Microtubule distribution during meiosis I in flea-beetle [*Alagoasa* (*Oedionychus*)] spermatocytes: evidence for direct connections between unpaired sex chromosomes

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Summary

The meiosis-I spindle in flea-beetle spermatocytes is unusual in that the autosomes and univalent sex chromosomes are separated by a mitochondrial sheath and move polewards at different times. To help understand the basis for this interesting chromosome behaviour, and to gather more detailed information about it, we studied microtubule distributions throughout meiosis I using immunofluorescence and confocal microscopy, and took careful measurements of pole and kinetochore positions at all stages of division. Our results show that, by late prophase, there is a spindle-shaped cytoplasmic array of microtubules in the central part of the cell, with the nucleus at the periphery. Following nuclear envelope breakdown, both autosomes and sex chromosomes become associated with cytoplasmic microtubules, although only the autosomes move centrally to the 'cytoplasmic spindle'. The two unpaired sex chromosomes remain at the cell periphery and appear to be connected to each other by a microtubule bundle extending between their kinetochores. These bundles often persist into anaphase. Analysis of measurements taken from fixed/stained cells supports previous observations that sex chromosomes move part way to the pole in early prometaphase and then stop. The measurements also suggest that during autosomal anaphase, spindle elongation precedes autosome movement to the poles and polewards movement of sex chromosomes is limited or absent when autosomes are moving polewards.

Key words: Microtubules, Meiosis, Chromosome orientation, Acetylated tubulin

Introduction

Models of cell division are generally based on studies of cultured vertebrate somatic cells, in which chromosomes behave uniformly and the direction of anaphase movement is determined by the orientation of sister kinetochores to opposite poles. However, many other interesting and unusual divisions exist, and several examples are found in male meiotic cells (White, 1973). Non-uniform chromosome behaviour occurs in grasshopper spermatocytes, where the single univalent (X) chromosome remains unpaired and moves to one of the two poles before the start of autosomal anaphase (Nicklas, 1961). In the spermatocytes of other species, single univalents might act differently, such as moving polewards after the autosomes reach the poles or dividing equationally in meiosis I (Stevens, 1909). Non-random chromosome segregation has also been described. In mole-cricket spermatocytes, for example, the larger partner of a heteromorphic bivalent invariably segregates to the same spindle pole as the univalent sex chromosome (e.g. Payne, 1916; Camenzind and Nicklas, 1968; Kubai and Wise, 1981). In *Sciara* spermatocytes (e.g. Gerbi, 1986; Goday and Esteban, 2001), mealy bug spermatocytes (e.g. Brown and Nur, 1964; Brown and Weigmann, 1969) and *Poeciliopsis* oocytes

(e.g. Schultz, 1966; Schultz, 1973; Cimino, 1972), the entire complement of male-derived chromosomes segregates to one pole during anaphase, while the female-derived chromosomes move to the other pole. These examples are just a few of many.

Mechanisms that give rise to non-uniform behaviours of the kinds described above are largely unknown. One possible exception is the coordinated movements of univalent sex chromosomes in crane-fly spermatocytes. In these cells, the two sex chromosomes remain univalent (unpaired) throughout meiosis I. They congress to the metaphase plate with the bivalent autosomes and then remain at the equator until the autosomes complete anaphase, at which time they begin their own anaphase motion to opposite poles (e.g. Forer, 1980). Experiments suggest that this independent behaviour involves local signalling between the two univalents. For example, in micromanipulation experiments, when one of the segregating univalents is pushed opposite to its direction of anaphase motion until it overtakes the other univalent, both univalents reverse their directions of motion (Forer and Koch, 1973), suggesting that, as the two move to opposite poles, there is continuous signalling between them. Signalling also seems to occur between univalents and autosomes or between their

spindle fibres (cf. Dietz, 1969; Sillers and Forer, 1981), and local signals seem to coordinate the movements of partner half-bivalents to opposite poles during anaphase (Yin and Forer, 1996).

The structural and chemical bases for these local 'coordination' mechanisms are unknown, but they serve to remind us that individual chromosomes respond to local as well as to global signals, and that understanding these unconventional divisions might provide clues about mechanisms underlying more conventional divisions. Unfortunately, they have been studied less than conventional tissue culture cells and, aside from *Sciara* spermatocytes (Gerbi, 1986), no one has studied them genetically. In this article, we describe observations of one of these unique divisions: meiosis-I in spermatocytes of flea beetles.

Spermatocyte divisions in flea-beetle species including *Alagoasa* (*Oedionychus*) *bicolor* and *Omophoita cyanipennis* have several unique features described in some detail by Virkki (Virkki, 1970; Virkki, 1971; Virkki, 1972; Virkki, 1973; Virkki, 1985; Virkki, 1990). He described meiotic cells with ten small autosomal bivalents and two large univalent sex chromosomes, X and Y. The most distinguishing feature of these cells was that early in prometaphase I the autosomes became separated from the unpaired sex chromosomes, with the sex chromosomes remaining at the cell periphery and the autosomes moving centrally. The two groups of chromosomes then seemed to form separate spindles and the autosomes proceeded through the normal stages of meiosis I. Before the autosomes entered anaphase, the two sex chromosomes oriented to opposite poles, moved polewards and then stopped part way to the pole and remain stopped until the autosomes entered anaphase.

Recent data (Forer and Wilson, 2000; Green-Marroquin et al., 2001) have shown that, in fact, the sex-chromosome spindle shares poles with the autosomal spindle. Nevertheless, the behaviour of the chromosomes in flea-beetle primary spermatocytes gives rise to several unanswered questions. For example, what are the early events that give rise to the central autosomal spindle? How are the unpaired sex chromosomes excluded from the central spindle? How do they orient to opposite poles rather than to the same pole? To what extent are the movements of the autosomes and sex chromosomes independent? And, finally, if the sex chromosomes indeed do not move polewards when the autosomes do, how do they avoid the metaphase/anaphase triggers that presumably govern the autosomes?

Virkki and others have attempted to answer some of these questions. Their work indicates that prior to prophase the nucleus is in the middle of the cell; by late prophase I the nucleus is to one side of the cell (Virkki, 1970; Virkki, 1971; Virkki, 1972). A clear zone that can be seen around the nucleus in prophase (Virkki, 1972) seems to disappear before breakdown of the nuclear membrane, and by late prophase there is evidence of asters and of mitochondrial alignment in the central part of the cell (Virkki, 1972), where the autosomal spindle will eventually be established. Virkki referred to this early cytoplasmic alignment as a 'cytoplasmic spindle', but he based his interpretation on the alignment of the mitochondria, without information regarding microtubules. In later publications (e.g. Virkki, 1985), he suggests that asters push the nucleus to the cell periphery.

