

Microtubule assembly and kinetochore directional instability in vertebrate monopolar spindles: implications for the mechanism of chromosome congression

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SUMMARY

We have proposed previously a kinetochore motor-polar ejection model for chromosome congression to the metaphase plate where forces generated at the kinetochore are antagonized by away-from-the pole forces generated within each half-spindle on the chromosome arms. This model was based in large part on observations of the behavior of chromosomes on monopolar spindles. In these cells chromosomes typically become attached to the pole by only one kinetochore fiber. These mono-oriented chromosomes move to positions away from the pole even though they are pulled poleward at their kinetochores. Their arms are also ejected away from the pole when severed from the centromere. Here we have characterized further the properties of monopolar spindles in newt lung epithelial cells to determine the similarities between monopolar and bipolar spindles. We found no significant differences between monopolar and bipolar spindles over the parameters examined, which included: microtubule dynamics as measured by fluorescence redistribution after photo-bleaching; the ability of polar microtubule arrays to push chromosome arms away from the pole; the dependence of chromosome position relative to the pole on microtubule assembly; the number of kinetochore microtubules per kinetochore; and the directional instability of kinetochore motion during chromosome oscillations poleward and away-from-the-pole. As in bipolar spindles, kinetochore directional instability is characterized by abrupt switching

between constant velocity phases of poleward and away-from-the-pole motion. From these data we conclude that the mechanism(s) responsible for chromosome positioning in monopolar spindles are fundamentally the same as those in bipolar spindles; only the geometry of the two spindle forms and the interplay between sister kinetochore directional instabilities are different. We also found no correlation in the kinetochore-to-pole distance with kinetochore microtubule number in monopolar spindles, but a strong qualitative correlation with microtubule density. This finding indicates that oscillations of mono-oriented chromosomes in both monopolar and bipolar spindles occur because chromosomes persist in poleward motion until they reach a density of polar microtubules sufficiently high to promote switching to away-from-the-pole motion. As the kinetochore and chromosome arms move away-from-the-pole, microtubule density decreases and the kinetochore switches to poleward motion, pulling the chromosome arms back into regions of higher microtubule density. The mechanism regulating kinetochore switching between poleward and away-from-the-pole motion is poorly understood, but may depend on tension at the kinetochore generated by pushing forces on the chromosome arms produced by the polar microtubule arrays.

Key words: monopolar spindle, kinetochore directional instability, microtubule, kinetochore, congression, chromosome position

INTRODUCTION

Spindle bipolarity is critical for segregating the replicated chromosomes into two equal genomes (e.g. Mazia, 1961). This bipolarity is established as the replicated spindle poles (centrosomes) separate to produce two overlapping microtubule (MT) arrays or 'half-spindles'. The MTs in each half-spindle are oriented with their plus, dynamic assembly ends distal and

their minus ends proximal to the centrosomal nucleation sites. The free plus ends of polar MTs exhibit dynamic instability, growing at 7-15 $\mu\text{m}/\text{min}$ for about 30-60 seconds, before switching to fast shortening (approx. 17 $\mu\text{m}/\text{min}$) back to the nucleation center where renucleation and another cycle of growth and shortening occurs (reviewed by Salmon, 1989b; Hayden et al., 1990). Sister (mitosis) or homologous (meiosis) kinetochores become tethered (oriented) to opposite spindle

poles by attachment to plus ends of polar nucleated MTs (reviewed by Rieder, 1991). Each kinetochore has a limited and defined number of binding sites (Rieder, 1982) and kinetochore-associated MTs (kMTs) form a kinetochore fiber (k-fiber) that tethers the kinetochore to the pole. In bipolar spindles, k-fiber formation on sister kinetochores is usually asynchronous. As a rule, one sister kinetochore attaches to the ends of MTs extending from the closest pole, while the distal kinetochore on the now 'mono-oriented' chromosome faces away from the pole and lacks MTs. When the distal kinetochore finally captures the ends of MTs from the opposite pole, the now 'bi-oriented chromosome' initiates motion towards the equator of the forming spindle in a process termed congression (Darlington, 1937; reviewed by Mitchison, 1989a; Salmon, 1989b; Rieder, 1991).

The popular view of chromosome congression is based on the model of Ostergren (1951) in which the process is thought to be produced solely by a tug of war between antagonistic pulling forces at the sister kinetochores that are directed toward opposite poles. Since the pulling force is proposed to increase with increasing distance from the pole, the pulling forces towards opposite poles are balanced when the chromosome achieves a position at the spindle equator (where the distance to opposite poles is equal). This force-versus-distance relationship is thought to be produced by the k-fiber, which is viewed as a 'traction' fiber where poleward force producers are equally distributed along its length (Ostergren, 1951; Hays et al., 1982) and somehow related to the number of kMTs (Hays and Salmon, 1990).

Although Ostergren's model is consistent with several aspects of chromosome behavior on bipolar spindles (e.g. Book, 1945; Wise, 1978; Hays et al., 1982; Hays and Salmon, 1990), it is not consistent with the positioning of chromosomes on monopolar spindles (Mazia, 1961). On monopolar spindles, all of the chromosomes are mono-oriented, i.e. attached to the only pole by a single k-fiber on the proximal kinetochore (Mole-Bajer et al., 1975; Mazia et al., 1981; Rieder, 1982; Salmon, 1989b). In spite of this, the chromosomes do not move all the way to the pole as predicted by the Ostergren model. Rather they achieve average positions distal to the pole that can be as great as the normal distance between the spindle equator and pole in bipolar spindles (Bajer, 1982; Rieder et al., 1986; Salmon, 1989a,b; Ault et al., 1991; Leslie, 1992). These are average positions because mono-oriented chromosomes exhibit constant oscillatory phases of pole-directed (P) and away-from-the-pole (AP) motion at velocities typical of congressing and anaphase chromosomes in bipolar spindles (Bajer, 1982; Skibbens et al., 1993). Laser microbeam studies have shown that the distal, untethered kinetochore does not contribute to the behavior of chromosomes on monopolar spindles (Rieder et al., 1986; Rieder and Salmon, unpublished). Therefore, contrary to Ostergren's model, the position of chromosomes on monopolar spindles is not determined only by antagonistic P pulling forces acting at sister kinetochores. In addition, recent evidence has shown that the great majority of kinetochore motion in bipolar spindles occurs at the ends of relatively stationary kinetochore MTs by forces produced at the kinetochore and not along the kinetochore fiber (reviewed by Rieder, 1991; McIntosh and Pfarr, 1991; Mitchison and Salmon, 1992).

Based on the above observations, and the similar behavior exhibited by mono-oriented chromosomes on bipolar spindles, we (Rieder et al., 1986; Salmon, 1989a; Rieder, 1991; Skibbens et al. 1993) and others (Leslie, 1992) have proposed a kinetochore motor/polar ejection model for congression. In this model, the MT arrays associated with each pole and half-spindle produce AP ejection forces on the chromosomes in the direction of MT growth with a strength proportional to MT density (Salmon, 1989a). We envision that the ejection force has two components: a steric resistance to chromosome penetration based on MT density and an active pushing force related to the dynamic growth of MTs impacting on the chromosome and/or by plus-end-directed motors associated with the surface of the chromosome (reviewed by Rieder, 1991; Leslie, 1992; Theurkauf and Hawley, 1992). Regardless of how the AP force is produced, the model proposes that attached kinetochores pull chromosomes poleward in monopolar spindles until the strength of the 'polar ejection' forces on the chromosome inhibit further movement. Unlike Ostergren's model, in the kinetochore motor/polar ejection model the strength of the P force is thought to be independent of distance from the pole.

A central tenet of the kinetochore motor/polar ejection model is that the congression forces on chromosomes on a bipolar spindle are the simple algebraic sum of the P and AP forces produced in association with each half-spindle. Thus, the model considers the bipolar spindle to be functionally equal to two oppositely oriented monopolar spindles that are held together by bi-oriented chromosomes, as argued by Bajer and Mole-Bajer (1972) and Mazia et al. (1981).

