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Phosphorylated proteins are involved in sister-chromatid arm cohesion during meiosis I

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SUMMARY

Sister-chromatid arm cohesion is lost during the metaphase I/anaphase I transition to allow homologue separation. To obtain needed information on this process we have analysed in grasshopper bivalents the sequential release of arm cohesion in relation to the behaviour of chromatid axes. Results show that sister axes are associated during early metaphase I but separate during late metaphase I leading to a concomitant change of chromosome structure that implies the loss of sister-kinetochore cohesion. Afterwards, homologues initiate their separation asynchronously depending on their size, and number and position of chiasmata. In all bivalents thin chromatin strands at the telomeres appeared as the last point of contact between sister chromatids. Additionally, we have analysed the participation of phosphoproteins recognised by the MPM-2 monoclonal antibody against mitotic phosphoproteins in arm cohesion in bivalents and two different kinds of univalents. Results show the absence of MPM-2 phosphoproteins at the interchromatid domain in mitotic chromosomes and meiotic univalents, but their presence in metaphase I bivalents. These phosphoproteins are lost at the onset of anaphase I. Taken together, these data have prompted us to propose a 'working' model for the release of arm cohesion during meiosis I. The model suggests that MPM-2 phosphoproteins may act as cohesive proteins associating sister axes. Their modification, once all bivalents are correctly aligned at the metaphase plate, would trigger a change of chromosome structure and the sequential release of sister-kinetochore, arm, and telomere cohesions.

Key words: Phosphoprotein, Chromatid axis, Metaphase/anaphase transition, Sister-chromatid arm cohesion, Meiosis I

INTRODUCTION

Mitotic metaphase chromosomes show sister chromatids attached to each other at the centromere and along their arms. The maintenance of sister-chromatid cohesion is essential to ensure a correct attachment of the chromosome to the spindle and therefore for its proper segregation. During the metaphase/anaphase transition the cohesive forces acting primarily at the centromere, and counteracting the splitting forces exerted by microtubules on sister kinetochores, are lost. Consequently, the sister chromatids separate and segregate to opposite poles (for review see Miyazaki and Orr-Weaver, 1994; Holloway, 1995; Biggins and Murray, 1998). Meiotic chromosome segregation greatly differs from that shown by mitotic chromosomes since two sequential cell divisions take place after a single round of replication. Meiosis I bivalents, as mitotic chromosomes, show sister-chromatid centromere and arm cohesions. These cohesions hold the bivalent integrity and are essential for proper homologue segregation. During the metaphase I/anaphase I transition the arm cohesion is lost so that homologues separate and initiate their segregation towards opposite spindle poles. However, the two chromatids of each homologue remain joined at the centromere. This centromere cohesion is released during the metaphase II/anaphase II transition allowing the separated chromatids to segregate to opposite poles (Suja et al., 1992). Thus, unlike mitotic chromosomes, whose centromere and arm cohesions are simultaneously dissolved, meiotic chromosomes first abolish arm cohesion during meiosis I, and then release centromere cohesion during meiosis II (for review see Moore and Orr-Weaver, 1998). Consequently, this sequential loss of cohesion during meiosis might be precisely regulated.

The nature of the molecular mechanisms regulating centromere and arm cohesion in mitosis and meiosis are still poorly understood. Nevertheless, it is believed that cohesive proteins may hold sister chromatids together during mitosis and meiosis. It has been demonstrated that the release of cohesion depends on the proteolysis of some proteins, a process that is mediated by a large complex with ubiquitin-ligase activity, the anaphase-promoting complex (APC) (for review see Cohen-Fix and Koshland, 1997). Studies in budding yeast have demonstrated that the Scc1p/Mcd1p chromosomal protein is a 'cohesin' protein since mutants show failure in the maintenance of cohesion between sister chromatids (Guacci et al., 1997; Michaelis et al., 1997). The dissociation of Scc1p/Mcd1p from chromosomes during the metaphase/anaphase transition depends

on the APC-mediated proteolysis of the anaphase inhibitor Pds1p (Cohen-Fix et al., 1996; Yamamoto et al., 1996). Recently it has been shown that the Pds1p degradation activates the sister-separating protein Esp1p, a protein that promotes, by an unknown mechanism, the dissociation of Scc1p/Mcd1p from chromosomes (Ciosk et al., 1998). This Scc1p/Mcd1p dissociation is not a consequence of its destruction at the metaphase/anaphase transition (Michaelis et al., 1997).

In the meiotic context, it has been proposed that remnants of the synaptonemal complex (SC) lying between sister chromatids of metaphase I chromosomes may be related to cohesion (for review see Maguire, 1995). In this sense, the rodent Cor1/SCP3 protein, a component of the axial elements and SC lateral elements that persists between sister chromatids at metaphase I, has been proposed to be a cohesive protein (Moens and Spyropoulos, 1995). Likewise, there are proteins such as Red1 in budding yeast, a component of axial elements and SC lateral elements (Smith and Roeder, 1997), and Rec8 in fission yeast, a component of linear elements considered to be equivalent to axial elements (Molnar et al., 1995), whose absence promotes defects in the maintenance of cohesion. Other cohesive proteins not related to SC elements have been identified in *Drosophila*. Thus, MEI-S332 maintains centromere cohesion until anaphase II (Moore et al., 1998; Tang et al., 1998), while ORD is essential in the maintenance of arm cohesion (Bickel et al., 1996). Similarly to Scc1p/Mcd1p in budding yeast, it has been reported that MEI-S332 is not degraded at the metaphase II/anaphase II transition, and it has been suggested that its phosphorylation may promote its dissociation from the centromere (Moore et al., 1998).

Protein dephosphorylation is essential for progression from metaphase to anaphase. The analysis of the phenotype in a fission yeast mutant with altered phosphatase activity (Ishii et al., 1996), together with results obtained by using phosphatase inhibitors both in mitosis (for review see Holloway, 1995; Yanagida, 1995) as in meiosis (Wiltshire et al., 1995), suggest that protein dephosphorylation is necessary to allow the separation of sister chromatids. Nevertheless, it is not known whether phosphatases may act directly in the dephosphorylation of cohesive proteins, or indirectly on a regulatory cascade implying the APC complex and that culminates in the separation of sister chromatids (Minshull et al., 1996; Ishii et al., 1996).

In this paper we have carefully analysed the loss of arm cohesion during the metaphase I/anaphase I transition in several grasshopper species. We have tested by immunofluorescence the participation of phosphoproteins in centromere and arm cohesion during meiosis I. For this purpose we have employed the MPM-2 mAb. In mammalian somatic cells this antibody reacts with over 40 or 50 different proteins that become phosphorylated at the G_2/M transition and that are dephosphorylated at the end of mitosis (for references see Renzi et al., 1997). This antibody mainly detects phosphorylated DNA topoisomerase IIα (topo IIα) on isolated mammalian metaphase chromosomes (Taagepera et al., 1993), as well as kinetochores in mammals and plants (for references see Renzi et al., 1997). We studied the distribution and behaviour of MPM-2 phosphoproteins on autosomal bivalents, two kinds of univalents (the X chromosome and a B-chromosome), and mitotic chromosomes. Additionally, we also investigated the participation of silver-stained chromatid axes (Rufas et al., 1987; Suja et al., 1991) in the maintenance of arm cohesion. The chromatid axis occupies an internal location inside mitotic metaphase chromatids (Howell and Hsu, 1979; Saitoh and Laemmli, 1994). However, in metaphase I chromosomes the axis is peripherally located in relation to the chromatid width, i.e. chromosomes show a meiotic organisation (Rufas et al., 1987), and thus the axes of sister chromatids could be involved in arm cohesion. In this sense we have studied their precise location in metaphase I bivalents by EM, and their behaviour during the metaphase I/anaphase I transition by light microscopy. The present findings have led us to propose a 'working' model for the release of arm cohesion during meiosis I. In this model MPM-2 phosphoproteins lying at the interchromatid domain are considered as cohesive proteins that initially tie sister axes together. The modification of these MPM-2 cohesive proteins at the metaphase I/anaphase I transition would promote the sequential release of sister-kinetochore, arm and telomere cohesions allowing the homologue separation.