Virkki (Virkki, 1971; Virkki, 1972) showed that the autosomes move centrally following nuclear membrane breakdown, establishing a central spindle, while the unpaired sex chromosomes remain behind. There is little information to explain why the sex chromosomes do not move with the autosomes, although Virkki argued that the autosomes are 'activated' for movement before the sex chromosomes (Virkki, 1971), or that sex chromosomes make connections with the poles later than the autosomes (Virkki, 1972). Although remaining at the cell periphery, the sex chromosomes do make connections to opposite poles and move towards opposite poles, but the details of this process are unknown. For example, it remains unclear how the unpaired sex chromosomes manage to orient to opposite poles; several studies reject the notion that there are connections or physical interactions between the sex chromosomes (Virkki, 1972; Virkki, 1985; Kupfer and Wise, 2000a; Green-Marroquin et al., 2001). With respect to sex-chromosome spindle fibres, Virkki (Virkki, 1971; Virkki, 1972; Virkki, 1985) described them as syntelic (i.e. each sex chromosome has fibres extending to only one spindle pole); more recently, Green-Marroquin et al. argued that the sex-chromosome spindle connections are initially amphitelic (i.e. each sex chromosome has fibres extending to both poles) and later become syntelic (Green-Marroquin et al., 2001). Unfortunately, none of the studies provide sufficient evidence to be convincing and none include a comprehensive study of microtubule organization within the two spindles.

Finally, there is the issue of the independent behaviour of autosomes and sex chromosomes. Virkki (Virkki, 1967; Virkki, 1970; Virkki, 1972) argued that as the autosomes proceed through prometaphase, metaphase and anaphase the univalent sex chromosomes orient and move part way to opposite poles to form a 'distance bivalent' by autosomal metaphase. [The only exception mentioned is a case of malorientation, in which both sex chromosomes temporarily faced the same pole and seemed to move in a coordinated fashion (Virkki, 1972).] Furthermore, although no direct measurements were available, it was Virkki's impression that the sex chromosomes moved polewards roughly around the time that the autosomes did in anaphase (Virkki, 1970). However, Forer and Wilson presented data on kinetochore fibre lengths that seemed to argue against the interpretation that sex chromosomes move polewards with the autosomes (Forer and Wilson, 2000). Kupfer and Wise also dispute this claim, arguing that there is little or no polewards movement of sex chromosomes during anaphase (Kupfer and Wise, 2000a). To date, no one has addressed the mechanism for coordinating sex chromosome movements to opposite poles, nor have there been measurements of autosomal and sex-chromosome movement during meiosis.

Related to the issue of anaphase chromosome motion is the relative contribution of spindle elongation. Virkki did not make sufficient measurements to address the relative contributions of movement to the pole ('anaphase A') and pole elongation ('anaphase B'), but Kupfer and Wise suggest from measurements on fixed cells that, whereas spindle length does increase between prometaphase and anaphase, spindle elongation in anaphase contributes little to autosome separation (Kupfer and Wise, 2000a).

In this article we address some of these unanswered issues and, in particular, present more detailed information about microtubule distribution and kinetochore-microtubule

interactions throughout the first division, as well as measurements of chromosome motion at different stages of division. Unfortunately the animals are difficult to rear in large numbers, so extended experimentation is difficult. Nevertheless, as a first step towards extending our understanding of this remarkable division, we have examined microtubule organization in these cells. We studied microtubules via indirect immunofluorescence and confocal microscopy, looking at both total tubulin and acetylated tubulin, the latter being an indicator of more 'stable' microtubules (Wilson et al., 1994; Wilson and Forer, 1997; Rosenbaum, 2000). We supplemented our observations with study of live cells when possible and made extensive measurements of chromosome and pole positions at all stages of division.

The results, reported below, confirm the presence of a cytoplasmic spindle prior to prometaphase, show that sex chromosomes have bidirectional kinetochore fibres that often persist into later stages of division, show that sex chromosomes are linked by microtubules and suggest that, during anaphase, spindle elongation precedes autosome-to-pole movement.

Materials and Methods

Stocks of *Alagoasa bicolor* were initially provided by Niilo Virkki, then maintained in cages in the greenhouse at York University, reared on *Aegiphila* and *Chlorodendron*, using methods essentially as described earlier (Virkki and Zambrana, 1983).

Spermatocytes in division were prepared for live observation or for immunofluorescence microscopy using methods described previously (Forer and Wilson, 2000). In brief, an animal was put under halocarbon oil and its single testis removed and placed under oil. For some preparations of living cells, portions of the testis were smeared under oil and cells observed by phase-contrast microscopy using an oil immersion objective (NA=1.3, 100×). For immunofluorescence preparations and other observations of living cells, portions of testes were placed in fibrinogen in modified Belar's Ringer's solution, spread, mixed with thrombin and then perfused with Belar's solution, using procedures described previously (Forer and Pickett-Heaps, 1998). Living cells affixed to the coverslip with the fibrin clot were studied live and then prepared for immunofluorescence, or they were directly processed for immunofluorescence studies. To process for immunofluorescence labelling, the coverslip was placed in lysis buffer and subsequently fixed and labelled with antibodies using procedures described elsewhere (Wilson et al., 1994). Cells generally were stained for both total tubulin (using YL1/2 antibody) and acetylated

tubulin (using 6-11B-1 antibody), though some preparations were only single stained (with YL1/2 antibody). Some preparations were treated additionally with acridine orange (0.001-0.010 mg ml^{-1} in phosphate-buffered saline), in order to label the chromosomes. In the case of triple-labelled cells, the acetylated tubulin and chromosomes were visualized in the same channel. Confocal microscope observations were made with a BioRad 600 confocal system attached to a Nikon Optiphot microscope, using a 60× lens (NA=1.4), as described previously (Wilson et al., 1994). Distances were measured from confocal microscope images using either BioRad (Comos) software or custom software described previously (Wilson et al., 1994).

As described previously (Forer and Wilson, 2000), we used composite images of the entire cell to locate spindle poles. We marked the spindle-pole positions with a cursor, marked the corresponding sex-chromosome and autosome kinetochore positions with a cursor, and used either BioRad (Comos) software or custom software (Wilson et al., 1994) to measure distances between the poles and between the kinetochores and the poles. In reporting sex-chromosome distances, we report the value for each chromosome in that cell; in reporting autosomal distances, we measured distances for five or six autosome pairs in each cell and report the average value for each cell. Also as described previously (Forer and Wilson, 2000; Virkki, 1972), in some cells, one or a few bivalents disjoin prematurely; we classified these cells as 'metaphase' and considered as 'anaphase' only those cells in which all autosomal bivalents were completely (and clearly) disjoined.