To evaluate this assumption, we have investigated several aspects of MT dynamics and chromosome behavior on monopolar spindles for comparison to existing data for bipolar spindles. We chose newt lung cells because previous studies provide data that allow for comparisons between monopolar and bipolar spindles in terms of MT organization and density, MT dynamics as measured by fluorescence redistribution after photobleaching (FRAP), average chromosome congression positions relative to the poles and their dependence on MT assembly, and the oscillation of kinetochores between P and AP phases of constant velocity motion (termed kinetochore directional instability by Skibbens et al., 1993). We found these parameters to be the same between monopolar and bipolar newt spindles. We also examined whether there was any correlation between distance from the pole and the number of kMTs (see Hays and Salmon, 1990) or the density of non-kMTs (see Ault et al., 1991). We found no correlation in the kinetochore-to-pole distance with kMT number as predicted by Hays and Salmon (1990) for the Ostergren (1951) traction fiber model of congression in bipolar spindles. Instead we found a strong qualitative correlation between chromosome position and MT density indicating that MT density near the chromosome arms more strongly influences kinetochore directional instability and congression position than does the number of kMTs. Our results lend strong support to the proposal of Bajer and Mole-Bajer (1972; see also Mazia et al., 1981) that monopolar spindles are functionally equal to one-half of a bipolar spindle. They are also consistent with the hypothesis that chromosome position, and thus congression, is partly mediated by MT-based ejection forces associated with each half-spindle.

MATERIALS AND METHODS

Newt lung culture

Primary cultures of newt lung epithelium were cultured as described by Rieder and Hard (1990). Cultures were screened by phase-contrast microscopy, and those with monopolar spindles were chosen for experiments. The percentage of mitotic cells forming monopolar spindles varied between cultures and was approximately 10-30% of the total number of mitotic cells in each culture.

Light microscopy

MT density was reduced by perfusion with nocodazole (prepared as a 10 mg/ml stock solution in DMSO and diluted to 10 μ g/ml in culture medium prior to perfusion). Cell cooling to 4°C was also used to reduce MT density. Coverslips were incubated in ice-cold culture medium for 15 minutes and then fixed at 4°C. For recovery studies, previously cooled cells were allowed to recover at room temperature for 30 minutes and then fixed at room temperature. Cells were fixed, processed for anti-tubulin immunofluorescence, and photographed as previously described (Cassimeris et al., 1986; Ault et al., 1991).

Kinetochores directional instability

The motions of mono-oriented chromosomes were recorded at 4 second intervals into an optical memory disk recorder (OMDR) using video-enhanced differential interference contrast microscopy (VE-DIC) as described by Rieder and Alexander (1990). We also used the semi-automatic computer tracking system developed by Skibbens et al. (1993) to measure the motility of tethered kinetochores with respect to their poles and the deformation of the centromere region generated by kinetochore motility. In this analysis, an 8 \times 8 pixel cursor is superimposed on the centrioles defining the pole and another cursor of the same size is placed over the kinetochore region at the edge of the centromere proximal to the pole. The computer retrieves images sequentially from the OMDR and measures the changes in distance between the kinetochore region and the pole for each video frame by moving the cursors until the best correlation is found (Skibbens et al., 1993). This tracking system has an accuracy of ± 1 pixel, or about ± 0.125 μ m for the images analyzed in this study. The phase velocities and durations of kinetochore directional instability were measured as described by Skibbens et al. (1993).

Fluorescence redistribution after photobleaching (FRAP)

Photometric and video FRAP experiments were conducted essentially as described by Wadsworth and Salmon (1985, 1986a). Appropriate cells were injected with dichlorotriazinyl-aminofluorescein-labeled tubulin (DTAF-tubulin; Leslie et al., 1984; Wadsworth and Salmon, 1986b) and allowed to equilibrate for at least 20 minutes prior to photobleaching. The microscope system used for photobleaching has been previously described (Wadsworth and Salmon, 1986a; Salmon and Wadsworth, 1986; Cassimeris et al., 1988). Three cells were examined for each type of FRAP experiment.

Electron microscopy

Cultures containing monopolar spindles were fixed either in 3% glutaraldehyde diluted in phosphate buffer (30 minutes; Rieder et al., 1985), or lysed for 15 seconds in PEM/0.5% Triton X-100 (PEM: 80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂, pH 6.8) and then fixed with 1% glutaraldehyde diluted in PEM (2 minutes) followed by 0.1% glutaraldehyde in PEM (20 minutes; Rieder and Bowser, 1985). After primary fixation, coverslips were osmicated in 1% OsO₄ for 10 minutes at 4°C, dehydrated in ethanol, and embedded in Epon-Araldite (Rieder et al., 1986). Selected cells containing monopolar spindles were serially thin sectioned. Sections on slot grids were stained with uranyl acetate and lead citrate and examined with a Phillips EM 301 electron microscope operated at 80 kV. The number of kMTs and the distance between each kinetochore and its pole were

determined from micrographs of serial sections. Serial-section reconstructions were made by tracing the MTs and chromosomes in photographic prints (final magnification of $\times 5500$) onto transparent acetate sheets, and then stacking the sheets using the chromosomes and the pole as fiducial markers.

RESULTS

Monopolar spindles are generated in newt and other vertebrate cells when the replicated centrosomes fail to separate prior to nuclear envelope breakdown (NEB), or when they are too far apart at NEB to allow for the formation of a bipolar spindle (Bajer, 1982; Waters et al., 1993). In the case of the second route, termed anaphase-like prometaphase (Bajer, 1982), a random number of chromosomes becomes associated with the two monopolar spindles that reside in the same cell. Often cells with monopolar spindles round to the point where they are unusable for experimentation. We therefore restricted our studies to cells that remained flat so that the centrosome at the spindle pole and many of the attached chromosomes were in or near the same focal plane.

Monopolar spindle structure: chromosome position and microtubule organization

The distribution of MTs in a monopolar spindle depended on the distribution of chromosomes around the monopole. When the chromosomes were clustered to one side of the centrosome, MT organization was similar in density and extent to the half-spindle of a bipolar prometaphase spindle (Figs 1C,D and 2b). On these asymmetrical monopolar spindles, chromosomes were positioned with their attached kinetochores oriented toward, and their arms pointing away from, the single polar area (Figs 1, 2, 8). Chromosomes positioned near the long axis of the monopolar spindle were often as far from the pole as fully congressed metaphase chromosomes on a bipolar spindle (about 20 μ m; Figs 1, 2b, 8). As the monopolar spindle slowly 'aged' the extent of MT assembly decreased, and the chromosomes became progressively closer to and more radially arranged around the monopole (e.g. Fig. 2a).

The number of kMTs per mono-oriented chromosome was determined from electron micrographs of serial sections cut from 4 cells. An example of a micrograph used to count kMTs is shown in Fig. 3. We found that the mean number of kMTs per chromosome was 18.2 ± 4.0 ($n=30$ kinetochores). Cells were fixed either with or without first lysing, and these different fixation protocols did not change the mean number of kMTs per chromosome. It has been shown previously for many other vertebrate cell types that the metaphase kinetochore is saturated with kMTs when its surface area is covered with MTs spaced 60 nm center-to-center (Rieder, 1982). Using this criteria, and our measured diameter of the attached newt kinetochore outer plate of 0.20 μ m, 18 MTs should saturate the newt kinetochore at metaphase. This number is similar to that measured here for the number of kMTs attached to kinetochores in monopolar spindles.

