Parthenogenesis in *Xenopus* Eggs Requires Centrosomal Integrity

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Abstract. *Xenopus* eggs are laid arrested at second metaphase of meiosis lacking a functional centrosome. Upon fertilization, the sperm provides the active centrosome that is required for cleavage to occur. The injection of purified centrosomes mimics fertilization and leads to tadpole formation (parthenogenesis). In this work we show that the parthenogenetic activity of centrosomes is inactivated by urea concentrations higher than 2 M. The loss of activity is correlated with a progressive destruction of the centriolar cylinder and extraction of proteins. This shows that centrosomes are relatively sensitive to urea since complete protein unfolding and solubilization of proteins normally occurs at urea concentrations as high as 8–10 M. When present, the parthenogenetic activity is always associated with a pelletable fraction showing that it cannot be solubilized by urea.

The parthenogenetic activity is progressively inactivated by salt concentrations higher than 2 M (NaCl or KCl). However, only a few proteins are extracted by these treatments and the centrosome ultrastructure is not affected. This shows that both parthenogenetic activity and centrosomal structure are resistant to relatively high ionic strength. Indeed, most protein structures held by electrostatic forces are dissociated by 2 M salt. The loss of parthenogenetic activity produced at higher salt concentrations, while the structure of the centrosome is unaffected, is an apparent paradox. We interpret this result as meaning that the native state of centrosomes is held together by forces that favor functional denaturation by high ionic strength. The respective effects of urea and salts on centrosomal structure and activity suggest that the centrosome is mainly held together by hydrogen and hydrophobic bonds.

The in vitro microtubule nucleating activity of centrosomes can be inactivated at salt or urea concentrations that do not affect the parthenogenetic activity. Since egg cleavage requires the formation of microtubule asters, we conclude that the extracted or denatured microtubule nucleating activity of centrosomes can be complemented by components present in the egg cytoplasm.

Both parthenogenetic and microtubule nucleating activities are abolished by protease treatments but resist nuclease action. Since we find no RNA in centrosomes treated by RNase, they probably do not contain a protected RNA.

Taken together, these results are consistent with the idea that the whole or part of the centrosome structure acts as a seed to start the centrosome duplication cycle in *Xenopus* eggs.

In *Xenopus*, the unfertilized egg is laid arrested at the second meiotic metaphase. The cell cycle is arrested by a cytostatic factor which has not yet been fully characterized (Meyerhof and Masui, 1977, 1979). During fertilization, the block is released by the penetration of the sperm. A rapid series of cell cycles ensues in which the egg cleaves regularly every 30 min. The release from mitotic block is mediated by a calcium wave (Busa and Nuccitelli, 1985; Kubota et al., 1987) that is believed to inactivate the cytostatic factor (Meyerhof and Masui, 1979; Newport and Kirschner, 1984). The calcium wave can also be induced by pricking the egg with a fine microinjection needle, again resulting in cytostatic factor inactivation and cell cycle reinitiation as revealed by the periodic occurrence of surface contraction waves, as well as oscillations of mitotic factors and kinase activities (Capony et al., 1986; Gerhart et al., 1984; Hara et al., 1980; Karsenti et al., 1987). However, no cleavage occurs. Cleavages can be restored if somatic cells (Bataillon, 1911), centriole-containing fractions (Maller et al., 1976), or purified centrosomes (Karsenti et al., 1984; Bornens et al., 1987) are injected in the eggs at the time of pricking. This strongly suggests that *Xenopus* eggs lack a functional centrosome or a centrosomal associated activity, and that a new centrosome cannot form spontaneously upon simple egg activation. Under normal conditions the centriole of the sperm

This paper is the synthesis of two independent manuscripts prepared by each of the first two authors. Therefore, the order of these authors is arbitrary.

probably brings factors necessary to initiate the successive cycles of centrosome duplication. It appears that although centrosomes are formed de novo in mouse eggs under physiological conditions (Szollosi et al., 1972), as well as in marine eggs after various treatments (Kuriyama and Borisy, 1983), this does not happen in frog eggs. Yet most components required for centrosomes assembly must be stored in the egg cytoplasm in order to allow the formation of 4,000 centrosomes in the first 7 h of development (Kirschner et al., 1985).

The question addressed in this paper is whether the whole centrosomal structure, including the centriole and its surrounding material, is required to start the centriole duplication cycle or if a particulate or soluble subfraction, lacking in the egg (a nucleic acid, for example), is sufficient. We have tested the effect of various chemical and enzymatic agents on the cleavage-inducing activity of purified centrosomes isolated from human cells. The results obtained are compared with the effect of the same agents on the ultrastructure, chemical composition, and microtubule nucleating activity of centrosomes in vitro. The data shed some light on the forces involved in the cohesion of the centrosome. Moreover, they indicate that at least some part of the centrosome structure is necessary for the activities since we were unable to isolate an active soluble fraction—in particular, a nucleic acid.

Materials and Methods

Animals

Xenopus laevis females were obtained from the Service d'Elevage d'Amphibiens du CNRS (France). Eggs were obtained from females injected 3 d before use with 100 U of pregnant mare serum gonadotropin and 1 d before with 1,500 U of human chorionic gonadotropin (Karsenti et al., 1984).

Centrosome Preparation and Immunofluorescence

Centrosomes were prepared from human lymphoid cells (KE 37) according to Bornens et al. (1987). Centrosome purification was monitored by indirect immunofluorescence. The centrosomes were diluted in 10 mM KPipes, pH 7.2, and centrifuged in 15-ml Corex tubes 12 min at 12,000 rpm in a centrifuge (Sorvall Instruments' Div., Newton, CT). The modified corex tubes contain a plexiglass adaptor which allows one to easily remove the coverslip (Evans et al., 1985). The centrosomes were fixed 5 min in methanol (−20°C) and processed for double-labeling immunofluorescence using an antipericentriolar material (PCM)[1] antibody (0132 serum, Gosti-Testu et al., 1986) and a monoclonal anti-β tubulin antibody which stains the centriolar cylinder.

Antibodies

Anti-α or -β tubulin and antiactin were from Amersham Corp. (England). Antifodrin was kindly provided by Drs. P. Mangeat (Mangeat and Burridge, 1984) and L. A. Pradel (Regnouf et al., 1985). Anticentrosome antibodies were from a rabbit serum: 0132 (Gosti-Testu et al., 1986).

Assay for Parthenogenetic Activity

Unfertilized eggs were dejellied with 2% cysteine, pH 7.8, and washed five times with MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA, pH 7.2). The eggs were transferred to MMR containing 5% Ficoll 400 and incubated for 10 min at room temperature before any injection. This makes the injection easier to perform. The purified centrosomes were injected after dilution in 10 mM KPipes, pH 7.2. 5-10 eggs were injected per assay.

1. *Abbreviations used in this paper*: PCM, pericentriolar material.

Assay for Microtubule Nucleating Activity

The microtubule nucleating activity of centrosomes were assayed according to Mitchison and Kirschner (1984). 10 μl of purified centrosomes were incubated with 100 μl of phosphocellulose-purified tubulin at 2.5 mg/ml in RGl buffer (80 mM KPipes, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8). The mixture was incubated 10 min at 37°C and fixed by adding 200 μl of 1% EM grade glutaraldehyde diluted in RGl. 3 min later 1 ml of RG2 (RGl without GTP) was added. The suspension was layered onto a 5-ml cushion of 25% glycerol in RG2, in a 15-ml modified corex tube (Evans et al., 1985). The asters were sedimented on coverslips for 15 min at 12,000 rpm using an HB4 swinging bucket rotor and a centrifuge (Sorvall Instruments Div.). The supernatant was discarded and the cushion washed twice with 1 ml of 1% Triton X-100 in RG2. The coverslips were removed and postfixed for 5 min in −20°C methanol. Microtubules were stained by immunofluorescence using anti-β tubulin.