MATERIALS AND METHODS

Materials

Adult males of the grasshopper species *Eyprepocnemis plorans*, *Chorthippus jucundus*, *Arcyptera fusca*, *Stethophyma grossum* and *Pyrgomorpha conica* (Orthoptera: Acrididae) collected from natural populations were used for the present study.

Orcein staining

Testes of *S. grossum* were removed and fixed in 3:1 ethanol/glacial acetic acid and stored at –20°C. Single seminiferous tubules were squashed in a drop of 2% lacto-propionic orcein (BDH).

Silver staining

Testes of *C. jucundus*, *A. fusca* and *E. plorans* were removed and processed for silver staining as previously described by Rufas et al. (1987).

Immunofluorescence microscopy

Testes were removed and then simultaneously fixed and lysed in 2% formaldehyde in PBS containing 0.1% Triton X-100 (Sigma). In some cases 5 μM microcystin LR (Alexis Corporation), a potent phosphatase inhibitor, was added to the fixation/lysis solution. During the first 10 minutes of fixation the different seminiferous tubules were separated and the fat tissue covering them eliminated. Afterwards, several seminiferous tubules were placed on a slide with a small drop of fixation/lysis solution and gently minced with tweezers. The tubules were posteriorly squashed and the coverslip removed after freezing in liquid nitrogen. The slides were then rinsed three times for 5 minutes in PBS and incubated for 45 minutes at room temperature in either a 1:1000 dilution of the MPM-2 mouse ascites fluid (kindly provided by Dr P. N. Rao), or a 1:1000 dilution of the 3F3/2 mouse ascites fluid (kindly provided by Dr G. J. Gorbsky). Following three washes in PBS, the slides were incubated for 30 minutes at room temperature with an FITC-conjugated goat anti-mouse IgG (Sigma) secondary antibody at a 1:50 dilution in PBS. The slides were posteriorly rinsed in PBS and counterstained for 3 minutes with 5 μg/ml DAPI (4′,6-diamidino-2-phenylindole). After a final rinse in distilled water, the slides were mounted with Vectashield (Vector Laboratories). Observations were performed using an Olympus BH-2 microscope equipped with epifluorescence optics and the images were recorded on Kodak T-Max 100 film. The negatives were scanned in a Polaroid SprintScan 35 scanner and the images were treated using the Adobe Photoshop 5.0 software. The resulting final images were then printed in a Fargo PrimeraPro Elite dye-sublimation printer.

Electron microscopy

For silver staining we employed the preembedding technique described by Rufas et al. (1994). Briefly, testes were fixed for 2 hours

at room temperature in 3% glutaraldehyde in 0.067 M Sörensen phosphate buffer (pH 7.2), thoroughly rinsed in distilled water, and incubated overnight at 70°C in 1 g/ml silver nitrate (Merck) in bidistilled water. The testes were then rinsed in distilled water, dehydrated through a graded ethanol series, and embedded in Epon 812 (Taab). Serial 150 nm thin sections were mounted on 0.5% Butvar recovered single-slot oval copper grids, and contrasted with 5% uranyl acetate for 5 minutes. Grids were examined in a Jeol 1010 transmission electron microscope operated at 80 kV.

RESULTS

Release of arm cohesion during the onset of anaphase I occurs sequentially

During the onset of anaphase I the homologues separate, however, there are not accurate cytological descriptions on how this loss of arm cohesion occurs. To analyse this point in detail we selected the grasshopper species *S. grossum* since it offered several advantages, namely: (i) it possesses long telocentric chromosomes, and (ii) their longer bivalents present a single proximal chiasma as a consequence of an extreme proximal chiasma localisation (Fig. 1A).

In orcein-stained metaphase I bivalents the sister chromatids were clearly discernible since there was a narrow cleft between them along their entire length (Fig. 1B,C). This interchromatid domain appeared frequently interrupted by discrete small patches that showed the same staining response as the condensed chromatin. The onset of anaphase I, i.e. the initiation of homologue separation, is a rapid process and thus a low number of spermatocytes were found at this stage. The initial separation of homologues did not imply a sudden loss of arm cohesion all along the chromatid length. Instead, a 'rhomboid hole' appeared at the previous chiasma site when homologous centromeres began to separate toward opposite poles (Fig. 1D). In this moment the interchromatid domain was larger than previously (compare Fig. 1C,D), and it appeared occupied by small patches of chromatin-like material connecting sister chromatids (Fig. 1D). With progression of homologue separation the rhomboid hole enlarged and consequently the sister chromatids only remained parallely attached to each other at their distal regions (Fig. 1E). Therefore, it seemed that the chiasma moved or was pushed toward the distal chromatid ends (Fig. 1F). In a more advanced stage the sister telomeres remained connected by thin chromatin strands (Fig. 1G). Finally, these telomeric connections might disappear allowing the complete separation of homologues and their segregation to the poles.

This sequence of separation, that resembles the opening of a 'zipper' from the chiasma site towards telomeres and centromeres, was observed in telocentric, acrocentric or submetacentric bivalents in all the grasshopper species that we analysed. However, not all bivalents separated simultaneously their chromosomes in a given spermatocyte. The homologue separation is not a synchronous process but depends both on the bivalent size and the number and location of chiasmata. Therefore, monochiasmate bivalents, in comparison with bivalents presenting two or more chiasmata, were the first to accomplish homologue separation. Among the monochiasmate bivalents the smallest ones separated first (Fig. 2H), and among similar size monochiasmate bivalents those showing more distal chiasmata were the first to separate their homologues.