The four cells used in the kMT number analysis were also reconstructed to determine the relationship between kMT number and the kinetochore-to-pole distance. It was not possible to include all non-kMTs in these reconstructions (Fig. 2). Regardless, the overall pattern of MT density corre-

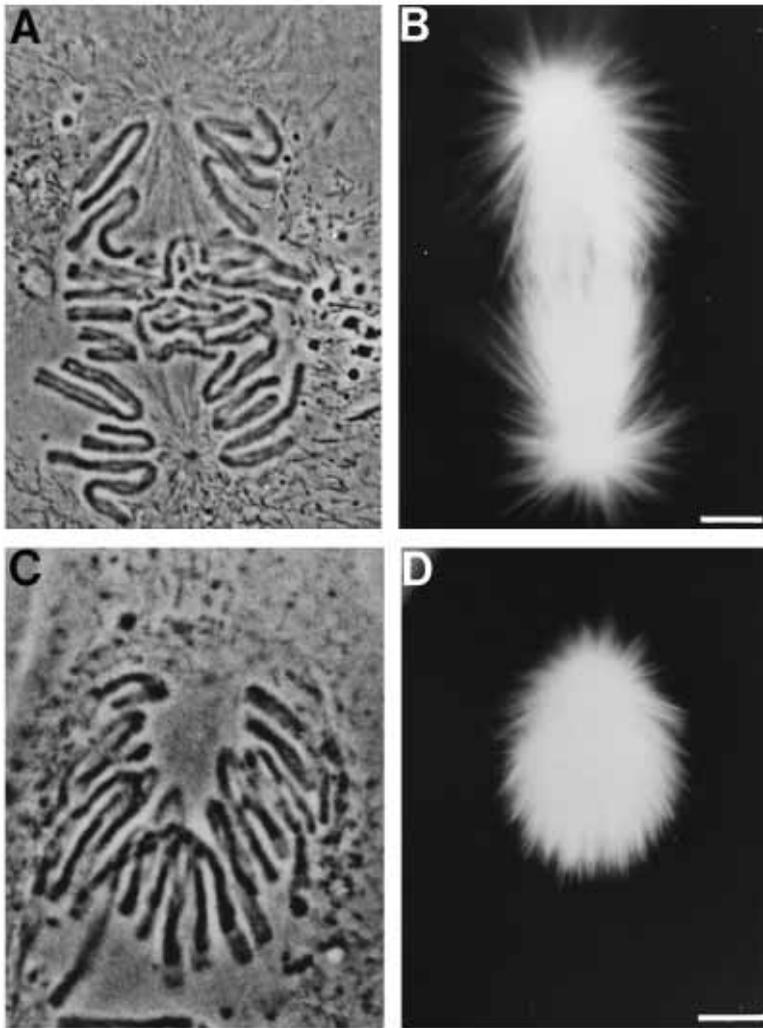


Fig. 1. Phase-contrast and immunofluorescent micrographs of newt lung cell prometaphase bipolar (A,B) and monopolar (C,D) spindles. In both spindles, the distance between the spindle pole and the metaphase plate are approximately equivalent. In each case chromosomes positioned towards the sides of the spindle are located closer to the pole. Bar, 10 μm .

lated well with the pattern of fluorescence intensity in immunofluorescence micrographs of monopolar spindles stained with an antibody to tubulin (Fig. 1). As shown in Figs 2 and 4, the distance of a kinetochore from the monopole showed little if any correlation with the number of its associated MTs.

In contrast to the lack of a relationship between kinetochore distance from the pole and the number of kMTs, we consistently observed a strong qualitative correlation between this distance and MT density between the chromosome and pole (Figs 1, 2). As illustrated by the reconstructions in Fig. 2, the chromosomes reside just in front of that point within the polar MT array where MT density is the highest. The dense array of MTs continues further away from the pole along the long axis of the asymmetrical monopolar spindle than it does along its sides (Figs 1, 2). In general, chromosomes near this central region (chromosomes a, b and c in Fig. 2b) are further away from the pole than chromosomes situated on either side of this region (chromosomes d, e, f in Fig. 2b). The chromosomes located closer to the pole (chromosomes d, e and f in Fig. 2b) are found on the sides of the monopolar spindle, where low densities of polar MTs occur closer to the pole.

Microtubule turnover in monopolar spindles measured by FRAP

Wadsworth and Salmon (1985, 1986a) measured MT turnover in the central half-spindle region between the chromosomes and the poles in metaphase newt spindles using FRAP methods. We used their photometric procedures to determine the rate and extent of MT turnover by measuring fluorescence recovery in a 2.8 μm diameter spot in the central monopolar spindle region. A typical computer record is shown in Fig. 5. As reported for bipolar newt spindles (Wadsworth and Salmon, 1985, 1986a), the kinetics of fluorescence recovery in monopoles involved three phases. There was an initial brief rapid phase thought to correspond to the diffusive movement of free tubulin subunits in the cytosol. The second phase accounted for the great majority of fluorescence recovery and it probably reflects the dynamics of the non-kMTs since they represent the bulk of MTs in the spindle (Wadsworth and Salmon, 1986a). Recovery in this second phase followed exponential kinetics with an average half-life of 63 seconds (Table 1), a value similar to the 73 second half-life measured for bipolar spindles. As with bipolar spindles, about 20-30% of the bleached fluorescence did not recover rapidly, but persisted longer than 2 minutes. This unrecovered fluorescence is

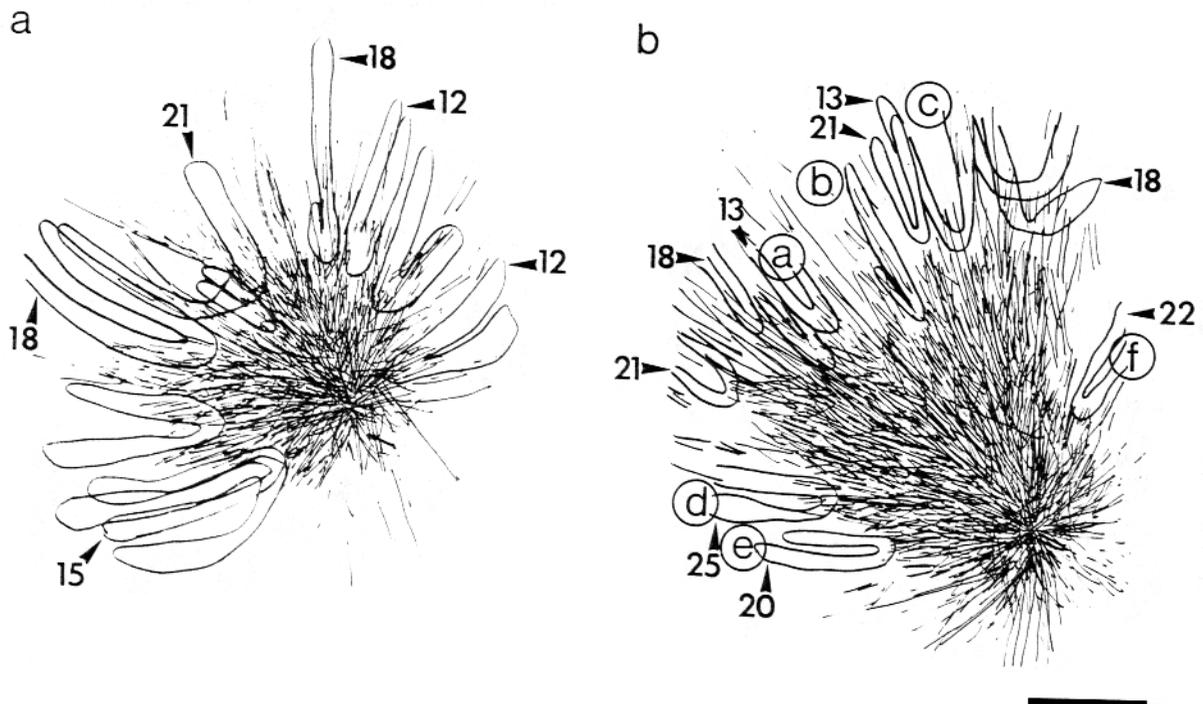


Fig. 2. Ultrastructural reconstructions of MT distribution and chromosome position in two monopolar spindles. The numbers of kMTs, determined from serial sections, are given for many chromosomes. In (b) chromosomes labelled a, b and c are located along the long axis of the spindle and are further from the pole than chromosomes labelled d, e and f located along the sides of the spindle. Bar, 10 μm .

