Chromosoma (1992) 101:493–501

CHROMOSOMA

Involvement of chromatid cohesiveness at the centromere and chromosome arms in meiotic chromosome segregation: A cytological approach

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Received August 1, 1991 / in revised form January 20, 1992
Accepted January 31, 1992 by D.P. Bazett-Jones

Abstract. Kinetochores and chromatid cores of meiotic chromosomes of the grasshopper species *Arcyptera fusca* and *Eyprepocnemis plorans* were differentially silver stained to analyse the possible involvement of both structures in chromatid cohesiveness and meiotic chromosome segregation. Special attention was paid to the behaviour of these structures in the univalent sex chromosome, and in B univalents with different orientations during the first meiotic division. It was observed that while sister chromatids of univalents are associated at metaphase I, chromatid cores are individualised independently of their orientation. We think that cohesive proteins on the inner surface of sister chromatids, and not the chromatid cores, are involved in the chromatid cohesiveness that maintains associated sister chromatids of bivalents and univalents until anaphase I. At anaphase I sister chromatids of amphitelically oriented B univalents or spontaneous autosomal univalents separate but do not reach the poles because they remain connected at the centromere by a long strand which can be visualized by silver staining, that joins stretched sister kinetochores. This strand is normally observed between sister kinetochores of half-bivalents at metaphase II and early anaphase II. We suggest that certain centromere proteins that form the silver-stainable strand assure chromosome integrity until metaphase II. These cohesive centromere proteins would be released or modified during anaphase II to allow normal chromatid segregation. Failure of this process during the first meiotic division could lead to the lagging of amphitelically oriented univalents. Based on our results we propose a model of meiotic chromosome segregation. During mitosis the cohesive proteins located at the centromere and chromosome arms are released during the same cellular division. During meiosis those proteins must be sequentially inactivated, i.e. those situated on the inner surface of the chromatids must be eliminated during the first meiotic division while those located at the centromere must be released during the second meiotic division.

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Introduction

Sister chromatids of mitotic chromosomes remain closely associated along their entire length until their disjunction at the onset of anaphase. Poleward forces acting on the centromere are not solely responsible for chromatid separation since chromatids from acentric fragments separate synchronously with chromatids of normal centric chromosomes (Carlson 1938). This classical observation indicates that factors present both at the centromere and along the chromosome arms account for the cohesiveness between sister chromatids until anaphase. However, as has been pointed out, this feature of chromosome behaviour has been overlooked in the past (Maguire 1990).

The centromere plays a major role during cell division during which it functions in connecting sister chromatids and as the interaction site for spindle microtubules. This last function is accomplished by the kinetochore, a proteinaceous structure located on the lateral surface of the centromere. However, the terms centromere and kinetochore have been used synonymously by many investigators resulting in considerable confusion (Earnshaw 1991a). Ultrastructural studies and the use of antibody probes against centromere-associated proteins have allowed the subdivision of the centromere into three structurally and functionally different domains designated the kinetochore, and the central and pairing domains (Earnshaw and Rattner 1989; Rattner 1991). The kinetochore domain consists of both the kinetochore and the subjacent chromatin upon which it rests. The kinetochore captures and stabilises spindle microtubules during prometaphase and seems to be the probable location of a mechanochemical motor for anaphase chromosome movement (reviewed in Nicklas 1988; Brinkley 1990; Pluta et al. 1990). The central domain represents the bulk of the area of the centromere. The pairing domain is the site of interaction between sister chromatids (Rattner 1991). It has been suggested that the centromere proteins recently found in these domains could be involved in sister chromatid cohesiveness at the centromere (Cooke

