The pairing of X and Y chromosomes during meiotic prophase in the marsupial species *Thylamys elegans* is maintained by a dense plate developed from their axial elements

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Summary

Unlike eutherian males, pairing of the sex chromosomes in marsupial males during the first meiotic prophase is not mediated by a synaptonemal complex. Instead, a specific structure, the dense plate, develops during pachytene between the sex chromosomes. We have investigated the development and structural nature of this asynaptic association in males of the marsupial species *Thylamys elegans* by means of immunolabelling and electron microscopy techniques. Our results show that the behaviour of male marsupial sex chromosomes during first meiotic prophase is complex, involving modifications of their structure and/or composition. Pairing of the sex chromosomes and formation of the dense plate take place in mid pachytene, paralleling morphological changes in the sex chromosomal axial elements. Components of the central element of the synaptonemal complex were not found in the sex body, in agreement with ultrastructural studies that reported the absence of a canonical tripartite synaptonemal complex between male marsupial sex chromosomes. Interestingly, the dense plate is labelled with antibodies against the SCP3 protein of the lateral elements of the synaptonemal complex. Moreover, as sex chromosome axial elements decrease in mass throughout mid-late pachytene, the dense plate increases, suggesting that material moves from the axial elements to the dense plate. Additionally, both sex chromosome axial elements and the dense plate have proteins that are specifically phosphorylated, as revealed by their labelling with the MPM-2 antibody, indicating that they undergo a chromosome-specific regulation process throughout first meiotic prophase. We propose that the unique modifications of the composition and structure of the axial elements of the sex chromosomes in meiotic prophase may result in the proscription of synaptonemal complex formation between male marsupial sex chromosomes, where the dense plate is an extension of the axial elements of sex chromosomes. This replaces synapsis to maintain X and Y association during first meiotic prophase.

Key words: Meiosis, Sex chromosomes, Pairing, Marsupials, *Thylamys*, SCP3, MPM-2

Introduction

During meiotic prophase homologous chromosomes pair, synapse and recombine. Synapsis is mediated by a meiosis-specific structure, the synaptonemal complex (SC) (Fawcett, 1956; Moses, 1956), which organises between homologous chromosomes and keeps them associated all along their length (Wettstein and Sotelo, 1967). Ultrastructural studies have demonstrated that the SC is a tripartite structure, composed of two lateral elements (LEs), one per homologue, a central element (CE) and a series of transverse filaments (TFs) that connect the LEs to the CE. The unpaired LEs of each homologue are called axial elements (AEs) before synapsis has been completed. In the past decade, the use of antibodies that recognise SC components has increased our knowledge of the organisation and dynamics of the SC in meiocytes of different species (Heyting, 1996). Some SC proteins have been identified in mammals. The best characterised are: SCP3 (Lammers et al., 1994), also called Cor1 (Dobson et al., 1994); SCP2 (Offenberg et al., 1998); and SCP1 (Meuwissen et al., 1992), also called Syn1 (Dobson et al., 1994). Whereas SCP3 and SCP2 are components of the LEs (and therefore of the AEs), SCP1 is a component of the TFs and the CE.

Solari, using serial sections and electron microscopy was the first investigator to show that an SC is formed between the heteromorphic sex chromosomes in male mice (Solari, 1970). Since then, the occurrence of SC has been demonstrated in the sex chromosomes of almost all mammalian species studied (Solari, 1993). It is currently known that sex chromosomes in eutherian mammals share regions of homology, the so-called pseudoautosomal regions (PARs) (Burgoyne, 1982), where

crossing-over and chiasma formation occur. It is in the PAR that the SC forms between sex chromosomes.

Most studies on meiotic sex chromosome pairing have been carried out in eutherian species. However, little attention has been paid to the meiotic structure and behaviour of sex chromosomes in the other mammalian taxa, the monotremes and the marsupials. There is only one report on monotreme meiosis (Murtagh, 1977), whereas the meiosis of only some American and Australian marsupial species has been thoroughly described (Koller, 1936; Solari and Bianchi, 1975; Pathak et al., 1980; Sharp, 1982; Roche et al., 1986; Seluja et al., 1987). Some of the features found in eutherian males are also observed in marsupial males. For instance, the sex chromosomes are delayed in pairing relative to the autosomes, and they form a dense chromatin mass, the sex body, which is thought to be a result of condensation and transcriptional inactivation of the sex bivalent during meiosis (Solari, 1974).

Other features of meiotic sex chromosomes differ between marsupials and eutherians, including the involvement of SC in mediating their association. Thus, while eutherian sex chromosomes synapse (i.e. form tripartite SC), marsupial sex chromosomes develop AEs that do not associate by a SC central element, but rather by a dense plate (DP) attached to the sex chromosome ends (Solari and Bianchi, 1975; Roche et al., 1986; Seluja et al., 1987). 'Balloon' structures between sex chromosome ends have also been described (Sharp, 1982; Roche et al., 1986). It has been suggested that the DP and the balloons are related structures (Roche et al., 1986), but their unequivocal correspondence has not been demonstrated so far. It is widely assumed that marsupial sex chromosomes do not share a region of homology (Graves and Watson, 1991). This lack of homology could prevent synapsis and SC formation between these chromosomes. However, recent studies have revealed that in some Australian marsupials the X and Y chromosomes share some sequences (Toder et al., 1997; Toder et al., 2000). Whether these sequences constitute a PAR is uncertain.

In this study we followed meiotic sex chromosome pairing in males of the marsupial species *Thylamys elegans* using immunolabelling and electron microscopy. We present immunocytological evidence that sex chromosomes do not form SC during first meiotic prophase, confirming the ultrastructural observations made in this and other marsupial species. We also show that the specific pairing structure of marsupial sex chromosomes, the dense plate, is labelled by antibodies against the SCP3 protein of the AEs. Additionally, we show that component(s) of both sex chromosomal AEs and the DP, but not of the autosomal LEs, are phosphorylated, as revealed by MPM-2 antibody labelling. We propose that DP is formed in mid pachytene as a modification of the sex chromosomal AEs. We discuss the biological significance of the structural and behavioural features displayed by marsupial sex chromosomes during first meiotic prophase.

Materials and Methods

Males of *Thylamys elegans* Waterhouse (Didelphidae) were collected in the central region of Chile. The specimens were castrated by a cut at the base of the scrotum, the seminiferous tubules were extracted and placed in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) for further processing.

Immunofluorescence

The seminiferous tubules were either squashed or spread prior to incubation with antibodies. For squashing we used a technique described previously (Page et al., 1998). Seminiferous tubules were fixed for 10 minutes in 2% formaldehyde in PBS containing 0.05% Triton X-100. Afterwards, several pieces of the tubules were placed on a slide and squashed by exerting pressure on the coverslip. The slides were immersed in liquid nitrogen and the coverslips were removed with a knife. The slides were washed in PBS for 15 minutes and incubated with primary antibodies. For spreading of spermatocytes, we followed the drying-down technique of Peters et al. (Peters et al., 1997). Briefly, a testicular cell suspension in 100 mM sucrose was spread onto a slide dipped in 1% paraformaldehyde in distilled water containing 0.15% Triton X-100 and left to dry for two hours in a moist chamber. They were subsequently washed with 0.08% Photoflo (Kodak), air dried and rehydrated in PBS.

