EXTENSIVE PAIRING OF THE XY BIVALENT IN MOUSE SPERMATOCYTES AS VISUALIZED BY WHOLE-MOUNT ELECTRON MICROSCOPY

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SUMMARY
Autosomes and sex chromosomes of mouse spermatocytes were examined during zygotene, pachytene, and diplotene by a whole-mount electron-microscope technique after cell dispersion in a detergent solution (Nonidet-P40). Zygotene, pachytene, and diplotene stages can be adequately identified in the preparations. Thus, asynchronous side-by-side pairing of homologous autosomes, some of them displaying attached nucleoli, defines zygotene. Pachytene is identified by complete pairing of homologues. Diplotene is characterized by disjunction of bivalents (autosomes and sex chromosomes), lack of autosomal-attached nucleoli, divergent expansions observed at lateral element endings of disassembled synaptonemal complexes, end-to-end association of the XY pair and well defined outward deformations ('bulges') along sex chromosomal axial cores. X and Y chromosomes display at pachytene an extensive side-by-side pairing segment which decreases in length as meiotic prophase advances. Each sex chromosomal axial core appears double and is formed by close apposition of 2 nearly parallel elements displayed separately along the entire length of the chromosomal core. This double structural feature suggests that each sex chromosomal axial core is presumably composed of 2 chromatid axial cores, each of which, in turn, constitutes the respective lateral elements of short synaptonemal complexes observed at the unpaired segment.

INTRODUCTION
Knowledge of structural organization of homologous chromosome pairing during male meiotic prophase can contribute significantly to understanding of molecular events leading to genetic recombination (crossing-over). Details of the structure of the synaptonemal complex and its role in chromosomal pairing and crossing-over during meiotic prophase in a wide variety of species have been reviewed (Moses, 1968; Comings & Okada, 1971; Westergaard & von Wettstein, 1972; Stubblefield, 1973; Moens, 1973, amongst others). Nonetheless, it is still unclear whether crossing-over takes place within the synaptonemal complex or outside it.

In most mammalian species, autosomal bivalents display a complete pairing between homologues of the same length. X and Y chromosomes have been identified as 2 associated chromosomes of unequal length, paired over a portion at one end, displaying a pairing segment much shorter than either chromosome. This information derives from earlier cytogenetic studies in man (Sachs, 1954), rats (Tjio & Levan, 1956; Ohno, Kaplan & Kinosita, 1958), mice (Sachs, 1955; Ohno, Kaplan & Kinosita, 1959), golden hamster (Ohno & Weiler, 1962; Fredga & Santesson, 1964) and cat...
(Ohno et al. 1962). More recently 3-dimensional reconstructions of the XY bivalent in serial thin sections (Solari, 1970; Solari & Tres, 1970) and whole-mount electron-microscope displays of the total set of autosomal and sexual bivalents in single spermatocytes (Kierszenbaum & Tres, 1974b) have shown a synaptonemal complex present at the terminal short paired region of the XY pair. The finding of a short synaptonemal complex developed at the XY pairing segment in the mouse suggests a relationship with synapsis and the possibility of partial sex linkage, first proposed by Koller & Darlington (1934).

Although available data indicate that the mouse XY bivalent has a short pairing segment, side-by-side pairing of the sexual bivalent has been observed in other species (Rattus natalensis: Hamerton, 1958); Eurasian Cricetinae (Matthey, 1961); Cricetus griseus (Ohno & Weiler, 1962); Cricetus cricetus (Fredga & Santesson, 1964).

A whole-mount electron-microscope technique based on that of Miller & Hamkalo (1972) and Miller & Bakken (1972) and adapted for mammalian testes (Kierszenbaum & Tres, 1974b, 1975), has overcome time-consuming problems associated with 3-dimensional reconstructions of sexual bivalents. The whole-mount technique provides a satisfactory display of chromatin fibres, ribonucleoprotein transcripts and synaptonemal complexes in a considerable number of spermatocytes. It was used in search of additional information concerning the spatial relationship of sex chromosomes and autosomes and yielded structural data, here presented, permitting the identification of diverse meiotic prophase stages and structural and pairing aspects of the mouse XY bivalent during zygotene, pachytene, and diplotene.