Chemical and Enzymatic Treatments of Centrosomes

50 μl of centrosomes (5×10^7/ml in 50% sucrose) were diluted with 50 μl of various extraction buffers or enzyme solutions. All solutions were made in 10 mM KPipes, pH 7.2. The treated centrosomes were then dialyzed against 10 mM KPipes, pH 7.2, for 1 h at 4°C. As a control, untreated centrosomes were incubated and dialyzed under the same conditions. The following reagents were used: KCl and NaCl at 0.5–4 M; KI at 1.0 and 2.0 M; urea at 0.5–8 M; sodium deoxycholate at 0.2 and 0.5%; 2-mercaptoethanol at 5 and 10% (vol/vol). As a control of the dialysis step, a blank dialysate obtained from each extraction buffer was added to native centrosomes which were further assayed for their microtubule nucleating and parthenogenetic activities. None of the dialysate affected centrosome activities. Trypsin was used at 1 μg/ml for 20 min at room temperature and at 4°C; RNAase A and micrococcal nuclease were applied for 30 min at a concentration of 10 μg/ml in the presence of 1 mM $CaCl_2$ at 4°C or at room temperature. The micrococcal nuclease activity was stopped by adding EGTA at a final concentration of 2 mM. Enzyme-treated centrosomes (100 μl) were centrifuged for 20 min at 30,000 rpm in 0.8-ml SW 50.1 tubes containing 300 μl of 70% sucrose and 400 μl of 30% sucrose in 10 mM KPipes, pH 7.2. The fractions were assayed routinely for parthogenetic and microtubule nucleating activity. In some experiments, the centrosomes treated with chemical agents were also centrifuged through similar sucrose gradients. The biochemical analysis was carried out on centrosomes treated in the same way, except that they were directly pelleted at 20,000 *g* for 20 min after the treatment. The supernatant was dialyzed against 0.06 M Tris, pH 6.8, in floating Sartorius dialysis tubes (Sartorius, Göttingen, FRG) for 1 h at 4°C and then for 30 min against the same buffer containing 30% glycerol in order to concentrate the sample. The pellet and the dialyzed supernatant were solubilized by boiling in 2.3% SDS, 1% 2-mercaptoethanol for 3 min. The proteins were analyzed by electrophoresis on a 6–15% polyacrylamide gradient gel in the presence of SDS (Porzio and Pearson, 1977). The gel slabs were either stained for proteins by a silver nitrate method (Switzer et al., 1979), or electrophoretically blotted on a 0.1-μm nitrocellulose filter (Schleicher and Schüel, Dassel, FRG) using a semi-dry system with 0.025 M Tris–0.19 M glycine containing either 20% methanol in the anodic side or 0.1% SDS in the cathodic side.

Immunodetection

The quality of the protein transfer was evaluated by staining the nitrocellulose filter with Ponceau Red S (0.1% in 0.3% TCA). The filter was then saturated in 10 mM Tris, pH 7.4, 0.15 M NaCl, 5% nonfat dry milk, 0.1% Tween-20, 0.02% sodium azide at room temperature for 1 h and all subsequent washes and antibody dilutions were performed in this buffer. The filter was incubated in the first antibody overnight at 4°C. After three 10-min washes, the filter was incubated for 30 min in the second antibody: either anti-mouse or anti-rabbit IgG labeled with alkaline phosphatase (Promega Biotech, Madison, WI). After three washes as before, the enzymatic activity was revealed by color development in 30 ml of 0.1 M Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ containing nitroblue tetrazolium (200 μl at 50 mg/ml in 50% dimethylformamide) and 5-bromo-4-chloro-3-indolyl phosphate (200 μl at 25 mg/ml in 50% dimethylformamide).

Ultrastructural Study

Controls or treated centrosomes were sedimented onto 12-mm round coverslips, fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4,

postfixed with osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Sections parallel to the coverslips were observed in an electron microscope (model 201; Philips Electronic Instruments, Mahwah, NJ) after contrasting with uranyl acetate and lead citrate. Observations of at least 150–200 centrosomes were routinely performed to obtain significant micrographs of the treated centrosomes.

RNA Extraction and Labeling

5×10^8 centrosomes were incubated in the presence of proteinase K (0.5 mg/ml) for 1 h at 37°C in 1% SDS, 100 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 8.0. The RNA was extracted with phenol/chloroform/isoamylalcohol (Scheer et al., 1976) and labeled with ^{32}P according to the method of Maniatis et al. (1982). After resuspension in 0.05 M Tris, pH 7.2, containing 0.01 M $MgCl_2$ and 0.005 M DTT, the RNA was incubated twice in the presence of 1 U calf intestine phosphate for 30 min at 37°C. The phosphate action was stopped by adding β-glycerophosphate at a final concentration of 20 mM and the RNA labeled by adding 2 U of polynucleotide kinase and 50 μCi of γ^{32} PATP (3,000 Ci/mM) for 30 min at 37°C. The reaction was stopped by the addition of 0.5 vol of 7.5 M ammonium acetate. The RNA was precipitated by 2.5 vol of ethanol overnight at −20°C. The unincorporated nucleotides were removed by washing the pellet three times with 70% ethanol. The RNA was run on a 1% agarose gel made on 0.04 M morpholino-propanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 2.2 M formamide as described by Maniatis et al. (1982).

Results

The Parthenogenetic Assay

The induction of cleavage by injection of centrosomes into *Xenopus* eggs was used as an assay to further fractionate the relevant centrosome components. To make the assay semiquantitative, we injected several dilutions of a given centrosome preparation into at least six eggs for each dilution. The centrosome concentration was independently determined by immunofluorescence as described by Mitchison and Kirschner (1984). Fig. 1 shows the aspect of eggs photographed 4 h after the injection of centrosome dilutions routinely used in the assay. The number of eggs cleaving correctly decreased with increasing numbers of injected centrosomes. This is due to the conflicting action of the many cytoplasmic microtubule asters generated by the centrosomes. When the optimum number of centrosomes was injected, some of the eggs developed into tadpoles as previously reported (Karsenti et al., 1984; Bornens et al., 1987).

