Telomere-independent homologue pairing and checkpoint escape of accessory ring chromosomes in male mouse meiosis

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We analyzed transmission of a ring minichromosome (MC) through mouse spermatogenesis as a monosome and in the presence of a homologue. Mice, either monosomic or disomic for the MC, produced MC+ offspring. In the monosomic condition, most univalents underwent self-synapsis as indicated by STAG3, SCP3, and SCP1 deposition. Fluorescent in situ hybridization and three-dimensional fluorescence microscopy revealed that ring MCs did not participate in meiotic telomere clustering while MC homologues paired at the XY-body periphery. Self-synapsis of MC(s) and association with the XY-body likely allowed them to pass putative pachytene checkpoints. At metaphase I and II, MC kinetochores assembled MAD2 and BUBR1 spindle checkpoint proteins. Unaligned MCs triggered the spindle checkpoint leading to apoptosis of metaphase cells. Other MCs frequently associated with mouse pericentric heterochromatin, which may have allowed them to pass the spindle checkpoint. Our findings indicate a telomere-independent mechanism for pairing of mammalian MCs, illuminate escape routes to meiotic checkpoints, and give clues for genetic engineering of germ line-permissive chromosomal vectors.

Introduction

Meiosis is a succession of two cell divisions by which the chromosome number is reduced from diploid to haploid to compensate for the genome doubling at fertilization. During the extended prophase of meiosis I, replicated homologous chromosomes (homologues) extend (leptotene), align, and initiate pairing (zygotene). Once pairing is complete (pachytene), there is only half the number of chromosomes than entered prophase I. During the first meiotic prophase, homologues undergo genetic exchange, which ensures their correct segregation during metaphase I (MI). During the second meiotic division, sister chromatids segregate to opposite poles, thereby forming haploid gametes or spores (von Wettstein et al., 1984; McKim and Hawley, 1995).

In most (if not all) organisms, meiotic telomeres cluster at the nuclear envelope during the leptotene/zygotene transition, which is known as the bouquet stage (Scherthan, 2001; Yamamoto and Hiraoka, 2001). Clustering of chromosome ends is thought to instigate homologue interactions and regional presynaptic alignment, which is transformed into stable pairing by the synaptonemal complex (SC; Zickler and Kleckner, 1998). The SC consists of two lateral elements (LE) that run along the sister chromatids of replicated homologues, and a central element that is formed by transverse filaments connecting the LEs (von Wettstein et al., 1984). Before synopsis, the LEs are called axial elements (AE). Three SC proteins have been characterized in mammals: SCP2 (Offenberg et al., 1998) and SCP3 (Lammers et al., 1994; Yuan et al., 2000), which both are components of the AE/LE (Schalk et al., 1998; Yuan et al., 2000), and SCP1 (Meuwissen et al., 1992), which is a major component of transverse filaments (Schmekel et al., 1996).

Abbreviations used in this paper: 3D, three dimensional; AE, axial element; DSB, DNA double-strand break; IF, immunofluorescent; LE, lateral element; MC, minichromosome; MI, metaphase I; MII, metaphase II; PNA, peptide nucleic acid; SC, synaptonemal complex.
AEs are built on cohesin cores that form after premeiotic DNA replication and consist of different subunits (SMC1α, SMC1β, SMC3, REC8, and STAG3; Prieto et al., 2001, 2002; Revenkova et al., 2001; Eijpe et al., 2003). The separation of homologues is achieved by removal of sister chromatid cohesion along chromosome arms at the onset of anaphase I, whereas cohesion at sister centromeres is lost at anaphase II (Buonomo et al., 2000; Petronczki et al., 2003). The complex events in meiotic differentiation are under checkpoint control, and meiotic disturbances elicit different responses in male and female mammals (Hunt and Hassold, 2002), which often prevent efficient germ line transmission of accessory (mini)chromosomes in murine spermatogenesis (Voet et al., 2001). Furthermore, knockout mice for genes involved in recombination and synapsis display sex-specific responses, with male gametogenesis often grinding to a halt during prophase I (Escalier, 2001; Hunt and Hassold, 2002). These observations have provided evidence for mammalian “pachyten” checkpoints that respond to defective meiotic recombination and/or synapsis in spermatocytes in a p53-dependent or -independent manner (Odorisio et al., 1998; Cohen and Pollard, 2001).