MATERIALS AND METHODS

Testes from adult male Swiss mice (2n = 40), 15-20 days old, were removed under ether anaesthesia and placed on a watch glass kept on crushed ice. After removing the tunica albuginea, seminiferous tubules were cut into small segments (2-3 mm in length) and suspended in 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 0.15 M NaCl (TBS buffer). Cells were dissociated from seminiferous tubules by shearing forces produced by repeated aspirations and ejections with a syringe. The suspension was transferred to centrifuge tubes (15 ml) precooled in ice and centrifuged at 1000 rev/min for 4 min in a refrigerated centrifuge at 4 °C. The supernatant was discarded and replaced with the same amount of fresh TBS. After the pellet had been gently resuspended with a Pasteur pipette, the suspension containing pieces of seminiferous tubules, cell aggregates, and isolated cells was strained through a stainless-steel mesh with openings 0.5 mm in diameter to trap pieces of seminiferous tubules. Resulting samples were used for whole-mount electron microscopy or for air-dried light microscope preparations.

Electron-microscope visualization of meiotic prophase chromosomes of spermatocytes

The procedure is an adaption for testicular cells (Kierszenbaum & Tres, 1974b, 1975) of the procedure of Miller & Hamkalo (1972) and Miller & Bakken (1972). Once the cell suspension was strained, cell aggregates and isolated cells were transferred with a pipette to a cold 0.3% solution of Nonidet-P40 (NP-40) (BDH Chemicals Ltd, Poole, England) in distilled water with the pH adjusted to 8.6 by adding suitable amounts from a stock solution of borate buffer (pH 9.0). NP-40 is a detergent which dissolves cell membranes and permits isolated nuclei to remain active in transcription (Tsai & Green, 1973). Cells were allowed to disperse for 5 min at 4 °C. Dispersed nuclear contents were transferred with a micropipette, Lang-Levy type, with a capacity of 500 μl, into a plastic trough containing electron-microscope hydrophilic carbon-coated copper grids (300-mesh) and a solution of 0.1 M sucrose with 10 % formaldehyde
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(pH 7.0), and centrifuged at 3000 rev/min for 5 min at 4 °C. Grids were rinsed in 0.4 % Kodak Photo-flo and air-dried. Preparations were stained with 1 % phosphotungstic acid in 95 % ethanol (pH 2.5), rinsed in 95 % ethanol and air-dried. Electron micrographs were taken with a Siemens Elmiskop 101 or a JEM 100B operated at 60 kV.

Air-dried preparations of testicular cells

The procedure was described in detail elsewhere (Kierszenbaum & Tres, 1974a). Briefly, the testicular cell suspension obtained as indicated above was centrifuged at 1000 rev/min and the resulting pellet fixed for 30–60 min in a mixture of absolute alcohol-glacial acetic acid, 3:1 (Carnoy fixative). After centrifugation, the fixed pellet was resuspended in fresh fixative and preparations were made by rapidly drying a drop of the cell suspension on a microscope slide with the help of a fan. Slides were stained with 1 % Giemsa stain in phosphate buffer (pH 7.0) for 2 min or with methyl green-pyronin for histochemical observations.

RESULTS

Mouse spermatocytes displayed in air-dried preparations stained with Giemsa reveal chromosomal features characteristic for pachytene (Fig. 1) and diplotene (Fig. 2). In pachytene nuclei, the sex chromosomes are represented by a uniformly condensed chromatin mass displaying 2 curved dense profiles corresponding to associated X and Y chromosomes (Fig. 1). For correlation purposes, Fig. 3 depicts a whole-mounted electron-microscope preparation of a pachytene spermatocyte in which the sexual bivalent displays similar curved profile corresponding to chromosomal axial cores and associated chromatin fibres. Less-dense regions parallel to the dense profiles in Fig. 1 are interpreted as interchromosomal spacings. Autosomal bivalents are represented by threads of variable length with fuzzy boundaries due to a ‘lampbrush’ configuration determined by looping chromatin fibres (Kierszenbaum & Tres, 1974b).