Results

Living flea-beetle metaphase-I spermatocytes contain two spindles. Ten pairs of autosomes are arranged on a central spindle, and the large sex chromosomes are on a spindle at the periphery of the cell, separated from the autosomes by a mitochondrial sheath (Fig. 1). Cells to be stained with antibodies were first lysed in a microtubule-stabilizing buffer and then fixed. The relative positions and orientations of the chromosomes were unaltered by lysis (Fig. 1A-D); subsequent immunofluorescence images revealed an abundance of microtubules in cells at all stages, as described below. In other spermatocytes, the same stabilizing buffer preserves spindle structures, including irradiated areas of reduced birefringence (e.g. Wilson et al., 1994; Czaban and Forer, 1994), indicating that the basic spindle cytoskeletal architecture is preserved by these procedures.
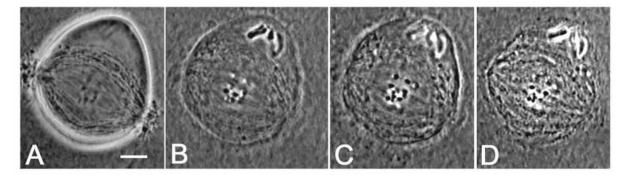
Because sex-chromosome behaviour in flea-beetle

Fig. 1. Lysis of a living cell in metaphase I. Cells to be processed for immunofluorescence staining were first lysed in a microtubule stabilizing buffer, then fixed in 0.2% glutaraldehyde in phosphate-buffered saline. General cell morphology was preserved by this procedure. (A) Cell 5 minutes prior to lysis. (B) 50 seconds after perfusion of lysis buffer: the lysis buffer is beginning to reach the cell. (C) 1 minute after perfusion: lysis starting. (D) 6 minutes after perfusion: lysis is completed. Bar, 10 μm.

spermatocytes is 'out of phase' with autosomal behaviour, we assessed the stage of lysed cells according to the positions of the autosomes. Thus, in describing 'metaphase', for example, we refer to the stage in which the autosomes are at the metaphase plate. Observations in this report cover the period from late prophase I to late anaphase I, and focus on the creation of the central autosomal spindle, the behaviour of the sex chromosomes and the relationship between autosomal movement and sex-chromosome movement.

Establishing the central autosomal spindle

The spindle is established prior to nuclear envelope breakdown (NEB)

Late prophase cells have nuclei that are at the cell periphery (Fig. 2A-C), as described previously (Virkki, 1971; Virkki, 1972). The peripheral nucleus creates a bulge or out-pocketing of the cell membrane (e.g. Fig. 2B), which usually persists throughout later stages of division (e.g. Fig. 1A).

Phase-contrast observations of living prophase cells confirm the existence of linear cytoplasmic elements in the central area of prophase cells, arranged in the shape of a mitotic spindle (Fig. 2A-C), presumably the structure that Virkki referred to as the 'cytoplasmic spindle' (Virkki, 1972). Anti-tubulin labelling of fixed cells reveal that these spindle-shaped structures contain microtubule arrays, with poles at either end (Fig. 2D,E). In most cases the microtubules appear to be unacetylated, and in some cells one pole is associated with (or abuts) the nucleus (Fig. 2C,E). Thus, our results confirm the existence of cytoplasmic microtubules arrayed in the shape of a spindle, organized before the chromosomes are released by the dissolution of the nuclear membrane.

That a central spindle is present prior to NEB means that the autosomes must migrate to the forming spindle following NEB, rather than the spindle organizing around the chromosomes, as is common in animal cells. Our immunofluorescence preparations contained many cells in which the sex chromosomes were at the cell periphery and autosomes were scattered between them and the central spindle area (e.g. Fig. 3A,B), presumably in transit to the central spindle. Prometaphase therefore includes the time during which autosomes move to the central spindle, enter the central spindle and congress to the metaphase plate. For purposes of discussion, we have subdivided prometaphase cells into three stages: (1) early prometaphase, the period when most autosomes are moving centrally, as deduced from fixed cells

whose autosomes are located between the cell periphery and central spindle; (2) mid-prometaphase, the period when most autosomes have reached the central spindle but have not yet congressed to the equator; and (3) late prometaphase, the period when most but not all of the autosomes have reached the metaphase plate. Cells in which all autosomes are at the equator, with well-developed connections to the poles, are considered metaphase.

Microtubule interactions with migrating autosomes during early prometaphase

Immediately following NEB the autosomes and sex chromosomes are intermingled. Throughout prometaphase microtubules are present between the poles, with extensive microtubule arrays throughout the cell, surrounding both the autosomes and the sex chromosomes (Fig. 3A). Bivalents in transit from the cell periphery to the central part of the cell have various orientations and microtubule associations. Different microtubule associations include fibres extending from only one half-bivalent to a pole (Fig. 3B,D, closed arrowheads), fibres extending from only one half-bivalent but extending to both poles (Fig. 3B, open arrowheads) and fibres extending from both half-bivalents to both poles (Fig. 3C, open arrowheads). Interestingly, there are also examples of kinetochore fibres extending away from the central spindle (Fig. 3B,C, arrows). Bivalent orientation varies from perpendicular to parallel to the spindle axis, and bivalents of all orientations have microtubule attachments. Because examples of all types of attachments described above are seen in bivalents located between the cell periphery and the central spindle area, we assume that all were moving or had been moving. Most bivalents that are in the central spindle have normal bipolar microtubule arrangements (Fig. 3E, arrowheads). Thus, following NEB, autosomes appear to interact with microtubules extending from the poles and begin to move into the central spindle. Association of only one half-bivalent with microtubules from either one or both poles appears to be sufficient to permit movement.

The kinetochore fibres of autosomes in early prometaphase, as they move centrally, are weakly acetylated or unacetylated (Fig. 3C-E), and the attachments can appear quite delicate. By mid prometaphase, the central autosomal spindle is well established and separate from the sex chromosomes (Fig. 4A-C) and by late prometaphase and metaphase the kinetochore fibres are acetylated (Fig. 4D), indicating that the fibres
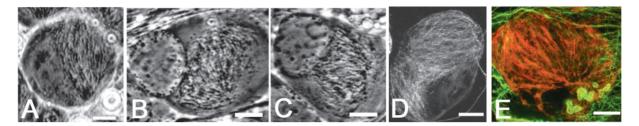