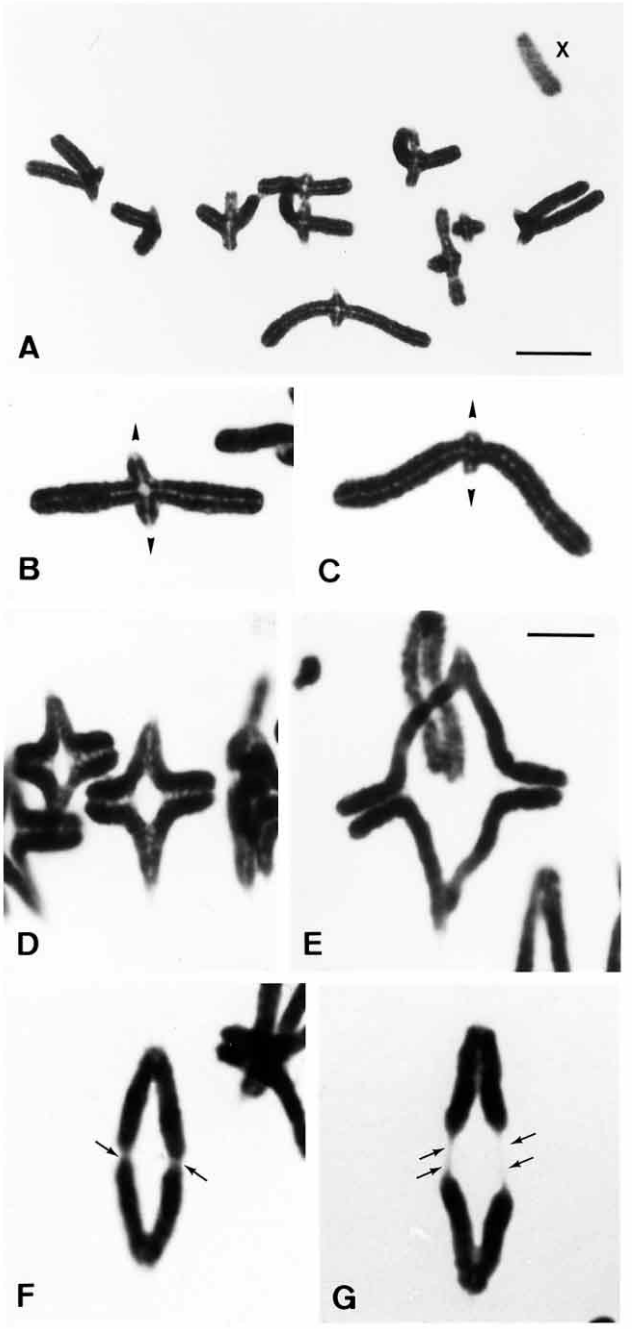
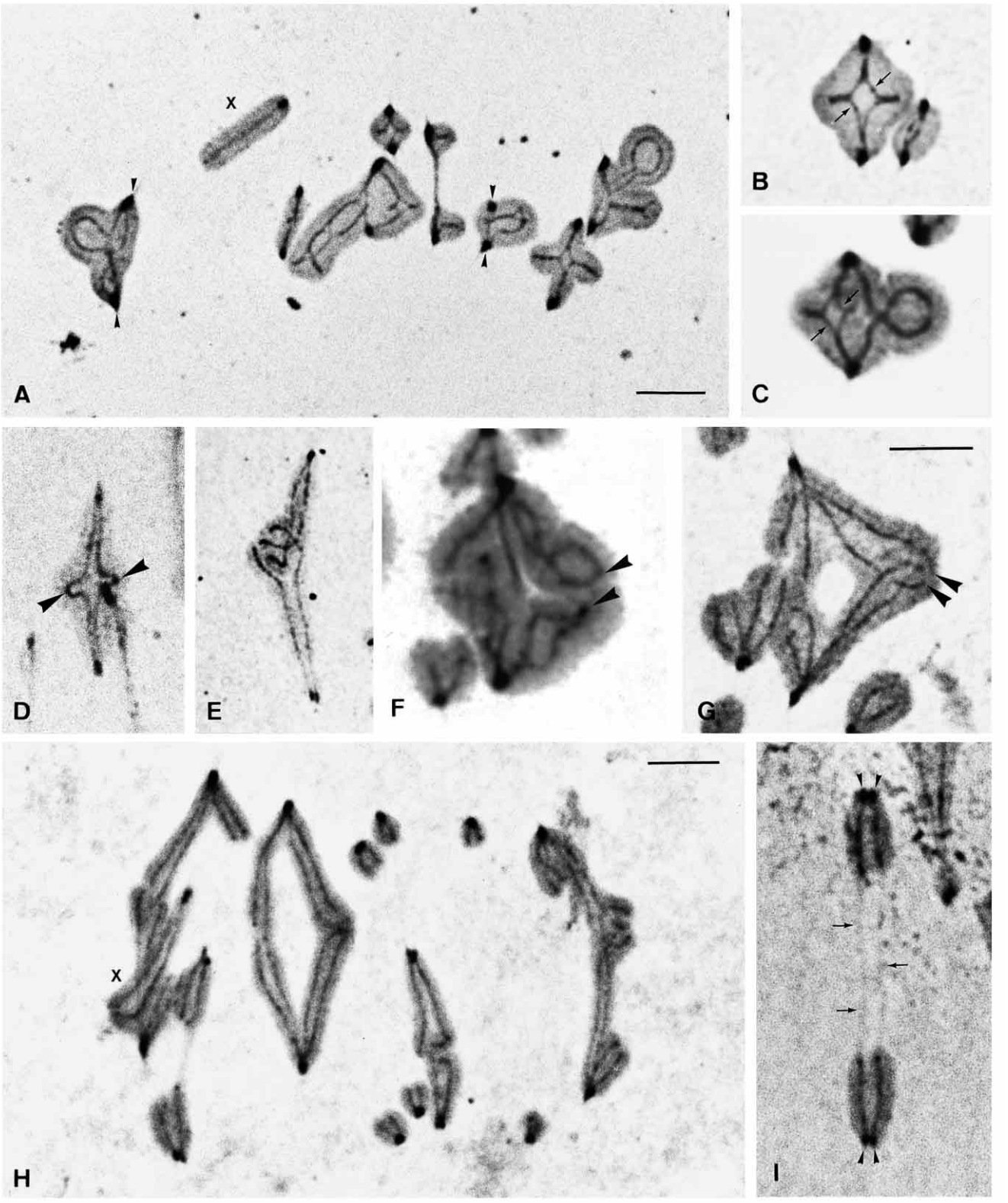


Fig. 1. Sequence of homologue separation during the metaphase I/anaphase I transition. Orcein-stained spermatocytes of *S. grossum*. (A) Metaphase I. All eleven telocentric bivalents are present as well as the telocentric univalent sex chromosome (X). (B-C) Selected metaphase I autosomal bivalents with a single proximal chiasma. Arrowheads indicate the positions of homologous centromeres. (D-G) Selected bivalents at the onset of anaphase I. Arrows in F and G point to telomeric associations. Bars: (A) 10 μm; (B-G) 5 μm.

Individualisation of sister axes precedes the release of arm cohesion

To analyse the participation of the chromatid axes in arm



cohesion we employed a silver staining technique demonstrating these chromosome structures, similar to mitotic chromosome axes, but in condensed meiotic chromosomes (Rufas et al., 1987; Suja et al., 1991).

In early metaphase I spermatocytes all bivalents showed one silver-stained round structure at the centromere region of each homologue (Fig. 2A). Ultrastructural observations have previously revealed that each round centromere structure corresponds to the two closely associated ‘ball and cup’ sister kinetochores (Rufas et al., 1994). On the other hand, the axes were observed as lines running along the chromosomes from kinetochores to telomeres although they did not reach the distal

Fig. 2. Change of chromosome structure during the metaphase I/anaphase I transition as detected by the rearrangement of the silver-stained chromatid axes. (A) Early metaphase I of *C. jucundus*. Arrowheads indicate the homologous centromeres, each one possessing the two closely associated sister kinetochores, oriented to opposite poles. (B) Selected telocentric and (C) submetacentric early metaphase I bivalents of *C. jucundus*. Arrows point to the separation of sister axes at chiasma sites. (D) Late metaphase I telocentric bivalent of *A. fusca* with a subdistal chiasma. Arrowheads indicate the association of sister axes at their distal ends. (E) Late metaphase I telocentric bivalent of *A. fusca* with completely separated axes. (F) Late metaphase I submetacentric bivalent of *C. jucundus*. Arrowheads indicate the distal association of axes. (G) Early anaphase I submetacentric bivalent of *C. jucundus*. Arrowheads indicate the individualised distal axis ends. (H) Anaphase I of *C. jucundus*. Note that the shorter chromosomes have separated while the longer ones are still connected. (I) Selected segregating telocentric homologues at anaphase I in *C. jucundus*. Arrowheads point to individualised sister kinetochores at each centromere, and arrows indicate thin chromatin strands connecting telomeres. Bars: (A and H) 5 μm; (B-G and I) 5 μm.

tips (Fig. 2A). These axes were observed as single lines except in chiasma sites where each line bifurcated into two thinner lines, each one representing a sister chromatid axis. This arrangement was found not only in favourable situations such as in telocentric bivalents with a single chiasma (Fig. 2B), but also in submetacentric bivalents presenting several chiasmata (Fig. 2A,C). Thus, the single line found between kinetochores and the first chiasma, between chiasmata, and between the last chiasma and the telomere, seems to represent the two closely associated sister axes. Therefore, sister axes occupy a peripheral location in relation to the chromatid width. This axes arrangement corresponds to a meiotic chromosome organisation, and was invariably found in bivalents of all the analysed species.

Nevertheless, in late metaphase I spermatocytes sister axes did not appear tightly associated. In some bivalents sister axes were not only individualised at chiasmata sites, but also all along their entire length although showing a persisting distal association (Fig. 2D). This change of axis location within chromatids was observed in telocentric (compare Fig. 2B,D) and in submetacentric bivalents (compare Fig. 2C,F). In other bivalents the axes were completely individualised even at their distal ends (Fig. 2E). In such bivalents the sister kinetochores were frequently observed as close but individualised structures (lower centromere in Fig. 2E). It is important to point out that the distal ends of sister axes represent the last point of contact between them, and that both kinds of axes arrangements were found in spermatocytes whose bivalents were perfectly aligned at the metaphase plate. These results thus indicate that the individualisation of sister axes only takes place when all bivalents are subjected to tension towards opposite poles. It is worth noting that even although such changes in the axes disposition take place, the homologues show no sign of separation. Thus, the individualisation of sister axes does not imply a concomitant release of cohesion, since the bivalent morphology is maintained, but precedes the loss of arm cohesion.