In diplotene nuclei (Fig. 2), the XY bivalent stands out from chromosomal bivalents by its heteropycnosis. The U-shaped sexual bivalent displays dense staining at resolved free endings. Staining is interpreted as indicating paracentromeric heterochromatin regions of acrocentric sex chromosomes. Autosomal bivalents exhibit similar deeply stained free endings and structural evidences for chiasmata.

These structural chromosomal features and other chromosomal components not resolved with the light microscope become better defined when similar meiotic prophase spermatocytes are displayed for study with the electron microscope. Whole-mount electron-microscope preparations permit the identification of complete bivalent complements of mouse spermatocytes (Kierszenbaum & Tres, 1974b). A pachytene spermatocyte (Fig. 4) displays completely paired homologues exhibiting synaptonemal complexes at the pairing region. Autosomal synaptonemal complexes have different lengths. This feature represents an indirect marker for chromosomal length. Each autosomal bivalent has 2 endings. One ending is equivalent to the basal knob region described in sections by Woollam & Ford (1964) and appears in air-dried preparations as deeply stained blocks of heterochromatin (Fig. 2). These endings have been described in mouse metaphase karyotypes by Hsu, Cooper, Mace & Brinkley (1971). Identification of the basal knob region in acrocentric bivalents permits chromosome
Figs. 1, 2 are light micrographs of Giemsa-stained, air-dried preparations.

Fig. 1. Pachytene mouse spermatocyte. Arrows indicate 2 curved profiles corresponding to paired XY chromosomes (cf. Fig. 3).

Fig. 2. Early diplotene mouse spermatocyte. Arrows indicate intensely stained free ends of a sex bivalent. Similar dense stained terminal regions are observed on autosomal bivalents involved in a disjunction process.

Figs. 3–13 display spermatocyte meiotic prophase chromosomes visualized in electron-microscope preparations made according to the Miller technique (1972) adapted for the testis (Kierszenbaum & Tres, 1974b, 1975).

Fig. 3. Moderately dispersed pachytene spermatocyte nucleus. The condensed mass of the XY bivalent displays 2 curved profiles corresponding to axial chromosomal cores (arrows) surrounded by chromatin fibres. A less-dense region is observed between the 2 curved densities and corresponds to interchromosomal spacing (cf. Fig. 1). Arrowheads indicate synaptonemal complexes of autosomal bivalents.
Fig. 4. Pachytene spermatocyte. Autosomal synaptonemal complexes display variable length. Some of them are twisted (arrows). The XY pair displays axial cores of different lengths, paired over a short segment (ps). Overlapped chromatin fibres do not permit clear display of the central element of some synaptonemal complexes.
Fig. 5. Late zygotene spermatocyte. The arrow points an autosomal bivalent with synapsis less complete than the synapses of other autosomal bivalents displayed in the micrograph. *nu*, nucleolus attached to the basal knob region (*bk*) of an autosome. The XY pair displays a pairing segment (*ps*). Notice the difference in thickness between the X and Y axial chromosomal cores.
Fig. 6. Early pachytene spermatocyte. A, the sexual bivalent (XY) exhibits extensive side-by-side pairing. Autosomes are completely paired. Arrows indicate the region scanned with the microdensitometer (see Fig. 6B). B, widths and heights of peaks indicate differences in thickness and density of X and Y chromosomal cores. Tracings were made with a Joyce-Loebl Mark III automatic recording microdensitometer from a positive transparent print corresponding to Fig. 6A. The microdensitometer was operated with a 40-μm slit, 48-mm lens, wedge B and open iris. C, diagrammatic representation of the XY pair illustrated in A indicating length values (see Results for details).