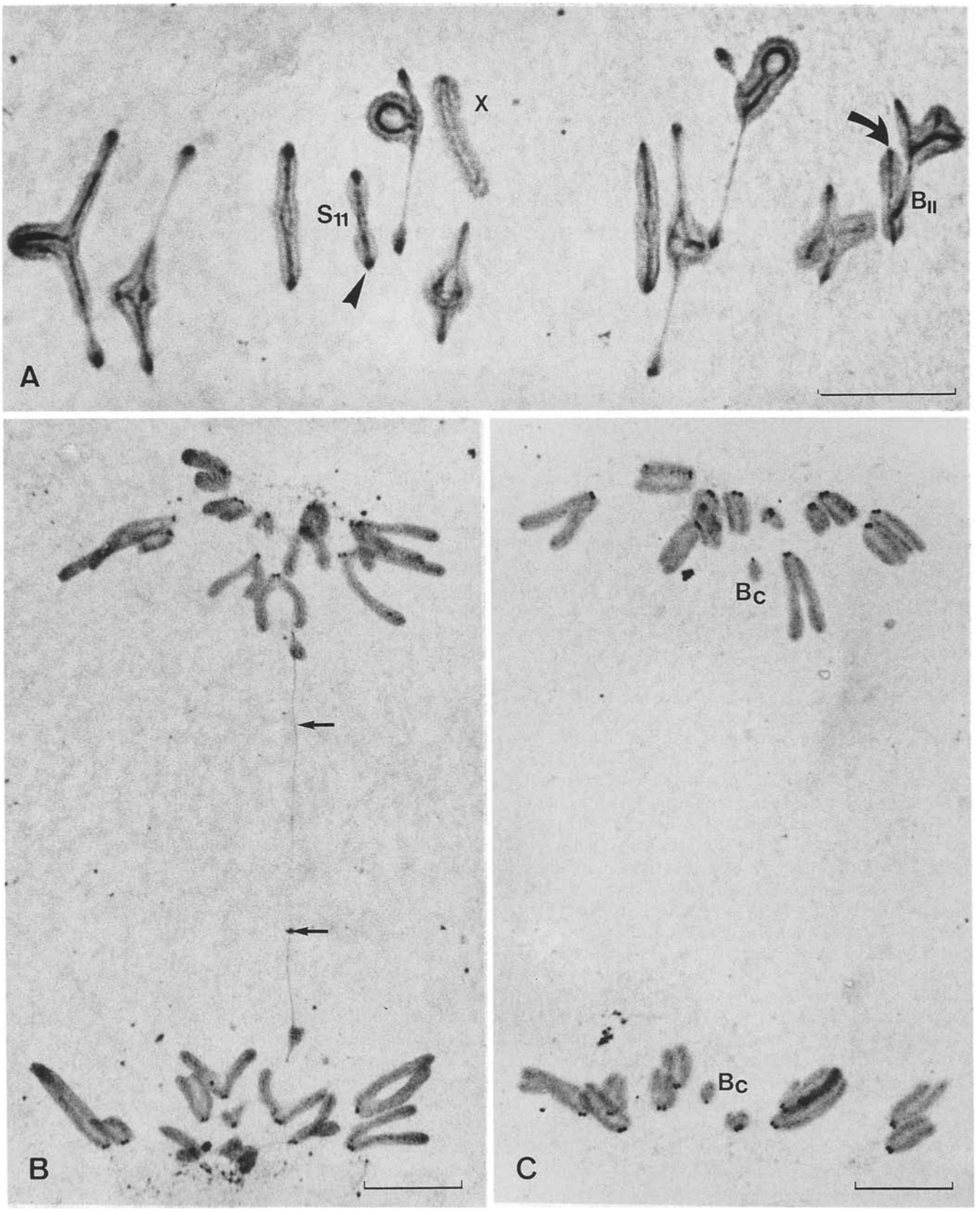

Fig. 1 A–C. First meiotic division spermatocytes of *Arcyptera fusca*. **A** Metaphase I with a B bivalent (B_{II}). Kinetochores of B chromosomes (*arrow*) are smaller than those of autosomes (*arrowhead*) while the chromatid cores have the same width. The chromatid cores of homologues are associated except where chiasmata occur. In contrast, the cores of the sex chromosome (X) are individualised except at their ends. **B** Anaphase I with one B univalent. The B chromatids have almost reached opposite poles but are still connected by a silver-stained strand (*arrows*) that joins stretched sister kinetochores. Half-bivalents present two round sister kinetochores. **C** Anaphase I. Each cellular pole shows one B chromatid (B_c). Note the smaller size of the B chromatid kinetochore compared with the sister kinetochores present on each autosome. Bars represent 10 μm

et al. 1987; Rattner et al. 1988; Earnshaw and Cooke 1989, 1991). On the other hand, Rattner et al. (1988) have identified some proteins localised in patches along the entire length of the junction between sister chromatids at metaphase. These authors have proposed that these 'junction patch antigens' could play a role in the maintenance of sister chromatid cohesiveness.

These results refer to the mitotic chromosome where the separation of sister chromatids at anaphase implies their individualization at the centromere and all along the chromosome arms. During meiosis, however, the sequence of sister chromatid separation is different. Sister chromatid cohesiveness at the chromosome arms is lost at anaphase I, when homologues segregate to opposite poles, while their connection at the centromere persists until anaphase II. Tests for the presence of proteins similar to those found in mitosis have not yet been carried out on meiotic chromosomes.

The ultrastructural morphology of kinetochores in chromosomes with a localised centromere fits into two distinct classes: the "trilaminar disk", which is characteristic of mammals, and the "ball and cup" commonly found in higher plants and certain insects, i.e. in grasshoppers (Esponda 1978). Rufas et al. (1987) have developed a silver impregnation technique that stains differentially the ball and cup kinetochores and the chromatid cores of grasshopper meiotic chromosomes. Recently Rufas et al. (1989) have analysed the morphological changes observed in kinetochores during both meiotic divisions. These authors have proposed that these morphological changes could be caused by tensions created by the interaction between the centromere region and the spindle microtubules.

In this study we analysed the meiotic behaviour of kinetochores and chromatid cores in grasshopper univalents. For this purpose we focussed our attention on the sex chromosome, which undergoes reductional segregation during the first meiotic division, and on two different kinds of B univalents. The B univalents found in *Eyprepocnemis plorans* exhibit preferential reductional segregation during the first meiotic division (Suja et al. 1989; Rufas et al. 1989), while those found in *Arcyptera fusca* show equational segregation (Suja et al. 1991).

Materials and methods

Adult males of *E. plorans* and *A. fusca* collected from natural populations at Salobreña (Granada, Spain) and the Alpes Maritimes (France) respectively were used for the present study.

Testes were removed and fixed in 3:1 ethanol:acetic acid and stored at −20° C. Lacto-propionic orcein staining was employed to select individuals carrying B chromosomes. The male karyotype of both species is composed of 11 pairs of autosomes plus a single sex (X) chromosome. All chromosomes, including the B chromosomes, present terminal centromeres.

For the silver staining technique single follicles were squashed in a drop of 50% acetic acid. Coverslips were removed after freezing slides in liquid nitrogen and the preparations were then air-dried. Slides were incubated in double strength saline citrate solution (2×SSC; 0.3 M NaCl, 0.03 M sodium citrate) at 60° C for 15 min, rinsed thoroughly in tap water and air-dried. A drop of an $AgNO_3$ solution (0.1 g of $AgNO_3$ in 0.1 ml of distilled water adjusted to pH 3 with formic acid) was placed on each slide, then covered with a coverslip and incubated in a moist chamber at 80° C. After 3 min the degree of staining was monitored under the light microscope. Finally, slides were rinsed in tap water, air-dried and mounted in Euparal.

