander (Eds.), "Microtubules and Microtubule Inhibitors." Amsterdam: North Holland Publishing Co., pp. 3–18 (1975).
19. McGill, M., Pathak, S., and Hsu, T. C.: Chromosoma 47:157–167 (1974).
20. Weisenberg, R. C., and Rosenfeld, A. C.: J. Cell Biol. 64:146–158 (1975).
21. Brownhill, T. J., Jones, A. S., and Stacey, M.: Biochem. J. 73:434–438 (1959).
22. Levy, C. C.: Life Sci. 17:311–316 (1975).
23. Bryan, J.: Amer. Zool. 15:649–660 (1975).
24. Farrell, K. W., and Burns, R. G.: J. Cell Sci. 17:669–681 (1975).
25. Sakai, H.: J. Biol. Chem. 242:1458–1461 (1967).
26. Kimura, I.: Exp. Cell Res. 79:545–546 (1973).
27. Jacobs, M., Bennett, P. M., and Dickens, M. J.: Nature 257:707–709 (1975).
28. Benke, O.: Nature 257:709–710 (1975).
29. Erickson, H. P.: J. Cell Biol. 67:110a (1975).
30. Kusuoki, S., and Yasumasu, I.: Biochem. Biophys. Res. Comm. 68:881–885 (1976).
31. Kuangyu, C., Heller, J., and Canellakis, E. S.: Biochem. Biophys. Res. Comm. 68:401–408 (1976).
32. Lazarides, E., and Lindberg, U.: Proc. Nat. Acad. Sci. USA 71:4742–4746 (1974).
33. Mannherz, H. G., Barrington Leigh, J., Leberman, R., and Pfrang, H.: FEBS Letters 60:34–38 (1975).
34. Hitchcock, S. E., Carlsson, L., and Lindberg, U.: Cell 7:531–542 (1976).