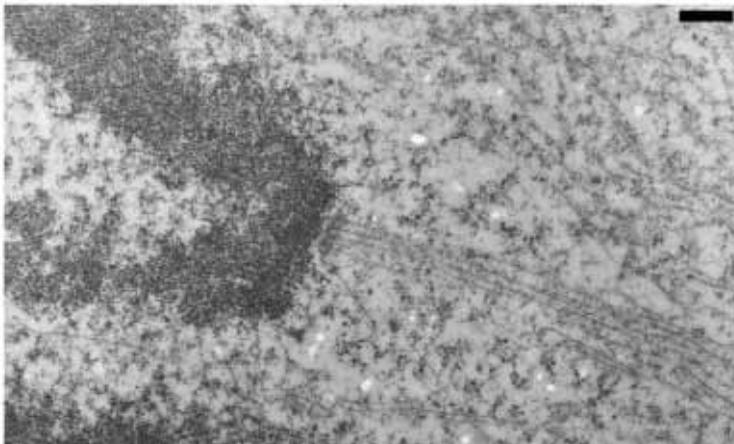


Fig. 3. Thin-section electron micrograph of a chromosome on a monopolar spindle. MTs are only associated with the attached kinetochore facing the pole (the unattached kinetochore is not shown). Bar, 0.5 μm .

Table 1. FRAP in bipolar and monopolar newt lung cell spindles

	k (s^{-1})*	$t_{1/2}$ (s)*	% Recovery
Bipolar spindle†	0.008	86.6	73%
Monopolar	0.012	63 \pm 10	81 \pm 14%

* k , first order constant and $t_{1/2}$, half time of fluorescence recovery, determined from plots of $\ln(F_{\infty} - F(t))$ versus time as described previously (Wadsworth and Salmon, 1986a). F_{∞} is the average fluorescence at 450 seconds.

†From Wadsworth and Salmon (1985).

thought to correspond to bleached subunits within the differentially stable kMTs (Wadsworth and Salmon, 1986a).

We also used video microscopy methods to visualize the

FRAP pattern in monopolar spindles. Previous studies on bipolar newt spindles found that when a narrow bar was photobleached across the spindle midway between the chromosomes and a pole, the great majority of fluorescence recovery occurred without measurable movement of the bleached pattern (Wadsworth and Salmon, 1985). The same experiments on monopoles gave similar results as shown in Fig. 6. The first phase of fluorescence recovery was not seen in these micrographs because it occurred too rapidly. Most of the fluorescence recovery occurred without any apparent movement of the bleached pattern. The boundaries of the bar pattern become diffuse after several minutes, but a faint photobleach pattern was detectable for at least 4 minutes.

As Wadsworth and Salmon (1985, 1986a) found for bipolar spindles, we were unable to detect significant poleward motion

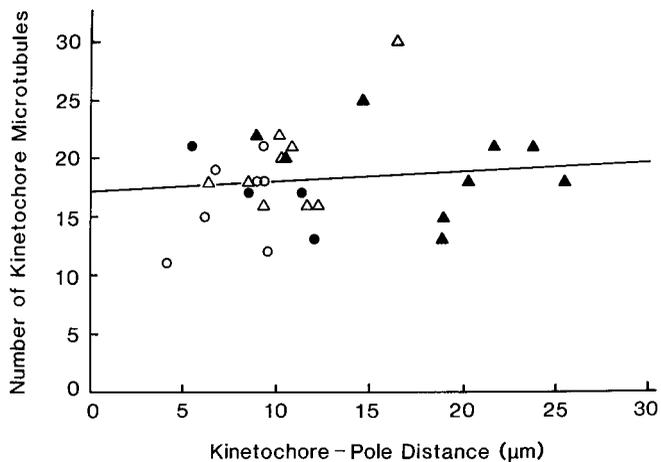


Fig. 4. kMT number versus kinetochore distance from the pole. Different symbols represent data from different cells. The solid line is a linear regression analysis of the data (slope, 0.08) and reveals little, if any, relationship between the number of kMTs and kinetochore-to-pole distance.

of photobleached regions of the fluorescent spindle fibers during fluorescence recovery. In contrast, Mitchison and Salmon (1992) were able to observe slow (0.5 $\mu\text{m}/\text{min}$) poleward flux of marks on kinetochore fibers when the marks were made using local photoactivation of caged fluorescein bound to tubulin within the fibers. The fluorescent signal from kinetochore fibers within a single plane of focus was estimated to be less than 10% to 15% of the fluorescence initially photoactivated by 366 nm irradiation in a narrow slit pattern across the central half-spindle region. These fluorescent marks on the kinetochore fibers were only visible several minutes following photoactivation, after the majority of non-kMTs had turned over and released their labeled subunits into the cytoplasmic tubulin pool. In addition, marks on adjacent fibers moved poleward at different rates. Thus, it is likely that our FRAP methods were unable to detect poleward flux of kMTs because kMTs contribute only a minor part of the fluorescent signal after fluorescence recovery of the non-kMTs and because flux, if it occurs in monopolar spindles, may be asynchronous. For these reasons, the issue of flux in monopolar spindles in living

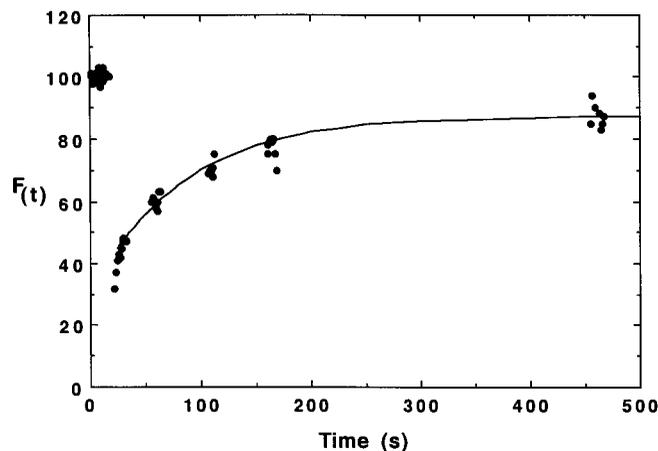


Fig. 5. Computer-generated photometric record of fluorescence recovery in a newt lung monopolar spindle. Photons were counted at 1 second intervals. Fluorescence measurements, $F(t)$, were normalized to 100 using the average number of photon counts for five samples prior to recording the first data point. The solid line plotted through the data is an exponential regression line used to calculate the half-time of fluorescence recovery (see Wadsworth and Salmon, 1986). The initial rapid phase of fluorescence recovery is due to the diffusion of subunits into the bleached region.

cells needs to be addressed using the photoactivation marking methods of Mitchison (1989b).

Changes in chromosome position induced by changes in microtubule assembly

We next tested how chromosome position in monopolar spindles depends on the extent of MT assembly by inducing MT disassembly with nocodazole (10 $\mu\text{g}/\text{ml}$, data not shown; see also Ault et al., 1991) or cooling to 4°C (Fig. 7) for 15 minutes. In both cases, the kinetochore-to-pole distance of tethered chromosomes shortens as occurs for metaphase bipolar spindles (reviewed by Salmon, 1989b). Short k-fibers were clearly seen by immunofluorescence microscopy in cells fixed 15 minutes after either treatment (e.g. see Fig. 7B). We then investigated if the chromosomes would move back to their normal positions from the pole if the block to MT assembly was removed. In these experiments, cells were initially treated with nocodazole or 4°C for 15 minutes, then returned to normal

