Meiotic Spindle Assembly In *Drosophila* Females: Behavior of Nonexchange Chromosomes and the Effects of Mutations in the *nod* Kinesin-like Protein

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Abstract. Mature *Drosophila* oocytes are arrested in metaphase of the first meiotic division. We have examined microtubule and chromatin reorganization as the meiosis I spindle assembles on maturation using indirect immunofluorescence and laser scanning confocal microscopy. The results suggest that chromatin captures or nucleates microtubules, and that these subsequently form a highly tapered spindle in which the majority of microtubules do not terminate at the poles. Nonexchange homologs separate from each other and move toward opposite poles during spindle assembly. By the time of metaphase arrest, these chromosomes are positioned on opposite half spindles, between the metaphase plate and the spindle poles, with the large nonexchange X chromosomes always closer to the metaphase plate than the smaller nonexchange fourth chromosomes. Nonexchange homologs are therefore oriented on the spindle in the absence of a direct physical linkage, and the spindle position of these chromosomes appears to be determined by size. Loss-of-function mutations at the *nod* locus, which encodes a kinesin-like protein, cause meiotic loss and nondisjunction of nonexchange chromosomes, but have little or no effect on exchange chromosome segregation. In oocytes lacking functional *nod* protein, most of the nonexchange chromosomes are ejected from the main chromosomal mass shortly after the nuclear envelope breaks down and microtubules interact with the chromatin. In addition, the nonexchange chromosomes that are associated with spindles in *nod/nod* oocytes show excessive poleward migration. Based on these observations, and the structural similarity of the *nod* protein and kinesin, we propose that nonexchange chromosomes are maintained on the half spindle by opposing poleward and anti-poleward forces, and that the *nod* protein provides the anti-poleward force.

ACCURATE chromosome segregation at meiosis I generally requires recombination between homologs during meiotic prophase, which leads to the physical linkage of homologous chromosomes by chiasmata, which form at sites of meiotic recombination (for review see Hawley, 1988). It is this physical linkage, which forms the bivalents that are aligned on the spindle at metaphase I, that is thought to assure meiotic chromosome disjunction in most systems. A simple mechanical model (Nicklas, 1974), supported by a series of micromanipulation studies (Nicklas and Staehly, 1967; Nicklas, 1967; Nicklas and Koch, 1969), explains the need for physically linked homologs during meiosis I. The kinetochores associated with individual homologs are fused into single functional units which capture microtubules from one of the spindle poles. The bivalent then moves toward the pole, as a result of a microtubule-dependent poleward force acting at or near the kinetochore. When the two kinetochores associated with a bivalent are captured by microtubules from opposite poles, the chiasmata prevent homolog separation and the resulting mechanical tension moves the bivalent to the metaphase plate. Through an unknown mechanism, mechanical tension also stabilizes the microtubule–kinetochore interaction. When both kinetochores of a bivalent are captured by microtubules from the same pole, the bivalent moves to that pole. In most cases the microtubule–kinetochore bond is unstable in the absence of mechanical tension, however, and the bivalent then dissociates. The free kinetochores then go through a second round of microtubule capture and orientation. This process continues until the kinetochores capture microtubules from opposite poles and the resulting mechanical tension positions the bivalent at the metaphase plate and stabilizes the microtubule–kinetochore link. In this model, nonexchange chromosomes are meiotically unstable because the single functional kinetochore associated with each univalent can only interact with microtubules from one of the poles. As a result, mechanical tension cannot balance the univalent at the metaphase plate, and the univalent moves to the pole and dissociates.

Variations on this model have dominated discussions of

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meiotic and mitotic chromosome alignment (for recent reviews see McIntosh and McDonald, 1990; Mitchison, 1989*a*). However, a number of observations cannot be explained by balancing poleward forces. For example, mitotic chromosomes can stably attach to, and oscillate on, monopolar spindles (Mazia et al., 1981; Bajer, 1982), and nonexchange and univalent meiotic chromosomes segregate properly in several species (for examples see Ault, 1986; Nokkala, 1986; Hughes-Schrader, 1969). In these systems chromosomes that are not under mechanical tension between opposite poles nonetheless stably associate with the spindle.

Of the meiotic systems in which chiasma-mediated balancing of opposing poleward forces does not fully explain chromosome behavior, female meiosis in *Drosophila melanogaster* is the best studied genetically. During meiosis in *Drosophila* females, the small fourth chromosomes never exchange, X chromosomes fail to exchange in 5% of meioses, and chromosomes containing multiple inversions (Balancer chromosomes) rarely recombine with normal homologs. Nevertheless, these chromosomes segregate accurately at the first meiotic division. The available cytological data on female meiosis in *Drosophila* suggest that nonexchange homologs are not physically joined (Puro and Nokkala, 1977; Puro, 1991; Kimble and Church, 1983), but rather move precociously toward the poles. Nonexchange chromosome segregation in the oocyte, therefore, appears to be achieved by a system that does not require mechanical tension between opposite poles.

Genetic analysis of meiosis in *Drosophila* females has led to the proposal that disjunction of nonexchange chromosomes occurs through a process that is independent of that which assures exchange chromosome segregation; this process has been termed distributive segregation (for review see Grell, 1976). The phenotype of mutations at the *nod* locus provides strong evidence for a specific nonexchange chromosome segregation system in *Drosophila* oocytes. Mutations at this locus cause loss and nondisjunction of nonexchange chromosomes in females, but have at most a very weak effect on either the exchange process itself or on the disjunction of those chromosomes that have undergone exchange (Carpenter, 1973; Zhang and Hawley, 1990). In addition, segregation of nonexchange chromosomes during male meiosis is not affected by *nod* mutations.

Structural analysis of the *nod* locus provides an important insight into the mechanism of distributive segregation. This locus encodes a protein with significant homology to the ATP-binding mechanochemical domain of kinesin (Zhang et al., 1990). Kinesin, originally isolated from squid axons (Vale et al., 1985), is a protein that can translocate microtubules in vitro and is likely to mediate organelle movement along microtubules in vivo (Hirokawa et al., 1991, Saxton et al., 1991). The structural similarity of kinesin and *nod* raises the intriguing possibility that the *nod* protein is a microtubule motor that translocates chromosomes along meiotic spindle microtubules.

The cellular basis of distributive segregation and the effect of *nod* mutations on this process are not understood. High resolution cytological data on microtubule and chromatin organization in mature oocytes are required to elucidate the mechanism of meiotic chromosome segregation. This data has proved difficult to obtain, however, as mature oocytes are surrounded by vitelline membranes and the chorion, and these structures are impermeable to antibodies and thus prevent immunocytochemical studies. Mechanical sectioning can be used to allow antibody penetration, but the cytoskeleton is not well preserved in these preparations and the three-dimensional organization is difficult to reconstruct from the resulting images. The available cytological data on chromosome behavior during female meiosis in *Drosophila* were obtained on hypotonically treated oocytes (Puro and Nikkola, 1977; Puro, 1991), or oocytes fixed in a hypotonic buffer (Kimble and Church, 1983). Because hypotonic treatment activates *Drosophila* oocytes, inducing anaphase chromosome movements (Mahowald et al., 1983), these data are difficult to interpret. Positioning of nonexchange chromosomes toward the poles in so-treated oocytes could either reflect homolog alignment in the absence of direct physical association, or differences in the behavior of exchange and nonexchange chromosomes during anaphase.

We have therefore determined microtubule organization and reevaluated chromosome behavior in unactivated *Drosophila* oocytes, using whole-mount immunocytochemical labeling and laser scanning confocal microscopy (White et al., 1987). We show that the meiotic spindle is structurally atypical, and present cytological evidence suggesting that chromatin plays a key role in spindle microtubule organization. In addition, we show that nonexchange chromosomes separate from the exchange chromosomes during spindle assembly, and confirm that these chromosomes are positioned in a bilaterally symmetric pattern between the spindle plate and the poles in mature oocytes (Puro and Nokkala, 1977; Puro, 1991). Thus, homologs which are not directly linked nonetheless associate stably with the spindle in a manner that causes homologs to travel to opposite poles at anaphase.

We find that loss of *nod* function causes ejection of nonexchange chromosomes early in the spindle assembly process. In addition, nonexchange chromosomes lost during spindle assembly randomly reassociate with the spindle in mature oocytes, where they generally show excessive poleward migration. These observations support the proposal that *nod* provides an anti-poleward force that substitutes for chiasma by opposing forces directed away from the metaphase plate (Zhang et al., 1990). We also present evidence that nonexchange chromosome loss during spindle assembly, followed by random chromosome reassociation with spindles at metaphase, is the physical basis of genetically observed nonexchange chromosome loss and nondisjunction in *nod/nod* oocytes. Based on these observations we propose a model for meiotic chromosome behavior and discuss the mechanism of nonexchange chromosome orientation in *Drosophila* females.