At the onset of anaphase I, the sister axes were completely individualised and showed an internal location in relation to the chromatid width, i.e. the chromosomes presented a mitotic organisation (Fig. 2G). The homologue separation proceeded as previously described and consequently while the smaller chromosomes appeared completely separated the longer ones were still connected (Fig. 2H). It is important to point out that telomeres represent the last point of contact between homologues, and that although they initiated their separation, thin chromatin strands still persisted joining sister telomeres (Fig. 2I).

Sister axes are not joined in early metaphase I bivalents

Although the individualisation of sister axes, and a change in chromosome organisation takes place in late metaphase I spermatocytes, homologues do not initiate a concomitant separation. However, the apparent association of sister axes before their individualisation may act as a sort of homologue binder. To test this possibility we performed an ultrastructural analysis of their arrangement on serial sections of silver-stained early metaphase I bivalents. The observations showed that sister axes always appeared as parallel structures formed by rod-like accumulations of silver grains. Sister axes appeared separated, not only at chiasma sites (Fig. 3B), but also in regions comprised between the chiasma and the centromeres (Fig. 3A) and telomeres respectively. Thus, although light microscopical observations suggested a close association between sister axes in those regions, they are not physically joined.

Phosphoproteins are present at the interchromatid domain and kinetochores in late prophase I and metaphase I bivalents

We next tried to test the presence of phosphoproteins in meiosis I spermatocytes by indirect immunofluorescence by using the MPM-2 mAb against mitotic phosphoepitopes.

Pachytene spermatocytes showed a faint nuclear labelling, however a series of lines composed of tightly associated fluorescent spots traversing the nuclei were discerned (Fig. 4A-D). In each spermatocyte the number of fluorescent lines corresponded to the number of bivalents, and the ends of each line were associated with the nuclear envelope. These results suggest that MPM-2 was detecting some SC component. In late diplotene nuclei the MPM-2 labelling increased but some brightly fluorescent nuclear spots were observed. During diakinesis the nuclear labelling increased dramatically so that the condensing bivalents appeared clearly delineated (Fig. 4E-H). However, in spite of the brightly fluorescent nucleoplasm, the observation of different focal planes throughout the nuclei showed that the strongest signals were found at the centromere regions of all bivalents (Fig. 4E-G). In addition to centromeres, thin weak lines were observed running along the central inner chromosome regions (Fig. 4F). In some species, the separating centrosomes also appeared brightly labelled (Fig. 4E,G).

At the time of nuclear envelope breakdown the brightly labelled nucleoplasm spreaded throughout the cytoplasm. We tried to extract spermatocytes with detergent before fixation, as previously reported on somatic cells (Vandre et al., 1984), to avoid this diffuse cytoplasmic reactive material but we could not remove it. In any case, we obtained a bright labelling on several cell and chromosome structures. Thus, over the general cytoplasmic background, the spindle poles were the most intensely labelled structures in metaphase I spermatocytes (Fig.

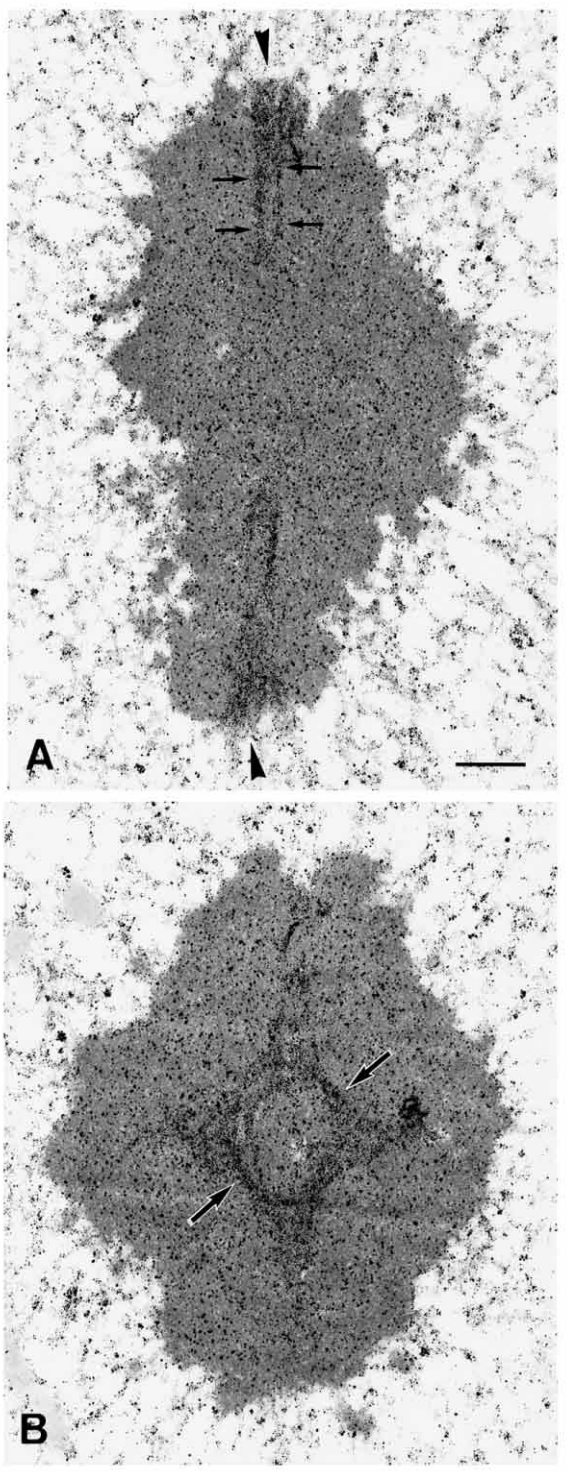


Fig. 3. Sister axes are not intimally associated. Electron micrographs of thin sections from two different silver-stained telocentric bivalents of *C. jucundus*. (A) The homologous kinetochores (arrowheads) and the slightly separated sister axes (arrows) emanating from the kinetochores are indicated. (B) The interstitial chiasma region with widely separated sister axes is indicated (arrows). Bar, 0.5 μm.

4K-M). Bivalents appeared negatively labelled except at their centromeres and inner regions. It must be emphasised that in all the analysed species, and independently of the presence of the phosphatase inhibitor microcystin in the fixing/lysis solution, the homologous centromeres were observed as bright fluorescent dots of similar size (Fig. 4I-M). Consequently, as previously reported for mitotic cells (Taagepera et al., 1995), MPM-2 phosphoepitopes are not sensitive to tension either in mitosis or meiosis I. In addition to centromeres, the interchromatid domain appeared preferentially and weakly labelled if compared with the intensity of labelling shown by the centromeres (Fig. 4I-M).

Because of the squashing protocol we have developed, metaphase I spermatocytes were frequently broken so that chromosomes were spreaded. This apparent disadvantage was useful since bivalents were more flattened and possessed a lower amount of diffusely labelled cytoplasm around them, and consequently, some details could be accurately observed. A careful observation at higher magnification demonstrated that the centromere MPM-2 labelling did not occupy all the centromere width, but that the round labelled region occupied a depression of its surface that appeared dimly fluorescent with DAPI (compare Fig. 5A-D). The labelling at the interchromatid domain was simultaneously observed in all bivalents but it was easily observed and understood in monochiasmate telocentric bivalents (Fig. 5). In these bivalents the interchromatid domain was occupied by a series of small round patches of similar size that frequently gave the impression of being straight lines with some interruptions (Fig. 5A-H). Nevertheless, in some species like *S. grossum*, these patches were not so tightly associated so that a real series of patches was observed (Fig. 6A,B). Independently of the degree of lateral association of these patches, this labelling was observed from the centromere to the chiasma site, and from this site towards the telomeres, although it never reached the chromosomal ends (Fig. 5). This pattern of MPM-2 labelling was reminiscent of that obtained by silver staining. However, the MPM-2 labelling at chromosome arms was always interrupted at chiasma sites where silver-stained sister axes appeared bifurcated but uninterrupted (compare Fig. 2B and 5C). This interruption of MPM-2 labelling at chiasma sites is clearly illustrated in Fig. 5 for bivalents with different chiasma locations. It is interesting to mention that this pattern of MPM-2 labelling was also observed with the 3F3/2 mAb, also recognising phosphoproteins, after the use of microcystin in the lysis/fixation solution.