Results

After silver staining, black round structures located terminally on dark yellow stained chromosomes are observed in both species. Electron microscopy observations demonstrate that these round silver-stained structures observed at the light microscope level are the ball and cup kinetochores (in preparation).

At metaphase I, in both species, each bivalent presents two kinetochore structures, one per half-bivalent, oriented to opposite poles (Figs. 1 A and 3). These round

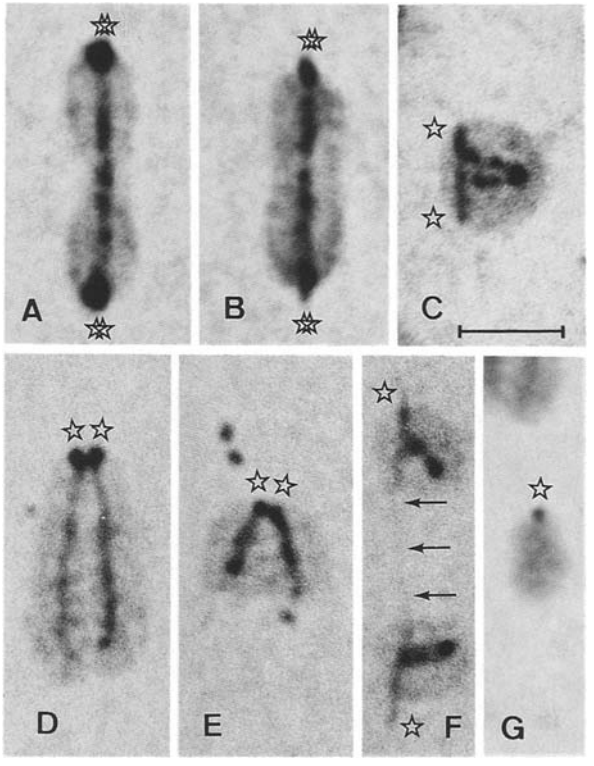

Fig. 2 A–G. Selected chromosomes from metaphase I (**A–C**) and anaphase I (**D–G**) spermatocytes of *Arcyptera fusca*. **A** Autosomal and **B** B bivalents with distal chiasma. Note the associated arrangement of chromatid cores within each homologue. The joined sister kinetochores in the B bivalent are smaller than those of the autosomal bivalent. *Superimposed stars* denote associated sister kinetochores. **C** B univalent. While sister chromatids are associated, chromatid cores are individualised except at their distal ends. Cores are continuous with the stretched sister kinetochores (*stars*) that are connected by a thin silver-stained strand. **D** Autosomal and **E** B half-bivalents. Note that B kinetochores are smaller than those of the autosome, while the width of the centrally located chromatid cores is the same. Individualised kinetochores are marked with *stars*. **F** Migrating B chromatids from a lagging B univalent. Note that the stretched sister kinetochores (*stars*) are connected by a thin silver-stained strand (*arrows*). **G** B chromatid that has reached the pole at anaphase I. Note the small sized kinetochore (*star*). Bar represents 3 μm