Materials and Methods

Egg Chamber Isolation and Culture

Oocytes were isolated by a modification of the procedure of Mahowald et al. (1983). 3–5-d-old *Oregon R* flies were anesthetized with CO_2 and transferred to a blender containing 200 to 300 ml of modified Robb's medium (Robb's; 55 mM potassium acetate, 40 mM sodium acetate, 100 mM sucrose, 10 mM glucose, 1.2 mM magnesium chloride, 1.0 mM calcium chloride, 100 mM Hepes, pH 7.4). The blender was pulsed three times for 2 s at low speed and the resulting mixture was passed through a loose mesh (~500 μm pore size) and collected in a 1 liter beaker. The material retained on the mesh was then returned to the blender with 200 ml of Robb's and

the blender was then again pulsed two to three times at low speed. This material was then passed through the same mesh and pooled with the previous filtrate.

The pooled filtrate was left undisturbed for 5 min to allow egg chambers to settle to the bottom of the beaker. The supernatant was then asperated off and the settled egg chambers were resuspended in 200 ml of fresh Robb's medium. The mixture was passed through a 250-μm nylon filter and the filtrate was collected in a 250-ml beaker. The 250-μm filter removes most of the larger contaminants. Egg chambers in this final filtrate were then allowed to settle for 2 to 3 min before the supernatant was asperated off.

Fixation and Indirect Immunofluorescence Labeling

Isolated egg chambers were fixed as follows: egg chambers were transferred to 10 × 75 mm test tubes and allowed to settle. The Robb's medium was then removed and replaced by 5 ml of fixation solution (100 mM potassium cacodylate, pH 7.2, 100 mM sucrose, 40 mM potassium acetate, 10 mM sodium acetate, 10 mM EGTA, 8% EM grade formaldehyde). This solution is slightly hypertonic, which assures that mature oocytes are not hypotonically activated during fixation. Fixation was allowed to proceed for 5 to 10 min at room temperature on a rotator. Fixed egg chambers were rinsed three times in PBS (Karr and Alberts, 1986), extracted with 1% Triton X-100 in PBS for 2 h, and then rinsed twice in 0.05% Triton X-100 in PBS (PBST)[1] before immunolabeling.

Alternatively, oocytes were fixed by a rapid procedure that eliminates incubation in artificial medium. Flies were anesthetized, transferred to a blender containing fixation solution, and immediately disrupted by several pulses of the blender. Fixed oocytes were then isolated from the crude mixture by the procedure described above for isolation of live oocyte.

Before immunolabeling follicle cells, chorion, and vitelline membranes were removed as follows: fixed egg chambers rinsed in PBS were transferred to the frosted surface of a glass slide. Most of the PBS was removed and a 22 mm × 50 mm cover glass was placed over the egg chambers, which were then rolled between the two surfaces. The edge of the cover glass must be drawn over the egg chambers to efficiently disrupt the chorion and vitelline membranes. The density of egg chambers on the slide is critical to efficient removal of vitelline membranes. At low density the oocytes are easily broken open, and at high density the egg chambers cannot roll, and membranes are not efficiently removed. "Rolled" egg chambers were then rinsed into a 10 × 75 mm test tube with PBST. The egg chambers were allowed to gravity settle and then the PBST was removed and the egg chambers were extracted 1% Triton X-100 in PBS and rinsed in PBST as described above.

Microtubules were labeled using a monoclonal anti-α-tubulin directly conjugated with rhodamine, prepared as described elsewhere (Theurkauf, W., S. Smiley, M. L. Wong, and B. M. Alberts, manuscript in preparation). Nuclei were visualized with 4,6-Diamino-2-phenylindole (DAPI) or immunolabeled using a monoclonal anti-histone antibody (Chemicon Inc., Temecula, CA).

Oocytes were double labeled with anti-histone and anti-α-tubulin antibodies as follows: extracted oocytes were transferred to 0.5-ml Eppendorf tubes (Brinkman Instruments Inc., Westbury, NY) and resuspended in 500 μl PBST containing monoclonal anti-histone (1:500 dilution), and were incubated overnight at 4°C with gentle mixing. Oocytes were then rinsed four times for 15 min each in PBST at room temperature and then incubated for 2 h at room temperature, or overnight at 4°C, in a 1:500 dilution of rhodamine-labeled goat anti-mouse or rhodamine-conjugated goat anti-rabbit secondary antibody preabsorbed against fixed *Drosophila* embryos, as previously described (Karr and Alberts, 1986). Unbound secondary antibody was then removed by four 15 min washes in PBST. Oocytes were then incubated for 1 h in a 1:100 dilution of normal mouse serum in PBST. This incubation is required to block the unoccupied binding sites on the secondary antibodies used to detect the histone antibody. The oocytes were then incubated with rhodamine-conjugated mouse monoclonal anti-α-tubulin overnight at 4°C. After four 15 min rinsed in PBST the labeled oocytes were resuspended in mounting medium (1 mg/ml *p*-phenylene diamine, 90% glycerol, in PBS), transferred to slides, and sealed under a cover glass with nail polish. Alternatively, labeled egg chambers were dehydrated in three changes of 100% methanol (5 min each) and transferred to a 2:1 mixture of benzyl benzoate/benzyl alcohol. This mixture matches the index of refraction of the yolk, clearing the egg chambers. Cleared egg chambers were transferred to glass slides and sealed under a cover glass as described above.

1. *Abbreviations used in this paper*: DAPI, 4,6,-Diamino-Z-phenylindole; PBST, Triton X-100 + PBS.

Microscopy

Standard epifluorescence microscopy was performed using a microphot FXA microscope (Nikon Inc., Garden City, NY) with epifluorescence attachment and a 20 × DIC, 0.5 NA lens. Laser scanning confocal microscopy was performed using the MRC 600 confocal head (Bio-Rad Laboratories, Cambridge, MA) mounted on a Nikon photoscope with a 60× Planapo, 1.4 NA lens. Linear projections and fluorescence intensity profiles were produced using the "project" and "length" utilities supplied with the Bio-Rad Laboratories confocal head. Video displays of confocal images were photographed using panatomic X film (Eastman Kodak Co., Rochester, NY). Conventional epifluorescence micrographs were taken using technical pan film (Eastman Kodak Co.).

Drosophila Stocks

All of the chromosomes used in this study are described in Zhang and Hawley (1990). The nod^{a} allele (Carpenter, 1973) is an ethylmethan sulfanate (EMS)-induced loss-of-function allele of *nod* which is borne by a normal sequence *X* chromosome. nod^{b27} is a gamma-ray induced allele of *nod* carried by the multiply inverted X chromosome *FM7a* (described in Zhang and Hawley, 1990).

Results

Microtubule Organization in Mature Oocytes

In *Drosophila* females the meiosis I spindle assembles during stage 13 and the mature stage 14 oocyte is arrested in metaphase of the first meiotic division (King, 1970). The meiotic divisions are completed after activation, which normally occurs as the oocyte enters the oviduct (for a review of oogenesis see Mahowald and Kambysellis, 1980). We have examined microtubule and chromatin organization in mature stage 14 oocytes using indirect immunofluorescence labeling and conventional epifluorescence and laser scanning confocal microscopy. A monoclonal anti-α-tubulin antibody (Blose et al., 1984) was used to localize microtubules and an anti-histone antibody was used to label chromosomes.

The meiotic spindle in mature oocytes is highly tapered, and relatively few spindle microtubules appear to terminate at or near the poles (Fig. 1 *b*). Similarly tapered meiotic spindles have been observed in ascidian eggs (Sawada and Schatten, 1988) and maize (Staiger and Cande, 1990). In most well-studied systems the majority of spindle microtubules terminate at or near the spindle poles (McIntosh and Landis, 1971), apparently as a result of microtubule nucleation at centrosomes which are positioned at the poles. Therefore, the meiotic spindles in several systems, including *Drosophila* oocytes, appear to be structurally atypical.

To confirm the qualitative impression that the majority of microtubules in the meiotic spindle do not terminate at or near the poles, we have used confocal microscopy to estimate the microtubule number profile along the spindle axis. Images containing all of the immunofluorescently labeled microtubules in a given spindle were obtained by constructing linear projections from optical sections taken on a confocal microscope. The intensity of anti-tubulin labeling was assumed to be proportional to the number of microtubules present. As a control, we have used this technique to determine the microtubule numbers profile of mitotic spindles in early embryos. A nuclear cycle 1 mitotic spindle in the embryo is shown in Fig. 2 *a*. This spindle has well defined spindle poles and astral microtubules. Anti-tubulin fluorescence intensity in this spindle reaches a maximum within 0.5 μm of the pole, remains relatively constant for 2 to 3 μm, and then decreases in the chromosome containing region near