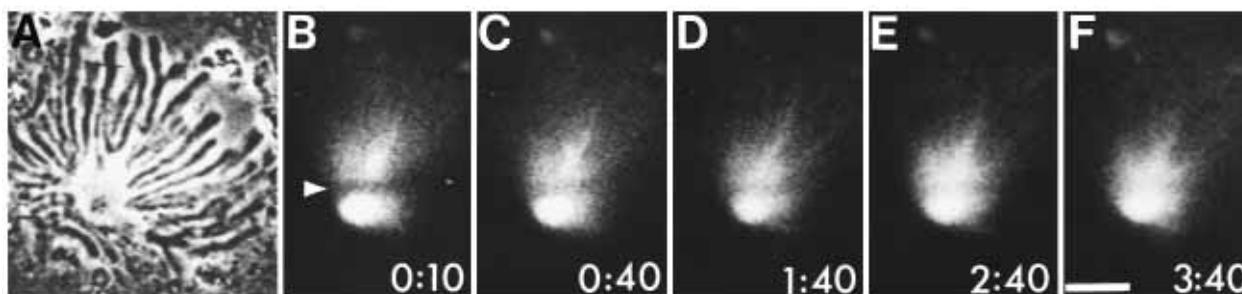


Fig. 6. Video recording of FRAP in a monopolar spindle. Phase-contrast micrograph of the cell before photobleaching (A). A narrow bar pattern (arrow) was photobleached across the spindle approximately 10 seconds after recording the phase-contrast image. Fluorescent images during FRAP (B-F) and the time after photobleaching is indicated on each frame. Before photobleaching the monopolar spindle was uniformly fluorescent (not shown). Bar, 10 μm .

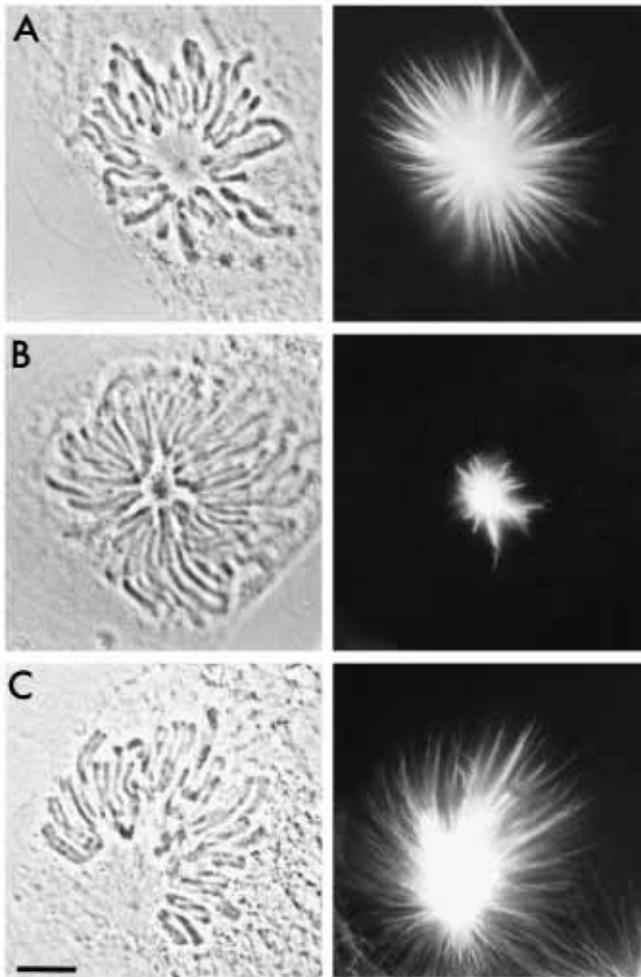


Fig. 7. Phase-contrast and immunofluorescent micrographs of monopolar spindles prior to treatment (A), after 15 minutes at 4°C (B) and after 15 minutes at 4°C and 30 minutes recovery at room temperature (C). In (A) the chromosomes are positioned away from the pole and there is a dense array of MTs in the spindle. After cooling (B) the chromosomes are located close to the pole and MT density is greatly reduced and only short MTs remain. After recovery from the cold (C), chromosomes are again located away from the pole and the dense array of MTs has reassembled. Bar, 10 μm .

conditions to allow spindle reassembly. No recovery of spindle assembly occurred for the nocodazole-treated cells (we have observed this lack of recovery in both bipolar and monopolar newt spindles; Cassimeris and Salmon, unpublished observations). However, rewarmed cells had normal-looking monopolar spindles after 30 minutes at room temperature and the chromosomes were again many micrometers from the pole (Fig. 7C). These results show that the kinetochore-to-pole distance in monopolar spindles is a reversible function of the extent of MT assembly as has been shown to occur for bipolar spindles (reviewed by Salmon, 1989b), and that a bipolar central spindle is not required for chromosomes to move away from the pole.

Kinetochore directional instability

To analyze the oscillations of chromosomes on monopolar

Table 2. Kinetochore directional instability in monopolar spindles: average phase velocities, V_{av} , durations, t_v , and displacements, d_{av}

	V_{av} ($\mu\text{m}/\text{min}$)	t_{av} (s)	d_{av}^* (μm)	n_p, n_{ch}, n_c^\dagger	% Time ‡
P phase	1.72 ± 0.74	1.42 ± 0.91	2.40 ± 1.48	38, 7, 2	46
AP phase	1.62 ± 0.63	1.58 ± 0.76	2.52 ± 1.31	39, 7, 2	51
N phase §	---	0.42 ± 17	---	8, 7, 2	3

*Displacements are distances between kinetochores and their poles.

† Number of phases, n_p , chromosomes, n_{ch} , and cells, n_c , analyzed.

‡ Percentage of time spent in a given phase during 118 minutes total time.

§ Indicates very short-lived movements or longer duration movements where direction could not be determined.

Data for V_{av} , t_{av} and d_{av} are the means \pm s.d.

spindles, we measured the motion of attached kinetochores using the VE-DIC and semi-automatic tracking methods described by Skibbens et al. (1993; see Materials and Methods). As previously reported by Seto et al. (1969) and Bajer (1982), chromosomes in newt monopolar spindles oscillated radially poleward (P) and away-from-the-pole (AP). These oscillations were most noticeable for chromosomes positioned furthest from the pole, i.e. situated near the central periphery of the monopolar spindle (e.g. chromosomes 1, 2 and 3 in Figs 8A and 9). High resolution kinetic plots of the distance between attached kinetochores and their poles showed that these oscillations were not sinusoidal as previously reported by Bajer (1982), but more 'saw-tooth' in profile (Fig. 9). These kinetochores often abruptly switched between persistent phases of P and AP motion and this behavior has been termed directional instability by Skibbens et al. (1993) for kinetochores on chromosomes attached to bipolar spindles.

Each P and AP phase of kinetochore motion typically consisted of 1 or 2 constant velocity domains. On average (Table 2), kinetochore P phase motion occurred at 1.7 $\mu\text{m}/\text{min}$ for about 1.4 minutes producing an average displacement of 2.5 μm before a switch to AP motion occurred. AP phase velocities and durations were similar to the P phase values so that little net displacement of the centromere region occurred when averaged over many minutes. Occasionally (only 3% of the time, Table 2), the kinetochore appeared not to be moving (N phase, see Fig. 9). However, 97% of the time, attached kinetochores on monopolar spindles persisted in either P or AP phases of motion.

The switching between P and AP phases for kinetochores on adjacent mono-oriented chromosomes occurred independently of each other. For example, chromosome 1 in the cell in Fig. 9 was between chromosomes 2 and 3. About 50% of the time, k1 (kinetochore region of chromosome 1) was out-of-phase or in-phase with the motions of k2 or k3.

