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Mitotic Spindle Assembly by Two Different Pathways in Vitro

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Abstract. We have used *Xenopus* egg extracts to study spindle morphogenesis in a cell-free system and have identified two pathways of spindle assembly in vitro using methods of fluorescent analogue cytochemistry. When demembrated sperm nuclei are added to egg extracts arrested in a mitotic state, individual nuclei direct the assembly of polarized microtubule arrays, which we term half-spindles; half-spindles then fuse pairwise to form bipolar spindles. In contrast, when sperm nuclei are added to extracts that are induced to enter interphase and arrested in the following mitosis, a single sperm nucleus can direct the assembly of a complete spindle. We find that microtubule arrays in vitro are strongly biased towards chromatin, but this does not depend on specific kinetochore-microtubule

interactions. Indeed, although we have identified morphological and probably functional kinetochores in spindles assembled in vitro, kinetochores appear not to play an obligate role in the establishment of stable, bipolar microtubule arrays in either assembly pathway. Features of the two pathways suggest that spindle assembly involves a hierarchy of selective microtubule stabilization, involving both chromatin-microtubule interactions and antiparallel microtubule-microtubule interactions, and that fundamental molecular interactions are probably the same in both pathways. This in vitro reconstitution system should be useful for identifying the molecules regulating the generation of asymmetric microtubule arrays and for understanding spindle morphogenesis in general.

THE mitotic spindle of higher eukaryotes must rapidly assemble from preexisting subcellular components at the onset of mitosis and disappear in late telophase. Between its creation and dissolution the spindle must effect the proper segregation of chromosomes, and in recent years progress has been made towards understanding the molecular mechanisms of chromosome movement during mitosis (Gorbsky et al., 1988; Mitchison, 1988; Nicklas, 1989). However, basic questions concerning the assembly of the spindle as a morphological structure and the relation between morphogenesis and mechanochemistry remain to be elucidated. A number of coordinated events and interactions have been proposed to be involved in spindle assembly (Mazia, 1961; Nicklas, 1971; McIntosh and Koonce, 1989), and recent work has emphasized microtubule dynamics in the spindle (Salmon et al., 1984; Saxton et al., 1984; Mitchison et al., 1986). The recognition that the fundamental behavior of spindle microtubules is one of rapid turnover generated by dynamic instability has led to the idea that selective microtubule stabilization may be the principal mechanism for generating asymmetric microtubule distributions in cells (Kirschner and Mitchison, 1986). The nature of stabilizing factors and how they are spatially organized, however, remains obscure. Ultimately we would like to understand the organizational principles involved in the assembly of the mitotic spindle and to identify the molecules that mediate important interactions, in particular those that result in particular microtubule conformations. What regulates the formation

of a stable, fusiform (i.e., spindle-shaped) microtubule array, for example, after centrosomes have migrated to opposite sides of the nucleus? Our current molecular picture of the spindle is limited, in part because of the lack of assays correlating structure and function. While genetic analysis in simple eukaryotes may allow us to identify components of the spindle and interactions among components (Enos and Morris, 1990; Meluh and Rose, 1990), these systems have been less useful for studying molecular dynamics and the forces of morphogenesis. More functional experiments with lysed or living cells or with isolated spindles have contributed to our understanding of mitotic mechanism, but reactivation of many of the complex processes of mitosis in vivo has been difficult to attain in vitro (reviewed in Cande, 1989), and isolated spindles have typically defied any detailed correlation between biochemical and functional analyses (see, for example, Salmon and Segall, 1980; Wordeman and Cande, 1987; Dinsmore and Sloboda, 1988). Ideally, to address these problems one would like to establish a system in which both spindle morphogenesis and function can be reconstituted in vitro and then dissected biochemically.

Following the pioneering work of Lohka and Masui (1983a), cell-free *Xenopus* egg extracts in defined cell cycle states have been used to study the biochemistry of cell cycle regulation (Lohka et al., 1988; Murray and Kirschner, 1989a), as well as cellular events under mitotic control (Gard and Kirschner, 1987; Newport and Spann, 1987; Verde et al., 1990; Ward and Kirschner, 1990; Belmont et

al., 1990). Lohka and Maller (1985) noted that cytoplasmic egg extracts arrested in mitosis were capable of organizing spindles when incubated with demembrated *Xenopus* sperm nuclei, and that a particulate fraction was required for spindle assembly. Murray and Kirschner (1989a) also observed spindle formation in extracts capable of undergoing multiple cell cycles in vitro. In this paper we extend these observations with methods of fluorescence analog cytochemistry. We demonstrate two different pathways of spindle formation in *Xenopus* egg extracts. The nature of these assembly pathways as elucidated by reconstitution in vitro has important implications for the respective roles of chromatin, kinetochores, and centrosomes in spindle morphogenesis. In the paper that follows (Sawin and Mitchison, 1990), we address dynamic properties of metaphase spindles assembled in vitro.

Materials and Methods

Mitotically Arrested Extracts ("Mitotic Extracts")

Crude cytoplasmic extracts arrested in a mitotic state ("mitotic extracts") were prepared from unfertilized eggs of *Xenopus laevis*, following exactly the modification made by Murray et al. (1989) to the original centrifugal crushing method of Lohka and Masui (1983a). Unfertilized eggs are naturally arrested in metaphase of meiosis II, through stabilizing effects of cytostatic factor (CSF) (Masui and Markert, 1971) on the activity of maturation promoting factor (Murray and Kirschner, 1989b), so extracts prepared in this manner might more correctly be called "meiotic extracts." Murray et al. (1989) have termed these extracts CSF extracts, while Lohka and Maller (1985) have referred to them as EGTA extracts, because of the requirement for EGTA in the maintenance of CSF activity (Sagata et al., 1989; Watanabe et al., 1989). Because of similarities between meiosis II and mitosis, and because of the ability of these extracts to induce mitotic effects on interphase nuclei (see below), we will refer to them as mitotic extracts. After preparation, extracts were held on ice before use up to 10 h, during which time they remained competent to assemble spindles.

Extracts That Enter Mitosis after Interphase ("Interphase-to-Mitotic Extracts")

Mitotic extracts prepared as described above were activated into an interphase state by the addition of submillimolar calcium (0.2–0.4 mM), before use (Lohka and Maller, 1985; Murray et al., 1989) 1 h after calcium activation, an equal volume of mitotic extract was added back to calcium-activated interphase extracts in order to drive them back into mitosis (Lohka and Maller, 1985). Mitotic induction by this method invariably results in irreversible mitotic arrest in the final mixture, probably because of the high levels of cytostatic factor present in mitotic extracts. Spindles formed in these "interphase-to-mitotic" extracts are therefore stable over time and do not undergo a metaphase-to-anaphase transition. Alternatively, extracts activated into interphase by calcium addition can enter mitosis without the further addition of mitotic extract, often (but not reproducibly) arresting in the first mitosis after interphase (A. Murray and K. Sawin, unpublished observations). These two methods of generating mitotically arrested extracts from interphase extracts produce identical structures in mitosis: we will refer to such extracts interchangeably as interphase-to-mitotic extracts.

Fluorescent Labeling of Tubulin

Tetramethylrhodamine (TMR)¹-labeled bovine brain tubulin was prepared exactly as described by the high pH labeling method (Hyman et al., 1990), which involves two cycles of temperature-dependent assembly/disassembly to select functional subunits after labeling. Fluorescein and TMR-labeled tubulin were aliquoted in tubulin injection buffer (10–30 mg/ml in 50 mM Kglutamate, 0.5 mM MgCl₂, pH 6.5) and stored at –80°C. In most experiments the labeling stoichiometry after two cycles was between 0.8 and 1.8

fluorochromes per tubulin dimer, assuming extinction coefficients of 80,000 M⁻¹ cm⁻¹ and 60,000 M⁻¹ cm⁻¹ for fluorescein and tetramethylrhodamine, respectively (Haugland, 1989), and 0.8 (mg/ml)⁻¹ cm⁻¹ for tubulin (Kristofferson et al., 1986).

Isolation of Sperm Nuclei

Sperm nuclei were isolated from *Xenopus laevis* male testes by the SuNaSp-lysolecithin procedure of Gurdon (1976), as modified by Murray and Kirschner (1989a), as follows. Testes were dissected and minced with a razor blade to free sperm from the tissue, washed in HSP (250 mM sucrose, 15 mM Hepes pH 7.4, 1 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.1% βME, 10 μg/ml leupeptin, pepstatin, and chymostatin, 0.3 mM PMSF), and treated with 0.5 mg/ml lysolecithin for 5 min at room temperature. After the addition of 10 vol of ice-cold HSP/3% BSA (fraction V) to adsorb lysolecithin, demembrated nuclei were taken through additional washes in HSP/0.3% BSA before being stored in HSP/0.3% BSA/30% glycerol at –80°C (Lohka and Masui, 1983b).

Fluorescent Labeling of Sperm Nuclei

We were unsuccessful in our attempts to prelabel nuclei in extracts with fluorescent DNA-binding dyes such as ethidium bromide; under all conditions, specific staining was lost from prelabeled nuclei within minutes after the addition of nuclei to extracts. We found that we could label demembrated sperm nuclei covalently with N-hydroxysuccinimide (NHS) esters of fluorescent dyes. Sperm to be labeled with NHS-TMR were isolated as above, except that 5 mM MgCl₂ was substituted for spermidine and spermine in HSP, since polyamines react with NHS esters. After lysolecithin treatment and BSA adsorption, sperm nuclei were washed in HSP without BSA. Nuclei were labeled with 50 μM NHS-TMR for 30 s at room temperature, and the reaction was immediately quenched by the addition of 10 mM Kglutamate and taken through repeated washes in HSP/0.3% BSA as above, before storage at –80°C in HSP/0.3% BSA/30% glycerol. Nuclei labeled by this method were competent to form interphase nuclei and mitotic chromosomes at the same rate and with the same efficiency as unlabeled nuclei. Much higher levels of labeling produced sperm nuclei that were less competent in reconstitution assays.

Reconstituting Spindle Assembly in Extracts

Isolated sperm nuclei were diluted into sperm dilution buffer (100 mM KCl, 1 mM MgCl₂, 150 mM sucrose, 10 μg/ml cytochalasin D) (Murray and Kirschner, 1989a) to a concentration of ~1,000 nuclei/μl, and this dilution was added to extracts on ice at a 1:10 dilution. In all cases sperm was added to extracts immediately before use; when mitotic extracts were activated into interphase, calcium and sperm nuclei were added simultaneously. In sperm mixing experiments, labeled and unlabeled nuclei were diluted together into sperm dilution buffer, and an aliquot was stained with Hoechst 33258 to determine the ratio of labeled/unlabeled nuclei. This was adjusted to within 10% of a 50:50 ratio, counting a minimum of 200 nuclei. Where indicated, labeled tubulin was added to extracts at a final concentration of 0.3 mg/ml or less, aphidicolin (5 mg/ml stock, frozen in DMSO) at 10 μg/ml, and calcium as a 1:10 dilution of (2–4 mM CaCl₂, 100 mM KCl, 1 mM MgCl₂). When sperm, tubulin, etc. were added to extracts we were careful to limit the total dilution to 25%, and control extracts were similarly diluted, because extracts that are extensively diluted (e.g., 400% or more) do not form normal spindles. Spindle assembly was initiated by incubating extracts in eppendorf tubes in volumes of 50 μl or less in a water bath at 20°C. Incubating extracts in small volumes was necessary to produce consistent results; this may be due to a requirement for efficient gas exchange.