Furthermore, there is evidence for a spindle assembly checkpoint that operates at MI in mice, which responds to kinetochores that fail to attach or are not under tension (Woods et al., 1999; Sluder and McCollum, 2000). Thus, introduction of artificial chromosomes into a mammalian genome can be used to test crucial aspects of meiotic chromosome behavior and checkpoints.

Recently, we have generated “transchromosomal” mice carrying a chromosomal vector in the form of a small accessory human minichromosome (human MC, previously termed HCV; Voet et al., 2001). This MC consists mainly of alphoid sequences of the human chromosome 20, a fragment of the human chromosome 1p22 region, and lacks telomere sequences (Voet et al., 2001). Most surprisingly, both male and female mice carrying the MC (MC⁺ mice) efficiently transmit the univalent MC to the offspring (Voet et al., 2001), even when a large transgene insert is present in the MC (Voet et al., 2003). Here, we analyze which checkpoint responses are elicited by the presence of a monosomic or disomic circular MC in spermatogenesis, test the role of telomeres for the pairing of ring minichromosomes, and disclose mechanisms that enable MCs to bypass meiotic checkpoints.

### Results

#### MC⁺ mice and offspring

MC⁺ mice, generated previously (Voet et al., 2001), were used to breed the MC into the C57Bl/6 and NMRI genetic background. Up to now, a total of 17 male mice, containing one copy of the MC, have produced 1,115 offspring, 308 of which inherited the MC. Mice with a second copy of the MC were generated by mating MC⁺ mice (Table I). For simplicity, mice (or cells) containing one copy of the MC are referred to as monosomic mice, and mice harboring two MC copies in most of their cells are designated as disomic mice.

All monosomic and disomic MC⁺ mice of both sexes tested so far produced live MC⁺ offspring when mated to wild-type mice. Additionally, meiotic MC missegregation occurred in disomic mice of both sexes because part of their offspring harbored two MCs when mated to wild-type mice (Table II) and because 8–37% of their sperm nuclei (∼100/animal) contained two or more MCs (Table III; Fig. 1 A). However, the MC-carrying males displayed similar testes weights (ANOVA combined with post-hoc test; P values > 0.16; Fig. 1 B).

### Table I. Frequency of MC⁺ primary tail fibroblast metaphases

| Mice         | 0 MCs | 1 MC | 2 MCs | 3 MCs | 4 MCs |
|--------------|-------|------|-------|-------|-------|
| Monosomic    |       |      |       |       |       |
| 1MC⁺-28      | 13    | 87   |       |       |       |
| 1MC⁺-39      | 17    | 77   | 4     | 2     |       |
| 1MC⁺-45      | 45    | 54   | 1     |       |       |
| 1MC⁺-20      | 12    | 86   | 2     |       |       |
| 1MC⁺-11      | 22    | 78   |       |       |       |
| 1MC⁺-23      | 26    | 68   | 6     |       |       |
| 1MC⁺-7       | 34    | 62   | 4     |       |       |
| 1MC⁺-6       | 28    | 69   | 3     |       |       |
| 1MC⁺-30      | 22    | 78   |       |       |       |
| Disomic      |       |      |       |       |       |
| 2MC⁺-7       | 6     | 38   | 52    | 3     | 1     |
| 2MC⁺-10      | 6     | 28   | 59    | 6     | 1     |
| 2MC⁺-35      | 5     | 46   | 48    | 1     |       |
| 2MC⁺-4       | 4     | 19   | 29    | 46    | 2     |
| 2MC⁺-6       | 5     | 24   | 41    | 30    |       |
| 2MC⁺-13      | 12    | 46   | 39    | 3     |       |
| 2MC⁺-16      | 16    | 48   | 32    | 2     | 2     |

### Table II. Transmission of MCs in disomic male and female NMRI wild-type crosses

| Parents                 | Disomic offspring | MC⁺ primary tail fibroblast cells |
|-------------------------|-------------------|----------------------------------|
|                         | 0 MCs | 1 MC | 2 MCs | 3 MCs | 4 MCs |
| Disomic male mated with |       |      |       |       |       |
| NMRI female            | Mouse A | 11 | 39 | 50 |       |       |
|                        | Mouse B | 7 | 37 | 57 |       |       |
|                        | Mouse C | 15 | 44 | 38 | 3 |       |
| Disomic female mated   |       |      |       |       |       |
| with NMRI male         | Mouse D | 15 | 41 | 44 |       |       |
|                        | Mouse E | 14 | 36 | 50 |       |       |
|                        | Mouse F | 13 | 49 | 38 |       |       |

*One litter of nine pups was analyzed. Four descendants had one MC, three (A–C) had predominantly two MCs.