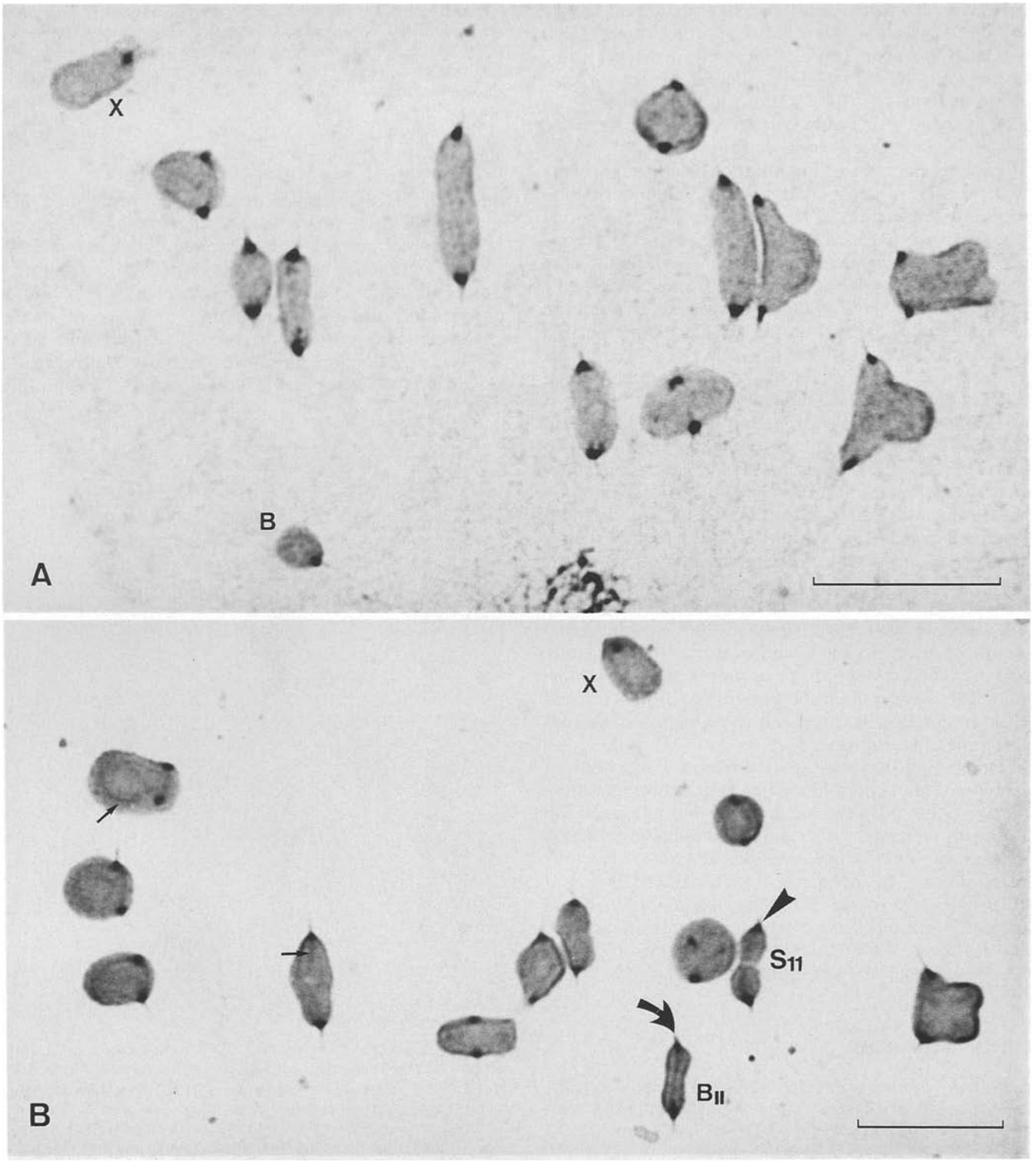

Fig. 3A, B. First meiotic division spermatocytes of *Eyprepocnemis plorans*. **A** Metaphase I with one reductionally segregating B chromosome (*B*). The sex chromosome is marked (*X*). Bar represents 10 μm. **B** Metaphase I with one B bivalent (B_{II}). The size of joined sister kinetochores of each B chromosome (*arrow*) is similar to that of the autosomes (*arrowhead*). The chromatid cores are slightly contrasted in some bivalents (*small arrows*) Bar represents 10 μm

to cone-shaped structures represent the two closely associated sister kinetochores. In *A. fusca,* in addition to the kinetochores, silver staining reveals the chromatid cores. These chromosomal axes run along the chromatids from the kinetochores to the telomeres although they do not reach the distal tips. At metaphase I the chromatid cores within a homologue are associated. Only where proximal or interstitial chiasmata occur, are four cores (one per chromatid) clearly discerned (Fig. 1 A). This arrangement implies that the cores are peripherally located with

respect to the width of the chromatids (for detailed description see Rufas et al. 1987).

The univalent sex chromosome shows associated sister chromatids, syntelic orientation and paired sister kinetochores at metaphase I (Figs. 1A and 3). In *A. fusca* it is clearly observed that while the chromatid cores of metaphase I half-bivalents are associated, those of the sex chromosome are individualised except at their distal ends (Fig. 1A).

B chromosomes in A. fusca

At metaphase I B univalents of *A. fusca* always appear amphitelically oriented in the equatorial plate with aligned sister chromatids. After silver staining their sister kinetochores appear stretched and separated facing opposite poles (Fig. 2C). In addition, a thin silver-stained strand joining the sister kinetochores is observed (Fig. 2C). Chromatid cores (that are thinner than and continuous with kinetochores) are individualised except at their distal ends (Fig. 2C).

B bivalents are commonly formed when two B chromosomes are present in a spermatocyte (Fig. 1A). As in autosomal chromosomes chromatid cores of B homologues are associated at metaphase I. A striking characteristic is that B kinetochores are smaller than those of autosomes and the sex chromosome (Fig. 1A; compare Fig. 2A and 2B).

At anaphase I the sex chromosome and autosomal half-bivalents separate their chromatid arms and show two round and individualised sister kinetochores. In both types of chromosome, chromatid cores are centrally located within the separated chromatids (see selected half-bivalent of Fig. 2D). At the onset of anaphase I B univalents with associated sister chromatids lag at the equatorial plate. These univalents exhibit stretched sister kinetochores. As anaphase I proceeds it is frequently observed that sister chromatids of B univalents begin to segregate in parallel towards opposite poles. In this case each chromatid posesses a stretched kinetochore continuous with the chromatid core. Moreover, both kinetochores are connected by a thin silver-stained strand (Fig. 2F). At late anaphase I B chromatids are still con-