Kinetochore directional instability for those chromosomes positioned on the side of the polar MT array (e.g. chromosome 4 in Fig. 8A) was different from that exhibited by chromosomes located along the long axis of the spindle (e.g. chromosomes 1, 2 and 3 in Fig. 9A). As seen in Fig. 9, k4 switching between P and AP phases appears more frequent and the displacements smaller in comparison to the directional instability of k1, k2 and k3. k4 on average was about 5 μm from the pole whereas k1, k2 and k3 were between 16–19 μm . We did not attempt to quantitate the P and AP motions of kinetochores like

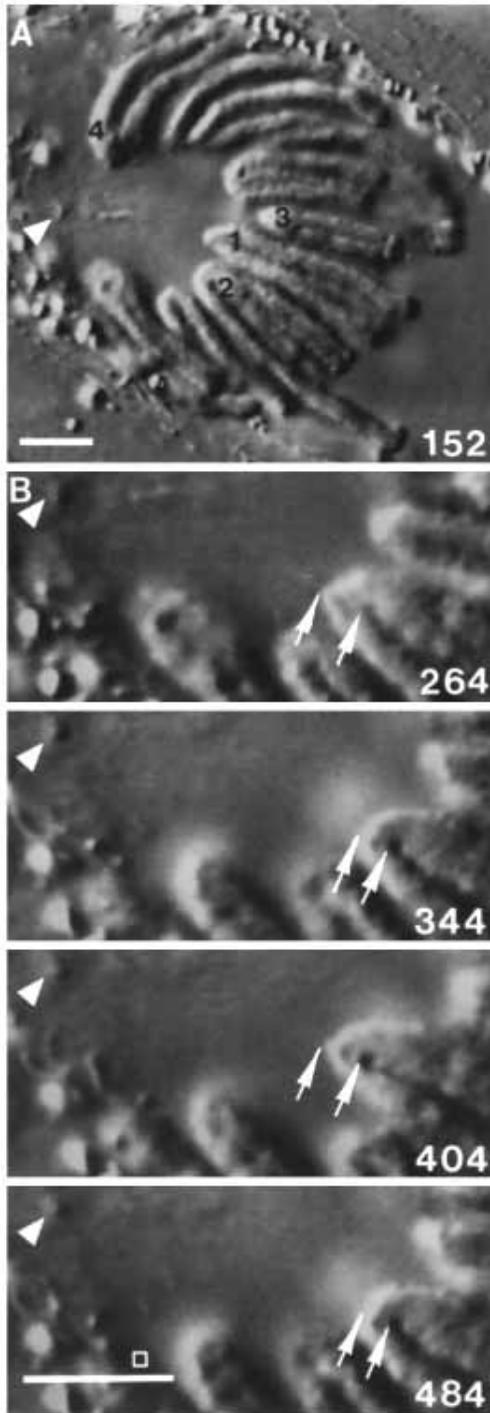


Fig. 8. VE-DIC images of chromosome oscillations and kinetochore directional instability on a monopolar spindle. (A) Low magnification view of a monopolar spindle. The motion of kinetochores with respect to the centrosome (large arrowheads in (A) and (B)) is plotted in Fig. 9 for the chromosomes labeled 1, 2, 3 and 4. Bar, 10 μm . (B) High magnification views of centromere deformation on chromosome 2 produced by the pulling and pushing forces generated by kinetochore directional instability. The narrow arrowheads mark the positions of the proximal and distal kinetochore regions. The box represents the size of the 8x8 pixel cursor used for tracking the motion of the centrosome and kinetochore regions. Bar, 10 μm . Time in seconds on each frame corresponds to measurements plotted in Fig. 9.

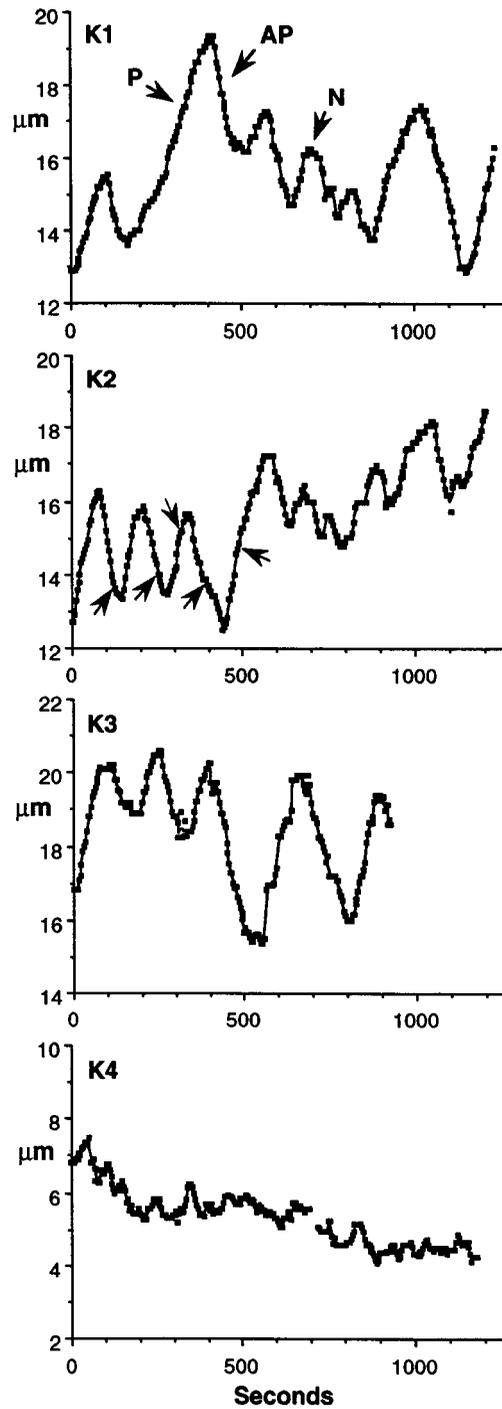


Fig. 9. Kinetochore directional instability for 4 chromosomes in the cell shown in Fig. 8. Each dot in a plot represents a measured position while the line through the data points was obtained by averaging values of three adjacent points. The positions within the monopolar spindle of chromosome 1, 2, 3 and 4 are indicated in Fig. 8. Chromosome 1 (k1) is located between 2 (k2) and 3 (k3) and these chromosomes are furthest from the pole. Chromosome 4 (k4) is on the side of the monopolar spindle, not adjacent to the other chromosomes, and much closer to the pole. Examples of poleward (P), away-from-pole (AP) and no motion (N) phases are indicated by arrows in the k1 plot. The arrows in the k2 plot indicate the times for the VE-DIC micrographs shown in Fig. 8B.

k4 because their magnitudes were often too close to our tracking resolution of 0.125 μm .

We also examined whether the switch between P and AP phases of kinetochore directional instability was a switch from pulling to pushing on the centromere. This pull-push mechanism was proposed by Bajer (1982) for kinetochore oscillations on monopolar spindles, and demonstrated from analyses of centromere deformation by Skibbens et al. (1993) during kinetochore directional instability in bipolar spindles. High resolution video images, like those in Fig. 9B, showed that the kinetochore region was stretched poleward during P motion, while it was 'flattened' or 'punched-in' during AP motion. Thus, like the attached kinetochores on bipolar spindles (Skibbens et al., 1993), kinetochore directional instability on monopolar spindles also involves switching between (P) pulling and (AP) pushing on the centromere.

DISCUSSION

The experimental results presented here demonstrate the similar properties of monopolar and bipolar spindles in vertebrate animal cells. In this regard, we found that the MT assembly properties of monopolar and bipolar spindles and the motility of mono-oriented chromosomes on both of these spindle types are, in general, the same for all the parameters we measured. In particular, our FRAP studies revealed no significant difference in the rate of MT turnover between monopolar spindles (our study) and that previously reported for bipolar newt spindles (Wadsworth and Salmon, 1985). We also found that the numbers of kMTs associated with the chromosomes on monopolar spindles is similar to that predicted for bi-oriented metaphase chromosomes. In addition, in monopolar (this study) and bipolar spindles (Centonze and Borisy, 1991; Cassimeris and Salmon, 1991), conditions that preferentially depolymerize non-kMTs produce a shortening of the kinetochore-to-pole distance that is reversible. Finally, a comparison of our data on monopolar spindles, with that of Skibbens et al. (1993) for chromosomes on newt bipolar spindles, reveals that the attached kinetochores in both spindle types exhibit a similar oscillatory behavior that is defined by kinetochore directional instability.