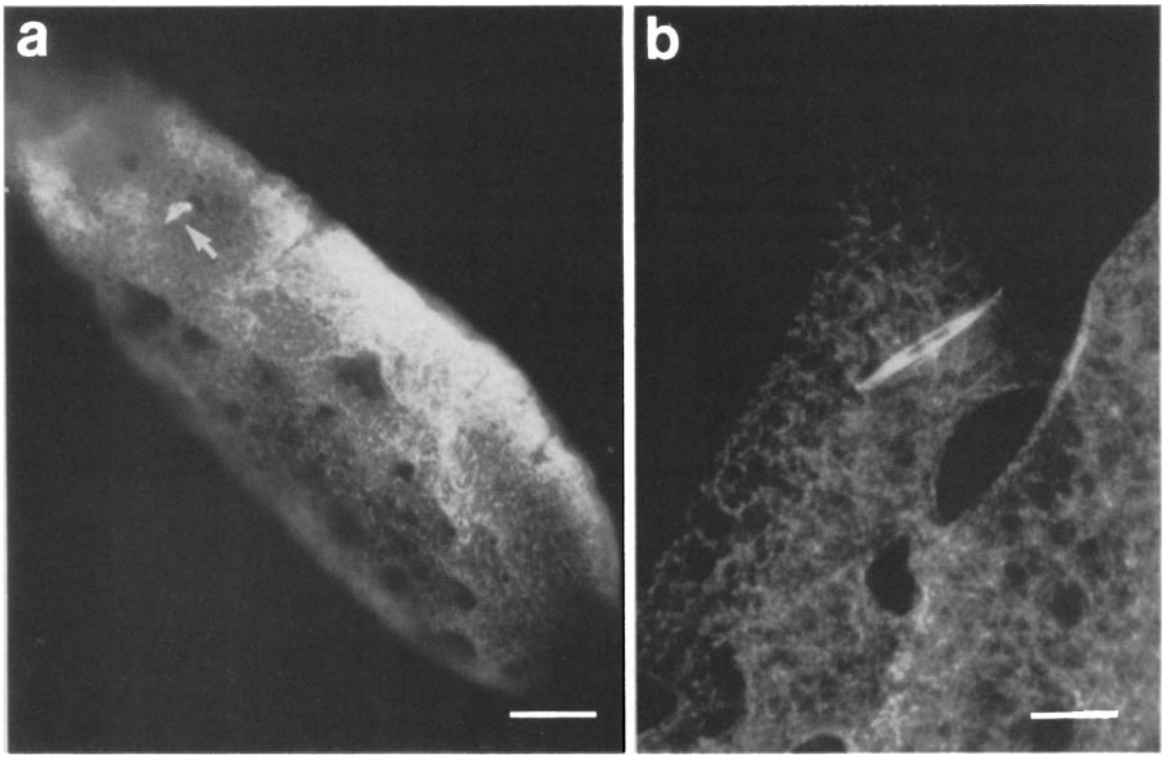

Figure 1. Microtubule organization in mature oocytes. (*a*) Conventional epifluorescence micrograph showing microtubule distribution in a stage 14 oocyte. Microtubules are present throughout the oocyte and surround the spindle, which is located near the anterior pole (*arrow*). (*b*) Linear projection of serial optical sections obtained using a laser scanning confocal microscope showing details of microtubule organization in the meiotic spindle. The spindle tapers dramatically toward the poles and is surrounded by a mesh of cytoplasmic microtubules. This projection was constructed from five optical sections taken at 1-μm intervals. Microtubules were labeled with a rhodamine conjugated anti-α-tubulin antibody. Bars: (*a*) 50 μm; (*b*) 10 μm.

the metaphase plate (Fig. 2 *a*). This profile is very similar to microtubule number profiles determined by serial section electron microscopic analysis of mitotic spindle numbers (McIntosh and Landis, 1971) and is consistent with nucleation of the majority of spindle microtubule at or very near the poles. In the meiotic spindles which form in the oocyte, however, only 10 to 20% of the peak anti-tubulin fluorescence is detected within 1 μm of a pole (Fig. 2 *b*, *arrows*). Fluorescence intensity gradually increases away from the poles, and is highest adjacent to chromatin at the metaphase plate (Fig. 2 *b*). Based on these observations, we conclude that the vast majority of spindle microtubules in mature oocytes do not terminate at the poles.

Chromosome Organization in Mature Oocytes

Previous cytological studies of meiotic chromosome organization in *Drosophila* females suggest that the small fourth chromosomes, which never undergo exchange, are positioned between the metaphase plate and spindle poles in mature oocytes (Puro and Nokkala, 1977; Puro, 1991; Kimble and Church, 1983). As discussed above, interpretation of these data is complicated by the use of hypotonic shock before fixation (Puro and Nokkala, 1977; Puro, 1991), or the use of hypotonic fixation buffers (Kimble and Church, 1983). Hypotonic treatment activates mature oocytes, inducing the onset of anaphase (Mahowald et al., 1983). We have fixed *Drosophila* oocytes under conditions designed to minimize the possibility of activation (see Materials and Methods). In contrast to previous reports (Nokkala and Puro, 1977; Puro, 1991; Kimble and Church, 1983), we find that individual exchange chromosomes cannot be resolved in the mature oocyte (Fig. 3), and that separation of exchange bivalents occurs only after hypotonic shock (Theurkauf, W., unpublished data). We therefore conclude that the conditions used in earlier studies activate mature oocytes, and that the chromosome configurations reported in these studies represent anaphase figures.

The position of nonexchange chromosome in unactivated oocytes was therefore of particular interest. We have found that nonexchange chromosomes are positioned between the metaphase plate and the spindle pole in a bilaterally symmetric manner (Fig. 3), confirming the conclusion that these chromosomes are prepositioned toward the spindle poles (Puro and Nokkala, 1977; Puro, 1991; Kimble and Church, 1983). The two fourth chromosomes, identifiable by their small size, are positioned between the metaphase plate and the poles, while the remaining chromosomes are tightly massed at the metaphase plate (Fig. 3 *a*). In rare cases the X chromosomes, which fail to crossover in approximately 5% of normal meioses, are also observed separated from the main chromosomal mass and are positioned between the fourth chromosomes and the metaphase plate. We have also examined oocytes in which X chromosomal exchange is strongly suppressed as a consequence of herterozygosity for a multiply inverted (balancer) X chromosome known as *FM7a*. In *FM7a*/+ females, the two X chromosomes are consistently found between the metaphase plate and fourth chro-

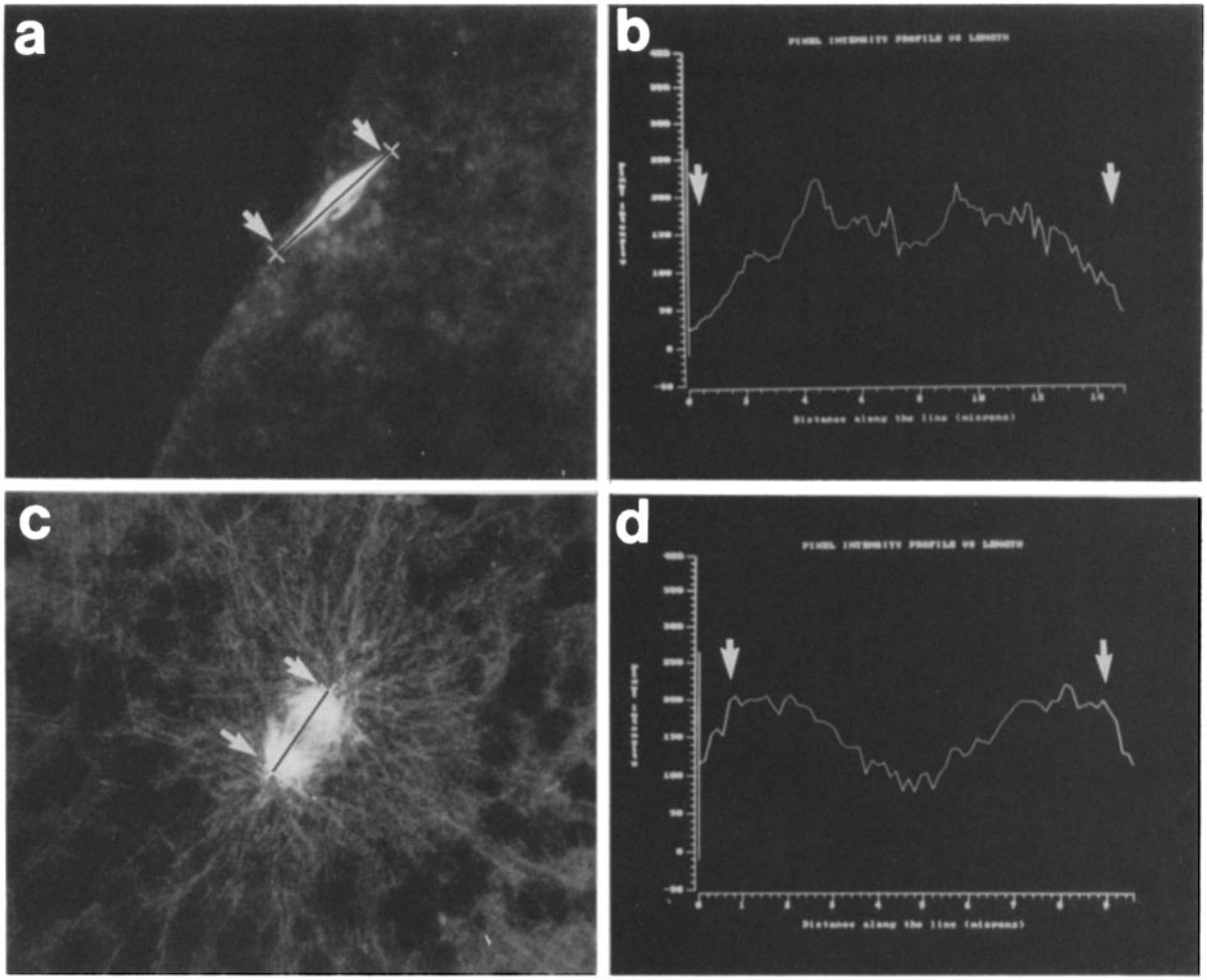

Figure 2. Microtubule number profiles of meiotic and mitotic spindles estimated from anti-α-tubulin immunofluorescence labeling intensity. (*a*) Meiotic spindle microtubules in a stage 14 oocyte arrested in metaphase of the first meiotic division. The image is a linear projection of optical sections obtained with a scanning confocal microscope (see Materials and Methods). (*b*) Pixel by pixel intensity of anti-tubulin staining along a line bisecting the spindle shown in (*a*). Anti-tubulin staining is most intense near the center of the spindle and decreases toward the poles. (*c*) Microtubule organization in the first mitotic spindle in an early embryo. This image was generated from optical sections as described for the meiotic spindle in *a*. (*d*) Anti-tubulin staining intensity along the axis of the spindle shown in *c*. Anti-tubulin staining is highest at the poles and decreases toward the metaphase plate. Lines bisecting the spindles indicate the regions sampled to produce the profiles in *b* and *d*. Bar, 10 μm.

mosomes (Fig. 3 *b*). Therefore, nonexchange homologs are not directly linked at metaphase.