Both squashes and spreads were incubated with the following primary antibodies diluted in PBS: rabbit serum A1, which recognises SCP3 protein of the SC lateral elements (Lammers et al., 1994) at a 1:500 dilution; rabbit serum A2, which recognises SCP1 protein of the transverse filaments and the central element of the SC (Meuwissen et al., 1992) at a 1:200 dilution; mAb MPM-2, which recognises mitotic phosphoproteins (Davis et al., 1983), kindly provided by A. Debec (Paris, France) at a 1:1000 dilution; and human CREST serum 098C7875, which recognises centromeric proteins, kindly provided by Chantal Andre (Hospital Henry Mondor, Paris, France) at a 1:100 dilution. The incubations were carried out for 1 hour at 20°C in a moist chamber. Then, the slides were rinsed in PBS for 3×5 minutes and incubated with the appropriate secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson) at a 1:100 dilution, Texas Red (TR)-conjugated goat anti-mouse IgG (Jackson) at a 1:100 dilution, and TR-conjugated goat anti-human IgG (Jackson) at a 1:150 dilution. 45 minutes of incubation at 20°C in a moist chamber was followed by 3×5 minutes rinse in PBS and staining with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole). After a final rinse in PBS, the slides were mounted with Vectashield (Vector Laboratories). For double immunolocalisation the primary antibodies were incubated simultaneously except for the double localisation of SCP1 and SCP3. In this case the slides were first incubated for 1 hour with serum A2 against SCP1, rinsed in PBS and incubated overnight at 4°C with a FITC-conjugated Fab′ fragment goat anti-rabbit IgG (Jackson) at a 1:100 dilution. After 3×10 minutes rinse in PBS the slides were incubated with serum A1 against SCP3 for 1 hour, rinsed 3×5 minutes in PBS and incubated with TR-conjugated goat anti-rabbit IgG (Jackson) at a 1:150 dilution.

Observations were made on either a Nikon Optiphot or an Olympus BH2 microscope equipped with epifluorescence optics and the images were photographed on Fujichrome Provia 400*F* or Kodakchrome 100. Colour slides were scanned in an Agfa DuoScan T-1200 or a Polaroid SprintScan 35 scanner, and images were processed with Adobe Photoshop 6.0 software on a Power Macintosh G3.

Electron microscopy

Seminiferous tubules were processed with conventional techniques for electron microscopy. They were fixed in 3% glutaraldehyde in 0.067 M Sörensen phosphate buffer (pH 7.2) for 90 minutes, rinsed and postfixed in 2% OsO_4 for 1 hour and embedded in Embed 812 (EMS). Serial ultrathin sections were obtained with a DuPont MT2-B ultramicrotome and contrasted with uranyl acetate and lead citrate.

For spreading we followed the technique described by Solari (Solari, 1982). A testicular cell suspension enriched in pachytene spermatocytes was dropped over a 0.5% NaCl solution. Spread cells were picked up on plastic-coated slides and fixed in 4% paraformaldehyde in 0.2 M $Na_2B_4O_7$ buffer (pH 8) containing 0.03% SDS for 10 minutes. The slides were then washed in 0.04% Photoflo

(Kodak), air-dried and stained with 1% PTA in ethanol. Selected cells were transferred to parallel bar copper grids. Observations were made on a Zeiss EM 109 transmission electron microscope operated at 80 KV. Photographs were recorded on Kodalite negative film (Kodak).

For immunocytochemistry, seminiferous tubules were fixed in 4% paraformaldehyde in 0.1 M Sörensen buffer (pH 7.3) for 2 hours. Dehydration was followed by embedding in LR White (London Resins). Silver-gold sections were obtained in a Reichert-Jung Ultracut ultramicrotome and mounted on nickel grids. The grids with the sections were incubated with serum A1 against SCP3 at dilutions of 1:200 and 1:500 for 1 hour, washed in PBS for 4×5 minutes, incubated with 10 nm gold-conjugated goat-anti rabbit IgG (EMS) for 1 hour, washed in PBS, postfixed in 2% glutaraldehyde for 2 minutes and contrasted with uranyl acetate. Observations were made on a Jeol 1010 transmission electron microscope operated at 80 kV.

Results

The chromosome complement of *Thylamys elegans* (2*n*=14) is composed of six autosomal pairs and a pair of sex chromosomes (XX in females and XY in males), the Y being a dot-like chromosome (Reig et al., 1972). Anti-SCP3 serum labelled axial structures in the nuclei of first meiotic prophase spermatocytes (Figs 1 and 2). This pattern of labelling is thought to represent the unpaired axial elements (AEs) of autosomes and sex chromosomes as well as the lateral elements (LEs) of the synaptonemal complex (SC). Anti-SCP3 labelling allowed us to deduce the sequence of meiotic chromosome pairing in *T. elegans* spermatocytes during first meiotic prophase.

Differentiation of chromosome AEs

The only labelling detectable with anti-SCP3 serum in leptotene spermatocytes are small spots homogeneously distributed in the nucleus (data not shown). Axial structures are first detectable during zygotene, when the autosomal AEs are seen as faint threads (Fig. 1A). At this stage, the chromosomes show a 'bouquet' arrangement with the telomeres clustered in one region of the nucleus. Synapsis of autosomes initiates at telomeres and extends to the interstitial regions. The synapsed