*One litter of fourteen pups was analyzed. Four descendants had one MC, three (D–F) had predominantly two MCs.
Meiotic MCs contain active centromeres, but no telomeres
Because the data above show that MCs are able to bypass the stringent male meiotic control checkpoints, a feature that is rarely encountered in mammalian spermatogenesis (see Introduction), we next analyzed how the MC segregated through male meiosis in the monosomic or disomic condition.

First, we determined whether the MC is endowed with a functional centromere during meiosis and thus subject to checkpoint response. To this end, we performed combinatorial MC-specific FISH and immunofluorescent (IF) staining with either CREST antiserum or an anti-CENP-C antibody. Both types of experiments yielded dotlike signals on MCs in meiotic MI and metaphase II (MII; Fig. 1, C–F). Furthermore, we observed the presence of MAD2 and BUBR1 at MC kinetochores (see below).

FISH with a peptide nucleic acid (PNA) telomere probe to mitotic and meiotic metaphase chromosomes failed to detect telomere FISH signals at the MC (Voet et al., 2001). Similarly, IF staining with antibodies to TRF1 telomere repeat-binding factor (Broccoli et al., 1997) or mRap1 (Li et al., 2000) telomere proteins, which both stain meiotic telomeres (Scherthan et al., 2000), generated telomere signals at mouse SC ends, but failed to reveal telomere protein signals at the MCs (unpublished data). Furthermore, the MC resisted attempts to resolve it by pulsed-field gel electrophore-
sis, which is expected for a ring chromosome of this size. These and the following observations indicate that the artificial chromosome is a ring chromosome that contains a functional centromere.

**MCs assemble SC protein structures**

The fertility of MC<sup>+</sup> mice suggests germ line transmission in the presence of one and two (or multiple) MC copies. However, unpaired chromosome cores and absence of recombination appear to have the potential to trigger apoptosis through checkpoint activation in mammalian prophase I (for review see Cohen and Pollard, 2001). Two proteinaceous chromosome core structures, the AE/LE and the cohesin core (Pelttari et al., 2001; Prieto et al., 2002), contribute to correct meiotic chromosome segregation (Petronczki et al., 2003). To investigate whether the MC assembles meiotic chromosome cores, we combined MC-specific FISH with IF staining of STAG3 (a component of meiotic cohesin cores), and SCP3 or SCP1 (SC components) on monosomic and disomic pachytene nuclei. Dotlike STAG3 signals were seen at 82% of the monosomic nuclei (Fig. 2 A), whereas in 15% of the nuclei, a STAG3 signal spanned between the univalent MC and the cohesin core of an adjacent bivalent (Fig. 2 B).

SCP3 IF staining produced signal dots at the MCs of monosomic and disomic spermatocytes (Fig. 2, C and F). 18% of disomic pachytene nuclei showed SCP3 rods between two closely associated MCs (Fig. 2, D and F) that measured 0.6 µm (SD ± 0.06 µm). Interestingly, in 14% of monosomic pachytene nuclei, SCP3 rods spanned between the univalent MC and the SC of a nearby bivalent (Fig. 2, E and F). The rodlike SCP3 structures suggest that the cohesin and SCP3 dot-cores of individual MCs may serve as a nucleation point for AE polymerization until contact is made to an AE/LE of a neighboring chromosome or to another dot-core on a homologous MC.

**Immunostaining of SCP1**

Immunostaining of SCP1, a marker for synapsis (Meuwissen et al., 1992; Dobson et al., 1994), revealed a dotlike SCP1 signal at the MC (Fig. 3, F and I) in 73% of the monosomic pachytene nuclei (n = 100), which suggests that univalent MCs undergo self-synapsis and thereby evade checkpoint control.