Parthenogenetic Activity and Centrosomal Structure Are Resistant to Salt Treatment Up to 2 M

As shown in Fig. 2, the parthenogenetic activity was surprisingly resistant to salt treatments. After treatment with 2 M KCl, 80% of the eggs injected with two centrosomes each still developed into blastulas. This is similar to the activity of untreated centrosomes. At higher salt concentrations, the parthenogenetic activity was progressively inhibited. After treatment with 4 M KCl, 10% of the eggs injected with 100 centrosomes each developed into blastulas. This decrease in

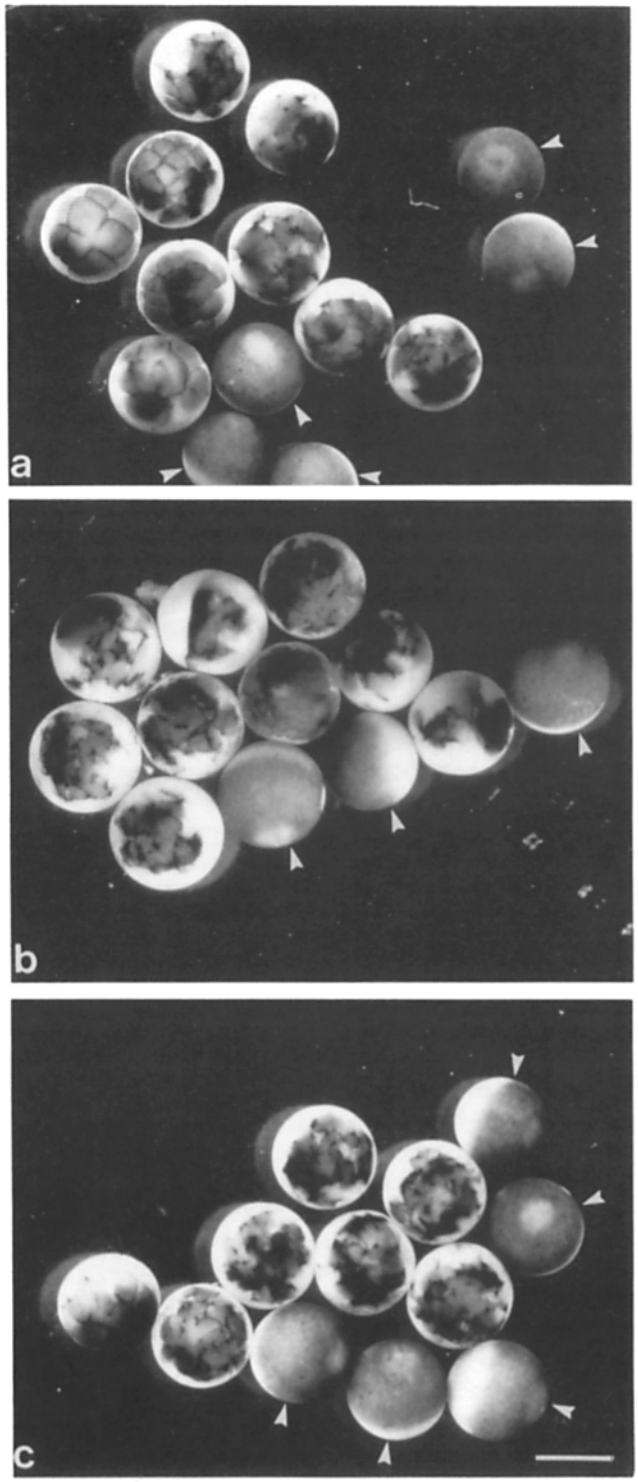

Figure 1. Parthenogenic activity of centrosomes at various concentrations. Purified centrosomes injected into unfertilized eggs after (*a*) 1:2,000 dilution, (*b*) 1:500 dilution, and (*c*) 1:10 dilution. The number of eggs cleaving correctly decreases with increasing numbers of injected centrosomes. Arrowheads show eggs which have not been injected. The 1:2,000 dilution corresponded to a theoretical injection of one centrosome per egg (initial concentration: 5×10^7 centrosomes/ml, 50 nl injected). Three out of nine injected eggs developed into blastulas. Two other eggs cleaved rather irregularly and the last four eggs showed only the typical pigment reorganizations that occur after egg activation. Only one of the eggs injected with about four centrosomes each developed into a blastula (Fig. 1 *b*). No cleavage occurred when the eggs were injected with too many centrosomes (350 per egg, Fig. 1 *c*). Instead, black dots and cortical contractions appeared at the surface of the eggs. Bar, 1 mm.

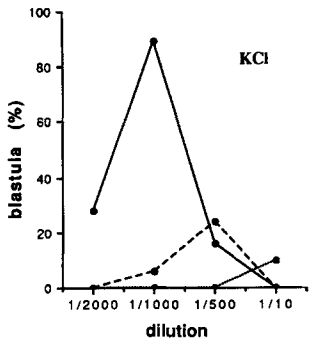

Figure 2. Effect of KCl on the parthenogenetic activity of centrosomes. Centrosomes were extracted for 30 min at 4°C with KCl. Parthenogenetic activity of control centrosomes and centrosomes extracted with 1 M and 2 M (*solid lines*); 3 M (*dashed lines*); and 4 M (*dotted lines*). Abscissa: dilution of injected centrosomes. Ordinate: percentage of blastulas formed 6 h after injection (5–10 eggs injected/point).

parthenogenetic activity could be formally interpreted either as a reduction in the proportion of active centrosomes, or as a decrease in the activity of each centrosome. The second interpretation is unlikely, since each centrosome seems to behave as a functional unit. Indeed, when too many native centrosomes are injected several asters develop and the egg cannot cleave. The injection of many damaged units does not substitute for the injection of one active unit. The dilution at which a given centrosome suspension is fully active reflects, therefore, a content in units which are competent to initiate successive cycles of centrosome duplication.

According to this interpretation, the results presented in Fig. 2 mean that 1 centrosome out of 1,000 retained a parthenogenetic activity after exposure to 4 M KCl. Similar results were obtained after extraction of the centrosomes with NaCl.

In an effort to determine the nature of the salt effect, we studied the ultrastructure and biochemical composition of treated and untreated centrosomes. As previously reported (Bornens et al., 1987), untreated centrosomes had two centrioles surrounded by a complex network of fibers that link them together. The PCM showed a proximo-distal organization with a 40-nm thick sheath running from the proximal end through half of the centriole length (Fig. 3 *a*). At the other end, the PCM displayed radial arms distributed according to a nine-fold axial symmetry (Fig. 3, *a* and *c*). Each arm possessed a multilayered transverse plate visible when longitudinal sections of centrioles were observed (Fig. 3 *a*). Finally the lumen of the nine microtubule triplets contained a cylindric hub over three fifths of the length of each centriole. The overall organization of centrosomes was not significantly modified by treatments with 2 M NaCl or KCl (Fig. 3, *d* and *e*). In particular, the radial arms of the distal PCM and the centriolar structure were well preserved. However, the A tubule of each triplet was consistently extracted at the proximal end. The B and C tubules were occasionally ruptured at the distal end (Fig. 3 *f*).

The protein composition of these centrosomes was complex (Fig. 4 *A*). More than 50 different molecular species were found, the molecular mass of which ranged between 300 and 14 kD. There were four main features. (*a*) There was a group of faint bands of ~280 kD. These proteins were specifically recognized by the rabbit serum 0013. They were found at the periphery of centrosomes (Gosti-Testu et al., 1986), and represent good markers for the PCM. (*b*) There was a doublet of 220–240 kD (arrowhead in Fig. 4 *A*) identified as fodrin by the use of antifodrin antibodies. (*c*) There was a group of bands of 200 kD (star in Fig. 4), one of which is the myosin heavy chain (Klotz, unpublished observations). And (*d*) there were several bands of 170, 135, 110, 90, 64, and 57 kD and the α and β tubulins which were used as markers for the centriolar structure of the centrosomes. Only a few bands were observed in the 45–14 kD range, one component being identified as actin by the use of antiactin antibodies. NaCl or KCl had little effect on the protein composition of centrosomes (Fig. 4 *A*). The 0.15 M salt treatment removed a 64-kD protein, a group of ~55–57 kD proteins, and a band of 45 kD. These are probably contaminants or weakly associated proteins. Some of the proteins that were not removed by this salt wash, like fodrin and myosin heavy chains, may not be true centrosomal components. They may simply be insoluble in these conditions. Fodrin, part of the 200-kD band and a 110-kD protein were solubilized by 0.5 M salt (Fig. 4 *A*). At concentrations of KCl or NaCl higher than 1 M, the pattern of extracted proteins did not change any more. A slight difference was noted between the effect of NaCl and KCl: a high molecular weight protein was extracted by KCl but not by NaCl (Fig. 4 *A*). An immunoblot analysis of these samples with antitubulin antibodies (Fig. 4 *B*) showed that the antigen remained associated with the insoluble fraction after treatment with 4 M KCl. The potential involvement of a nucleic acid in centrosomal structure was tested by treating the centrosomes with micrococcal nuclease before or after incubation in 1 M salt (Kaufmann et al., 1981). This had no effect on the pattern of proteins extracted by the salts (data not shown).