MPM-2 phosphoepitopes are released from the interchromatid domain during the metaphase I/anaphase I transition

Next, we examined whether the MPM-2 labelling at the interchromatid domain disappeared or remained bound to the chromatids during the metaphase I/anaphase I transition. Spermatocytes at the onset of anaphase I were identified since, as described above, an enlargement of a rhomboid hole at the previous chiasma site began to be observed (Fig. 6C-J). In these bivalents the intensity of MPM-2 labelling at centromeres was similar to that found at metaphase I. Nevertheless, most bivalents did not show significant labelling at the interchromatid domain even although the sister chromatids were still closely attached along most of their length (Fig. 6G). Only a small proportion of these early anaphase I bivalents presented some MPM-2 labelling

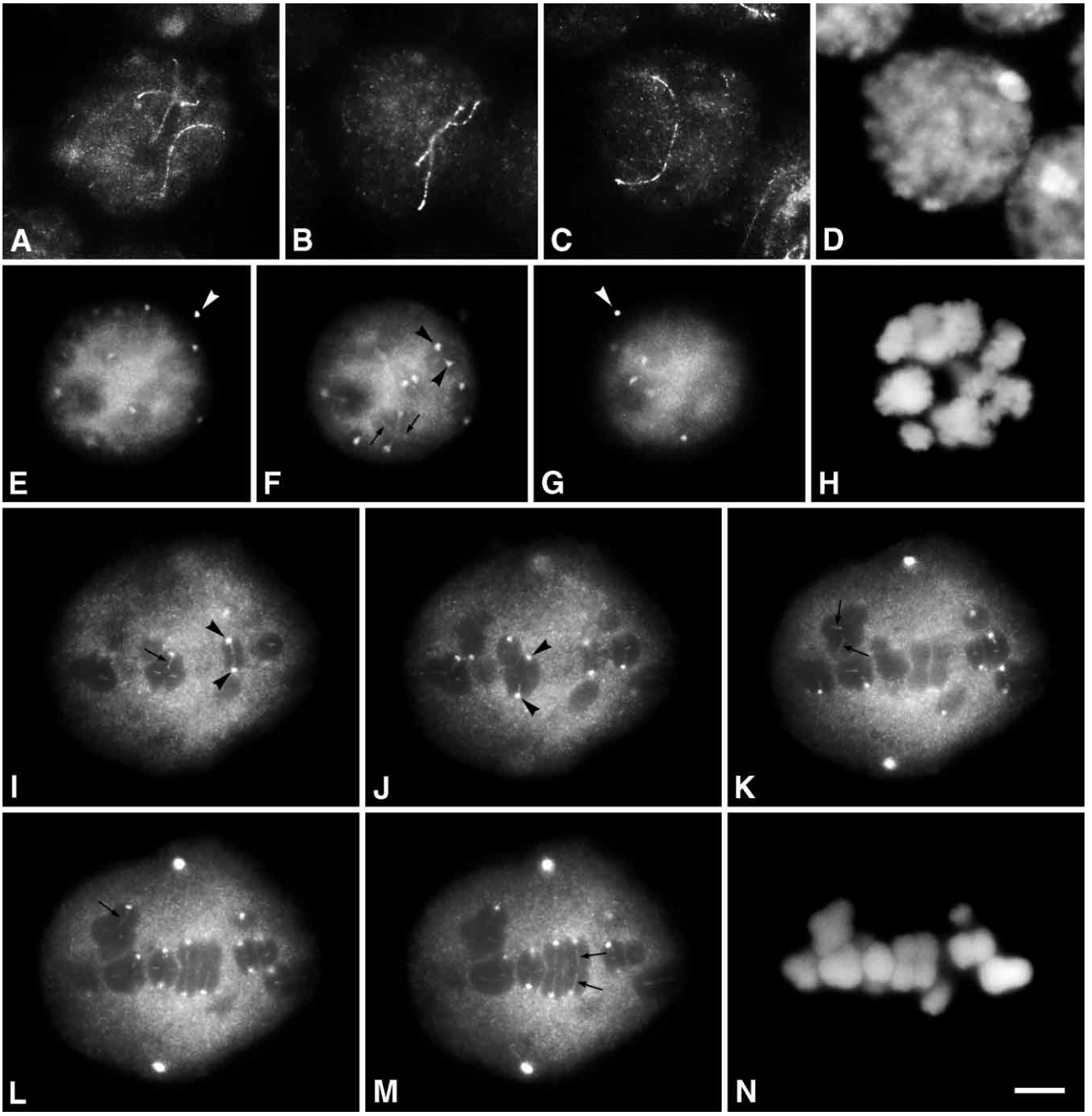


Fig. 4. Immunofluorescence labelling of MPM-2 phosphoepitopes in pachytene, diakinesis and metaphase I spermatocytes of *E. plorans*. (A-C, E-G, and I-M) MPM-2 labelling. (D,H,N) DAPI counterstaining. (A-C) Single focal planes of three different pachytene spermatocytes. A series of aligned fluorescent spots reminiscent of SCs are observed in faint labelled nuclei. (E-G) Three focal planes throughout a diakinesis spermatocyte. The nucleoplasm, the separating centrosomes (white arrowheads in E and G), and the homologous centromeres (black arrowheads in F) are brightly labelled. The interchromatid domain shows a faint labelling (black arrows in F). (I-M) Five focal planes throughout a metaphase I spermatocyte. In addition to the spindle poles (observed in K-M), the homologous centromeres facing opposite poles are intensely labelled (arrowheads in I and J). Some faint lines at the interchromatid domains are also labelled (arrows in I and K-M). Bar, 10 μm.

between sister chromatid arms (Fig. 6C,E). In those bivalents the labelling at the interchromatid domain was visualised as round patches of different size, some of them larger than those observed at metaphase I (compare Fig. 6A,E). In more advanced anaphase I spermatocytes the homologues continued their separation, and the MPM-2 labelling was not observed either associated to the chromatid surfaces or inside them (Fig. 6I). These findings support that MPM-2 does not recognise the axes since these structures are observed by silver staining in an internal location in relation to the chromatid width (compare Figs 6I and 2I). In segregating homologues MPM-2 labelled two round and closely

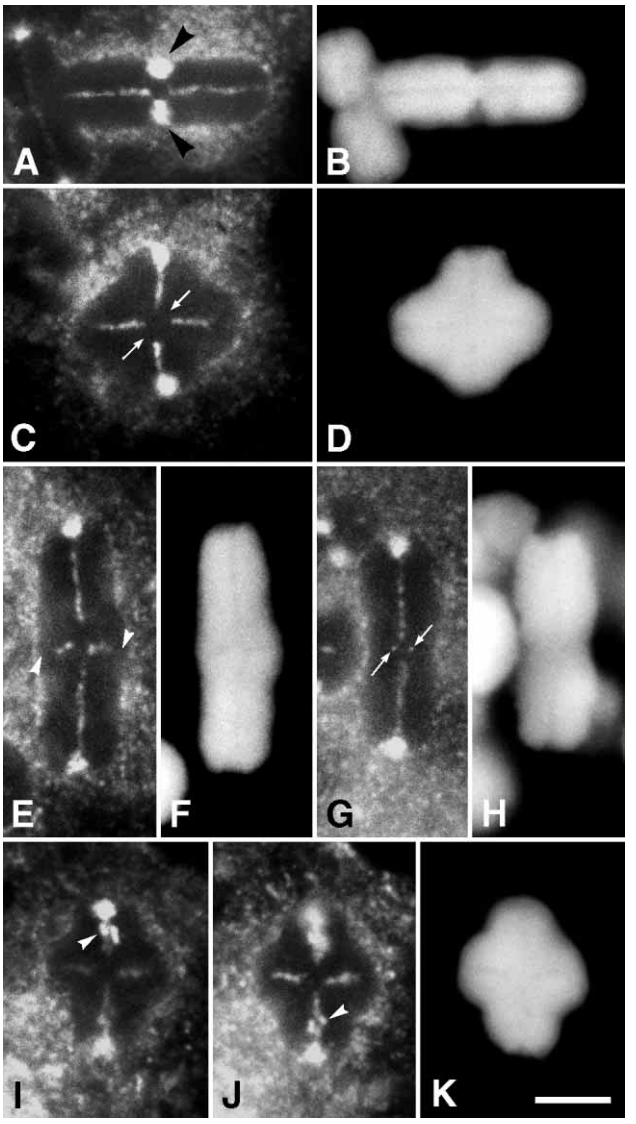


Fig. 5. Selected metaphase I monochiasmate telocentric bivalents of *E. plorans* labelled with MPM-2 (A,C,E,G,I-J), and counterstained with DAPI (B,D,F,H,K). In all bivalents the homologous centromeres (arrowheads in A), as well as the interchromatid domains, are labelled. Note that this labelling interrupts at chiasma sites independently of their location (arrows in C), and that it never reaches the distal chromatid ends (arrowheads in E). In bivalents with a distal chiasma the labelling between the chiasma and the chromatid ends may be so reduced that only small MPM-2 spots are detected (arrows in G). (I and J) Two focal planes through a nucleolar bivalent show the labelling at nucleolar organiser regions (arrowheads). Bar, 4 μm.