Spindle assembly was usually assayed by spotting 1–2 μl of extract onto a microscope slide, adding 3–4 μl formaldehyde fixative (Murray and Kirschner, 1989a), and squashing gently under a coverslip. Alternatively, extracts were diluted at least 50-fold into 80 mM KPipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA (BRB80)/30% glycerol/1% Triton X-100 and layered over a cushion of BRB80/40% glycerol in Corex tubes modified to contain a 12-mm round coverslip at the bottom (Evans et al., 1985). Tubes were spun in a swinging bucket rotor 8,000 g, 30', 20°C. The addition of Triton X-100 allowed centrifugation of up to 50–100 μl of extract on a single coverslip without any significant background fluorescence. After centrifugation, coverslips were either fixed in ice-cold methanol or mounted directly onto microscope slides in chambers containing BRB80/40% glycerol. Spindles in BRB80/40% glycerol are stable at room temperature at least overnight.

1. Abbreviations used in this paper: NHS, N-hydroxysuccinimide; SIT, silicon-intensified target; TMR, tetramethylrhodamine.

Fixed spindles were washed in TBS/0.1% Triton X-100 and mounted on microscope slides.

Light Microscopy and Photography

Light micrographs were taken on a Photomicroscope III (Zeiss; Oberkochen, FRG) with a 25×/0.8 N.A. Neofluar (Zeiss) or a 60×/1.4 N.A. S Plan Apo (Olympus) objective, 1.25 optovar, using Kodak Technical Pan film or Technical Pan film that had been hypersensitized by exposure to hydrogen (Schulze and Kirschner, 1986). Development was in D-19. Fig. 10 was shot with Kodak T-MAX400 film pushed to 1600 ASA in TMAX developer, because the rapid bleaching of fluorescein-labeled tubulin makes it more difficult to photograph than TMR-tubulin. Photographs were printed on low-contrast paper.

Quantitative Fluorescence Microscopy

Spindles were assembled in mitotic extracts containing 0.3 mg/ml TMR-tubulin and fixed in situ by the coverslip squash assay after 70 min incubation. Spindles and half-spindles were observed with a Zeiss Universal fluorescence microscope equipped with a 25×/0.8 N.A. Neofluar (Zeiss) objective and mercury-arc illumination. Fluorescent images were collected through a silicon-intensified target (SIT) video camera (Cohu, San Diego, CA) under the control of a Maxvision image processor (Datacube, Peabody, MA) driven by a 386 AT microcomputer and custom-written software (Sawin and Mitchison, 1990). Before collecting images we adjusted the light source and the SIT camera gain such that pixel intensity values in the brightest spindles did not exceed 80% of saturation (i.e., intensity values <200 in a 256 grey scale); lamp and camera gain remained constant, at these levels, throughout data collection. The SIT camera was calibrated with a set of neutral density filters and found to be virtually linear at these gain settings throughout the tested range of illumination (intensity values from 0 to 200, in a 256 grey scale).

Successive rhodamine and Hoechst images of half-spindles and spindles were recorded with an optical memory disk recorder (TQ-3038F; Panasonic; Secaucus, NJ) as 64-frame averages, taking care to avoid photobleaching. After recording, the average rhodamine fluorescence intensity in the vicinity of chromatin was measured for three 12×10 -pixel regions in each spindle and half-spindle (areas chosen at random on the basis of Hoechst staining) and averaged to a single value. At the same time, background fluorescence, which varied considerably and was dependent on the thickness of the squash, was also measured and subtracted from the average

intensity value to yield a corrected value. The data in Fig. 4 are averages of corrected values for 20 spindles and 16 half-spindles.

EM

For EM, spindles assembled in extracts were diluted in BRB80/30% glycerol/1% Triton X-100 and centrifuged through cushions of BRB80/40% glycerol as described above, except that coverslips were made of mylar (Acklar; DuPont) and were ethanol washed, glow discharged, and incubated in 1 mg/ml polylysine (400,000 mol wt; Sigma Chemical Co., St. Louis, MO) before use. Spindles on mylar coverslips were immobilized by adding a drop of low-gel agarose (1% NuSieve, FMC, in BRB80/40% glycerol) and fixed in BRB80/40% glycerol/2.5% glutaraldehyde at least 2 h at room temperature. After fixation spindles were rinsed, stained in tannic acid (30', 1% tannic acid in BRB80), osmicated, and flat-embedded in Epon-Araldite. 60- and 150-nm sections were cut from blocks and stained with 2.5% uranyl acetate and 0.02% lead citrate and viewed at 60 and 100 kV, respectively.

Calculation of Values in Table II and Error Analysis of Sperm Mixing Experiments

The calculated ratios of (unlabeled only):(labeled only):(labeled and unlabeled, i.e., hybrid) spindles shown in Table II were obtained by evaluating binomial expressions $(a + b)^n$ of different order n , as follows. If a is the fraction of labeled nuclei at time zero and b is the fraction of unlabeled nuclei ($a + b = 1$), then for a random assembly mechanism of order n ($n = 1$ for "unitary" assembly, $n = 2$ for "binary"), the ratio of (unlabeled):(labeled):(hybrid) spindles will be $a^n:b^n:(1 - a^n - b^n)$. Because equal amounts of labeled and unlabeled sperm were used in each experiment, for the values calculated in Table II, $a = b = 0.5$. The discussion of error (below) considers alternate values for a and b .

Since the ratios of (unlabeled):(labeled):(hybrid) spindles observed in mitotic extracts do deviate somewhat from ideal (i.e., 100% binary) values, it is important to consider potential sources of error in these experiments. Random error should not contribute significantly, since in all cases over 100 spindles were counted for each data point. A more significant source of error may be the ratio of (unlabeled):(labeled) input DNA (i.e., a/b). From counting sperm nuclei at time zero we are confident that this ratio is between 45:55 and 55:45, but we note that this range would yield final ratios of (unlabeled):(labeled):(hybrid) spindles anywhere between 20:30:50 and 30:20:50, even with 100% binary assembly, so this might account for some of the variation between experiments as well as deviations from ideal values.

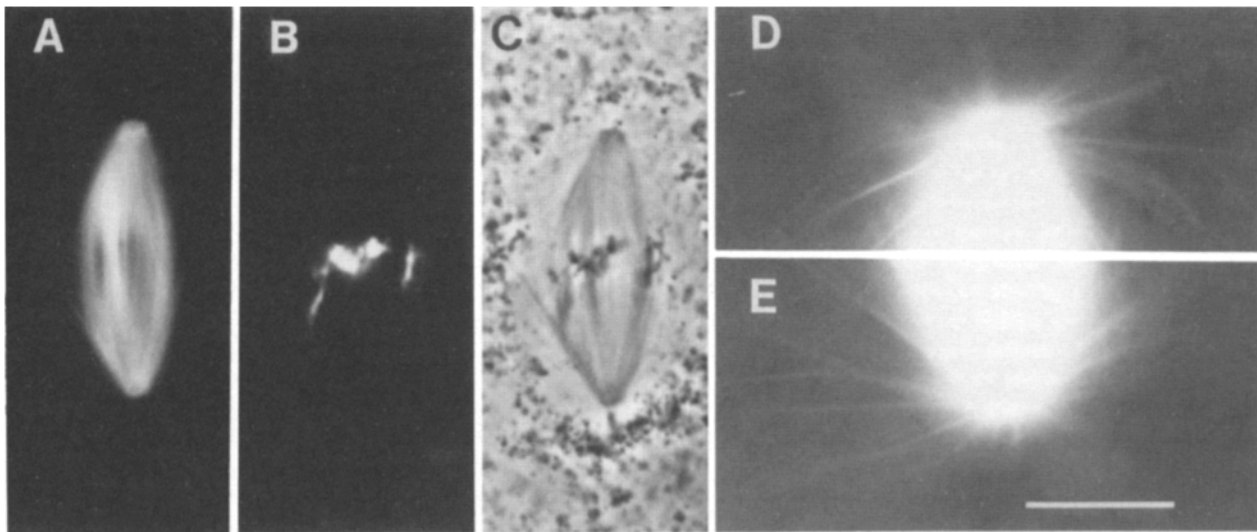


Figure 1. Mitotic spindle assembly in crude mitotic *Xenopus* oocyte extracts after incubation with demembrated sperm nuclei and 0.3 mg/ml TMR-tubulin. (A) TMR-tubulin; (B) Hoechst (DNA); and (C) phase-contrast images of a spindle assembled in vitro and fixed after 90' incubation. Note the central position of chromatin within the spindle (B) and microtubule bundles in the spindle midzone (A and C); bundles were seen in many different focal planes, as determined by laser scanning confocal microscopy (not shown). Longer exposures of TMR-tubulin fluorescence (D and E) reveal a small number of astral (i.e., nonspindle) microtubules radiating from each spindle pole. Bar: (A-C) 30 μ m; (D and E) 20 μ m.

The greatest potential source of error in these experiments stems from the possibility that not all nuclei in a given extract follow the same mechanism of assembly. The effects of a fraction of nuclei following a unitary assembly mechanism and the remainder following a binary mechanism are shown in Table II and are calculated as follows, always assuming that $a = b = 0.5$: Consider $p + q$ nuclei, such that p nuclei follow a unitary mechanism and make p spindles, and q nuclei follow a binary mechanism and make $q/2$ spindles. The ratio of (unlabeled):(labeled):(hybrid) spindles will then be $p/2 : p/2 : 0$ for the p unitary spindles, and $q/8 : q/8 : q/4$ for the $q/2$ binary spindles. Adding these together and normalizing to 100, one obtains a final ratio of $((p/2) + (q/8))/(p + (q/2)) : ((p/2) + (q/8))/(p + (q/2)) : ((q/4)/(p + (q/2)))$. Accordingly, if one assumes that 10% of the total input nuclei form spindles following a unitary assembly mechanism (say, $p = 10$), while 90% of the nuclei follow a binary pathway ($q = 90$), the calculated ratio of (unlabeled):(labeled):(hybrid) spindles shifts from 25:25:50 to 29:29:41, which matches the average data in Table II for mitotic extracts quite closely. Thus, irrespective of what mechanisms may be responsible for unitary assembly in mitotic extracts, it is quantitatively likely that ~90% of spindles in mitotic extracts are formed from binary assembly mechanisms. A likely reason for partial unitary assembly in mitotic extracts is that most sperm preparations are contaminated by immature spermatocyte nuclei (10–15% of total input nuclei; data not shown). These nuclei are easily distinguished from mature spermatozoa by their spherical shape at time zero, and observations at early time points suggest that at least some of these nuclei may form spindles by a unitary pathway (data not shown).