Parthenogenetic Activity and Centrosomal Structure Are Sensitive to Moderate Concentrations of Chaotropic Agents

The parthenogenetic activity began to be affected around 2 M and was almost completely inhibited at 4 M urea (Fig. 5). This activity was readily destroyed after treatment by 0.5 M KI, another chaotropic agent (not shown).

The whole centrosomal structure was slightly affected by 1 M urea (Fig. 6). The centriole proper still had microtubule triplets at the proximal end although not always complete (Fig. 6 *b*). The microtubule doublets at the distal extremity were absent (Fig. 6, *a* and *c*). A partial disappearance of the centriolar triplets occurred in 2 M urea starting on the internal side of the centriolar triplets in such a way that, depending on the level of the transverse section, a more or less continuous ring was still observed (Fig. 6). Treatment with increasing urea concentrations led to the progressive destruction of the centriole cylinder which was complete in 4 M urea. Complete disruption of the centrioles was also achieved by 0.5 M KI (Fig. 6, *d–f*).

Centrosomal proteins were progressively solubilized by increasing urea concentrations (Fig. 7 *A*). Between 0.5 and 2 M urea, four different bands or groups of bands (280 kD, 240–220 kD, 57 kD, and 45 kD) were solubilized. At 4 M urea, an abrupt solubilization of ~50% of the total sample proteins occurred and only a few proteins were still insoluble in 8 M urea (220, 200, 60, 57, 47, and 45 kD). The 60-kD protein was obviously partly artifactual since it increased in the insoluble fraction with the concentration of urea and was also observed in the absence of added proteins (not shown). Fig. 7 *B* shows the immunoblot of centrosomal proteins stained with anti-α and -β tubulin antibodies before and after extraction of the centrosomes with 2, 3, and 8 M urea. In un-

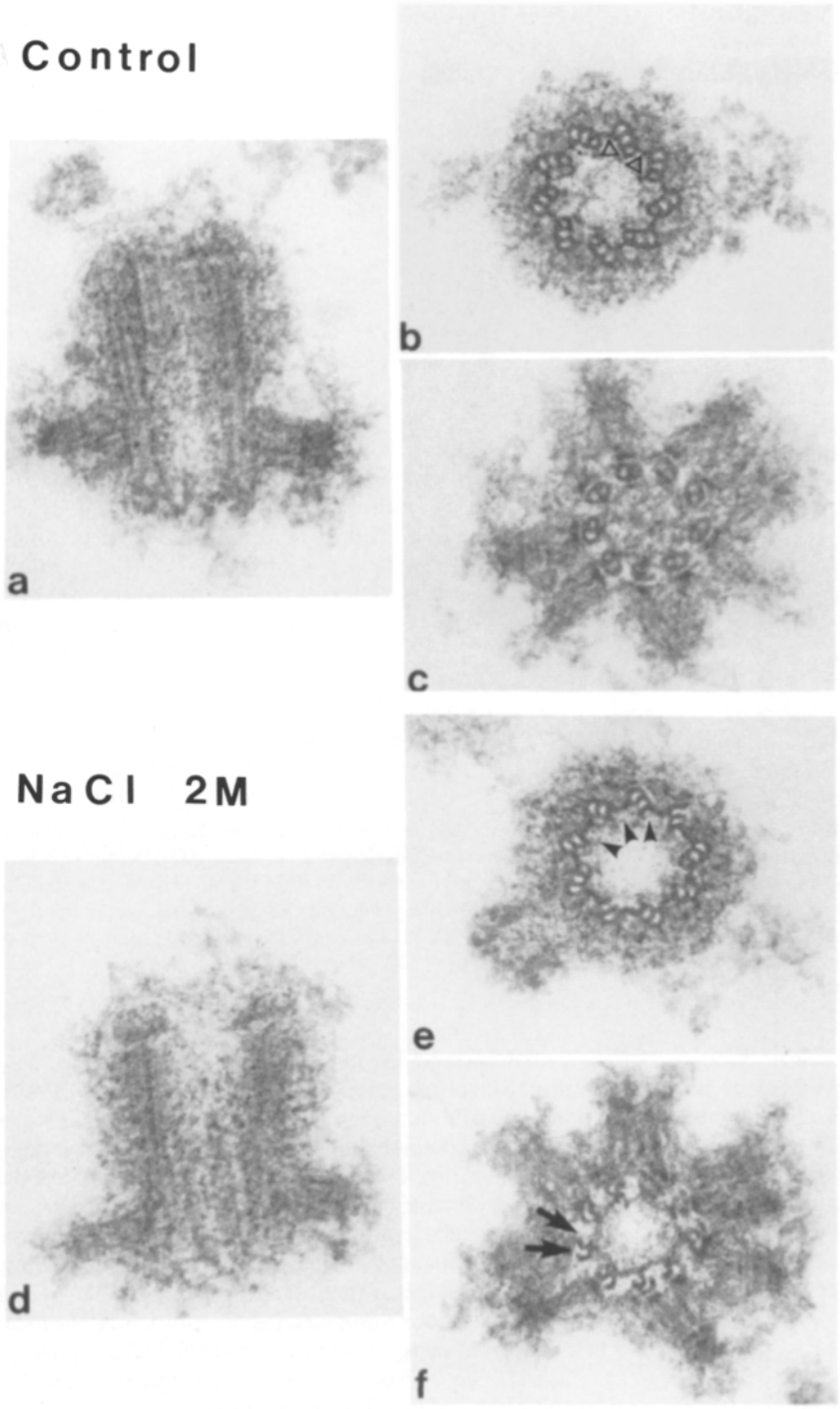

Figure 3. The ultrastructure of centrosomes is not modified by treatment with NaCl or KCl. Ultrathin sections, either longitudinal (*a* and *d*) or transversal at the proximal part (*b* and *e*) or at the distal part (*c* and *f*) of untreated centrosomes and of centrosomes treated with 2 M NaCl. Note the good preservation of the overall structure in 2 M NaCl: the nine triplets of microtubules bound by a link in the centrioles (*b*, *open arrowheads*), and the nine radial arms of the proximal PCM are still present. The A tubule of each centriolar triplet appears slightly extracted at the proximal end (*e*, *arrowheads*), and the B and C tubules are disrupted at the distal end (*f*, *arrows*).

treated centrosomes, the tubulin was exclusively found in the pellet. Above 2 M, the soluble fraction contained some tubulins, but the pellet of centrosomes treated with 8 M urea still contained at least half of the total tubulins. The centrosomal proteins recognized by the serum 0013 were only extracted in 8 M urea (Fig. 7 *C*).