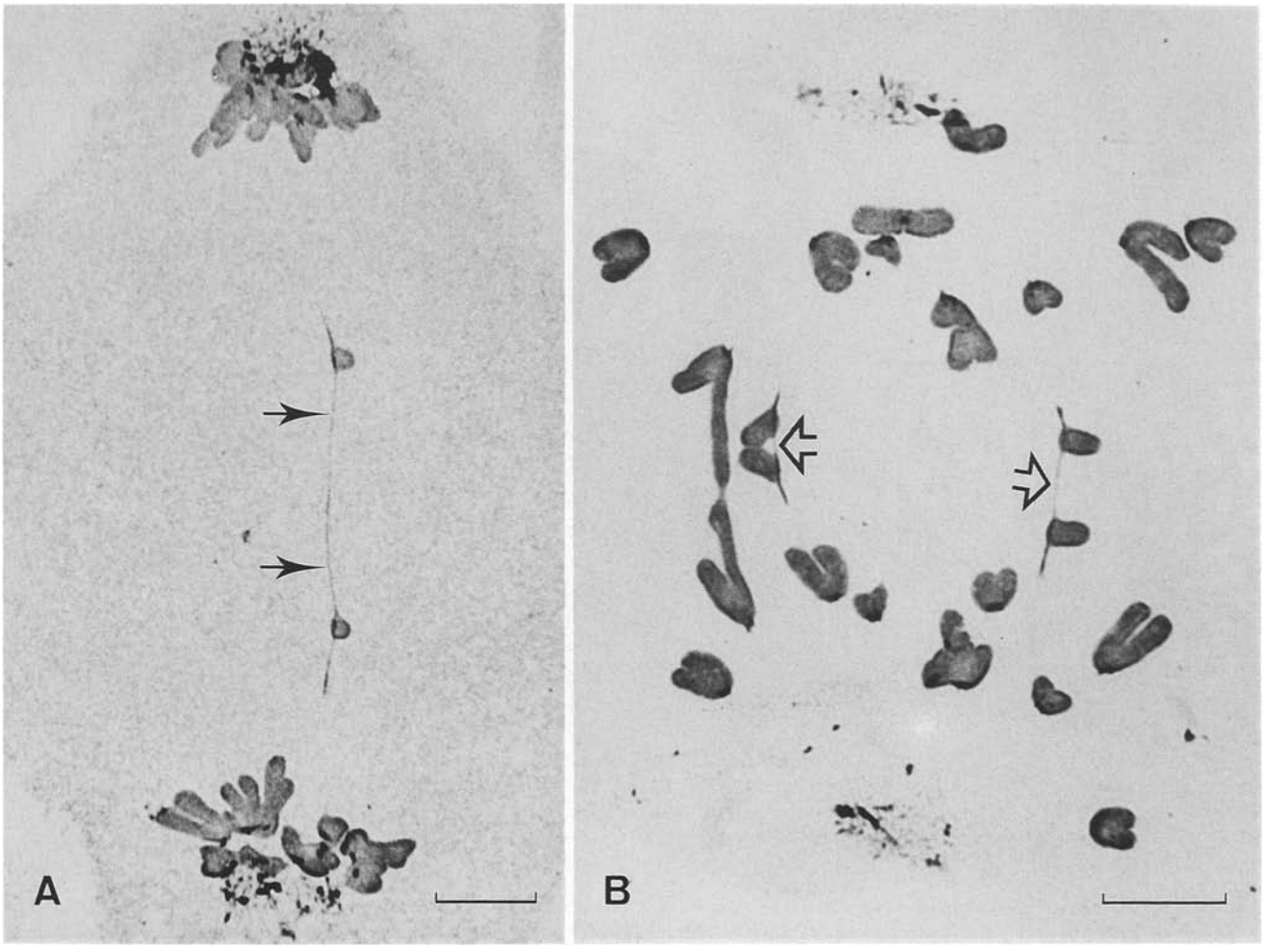

Fig. 4A, B. First meiotic division spermatocytes of *Eyprepocnemis plorans*. **A** Telophase I with one lagging B univalent. Both chromatids have a stretched kinetochore and remain connected by a silver-stained strand that joins sister kinetochores (*arrows*). Bar represents 10 μm. **B** Anaphase I with two lagging autosomal univalents. Note that these univalents have stretched kinetochores connected by a silver-stained strand (*arrows*). Bar represents 10 μm