Spindle Assembly

We have determined chromatin and microtubule organization as the meiotic spindle assembles, during stages 12 through 14. At stage 12 the meiotic chromosomes have condensed into a tight mass within the intact nuclear envelope (Fig. 4 *a*), and relatively long microtubules are present throughout the cytoplasm. There is no centrosome-like microtubule organizing center in the oocyte at this time, instead, the majority of microtubules appear to originate at the anterior cortex (Theurkauf, W., S. Smiley, M. L. Wong, and B. Alberts, manuscript submitted for publication). Early in stage 13, the nuclear envelope breaks down and microtubules appear to interact end-on with the mass of condensed chromosomes, associating with their entire surface (Fig. 4 *b*). There is no apparent bipolar organization to the microtubules associated with the chromosomes at this stage.

Late stage 13 and stage 14 oocytes cannot be distinguished after the chorion has been removed. These oocytes fall into two classes based on microtubule and chromatin organization, however. In one class, long tapered spindles are present, and nonexchange fourth chromosome are always present between the metaphase plate and the pole (Fig. 1 and Fig. 4 *d*). In the second class, very short bipolar microtubule arrays are present (Fig. 4 *c*), and small knobs are often found on the main mass of chromatin, which appear to be the fourth chromosomes (Fig. 4 *c'*). We believe that the simplest interpretation of these data is that the short spindles are assembly intermediates in which the nonexchange chromosomes are beginning to separate, and that the longer spindles with well-separated nonexchange chromosomes represent the metaphase arrest configuration. This model explains the position of nonexchange chromosomes in activated oocytes, in which the nonexchange chromosomes are positioned toward the spindle poles (Puro and Nokkala, 1977; Kimble and Church, 1983; Puro, 1991).

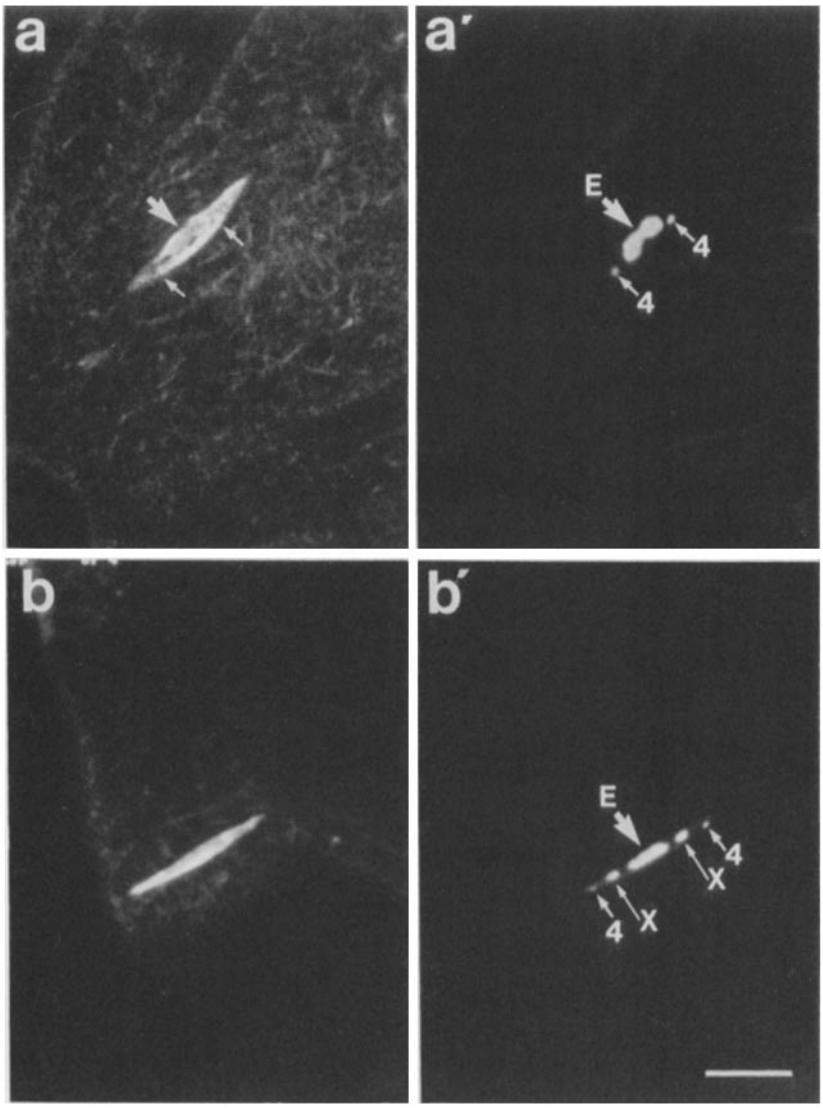

Figure 3. Organization of microtubule and chromatin in meiotic spindles at metaphase. Oocytes were double immunolabeled with anti-tubulin antibody (plain lower case letters) and anti-histone antibody (*a'* and *b'*). (*a* and *a'*) Wild-type oocytes. The small fourth chromosomes (*4*), which never exchange, are positioned between the main mass of exchange chromosomes (*E*) at the metaphase plate and the spindle poles. (*b* and *b'*) (*FM7a*/+) oocytes. The X chromosomes (*X*) rarely exchange in these oocytes, and are positioned between the fourth chromosomes and the exchange chromosomes. Oocytes were double labeled and projections were constructed from confocal micrographs as described in the legend to Fig. 2. Panels are designated as described in Fig. 2. Bar, 10 μm.

The timing of nonexchange chromosome separation from the exchange chromosome mass appears to be determined by size. When relatively short spindles are present, in oocytes with nonexchange X chromosomes, fourth chromosomes are often observed away from the metaphase plate while the X chromosomes are not visible. On more elaborate spindles displaying X chromosomes separated from the exchange chromosomes, however, the fourth chromosomes are always visible. These observations suggest that the small fourth chromosomes move away from the metaphase plate before the larger X chromosomes move.

Chromosome Behavior in nod/nod Oocytes

The *nod* locus encodes a kinesin-like protein required for nonexchange chromosome segregation (Carpenter, 1973; Zhang and Hawley, 1990; Zhang et al., 1990). To gain further insights into the mechanism of nonexchange chromosome segregation and the function of the putative microtubule motor encoded by the *nod* locus in this process, we have examined spindle organization in oocytes lacking functional *nod* protein. We have examined oocytes of the genotype $nod^{a}/FM7a$, nod^{b27} in detail. There are two distinct advantages to analyzing oocytes with this genotype: first, the X chromosomes rarely exchange in these oocytes, allowing analysis of the behavior of two sets of nonexchange chromosomes, which differ in size, in the absence of nod^{+} function. Second, the possibility that the observed defects are a result of other second site recessive mutations, borne by either stock, is reduced by using trans-heterozygotes.

Spindle microtubule organization in *nod* oocytes is generally similar to wild type, although asymmetric spindles are occasionally observed (Fig. 5 *a*), as well as normal spindles with spurs associated with one pole (Fig. 5 *d*).