A similar analysis applied to mixing experiments in interphase-to-mitotic extracts, shown in Table II, indicates that at least the majority of spindles formed under these extracts conditions result from unitary assembly.

Results

Fluorescence Assay for Spindle Assembly

Lohka and Maller (1985) originally observed in paraffin sections that demembrated sperm nuclei added to unfertilized egg extracts were capable of organizing into mitotic spindles; however, by phase-contrast microscopy it is difficult to recognize spindles in crude extracts with any consistency (see Fig. 1, 2, and 5). To reduce the time required to visualize spindles, we developed a fluorescence assay for spindle assembly. Tetramethyl-rhodamine-labeled bovine brain tubulin and demembrated sperm were added to extracts and spindle assembly assayed directly by fluorescence microscopy after incubation at 20°C. By 90 min, bipolar, fusiform microtubule arrays with chromatin in their centers formed in extracts, as is shown in Fig. 1; these were identical in appearance to the bipolar structures observed by Lohka and Maller (1985), and we refer to them as spindles. Antitubulin immunofluorescence was coextensive with TMF-tubulin fluorescence when spindles were stained with antitubulin antibodies (data not shown). To determine whether the addition of labeled tubulin affected spindle morphology or the efficiency of spindle formation, we added sperm nuclei and different concentrations of TMF-tubulin aliquots of the same extract and assayed spindle assembly by fluorescence. As shown in Table I the efficiency of spindle formation in the absence of exogenous tubulin, expressed as the percent spindles over total countable nuclei, was typically 20–40%, although somewhat variable among different extract preparations (see Fig. 3). This percentage decreased at maximal concentrations of added tubulin (1.2 mg/ml, 50% of endogenous tubulin), and at the highest concentration, spindles also tended to be smaller than average (data not shown). Both of these effects were less pronounced at lower concentrations of added tubulin; we therefore decided to add tubulin to extracts at a final concentration of 0.3 mg/ml or less, as this provided a strong

Table I. Spindle Assembly Is Unaffected by the Addition of Low Concentrations of TMRd-labeled Tubulin

Added tubulin mg/ml	Nuclei present as:			n
	Spindles*	Half-spindles†	Free nuclei‡	
		%		
1.2	13	74	13	67
0.6	13	79	8	111
0.3	23	77	0	65
0.15	23	77	0	119
0	27	73	0	127

* Bipolar fusiform arrays.

† Monopolar arrays; described in text.

‡ Not associated with microtubules.

signal without any obvious effects on spindle morphology. By Western blotting we have found the concentration of endogenous egg tubulin to be 2.0–2.5 mg/ml (not shown), in agreement with previous data (Gard and Kirschner, 1987). These results indicate that spindle formation can occur in egg extracts with high efficiency and that TMR-labeled bovine brain tubulin can serve as a microtubule probe at a concentration 12–15% that of endogenous *Xenopus* egg tubulin, without significant effects on microtubule organization.

Spindle Assembly from Sperm Nuclei in Mitotic Extracts

The fluorescence assay allowed us to observe apparent intermediates in spindle assembly, as shown in Fig. 2. Labeled tubulin and demembrated sperm nuclei were added to mitotic extracts and incubated until fixation in situ. We found that demembrated nuclei, while free of membranes and flagellar axonemes (Lohka and Masui, 1983a), maintain a functional centrosome at the base of the nucleus. Microtubule growth was initially radially symmetric from the sperm centrosome (Fig. 2, A–C). At slightly later times (10–20 min) microtubule arrays no longer exhibited radial symmetry, and more centrosomally nucleated microtubules were seen towards the chromatin than in the opposite direction. At the same time, chromatin began to reorganize into a condensed mitotic state and to move away from the centrosome, which by TMR-tubulin fluorescence appeared to swell into a more diffuse sphere-like structure, suggestive of a structural reorganization (Fig. 2, D–F). By 30-min, the separation of chromatin and the centrosome was complete (~20 μ m on average; data not shown), and the asymmetry in microtubule distribution towards chromatin became highly pronounced. By this time, microtubules became focused to a more point-like pole, resulting in the formation of a polarized microtubule array with the sperm centrosome at one end and chromatin in a condensed, mitotic state at the other (Fig. 2, G–I; see Fig. 4, A and a); we refer to this as the “half-spindle.” In many half-spindles the pole seemed to split into two point-like poles (Fig. 2 G, arrows). Finally, at 40–90 min, spindles appeared in the extracts (Fig. 2, J–L), gradually increasing in number with time (see Fig. 3). Like half-spindles, spindles also often had split poles (Fig. 2 J, arrows). Mitotic chromatin was highly condensed and usually at the center of the spindle. However, in many cases a canonical metaphase plate configuration was not achieved and/or

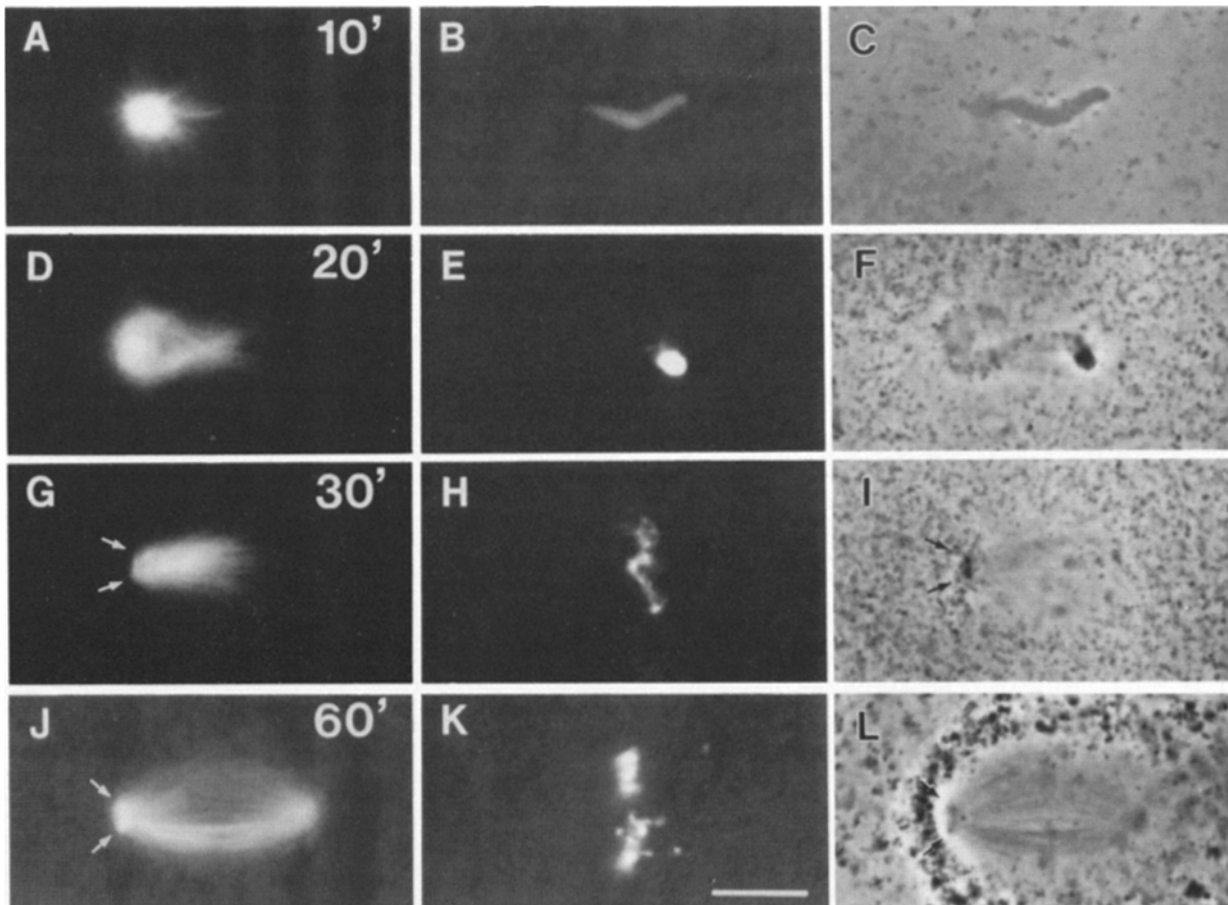


Figure 2. Time course of spindle assembly in mitotic extracts incubated with sperm nuclei and TMR-tubulin. Conventional photomicrographs. (A, D, J, and G) TMR-tubulin; (B, E, H, and K) Hoechst; and (C, F, I, and L) phase-contrast images of spindle assembly intermediates fixed after different incubation times, given in minutes. Microtubule arrays become increasingly asymmetric over time. Arrows indicate separating half-spindle (G and I) and spindle (J and L) poles. The right-hand spindle pole in J has not separated, and chromosomes in the central spindle (K) appear as two distinct clumps of mitotic chromatin. Negatives were printed at varying exposures and therefore do not represent microtubule fluorescence quantitatively (see Fig. 4). Bar, 20 μ m.

it was difficult to resolve individual chromosomes from the bulk of chromatin, which persisted as large clumps from which condensed threads emerged (compare Fig. 1 and Fig. 2 J-L). For this reason we will refer to chromatin in spindles assembled in mitotic extracts simply as "chromatin," rather than "chromosomes." On careful through-focusing, many spindles seemed to contain two distinct clumps of chromatin, which we believe originate from separate nuclei (Fig. 2, J-L; see below), but this was often obscured by the condensation state of chromatin. Microtubules in spindles were usually highly bundled (Fig. 1, A and C). In both mature half-spindles and spindles the overall microtubule distribution was highly biased towards the chromatin, although we observed a few microtubules radiating from the centrosome in the opposite direction (Fig. 1, C and D). This organization is different from that observed in early embryonic *Xenopus* spindles, which have well-formed asters (Karsenti et al., 1984a). We do not yet have an explanation for this discrepancy; it may reflect a qualitative difference in the three-dimensional structure of microtubule nucleating centers (Mazia, 1984, 1987) in vivo vs. in vitro, and/or quantitative differences in the extent of microtubule stabilization or bundling towards vs. away from the central spindle. At very long

times and at high sperm concentrations we often observed multipolar spindles (data not shown). Apart from the structures described, we observed no other stereotyped microtubule arrays during spindle assembly.