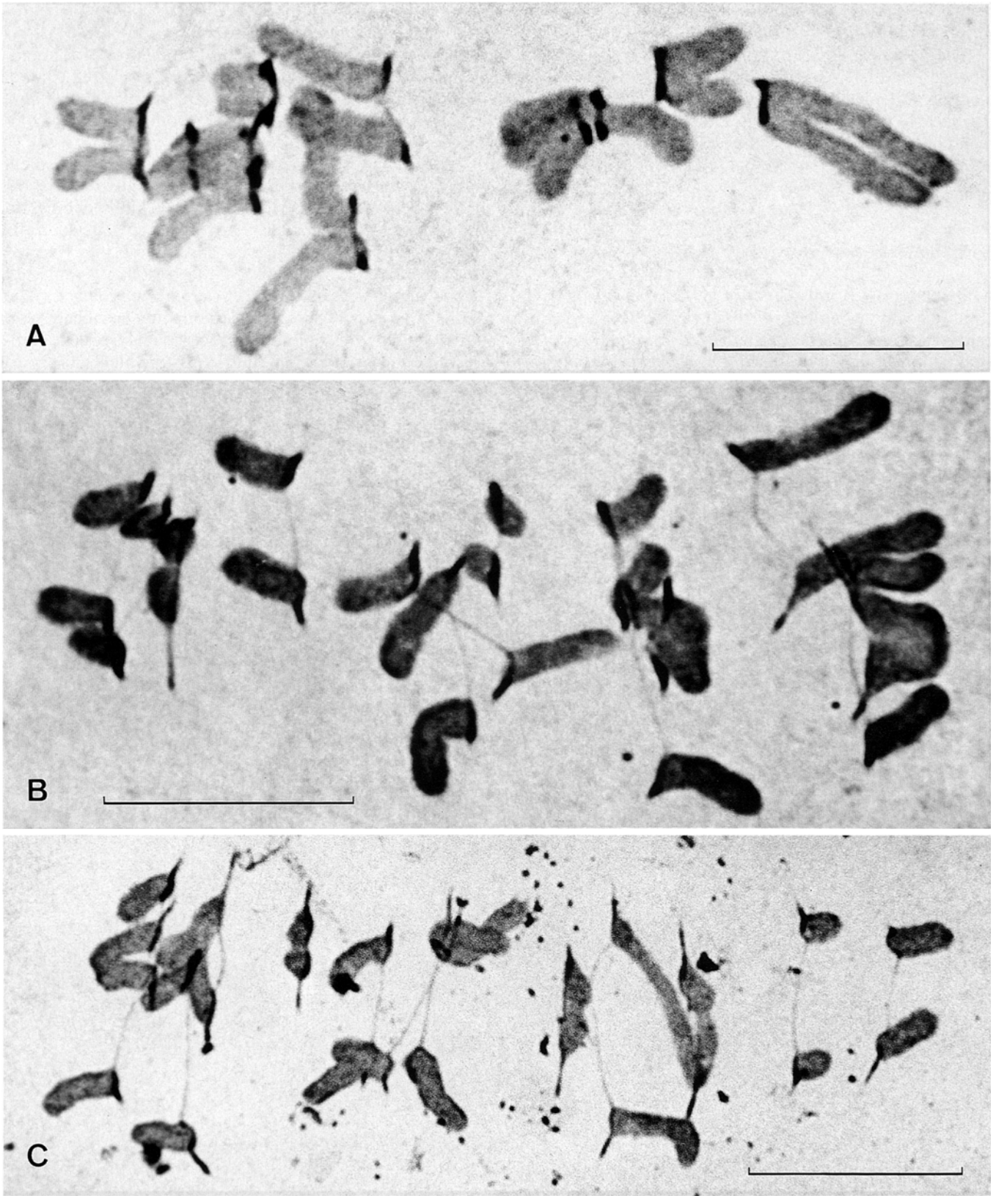

Fig. 5 A–C. Second meiotic division spermatocytes of *Eyprepocnemis plorans*. **A** Metaphase II. Each chromosome shows two stretched sister kinetochores. **B, C** Early anaphase II. The chromatids are parallel and have separated but are still joined by a thin silver-stained strand that connects stretched sister kinetochores. Bar represents in **A** and **B** 10 μm, in **C** 10 μm

nected by the thin strand, which is continuous with their stretched kinetochores. This strand is as long as the distance between B chromatids (Fig. 1B). In some cases this strand can be disrupted so that B chromatids, showing single round kinetochores, are included in the cellular poles (Figs. 1C and 2G). On the other hand, at anaphase I B bivalents show normal segregation and each B chromosome presents two round sister kinetochores. The smaller size of B kinetochores compared with those of autosomes and the sex chromosome is not only observed during metaphase I, when sister kinetochores are joined, but also at anaphase I (compare Fig. 2D and 2E; Fig. 2G). The amphitelic orientation of B univalents at metaphase I and their lagging during anaphase I cannot be due to the small size of their kinetochores since: (i) at metaphase I B univalents with non-functional kinetochores would be located elsewhere, while the B univalents that we have observed are always in the equatorial plate; (ii) the stretching of B univalent sister kinetochores indicates that they are able to capture and stabilise kinetochore microtubules and are subjected to traction forces from opposite poles (Fig. 2C) and (iii) B bivalents segregate correctly at anaphase I (Fig. 2E).

B chromosomes in E. plorans

At metaphase I, and contrary to what is found in *A. fusca,* B univalents are frequently displaced towards one pole (Fig. 3A). During that stage B univalents show syntelic orientation in 89% of cells analysed (129 out of 145 cells). In the remaining 11% (16 out of 145 cells) B univalents are located in the equatorial plate and show amphitelic orientation with sister kinetochores facing opposite poles. The size of B kinetochores is similar to that of autosomes and the sex chromosome (Fig. 3B).

Sister kinetochores of B univalents of *E. plorans,* and those of the X chromosome of both species, probably associate during prometaphase I to facilitate syntelic orientation, followed by the reductional division of these chromosomes during the first meiotic division. By contrast the sister kinetochores of B univalents of *A. fusca* remain individualised during early prometaphase I to allow amphitelic orientation.

Lagging B univalents, presumably derived from amphitelically oriented B univalents at metaphase I, have been observed in some ana/telophase I spermatocytes (Fig. 4A). These lagging B univalents show stretched kinetochores connected by a thin silver-stained strand like those found in *A. fusca* (Fig. 4A). Moreover, when autosomal lagging univalents are observed in some anaphase I spermatocytes from standard individuals, their kinetochore morphology and the presence of the connecting strand is similar to that found in lagging B univalents of both species (Fig. 4B).

The stretched morphology of the kinetochores and the connecting strand between them are characteristically found in metaphase II chromosomes (Fig. 5A). At early anaphase II these structures are more clearly recognized. The chromatids that begin the migration in parallel towards opposite poles are connected by the thin silver-stained strand (Fig. 5B and 5C). Shortly afterwards when this strand is disrupted the chromatids have round kinetochores and no longer appear parallel to the equatorial plate but longitudinal to the spindle axis.

Discussion

Cohesiveness along chromosome arms

During prophase I and metaphase I sister chromatids remain closely associated. Figure 6A shows a metaphase I bivalent composed of chromosomes with terminal centromeres, and one interstitial chiasma. Sister chromatids are tightly associated along their entire length. At anaphase I the chromatids of the half-bivalent appear individualised except at the centromere (Fig. 6B). How bivalents are maintained as a single structure with associated sister chromatids at metaphase I, and how half-bivalents segregate at the onset of anaphase I, are still open questions. Current opinion suggests that the factors involved in the maintenance of bivalent structure must be released or modified to allow chromosome segregation. Factors such as synaptonemal complex remnants (Maguire 1990), chromatid cores (Rufas et al. 1987), intertwining of sister chromatids (Rose et al. 1990) and proteins similar to those identified on mitotic chromosomes (Rattner et al. 1988) could be involved in sister chromatid cohesiveness at the chromosome arms.