Fig. 2. The cytoplasmic spindle. (A-C) Phase-contrast images of living cells in prophase, revealing spindle-shaped cytoplasmic alignments outside the nucleus. (A) Bar, 10 μm. (B,C) Bar, 5 μm. (D) Labelling of a prophase cell with antibodies to tubulin reveals a spindle-shaped array of microtubules in the cytoplasm. The nucleus is at the bottom of the frame. Bar, 10 μm. (E) Prophase cell triple labelled to reveal acetylated tubulin (green), tyrosinated tubulin (red) and chromosomes (acridine orange labelling, which appears in the green channel). A spindle-shaped structure containing unacetylated microtubules is evident. The nucleus is at the bottom of the frame. Bar, 10 μm.
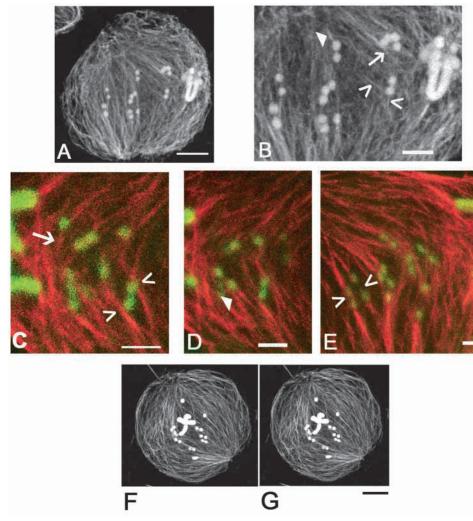
Fig. 3. Confocal serial section reconstructions of cells in early prometaphase I, showing kinetochore-microtubule associations of autosomes. (A) Prometaphase cell labelled with antibodies against tubulin. Bar, 10 μm. (B) Higher magnification image of cell in (A). The closed arrowhead points to a bivalent with associations to only one pole. Open arrowheads point to a bipolar connection made by a single half-bivalent. The sister half-bivalent has microtubule associations only to the top pole. The arrow points to a half-bivalent with bidirectional microtubule associations, only one of which points towards a pole. Bar, 5 μm. (C-E) Triple-labelled prometaphase cells, stained for acetylated tubulin (green), tyrosinated tubulin (red) and chromosomes (green). Co-localization of acetylated and tyrosinated tubulin would result in a yellow or orange image. (C) Arrowheads point to microtubule associations with a bivalent that has connections to both poles. The arrow points to a kinetochore fibre pointed away from the poles. Bar, 5 μm. (D) Different series of sections from the same cell shown in (C). The closed arrowhead points to a bivalent with attachments only to one pole: notice the two fibres coming from one half-bivalent, one from each sister chromatid. Bar, 5 μm. (E) Arrowheads point to bivalents that have reached the central spindle and established bipolar connections. Bar, 5 μm. (F,G) Stereo images of a prometaphase cell showing the arrangements of microtubules and chromosomes. Bar, 10 μm.

become more stable once the bivalents develop typical bipolar attachments within the central spindle.

Metaphase and anaphase microtubule arrays

By metaphase, the autosomes have congressed to the metaphase plate and the kinetochore fibres are highly acetylated (Fig. 5C). The kinetochore fibre structure at this stage is bimorphic, as described previously (Forer and Wilson, 2000), in that the kinetochore fibre is thick and highly acetylated from the kinetochore to roughly halfway to the pole but polewards from this point the bundle splays into several smaller microtubule bundles that extend towards the pole. Astral microtubules either are focused at the poles [Fig. 4D; see also Figs 3, 4 in Forer and Wilson (Forer and Wilson, 2000)] or form a broad 'fringe' of microtubules extending from the membrane across the polar ends of the cell [e.g. Fig. 5C; see also Fig. 2 in Forer and Wilson (Forer and Wilson, 2000)]. Astral microtubules are acetylated by late prometaphase and unacetylated by late anaphase (Fig. 4E).

In summary, we have confirmed the existence of a spindle-like structure in the prophase cytoplasm and shown that it contains microtubules organized between two poles. Our observations are consistent with the autosomes moving into the

pre-existing cytoplasmic spindle from the periphery of the cell by virtue of interactions with microtubules oriented toward the spindle. Microtubule associations with a single half-bivalent appear to be sufficient to give rise to movement. Once the autosomes reach the central spindle and develop bipolar attachments, the kinetochore fibres become thicker and acetylated, and the astral microtubules become acetylated.

Sex chromosomes

Our study of the univalent sex chromosomes focused on three issues: (1) their failure to move with the autosomes to the central spindle; (2) their independent orientation to opposite poles; and (3) their polewards motion, especially with respect to autosomal motion and pole-to-pole elongation. We paid special attention to the timing of the orientation events and to interactions of microtubules with the sex-chromosome kinetochores.

Failure to move with the autosomes

During prometaphase, the autosomes move into the central spindle but the sex chromosomes remain at the periphery. A possible explanation for this behaviour would be the absence
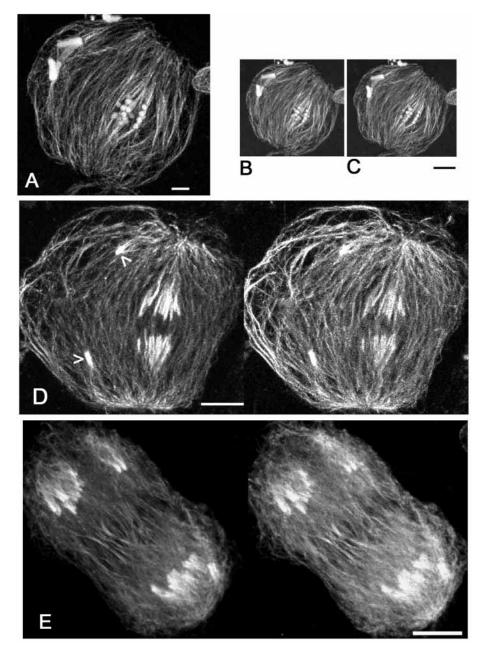
Fig. 4. Confocal Z-series showing microtubule organization at different stages of meiosis I. (A-C) Mid-prometaphase cell labelled with antibodies against tubulin and with acridine orange to reveal the chromosomes. (B) and (C) are stereo images. (A) Bar, 5 μm. (B,C). Bar, 10 μm. (D) Late prometaphase cell stained for acetylated tubulin (left) and tyrosinated tubulin (right). The arrowheads indicate the kinetochore fibres of the two sex chromosomes. Bar, 10 μm. (E) Anaphase cell stained for acetylated tubulin (left) and tyrosinated tubulin (right). Bar, 10 μm.

Independent orientation to opposite poles

We studied the interactions of microtubules with sex-chromosome kinetochores during prometaphase, when the sex chromosomes become oriented to opposite poles. There is substantial heterogeneity in the types of interactions but, in general, they fall into three broad groups. In the first group, termed 'bidirectional', thin microtubule bundles extend from the kinetochore region in two directions, usually but not exclusively in the direction of the two poles (Fig. 5A,B, arrowheads). In some cases the fibre extending to the closest pole is slightly thicker than the fibre extending to the further pole but, overall, the fibres are relatively thin and either unacetylated or very weakly acetylated. In the second group of kinetochore-microtubule interactions, termed 'syntelic', a single fibre extends from the kinetochore to a single pole. The fibre typically is relatively thick and acetylated (Fig. 5C, arrowheads).