Half Spindles Are Intermediates in Spindle Assembly

Are half-spindles actual intermediates in spindle assembly, or merely end products of a side reaction? Observations from many experiments suggested that the number of spindles increases slowly with time during assembly while half-spindle number seem to decrease, consistent with half-spindles being structural intermediates. We confirmed this by centrifuging extracts onto coverslips and counting the types of chromatin-containing figures at different time points. The results of this kinetic analysis are given in Fig. 3, which shows that nearly all sperm nuclei develop into asters and, in turn, half-spindles. Furthermore, as spindles increase in number, half-spindles do indeed decrease in number. While these data are not quantitative enough to allow any inferences about the mechanism of conversion from half-spindles into spindles, they demonstrate that chromatin in half-spindles eventually ends up in spindles and/or multipolar ar-

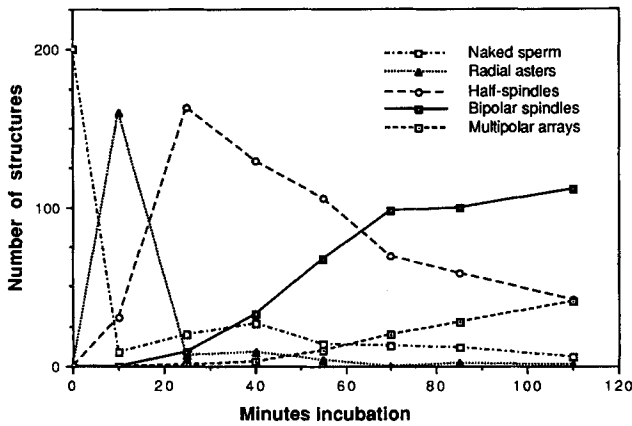


Figure 3. Kinetic analysis of spindle assembly in mitotic extracts. Extracts were incubated with sperm nuclei and TMR-tubulin and diluted into a microtubule stabilizing buffer at the indicated times. They were then centrifuged onto coverslips and mounted on slides, as described in Materials and Methods. For each time point, 200 chromatin-containing structures were counted and categorized into the five classes shown.

rays, suggesting that half-spindles are kinetic intermediates in spindle assembly.

Microtubule Density in Half-spindles and Spindles

Conventional photomicrographs and observation under the microscope suggested that the total tubulin fluorescence was qualitatively much greater in spindles than in half-spindles. (Photographs in Fig. 2 were exposed for varying times, to emphasize the particular structures in each image.) To investigate this apparent difference more quantitatively, we measured fluorescence in half-spindles and spindles with an intensity-calibrated SIT camera. Fig. 4, *A* and *B* show SIT camera images of a spindle and a half-spindle shot and printed under identical exposures. Quantitative fluorescence measurements from a number of independent structures indicated that spindles contained more than fourfold more microtubules per unit area than half spindles, measuring

comparable regions (the spindle midzone, defined by proximity to chromatin) at the same time point, as shown in Fig. 4 *C*. We consider the reasons for this increase in microtubule density in the Discussion.

Spindle Assembly in Interphase-to-Mitotic Extracts

In the procedure described above, sperm nuclei containing unreplicated DNA are added directly to mitotic extracts and are therefore forced to make spindles in the absence of DNA replication (Blow and Watson, 1987; Hutchison et al., 1987). We confirmed this by assaying incorporation of radio-labeled dCTP into TCA-precipitable counts and observed no appreciable DNA synthesis in mitotic extracts throughout the course of experiments (data not shown). To assemble spindles using a more physiological (i.e., replicated) chromatin substrate, mitotic extracts containing sperm nuclei and TMR-tubulin were activated into interphase by the addition of calcium (Lohka and Maller, 1985), and followed through fixed time points. As in mitotic extracts, the sperm centrosome organized a radial aster within 10 min. However, as the extract entered interphase, the sperm aster expanded rapidly, accompanied by an opening-up of the centrosome into a more sphere-like structure, which eventually fell apart (data not shown). Between 30 and 60 min, the sperm chromatin decondensed, forming an interphase nucleus, and the number of free microtubules in the extract increased significantly compared to mitotic extracts (Fig. 5, *A-C*). The interphase nucleus was often associated with microtubule arrays, but these were easily deformed by the coverslip squash (Fig. 5, *D-F*). Interphase extracts were then driven into mitosis by the addition of an equal volume of mitotic extract (Lohka and Maller, 1985) (see Materials and Methods); alternatively, they entered mitosis spontaneously, with similar results. Within minutes after addition of mitotic extract, the number of free microtubules in interphase extracts decreased significantly, and microtubule arrays formed around the condensing chromatin (Fig. 5, *D-F, G-I*). We did not observe typical half-spindles in extracts that converted from an interphase to a mitotic state. Rather, intermediates in spindle assembly appeared to be bipolar but

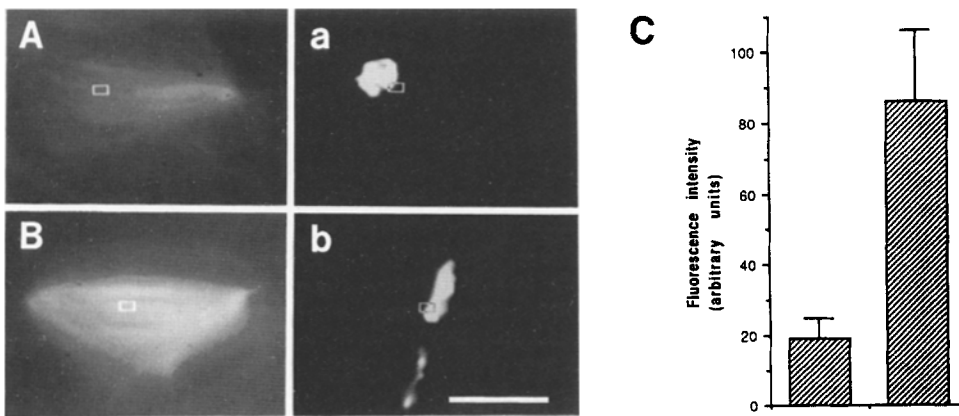


Figure 4. Microtubule density is significantly greater in spindles than in half-spindles. (*A* and *B*) TMR-tubulin and (*a* and *b*) Hoechst images of a half-spindle (*A* and *a*) and a spindle (*B* and *b*) assembled in mitotic extracts. These images were collected with a SIT camera and then photographed off the monitor and printed under identical conditions. Small regions chosen on the basis of proximity to chromatin were superimposed onto digitized images of microtubule arrays (shown as representative boxes), and the average

fluorescence intensity within regions of interest was determined (Materials and Methods). (*C*) Average microtubule density in spindles vs. half-spindles, measuring three independently chosen regions per microtubule array. Average fluorescence intensity + SD, for 16 half-spindles and 20 spindles. Bar, 20 μ m.

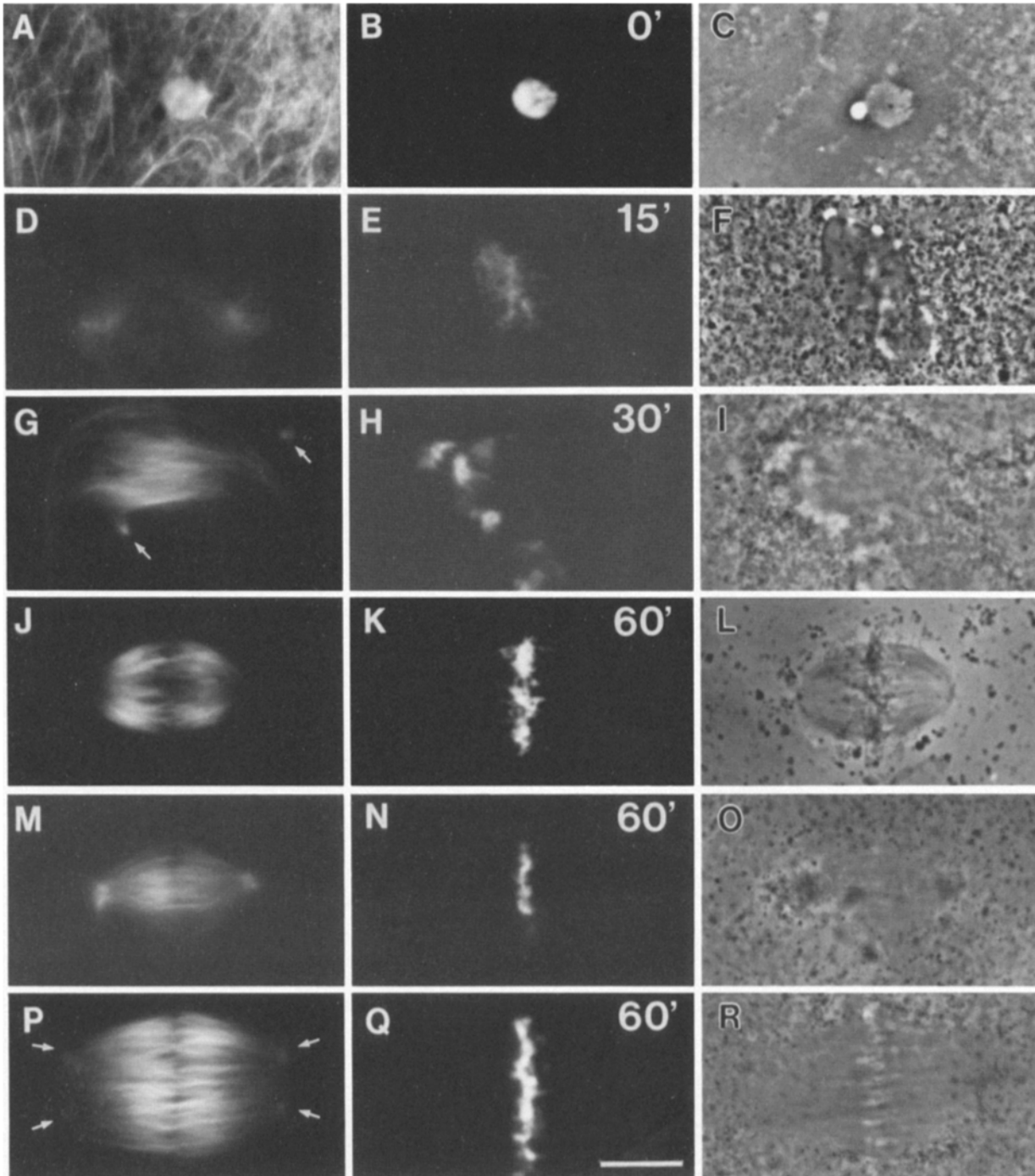


Figure 5. Time course of spindle assembly in interphase-to-mitotic extracts incubated with sperm nuclei and TMR-tubulin. Conventional photomicrographs. Mitotic extracts were activated into interphase by the addition of calcium and driven back into mitosis after formation of interphase nuclei by further addition of mitotic extract (Materials and Methods). (A, D, G, J, M, and P) TMR-tubulin; (B, E, H, K, N, and Q) Hoechst; and (C, F, I, L, O, and R) phase-contrast images of extracts fixed at increasing times after readdition of mitotic extract, given in minutes. Free microtubules are abundant during interphase (A-C); after the onset of mitosis, chromosomes condense inside the nuclear envelope into a prophase-like state, and paired centrosomes occasionally can be observed on the nuclear surface (D-F). Spindles form in interphase-to-mitotic extracts without any obvious half-spindle intermediates (G-I). Spindle pole structure varies considerably among the end-products of assembly (J-R); by tubulin fluorescence, centrosomes can be either poorly visible (J), closely apposed (M), or well-separated (P), although under phase-contrast, microtubules converge towards spindle poles in all cases (L, O, and R). Chromosomes are in spindle centers (K, N, and Q). Negatives printed at varying exposures. Bar, 20 μ m.