associated dots at the centromere region representing sister kinetochores as detected by silver staining (compare Figs 6K and 2I). Although sister kinetochores were also observed in some early anaphase I chromosomes (Fig. 6E), is in a more advanced stage when they became clearly discernible (Fig. 6I,K). The MPM-2 labelling at sister kinetochores declined with progression through anaphase I, and each spindle pole separated into two ones (not shown). By telophase I only the poles and the midbody were labelled.

The interchromatid domain of metaphase I univalents does not show MPM-2 phosphoepitopes

We then focused our attention on the possible MPM-2 labelling in two different kinds of univalents at metaphase I. We first examined the labelling in the univalent X chromosome commonly found in males of most grasshopper species. In all the analysed species MPM-2 revealed a centromeric dot similar to those found at homologous centromeres (Fig. 7A,D). An MPM-2 labelling at chromosome arms similar to that observed at the interchromatid domain of bivalents was never observed. In some species both sister chromatids appeared brightly labelled except at the pericentromeric region (Fig. 7A). In this regard, it is worth mentioning that this bright labelling also persisted along the chromatid arms through anaphase I. Therefore, this MPM-2 labelling seems to be related to the peculiar chromatin structure found in these chromosomes. In the X univalent of other species only the centromere region appeared labelled (Fig. 7D).

The second kind of univalent studied was a B chromosome found in some individuals of *E. plorans* (Henriques-Gil et al., 1984). B-Chromosomes are additional chromosomes that appear in the standard complement of some individuals in a wide range of species. In most species their origin is unknown, and they are dispensable for correct growth, development, and reproduction (Jones and Rees, 1982). The B chromosome only showed MPM-2 labelling at its centromere region (Fig. 7G). Interestingly, in silver-stained preparations the X (Fig. 7C,F) and the B-univalents (Fig. 7I) presented separated sister axes except at the centromere and telomeres. Consequently, the MPM-2 labelling does not correspond to the chromatid axes. Thus, although sister chromatids are attached, no MPM-2 labelling is found between them.

Mitotic chromosomes do not show MPM-2 phosphoepitopes at the interchromatid domain

We also tested the presence of MPM-2 phosphoepitopes at the interchromatid domain of mitotic chromosomes. For such purpose we employed mitotic cells from two different sources: neuroblast somatic cells and spermatogonial cells. In both kinds of mitotic cells the spindle poles were the brighter structures. Additionally, two intensely labelled dots at the centromere region of each chromosome, representing sister kinetochores facing opposite poles, were detected (Fig. 8). Interestingly, labelling between sister arms was never observed. Thus, MPM-2 phosphoepitopes at the interchromatid domain in metaphase I bivalents are not present in mitosis and consequently they seem to be exclusive of meiotic cells.

DISCUSSION

Homologue separation during the metaphase I/anaphase I transition

Our study on the behaviour of the chromatid axes through metaphase I in different grasshopper species offers some important clues related to homologue separation. In early metaphase I spermatocytes the sister axes are closely associated along their length except at chiasma sites where they bifurcate. However, in late metaphase I spermatocytes the sister axes are separating or are completely separated although the

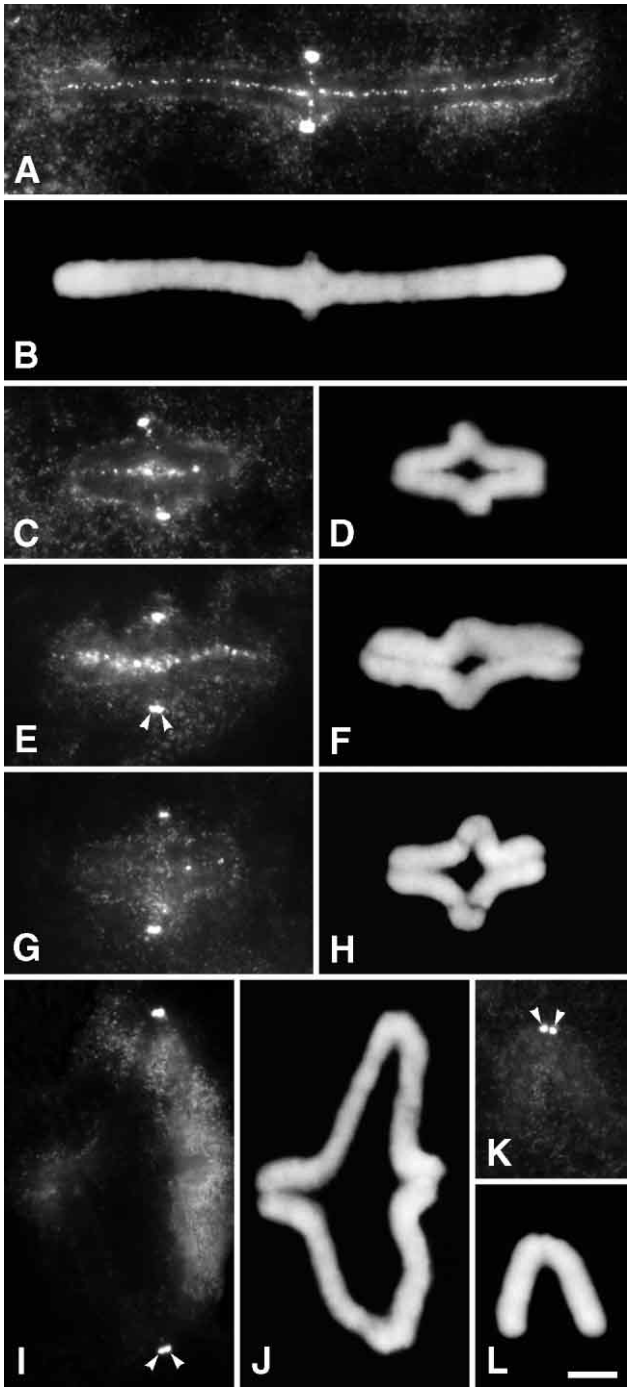


Fig. 6. Loss of MPM-2 phosphoepitopes at the interchromatid domain during the metaphase I/anaphase I transition in *S. grossum*. Selected bivalent at metaphase I, and separating homologues at anaphase I labelled with MPM-2 (A,C,E,G,I,K), and counterstained with DAPI (B,D,F,H,J,L). (A) At metaphase I MPM-2 labels the homologous centromeres and the interchromatid domain. (C and E) At early anaphase I the homologues begin to separate so that a 'rhomboid hole' appears at previous chiasma sites. The centromere labelling persists but the interchromatid domain between the centromeres and the chiasma is no longer detected, while the regions between the chiasma and telomeres progressively lose the labelling. In E sister kinetochores (arrowheads) are observed in the lower centromere. (G) At early anaphase I when homologues still remain attached along most of their length the MPM-2 labelling at the arms has disappeared. (I and K) In a more advanced anaphase I stage only sister kinetochores (arrowheads) remain labelled. Bar, 5 μm.

bivalent morphology does not indicate signs of homologue separation. This separation of sister axes indicates a change of chromosome structure from a meiotic organisation to a mitotic one (Rufas et al., 1987; Suja et al., 1991). Thus, the metaphase I bivalent is not so static, from a structural point of view, as conventional stainings could suggest. It could be argued that the closely association between sister axes found in early metaphase I bivalents is responsible for the maintenance of the bivalent integrity by holding sister arms together. Nevertheless, several findings argue against such possibility. First, although by light microscopy the sister axes appear closely associated, the observation of serial sections of silver-stained bivalents by EM clearly shows that they are separated even in early metaphase I bivalents. Second, the sister axes found in the X and B-univalents during early metaphase I are always separated although the chromatids remain associated. Thus, we propose that a first step in a sequence of events leading to the loss of arm cohesion implies a change of chromosome structure mediated by the separation of sister axes. Consequently, the chromatid axes are not themselves directly responsible for the maintenance of arm cohesion, but their separation precedes the loss of cohesion between sister chromatids.