Fig. 7. Early pachytene. XY pair displaying extensive pairing and divergent segment of the Y chromosomal axial core. The arrow points a twist at the pairing region.
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orientation since this region is located close to the centromere. Depending on the degree of nuclear dispersion in the preparations, the XY pair usually displays a characteristic chromatin condensation pattern which differs from that observed in autosomal bivalents (Fig. 3). However, the differing lengths of X and Y axial chromosomal cores provide reliable criteria for identifying sexual bivalents in whole-mount preparations (Figs. 4, 5, 6a, 7-13).

Mouse spermatocytes at zygotene, pachytene, and diplotene stages can be identified by following assembling features of synaptonemal complexes and structural aspects of axial chromosomal cores of XY pairs. Additional criteria are the display of nucleoli attached to the basal knob region of some autosomal bivalents (Fig. 5 and Kierszenbaum & Tres, 1974a, b), and the amount and distribution of non-nucleolar ribonucleoproteins in the complete chromosomal complement (Tres & Kierszenbaum, 1976). Zygotene spermatocytes are recognized by asynchronous chromosomal pairing, with some of the homologous chromosomes displaying complete synapsis and completion of synaptonemal complex formation (Fig. 5). Moreover, the presence of a nucleolus still attached to an autosomal bivalent indicates synapsis and not disjunction (as at diplotene), since autosomal bivalents bearing nucleoli are no longer observed at diplotene (Kierszenbaum & Tres, 1974a).

During pachytene, while homologous autosomes remain associated side by side along their entire length (Fig. 4), the XY bivalent displays temporal and spatial variations in the extent of pairing and in conformation of axial chromosomal cores. Thus, presumably during late zygotene and early pachytene, X and Y chromosomes are paired side by side along most of the length of the Y chromosome (Figs. 6, 7). Length measurements of sex-chromosomal cores indicate maximum values of 9.5 μm for the X and 3.6 μm for the Y. The length of the paired segment is about 3.3 μm (Figs. 6B, 14). Along the paired segment the thick axial core (about 90 nm) of the X chromosome is aligned with the less-thick (about 50 nm) of the Y chromosome. Each sex chromosomal core is double (Figs. 5, 6A, 7, 10-12) and forms short synaptonemal complexes at unpaired segments of the XY bivalent (Solari, 1970; Kierszenbaum & Tres, 1974b).

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Fig. 8. Middle to late pachytene. Thickening of chromosomal cores is observed along the paired segment of the X and Y.

Fig. 9. Stage as in Fig. 8. X and Y chromosomal axial cores are composed of 2 separate elements in the region of pairing.

Fig. 10. Stage similar to Figs. 8, 9. An autosomal bivalent is highly twisted, its lateral elements displaying 3 crossing points and forming the synaptonemal complex. The cores of the X and Y are divided into fascicles in the region of pairing. At core subdivision points, X and Y cores are close at one point and diverge at the opposite endings. Inset: detail of the pairing region.

Fig. 11. End of late pachytene. The XY paired region is short. Unpaired chromosomal cores are ribbon-like and display their dual nature.

Fig. 12. End of late pachytene-beginning of diplotene. End-to-end association of the XY pair. Distal free endings of autosomal synaptonemal complexes display dense and expanded lateral elements. A similar feature is observed in the sexual bivalent. The arrows indicate that the Y chromosomal core consists of 2 elements.
Fig. 13. Diplotene. The XY pair in this spermatocyte remains associated by a small, dense pairing region. Bulges (arrows) are observed along the X and Y chromosomal cores. Autosomal bivalents are disjoined. Inset: autosomal bivalent displaying side-by-side (arrow) and end-to-end attachments.

One can assume that each core subdivision constitutes a lateral element of the complex.