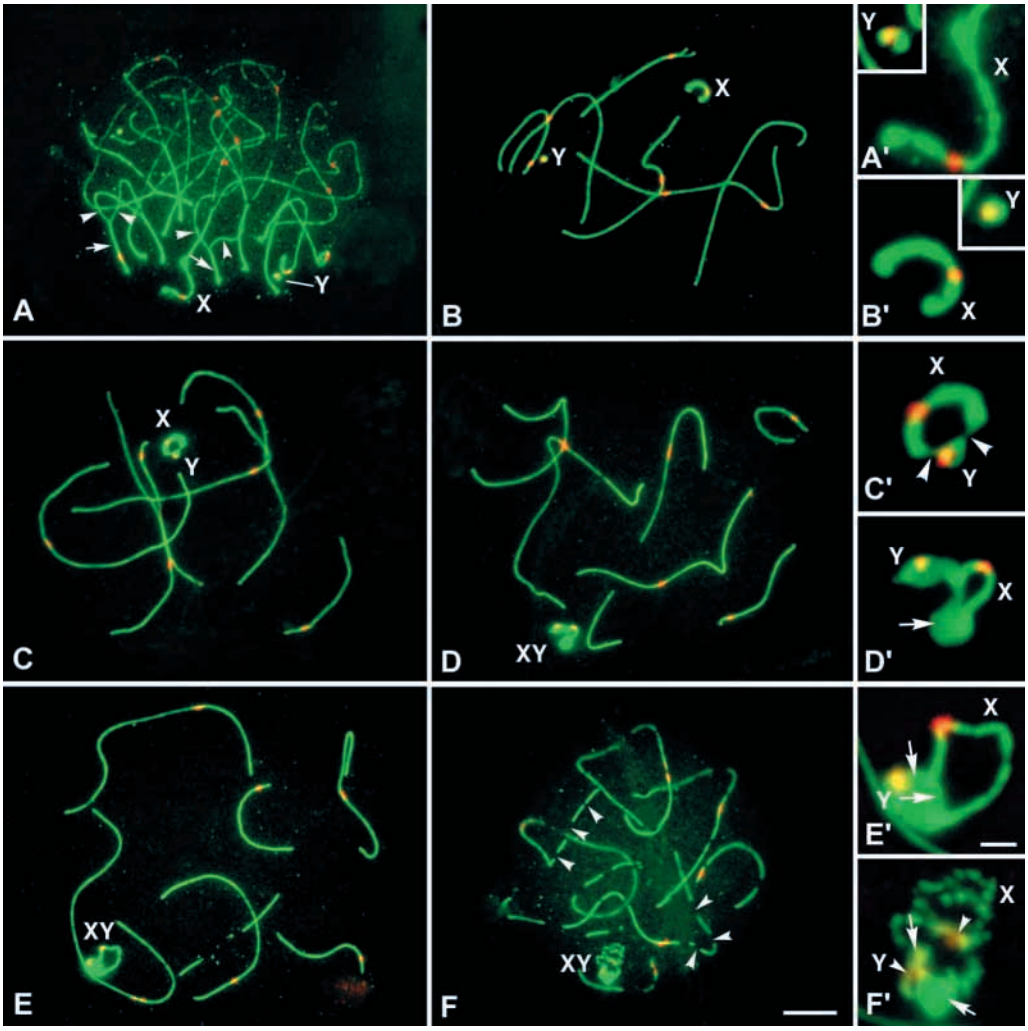

Fig. 1. Double immunolabelling on spread *T. elegans* primary spermatocytes with anti-SCP3 (green) and anti-centromere CREST (red) sera.
(A) Zygotene. The anti-SCP3 antibody labels axial structures that are polarised in a 'bouquet' configuration. Paired regions (arrows) appear thicker than the unsynapsed autosomal AEs (arrowheads). The sex chromosomal AEs (X,Y) are recognisable in the bouquet area, and they are separated. At higher magnification (A′) the irregular outline of the X AE can be seen. The AE of the Y chromosome (inset) is regular and the centromere is subterminal. (B-F) Pachytene. The bouquet arrangement is lost and the six autosomal pairs are fully synapsed. (B,B′). Early pachytene. The sex AEs are thickened and separated. The X AE loses its irregular outline. (C,C′) Early-mid pachytene. Sex chromosomes start to make contact by means of fine threads labelled in green with anti-SCP3 (arrowheads in C′). (D,D′) Mid pachytene. Sex chromosomal AEs (XY) become thinner. An anti-SCP3-labelled structure (arrow) with a horseshoe plate appearance is associated with the ends of the sex AEs. The AE of the Y chromosome is immersed in this plate. (E-E′) Mid pachytene. The AE of the X chromosome loses its stiffness and folds. The AE of the Y chromosome is immersed in the anti-SCP3 labelled plate (arrow). (F) Late pachytene. The autosomal SCs are fragmented (arrowheads). (F′) Enlargement of the XY body. The AE of the X chromosome is seen as a fine and fragmented thread tracing several loops. X and Y AEs are still associated with the anti-SCP3-labelled plate (arrow). Centromeres are still detectable but are fainter than in previous stages (arrowheads). Bars, 10 μm in (A-F); 2 μm in (A′-F′).

regions appear as short terminal segments where the anti-SCP3 labelling is thicker than that observed along the unpaired AEs, which radiate from the bouquet area as arcs that occupy the whole nuclear space (Fig. 1A). The bouquet arrangement loosens as zygotene progresses and is completely lost by the beginning of pachytene, when autosomes are completely synapsed (Fig. 1B-F).

AEs of sex chromosomes are strongly labelled with the anti-SCP3 serum during zygotene (Fig. 1A). They are thicker than expected for a single AE and appear even thicker than the synapsed regions of autosomes. The sex chromosomal AEs are arc-shaped, with their tips broadening at the attachment plates at the nuclear envelope. The outline of the AE of the X chromosome is irregular, showing some narrow regions (Fig. 1A′). The Y chromosome AE is very short and forms a small arc (inset in Fig. 1A′). In this phase the sex chromosomes are located in the bouquet area and usually lie apart from each other. Occasionally they appear end-to-end associated, as shown in Fig. 3G, but they disperse over the nuclear envelope as bouquet polarisation loosens.

Pairing of sex chromosomes

Pachytene was divided into substages (early, mid and late) in relation to the structure and behaviour of sex chromosomes. We did not follow the classification of meiotic stages of Rattner because in spreads and squashes the structure of the seminiferous epithelium is disrupted (Rattner, 1972). However, our classification is consistent with the pachytene sequence described by Solari and Bianchi (Solari and Bianchi, 1975).

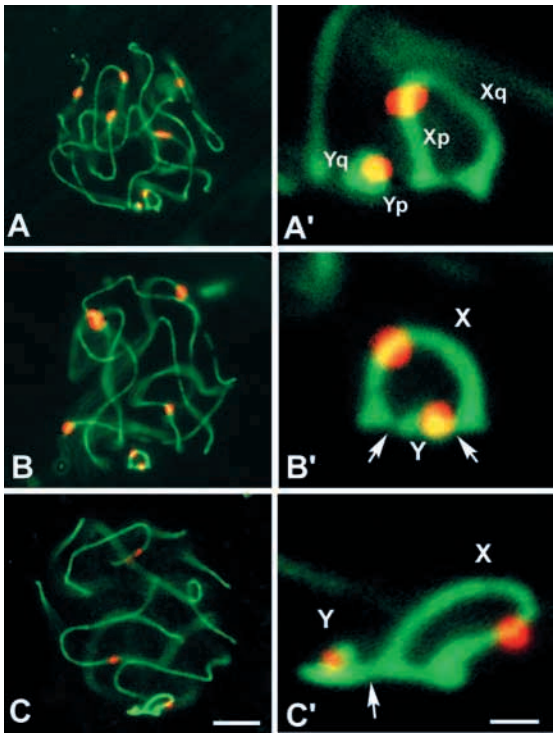

Fig. 2. Double immunolabelling on squashed primary spermatocytes with anti-SCP3 (green) and anti-centromere CREST (red) sera. (A-A′) Single focal plane through an early pachytene spermatocyte. Sex chromosomes lie on the nuclear periphery. Five autosomal centromeres and the sex chromosomes centromeres are visible. X and Y short (Xp and Yp respectively) arms face each other. (B-B′) Single focal plane of an early pachytene spermatocyte in which the sex chromosomes are in contact. Here the association is Xq-Yp and Xp-Yq (arrows). (C-C′) A mid pachytene spermatocyte in which both X and Y long arms face each other (arrow). Bars, 5 μm in (A-C); 2 μm (A′-C′).