lacked well-defined poles. Punctate tubulin fluorescence was often found near these microtubule arrays; this may represent spindle poles broken away from the primary microtubule array during the coverslip squash (Fig. 5, G-I). 30-60

min after the addition of mitotic extract we observed bipolar spindles with more well-defined poles, visible by either phase-contrast or fluorescence (Fig. 5, J-R). As in mitotic extracts, poles were sometimes split (Fig. 5, M-B), and

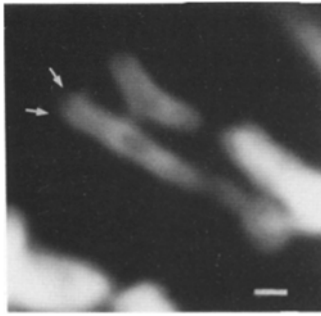


Figure 6. DNA replication occurs in interphase-to-mitotic extracts. Hoechst image of two chromosomes, each containing paired sister chromatids (arrows on left-hand chromosome). From an interphase-to-mitotic extract, prepared as in Fig. 5. Bar, 1.0 μm .

microtubule bundles were apparent (Fig. 5 *P*). Also, as in mitotic extracts, chromosomes were localized to the center of the spindle. However, unlike mitotic extracts, in interphase-to-mitotic extracts input DNA had replicated (Blow and Watson, 1987; Hutchison et al., 1987) (assayed by dCTP incorporation, data not shown), and we were usually able to recognize chromosomes as paired sister chromatids, as shown in Fig. 6. For this reason we refer to replicated chromatin in interphase-to-mitotic extracts as "chromosomes," as distinguished from "chromatin" in mitotic extracts. We also found that chromosomes in interphase-to-mitotic extracts were more tightly aligned at the spindle equatorial plane than chromatin in mitotic extracts, in a configuration reminiscent of a metaphase plate (compare Fig. 5, *K*, *N*, *Q* to Figs. 1 *B* and 2 *K*). Sometimes spindles appeared to fuse laterally, creating structures that were as wide or wider than they were long (Fig. 7); these contained multiple centrosomes, but lacked singular well-focused poles, reminiscent of mitotic spindles in some higher plants (Bajer and Mole-Bajer, 1972). Interestingly, chromosomes in laterally-fused spindles were also uniformly in the spindle center (Fig. 7 *B*).

Kinetochores Are Formed in Interphase-to-Mitotic Extracts but Not in Mitotic Extracts

To better characterize structural differences between spindles formed in mitotic and interphase-to-mitotic extracts, we centrifuged assembled spindles onto coverslips and exam-

ined them by EM. General microtubule organization at the EM level, shown in Fig. 8 *A*, was as expected from light-level observation, and we did not find any significant differences between mitotic and interphase-to-mitotic extracts. In both cases we found centrosomes at spindle poles (Fig. 8 *B*; data not shown), and in EM we also observed the occasional splitting of poles that had been seen by fluorescence at the light level (Fig. 8 *B*). We have not determined whether each half of split poles contains a centrosome, although we consider this a likely possibility.

We observed three kinds of interactions of microtubules and chromatin in spindles by EM, shown in Fig. 9. (1) In both mitotic and interphase-to-mitotic extracts single microtubules or microtubule bundles were seen running alongside chromatin without any obvious point of insertion into chromatin (Fig. 9 *A*). (2) Occasionally, also in both kinds of extracts, we observed microtubules that appeared to enter chromatin, but without any differentiated structure at the site of insertion (Fig. 9, *B* and *b*). In adjacent sections, however, it became clear that microtubules were not actually entering chromosomes but rather only grazing chromatin laterally (Fig. 9, *b'*). (3) Only in interphase-to-mitotic extracts did we observe microtubules terminating in small evaginations of chromatin which stained more darkly than bulk chromatin (Fig. 9, *C-F* and *c-f*). In many sections these blobs of more densely staining chromatin were present in opposed pairs; we interpret these structures to be kinetochores. In these preparations we did not observe the trilaminar plate structure characteristic of many kinetochores (Rieder, 1982), but similarly unstructured kinetochores are typical of *Haemaphysalis* and other species (Bajer and Mole-Bajer, 1972), and kinetochore morphology may depend strongly on fixation conditions. In a few instances we did observe what could be interpreted as a single plate (Fig. 9 *e*, arrow), but such structures were atypical.

Kinetochores were specific to spindles in interphase-to-mitotic extracts; despite extensive searching under a number of different fixation conditions we never observed kinetochores in spindles formed in mitotic extracts. The EM identification of kinetochores in interphase-to-mitotic extracts represents the first demonstration of the reconstitution of

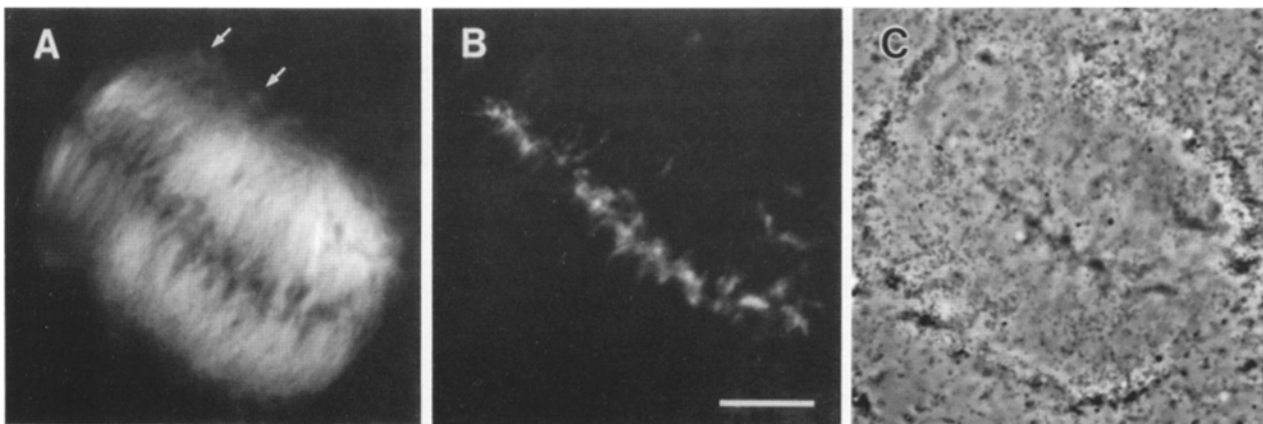


Figure 7. Lateral aggregates of spindles in interphase to mitotic extracts after long incubation times. (A) TMR-tubulin; (B) Hoechst; and (C) phase-contrast images. Arrows indicate examples of multiple punctate tubulin fluorescence, probably centrosomes, at spindle poles. Chromosome congression is nearly uniform even in these aberrant structures, which contain DNA from a number of independent nuclei (determined in sperm mixing experiments, not shown). Bar, 20 μm .

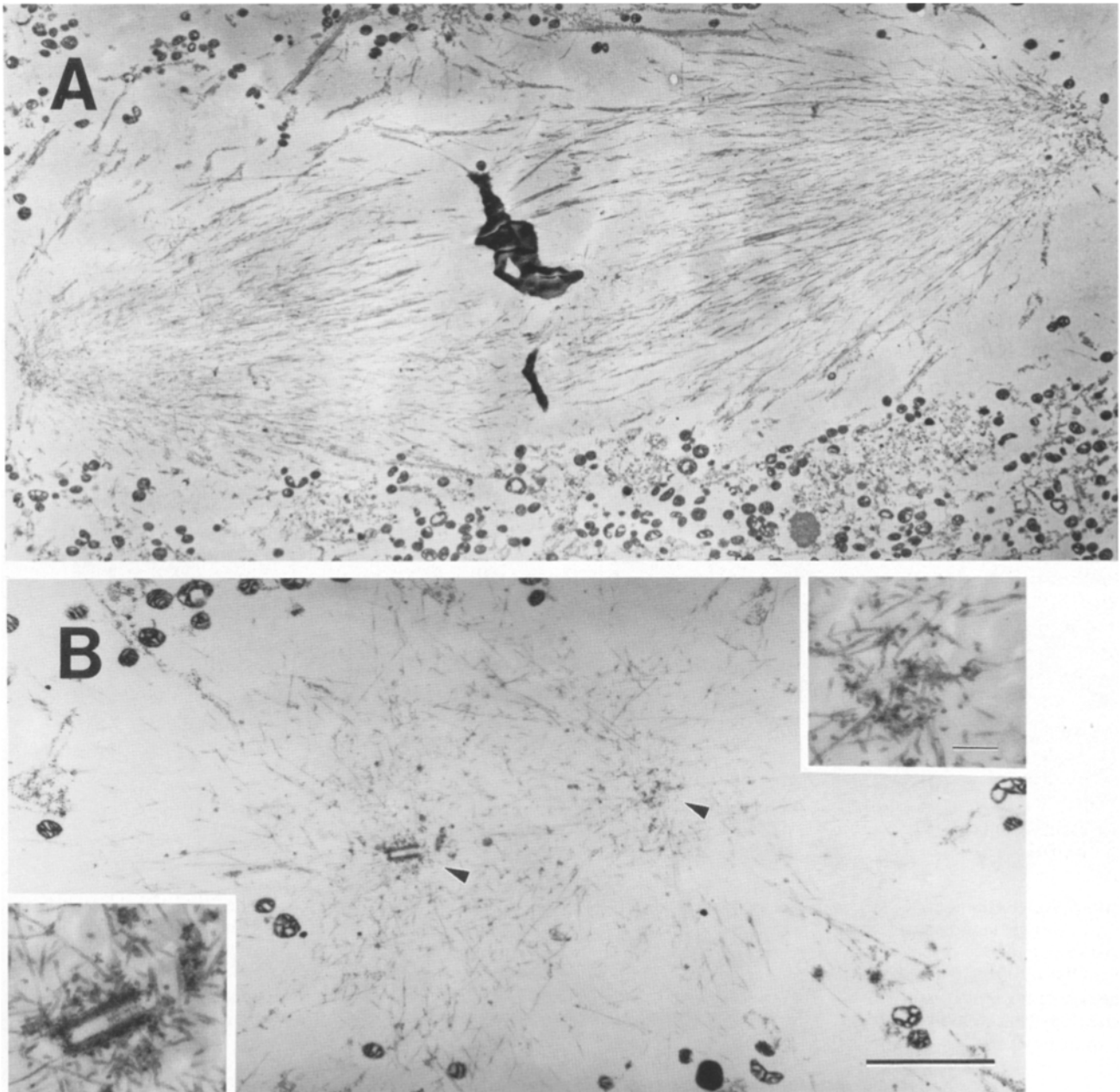


Figure 8. Electron microscopic visualization of microtubule organization in spindles assembled *in vitro*. (A) Low-magnification EM view of a spindle formed in an interphase-to-mitotic extract. Microtubule density appears lower near chromatin. This particular spindle was fixed in the presence of polyamines, which condense chromatin considerably. 60-nm section. (B) End-on view of a split pole in an interphase-to-mitotic extract. Microtubules can be seen to radiate from two distinct centers (*arrowheads, insets*), one of which contains a centriole in this section. 60-nm section. Bars: (A) 5.0 μm ; (B) 2.0 μm ; (*inset, B*) 0.2 μm .

kinetochore structure in *Xenopus* egg extracts *in vitro*. We have been unable to use autoimmune sera as a marker for kinetochores in extracts (Brenner et al., 1981), as all sera tried to date have been nonreactive in positive (i.e., interphase-to-mitotic) controls, including some sera which do cross-react with kinetochores in *Xenopus* tissue culture cells (cell line A6; data not shown).