As pachytene progresses, the synaptonemal complex region between X and Y chromosomes shortens. At late pachytene-diplotene, the sexual pair either maintains a very short pairing region (Figs. 4, 8–10) or X and Y chromosomes remain attached end to end (Figs. 12, 13). Another conspicuous feature at middle-late pachytene is a thickening and lengthwise splitting of each lateral element into 2 major fascicles, forming the synaptonemal complex at the pairing segment (Figs. 8–10). The double
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feature of each axial chromosomal core displayed at this region supports the assumption that at least 2 axial filaments constitute each sex chromosomal core along its entire length. Furthermore, when unpaired axial cores adopt a ribbon-like configuration (Figs. 11, 12), the 2 subunits are conspicuously displayed. Coinciding with these changes, autosomal synaptonemal complex free endings increase in density as a consequence of developing lateral elements deltoid expansions (Figs. 12, 13).

Fig. 14. Lengths of X and Y axial cores and of XY paired segments (ps), measured from prints of micrographs of 200 spermatocytes in various meiotic prophase stages as displayed in preparations made according to the procedure described in Materials and methods. The length of each axial chromosomal core was obtained by matching pieces of thin copper wire with the core density. The graph indicates that during zygotene, pachytene, and diplotene, the sex chromosomal cores shorten. The length of the paired region approaches the length of the Y chromosomal core at early pachytene and decreases progressively until disappearing at diplotene. Z, zygotene; eP, early pachytene; mP, middle pachytene; IP, late pachytene; D, diplotene.

During diplotene, sex chromosomal axial cores display at variable points outward expansions ('bulges'), each limited by a pair of thickened, dense walls having a less-dense centre (Fig. 13). Autosomal bivalents disjoin asynchronously and adopt elliptic or circular conformations (Fig. 13), remaining associated at terminally expanded segments by both side-by-side and end-to-end association (Fig. 13, inset).

Discussion

The conformation of mouse spermatocyte autosomal and sexual bivalents during zygotene, pachytene, and diplotene has been studied by whole-mount electron microscopy. It emerges that certain meiotic prophase stages can be identified by evaluating structural changes in axial cores of the sexual bivalent. Structural changes at synaptonemal complex endings of autosomes are also evident and permit identification of diplotene. These changes are characterized by terminal deltoid expansions of lateral elements of complexes which persist during disjunction of homologous
autosomes. These findings aid in recognition of male meiotic prophase stages in whole-mount preparations since determination of stage by cellular association is not possible with this method.

Spatial relationships and core length measurements of X and Y chromosomes in mouse spermatocytes have already been described on the basis of reconstructions of sexual bivalents from serial sections (Solari, 1970). Since a considerable number of well displayed spermatocytes can be studied in whole-mount electron-microscope preparations, it is now possible to re-evaluate intrinsic characteristics of XY pairing, known to be remarkably variable, not only in the mouse, but also in other species, including man (Solari & Tres, 1970; Tres, 1975).

Mode of association of the sexual pair

Earlier studies have indicated that X and Y chromosomes of the mouse were associated either end to end (Ohno et al. 1959) or side by side throughout the entire length of the Y (Slizynski, 1955), or along a short common region in which a synaptonemal complex is displayed (Solari, 1970; Kierszenbaum & Tres, 1974b). The possibility of side-by-side pairing was soon disregarded on the grounds of a mis-identification of the XY pair (Geyer-Duszyńska, 1963). End-to-end association was recognized at pachytene, remaining until first metaphase, and thought to be a consequence of chiasmata formation (Ohno et al. 1959). Evidence provided by a staining procedure which permits localization of centromeric regions indicated that X and Y chromosomes in mouse spermatocytes are associated at their ends opposite to the centromeres (Schnedl, 1972). Electron-microscope studies have shown that from zygotene on, the sexual bivalent contains 2 unpaired chromosomal cores of unequal length, paired at one end by a short synaptonemal complex which is longest (about 1.9 µm) at zygotene (Solari, 1970). Findings presented in this paper show a greater length value (about 3.3 µm) for the pairing segment as displayed presumably at early pachytene. According to Koller & Darlington (1934), each sex chromosome contains a homologous segment by which they pair and form chiasmata during meiotic prophase. Formation of chiasmata implies that genes located in the pairing segment can be exchanged between X and Y chromosomes, thus displaying partial sex linkage. Differential segments (Koller & Darlington, 1934) represented by unpaired X and Y chromosomal regions, imply that genes in these unpaired regions cannot be exchanged and are therefore totally sex-linked. However, partial sex linkage has not been clearly demonstrated genetically in the mouse (Sachs, 1955) and the significance of a synaptonemal complex at the pairing region is still open to interpretation.