Parthenogenetic Activity Remains Particulate after Salt or Urea Treatment

The previous set of experiments showed that parthenogenetic activity was unaffected by salt or urea concentrations lower than 3 M. To correlate the activity of the treated centrosomes with the structural data, it was important to determine if the activity remained particulate or was solubilized by these treatments. Centrosomes extracted with 2 M KCl or urea were centrifuged through a two-step gradient composed of a 30 and 70% layer. The parthenogenetic activity of control and treated centrosomes was then assayed in the various fractions. The maximum activity for control centrosomes was recovered at the 30–70% sucrose interface. The peak of activity was found at a lower dilution (1:250) than in the origi-

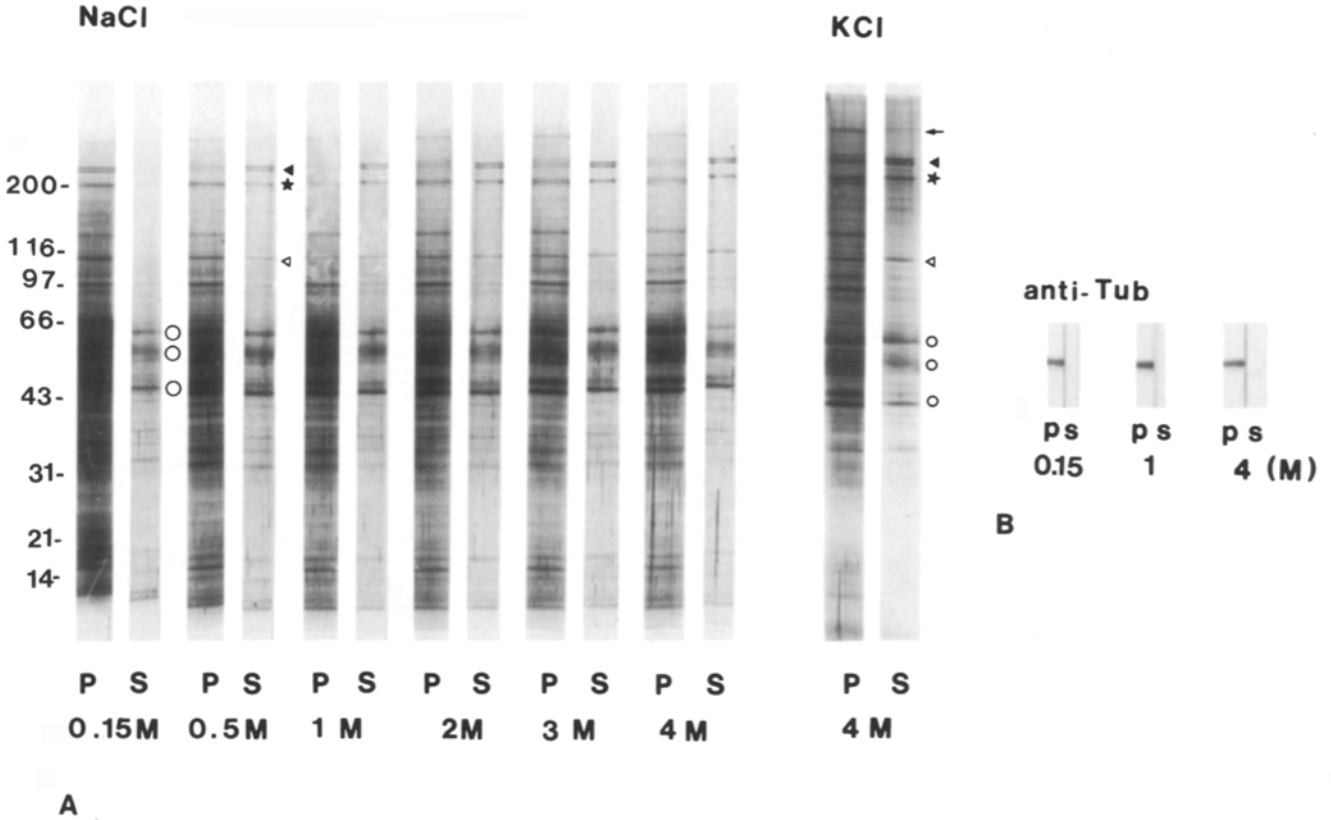

Figure 4. Centrosomal proteins are not solubilized by treatment with NaCl or KCl. 2×10^7 centrosomes were incubated in different concentrations of NaCl or KCl (0.15–4 M) in 10 mM Pipes buffer, pH 7.2, during 30 min at 4°C. Centrifugation at 20,000 *g* for 15 min separated the supernatant (*S*) containing solubilized proteins from the pellet (*P*). Fractions S and P were analyzed on a 6–15% polyacrylamide gradient in the presence of SDS and stained with silver nitrate (*A*) or transferred onto nitrocellulose filter (*B*). Only a few proteins were solubilized by NaCl or by KCl: the fodrin doublet (*arrowhead*), the myosin heavy chain (*star*), a 110-kD band (*open arrowhead*), and a group of proteins in the 60–45-kD region (*open circles*). Note the solubilization of a protein band of ~300 kD with KCl (*arrow*) but not with NaCl. (*B*) Immunoblot performed with antitubulin antibodies.

nal centrosome preparation because centrifugation through the gradient led to some dilution. No activity was recovered in the 30% fraction or in the supernatant. The parthenogenetic activity of centrosomes treated with 2 M urea or KCl was also recovered at the 30–70% sucrose interface, indicating that it was not solubilized by these treatments (Fig. 8).

Parthenogenetic Activity of Centrosomes Is Preserved under Conditions that Abolish Their Microtubule Nucleating Activity

Most of the treatments used in this study had been tested before on the microtubule nucleating activity of isolated centrosomes (Mitchison and Kirschner, 1984; Kuriyama, 1984). We have checked that our centrosomes reacted in a similar way in order to compare directly the effect of these treatments on microtubule nucleation and parthenogenesis. When the centrosomes were incubated with purified tubulin at 37°C for 10 min, microtubule asters assembled (not shown). In the same assay, centrosomes treated by 1 M urea had lost almost all activity and none of them remained active after a 2-M urea treatment (not shown). The same effect was observed when centrosomes were treated with 1 M KCl.

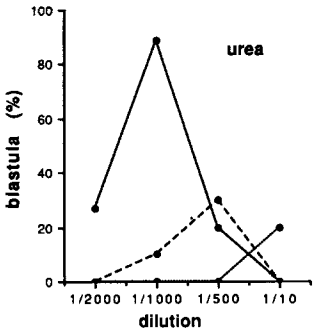

Figure 5. Effect of urea on the parthenogenetic activity of centrosomes. Centrosomes were extracted for 30 min at 4°C with urea. Parthenogenetic activity of control centrosomes and centrosomes extracted with 1 and 2 M (*solid lines*); 3 M (*dashed lines*); and 4 M (*dotted lines*). Abscissa: dilution of injected centrosomes. Ordinate: percentage of blastulas formed 6 h after injection (5–10 eggs injected/point).