Early pachytene

Pachytene is identified by the complete synapsis of autosomes (Fig. 1B). Labelling with anti-SCP3 shows a single line along each bivalent representing the two LEs, one per homologue, of the SC. The position of the centromeres along the bivalents, revealed by immunolabelling with an anti-centromere CREST serum, allowed us to identify three long submetacentric bivalents, one mid-sized submetacentric and two small acrocentrics (Fig. 1B-F), in agreement with the somatic karyotype described previously (Reig et al., 1972). At the beginning of pachytene, sex chromosomal AEs appear shorter and thicker than in zygotene, although the outline of the AE of the X chromosome still shows some irregularities near the telomeres. Sex chromosomes lie separated from each other and may frequently occupy very distant domains within the nucleus (Fig. 1B).

However, the behaviour and structure of sex chromosomal AEs change as early pachytene proceeds. They become thinner and the AE of the X chromosome becomes regular. These changes in the morphology of AEs are coincident with a progressive approach of the sex chromosomes, until they eventually touch each other (Fig. 1C′). The contact is always made at the chromosome ends and occurs at the nuclear periphery. This is especially evident in preparations of squashed spermatocytes, where the three-dimensional organisation of chromosomes within the nucleus is maintained (Figs 2 and 4). Contact between sex chromosomal AEs marks the transition to mid pachytene.

Double immunolabelling with anti-SCP3 and the anti-centromere serum revealed that the X chromosome is submetacentric, and the Y chromosome is acrocentric (Fig. 1A′ and B′). Therefore, we were able to distinguish Xq from Xp and Yq from Yp. We found that all possible configurations of contact occurred: sex chromosomal AEs can make contact by means of one (Fig. 2A′,C′) or both ends (Fig. 1C′, Fig. 2B′), and the contact may be established by both short arms (Fig. 1C′, Fig. 2A′), both long arms (Fig. 2C′) or by X long arm with Y short arm (Fig. 2B′). Thus, these first contacts do not seem to be arm specific.

Mid pachytene

During mid pachytene sex chromosomal AEs become thinner and more regular than in early pachytene (Fig. 1D,E). Their ends lose direct touch, becoming associated instead by a structure labelled by anti-SCP3 (Fig. 1D′). This plate-shaped structure is continuous with the sex chromosomal AEs and lies in the region where the chromosome ends are attached to the nuclear envelope (Fig. 2C′). It starts to form around the tips of the sex chromosomal AEs and increases in size throughout mid pachytene, until it eventually includes all four ends of sex chromosomal AEs (Fig. 1E′).

Late pachytene

Sex chromosomal AEs elongate and become thinner during late pachytene (Fig. 1F,F′). The AE of the X chromosome folds, usually forming several loops (Fig. 1F′). The AE of the Y chromosome is difficult to distinguish because it is immersed in the plate labelled by anti-SCP3, but the centromere signal indicates its presence. The morphological changes of the sex chromosomal AEs in late pachytene are accompanied by changes in the autosomal SCs. The labelling with anti-SCP3 starts to fade along the LEs of the bivalents and they appear fragmented (Fig. 1F). At the end of pachytene, the spermatocytes enter a diffuse stage characterised by decondensation of the chromatin and an almost complete disappearance of labelling with anti-SCP3 and the other antibodies. No later meiotic stages (diplotene onwards) were found on the spread slides. They were present, but rare, on squash slides, indicating that they are short-lived phases.

Given that sex chromosomal AEs are not in side-by-side contact during most of meiotic prophase, it seems that they are not kept together by a tripartite SC structure. To detect whether a SC central element is formed between sex chromosomal AEs during any of the stages of first meiotic prophase, we carried out double immunolabelling with anti-SCP3 and a serum that recognises the SCP1 protein, a component of the CE and the TFs of the SC (Meuwissen et al., 1992). During zygotene, anti-SCP1 label covers only those regions where the homologues are synapsed, whereas the anti-SCP3 serum labels both the AEs and LEs of autosomes (Fig. 3A,D). Sex chromosomal AEs, revealed by anti-SCP3 labelling, are devoid of signal with the anti-SCP1 serum, even when sex chromosomal AEs appear occasionally end-to-end associated (Fig. 3G). In pachytene, autosomes are fully synapsed and the signals of anti-SCP1 and anti-SCP3 sera are completely coincident on the autosomal bivalents (Fig. 3B,C,E,F). No labelling with anti-SCP1 is found on the sex chromosomes during pachytene, either when they contact by means of their ends (Fig. 3H) or by the anti-SCP3-labelled plate (Fig. 3I).

These results indicate that no canonical SC central element exists between sex chromosomes, even though the sex chromosomes are associated. Instead, the ends of sex chromosomal AEs associate over the nuclear envelope by means a dense plate-like structure, which holds the sex chromosomes in close apposition.

Presence of phosphoproteins on the sex chromosomal AEs

We also followed the behaviour of the sex chromosomes by means of immunolabelling of squashed spermatocytes with MPM-2, an antibody that recognises phosphoproteins. This antibody has proved to reveal some component(s) of the SC in insects (Suja et al., 1999) and fungi (van Heemst et al., 1999). We used MPM-2 in combination with the anti-SCP3 serum, and we found that whereas anti-SCP3 serum labels the autosomal LEs of SC and the sex chromosomal AEs (as described above), MPM-2 yields intense labelling only on the sex chromosomal AEs. This labelling appears in zygotene (data not shown), and it reveals a pattern of morphological and temporal changes of the sex chromosomal AEs identical to that described with anti-SCP3 serum. During early-mid pachytene the thickened sex chromosomal AEs approach each other and make contact (Fig. 4A-D). The labelling of both antibodies matches perfectly on the sex chromosomal AEs and the thread that connects them (Fig. 4C,D). In late pachytene (Fig. 4E-H), MPM-2 labelling on the X and Y AEs becomes fainter and an intense labelling, which colocalises with the anti-SCP3 labelled plate, appears in the area of association of sex chromosomes with the nuclear envelope. The MPM-2 labelling of sex chromosomes indicates that either sex chromosomal AEs contain exclusive component(s) or that a component shared with the autosomes is specifically phosphorylated in sex chromosomal AEs.