Once sister axes have separated, the homologues may initiate their separation. The first evidence that homologue separation has been triggered is the appearance of a 'rhomboid hole' at the chiasma site. This 'hole' obviously appears because of splitting forces exerted on homologous centromeres, and the concomitant loss of cohesion factor(s) located between sister chromatids. The diameter of the 'hole' widens upon progressive homologue separation so that finally the sister chromatids only remain attached by their telomeres. Our results also demonstrate that homologue separation is not a synchronous process as it is frequently reported to occur. The signal(s) triggering the onset of anaphase I, and probably allowing the loss of cohesive proteins, may simultaneously act on all bivalents. However, the rate of homologue separation depends on intrinsic chromosome factors such as their size, and bivalent characteristics such as the number and location of chiasmata.

Centromere and sister-kinetochore cohesion at meiosis I

Our present and previous findings obtained by silver staining by light and EM show that sister kinetochores are tightly associated during early metaphase I, while during late metaphase I the separation of sister axes allows their individualisation (Rufas et al., 1989, 1994; Suja et al., 1991, 1992). We have obtained the same result with MPM-2. Thus, a single bright spot is observed at homologous centromeres of early metaphase I bivalents, whereas two fluorescent spots are observed at each centromere from late metaphase I to early anaphase I. Consequently, as previously reported for mitotic mammalian and plant chromosomes, MPM-2 detects kinetochores but not the centromere as a whole chromosome domain.

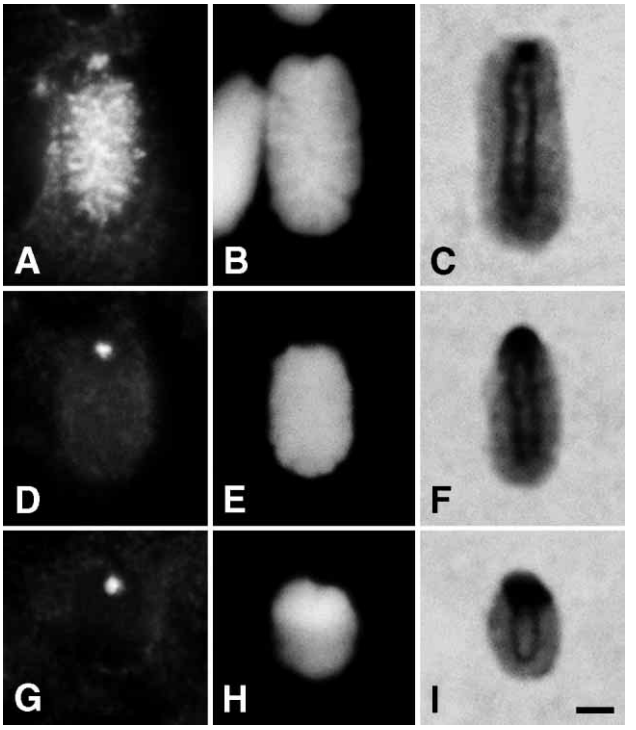


Fig. 7. Comparison between the immunofluorescence labelling of MPM-2 phosphoepitopes and silver staining in metaphase I univalent sex chromosomes of *C. jucundus* (A-C) and *E. plorans* (D-F), and a B-univalent of *E. plorans* (G-I). (A, D, and G) MPM-2 labelling. (B,E,H) DAPI counterstaining. (C,F,I) Silver staining. (A) The closely associated sister kinetochores are intensely labelled as well as the chromatids except at the pericentromeric region and their peripheral margins. (D and G) In both the X and B-univalents of *E. plorans* only the centromere region appears labelled. (C,F,I) In all these univalents silver staining demonstrates the associated sister kinetochores and separated sister axes. Bar, 2 μm.

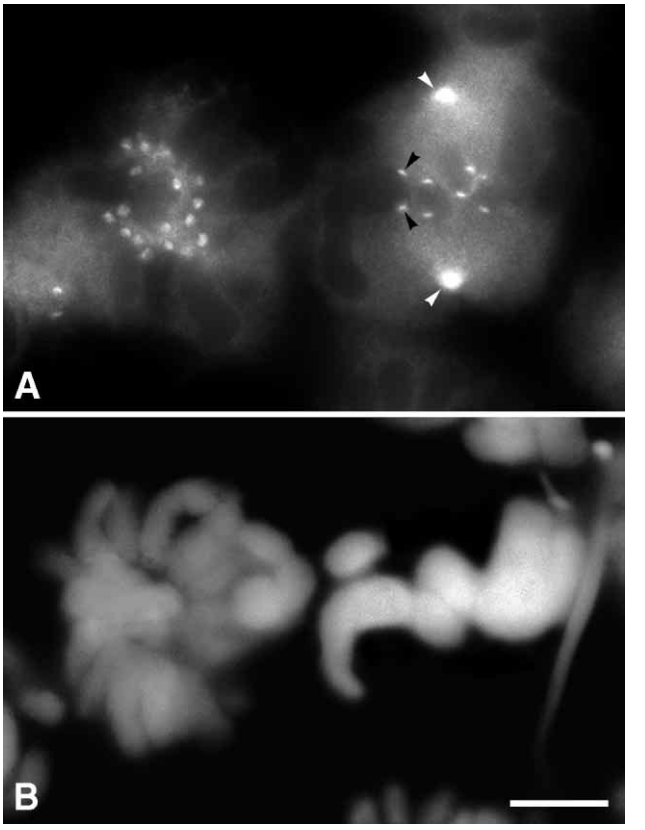


Fig. 8. Immunofluorescence labelling of MPM-2 phosphoepitopes in a spermatogonial metaphase of *P. conica*. (A) MPM-2 labelling. (B) DAPI counterstaining. The interchromatid domain is not labelled as observed in either an end view (left cell) or a side view (right cell). By contrast, the spindle poles (white arrowheads) and pairs of sister kinetochores (black arrowheads) are brightly labelled. Bar, 10 μm.

The presence of individualised sister kinetochores during late metaphase I and anaphase I has also been observed in different organisms (Rufas et al., 1989, and references therein). The close association of sister kinetochores during prometaphase and early metaphase I indicates that sister-kinetochore cohesion is needed to allow the correct biorientation of bivalents. Once all bivalents are aligned at the metaphase plate sister-kinetochore cohesion is lost and consequently, as observed by EM (Goldstein, 1981; Rufas et al., 1994), both kinetochores individualise. Thus, while centromere cohesion persists until anaphase II, sister-kinetochore cohesion is released in late metaphase I.

Telomere cohesion

Another interesting result refers to the behaviour of telomeres during the metaphase I/anaphase I transition. We have found that telomeric chromatin strands appear as the last point of contact between sister chromatids during anaphase I. The axes are not involved in these telomere associations that may represent the last chromatin domains to resist splitting forces so that stretched thin strands are observed (Fig. 1F,G and 2I). Similar telomeric strands have been ultrastructurally observed in separating homologues in the crane fly *Pales ferruginea* at early anaphase I (Fuge, 1978), and during mitotic anaphase (Wilson, 1925; Lima-de-Faria and Bose, 1962). We have observed by FISH that the telomeric DNA repeats are not present in such telomere strands (J. A. Suja et al., unpublished). Thus, we propose that some non-telomeric DNA located at telomeres may be involved in such distal strands and consequently in telomere cohesion. Furthermore, some telomeric proteins could also mediate such telomeric associations. For instance, the absence of the budding yeast telomere protein Ndj1/Tam1, only present at the telomeres of pachytene bivalents, increases the frequency of precocious sister-chromatid separation and nondisjunction during meiosis I, so that a role for this meiotic protein in telomere cohesion has been suggested (Conrad et al., 1997; Chua and Roeder, 1997). Moreover, Cenci et al. (1997) have reported that mutations in the *UbcD1* gene of *Drosophila*, a gene that encodes an ubiquitin-conjugating (E2) enzyme, prevents telomere separation during mitotic anaphase and anaphase I. These findings and our present observations point to an unsuspected role of the telomere in the release of arm cohesion during meiosis I.