In disomic MC<sup>+</sup> spermatocytes, combined SCP1 IF staining and MC-FISH disclosed that SCP1-positive structures connect two closely associated MCs in 78% of spermatocytes (n = 109). The SCP1 mini-cores (Fig. 3, A–D and J) measured 0.64 µm (SD ± 0.066 µm) in 61% of cases (n = 66; Fig. 3, B, C, and J), which agrees with the size of the SCP3 rods noted above. In
the remaining nuclei, a dotlike SCP1 signal (<0.5 μm; Fig. 3, A, D, and J) was present between two MCs, whereas 11% of juxtaposed MCs failed to exhibit a SCP1 connection. Nuclei with two individual, closely spaced MC signals showed either one SCP1 dot at each MC (Fig. 3, E, G, and J) or no signal at all (Fig. 3 J). The high frequency of the SCP1 rods suggests that SCP1 can extend between SCP3 dot cores because SCP3 rods were only seen in 18% of nuclei.

Because MCs without SCP1 labeling have the potential to trigger the putative pachytene checkpoint, we investigated seminiferous tubules of stages I to XI of testes tissue sections (that harbor pachytene spermatocytes; Oakberg, 1956) for apoptotic cells by TUNEL assay. However, no significant differences were detected between the frequencies of apoptotic cells between monosomic, disomic, and wild-type stage I–XI tubules (Table IV). This suggests that monosomic and disomic MCs efficiently transit through prophase I, with the disomic MCs obviously undergoing an authentic mode of synaptic pairing.

Telomere-independent homologue pairing of MCs

The pairing of the small MCs (estimated size of 10–15 Mb) in the huge mouse spermatocyte nucleus poses the question of how the small MCs associate with each other, especially because MC-FISH revealed that they are spatially separated in premeiotic cells (in >80% of disomic MC+ spermatogonia). At preleptotene, centromeres relocate to the nuclear periphery (Scherthan et al., 1996). In preleptotene nuclei of HMC+ mice, MCs were also found to follow these movements: three-dimensional (3D) microscopy revealed that 88% of the monosomic and 75% of the disomic preleptotene spermatocytes displayed peripheral MC(s) (unpublished data). In disomic preleptotene spermatocytes, only 13% of peripheral MCs were closely associated. Thus, MCs, just like authentic centromeres, transit to the nuclear envelope during preleptotene, and they apparently pair later in prophase I.

A potential mechanism to instigate meiotic chromosome pairing is meiotic telomere clustering or bouquet formation (Scherthan, 2001; Yamamoto and Hiraoka, 2001). Thus, we prepared three-dimensionally preserved nuclei from testes of monosomic and disomic MC+ mice 12 d post partum, which are moderately enriched in bouquet stage nuclei. Two-color FISH with a (T2AG3)n telomere and an MC-specific alphoid probe was used to identify bouquet nuclei with telomeres clustered in a limited region of the nuclear periphery, which also contain a few peripheral DAPI-bright heterochromatin masses (Scherthan et al., 1996). It was found that 97% of monosomic bouquet stage nuclei (n = 34) displayed the MC away from the bouquet base (Fig. 4, A and E). In the remaining nucleus, the MC was detected at the telomere cluster (Fig. 4, B and E). In all but one disomic bouquet nuclei, both MCs located away from the telomere-cluster site (n = 16; Fig. 4, C–E). Although the MCs failed

![Figure 3. MCs contain SCP1.](image-url)
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Figure 4. **MCs locate remote from the telomere cluster in bouquet nuclei.** (A and B) FISH with a \((C_3TA_2)_3\) PNA telomere probe (green) and an \(\alpha\)-satellite DNA MC probe (red; white arrowhead) on monosomic bouquet stage nuclei (1MC–30; 12 d post partum). (A) A spermatocyte I with one MC located remote from the telomere cluster at the top of the nucleus (the latter faces the observer). (B) Spermatocyte with the MC located among the tightly clustered telomeres at the bouquet base (yellow arrowhead). Focal plane near the nuclear top. (C and D) Bouquet stage nuclei of a 12-d post partum disomic mouse (2MC–7). (C) Two separate MCs (white arrowheads) located away from the bouquet base (yellow arrowhead). Focal plane at nuclear equator. (D) Spermatocyte I nucleus with relaxed telomere clustering and two paired MCs (white arrowheads) that create a single large MC signal in the nuclear interior below. Focal plane at the nuclear top. The bar in A represents 5 \(\mu\)m and applies to A–D. (E) Distribution frequencies of MCs with respect to the telomere cluster site. Two MCs were generally absent from the telomere cluster region.