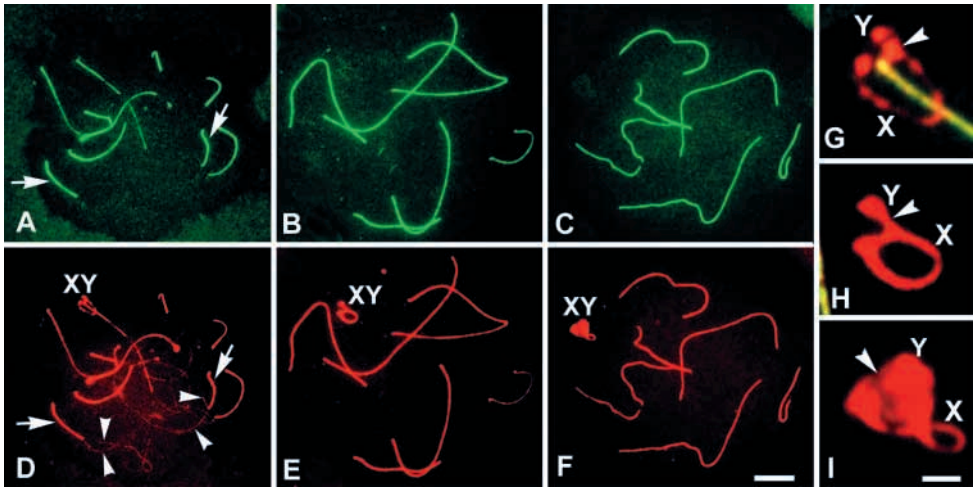

Fig. 3. Double immunolabelling on spread primary spermatocytes with anti-SCP1 (green) (A-C) and anti-SCP3 (red) (D-F) sera. (G-I) Enlargement of the sex chromosomes shown in A-F, where the anti-SCP1 and anti-SCP3 labelling have been superimposed and appear yellow where they colocalise. (A,D) Zygotene spermatocyte where synapsed autosomal regions are labelled with both SCP1 and SCP3 (arrows in A and D), whereas unsynapsed autosomal and sex (XY) AEs are only labelled with anti-SCP3 (arrowheads in D). (G) Enlargement of the sex chromosomes shown in A and D. The end of an autosomal SC appears yellow, whereas the unsynapsed sex chromosomal AEs, associated by one of their ends (arrowhead), only show red labelling corresponding to anti-SCP3. (B,E) Mid pachytene spermatocyte. Autosomes are fully synapsed as indicated by colocalisation of anti-SCP1 and anti-SCP3 labelling. (H) Detail of the sex chromosomes shown in B and E where they are associated by an anti-SCP3-positive structure (arrowhead) that is not labelled with anti-SCP1. (C,F) Late pachytene spermatocyte. Autosomes are fully synapsed. (I) Detail of the X and Y association shown in C and F. The anti-SCP3 positive plate (arrowhead) maintains the association between the sex AEs but no anti-SCP1 labelling is present. Bar, 10 μm in A-F; 2 μm in G-I.

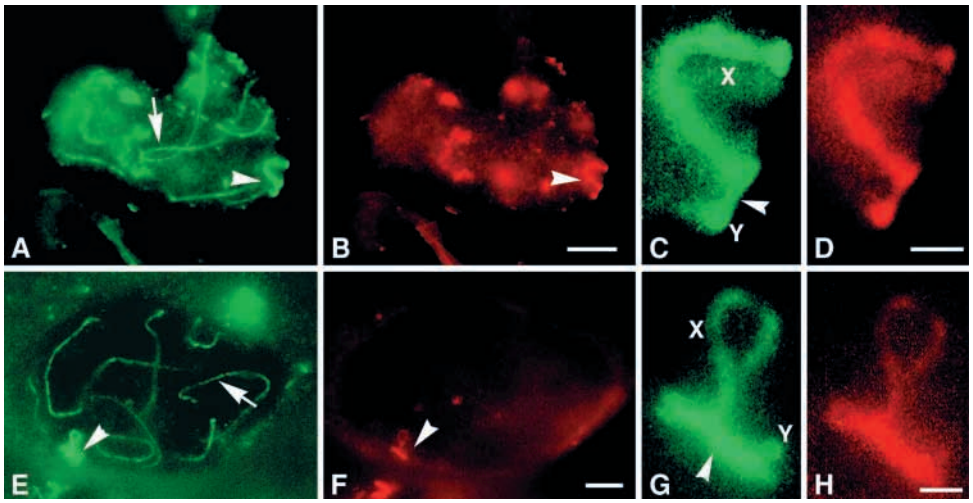

Fig. 4. Double immunolabelling on squashed primary spermatocytes with anti-SCP3 serum (green) and MPM-2 antibody (red). (A-B) Projection of three focal planes through a middle pachytene spermatocyte to reconstruct the whole XY body. (A) Anti-SCP3 labelling appears on the autosomal SCs (arrow) and the sex AEs (arrowhead). (B) MPM-2 labelling mainly appears on the sex chromosomal AEs (arrowhead) and as diffuse nuclear foci that do not correlate with autosomal SCs. At higher magnification (C,D) the outline of sex chromosomal AEs is clear. The chromosomes are connected at their ends by a fine thread that is recognised by both antibodies (arrowheads in C). The labelling with anti-SCP3 matches perfectly with that of MPM-2. (E,F) Projection of three focal planes through a late pachytene spermatocyte to reconstruct the whole XY body. (E) Autosomal SCs (arrow) are faintly labelled with anti-SCP3. The plate connecting sex chromosomal AEs (arrowhead) has increased in size and includes the four chromosome ends. (F) MPM-2 labelling only appears on the sex body. Higher magnification (G-H) shows details of the XY association. The intensity of labelling is so high on the plate that joins sex chromosomal AEs that it outshines the outline of chromosome AEs. This plate is larger than the region of attachment of AEs to the nuclear envelope. The two labels almost completely overlap. Note that in mid and late pachytene, sex chromosomes are located at the nuclear periphery. Bars, 5 μm in A,B,E,F; 1 μm in C,D,G,H.

Ultrastructure of the sex body

The structure of the sex chromosomes was also studied by means of electron microscopy. In serial sections of mid-late pachytene spermatocytes, the sex chromosomes are already paired and can be identified as a condensed chromatin mass, the sex body, located at the periphery of the nucleus (Fig. 5A,B). Inside the sex body, axial structures corresponding to the thickened sex chromosomal AEs can be discerned, but we never found any SC central element structure associated with them. The sex chromosomal AEs present conspicuous expansions, the attachment plates, where they associate with the nuclear envelope (NE) (Fig. 5B). Additionally, an electron-dense material, the dense plate (DP), is deposited on the inner face of the NE. The DP is a granular structure that lies on the inner membrane of the NE, which in this region appears to be stiff and is devoid of nuclear pores. The DP shows the same electron density as the sex chromosomal AEs. Indeed, both structures are continuous, as if the DP had been formed by the expansion of the sex chromosome AEs. An interesting feature of this nuclear region is the repeated association of the centrioles and the Golgi apparatus in the adjacent cytoplasmic region (Fig. 5A,B).

In electron microscopic spreads (Fig. 5C-E) we detected that in early pachytene the sex chromosomal AEs are thickened and lie apart from each other (Fig. 5C). The outline of X AE is irregular, with expansions at the centromeric region and its tips, the latter representing the attachment plates to the NE. As pachytene proceeds (Fig. 5D), sex chromosomes approach, their AEs elongate and become thinner and electron-dense material is detected at their tips. This material appears at first as fine threads connecting the AEs ends (arrowheads in Fig. 5D), and as pachytene proceeds, it becomes a round plate in which the four AEs ends are embedded (Fig. 5E). It is particularly noteworthy that this structure observed on spread spermatocytes by electron microscopy, which some authors refer to as ‘balloons’ (Sharp, 1982), and the plates revealed by immunolabelling with anti-SCP3 serum, are very similar (compare Fig. 1 to Fig. 5), indicating that they may in fact represent the same structure.

Finally, we analysed labelling with anti-SCP3 serum at the ultrastructural level. Fig. 6A,B shows two consecutive sections of a pachytene spermatocyte, in which the sex body is clearly discernible as a dense mass at the nuclear periphery. At the region of association of the sex body to the NE, the DP can be discerned as a structure with higher electron density than the rest of the NE. Inside the sex body the AE of the X chromosome can be seen. The anti-SCP3 labelling mainly appears over the AE and the DP (Fig. 6C,D).