Parthenogenetic Activity Does Not Require Nucleic Acids

Nucleic acids and in particular RNA have been often proposed to be components of basal bodies and centrosomes (for reviews see Vorobjev and Nadezhdina, 1987; Bornens and Karsent, 1984). We have tested the effect of two different nucleases: RNase A which is specific for RNA, and micrococcal nuclease which hydrolyzes both DNA and RNA. We compared the effect of these two enzymes to that of trypsin. The results are shown in Fig. 9. The activity of the RNase A and micrococcal nuclease–treated centrosomes was similar to that of control centrosomes incubated under similar conditions in the absence of an active enzyme. By contrast,

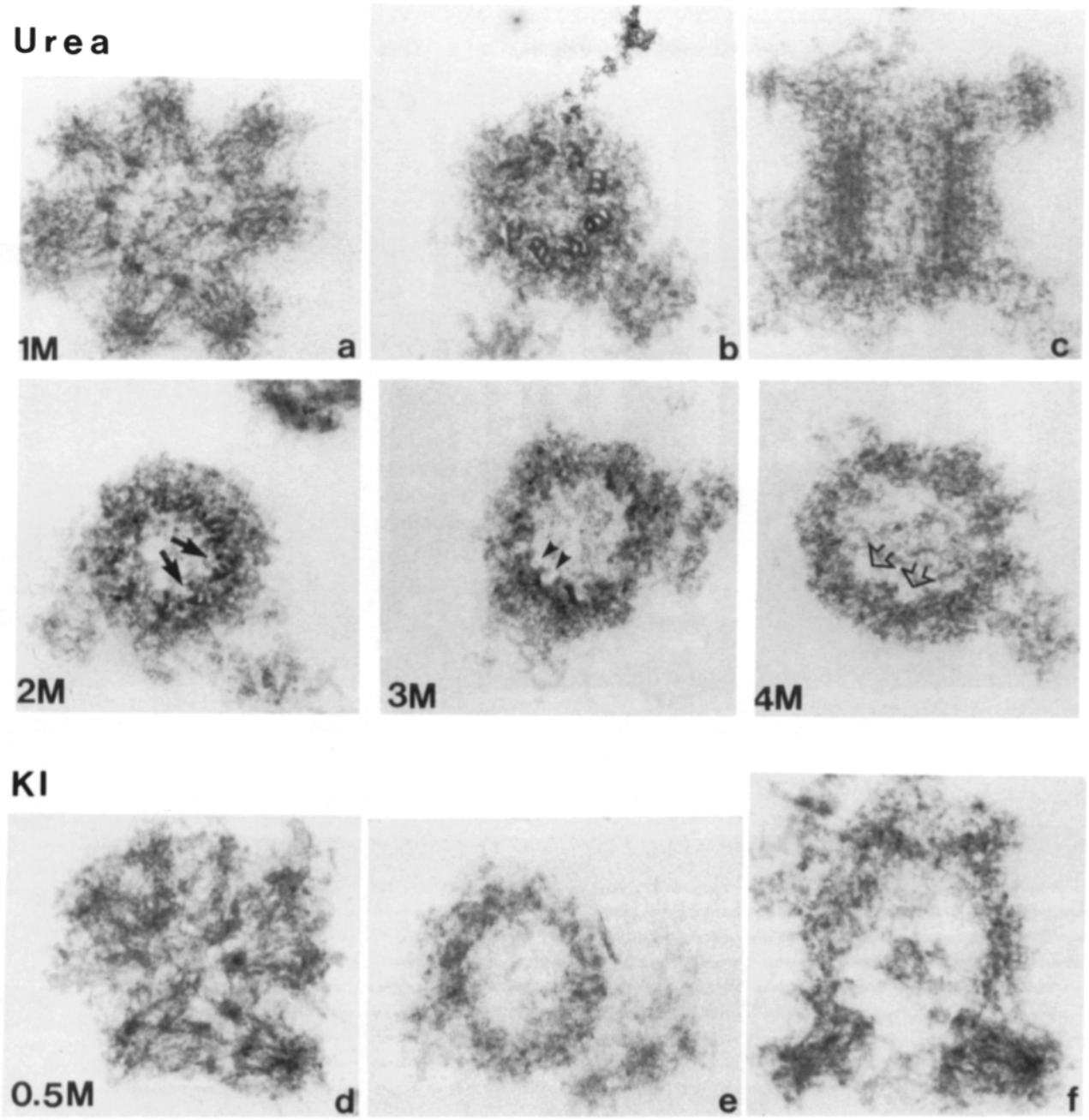

Figure 6. The ultrastructure of centrosomes is disorganized by urea and KI treatments. Ultrathin sections of centrosomes treated with 1 (*a–c*), 2, 3, and 4 M urea. Transverse section at the distal end (*a*) shows that microtubules have been solubilized, whereas the PCM organization remains similar to the native one (compare with Fig. 2 *c*). Transverse section at the proximal end (*b*) shows a good preservation of the structures, particularly of the centriolar organization (compare with Fig. 2 *b*). Longitudinal section (*c*) shows the structure of native centrosomes, particularly the radial arms (compare with Fig. 2 *a*). Increasing concentrations of urea induced a progressive disappearance of the microtubule triplets at the proximal end of the centrosome; 2 M urea solubilizes the internal part of the microtubule triplets (*2 M, arrows*). The solubilization is increased in 3 M urea (*3 M, arrowheads*) and is completed in 4 M urea (*4 M, open arrows*). The centriolar structure is entirely destroyed in centrosomes treated with 0.5 M KI (*d–f*), whereas the PCM remains barely recognizable on transverse sections at the distal (*d*) and proximal (*e*) ends as well as on longitudinal sections (*f*).

trypsin completely abolished the activity. The lack of nuclease effect strongly suggested that nucleic acids were not components of the structure required to initiate centrosome duplication. The possibility remained, however, that the nucleic acid was somehow protected from nucleases by proteins. We therefore attempted to combine nuclease action with treatments known to weaken protein or ribonucleoprotein interactions. It has been reported that sodium deoxycholate dissociates ribonucleoproteins from chromatin. This agent had no effect on the parthenogenetic activity of centrosomes even at concentrations as high as 0.5%, and it did not potentiate the activity of micrococcal nuclease (not shown). The activ-