Mechanisms of Spindle Assembly

In mitotic extracts, spindles often appeared to contain two distinct clumps of chromatin (Fig. 2, *J-L*), and the conver-

sion of half-spindles into spindles occurred without any obvious structural intermediates (Fig. 3). Taken together, these observations suggested that spindles might form in mitotic extracts through the fusion of two half-spindles. In contrast, because interphase-to-mitotic extracts seemed to make spindles without half-spindle intermediates, we wondered whether the mechanics of spindle assembly might be different between the two kinds of extracts. To test the fusion hypothesis we assayed whether spindles in each type of extract generally contained chromatin from one or from two sperm nuclei. Sperm nuclei were covalently labeled with TMR and

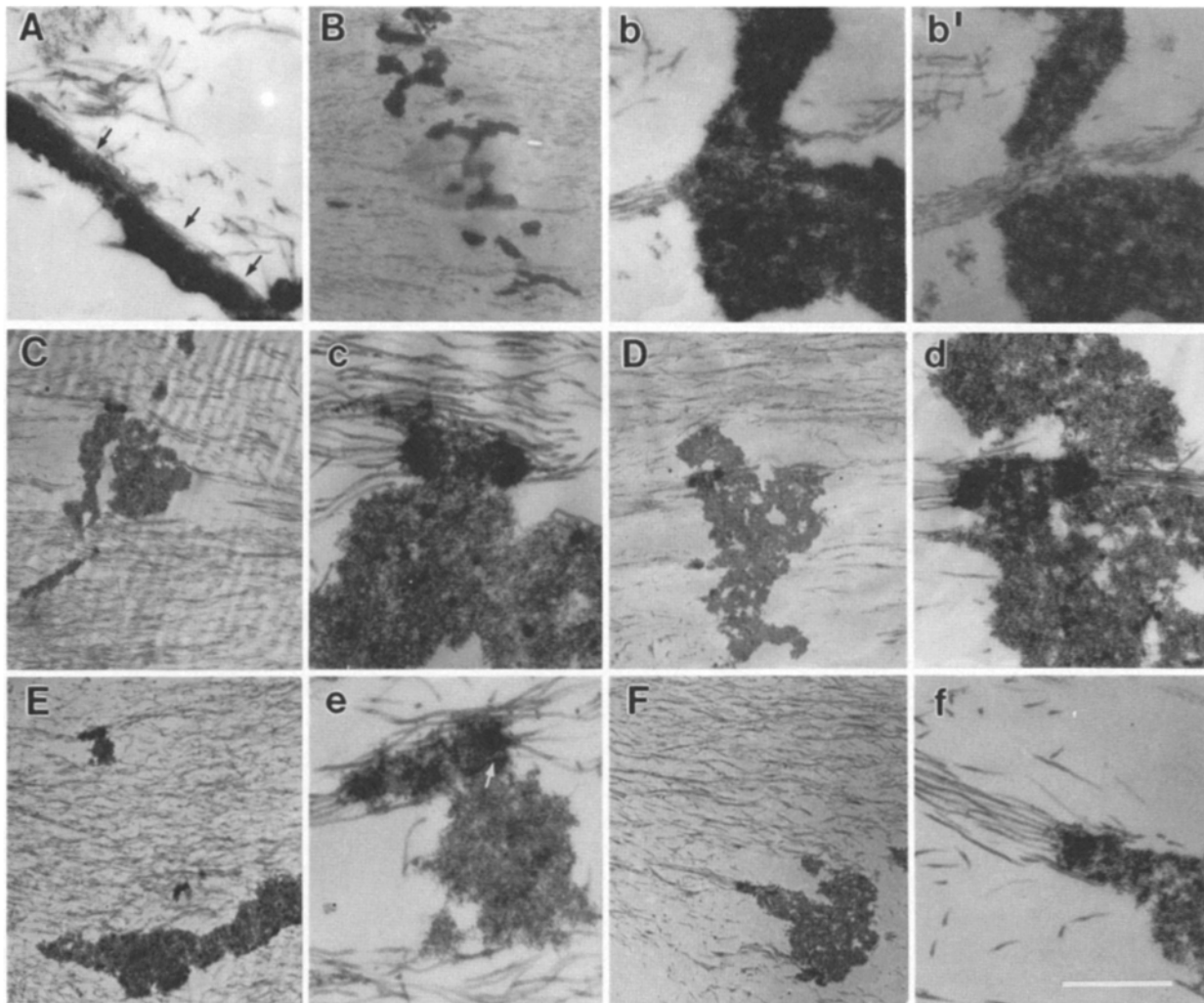


Figure 9. Kinetochores are present in interphase-to-mitotic extracts but not in mitotic extracts. (A) Microtubules (arrows) running tangentially to chromatin in a spindle from a mitotic extract. 60-nm section. (B, b, and b') Low-magnification view and two higher magnification serial sections of microtubules falsely appearing to terminate in chromatin. Note the absence of any differentiated chromatin at the insertion point. Spindle from a mitotic extract; 60-nm section. (C-F and c-f) Paired low- and high-magnification views of kinetochores and kinetochore microtubules in spindles from interphase-to-mitotic extracts. Note the typical dark chromatin staining at the insertion point and the apparent plate-like structure in the right-hand kinetochore of *e* (arrow); such plates were observed rarely. 150-nm sections. Bar: (A, d, and f) 1 μm ; (B-E) 5 μm ; (b and b') 0.5 μm ; (c and e) 0.75 μm .

mixed with an equal amount of unlabeled nuclei before being added to extracts and incubated in the presence of fluorescein-labeled tubulin. In these mixing experiments, rhodamine fluorescence was specifically retained on chromatin originating from prelabeled nuclei, without any effects on biological function; in mitotic extracts, labeled sperm nuclei formed mitotic chromatin as well as normal half-spindles, independent of half-spindles assembled around unlabeled chromatin (data not shown). In interphase-to-mitotic extracts, labeled sperm nuclei formed interphase nuclei, and then mitotic chromosomes upon reentry into mitosis (data not shown). After assembly, spindles were identified in the fluorescein channel without prior observation in the rhodamine channel, to prevent any observer bias. They were then scored as having (a) only unlabeled chromatin; (b) only labeled chromatin; or (c) both labeled and unlabeled chromatin (i.e., hybrid spindles) on the basis of TMR and Hoechst staining.

In mitotic extracts, many hybrid spindles were observed; an example of such a hybrid is shown in Fig. 10. Under the conditions of the experiment, if all spindles contained chromatin from two nuclei (i.e., "binary" assembly), one would expect the ratio of spindles containing only unlabeled chromatin, only labeled chromatin, or both labeled and unlabeled chromatin to be $\sim 25:25:50$ (see Materials and Methods). In contrast, if all spindles contained chromatin from a single nucleus ("unitary" assembly), this ratio should be closer to 50:50:0. As shown in Table II, the proportion of hybrid spindles observed in mitotic extracts was close to the value calculated for close to 100% binary assembly, within reasonable experimental error (see Table II and Materials and Methods for further discussion). Thus, while a small fraction of spindles in mitotic extracts may form through a unitary assembly mechanism, most do not. Since half-spindles appear to be true intermediates in spindle assembly, this result strongly implies that spindle assembly in

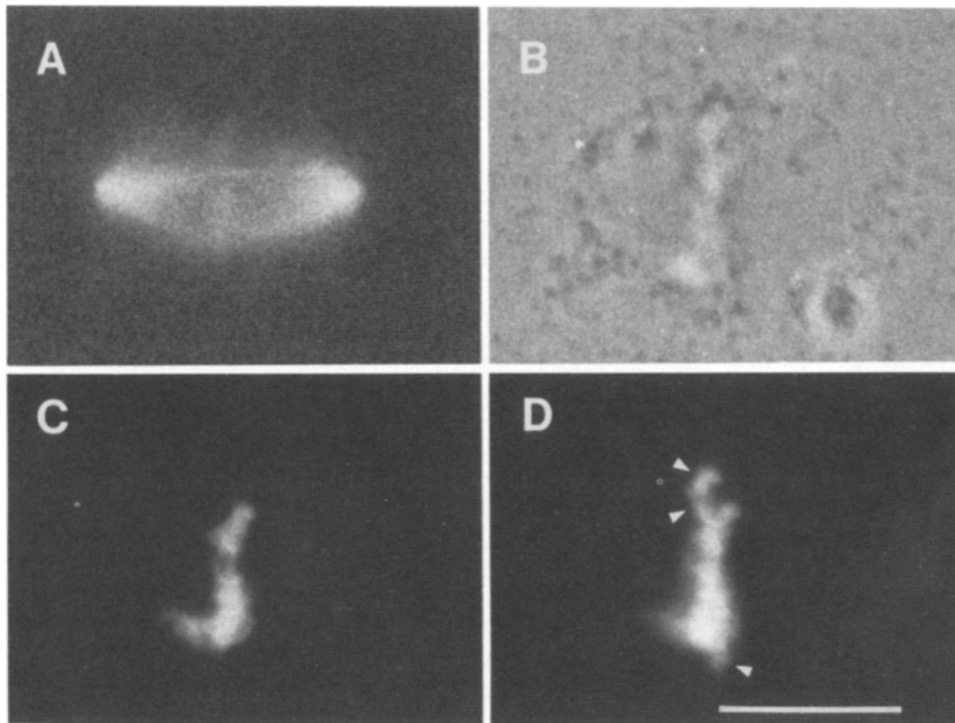


Figure 10. Hybrid spindle from a sperm mixing experiment. Mitotic extracts containing 0.3 mg/ml fluorescein-tubulin were incubated with equal numbers of TMR-labeled and unlabeled sperm nuclei and fixed in situ after 90 min. (A) Fluorescein-tubulin; (B) phase-contrast (C) TMR (labeled chromatin); and (D) Hoechst (total chromatin) images of a hybrid spindle containing both labeled and unlabeled chromatin. This particular spindle was chosen to emphasize the proximity of chromatin derived from independent sperm nuclei. Arrowheads indicate specific regions of chromatin derived from the unlabeled nucleus that do not lie directly over the labeled chromatin. Bar, 20 μ m.

mitotic extracts is predominantly the result of pairwise fusion of half-spindles.