In the third group, termed 'semi-syntelic', kinetochores are associated with a well-developed, acetylated fibre running from the kinetochore to the nearest pole, but there is a second, smaller fibre extending from the kinetochore in the opposite direction (Fig. 5D, arrowheads). The distinction between 'semi-syntelic' and 'bidirectional' is based on our evaluation of the relative development of the primary kinetochore fibre and its acetylation relative to the 'secondary' (poorly acetylated) fibre: in bidirectional fibres, the two microtubule bundles are more similar in size, thinner and poorly acetylated; in semi-syntelic fibres, there is a clearly dominant acetylated fibre in one direction. Not all fibres fell easily into one of the two categories and therefore the data presented include intermediates.

The frequencies of the different kinetochore-microtubule associations at various stages of division are shown in Fig. 6, the stages being assigned by scoring autosomes, as described earlier. The graph shows that bidirectional orientation is characteristic of the earlier part of prometaphase, whereas semi-syntelic and syntelic fibres are more common in later stages. Thus, orientation to opposite poles is accompanied by a shift from bidirectional kinetochore-microtubule associations to a mainly unidirectional kinetochore fibre.

It is worth noticing that in many examples of bidirectional fibres the microtubules appear to interact with the kinetochores at various angles. In some cases the sides of the microtubules

of kinetochore interactions with microtubules at this stage. Confocal images of cells labelled with anti-tubulin antibodies, however, reveal that the sex-chromosome kinetochores are associated with microtubules during the period when the autosomes are moving (e.g. Fig. 5A). In fact, we saw no examples where autosomes had kinetochore fibres and sex chromosomes did not. Therefore, a lack of microtubule associations cannot be the reason for the exclusion of the sex chromosomes from the central spindle.
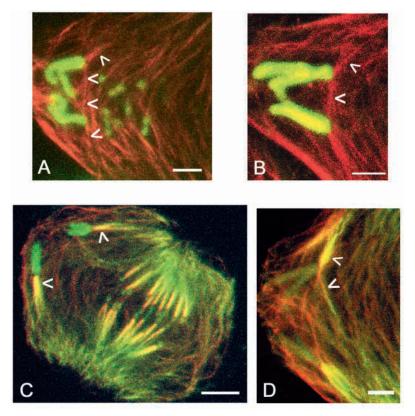
Fig. 5. Triple-labelled cells revealing microtubule-kinetochore associations of sex chromosomes. Acetylated tubulin and chromosomes are green, tyrosinated tubulin is red: colocalization results in yellow or orange images.
(A,B) Prometaphase cells showing bidirectional associations (arrowheads). Bar, 5 µm. (C) Metaphase cell showing syntelic associations (arrowheads). Bar, 10 µm. (D) Metaphase cell showing semi-syntelic associations (arrowheads). Bar, 10 µm.
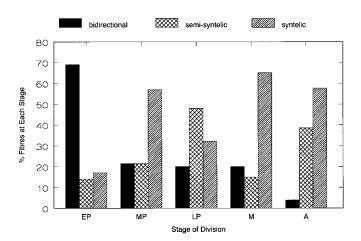

Fig. 6. Graph showing the distribution of different types of sex-chromosome/microtubule associations at different stages of division. We scored 29 sex chromosomes in early prometaphase (EP), 14 sex chromosomes in mid prometaphase (MP), 25 sex chromosomes in late prometaphase (LP), 20 sex chromosomes in metaphase (M) and 26 sex chromosomes in anaphase (A). (When the number of chromosomes is uneven, one sex chromosome in the group could not be analysed.)

appear to interact with the face of the kinetochore, consistent with microtubules sliding along the kinetochore rather than inserting into it.

Sex chromosomes are linked by microtubules

While analysing microtubule associations with sex chromosomes, we unexpectedly found some prometaphase cells with clear microtubule bundles linking the kinetochores of the two sex chromosomes (e.g. Fig. 5A,B). This observation prompted us to reanalyse all of our confocal images, looking specifically for evidence of microtubules extending between sex-chromosome kinetochores. We identified unambiguous interkinetochore connections in 6/15 cells in early prometaphase, 1/13 cells in late prometaphase and 1/10 cells in metaphase. Furthermore, in most of the other cells, we could identify putative connections between the sex chromosomes (thin fibres or single microtubules extending from one sex-chromosome kinetochore in the direction of the other sex-chromosome kinetochore). Putative connections were seen even in later stages of division, when the sex-chromosome spindle fibres were obvious, well-developed, 'syntelic' and acetylated. Such putative interkinetochore connections were observed in cells in prometaphase, metaphase and anaphase (Fig. 7). The high frequency of both unambiguous and putative connections suggests that microtubules might connect the sex chromosome univalents through all stages of division.

Sex-chromosome arm orientation is extremely variable in dividing cells, ranging from perpendicular to parallel to the long axis of the spindle. We tested whether the sex-chromosome arms change during division from perpendicular to parallel to the spindle axis, as discussed by Kupfer and Wise (Kupfer and Wise, 2000a), by examining the relationship between the angle made between the chromosome arm and its associated spindle fibre at different stages of division. There is very little change in average angle, regardless of stage (Fig. 8), so we conclude that the orientation of the sex-chromosome arms is not indicative of stage as we measured it, and we have not found such analysis useful in analysing sex chromosome behaviour at different stages.

Independent movement of sex chromosomes and autosomes

In 1970 Virkki suggested that the sex chromosomes move polewards in anaphase at about the same time that the autosomes do (Virkki, 1970). More recent data (Forer and Wilson, 2000) dispute this claim. To help resolve the issue, we measured interkinetochore and kinetochore-to-pole distances of the sex chromosomes and autosomes at different stages of division, classifying stage as described above – by the disposition of the autosomes. For autosomes, interkinetochore distance between autosomal half-bivalents remains essentially unchanged throughout prometaphase and metaphase, then increases during anaphase (Fig. 9).