Discussion

Pairing and recombination of homologous chromosomes during first meiotic prophase are steps that ensure their correct segregation during the first meiotic division. This also applies to heteromorphic sex chromosomes. In eutherian mammals this is accomplished by the formation of an SC, recombination and chiasma formation in the PAR. This ensures that sex chromosomes are properly associated until they segregate during anaphase-I. It has been shown in this and previous works that sex chromosomes in marsupials do not form SC (Solari and Bianchi, 1975; Sharp, 1982; Roche et al., 1986; Seluja et al., 1987). This is in contrast to the way sex chromosomes behave in eutherian mammals, and it implies that marsupials have alternative mechanisms by which sex chromosomes associate during first meiotic prophase. Such mechanisms must involve two steps: first, sex chromosomes have to come together; and second, they have to be held together until they segregate in anaphase-I. We present here evidence for how these processes are accomplished during first meiotic prophase in *Thylamys elegans*.

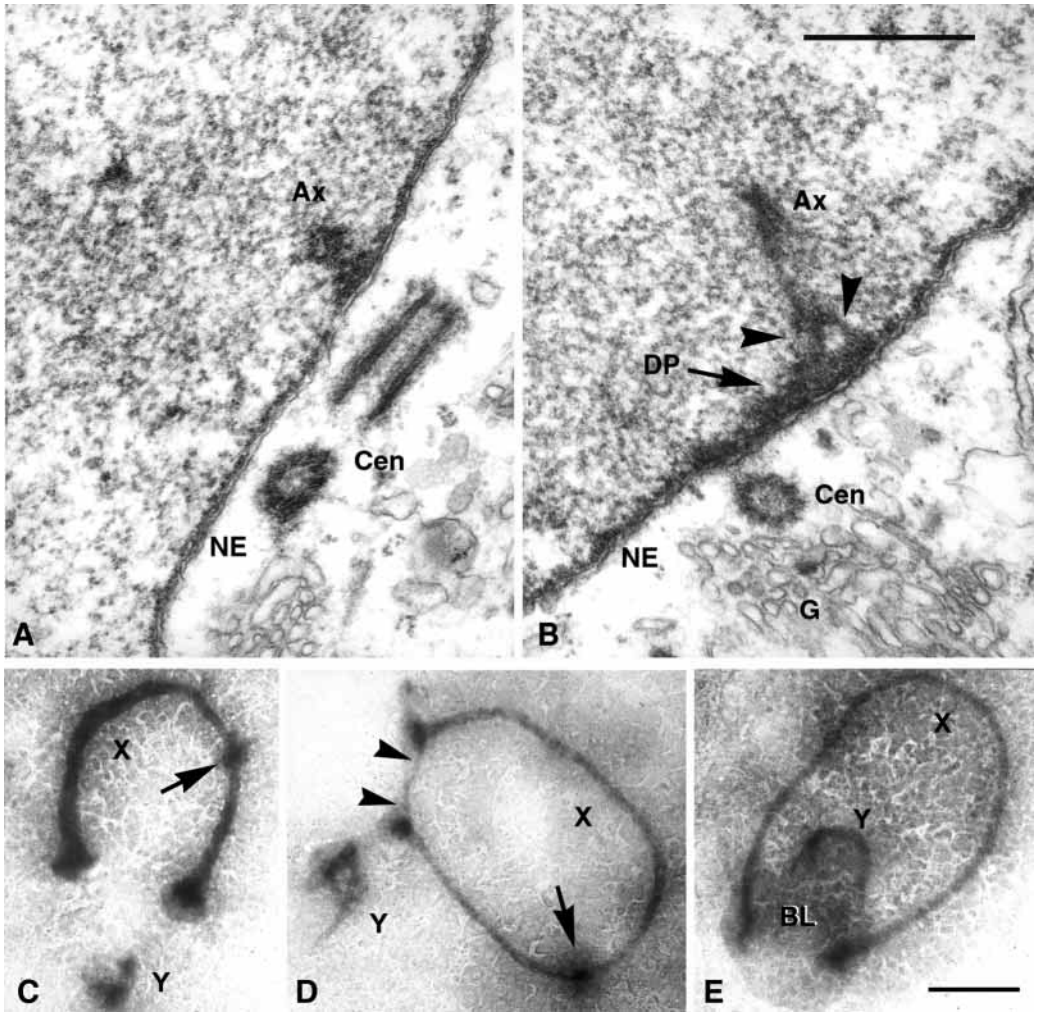

Fig. 5. (A-B) Electron micrographs of two consecutive sections of a mid-late pachytene spermatocyte. Sex chromatin has higher electron density than the rest of the chromatin. The sex body is associated with the nuclear envelope (NE). An axis (Ax) emanates from the NE showing an expansion at its base (arrowheads). A layer of electron-dense material corresponding to the dense plate (DP) (arrow) is present on the nucleoplasmic side of the NE. The DP has a granular structure and is continuous with the axis (Ax). The centrioles (Cen) and Golgi (G) are adjacent. (C-E) Electron micrographs of XY pairs from three different spread pachytene spermatocytes. (C) Early pachytene. The AEs are thickened. The AE of the X chromosome shows an irregular outline, with expansions at its ends and in the centromeric region (arrow). The AE of the Y chromosome is less distinct. (D) Mid pachytene. Both AEs are more elongated and thinner than in previous stages. An electron-dense material is present at the ends of AEs (arrowheads). It is composed of fine threads that extend from one extreme to the other. The centromere knob of the X chromosome is visible (arrow). (E) Late pachytene. The sex chromosomal AEs do not touch each other. An electron-dense material, the balloon (BL), mediates the association of AE ends. Bars, 0.5 μm in A,B; 1 μm in C,E.

The pairing of sex chromosomes

The differentiation of sex chromosomal AEs in *T. elegans*, as revealed by the labelling with the anti-SCP3 serum, starts during zygotene and is coincident with the beginning of autosome synapsis. In zygotene, sex chromosomes lie in the bouquet area where they are clearly distinguishable from the autosomal AEs or SCs, but they are usually separated from each other, although they are occasionally found joined by their ends (Fig. 3G), or even joined to autosomes (data not shown). These associations seem to be unstable since by the beginning of pachytene sex chromosomes are always separated and without contact and remain so throughout early pachytene. However, sex chromosomes approach and pair during the transition between early and mid pachytene. This is accompanied by changes in the organisation of sex chromosomal AEs: they become thinner at the time of pairing, the outline of X chromosome AE becomes regular and the trajectory of Y chromosome AE is clearly discernible. In contrast to the occasional zygotene associations, pairing of sex chromosomes during early-mid pachytene occurs in every nucleus, is stable and lasts (at least) until the entry into the diffuse stage.