In contrast, when the same mixing experiments were performed in interphase-to-mitotic extracts, very few spindles were found to be hybrids. This is shown in Table II. The ratio of spindles containing only labeled chromatin, only unlabeled chromatin, or both labeled and unlabeled chromatin was close to the calculated ratio 50:50:0, suggesting, again within experimental error (see Tables II and Materials and

Methods), that each spindle contains chromatin from a single sperm nucleus. In contrast to mitotic extracts, spindle formation in interphase-to-mitotic extracts appears to follow what is primarily a "unitary" assembly mechanism. That is, individual sperm nuclei are capable of directing the assembly of complete bipolar spindles upon progressing from interphase to mitosis in vitro, and this occurs without half-spindle intermediates.

Table II. Unitary and Binary Mechanisms of Spindle Assembly

		Spindles containing:			<i>n</i>
		Unlabeled nuclei only	Labeled nuclei only	Labeled and unlabeled nuclei	
		%			
Extract:*					
Mitotic	1	31	31	38	104
	2	29	18	53	192
	3	29	34	37	334
Interphase-to-mitotic	1	39	53	8	500
	2	39	57	4	489
Calculated values†					
100% unitary assembly		50	50	0	
100% binary assembly		25	25	50	
100% tertiary assembly		12	12	76	
10% unitary, 90% binary		29	29	41	
30% unitary, 70% binary		37	37	26	
70% unitary, 30% binary		45	45	9	
90% unitary, 10% binary		49	49	2	

* Data from independent experiments.

† Calculated as described in Materials and Methods.

Table III. Inhibition of DNA Synthesis Does Not Affect Spindle Assembly Mechanisms

Extract*	Spindles containing:			n
	Unlabeled nuclei only	Labeled nuclei only	Labeled and unlabeled nuclei	
		%		
Mitotic, + aphidicolin	17	25	58	137
Interphase-to-mitotic, control	45	45	10	513
+ DMSO‡	43	47	11	447
+ aphidicolin§	46	42	12	459

* Different treatments of the same extract, originally mitotic.

‡ 0.3% DMSO.

§ 15 µg/ml, from stock in DMSO (final 0.2% DMSO).

“Unitary” Spindle Assembly Does Not Require DNA Replication

To test whether DNA replication is required for bipolar spindles to form from individual nuclei, we performed sperm mixing experiments in interphase-to-mitotic extracts containing 10 µg/ml aphidicolin, a specific inhibitor of eukaryotic alpha DNA polymerase (Ikegami et al., 1978). Aphidicolin blocked DNA synthesis in interphase extracts nearly completely, compared to control extracts lacking aphidicolin, and mitotic extracts showed no appreciable DNA synthesis either in the presence or in the absence of aphidicolin (as measured by dCTP incorporation; data not shown). When mitotic extracts were added to aphidicolin-treated interphase extracts, spindles formed with somewhat lower than typical efficiencies, and chromatin morphology was distorted and more globular than thread-like. Replicated sister chromatids such as those shown in Fig. 6 were never seen under these conditions, nor were kinetochores observed in EM sections of spindles assembled in aphidicolin-treated interphase-to-mitotic extracts (data not shown). Interestingly, as shown in Table III, in sperm mixing experiments interphase-to-mitotic extracts did not produce significant numbers of hybrid spindles either in the absence or in the presence of aphidicolin, indicating that spindle assembly follows a unitary assembly mechanism in both cases. In contrast, control mitotic extracts produced ~50% hybrid spindles, as expected. Thus, neither DNA synthesis nor the formation of sister chromatids is required for spindle assembly in either mitotic or interphase-to-mitotic extracts. Furthermore, the differences in the mechanics of spindle assembly in the two types of extracts must be caused by something other than differences in DNA replication.

Discussion

The original experiments of Lohka and Maller (1985) indicated that *Xenopus* egg extracts might serve as a model biochemical system for studying the molecular interactions involved in the assembly of the mitotic spindle. Using fluorescence analogue cytochemistry we have developed a rapid and sensitive assay for spindle morphogenesis in these extracts and reconstituted pathways of spindle assembly in vitro under different conditions. Spindle assembly is an efficient process in vitro, and fluorescently labeled bovine brain tubulin has minimal effects on microtubule behavior in

egg extracts. As such, labeled tubulins can serve probes for microtubule structure and dynamics in live extracts (Belmont et al., 1990; Sawin and Mitchison, 1990) as well as fixed samples. Our results from spindle reconstitution in vitro suggest a model for the interactions involved in spindle morphogenesis, shown in Fig. 11. We address specific points concerning this model and implications for spindle assembly in vivo below.

Half-spindles and the Role of Mitotic Chromatin in the Establishment of Asymmetric Microtubule Arrays

The fluorescence assay allowed us to identify the half-spindle, a relatively stable structure in the course of spindle assembly, and a kinetic analysis of mitotic extracts showed that half-spindles are intermediates in spindle assembly. Mixing experiments with labeled and unlabeled sperm nuclei further demonstrated, within acceptable error (see Table II and Materials and Methods), that spindles assembling in mitotic extracts contain chromatin from two separate nuclei. Together, these results indicate that the primary mechanism of bipolar spindle formation in mitotic extracts is one of half-spindle fusion. The fusion of half-spindles in vitro is an obviously nonphysiological pathway of spindle formation, and spindles lacking kinetochores should be deficient in at least some aspects of spindle function, such as anaphase chromosome segregation. However, spindles assembled by fusion are morphologically quite similar to and retain at least some functional properties of spindles assembled in interphase-to-mitotic extracts, such as poleward microtubule flux (Sawin and Mitchison, 1990). Furthermore, the efficiency of formation of bipolar, fusiform, spindles from polar half-spindles suggests that at least some of the underlying, molecular interactions that drive spindle assembly in vivo are recapitulated during half-spindle fusion in vitro.

It is interesting in this light to note that in vivo, Sluder and Begg (1983) observed the occasional fusion of experimentally produced half-spindles after bisecting sea urchin embryonic spindles with a microneedle. Although it is likely that what they observed is analogous to half-spindle fusion in vitro, there may be significant differences, since their half-spindles were derived from preassembled spindles and clearly had both kinetochores and kinetochore microtubules, and needed to be pushed together for fusion to occur. Furthermore, their observations of birefringence in vivo sug-

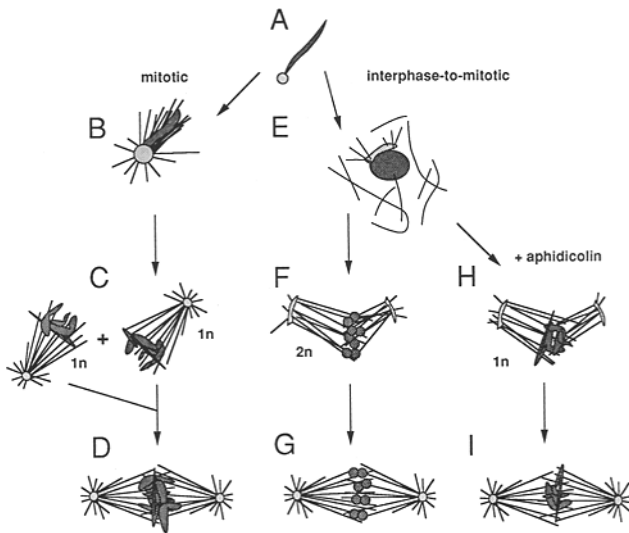


Figure 11. Spindle assembly by distinct pathways in vitro. (A–D) Mitotic extracts; (A, E–G) interphase-to-mitotic extracts; and (A, E, H–I) aphidicolin-treated interphase-to-mitotic extracts. Lines are microtubules, light-stippled regions are centrosomes, and dark-stippled regions are chromatin. Mitotic extracts: (1) dynamically unstable microtubules are nucleated by the sperm centrosome in a radially symmetric manner (A and B). (2) Interactions between dynamic microtubules and chromatin stabilize microtubules locally, creating an initial bias of microtubules towards chromatin that develops into a half-spindle with a defined polarity (B and C). (3) Half-spindles fuse together into bipolar spindles, probably through antiparallel microtubule bundling (C and D). Interphase-to-mitotic extracts (and aphidicolin-treated extracts): (1) upon activation, the sperm nucleus converts to an interphase nucleus, with concomitant changes in centrosomal organization (replication/spreading) and microtubule dynamics (A and E). In the absence of aphidicolin, DNA synthesis initiates. (2) Upon mitotic induction, the nuclear envelope disassembles, chromosomes condense, and paired half-spindles form around the same chromatin (E and F; E and H), through chromatin–microtubule interactions that are not kinetochore specific. Replicated DNA is present as paired sister chromatids with kinetochores (F), while unreplicated chromosomes (“+ aphidicolin”) are singlets (H). (3) Further interactions between antiparallel microtubules stabilize an initial bipolar orientation of half-spindle pairs, and centrosomes condense into more defined structures, forming bipolar spindles (F and G; H and I).

gested that microtubule density was not significantly different between spindles and half-spindles, although they did not quantitate birefringence in this study. In contrast, in our system, the efficiency of spindle assembly is rather high, and microtubule density in half-spindles appears to increase over fourfold upon fusion, as is discussed below.