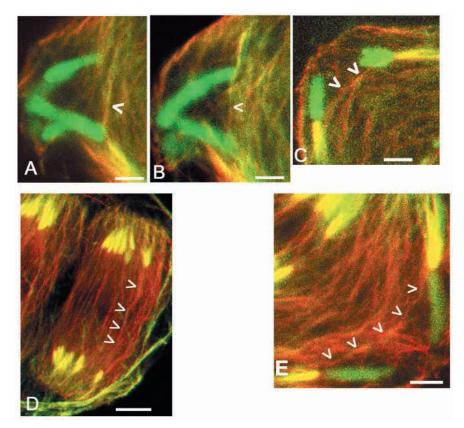A different pattern is seen for sex chromosomes (Fig. 9). By

Fig. 7. Confocal Z-series of triple-labelled cells revealing putative microtubule linkages between sex chromosomes (arrowheads). Acetylated tubulin and chromosomes are green, tyrosinated tubulin is red. Areas of colocalization are yellow or orange. (A) Anaphase cell. Bar, 5 μm. (B) Different sections of the same cell shown in (A). Bar, 5 μm. (C) Close up of metaphase cell from Fig. 5C, revealing putative connections between sex chromosomes that were initially classified as having syntelic fibres. Bar, 5 μm. (D) Late anaphase-I cell. Bar, 10 μm. [Flea-beetle spermatocytes are in cysts in the testes and sometimes the cysts survive the spreading procedure; the spindle partially seen in the upper left hand corner is the sister cell (in the cyst) to the one illustrated. Both are primary spermatocytes.] (E) Early anaphase-I cell. Bar, 5 μm. Panels D and E are of cells illustrated in different format (and composed of a different set of Z-series sections) in Fig. 4 of Forer and Wilson (Forer and Wilson, 2000). Only a subset of the original Z-series is shown here.

metaphase the sex chromosomes have moved ~19 μm apart, with most of the motion occurring when the autosomes are moving into the central spindle (before mid-prometaphase).
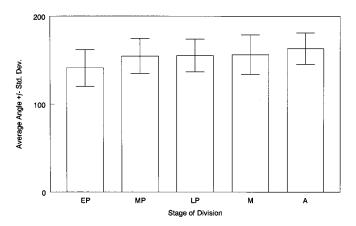

Fig. 8. Graph of the average angle made between the sex-chromosome arms and their associated fibres ± standard deviation, at different stages of division. We scored 30 sex chromosomes in early prometaphase (EP), 13 sex chromosomes in mid prometaphase (MP), 25 sex chromosomes in late prometaphase (LP), 20 sex chromosomes in metaphase (M) and 26 sex chromosomes in anaphase (A). Using the Student's t test with $\alpha=0.05$, there is no statistical difference between the average angles at different stages from midprometaphase to anaphase. The average angle in early prometaphase is not statistically different from that for midprometaphase, but is different from the other stages. [When the number of chromosomes is uneven, one sex chromosome in the group could not be analysed.]

There is another burst of separation in anaphase (on average an increase of 8.2 μm), but comparison with interpolar distances (which, on average, increased by 9.7 μm between metaphase and anaphase) suggests that the anaphase separation is due mostly to spindle elongation. To test whether sex-chromosomes move closer to the poles at this time, we compared average kinetochore-to-pole distances for sex chromosomes between metaphase (22.3 μm) and anaphase (19.1 μm); the difference is statistically significant using Student's t test ($P=0.04$) but, given the size and degree of overlap in the standard deviations and that the difference is only barely significant at the 5% level, we do not know whether the difference is real. We therefore attempted to gain further insight by arranging the data in a time series. We do not know when pole-to-pole elongation occurs relative to autosomal and sex-chromosome movements, but we do know that during anaphase chromosomes normally do not separate and then stall or move backwards, but continuously move polewards over time. Thus, following the approach of Ris in his classic study of chromosome movement in *Tamalia* (Ris, 1943), we used interkinetochore distance of the autosomes as a measure of time during anaphase. By plotting pole-to-pole distances and sex-chromosome-to-pole distances against autosomal interkinetochore distances in the same cells (Fig. 10A), or autosome distances from the poles against autosomal interkinetochore distances in the same cells (Fig. 10B), we deduce how sex chromosomes and autosomes move with respect to the spindle poles. Our interpretations of these data, indicated by the lines drawn in Fig. 10A,B, are: that the poles separate in early anaphase but not in later anaphase; that separation of autosomal half-bivalents during early anaphase is
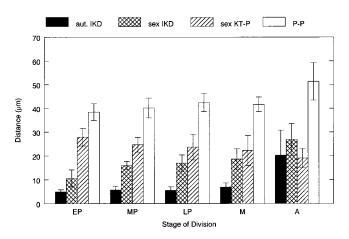
Fig. 9. Plot of average interkinetochore distances for autosomes and sex chromosomes, kinetochore-to-pole distances and pole-to-pole distances at different stages of division, with standard deviations. To generate the autosomal data, five bivalent pairs per cell were measured and an average distance calculated for each cell. The average distance for data from all cells was then calculated from the individual cell averages. Sex-chromosome data are simply the averages of all data taken. We scored 15 cells in early prometaphase (EP), 7 cells in mid prometaphase (MP), 13 cells in late prometaphase (LP), 10 cells in metaphase (M) and 13 cells in anaphase (A).

due primarily to spindle elongation, with little chromosome-to-pole motion; that after the initial spindle elongation the autosomes move to the poles as spindle length remains more-or-less constant; and that, while autosomes are moving polewards, the sex chromosomes appear not to move polewards. The data in Fig. 10A suggest that, if there is any polewards movement of sex chromosomes, it appears to occur early in anaphase, when the autosomes are not moving polewards, or to occur at stages in anaphase later than those in our sample.

In conclusion, early in prometaphase, most sex chromosomes are associated with microtubules that extend in two directions and, in many cases, microtubules connect the two chromosomes. As prometaphase proceeds, the fibre extending toward the farther pole is lost or diminished and the fibre oriented to the closer pole becomes larger, more substantial and highly acetylated, resulting in a syntelic, or syntelic-like orientation. Because smaller microtubule bundles often extend towards the other sex chromosome at all stages of division, it is possible that, at all stages of division, the sex chromosomes continue to maintain attachments with each other via small microtubule bundles. With respect to anaphase motion, sex-chromosome kinetochore-to-pole motion occurs primarily during the latter part of prometaphase. The sex chromosomes separate further during autosomal anaphase primarily as a result of spindle elongation. Autosomal half-bivalents, by contrast, seem to separate first through spindle elongation and then through chromosome-to-pole motion as spindle length remains constant.

Discussion

Our findings from combined immunofluorescence and study of living cells add significantly to our understanding of meiosis-I events in *Alagoasa bicolor*. The picture that emerges from our data confirms that offered by Virkki (Virkki, 1970; Virkki, 1971; Virkki, 1972; Virkki, 1985) in many regards and extends his observations by providing detailed information about microtubule organization and offering new insights about chromosome motion.