Since during early pachytene sex chromosomes lie apart from each other, sometimes occupying very different nuclear domains, the confluence of the sex chromosomes during the transition from early to mid pachytene is the result of a secondary polarisation. In this sense, the close location of the centrioles and Golgi complex in the adjacent cytoplasm is noteworthy (Solari and Bianchi, 1975; Roche et al., 1986) (this work). Association of the centrioles with the zygotene bouquet polarisation has been found in man (Berrios and Fernández-Donoso, 1990) and other species (for a review, see Zickler and Kleckner, 1998). It is likely that the centrioles play some role in the late polarisation of sex chromosomes, leading to the formation of a pachytene ‘late bouquet’ where the sex chromosomes ultimately find each other. It is remarkable that this late polarisation affects sex chromosomes but not autosomes. The remaining question is how do sex chromosomes recognise each other. We found that during pachytene in *T. elegans* the first contact of sex chromosomal AEs is established at their ends. One possibility is that sex chromosomes bear terminal homologous regions involved in their recognition. However, the fact that the association of sex chromosome AEs could involve any of their ends indicates that

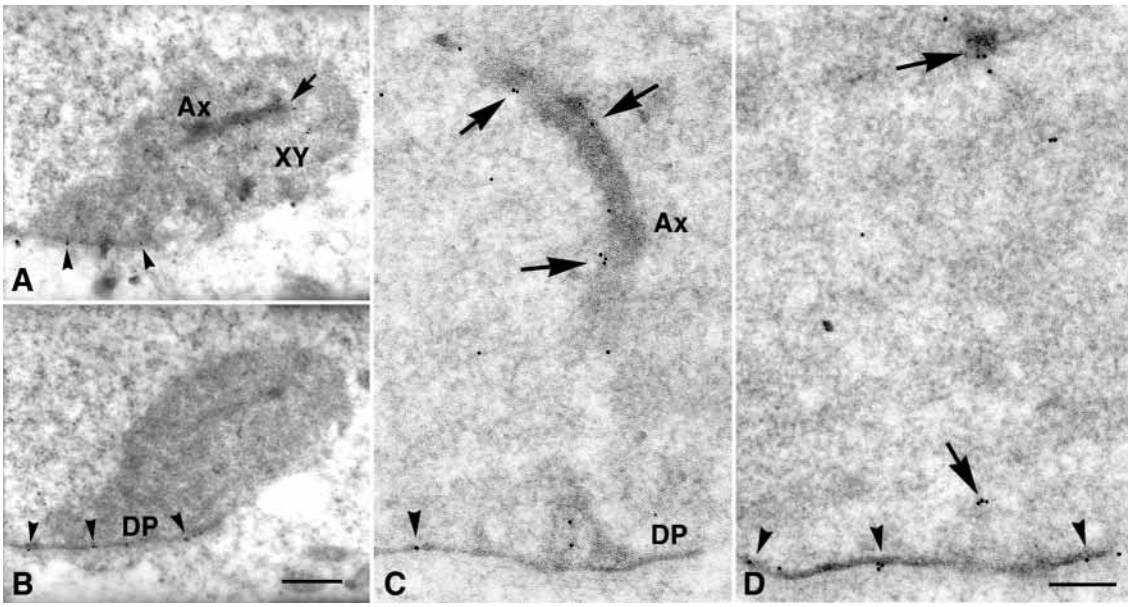

Fig. 6. Electron microscopic labelling with anti-SCP3 serum in pachytene spermatocyte sections. (A,B) Two serial sections of the same spermatocyte. The sex body (XY) is recognisable by its high electron density. Inside the sex body, an axial structure (Ax) is labelled with gold grains (arrow), so is the dense plate (DP) (arrowheads). (C,D) Higher magnification of two serial sections of another spermatocyte. Gold grains appear on the axial structure (Ax) (arrows) and on the dense plate (DP) (arrowheads). Bars 0.5 μm in A,B; 0.2 μm in C,D.

it does not depend upon specific chromosome arm recognition. Therefore, sex chromosomes seem to recognise each other, but this recognition is not based on the pairing of a specific chromosome region. This strongly supports the absence of a pseudoautosomal region.

The asynaptic nature of the XY association

In *T. elegans*, we did not find sex chromosomal SC either by light or electron microscopy. In spreads and serial sections for electron microscopy the sex chromosomal AEs were always found too far apart to be connected by SC central region, and no structural components of the CE were detected. Moreover, sex chromosomes are devoid of labelling with the anti-SCP1 serum.

Several hypotheses have been postulated to explain the absence of SC in marsupial sex chromosomes. Solari and Bianchi proposed that the small size of the Y chromosome could be an obstacle for a proper alignment of the sex chromosomes (Solari and Bianchi, 1975). However, SC is not formed even in species with a long Y chromosome (Roche et al., 1986), suggesting that chromosome size is not a determinant factor. On the other hand, some authors have postulated that the lack of SC is due to the absence of shared homologous sequences between both sex chromosomes. It is currently accepted that sex chromosomes in marsupials would have undergone a sequential process of differentiation from each other, leading eventually to a complete loss of homology (Graves and Watson, 1991). In this context, the absence of homology may prevent the formation of SC and therefore recombination and chiasma formation (Hayman, 1990). However, it has been reported that shared sequences between sex chromosomes are present in some marsupials as a result of translocation of the nucleolar organiser regions (NORs) to both sex chromosomes (Hayman, 1990; Toder et al., 1997; Toder et al., 2000). NORs usually form a normal SC when they are present in autosomal chromosomes in most mammalian species, including marsupials (S.B., unpublished), but in marsupials no SC is found between NORs when they are present in sex chromosomes (Sharp, 1982; Hayman, 1990).

Thus, it seems that marsupial sex chromosomes inhibit the formation of an SC, even in the presence of putative homologous regions (Hayman, 1990). This contrasts with the behaviour of eutherian sex chromosomes, in which SC frequently extends from the PAR to the non-homologous segments during early pachytene (Solari, 1970). We suggest that the lack of SC in marsupial sex chromosomes may result from structural modifications of their AEs. One of the features present in all marsupial sex chromosomes studied up to now is the conspicuous thickening of their AEs. This feature has been reported in a variety of eutherian mammals (Solari, 1974; Solari, 1993). In these cases, the thickening mainly involves the unpaired regions of chromosomes, whereas the LEs that participate in the formation of SC in the PAR are about as thick as autosomal LEs. One possible explanation is that structural modifications of sex chromosomal AEs, starting in zygotene, prevent the SC formation.

Our findings that sex AEs are the only axial structures labelled with MPM-2 antibody in *T. elegans* spermatocytes supports such a model. Because SCP3 and MPM-2 colocalise on the sex chromosomes throughout meiotic prophase, it could be possible that MPM-2 recognises a hyperphosphorylated form of SCP3. Alternatively, MPM-2 could recognise a different component that is exclusive to sex chromosomes. In either case, the specific presence and/or phosphorylation of some proteins on the sex chromosomal AEs could contribute to an inhibition of SC formation. All these structural and biochemical modifications of the sex chromosomal AEs could

be related to the programme of meiotic sex chromosome inactivation, preventing the establishment of heterologous interactions between sex chromosomes and other chromosomes, and also contributing to by-passing the meiotic arrest that should be induced by the unsynapsed sex chromosomal AEs (Handel and Hunt, 1992; McKee and Handel, 1993).