Synaptonemal complex. Ultrastructural studies in several grasshopper species have demonstrated the presence of synaptonemal complex remnants between the chromatids of metaphase I bivalents (Esponda and Krimer 1979; Moens and Church 1979). These observations and the results obtained by analysing the meiotic behaviour of univalents found in different maize meiotic mutants and trisomics (see Maguire 1990) have led this author to suggest that, perhaps, these synaptonemal complex remnants could reinforce some other factors "to assure the sister chromatid cohesiveness presumably required for maintenance of chiasmata until anaphase I, and possibly for the maintenance of dyad integrity between metaphase I and anaphase II". We have observed that sister chromatids from the univalent sex chromosome (Fig. 6C) and B univalents (Fig. 6E) are always associated independently of their orientation at metaphase I. Thus we propose that at least in these grasshopper species the synaptonemal complex, as a "tripartite structure", is not directly involved in the maintenance of sister chromatid cohesiveness until anaphase I.

Chromatid cores and intertwining of chromatids. The association of chromatid cores in metaphase I homologues and their central location within chromatids of anaphase I half-bivalents (Rufas et al. 1987) could indicate that these structures are involved in chromatid cohesiveness. Our results show that at metaphase I the cores of the univalent sex chromosome and B univalents are individualised while their chromatids are associated. Recently we have reported that, at least in *A. fusca,* the change from meiotic (associated cores and kinetochores) to mitotic (individualised cores and kinetochores) chromosome organization, is a gradual process that takes place during metaphase I when the homologue chromatids still

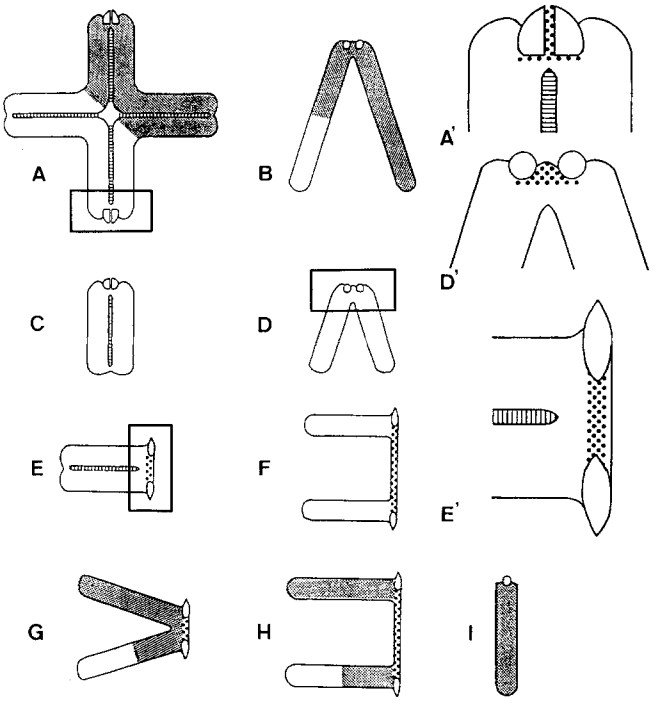

Fig. 6A–I. Schematic drawings depicting the possible location of cohesive proteins at the chromosome arms and at the centromere in different meiotic chromosome situations. All represented chromosomes possess terminal centromeres. The poles would be located at upper and lower positions within each drawing. **A** Metaphase I autosomal bivalent with one interstitial chiasma. Note that sister chromatids are associated by cohesive proteins, which are depicted by *parallel lines* (one chromosome is *blank*, its homologue is *dotted*). Sister kinetochores from each chromosome are joined. **A'** Enlargement of sector framed in **A**. The cohesive proteins on the chromosome arms (*parallel lines*) are on the surface of contact between sister chromatids, while those located in the centromere (*dots*) are around and connecting sister kinetochores. **B** Anaphase I autosomal half-bivalent. The cohesive proteins on the chromosome arms have been released and the chromatids are separated except at the centromere regions where round sister kinetochores are individualised. **C** Metaphase I syntelically oriented sex univalent. Sister chromatids are associated by chromosome arm cohesive proteins (*parallel lines*). Sister kinetochores are joined. **D** Anaphase I sex univalent. Chromatids are separated except at the centromere region where sister kinetochores are individualised. **D'** Enlargement of sector framed in **D**. At anaphase I the sister kinetochores of both sex univalent and autosomal half-bivalents, are round, individualised and connected by centromere cohesive proteins (*dots*). **E** Metaphase I amphitelically oriented B univalent. Sister chromatids are associated by a set of cohesive proteins (*parallel lines*). **E'** Enlargement of sector framed in **E**. The location of cohesive proteins between sister chromatids (*parallel lines*), and in the band that connects stretched sister kinetochores (*dots*) is depicted. **F** Anaphase I lagging B univalent. The proteins located between the sister chromatids have been released. A connecting strand containing chromatin and centromere cohesive proteins joins sister kinetochores and impedes the individualization of sister chromatids. **G** Metaphase II autosomal half-bivalent. The chromatids only show contact at the centromere where there are proteins (*dots*) connecting the stretched sister kinetochores that ensure chromosome integrity. **H** Early anaphase II autosomal half-bivalent. The strand connecting sister kinetochores (*dots*) appears slightly stretched. **I** Anaphase II autosomal chromatid. The cohesive proteins connecting the sister kinetochores have been released, thus allowing the poleward movement of the chromatids

remain associated (Suja et al. 1991). We suggest that the chromatid cores do not themselves serve as binders between sister chromatids, either in bivalents or in univalents, and consequently, their individualisation may be necessary but not sufficient to explain the segregation during anaphase I.