The spindle orientation of nonexchange chromosomes in *nod* oocytes, in contrast to microtubule organization, is highly variable and always abnormal. In wild-type oocytes with nonexchange X chromosomes (*FM7a*/+), the fourth chromosomes are found nearer to the poles than the X chromosomes, which are positioned between the fourth chromosomes and the metaphase plate (Fig. 3 *b*). In *nod* oocytes, however, nonexchange chromosome positioning is variable. The fourth and X chromosomes are observed both free in the cytoplasm and on the spindle (Fig. 5, *c* and *e*), and X chromosomes can be found closer to the poles than fourth chro-

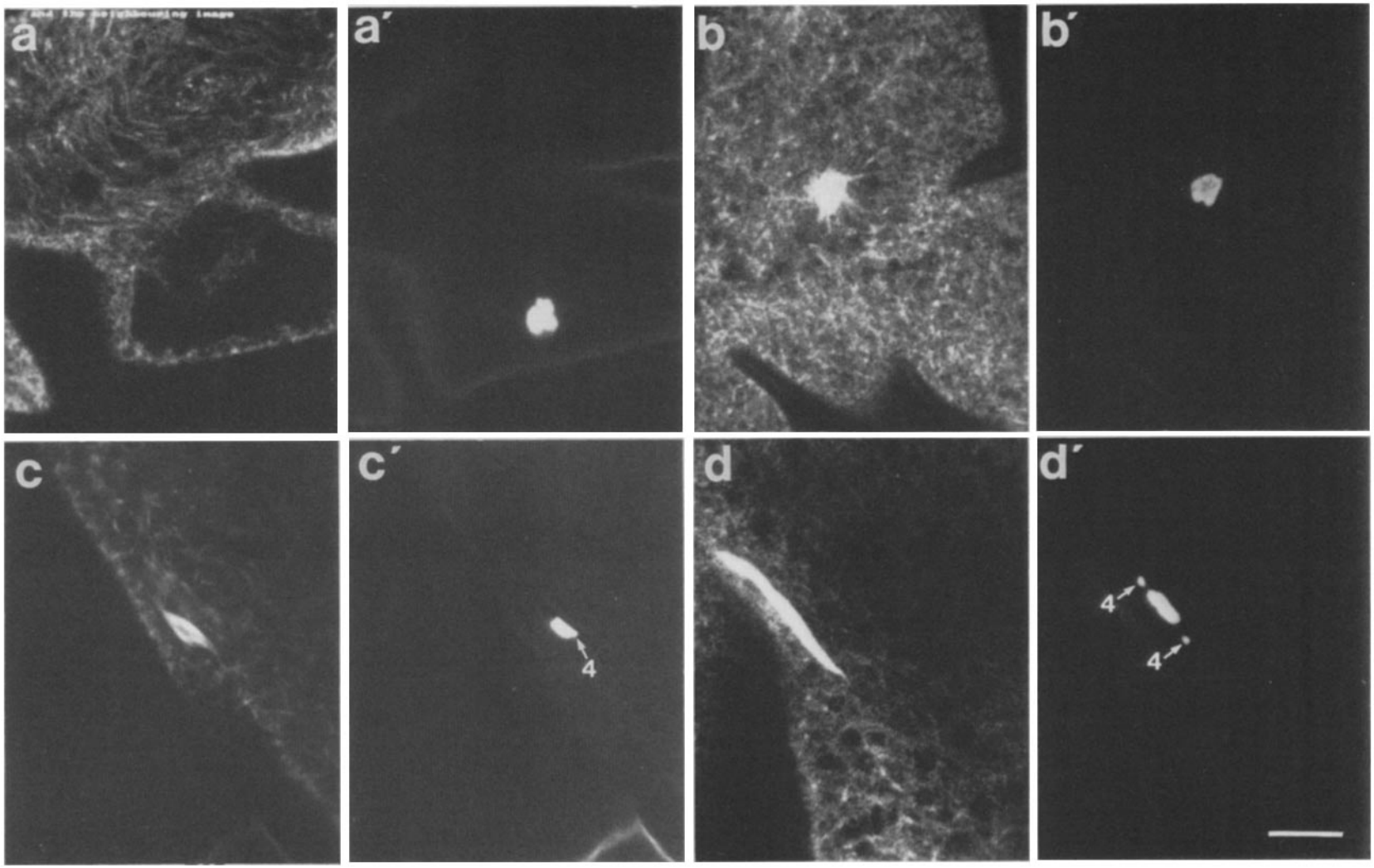

Figure 4. Microtubule and chromatin reorganization during meiotic spindle assembly. (*a* and *a′*) Stage 12 oocyte. The meiotic chromosomes form a tight mass within the nuclear envelope, which excludes cytoplasmic microtubules. (*b* and *b′*) Early stage 13. The nuclear envelope breaks down and microtubules associate with the chromatin mass. (*c* and *c′*) Late stage 13 or early stage 14. A short bipolar spindle is organized around the condensed chromosomes. (*d* and *d′*) Mature stage 14 oocyte. An elongated meiotic spindle is present and nonexchange fourth chromosomes are positioned between the metaphase plate and the spindle poles. Oocytes were double labeled and projections were constructed from confocal micrographs as described in the legend to Fig. 3. Panels are designated as described in Fig. 3. Bar, 10 μm.

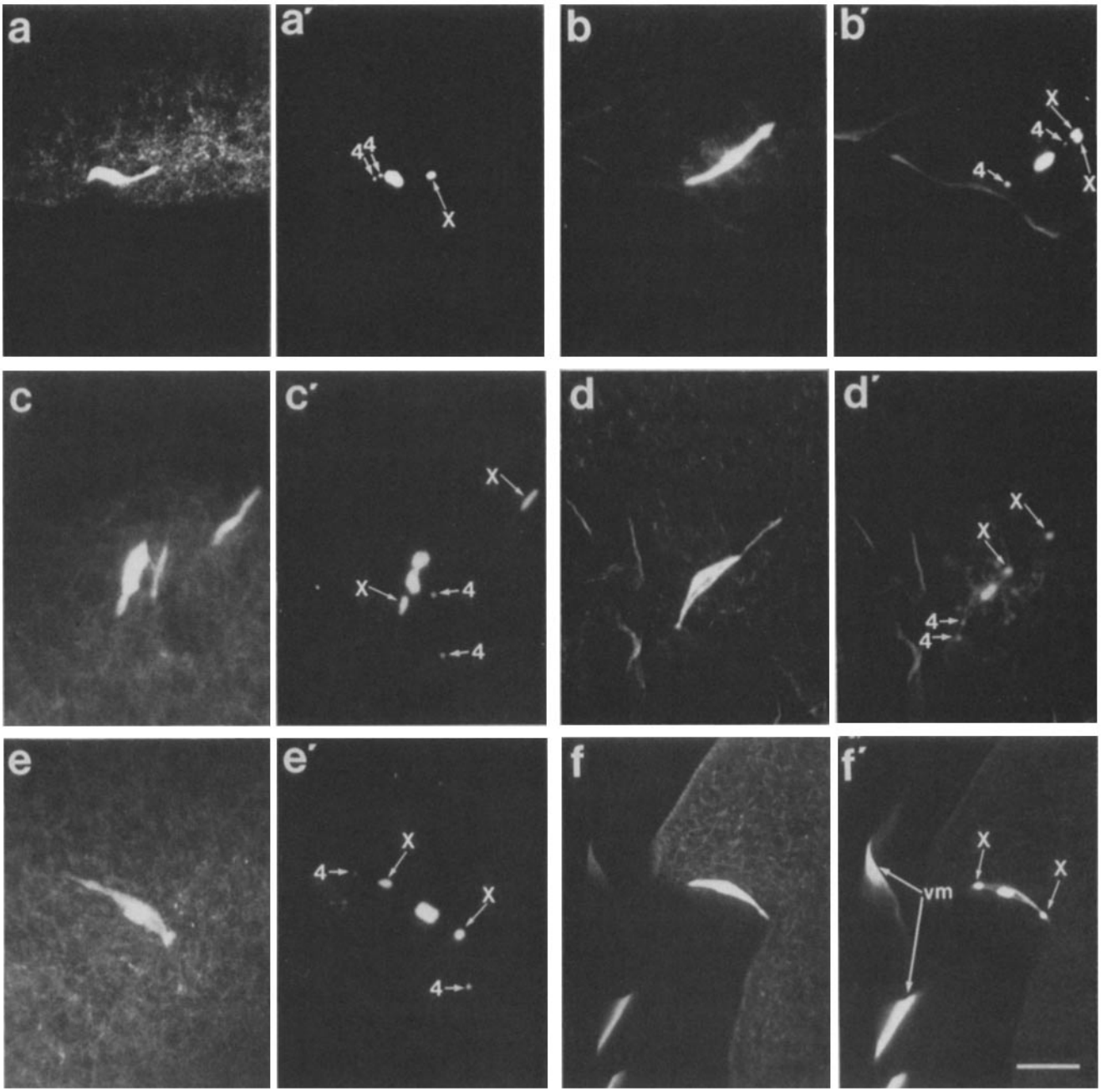

Figure 5. Gallery of metaphase spindles in *FM7a, nod/nod* oocytes. The position of fourth (4) and X (*X*) chromosomes varies from oocyte to oocyte. (*a* and *a'*) Both pairs of fourth chromosomes are oriented toward the same pole. One X chromosome is at the opposite pole and the second X chromosome is off the spindle and is not visible. (*b* and *b'*). Both X chromosome homologs are at the same pole, while the fourth chromosomes are oriented toward opposite poles. The fourth chromosome on the half spindle with the X chromosomes is positioned between the pole and the exchange chromosomes. (*c* and *c'*) Both fourth chromosomes and one X chromosome are off the spindle containing the exchange chromosomes. The second X chromosome is at one of the spindle poles. Microtubule bundles are associated with the free chromosomes. (*d* and *d'*) Both fourth chromosomes are oriented toward one pole, with one at the pole and the second displaced somewhat toward the metaphase plate. One X chromosome is positioned at the opposite pole, while the second is at the tip of a microtubule spur associated with that pole. (*e* and *e'*) One fourth chromosome is off the spindle and the second fourth chromosome is at a spindle pole. The X chromosomes are associated opposite poles, however, the X chromosome on the same half spindle as the fourth chromosome is positioned between the exchange chromosomes and the pole, while the X chromosome that is alone on the opposite half spindle is at the pole. (*f* and *f'*). Both fourth chromosomes are off the spindle and are not visible in the micrograph. The X chromosomes are present at opposite poles of the spindle. Oocytes were double labeled and projections were constructed from confocal micrographs as described in the legend to Fig. 3. Panels are designated as described in Fig. 3. Bar, 10 μm.