Maintaining marsupial sex chromosomes together: the dense plate

In the absence of SC, it is clear that the DP maintains the association of sex chromosomes. The DP was first described in spermatocyte sections of *Monodelphis dimidiata* (Solari and Bianchi, 1975). Afterwards, Sharp described the presence of 'balloons' in spermatocyte spreads of about twenty marsupial species (Sharp, 1982). Solari proposed that the balloons could represent remnants of the nuclear envelope that would remain attached to the sex chromosomal AEs in spreads (Solari, 1993). From our results it seems clear that both the DP and the balloon are different manifestations of the same structure, as suggested previously (Roche et al., 1986). The detailed analysis throughout first meiotic prophase of sex chromosomes structure and behaviour by means of immunofluorescence, electron microscopy spreads and sections and immunocytochemistry techniques enabled us to establish a morphological, temporal and compositional correlation between the SCP3-labelled structures (Figs 1, 4 and 6), the balloons (Fig. 5C-E) and the DP (Fig. 5A-B, Fig. 6). Therefore, we can conclude that all these structures are in fact the same, and we propose to refer to them as the dense plate, as they were first named (Solari and Bianchi, 1975).

Our data also establish an unequivocal correlation between the sex chromosomal AEs and the DP. We show that both structures share some components, since they are labelled with anti-SCP3 and also with MPM-2, and that the development of the DP is coincident in time with the progressive thinness of the sex chromosomal AEs. Furthermore, the DP seems to be originated by the expansion of the sex chromosomal AEs ends attached to the nuclear envelope. This agrees with observations made in spermatocyte sections (Fig. 5) that show both structures are continuous. It is likely that the DP arises as a specific modification of the sex chromosomal AEs. Such a model is represented in Fig. 7. It is interesting to note that there is an inverse correlation between the anti-SCP3 labelling on sex chromosomal AEs and that found on the DP. Our results with MPM-2 also support this inverse correlation. The DP seems to be formed by the material detached from the AEs, but we cannot reject the hypothesis that the DP is also formed by the accumulation of newly synthesised components.

The presence of SCP3 in the DP is of special interest. Despite the polyclonal origin of the rabbit anti-SCP3 serum, the labelling on meiotic chromosomes of *T. elegans* follows the same pattern described for eutherian mammals (Lammers et al., 1994; Dobson et al., 1994). Thus, anti-SCP3 labels the unpaired AEs during zygotene and the paired LEs in pachytene. Moreover, the presence of SCP3 in the DP has been corroborated by the use of a guinea pig polyclonal serum against the SCP3 protein (Alsheimer and Benavente, 1996) (data not shown). SCP3 has been related to the organisation of the AEs and LEs of the SC, and also, as remnants of these structures, in the maintenance of sister chromatid cohesion during meiosis (Moens and Spyropoulos, 1995). We demonstrate that SCP3 participates in the formation of the DP, which is a non-axial structure. Therefore, we report a novel unsuspected role for this protein in marsupial meiosis.

A remaining question to be answered is whether the DP maintains the association of sex chromosomes until they segregate during anaphase-I. Solari and Bianchi described a folded sheet associated to the sex chromosomes in metaphase-I in *Monodelphis dimidiata* (Solari and Bianchi, 1975). This structure most probably represents the remains of the DP. However, these authors did not attribute any role to the folded sheet in the maintenance of the integrity of the sex bivalent, since they assumed that a chiasma must exist between sex chromosomes. Further studies on the orientation and segregation of marsupial sex chromosomes during first meiotic division are under way in order to test these hypotheses (J.P., unpublished).

From the results presented here it is clear that marsupials and eutherians have striking differences in the meiotic process concerning the structure and behaviour of sex chromosomes. Perhaps the most important difference is the development of the DP as a differentiation of the sex chromosomal AEs, which

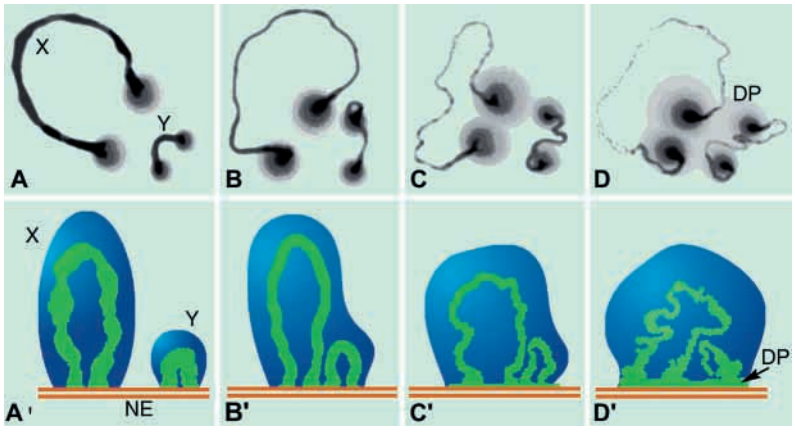

Fig. 7. Schematic representation of sex chromosome (X,Y) structure and behaviour throughout pachytene. The upper row depicts sex chromosomes as seen in spread spermatocytes, either by electron microscopy or by labelling with anti-SCP3 serum or MPM-2. AEs are initially short and thick and do not touch each other (A). In later stages, AEs become thicker and more loosely organised, and dense material starts to be deposited at their ends (B). Eventually, this material includes the ends of both chromosomes, and the AEs appear as faint threads (C,D). The lower row shows the sex chromosomal AEs as seen in electron microscopy sections and in squashes labelled with the anti-SCP3 serum or MPM-2 antibody. Initially X and Y AEs lie apart from each other, showing a stiff appearance and an irregular outline (A′). Subsequently, sex chromosomes approach and form the sex body, revealed as a dense chromatin mass, and their AEs touch (B′). Thereafter, AEs start to loose their stiffness, and the material composing the AEs is deposited on the nuclear envelope (NE) while the label on the AEs decreases (C′). Finally, AEs get thinner and looped, and the dense plate (DP) forms, extending from the attachment plates of sex chromosomal AEs to the nuclear envelope. The DP extends until it includes the ends of both sex chromosomes (D′).

ensures their association at least during first meiotic prophase. This indicates that some components of the SC, in this case their AEs, are capable of a surprising plasticity, generating new structures that have been useful to respond to new situations such as maintaining the association of two chromosomes that have become non-homologous. Examples of asynaptic sex chromosomes have also been reported in eutherian mammals (Solari and Ashley, 1977). However the DP seems to be an exclusive marsupial feature that has no counterpart in any other vertebrate. In the current context of knowledge, these observations open new ways of interpretation for the evolutionary history of mammalian sex chromosomes, their meiotic behaviour and how meiotic cells deal with non-exchange chromosomes (Wolf, 1994). The remarkable fact that marsupial sex chromosomes do not form SC, together with observations of the conspicuous modifications of the sex chromosomal AEs, indicate that the formation of the DP may be part of an asynaptic programme that cannot be overcome easily, even in the presence of homologous sequences. This could have placed marsupial sex chromosomes in an evolutionary pathway completely different from that followed by the eutherian sex chromosomes.

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