Involvement of MPM-2 phosphoepitopes in arm cohesion in bivalents

The most relevant result obtained with MPM-2 refers to the labelling found at the interchromatid domain in diakinesis and

metaphase I bivalents. Interestingly, we have obtained the same labelling with the 3F3/2 mAb. In this regard, Nicklas et al. (1995) reported some 3F3/2 labelling along the arms of metaphase I bivalents in another grasshopper species. It has been reported that topo IIα, a component of the chromosome scaffold, appears in the SC lateral elements (Moens and Earnshaw, 1989), and that it is the major chromosome protein recognised by MPM-2 in isolated mammalian mitotic chromosomes (Taagepera et al., 1993). Thus, it is tempting to speculate that the MPM-2 labelling that we have observed at the interchromatid domain really reflects the distribution of topo IIα at chromatid axes. This represents an interesting possibility since it has been proposed that phosphorylated topo II plays an important role during the separation of sister chromatids during mitosis (for review see Holloway, 1995; Warburton and Earnshaw, 1997) and meiosis (Cobb et al., 1997). However, we have obtained several results that argue against such possibility. First, in metaphase I bivalents sister axes bifurcate at chiasma sites appearing as thin lines whereas the MPM-2 labelling always interrupts at those sites. Second, we have never observed a parallel separation of the MPM-2 labelling at the interchromatid domain similar to the separation of axes at late metaphase I. Third, while sister axes are separated in X and B-univalents, MPM-2 either labels almost all the chromatid width or does not label at all. Fourth, while the axes are internally located inside the anaphase I chromatids, MPM-2 does not label the chromatid arms. Despite these reasons, we do not rule out that MPM-2 may detect a topo II population not involved in the chromatid axis since, as reported in *Drosophila*, several chromosomal populations of topo II might exist (Swedlow et al., 1993).

We have observed that MPM-2 labels the interchromatid domain in bivalents but not in mitotic chromosomes. Thus, the MPM-2 phosphoproteins recognised at the interchromatid domain seem to be only expressed during meiosis. Since in our materials MPM-2 seems to label some SC component at pachytene (Fig. 4A-D), and the interchromatid domain in bivalents until the metaphase I/anaphase I transition, it is tempting to suggest that MPM-2 could be detecting some SC component(s) that acts as a cohesive protein holding sister chromatids together. For instance, our results are similar to those reporting the presence of the SC lateral element Cor1/SCP3 protein, a phosphorylated protein (Lammers et al., 1995), in metaphase I mouse bivalents (Moens and Spyropoulos, 1995). These authors proposed a role for this protein in arm cohesion because it was lost from arms during anaphase I. Moreover, it has been recently demonstrated that sister-chromatid cohesion during the budding yeast meiosis requires a Mek1-dependent phosphorylation of the SC lateral element protein Red1 (Bailis and Roeder, 1998). Thus, we propose that the MPM-2 phosphoprotein(s) detected at the interchromatid domain in grasshopper bivalents could reveal some SC lateral element protein(s) associating the underlying sister axes up to late metaphase I.

Arm cohesion in univalents

The univalents we have studied, at least the X chromosome, forms an axial element that appears during leptotene and persists up to pachytene (Solari and Counce, 1977). Assuming that MPM-2 phosphoproteins, similarly as it has been observed for Cor1/SCP3 (Moens and Spyropoulos, 1995), could be present at axial elements, their absence at the interchromatid domain in metaphase I univalents suggests that if a SC is not formed the MPM-2 phosphoproteins would be lost before metaphase I. The loss of these cohesive proteins would promote the separation of sister axes as is observed in metaphase I univalents. However, the question that immediately arises is how arm cohesion is maintained in univalents up to metaphase I. One possibility is that proteins similar to those responsible for arm cohesion in mitotic chromosomes, and consequently not related to SC elements, could maintain arm cohesion in univalents.

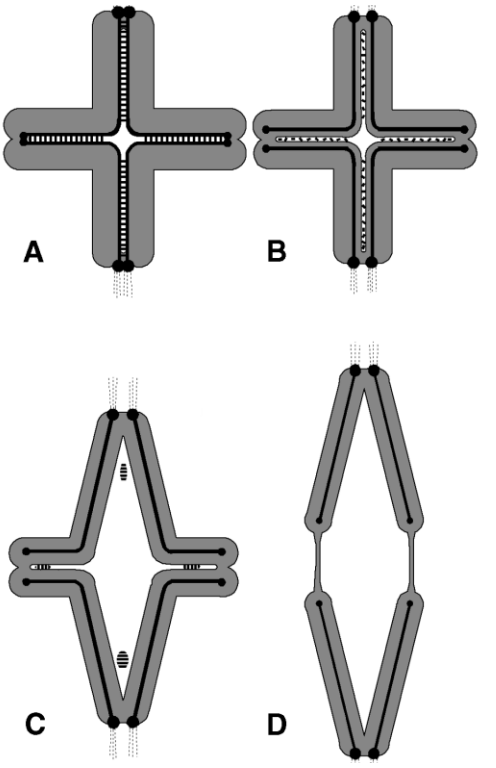


Fig. 9. Model for the release of arm cohesion during the metaphase I/anaphase I transition. (A) In early metaphase I bivalents the sister axes (black solid lines) are associated by MPM-2 phosphoproteins (hatched lines between the axes). (B) In late metaphase I bivalents MPM-2 phosphoproteins begin to be dephosphorylated (small patches at the interchromatid domain) so that sister axes separate, as well as sister kinetochores (round black spheres at homologous centromeres). (C) At the onset of anaphase I the homologues initiate their separation and a rhomboid hole at the previous chiasma site begins to be observed. (D) Early anaphase I situation where the homologues remain connected at sister chromatid ends by thin chromatin strands. Note that the axes ends are not involved in such telomere strands. See text for a more detailed description.

Model for the release of arm cohesion in bivalents

Taking into account the cytological observations on homologue separation, the behaviour of sister axes, and the pattern of MPM-2 labelling at the interchromatid domain, it is tempting to propose a ‘working’ model for the release of arm cohesion during meiosis I (Fig. 9). In early metaphase I bivalents MPM-2 phosphoproteins could function as cohesive ‘staples’ facilitating the association of sister axes (Fig. 9A). Once tension through the chromatid arms, generated by splitting

forces acting on homologous centromeres, is attained in all bivalents, the metaphase/anaphase checkpoint would be turned off. Similarly to what happens in budding yeast (Ciosk et al., 1998), the initial APC-mediated proteolysis of an anaphase inhibitor could finally lead to the dephosphorylation and/or degradation of the MPM-2 cohesive 'staples' so that sister axes would loss their association. This speculation is supported by the fact that phosphatase inhibitors such as okadaic acid do not allow the initiation of anaphase in both mitosis and meiosis I (for review and references see Holloway, 1995; Yanagida, 1995). Moreover, it is known that some of such toxins inhibit serine-threonine-specific protein phosphatases, and in this sense MPM-2 phosphoproteins could be a target for these phosphatases since this antibody seems to recognise phosphothreonine containing epitopes (for references see Renzi et al., 1997). Concomitantly, chromosome structure would sequentially change from a meiotic to a mitotic organisation and sister-kinetochore cohesion would be released (Fig. 9B). Because of the ongoing dephosphorylation of the MPM-2 cohesive phosphoproteins arm cohesion would be released, and homologues could initiate their separation giving rise to the visualisation of the rhomboid hole (Fig. 9C). Finally, only thin chromatin strands would associate sister telomeres (Fig. 9D). These telomeric strands would be then disrupted by the dissociation and/or degradation of putative telomere cohesive proteins. Therefore, this 'working' model implies that during the metaphase I/anaphase I transition there is a sequential loss of sister-kinetochore, arm and telomere cohesions. Obviously further research is needed to better define the mechanisms regulating arm cohesion both in univalents and bivalents.

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