It can be argued that extensive side-by-side pairing of X and Y chromosomal cores can be facilitated by mechanical forces incurred during technical manipulation. Yet several structural features support the concept of extensive XY pairing. First, the distance between paired axial cores forming lateral elements of a synaptonemal complex is equivalent to the distance existing between lateral elements of synapsed homologous autosomes displayed in the same chromosomal complement. Second, a small terminal segment assumed to represent the centromeric end of the Y does not participate in pairing and is displaced from the X chromosomal core, forming an
angle of approximately 45° with respect to a projection of the axis of pairing. The angle of divergence changes as the paired segment decreases in length. Third, although the behaviour of the XY bivalent of different species varies greatly, side-by-side pairing is a recognized feature in the Chinese hamster (Cricetus griseus: Ohno & Weiler, 1962; Fredga & Santesson, 1964). Side by side pairing of the XY bivalent of the Chinese hamster has been attributed to the large size of the Y chromosome, which is almost as large as the X (Ohno & Weiler, 1962), resulting from a translocation involving a small Y, a large X, and a homologous autosomal pair (Matthey, 1961).

In a previous study it was noted that whole-mount XY bivalents of pachytene mouse spermatocytes display double axial cores (Kierszenbaum & Tres, 1974b). Axial cores of the unpaired segments diverge and form short synaptonemal complexes. Therefore, it can be assumed that the doubleness of the chromosomal axial core is the result of closely apposed pairs of chromatid cores. Thus, the synaptonemal complexes of the unpaired X and Y segments can be interpreted as formed by contributions of appropriately coaligned axial cores from sister chromatids and not by abnormal branching of a single chromosomal core (Solari, 1970). Since one generally thinks of synaptonemal complexes as forming between homologous chromosomes and leading to genetic exchange (Moses, 1968), one faces possibilities of exchanges of genetic information between sister chromatids, though some may prefer to explain this finding in terms of a mechanism by which paired chromatids are locked together to prevent embarrassing lateral displacements. In this respect, it has been shown recently in metaphase I mouse spermatocytes that sister chromatid exchange occurs in the X chromosome after an in vivo treatment with 5-bromodeoxyuridine-33258 Hoechst (Allen & Latt, 1976).

The behaviour of sex chromosomal axes at late pachytene-diplotene

In mouse spermatocytes the double regions of chromosomal axial cores can show deformed, fusiform bulges along sexual chromosomal cores at late pachytene-diplotene. Similar core deformations have been observed in lateral elements of synaptonemal complexes of Lilium polyploids ('deformed lateral elements': Moens, 1968), of pollen mother cells of Phaedranassa viridiflora ('hollow-centred bulges': La Cour & Wells, 1973) and in chromosomal cores of the XY pair of the Armenian hamster (Cricetus migratorius) observed at middle pachytene ('bulging': Solari, 1974). We suggest that the mechanism leading to focal deformation of cores may be related to core doubleness and increasing chromatin condensation of the sexual bivalent as meiotic prophase advances. This concept is similar to the view of La Cour & Wells (1973). As a result of chromatin condensation, each double axial core, acting as an elastic tube-like structure, is deformed along its axis and at its endings, forming outward expansions distributed at variable intervals. Since a relatively constant number of deformations are seen for each X and Y chromosomal core, it can be assumed that the reduction in length of each core is related to the number of bulges.
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