From the above evidence we conclude that monopolar spindles contain the same molecular mechanisms for chromosome movement as are present in bipolar spindles. But there are several important exceptions to this general conclusion, including differences in spindle geometry and co-ordination of sister kinetochore motility (discussed below). In monopolar spindles there is only one polar MT array producing AP forces on the chromosome arms and chromosomes remain mono-oriented. The distal, unattached sister kinetochore only passively follows the P and AP motion of the attached sister kinetochore and thus the unattached kinetochore contributes nothing to chromosome behavior. In contrast, in bipolar spindles there are two overlapping MT arrays producing AP forces on the chromosome arms that antagonize each other. Bipolarity also allows chromosomes to become bi-oriented and in this case sister kinetochores influence each other's directional instability and this is ultimately responsible for congression to the metaphase plate.

Before considering further the implications of our data for

the mechanisms of chromosome congression, we first consider mechanisms influencing chromosome movements in monopolar spindles. We then discuss the differences in MT organization between monopolar and bipolar spindles and the significance of these differences for both polar ejection forces and chromosome movement. Finally, we look at the differences between kinetochore directional instability in monopolar and bipolar spindles that are responsible for chromosome congression to the equator of a bipolar spindle, a position further from the pole than that typically achieved by mono-oriented chromosomes on either monopolar or bipolar spindles.

How is chromosome position on the monopolar spindle established?

Our high resolution VE-DIC records of centromere deformation clearly show (Fig. 9B) that when an oscillating chromosome switches between P and AP phases of motion, the kinetochore switches between a P pulling to an AP motion that can push on the centromere. Similar but less compelling observations were reported by Bajer (1982) in his initial low resolution studies of chromosome oscillations on monopolar spindles. These observations show that the AP motion of a kinetochore is not produced simply by the ejection forces overcoming a constant pulling force at the kinetochore (Rieder et al., 1986; Salmon, 1989a,b; Ault et al., 1991), which would be expected to continue to stretch the centromere during AP motion.

As argued by Skibbens et al. (1993) for kinetochore directional instability on bipolar spindles, the positions achieved by chromosomes in monopolar spindles must depend on the control of switching of kinetochores between P and AP phases of motion. Since the average velocities of P and AP motions are similar, the average positions achieved by chromosomes relative to the pole must depend on the relative durations of P and AP motion. The probability of switching may depend on the balance of forces at the kMT attachment sites in the way proposed in the previous static force balance models (Rieder et al., 1986; Cassimeris et al., 1987; Salmon, 1989a,b; Ault et al., 1991). However, in this context, the velocity of P and AP phases of motion is independent of the net force at the attachment site since P and AP velocities are usually constant, or shift to different constant velocity domains during several μm displacements.

Our results provide two important structural findings that are relevant to the mechanism(s) controlling kinetochore switching and, thus, chromosome position from the pole. First, there was no correlation between the number of kMTs and chromosome to pole position. Second, there was a significant qualitative positive correlation between the density of non-kMTs in the region of the chromosome and its position with respect to the pole.

Previous experiments on bipolar meiotic spindles, in which part of a kinetochore on a metaphase chromosome was destroyed by a laser microbeam, suggested a relationship between kMT number and chromosome position from the pole (Hays and Salmon, 1990). Such a relationship implied that the P force acting on a kinetochore is somehow related to the number of kMTs. However, we found no correlation between kinetochore distance from the pole and the number of associated kMTs for monopolar spindles. One possible explanation for these apparently conflicting conclusions is that kinetochore

position relative to the pole is independent of kMT number, but dependent on some other component of the kinetochore that becomes diminished when the kinetochore is partly destroyed, as in the experiments of Hays and Salmon (1990). Such an explanation is consistent with the accumulating evidence that the force producers for the great majority of kinetochore P motion are located at the kinetochore (reviewed by Rieder, 1991; McIntosh and Pfarr, 1991; Mitchison and Salmon, 1992). Destroying part of these force producers would be expected to shift the bi-oriented chromosome towards the pole to which the intact kinetochore is attached.

In monopolar spindles, chromosomes appear to move poleward until they contact some threshold density of non-kMTs independent of their numbers of kMTs. If this density is lowered by treatments that preferentially disassemble non-kMTs (cold or nocodazole), the chromosomes move even closer to the pole. Chromosomes located peripheral to the long axis of the monopole exhibited less significant fluctuations in distance from the pole than did chromosomes located along the long axis (Fig. 9). One possibility for this difference is that kinetochore switching between P and AP phases is sensitive to the gradient in MT density, which is much steeper on the side of the monopolar spindle in comparison to the central region (Fig. 2). We envision that at higher MT density there is a higher probability that a kinetochore will switch to AP motion. By this mechanism, kinetochores exhibit P motion until they reach a region of sufficient MT density to induce switching to AP motion. AP motion of the kinetochore and chromosome arms moves the chromosome to a region of lower MT density, and thus increases the probability of switching back to P motion and moving closer to the pole. Since the gradient of MT density on the sides of the monopolar spindle appears much steeper than occurs along the long axis, a difference in kinetochore directional instability would be expected between the two regions (compare chromosome 4 with chromosomes 1, 2 and 3 in Fig. 9). Testing this model will require correlating kinetochore behavior with quantitative measurements of MT density within different regions of the half spindle.

How higher densities of polar spindle MTs increase the probability of kinetochore switching from P to AP motion is not known. Possible hypotheses include increasing: (1) kinetochore phosphorylation, which produces switching of kinetochore motor activity between minus-end (P) and plus-end (AP) activity as has been proposed by Hyman and Mitchison (1991) based on their *in vitro* studies; (2) the number of kMTs, which in turn would promote switching between P and AP motor activity (Hyman and Mitchison, 1991); (3) the probability of switching MT assembly at the kinetochore between the shortening and growth phases of dynamic instability by factors such as those listed in (1) and (2); and (4) the strength of polar ejection forces on the chromosome, which produces higher tension at the kinetochore and, as a result, a higher probability of switching from P to AP motion. All four of these hypotheses are possible explanations of kinetochore directional instability as discussed in more detail by Skibbens et al., 1993. There is no doubt that the chromosome arms are pushed AP by polar ejection forces; they move AP during kinetochore AP motion and they move AP independently of the kinetochore motion when severed from the centromere (Rieder et al., 1986). As a result, we currently favor the tension hypothesis

as the dominant mechanism regulating kinetochore directional instability in monopolar spindles while other molecular mechanisms, like phosphorylation control of motor activity or regulation of kinetochore MT dynamic instability, may be involved as downstream events.

Although we have not made quantitative measurements of MT density, we did observe a strong qualitative correlation between the concentration of chromosomes in a region of the monopolar spindle and the density and extent of MT assembly. A similar correlation has been observed for the reconstituted spindles in *Xenopus* meiotic extracts (Sawin and Mitchison, 1991a,b) and in other types of meiotic cells (Karsenti et al., 1984a,b; Church et al., 1986). Clearly, chromosomes by themselves are not sufficient to cause MT assembly in mitotic cells (Brinkley et al., 1988; Rieder and Alexander, 1990; see Rieder et al., 1993 for a discussion of the differences between meiotic and mitotic cells), otherwise monopolar spindles would not form. While the mechanism responsible for the chromosomal enhancement of MT assembly is not known, the extent of MT assembly observed may require functional kinetochores. Monopolar spindles assembled in *Xenopus* extracts, under conditions in which the chromosomes lack functional kinetochores, show less MT density than spindles with functional kinetochores (Sawin and Mitchison, 1991a,b). This is consistent with the proposal of Rieder et al. (1993) that the stability of non kMTs is enhanced in the vicinity of kMTs.

The chromosomal enhancement of MT assembly may play a role in regulating chromosome position. By enhancing the density and extent of MT assembly, chromosomes could indirectly promote AP forces that would increase tension at the kinetochore, promoting switching to AP motion. As a result, chromosomes would achieve average positions further from the pole than would occur if they did not promote MT assembly.