Half-spindle formation per se is particularly interesting as an experimental tool, in that it may serve as a model for distinguishing structural events of spindle assembly that are directly coupled to the generation of bipolarity from those that are not. While observations of half-spindles in vivo (also termed monopolar spindles) have already contributed to our understanding of mitosis (Bajer, 1982; Mazia et al., 1960; Mazia et al., 1981; Sluder and Begg, 1983; Sluder and Rieder, 1985a), the structure of the half-spindle itself raises an interesting question: what directs the strong bias of microtubules towards chromatin? Microtubule dynamic instability (Mitchison and Kirschner, 1984) has been proposed

to provide a mechanism for the reorganization of cytoskeletal structure (Kirschner and Mitchison, 1986). However, the rapid turnover of microtubules in mitosis requires that additional mechanisms exist to stabilize particular microtubule conformations. For example, in *Xenopus* egg extracts, the half-life of single microtubules nucleated from centrosomes is on the order of 10 s (Belmont et al., 1990; Verde et al., 1990), while microtubules in spindles assembled in vitro are considerably more stable (Sawin and Mitchison, 1990). It has been postulated that this requirement might be met through the selective stabilization of dynamically unstable microtubules (Kirschner and Mitchison, 1986), as exemplified by the capture of microtubules by kinetochores both in vivo and in vitro (Mitchison and Kirschner, 1985; Mitchison et al., 1986). However, we propose that mitotic chromatin stabilizes microtubule turnover through an activity that is local to chromatin but not specific to kinetochores, which appear to be absent from spindles and half-spindles in mitotic extracts. Similar effects have been seen in vivo by Karsenti et al. (1984a), who found that centrosomes injected into meiotically arrested *Xenopus* eggs will nucleate microtubule arrays only in the proximity of chromatin. Interestingly, the injection of karyoplast nuclei lacking centrosomes or even high molecular weight prokaryotic DNA is sufficient to induce the formation of similar arrays in the meiotically arrested egg (Karsenti et al., 1984b). Nicklas and Gordon (1985) also observed what can be interpreted as a similar result in insect spermatocytes, performing the converse experiment; when individual chromosomes are removed from the spermatocyte spindle by micromanipulation, both kinetochore and nonkinetochore microtubules are reduced in number, in a manner that is quantitatively dependent on the number of chromosomes removed. The molecular nature of an activity in chromatin responsible for generating microtubule stability is not yet clear. From our EM, most microtubules in spindles and half-spindles assembled in vitro do not terminate in chromatin, so the effects of chromatin on microtubule stability may not require direct contact between chromatin and microtubule ends (i.e., a cap).

These results underscore an apparent requirement for chromatin in the generation of polarized microtubule arrays during mitosis, as was suggested by Harvey (1936; and references therein). In a number of organisms, physically or chemically enucleated early cleavage embryos organize large asters in mitosis, but fail to assemble a fusiform spindle, despite the proximity of asters in a common cytoplasm and the ability of many of these “spindle-less” asters to induce proper cleavage (Briggs et al., 1951; Harvey, 1936; Nagano et al., 1981; Picard et al., 1988; Raff and Glover, 1989; Rappaport, 1961; Sluder et al., 1986). Similarly, we have never observed the formation of polarized microtubule arrays after adding isolated mammalian centrosomes to either mitotic or interphase-to-mitotic extracts in the absence of chromatin (K. Sawin and T. Mitchison, unpublished observations). The failure to form spindles under these conditions has never been explained in molecular terms; we address the role of chromatin in spindle assembly further below.

Nonrequirement for Kinetochores in Spindle Formation

A number of investigators have suggested that kinetochores may play an important and specific role in the selective

stabilization and organization of spindle microtubules (Nicklas and Gordon, 1985; Church et al., 1986; Dietz, 1966; Kirschner and Mitchison, 1986). We identified microtubules terminating in kinetochores in interphase-to-mitotic extracts in the EM but failed to identify kinetochores in mitotic extracts, so we wondered whether this difference might account for the unimolecular spindle assembly observed in interphase-to-mitotic extracts. Sperm mixing experiments in interphase-to-mitotic extracts, however, showed identical results in the presence and in the absence of aphidicolin. Because aphidicolin inhibits DNA synthesis nearly completely, we consider it unlikely that kinetochores are replicating under these conditions. We also failed to find any kinetochores in EM preparations from aphidicolin-treated extracts. These results strongly suggest that opposed sister kinetochores do not play a significant role in generating spindle bipolarity in our system, and thus that specific kinetochore-microtubule interactions are not required for spindle morphogenesis in vitro. We note that Brinkley et al. (1988) observed trilaminar kinetochores in tissue culture cells prematurely induced into mitosis during DNA synthesis arrest, whereas we did not observe similar structures when DNA synthesis had not occurred, due to either premature mitosis (mitotic extracts) or inhibition of DNA synthesis (aphidicolin-treated interphase-to-mitotic extracts). Given the absence of clearly delineated trilaminar plates even in our replicated chromatin, we cannot rule out the possibility that partial kinetochores did assemble in the absence of DNA synthesis but that we were unable to recognize them by morphology. Alternatively, differences between our results and those of Brinkley et al. (1988) may simply represent differences in the systems under study. Nevertheless, the results of sperm mixing experiments imply that the different spindle assembly mechanisms observed in vitro are not strictly dependent on differences in DNA replication, and that components other than kinetochores may determine the pathway to be followed.

The most likely reason for the different assembly mechanisms observed in mitotic vs. interphase-to-mitotic extracts involves the replication or fragmentation of centrosomes during interphase, which can occur in the presence of aphidicolin (Nagano et al., 1981; Sluder and Rieder, 1985b). If fragmentation or replication were to occur on or near the surface of the nuclear envelope, the two daughter centrosomes, each capable of organizing a half-spindle, would interact with the same chromatin during mitosis. Half-spindles, as nearest neighbors, would fuse earlier in the process of morphogenesis, concomitant to half-spindle formation. We suspect that unitary spindle assembly in vitro (and in vivo) employs the same basic molecular mechanisms as does half-spindle fusion, the difference between mitotic extracts and interphase-to-mitotic extracts being primarily the initial conditions of the system, i.e., the state of the centrosome before mitotic induction. We have not followed the disposition of centrosomes over time in extracts, but we note that both mitotic and interphase-to-mitotic extracts show signs of centrosome separation, in particular in the splitting of half-spindle and spindle poles. Distinguishing centrosome replication from fragmentation in this system will require counting centrioles in serial sections; we note that specific replication of the centrosome has been shown to occur in *Xenopus* embryos in the absence of nuclear cell cycle progression (Gard et al., 1990).

Chromosome Congression and Kinetochore Function In Vitro

Does the localization of chromatin to the spindle midzone in mitotic extracts and (especially) interphase-to-mitotic extracts reflect chromosome congressional movements and the formation of a true metaphase plate in vitro? We do not know to what extent congression-like movements might be occurring in mitotic extracts. Our inability to resolve individual chromosomes at the light level routinely or to identify structural kinetochores at the EM level suggest that canonical prometaphase congression does not occur in mitotic extracts. Furthermore, we note that a mechanism of half-spindle fusion could falsely lead to the illusion of congression, since chromatin from a fusion event will typically end up in the spindle mid-zone by default. Nevertheless, in spite of the probable absence of "true" prometaphase congression in mitotic extracts, we suspect that the earlier movement of chromatin away from the centrosome occurring during half-spindle formation may be analogous to the microtubule-based transport properties of the spindle in vivo (also termed astral exclusion, polar winds, radial ejection forces, etc.) (Bajer, 1982; Bajer and Mole-Bajer, 1972). Ejection forces have been proposed to play a role in metaphase chromosome alignment and can act on chromosome fragments directly, independent of kinetochores (Rieder et al., 1986), so there is reason to think such forces may be recapitulated in mitotic extracts, which retain other aspects of in vivo microtubule-based motility (Belmont et al., 1990; Sawin and Mitchison, 1990). Probably more relevant to the question of chromosome congression, however, is the much tighter equatorial alignment of sister chromatids in spindles assembled in interphase-to-mitotic extracts (Fig. 5, *K*, *N*, and *Q*). This suggests to us that more sophisticated aspects of congression may be recapitulated in interphase-to-mitotic spindles, and since opposed sister chromatids/kinetochores are present only in these spindles, it seems likely that they may play a role in chromosome alignment. We note in this regard that chromosomes become well-centered only in later stages of spindle assembly (compare Fig. 5, *G* and *H* and *J* and *K*). Furthermore, in multipolar lateral fusion spindles, chromosomes appear to form one large metaphase plate-like structure (Fig. 7, *A* and *B*), reminiscent of chromosome congression in flattened *Haemanthus* spindles (Bajer and Mole-Bajer, 1972). The direct demonstration of prometaphase chromosome congression or metaphase chromosome oscillations in vitro will require time-lapse observation of chromosome movements during spindle assembly.

Hierarchies of Selective Microtubule Stabilization in the Spindle

What is the mechanism of half-spindle fusion, and what mechanisms might account for the increased microtubule density observed in spindles vs. half-spindles? It seems unlikely that chromatin-chromatin interactions mediate fusion directly, as chromatin from the two half-spindles can be spatially segregated within the spindle (see, for example, Fig. 2, *J-L*). We also consider it unlikely that microtubule-chromatin interactions alone could account for half-spindle fusion, since microtubule fluorescence intensity increases synergistically, over fourfold, when polar half-spindles form bipolar spindles. This implies that fusion involves additional

microtubule stabilization beyond that generated by chromatin-microtubule interactions alone, which might be expected to increase microtubule density in the spindle midzone at most twofold upon fusion. We also note, although we have not quantitated this directly, that the increase in fluorescence upon fusion is not restricted to the central spindle, but appears to be more global.

We suggest that specific interactions between antiparallel microtubules of individual half-spindles may account for this additional microtubule stabilization and provide the driving force for half-spindle fusion. Such interactions could lead not only to recognition and binding together of half-spindles but also to increased possibilities of microtubule stabilization compared to polarized half-spindles, and thus a higher microtubule density. Antiparallel microtubule interactions are clearly of importance in the highly organized central spindles of lower eukaryotes (Masuda et al., 1988; McIntosh et al., 1979), but are generally more difficult to study in animal cells, where the central spindle is most prominent only in later stages of mitosis (Saxon and McIntosh, 1987). Candidate molecules for mediating antiparallel interactions include microtubule-associated proteins localized to the spindle (McIntosh and Koonce, 1989; Olmsted, 1986) as well as motor proteins that might cross-bridge microtubules (Meluh and Rose, 1990; Shpetner and Vallee, 1989), and in the following paper we consider in greater detail the function of potential microtubule motors during spindle assembly in vitro (Sawin and Mitchison, 1990).

It might appear puzzling to postulate antiparallel microtubule interactions as mediating spindle formation or stability, given the inability of pairs of asters in a common cytoplasm to induce the formation of fusiform microtubule arrays in vivo, as discussed above. However, we suggest that generating antiparallel-microtubule bundles may be possible only within a hierarchy of microtubule stabilization, requiring the presence of chromatin. That is, stabilizing interactions or cross-bridges might be able to form only between microtubules that have been previously stabilized through interactions with chromatin, for example, if cross-bridge formation is slow relative to unattenuated microtubule turnover. Alternatively, antiparallel-microtubule interactions may require the direct participation of chromatin, perhaps in initiating or synergistically activating cross-bridges that stabilize microtubules.

In summary, we propose that spindle assembly may be driven by a hierarchy of interactions which cause progressive increases in microtubule stabilization: (a) nonspecific interactions between microtubules and chromatin; (b) interactions between antiparallel microtubules, that may be somehow dependent on chromatin; and (c) (where possible) interactions between kinetochores and microtubule plus ends. We expect that the identification and localization of specific microtubule-stabilizing factors and their assignment within this stabilization hierarchy will flesh out this model in molecular terms.

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