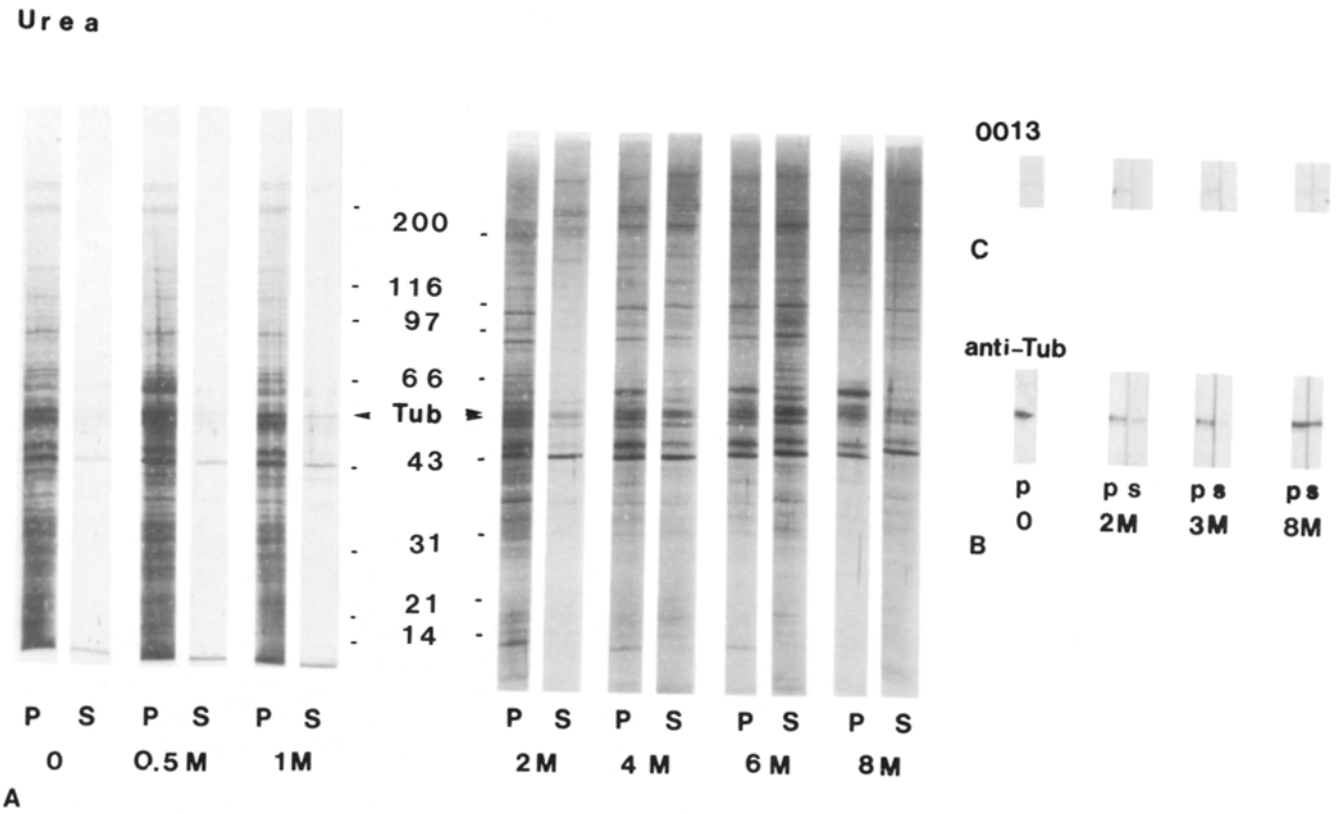

Figure 7. Centrosomal proteins are solubilized by urea. 2×10^7 centrosomes were incubated in different concentrations of urea (from 0.5 to 8 M) in 10 mM Pipes buffer, pH 7.2, during 15 min at 4°C. Solubilized proteins (*S*) were separated from the pellet (*P*) by centrifugation at 20,000 *g* for 15 min. Proteins were analyzed by electrophoresis on a 6–15% polyacylamide gradient in the presence of SDS and stained with silver nitrate (*A*), or transferred onto nitrocellulose filter and stained with antitubulin (*B*) or with the antiserum 0013 (*C*). The extractions with urea from 0 to 1 M and 2 to 8 M presented in *A* were analyzed onto different gels. Urea has a progressive effect on the dissociation of centriolar proteins, as judged by immunoblots of tubulin (*B*). On the contrary, only part of the 0013 antigen is solubilized in 8 M urea (*C*).

ity of centrosomes was not affected either by a successive treatment with 2 M urea and micrococcal nuclease (Fig. 9). Finally, the RNA was extracted from native centrosomes and from centrosomes treated with RNase A, labeled with radioactive phosphate using a polynucleotide kinase, and analyzed by electrophoresis on an agarose gel under denaturing conditions. Before RNase treatment, about five bands were detected at 6, 4, 2, 0.8, and 0.4 kb. After micrococcal nuclease digestion, only a very faint band was detected at 0.7 kb and most of the label was in the front of the gel (Fig. 10). The faint band at 0.7 kb has not been reproducibly found. We have verified that the parthenogenetic activity of the centrosomes used for this biochemical analysis was not altered. Micrococcal nuclease and RNase A also had no effect on the microtubule nucleating activity of purified human centrosomes, whereas trypsin readily destroyed the activity

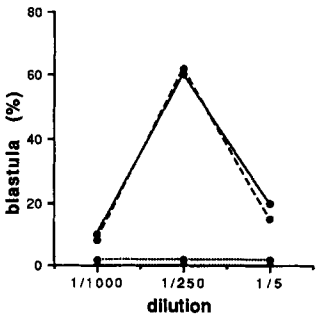

Figure 8. Parthenogenetic activity remains associated with particulate material after salt or urea extraction. Control centrosomes or centrosomes extracted with 2 M NaCl or urea were sedimented on two-step (70 and 30%) sucrose gradients as described in Materials and Methods. The parthenogenetic activity of both control (*solid line*) and extracted (*dashed line*) centrosomes was recovered at the 70–30% interface. No activity was recovered in the upper half of the 30% fraction or in the supernatant (*dotted lines*). Abscissa: centrosome dilutions. Ordinate: percentage of blastula formed 6 h after injection (5–10 eggs injected/point).

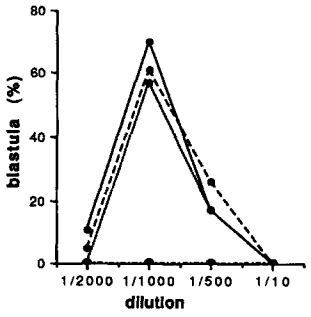

Figure 9. Effect of nucleases and proteases on the parthenogenetic activity of centrosomes. Centrosomes were treated with proteases and nucleases as described in Materials and Methods. A single treatment with 2 M urea, 5% sodium deoxycholate, micrococcal nuclease, or RNase A did not alter the parthenogenetic activity (*solid line*). Treatment with 2 M urea or 5% sodium deoxycholate, followed by micrococcal nuclease was not more effective (*dashed* and *dotted lines*, respectively). Trypsin completely abolished the parthenogenetic activity (*dashed-dotted line* on the x-axis). Abscissa: centrosome dilution. Ordinate: percentage of blastulas (5–10 eggs injected/point).

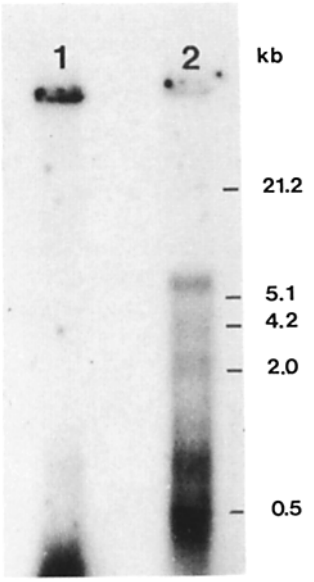

Figure 10. RNA composition of isolated centrosomes before and after micrococcal nuclease digestion. The RNA was extracted, labeled, and analyzed on an agarose gel as described in Materials and Methods. (Lane *1*) RNA extracted after RNaseA digestion of the centrosomes. (Lane *2*) RNA extracted before digestion.

(not shown). This confirms previous results reported by Kuriyama (1984).

Discussion

Parthenogenetic Activity Is Tightly Associated with Centrosomes

The aim of this work was to establish a correlation between the structure of isolated centrosomes and their functions as tested in vivo by following their parthenogenetic activity in *Xenopus* eggs. The approach was to progressively dissociate centrosomes, mainly using salt or chaotropic agents, to find conditions that would fractionate the parthenogenetic activity. We failed to find such conditions; as long as the parthenogenetic activity is not affected by urea or salt treatment, it remains associated with a material that sediments as native centrosomes do. This material contains centrosomes showing only slight alterations in their structure and biochemical composition.