Changes in spindle structure and polar ejection forces between monopolar and bipolar spindles

One of the differences we noted between monopolar and bipolar spindles is the actual shape of the spindle. Bipolar vertebrate mitotic spindles, defined by overlapping MT arrays of opposite polarity, ultimately adopt the familiar fusiform spindle shape, tapering and curving inward towards the poles. In contrast, the asymmetric monopolar spindle is shaped more like a fan, lacking the curvature of bipolar spindles (see Fig. 1). These differences in morphology can be clearly ascribed to the two obvious characteristics that distinguish monopolar from bipolar spindles: the presence of one instead of two poles and the lack of bi-oriented chromosomes on monopolar spindles. Indeed, separating spindle poles form two fan-like monopolar spindles at NEB if one or more chromosomes do not become bi-oriented to tether the poles (e.g. see Waters et al., 1993). Moreover, during the early stages of formation, the bipolar spindle often resembles two opposing fan-like arrays of MTs (e.g. see Rieder and Hard, 1990; Waters et al., 1993). Over time, however, the spindle becomes more compacted and fusiform-shaped. It is difficult to envision how the formation of k-fibers on sister kinetochores alone leads to the fusiform shape of the bipolar spindle, instead of the two opposing fan-like arrays seen during the early stages of spindle formation. Instead, it is likely that accumulating interactions between MTs derived from the opposing spindle poles impart a progressive

bend to what would otherwise be two radial arrays. In this respect it is noteworthy that Nislow et al. (1992) have recently identified a protein in vertebrate spindles that appears to bundle adjacent MTs of opposite polarity. However, since two mitotic asters fail to form a spindle in the absence of chromosomes (e.g. Sluder et al., 1986), chromosomes are also required for the morphogenesis of spindle shape. It is possible that the formation of bipolar kinetochore attachments tethers asters that would otherwise move apart until they were independent units, and that this tethering allows the MTs from each aster to then progressively interact.

Chromosomes achieve different orientations in monopolar and bipolar spindles that may be a consequence of the difference in spindle organization and polar ejection forces. In monopolar spindles, the arms of mono-oriented chromosomes become oriented parallel to the long axis of the MTs (Fig. 1C,D). This orientation is expected for ejection forces produced by only one polar MT array in a MT plus-end direction. In bipolar spindles, the arms of mono-oriented chromosomes are pushed over to the side of the spindle, while the arms of bi-oriented chromosomes near the equator tend to become arranged in a direction perpendicular to the spindle interpolar axis (Fig. 1A,B). These arrangements of chromosome arms on a bipolar spindle are the predicted result of the vectorial effects of the polar ejection forces produced by two oppositely oriented and overlapping polar MT arrays (Rieder et al., 1986; Salmon, 1989b). The vectorial components of the overlapping polar ejection forces oppose each other in the direction of the spindle interpolar axis, but sum with each other in directions perpendicular to the interpolar axis. As a result, mono-oriented chromosomes in a bipolar spindle would achieve average positions closer to the pole than occurs for mono-oriented chromosomes in the central periphery of monopolar spindles (compare Fig. 1C,D with A,B) because of the opposition of polar ejection forces along the interpolar axis. On the other hand, the combination of ejection forces in directions perpendicular to the interpolar axis act together to push chromosome arms away from the poles and out of the spindle so that the arms of bi-oriented chromosomes near the equator become aligned perpendicular to the spindle interpolar axis as seen in Fig. 1.

Changes in kinetochore directional instability between monopolar and bipolar spindles

The similarities and differences in kinetochore directional instability between monopolar and bipolar spindles can be seen by comparing our directional instability data for monopolar spindles with that obtained by Skibbens et al. (1993) for bipolar spindles (Table 3). Remarkably, kinetochore directional instability (as defined by the velocities and durations of P and AP phases) is similar for attached kinetochores on mono-oriented chromosomes in either monopolar or bipolar spindles and for sister kinetochores on bi-oriented chromosomes near the equator of bipolar spindles (Table 3). Skibbens et al. (1993) found that anaphase kinetochore velocities were not significantly different from those for prometaphase and metaphase. Anaphase is rare in monopolar spindles in newt lung cells (Bajer, 1982), so we have little information for comparison. We also found that kinetochores on adjacent mono-oriented chromosomes switched independently of one another as

Table 3. Comparison of kinetochore directional instability on monopolar and bipolar* spindles

	Monopolar mono-oriented	Bipolar mono-oriented	Bi-oriented (near equator)	Bi-oriented (congression)
Poleward (P)				
<i>V</i> (µm/min) ± s.d.	1.72±0.74	1.40±0.33	1.98±1.06	1.75±0.64
<i>t</i> (min) ± s.d.	1.42±0.91	1.47±0.68	1.25±1.03	0.64±0.61
<i>D</i> (µm)	2.40±1.48	2.1	2.5	1.1
Away (AP)				
<i>V</i> (µm/min) ± s.d.	1.62±0.63	1.24±0.42	1.98±1.42	2.08±1.02
<i>t</i> (min) ± s.d.	1.58±0.76	1.69±0.75	1.22±0.99	2.30±1.66
<i>D</i> (µm)	2.52±1.31	2.1	2.4	4.8
Indeterminate (N)				
<i>t</i> (min) ± s.d.	0.42±0.17	0.39±0.33	0.61±0.45	1.32±1.99

V, *t*, *D* and *N* are as defined in Table 2.
*Data from Skibbens et al. (1993).

reported for mono-oriented chromosomes in bipolar spindles (Skibbens et al., 1993).

There are two situations where a major difference occurs in kinetochore directional instability between mono-oriented and bi-oriented chromosomes: (1) when a bi-oriented chromosome is far off the equator towards one pole and (2) when a chromosome at the equator enters anaphase or is prematurely separated between kinetochores by cutting with a laser microbeam. Before bi-orientation, the attached kinetochore on a mono-oriented chromosome faces towards its pole (facing away from the equator) and oscillates between P and AP phases of similar durations. When the sister kinetochore attaches to MTs from the pole on the other side of the equator, it persists primarily in P motion and the kinetochore facing away from the equator becomes biased into AP motion until the chromosome moves to near the equator (Skibbens et al., 1993; Table 3). Near the equator, kinetochore directional instability again becomes similar to the directional instability of kinetochores on mono-oriented chromosomes. However, Skibbens et al. (1993) found that the motion of sisters remains 'out-of-phase' coordinated in that 70% of the time one sister is in P motion while the other is in AP motion or vice versa. If metaphase chromosomes near the equator are cut-apart between sister kinetochores, each sister persists in P motion, dragging their severed arms poleward (Skibbens et al., 1992). Closer to their poles, kinetochores again begin oscillating between P and AP phases of motion with velocities and durations typical of mono-oriented chromosomes. From these observations we conclude, as did Skibbens et al. (1993), that kinetochores persist in P motion when far from their spindle pole and that P motion of one sister kinetochore can bias the other into AP motion at MT densities where kinetochores on mono-oriented chromosomes would switch between P and AP motion.

How does the directional instability of one sister kinetochore bias the directional instability of the other sister on a bi-oriented chromosome? Since we do not yet understand the molecular mechanisms that produce and regulate kinetochore directional instability (tension, phosphorylation, dynamic instability, etc.), the answer to this question remains speculative, but tension may be a key factor as proposed by Skibbens et al. (1993). When initially mono-oriented, chromosome

position would be determined primarily by interactions with only one polar MT array as discussed above for monopolar spindles. When the unattached sister becomes attached to MTs from the opposite pole, it persists in P motion because it is too far from its pole to be under much tension from polar ejection forces. This P motion augments the tension generated on the other sister by polar ejection forces from the proximal pole and biases this kinetochore, which faces away from the equator, into AP motion. The centromeres move to, and oscillate back and forth near, the spindle equator because this is the position where the density of polar MTs and the strength of polar ejection forces from opposite poles are similar, making the probabilities over time of P or AP motion for each sister kinetochore similar. Upon chromosome disjunction, the P motions of sister kinetochores would no longer be able to antagonize each other and each sister would persist in P motion towards the pole until the density of polar MTs was sufficient to induce a switch to AP motion.

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