Autosomes move into a pre-existing spindle

We confirm the presence of a spindle body in the prophase cytoplasm and, for the first time, provide evidence that it is composed of microtubules with focused poles. Following NEB, the autosomes interact with microtubules from the
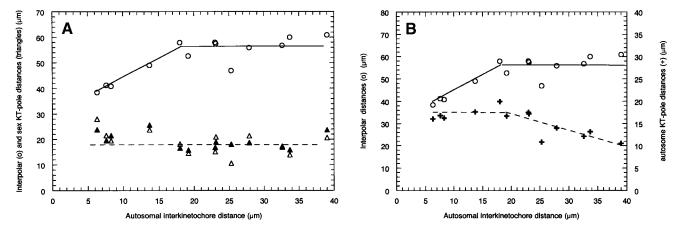

Fig. 10. (A) Plot of pole-to-pole distances (open circles) and of distances from the poles of sex-chromosome kinetochores (open and closed triangles, representing partner kinetochores) as a function of autosomal interkinetochore distance (abscissa), for which we used average distances as described in Fig. 9. The lines represent our interpretation: that the poles elongate early in anaphase but not later, and that the sex chromosomes do not move polewards (they remain a constant distance from the poles). (B) The same set of cells, with open circles representing pole-to-pole distances and crosses representing the average distances of the autosomal kinetochores from the spindle poles in these same cells. The lines indicate our interpretation: that autosomal separation during early anaphase is due to spindle elongation and separation later in anaphase is due to movement towards the pole.

central spindle and move into it. The 'mature' bipolar arrangement of the bivalents, in which a given autosome is syntelically oriented to the pole opposite to its sister homologue, is generally established later in prometaphase, once the bivalents are near or in the central spindle. Given the types of microtubule-kinetochore attachments observed for bivalents that are found between the periphery and the central spindle (and therefore presumably moving centrally), it appears that bivalent movement away from the periphery can be supported by any type of kinetochore-microtubule association and does not require bipolar attachments. Once in the central spindle, the autosomes assume bipolar orientation with well-developed acetylated kinetochore fibres, congress to the metaphase plate and then enter anaphase.

A cytoplasmic spindle in prophase that the autosomes move into during prometaphase seems unique with respect to animal cells, although something similar occurs in bryophytes (e.g. Brown and Lemmon, 1993; Brown and Lemmon, 1997). In these cells, a cytoplasmic spindle forms in prophase between the tips of two plastids (which act as microtubule-orienting centres); the single 'axial microtubule system' that they form is reminiscent of Virkki's 'cytoplasmic spindle'. The nucleus is to the side of the axial microtubule system and, after NEB, the released chromosomes and axial microtubule system combine to form a centrally located spindle [e.g. see Fig. 12 in Brown and Lemmon (Brown and Lemmon, 1993)].

Acetylated vs unacetylated microtubules

Our study for the first time provides information about the distribution of acetylated microtubules in flea-beetle spermatocytes at all stages of the first meiotic division, extending the observations of Wilson and Forer (Wilson and Forer, 2000). In general, microtubules that contain acetylated tubulin are more stable than others and are 'older', because there is a time lag between polymerization and acetylation [e.g. discussions in Wilson and Forer (Wilson and Forer, 1989; Wilson and Forer, 1997)]. Autosomal and sex-chromosomal kinetochore microtubules are initially not acetylated but become acetylated (and hence presumably more stable) by late prometaphase. Microtubules extending between sex-chromosome kinetochores are poorly acetylated or unacetylated. In metaphase, both the astral microtubules and the kinetochore microtubules are acetylated (Fig. 5C), although the astral microtubules lose their acetylation in later anaphase. Whereas acetylation of kinetochore microtubules is similar to other spermatocytes (e.g. Wilson and Forer, 1997), flea-beetle spermatocytes are unique in that from prometaphase onwards the astral microtubules are acetylated. It is likely that the loss of acetylation later in division is related to the need for cytoskeletal reorganization at the end of division.

Why are sex chromosomes excluded from the central spindle and how do they orient to opposite poles from the periphery?

It has been suggested that sex chromosomes might fail to move with the autosomes because they fail to make attachments to the spindle when the autosomes do (Virkki, 1971; Virkki, 1972). However, we see no evidence for this possibility – sex
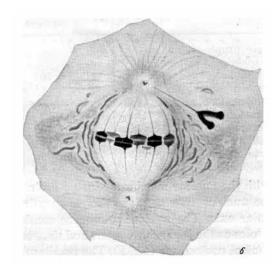

Fig. 11. Metaphase primary spermatocyte of *Humbertiella*, illustrating the resemblance to metaphase flea-beetle primary spermatocytes. Reproduced from Hughes-Schrader (1948) with permission from Springer Verlag (Hughes-Schrader, 1948).

chromosomes are associated with microtubules early in prometaphase, even though they do not move with the autosomes. It is also possible that sex chromosomes do not move with the autosomes because they are larger than the autosomes and would be blocked by the presence of mitochondria surrounding the central spindle, as suggested by one referee. However, evidence from mantid spermatocytes suggests otherwise. In primary spermatocytes of the mantid *Humbertiella* the autosomes are separated from the single sex chromosome (Fig. 11) in a spindle that is reminiscent of that of a flea-beetle spermatocyte. However, in the mantid cells the X chromosome seems to be expelled from the spindle (Hughes-Schrader, 1948), through the mitochondrial sheath, showing that mitochondria do not block the movement of large chromosomes.

Yet another possible explanation comes from our observations of sex-chromosome associations with microtubules. Our results show that the X and Y chromosomes are not always syntelic, as suggested by Virkki (Virkki, 1971; Virkki, 1972; Virkki, 1985), nor initially amphitelic, as suggested by others (Kupfer and Wise, 2000b; Green-Marroquin et al., 2001). Although there are bidirectional associations between the sex-chromosome kinetochores and microtubules in prometaphase, they appear in many cases to be lateral associations rather than truly amphitelic associations. Since the sex chromosomes are mobile in prometaphase (Virkki, 1971; Virkki, 1972), it is likely that the kinetochores are sliding along the microtubules, as observed in other cells (e.g. Reider and Alexander, 1990) and as suggested previously (Kupfer and Wise, 2000b). During prometaphase, the sex chromosomes move towards opposite poles, with the poleward fibre becoming thicker, more developed and acetylated, whereas the fibre in the opposite direction remains weak or disappears.

Equally interesting, and probably more important, is our observation of microtubule associations connecting the two sex chromosomes. As illustrated in Fig. 5, microtubules

unambiguously link sex chromosomes in at least a subset of spermatocytes, primarily in early prometaphase but also in later stages. Furthermore, similar 'putative' connections exist in most if not all of the cells, even in anaphase (Fig. 7). In many cases, we cannot tell whether the putative connections are real or simply represent non-kinetochore spindle microtubules that happen to lie in the path of the sex chromosomes. However, in the case of the unambiguous connections, we were able clearly to track the fibres from one kinetochore to the other, and we feel confident that they are real.