Table I. Nonexchange Chromosome Organization in FM7a, nod/nod Oocytes

Cytology	Number observed	Nonexchange chromosome associated with the spindle	
		4	X
	1	2	2
	1	2	1
	1	1	2
	2	2	2
	1	1	*2*
	2	*2*	*2*
	1	1	1
	4	0	1
	1	1	2
	2	1	2
	1	2	2
	1	2	1
	1	2	*2*
	1	0	2
	1	*2*	1

● Exchange chromosomes

| Nonexchange X-chromosomes

• Fourth chromosomes

Schematic diagrams indicate positions of nonexchange X and fourth chromosomes relative to the spindle. Only oocytes in which the spindle and all nonexchange chromosomes were visible were scored. Numbers in italics indicate that both homologs are on the same half spindle.

mosomes (Fig. 5 *b*). In addition, both homologs are often present on the same half spindle (Fig. 5, *a*, *b*, and *d*). Loss of nod^+ function therefore leads both to dissociation of nonexchange chromosomes from the spindle and improper orientation of those nonexchange chromosomes that are on the spindle.

We have also examined homozygotes for both nod^a and nod^{b27}. The defects observed in these oocytes are similar to those observed in the nod^a/nod^{b27} heterozygote. In these two homozygotes, however, free X chromosomes are observed at much lower frequency. This result is expected because nod^a and nod^{b27} homozygotes carry isosequential X chromosomes that recombine at normal frequencies and are expected to remain with the main mass of exchange chromosomes that are at the metaphase plate.

Several features of microtubule and chromosome organization in *nod/nod* oocytes bear comment. First, nonexchange chromosomes that are off the spindle containing the exchange chromosomes are often associated with spindle-like microtubules bundles (Fig. 5 *c*), suggesting that chromatin itself can organize spindle microtubules. Similar microtubule arrays are triggered by chromosomes mechanically removed from spindles in *Drosophila* spermatocytes (Church et al., 1986). Second, when a single nonexchange chromosome is present on a half spindle, it is always positioned at the pole (Fig. 5, *b*, *e*, and *f*), not at the wild type position between the plate and the pole (Fig. 4 *d*). *Nod* activity, therefore, prevents excessive poleward migration of nonexchange chromosomes. Third, when more than one nonexchange chromosome is present on a half spindle, one of these chromosomes is always at the pole (Fig. 5). The second nonexchange chromosome, however, is often positioned away from the pole, toward the metaphase plate (Fig. 5, *b*, *d*, and *e*). The presence of one nonexchange chromosome on a half spindle may therefore influence the behavior of additional nonexchange chromosomes on the same half spindle.

Does the extraordinary variability in spindle organization, observed in fixed *nod* oocytes, reflect in vivo variability? To address this issue we have used the cytological variability observed in *nod* oocytes to predict chromosome segregation, and compared these predictions to the behavior of nonexchange chromosomes in vivo as assayed genetically. The organization of chromosomes in 21 *nod/nod* oocytes, in which nonexchange chromosomes positions could be accurately determined, is summarized in Table I. As shown in Table II, this limited set of cytological data predicts rates of fourth and X chromosomal loss and nondisjunction that are in excellent agreement with estimates obtained by genetic means (Zhang and Hawley, 1990). These data indicate that the cytological preparations accurately reflect in vivo spindle organization.

How does inappropriate orientation of nonexchange chromosomes develop in *nod* oocytes, with both homologs frequently associated with the same half spindle? To address this question we have examined spindle assembly in *nod/nod* oocytes. The initial stages of spindle assembly appear normal: chromosomes condense into a tight mass within the nuclear envelope (Fig. 6 *a*), the nuclear envelope breaks down, and microtubules associate with the chromosomal mass (Fig. 6 *b*). In oocytes in which short bipolar spindles are present, however, the fourth chromosomes are essentially always (24 of 25 examples scored) found free in the cytoplasm (Fig. 6 *c*). X chromosomes are found free in the cytoplasm in *nod/nod* oocytes, although they appear to separate after the fourth chromosomes have done so, and at least one X chromosome is often found on short spindles. Commonly, one X chromosome is found at a spindle pole while the second X chromosome and both fourth chromosomes are free in the cytoplasm (Fig. 6 *d*). Because nonexchange fourth chromosomes are virtually always off the spindle early in assembly, while at least one fourth chromosome is associated with ~80% of spindles at metaphase (16 of 21 examples, Ta-

Table II. Cytological and Genetic Assays of X and fourth Chromosomal Nondisjunction in FM7a, nod/nod Females

Assay	X nondisjunction	Percent nullos	4 nondisjunction	Percent nullos
Cytology	45	74	84	81
Genetics	53	60	82	97

The cytological data are based on the 21 meiotic figures in Table II, while the genetic data are obtained from a classical mating experiment reported in Zhang and Hawley (1990). Cytological estimates of nondisjunction are based on the number of cases in which both homologs are attached to a spindle pole and those which lack either an X or fourth chromosome or both. After meiosis II those poles with two homologs attached will presumably produce diplo-X and diplo-4 gametes, while those poles which lack either an X or fourth chromosome will produce nullo-X or nullo-4 gametes. Genetic estimates of nondisjunction are based on direct measurement of the number of progeny derived from diplo- or nullo-bearing ova.

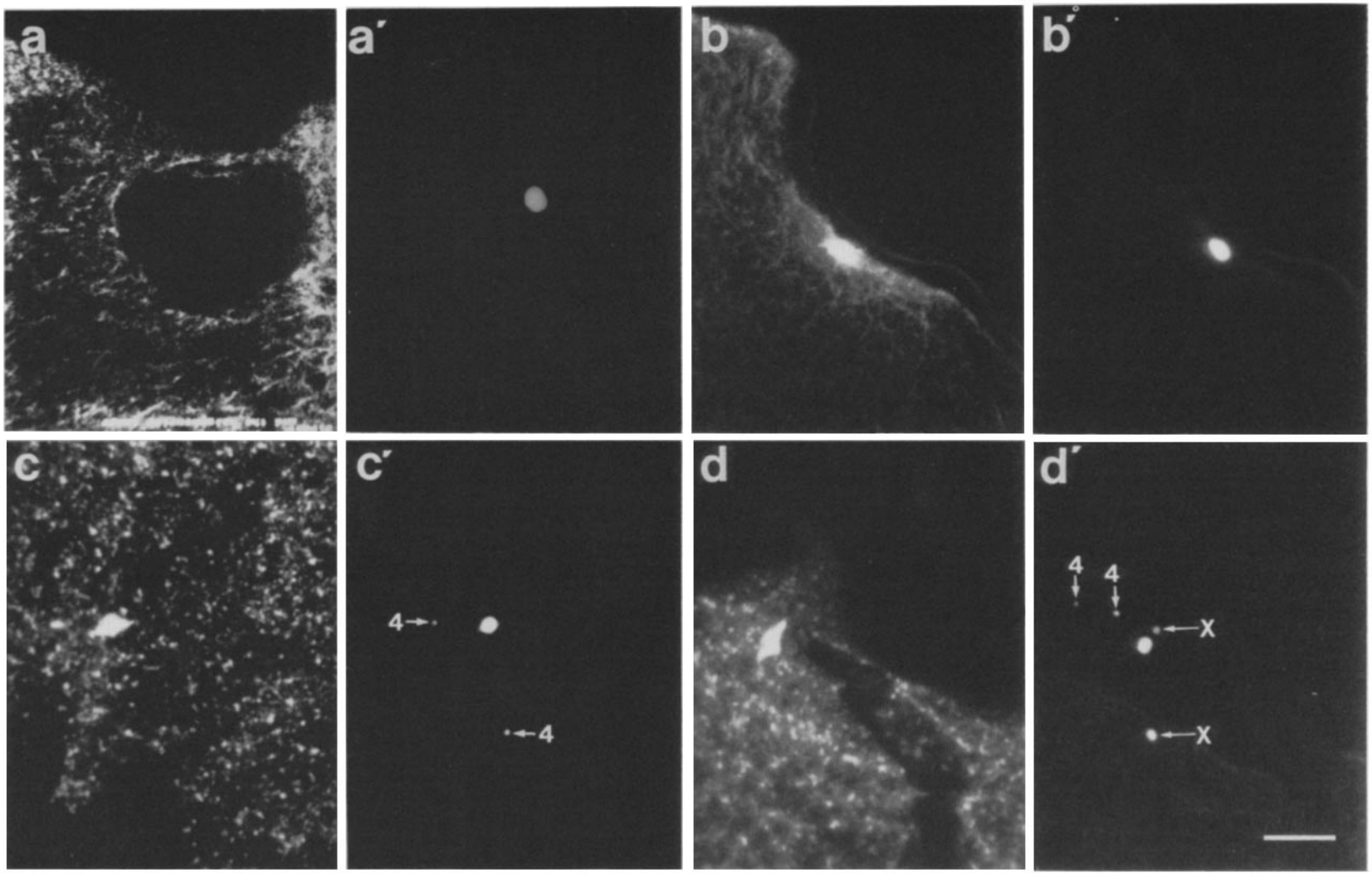

Figure 6. Spindle assembly in *FM7a, nod/nod* oocytes. Chromosome condensation (*a* and *a'*) and initial microtubule capture (*b* and *b'*) are essentially identical to wild type (see Fig. 4). Microtubules reorganize into a short bipolar spindle normally (*c*). The fourth chromosomes, however, are separated from the main mass of chromatin (*c* and *c'*). When a slightly longer spindle is present the X chromosomes are visible. The example shown in *d* and *d'* is typical, with one X chromosome at one of the poles and the second free in the cytoplasm. Both fourth chromosomes are essentially always off the spindle at this stage. Oocytes were double labeled and projections were constructed from confocal micrographs as described in the legend to Fig. 3. Panels are designated as described in Fig. 3. Bar, 10 μm.