Recently Rose et al. (1990) have developed a model of meiotic segregation from experiments on cold-sensitive topoisomerase II mutants of yeast. The failure of chromosome segregation in these mutants during meiosis I unambiguously proves the involvement of topoisomerase II in this process. However, if as these authors suggest (see Fig. 7 in Rose et al. 1990) the resolution of intertwined sister chromatids distal to sites of recombination facilitates chromosome segregation in meiosis I, many commonplace cytological observations would need a satisfactory explanation. For instance, our results clearly show that during anaphase I univalents undergo chromatid separation, except at the centromere region. In the case of B univalents this is dramatically shown in Fig. 1B. Likewise, one would expect that bivalents that commonly have a single distal chiasma would show paired chromatids during anaphase I and metaphase II. Such a situation, as far as we know, has never been described. Our opinion is that the role of topoisomerase II is, as in the case of the chromatid cores, necessary but not sufficient to explain the segregation of half-bivalents at anaphase I. Topoisomerase II might act both catalytically and structurally in chromosomes (Adachi et al. 1991; Earnshaw 1991b). This enzyme should disentangle intertwined sister DNA molecules (and not condensed chromatids) at the end of DNA replication during the premeiotic interphase and/or after recombination during the early stages of prophase I. During later stages of the meiotic process is should be involved in chromatin condensation (Wood and Earnshaw 1990; Adachi et al. 1991).

Proteins. Rattner et al. (1988) have described the presence of a set of CLiPs (Chromatid Linking Proteins) between chromatids of mammalian mitotic chromosomes. These authors have proposed that these proteins could play a role in the regulation of sister chromatid pairing. One could predict that they are also present in meiotic chromosomes to assure the maintenance of the bivalent structure (Fig. 6A and 6A'). These proteins would be released or modified at the metaphase/anaphase I transition to allow the separation of half-bivalent chromatids (Fig. 6B). The association at metaphase I of sister chromatids from the sex chromosome (Fig. 6C) and B univalents (Figs. 6E) would be due to the existence of these cohesive proteins. However, these proteins, like those located in bivalents, would be inactivated and probably released during the onset of anaphase I. Chromatid separation would take place independently of the syntelic or amphitelic orientation of univalents (Fig. 6D and 6F).

Cohesiveness at the centromere

Our results show that during anaphase I both autosomal and B univalents that were amphitelically oriented sepa-

rate their chromatids but remain connected at the centromere. During late anaphase I stretched sister kinetochores are joined through a strand that can be visualised by silver staining (Fig. 6F). This strand, which is normally observed in half-bivalents at metaphase II (Fig. 6G), elongates at the onset of anaphase II (Fig. 6H) and later disappears allowing the migration of chromatids to opposite poles (Fig. 6I). At the ultrastructural level a similar strand has been found between sister kinetochores of both mitotic and meiotic chromosomes of mammals and insects (for references see Suja et al. 1991). Moreover, Rattner et al. (1988) have used autoantibodies to detect a structure that connects sister kinetochores of mammalian metaphase mitotic chromosomes. These authors have suggested that the 'connecting strand antigen' could be responsible for sister chromatid association at the centromere.

We presume that until metaphase I cohesive proteins located between sister chromatids of half-bivalents are responsible for the maintenance of the bivalent structure (Fig. 6A and 6A'). Other proteins located at the centromere joining sister kinetochores are needed to preserve the integrity of half-bivalents until metaphase II (Fig. 6A' and 6D'). These centromere proteins would normally be released or modified during the transition from metaphase II to anaphase II to allow chromatid individualisation and movement to opposite poles (Fig. 6H and 6I). The appearance during anaphase I of the connecting strand between the sister kinetochores of separating but lagging chromatids of univalents (Fig. 6F) indicates that these cohesive centromere proteins are already present during the first meiotic division (Fig. 6E'). It also reinforces the assumption that these proteins are only inactivated during the second meiotic division. In this sense, poleward forces exerted on the kinetochores of amphitelically oriented univalents are counteracted by the existence of the connecting strand proteins. However, the increase in length that this connecting strand undergoes during late anaphase I reveals the 'elastic' nature of these proteins. In contrast it is shorter during anaphase II when the connecting strand would normally be inactivated.

During mitosis two sets of proteins, one located at the centromere between sister kinetochores, and another situated along the chromosome arms, are responsible for sister chromatid cohesiveness until anaphase and are released in a single division. During meiosis the proteins located on the inner surface of the chromatids would explain the maintenance of the bivalent structure until anaphase I, while the proteins localised at the centromere would assure the integrity of the half-bivalent until anaphase II.

Acknowledgements. We wish to express our sincere thanks to Prof. Godfrey M. Hewitt, and Drs. Richard Nichols, Jaime Gosálvez and Carmen López-Fernández for the collection of *A. fusca*. We thank Drs. Pilar Arana and Carlos García de la Vega for their critical reading and suggestions about the manuscript, and Claire Martin and Paloma Valverde for improving the English style. This paper was supported by grants PB 88/0437 and CE 91/0010 awarded by the Comisión Interministerial de Ciencia y Tecnología (Spain).

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