Given the apparently delicate nature of this connection, it is no surprise that it has not been previously reported. Virkki mentions the absence of a 'firm connection' between sex chromosomes, based primarily on observations using phase-contrast microscopy (Virkki, 1972). Three other publications claim to find no evidence for microtubule connections between sex chromosomes (Virkki, 1985; Kupfer and Wise, 2000a; Green-Marroquin et al., 2001). Unfortunately, these publications refer to unpublished work or do not present figures that clearly show the region between the sex chromosomes, and therefore there is little or no published evidence for or against a connection. We have found only one reference to connections between the sex chromosomes. 'Normally no visible link is seen between the distance-pairing sex chromosomes either in living or fixed cells. Sometimes a thin thread is seen, undoubtedly a consequence of a sticky contact in the contraction clump' [page 141 of Smith and Virkki (Smith and Virkki, 1978)]. Although this thin thread might or might not be the type of connection that we observe, the authors appear to attach no significance to it.

Based on the above observations, we present a third possible explanation for sex-chromosome behaviour in flea-beetle spermatocytes. In early prometaphase I, the sex-chromosome kinetochores become loosely associated with cytoplasmic microtubules (or bundles) that interact laterally with the kinetochore and extend in two directions from it. Some of these microtubules form a link between the two sex chromosomes, and we suggest that this link results in the sex chromosomes remaining at the cell periphery and orienting to opposite poles. How this link acts is unknown, but it represents a real difference between the sex chromosomes and the autosomes. We might speculate, for example, that the link forged between kinetochores is different from that formed between a kinetochore and pole, perhaps because motors that produce force in the two regions have opposite senses (e.g. kinesin and dynein) or are absent from one region. This difference, together with the connections made by the kinetochores to the poles, provides orientation information to the kinetochores, with the result that kinetochore fibres develop on the side that does not link the two chromosomes, which results in the sex chromosomes becoming oriented to opposite poles. The difference in the kinetochore interactions for these univalent chromosomes might also result in polewards rather than lateral motion in prometaphase. Once orientation to opposite poles is established, poleward kinetochore fibres become densely packed with microtubules, as indicated by their increasing thickness, and these microtubules are stable, as indicated by the fact that they are highly acetylated. The linking fibre between the two chromosomes remains relatively unstable and either poorly acetylated or unacetylated, and might eventually break as chromosomes move, resulting in the characteristic

syntely or semi-syntely that we have seen. This model provides a mechanism for establishing the 'distance segregation' of the sex chromosomes, their successful orientation to opposite poles and their coordinated motion.

There is some precedent for microtubule linkages occurring between chromosomes that appear to be physically unassociated but exhibit coordinated movement. For example, in the mole cricket *Neocurtilla* (*Gryllotalpa*) *hexadactyla*, during male meiosis I the univalent X_1 chromosome always segregates in a coordinated way with the heteromorphic X_2Y bivalent so that the X_1 and X_2 move to the same pole during anaphase. In a study of these cells using electron microscopic techniques, Kubai and Wise described a possible microtubule linkage between the X_1 and Y chromosome, and they argued that the link, if it existed, could provide a means for bringing about the nonrandom segregation of the two chromosomes (Kubai and Wise, 1981). A similar situation seems to apply in flea-beetle primary spermatocytes.

Anaphase segregation of sex chromosomes and autosomes

With respect to chromosome movement, our data (Fig. 9) indicate that the sex chromosomes segregate towards the poles during prometaphase and move very little (if at all) in anaphase. Using the separation of autosomes as an indicator of time in anaphase, we have also identified two possible relationships of interest. First, the autosomal half-bivalents appear to separate first by pole-to-pole elongation, moving polewards primarily after the poles stop separating (Fig. 10). This interpretation is different from that of Kupfer and Wise, who found little spindle elongation (Kupfer and Wise, 2000a). The difference is probably due to a different method of analysing the images of fluorescently stained cells. Kupfer and Wise lumped all stages together and looked for trends in autosome and sex-chromosome separations against spindle lengths, whereas we looked at individual stages, using the separation of autosomes as a measure of time in anaphase. Second, the sex chromosomes appear not to move polewards when autosomes do. Based on Fig. 10, it appears that any possible polewards motion takes place early in anaphase, when autosomes move the least. In addition, the perception of polewards movement is due primarily to the data points from one cell (the leftmost cell), and it is difficult to base solid conclusions on one cell. Admittedly, the data upon which Figs 9 and 10 are based are relatively crude in that they rely on straight-line distances between kinetochores and poles, and between the two poles, necessarily ignoring inaccuracies caused by the angle of the fibres with respect to the plane of the image, by curvature of the sex chromosome fibres and by variations in the degree of cell flattening. However, we doubt whether these inaccuracies systematically alter the data or are likely to affect the general conclusions (see also Forer and Wilson, 2000). Clearly, our or any other interpretation can only be confirmed by following living cells through the first meiotic division and studying how chromosomes and spindles behave in vivo, something which has not yet been achieved for these cells. Finally, with respect to further movement of the sex chromosomes to the poles, our analysis was restricted to those cells in which we could see individual autosome spindle fibres, so it is conceivable that sex chromosomes move polewards

later than cells in our sample, after the autosomes near the poles, as the sex chromosomes do in crane-fly spermatocytes (Forer, 1980).

There is now ample evidence for a checkpoint that monitors spindle readiness to enter anaphase by inhibiting the metaphase-anaphase transition until all kinetochores have formed normal attachments to spindle microtubules (e.g. Gorbsky and Ricketts, 1993; Campbell and Gorbsky, 1994; Rieder et al., 1995; Li and Nicklas, 1995; Li and Nicklas, 1997; Rieder and Salmon, 1998; Zhou et al., 2002). Although the exact molecular nature of this checkpoint is still being elucidated, data from PtK$_1$ cells with two spindles suggest that the inhibition of anaphase onset is local: once one spindle in the cell has all kinetochores attached, it will enter anaphase even if the second spindle has unattached kinetochores (Rieder et al., 1997). Once that spindle enters anaphase, however, the second spindle also enters anaphase, even with unattached chromosomes, suggesting that the molecular signal indicating that the checkpoint has been passed (and that anaphase should begin) is more global in nature. The results from flea-beetle spermatocytes support the notion of local inhibition, because improperly attached autosomes do not prevent the prometaphase polewards motion of the sex chromosomes. Our data also suggest that these cells, like crane-fly spermatocyte cells (in which autosomes and sex chromosomes move polewards at different times), are able to control kinetochore behaviour differently once the checkpoint has passed, meaning that the signal to enter anaphase in these cells is not global.

In conclusion, we have substantially extended our understanding of the microtubule arrangements in flea-beetle spermatocytes during the first meiotic division. Our study complements the early work of Virkki, providing information about microtubule arrangements missing in his many excellent phase-contrast studies, and providing new information about anaphase chromosome movement. It also provides evidence for the first time that a physical link exists between the apparently independent and unpaired sex chromosomes, providing a basis for a model to explain their orientation and movement to opposite poles.

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