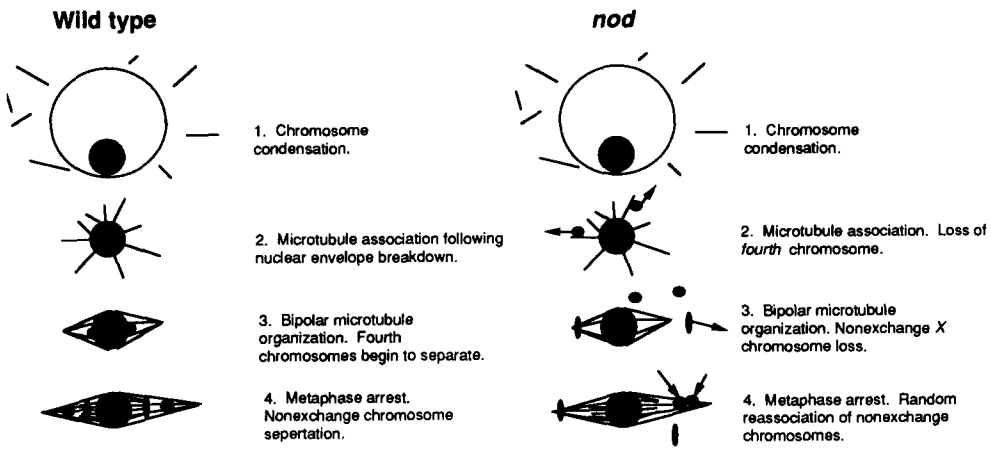

Figure 7. Summary of spindle assembly in wild-type and *nod/nod* oocytes. Wild type spindle assembly is divided into four phases: (*1*) Chromosome condensation. (*2*) Nuclear envelope breakdown and microtubule association. (*3*) Bipolar microtubule organization. (*4*) Metaphase arrest/nonexchange chromosome separation. In *nod/nod* oocytes the fourth chromosomes are essentially always lost and the X chromosome are frequently lost during spindle assembly. At metaphase some of these chromosomes reassociate randomly with the spindle.

ble I), we conclude that nonexchange chromosomes lost early in spindle assembly often reassociate with the spindle at metaphase. Abnormal nonexchange chromosome orientation in *nod/nod* oocytes, therefore, appears to result from inappropriate reassociation of nonexchange chromosomes with the spindle.

Discussion

Based on cytological analysis of chromatin and microtubule reorganization during oocyte maturation, we have divided meiotic spindle assembly in *Drosophila* oocytes into four phases (Fig. 7). The first phase, which begins during stage 11 and lasts through nuclear envelope breakdown at stage 13 (Mahowald and Kambysellis, 1981), consists of formation of a tight mass of chromatin in which individual chromosomes cannot be distinguished. The second phase, microtubule association, is characterized by microtubule capture or nucleation by the chromatin mass. During this phase there is no obvious bipolar microtubule organization or gross reorganization of the chromatin. During the third phase, bipolar microtubule organization, a short (<10 μm) bipolar microtubule array assembles around the chromatin. The fourth chromosomes can often be observed as buds on the main mass of exchange chromosomes during this stage. The fourth phase is metaphase arrest. This stage is characterized by a relatively long (20–25 μm) bipolar spindle with nonexchange chromosomes positioned between the metaphase plate and the poles. The temporal sequence of the first two phases is clear from oocyte morphology. During stages three and four the gross morphology of the oocyte is the same, however, making staging somewhat problematic. Analysis of chromosome organization in phase four oocytes homozygous for *nod* mutations (Fig. 5) accurately predicts the frequency of nonexchange chromosome loss and nondisjunction at anaphase (Table II). In addition, the positions of nonexchange chromosomes in wild type phase four oocytes predicts the positions of these chromosomes at anaphase, as observed in hypotonically activated oocytes (Puro and Nikkola, 1977; Puro, 1991). These observations strongly support the conclusion that phase four indeed represents the arrest configuration.

Similar spindle assembly pathways appear to function in ascidian eggs (Sawada and Schatten, 1988), and in some spore-forming higher plants (Osterhout, as described by Wilson, 1925). In these systems dispersed meiotic chromosomes appear to capture or nucleate random arrays of microtubules, which then reorganize into bipolar spindles. In *Drosophila* females, however, there is no diakinesis. As a result, the chromosomes are in a tight mass as spindle assembly begins, and only the nonexchange chromosomes leave this mass as the spindle assembly proceeds. Therefore, chromosomes do not congress to the metaphase plate. Rather, the chromosomes appear to define the position of the metaphase plate, and the spindle forms around this position. These observations suggest that chromosomes play a special role during spindle assembly in *Drosophila* oocytes.

Several additional observations suggest that chromatin is important in spindle microtubule organization in *Drosophila* oocytes: first, anti-tubulin fluorescence intensity in mature spindles is highest adjacent to the chromatin at the metaphase plate and dramatically decreases toward the poles (Fig. 2). Second, spindle formation appears to involve capture or nucleation of microtubules by the entire chromosomal mass, followed by reorganization of these microtubules into a bipolar spindle (Fig. 4). Third, free chromosomes in *nod/nod* oocytes organize spindle-like microtubule arrays (Fig. 5, *c* and *c'*). Based on these observations we suggest that chromatin is the primary microtubule organizing material in *Drosophila* oocytes. Further supporting this hypothesis, several centrosome-specific antigens, including γ-tubulin (Oakley et al., 1990), DMAP 60, and DMAP 190 (Kellogg et al., 1989) fail to localize to spindle poles in mature oocytes (Theurkauf, W., unpublished results). All of these antigens are found at spindle poles early in oogenesis and in early embryos, however. These observations suggest that centrosomes are not present at the spindle poles, consistent with the conclusion that most of the spindle microtubules in this system do not terminate at the poles.

Chromosomal Loss and Nondisjunction in nod/nod Oocytes

Loss-of-function mutations at the *nod* locus exhibit a female-specific meiotic defect in which nonexchange (achiasmate) chromosomes undergo loss at high frequencies and nondisjunction at lower frequency (Carpenter, 1973; Zhang and Hawley, 1990). In addition, the severity of *nod* induced chromosome loss and nondisjunction increases as chromosome

size diminishes (Carpenter, 1973; Zhang and Hawley, 1990). In contrast to their effects on nonexchange chromosome behavior, *nod* mutations have little or no effect on either the recombination process itself or on the segregation of exchange bivalents.

The cytological studies allow us to present a simple model that explains this rather complex phenotype. The fourth chromosomes are off the spindle in 96% of early spindles (24 of 25 examples), while at least one fourth chromosome is associated with ~80% of mature spindles (from data in Table I). Nonexchange chromosomes lost during spindle assembly therefore reassociate with the spindle after it has formed. We propose that meiotic chromosome loss results when one or both homologs fail to reassociate with the mature spindle, and that nondisjunction occurs when two homologs become associated with the same pole as a result of dissociation from the spindle followed by random re-association. Nondisjunction in *nod/nod* oocytes, therefore, is not the result of a failure in homolog separation at anaphase. Supporting this model, estimates of loss and nondisjunction frequencies in *FM7a*, nod^{b27}/nod^{a} females made from the cytological data are in excellent agreement with the frequencies observed in standard genetic crosses (see Table II).

An explanation for the effect of chromosome size on the severity of the *nod* phenotype is also suggested by the cytological observations. Loss of *nod* function causes dissociation of nonexchange chromosomes from the main chromosomal mass during spindle assembly, with the small fourth chromosomes virtually always separated from the main mass of chromatin by the time microtubules are organized into bipolar arrays. The larger nonexchange X chromosomes, in contrast, separate from the exchange chromosomes after the spindle has more fully formed, and are often retained at the developing spindle poles (Fig. 6, *d* and *d'*). We suggest that this reflects the presence of a structure which prevents chromosome dissociation from the spindle poles, and that this structure forms as the spindle matures. The diminished response of larger chromosomes to the *nod* phenotype would therefore reflect the increased probability that later separating chromosomes will be retained by this polar structure.