Surprisingly, the progressive inhibition of parthenogenetic activity by salts and urea is correlated with different effects on the centrosomal structure. Indeed, the whole structure and protein composition of centrosomes is poorly affected by NaCl and KCl at any concentration. Yet above 2 M salt the parthenogenetic activity begins to be affected and is severely inhibited above 4 M. By contrast, chaotropic agents, such as urea or KI, strongly affect the structure of centrosomes, and above 2 M urea the progressive loss of parthenogenetic activity correlates exactly with the progressive extraction of centriolar microtubules.

The lack of effect of high ionic strength on the structure of centrosomes indicates that electrostatic forces are not essential for their cohesion. Indeed, most supramolecular structures held together by electrostatic forces (like chromatin, for example) are dissociated by 2 M salt. By contrast, the high sensitivity of centrioles to urea suggests that protein–protein interactions involving mainly hydrogen and hydrophobic bonds are involved in the cohesion of this structure. Indeed, complete protein unfolding and solubilization usually occurs in the presence of 8–10 M urea. Therefore, the partial dissociation of centrioles that occurs between 2 and 4 M urea is probably due to the destabilization of weak protein–protein interactions without complete solubilization of the constituent proteins.

In this context, the effects of salts and urea on the parthenogenetic activity can be understood in the following way. Below 2 M salt, an ionic strength that would readily dissociate protein complexes held together by electrostatic forces, the centrosome structure and parthenogenetic activity remain unaffected. At higher ionic strength, an increasing proportion of the constituent proteins are denatured or precipitated ("salted out"). As a consequence, there is no extensive protein solubilization and no disorganization of the overall ultrastructure. It is easy to understand how such an effect could be irreversible and lead to inactivation of parthenogenesis.

The effect of urea suggests that parthenogenetic activity of centrosomes persists as long as enough structure is preserved to regenerate a new complete centrosome through complementation by material stored in the egg cytoplasm. Apparently, the proximal end of the centriole is more resistant to urea extraction. It is interesting to note that during normal development, the daughter centriole grows at a right angle from this region. Perhaps the structure of the centriole in the proximal region is essential for centrosome regeneration and duplication.

However, this interpretation might be an oversimplification since structural alterations are easier to detect in the precisely organized centrioles than in the PCM. Since we have not found a way to specifically dissociate the PCM from the centriole, we could not assay directly the parthenogenetic activity of the centriole proper. Moreover, we cannot eliminate the possibility that a molecule sufficient for controlling the initiation of assembly of a new centrosome is extracted at the same urea concentration. Nevertheless, our results strongly point to a mechanism by which the centriole, or the whole centrosome, acts as a template to induce the cycle of centrosome duplication which is necessary to embryogenesis.

Interestingly enough, urea and salt affect more severely the in vitro microtubule nucleating activity than the parthenogenetic activity. Centrosomes treated with 1 or 2 M urea, for example, do not nucleate microtubules in vitro, yet they induce egg cleavage normally. This means that the lost microtubule nucleating activity is reconstituted after microinjection in the egg by some cytoplasmic component. We can draw this conclusion because egg cleavage is blocked by microtubule disrupting drugs (Hara et al., 1980), showing that the capacity of centrosomes to nucleate microtubules is required for cleavage furrow formation. In fact, we have recently found that microtubule nucleating activity of urea-extracted centrosomes can be complemented by frog egg extracts (Verde, F., and E. Karsenti, unpublished observations). This further indicates that stored microtubule nucleating material can reassociate with damaged centrosomes and restore their nucleating activity.

Parthenogenetic Activity Does not Require a Nucleic Acid

Because of the apparent continuity of centrioles, inferred from their mechanism of duplication (Lwoff, 1950), nucleic

acids have been postulated to be important components of centrosomes. In the present work we did not find any effect of nucleases on the in vitro microtubule nucleating activity or on the parthenogenetic activity of purified human centrosomes. This was assessed by treating centrosomes with micrococcal nuclease or RNase A either alone or in addition to a dissociating agent. Although this is a negative result, it is strengthened by the finding that no major centrosome-associated RNA resists nuclease treatment. This strongly suggests that there is no centrosomal RNA involved in microtubule nucleating activity in vitro, in agreement with previous results from Heidemann et al. (1977) and Kuriyama (1984), or in vivo as implied by the parthenogenetic activity. This latter result is at variance with the RNase sensitivity of the aster-inducing activity of Tetrahymena basal bodies injected into *Xenopus* eggs (Heidemann et al., 1977). This may reflect a difference between basal bodies and centrosomes. There are however several procedural differences which could account for this discrepancy. Heidemann et al. (1977) used stratified eggs. This may have resulted in the separation of cytoplasmic components needed to complement RNase-treated centrosomes. Our results do not eliminate the possibility that RNase-treated centrosomes are complemented by some RNA present in the egg. They strongly suggest that there is no need for an RNA in the injected centrosome to reconstitute a functional centrosome in the egg.

The Centrosome: a Self-Duplicating Protein Structure?

The results reported in this work strongly suggest that the parthenogenetic activity of centrosomes (*a*) cannot be solubilized, (*b*) requires the whole centrosome or a structure that can reconstitute a centrosome in the egg cytoplasm, and (*c*) does not require a nucleic acid on the injected centrosome.

This raises the question of the duplication mechanism of a complex protein structure at each cell cycle. One possibility is that the centrosome, or part of it, simply acts as a seed to induce the growth of the daughter centrosome at each cell cycle. The timing of the duplication would then be determined by cell cycle signals, just as the initiation of nuclear envelope breakdown and spindle assembly are. It is not clear at present what phase of centrosome duplication is regulated during the cell cycle.

In marine eggs, centrioles assemble de novo after unspecific treatments (Kuriyama and Borisy, 1983; Kallenbach, 1985). It is likely that a seeding material is present but repressed or that the treatment induces an aggregation of a soluble or dispersed seeding structure. In mouse eggs, the first eight cleavages take place in the absence of centrioles (Szollosi et al., 1972). However, some aggregated material having microtubule nucleating activity is present and nucleates microtubule asters in anaphase, allowing cleavage to occur (Callarco-Gillam et al., 1983; Maro et al., 1985; Schatten et al., 1986). Around the eighth cell stage, typical centrioles appear (Szollosi et al., 1972). It is possible that before this developmental stage, specific centriolar components are missing. The assembly of centrioles at the 16th cell stage may be dependent on the synthesis of the missing components and initiated on the preexisting aggregated material.

Amphibian eggs appear to be a good system to study the initiation of centriole duplication since they apparently lack a functional seeding material. However, the parthenogenetic assay has some limitation, the egg being used as a "black box" in which we have no easy access to the injected centrioles. Further work on centriole duplication needs to be carried out in vitro. An attractive approach would be the use of well-characterized, concentrated frog egg extracts which support nuclear and mitotic spindle reconstitution (Lohka and Masui, 1983; Newmeyer et al., 1986; Newport, 1987).

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Note Added in Proof. During the preparation of this manuscript, strong experimental support for the presence of DNA in *Chlamydomonas* basal body has been provided (Hall, J. L., Z. E. Rananis, and D. J. L. Luck. 1989. *Cell.* 59:121–132). As mentioned in the Discussion, our results suggest that, just as for RNA, the presence of such a DNA in the infected chromosomes is not required for parthenogenesis.

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