The Cellular Basis of Distributive Segregation

How are nonexchange chromosomes oriented on the meiotic spindle? A previous genetic model (Grell, 1976) postulated that distributive segregation involved a second round of pairing and segregation which followed the segregation of exchange chromosomes. Our data and previous cytological studies (Puro and Nokkala, 1977; Kimble and Church, 1983; Puro, 1991) are not consistent with a second round of pairing, as nonexchange chromosomes are positioned toward opposite poles before the spindle has fully formed. More recently, Carpenter (1991) has proposed that nonexchange homologs are linked by a microtubule bridge which substitutes for chiasma. Nonexchange chromosomes in this model are aligned by the same opposing poleward forces that align exchange chromosomes in other systems (Nicklas, 1974). Models based on indirectly linked homologs, however, predict that univalent chromosomes will be lost at high frequency. Genetic analyses of univalent chromosome behavior during meiosis, however, indicates that this is not the case. The single Y chromosome in XXY females carrying exchange X chromosomes, and single copies of grossly deleted copies of the X chromosome, segregate randomly at meiosis I, but are not lost (Cooper, 1948; Linsley and Sandler, 1958). Univalent chromosomes could be stablized on the spindle through a microtubule link to exchange bivalents at the metaphase plate. Partner choice (exchange bivalent vs nonexchange homolog) and size-dependent positioning of nonexchange chromosomes (discussed below) are difficult to explain with this model.

How might nonexchange chromosomes be aligned on the spindle, if not through the action of opposing poleward forces? Puro (1991) has suggested that disjunction in oocytes is the result of the predetermined, non-random arrangement of chromosomes, not mechanical alignment of chromosomes on the meiotic spindle. Our data indicate that alignment of these chromosomes takes place before completion of spindle assembly, consistent with this hypothesis. We therefore favor models in which nonexchange chromosomes are aligned during meiotic prophase, either on the basis of heterochromatic homologies (Hawley, 1989, 1991; Puro, 1991), or nuclear position (Novitski, 1978), and those alignments or pairings coorient chromosomes on developing spindles.

We therefore propose that the function of *nod* is to maintain nonexchange chromosome orientation, established during meiotic prophase, as the spindle assembles. Mechanistically, we propose that *nod* achieves this by delaying dissociation of oriented nonexchange chromosomes from the exchange chromosomal mass until a bipolar spindle has formed, at which time association with the spindle itself maintains nonexchange chromosome orientation. Based on the structural similarity of the *nod* protein and kinesin (Zhang et al., 1990), and the cytological data presented here, we propose that *nod* delays nonexchange chromosome separation by generating a microtubule-dependent force which acts on the chromosomes, and is directed toward the main chromosomal mass. This force counteracts an opposing microtubule-dependent force which develops as the spindle forms. It is this opposing force which leads to ejection of nonexchange chromosomes in the absence of *nod* activity.

Our data show that, in wild type oocytes, the fourth chromosomes are always found closer to the poles than the larger nonexchange X chromosomes. In addition, a grossly deleted copy of the X chromosome, which is smaller than the fourth chromosome, is observed between the fourth chromosome and the spindle pole (W. Sullivan and G. Karpen, personal communication). Nonexchange chromosomes therefore appear to be positioned on the spindle by size. A complete description of nonexchange chromosome behavior must explain size-dependent positioning of these chromosomes on the meiotic spindle.

We have constructed a model for distributive segregation which accounts for size-dependent positioning of nonexchange chromosomes, and should serve as a basis for further experimentation. This model, outlined in Fig. 8, is based on the following assumptions: (*a*) Chromosomes are oriented prior to spindle assembly; (*b*) the polar organization of chromosomes determines spindle orientation; (*c*) opposing poleward and anti-poleward forces, which act within the half spindle, balance the nonexchange chromosomes on the spindle.

Size-dependent positioning of nonexchange chromosomes on the spindle suggests that either the poleward or anti-

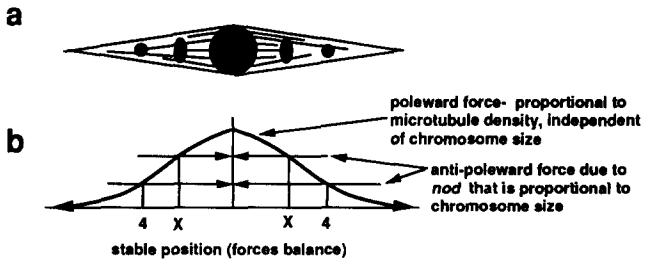

Figure 8. Model for nonexchange chromosome positioning on metaphase spindles. (*a*) Spindle organization, showing stable positions of nonexchange X and fourth chromosomes. (*b*) Graphical representation of the forces proposed to act on nonexchange chromosomes. Curves sloping away from the midline (position of the metaphase plate) indicate the magnitude of the microtubule number-dependent poleward force, as a function chromosome position on the spindle. The magnitude of the chromosome-size dependent forces, proposed to be generated by the *nod* protein, are indicated by the arrows. The positions at which these forces balance are indicated (*X* and *4*).

poleward force is proportional to chromosome size. Work in other systems indicates that an anti-poleward force, referred to as the polar ejection force (Rieder et al., 1986; Salmon, 1989), acts along the chromosome arms (for a review of the role of bulk chomatin in chromosome transport see Fuge, 1990). We therefore propose that the hypothesized anti-poleward force acts along the length of the chromosome arms, and that this force is proportional to chromosome size. For the purpose of modeling chromosome behavior, we propose that the poleward force in the oocyte is chromosome size independent, and likely acts at the kinetochore. The combination of a chromosome size proportional anti-poleward force and a size-independent poleward force would produce net poleward forces that are inversely proportional to size.

To account for stable positioning of nonexchange chromosomes on the half spindle, however, the poleward and anti-poleward forces must balance. To achieve balanced forces, we propose that the poleward force is proportional to spindle microtubule number, and therefore decreases toward the poles, while the anti-poleward force is independent of microtubule numbers. The anti-poleward force, which is proposed to act along chromosome arms, would be relatively independent of microtubule number if a chromosome arm could either interact with multiple microtubules, or form many contacts with one or a few microtubules. These modifications lead to a model that predicts the observed size-dependent positioning of nonexchange chromosomes on the meiotic spindle (Fig. 8). Nonexchange chromosomes will move away from the metaphase plate until the poleward force decreases to a level that matches the anti-poleward force. Because the anti-poleward force is proportional to chromosome size, the small fourth chromosomes migrate further toward the pole before the forces balance than the larger X chromosomes. Mitchison (1989*b*) has proposed that opposing poleward and anti-poleward forces, acting at the kinetochore, are involved in positioning mitotic chromosomes on spindles. Our model for meiotic chromosome behavior in *Drosophila* females differs in the site of action of the anti-poleward force, and in the mechanism which generates balanced forces.

The cellular phenotype of *nod* mutations, as discussed above, indicate that the *nod* protein provides the hypothesized anti-poleward force. The model therefore explains the excessive poleward migration of single nonexchange chromosomes in *nod/nod* oocytes. The behavior of multiple nonexchange chromosomes on the same half spindle in *nod/nod* oocytes, however, cannot be completely explained by this model. At least one nonexchange chromosome is always found at the pole in *nod/nod* oocytes, as predicted. Additional chromosomes, however, are often positioned closer to the metaphase plate (Fig. 5, *b* and *b'* and *e* and *e'*, for example). This observation suggests that the presence of a nonexchange chromosome at the pole can impede the poleward migration of a second chromosome. Nonexchange chromosomes can closely associate with one another at the spindle pole (Fig. 5, *b*, and *b'*), indicating that nonexchange chromosomes do not have an intrinsic "zone of exclusion" that prevents the close approach of other chromosomes. It is possible that the spacing of nonexchange chromosomes in *nod/nod* oocytes reflects the existence of an *nod*-independent force, and such a force could play an important role in determining chromosome behavior. Alternatively, these figures may simply represent intermediates in which chromosomes are fixed as they progress toward the pole. This issue is difficult to resolve from analysis of fixed samples.

A number of questions must be answered before the mechanism of distributive segregation and the function of the *nod* protein in this process can be fully understood: where is the nod protein located? Is *nod* a microtubule motor and if so what is its directionality? What is the orientation of microtubules in the meiotic spindle? When are the meiotic chromosomes oriented? These questions are experimentally approachable and the answers should provide valuable insights into the forces acting on chromosomes in this system.

Will the lessons learned from analysis of distributive segregation apply to chromosome segregation in other systems? Although the *nod* protein is only required during meiosis, and therefore only for distributive disjunction, two lines of evidence suggest that this protein also plays a role in mitosis. First, as shown by Zhang et al. (1990), the *nod* transcript is present throughout development. Second, an antimorphic allele of *nod*, known as nod^{DTW}, affects both meiotic and mitotic behavior (Wright, 1974; Rasooly et al., 1991). The finding that *nod* protein is present but not required in mitotically dividing cells suggests, at least mitotically, that the nod protein is either functionally or genetically redundant.

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