MCs associate with the XY body

The data above are suggestive for the presence of a bouquet-independent mode of chromosome alignment and pairing, at least for MCs. Similar numbers of apoptotic spermatocytes in stage I–XI seminiferous tubules of monosomic, disomic, and wild-type mice (Table IV) indicate that MCs manage to bypass checkpoints that putatively monitor synopsis and/or DNA double-strand break (DSB) repair (Roeder and Ballis, 2000; Cohen and Pollard, 2001). A subcompartment of the spermatocyte I nucleus that tolerates the presence of asynapsed chromosome cores is the XY body (Jablonka and Lamb, 1988; Turner et al., 2000). Thus, we tested whether the univalent MC associates with the XY body by X chromosome–specific FISH (Disteche and Adler, 1990) and an MC-specific alphoid probe. In 87–100% of late pachytene/diplo-tene monosomic nuclei (\(\geq 40\) nuclei analyzed in each of eight monosomic mice), the MC was associated with the morphologically distinct XY body. Furthermore, \(\sim 90\%\) of the MCs in disomic spermatocytes (\(\geq 29\) nuclei analyzed in each of two disomic mice) displayed an MC/sex body association. To scrutinize this association in more detail, we stained the MC by FISH and the sex-body chromatin with the XMR antibody (Calenda et al., 1994). 3D wide-field light microscopy disclosed that the MCs were located at the periphery or were partially submerged in the XMR-marked sex body (Fig. 5, A and B) in 97 and 94% of 35 monosomic and 34 disomic pachytene nuclei. Similar results were obtained when we stained the MC by FISH and the XY body with antibodies to phosphorylated histone (\(\gamma\)H2ax) (Fig. 5 C). \(\gamma\)H2ax IF staining first marks the onset of DSB repair at meiosis, and later is restricted to the XY body, where it is required for meiotic sex chromosome inactivation (Mahadevaiah et al., 2001; Fernandez-Capetillo et al., 2003). At leptotene/zygotene, MCs were covered with the abundant \(\gamma\)H2ax signals (unpublished data), whereas in pachytene nuclei, the \(\gamma\)H2ax–sex body signal and MC signal partially colocalized (Fig. 5 C). However, monosomic and disomic MCs that were not associated with the XY body did not display a \(\gamma\)H2ax signal (Fig. 5 D). This suggests that MCs likely follow the mode of DSB repair of their local chromatin environment.

Most interestingly, in 74% of disomic nuclei with an MC/sex body association both MC signals were paired (Fig. 5, B and C), whereas in the remaining 20% of the spermatocytes, the XY body–associated MCs were separated (Fig. 5 A). Localization of an MC completely inside the XY body was only seen in 3% of the nuclei analyzed, both in monosomic and disomic mice. Thus, it appears that the MC(s) undergoes an intimate interaction with the periphery of the XY body where it most likely “hitch-hikes” through male prophase I, thereby escaping potential checkpoints that control synapsis and/or DSB repair.

**MC bivalents frequently disjoin precociously in MI, but maintain sister centromere cohesion**

Next, we investigated, in disomic mice, whether an MC bivalent forms that aligns in the MI plate because disomic MCs in MI lacked the SCP3- and SCP1-positive “mini” cores as well as MLH1 foci at pachytene (unpublished data). This suggests that a fraction of MC bivalents may lack chiasmata and predicts that the MC bivalent could disjoin precociously. MC FISH to MIs showed that only 18–44% of the disomic MIs (\(n = 23; 28\) and 9, respectively) contained paired MCs with juxtaposed FISH signals with their DAPI outline touching or overlapping (Fig. 6 A). The remaining disomic MIs
contained separated MC signals (Fig. 6 B), which suggests that a significant number of MC bivalents separated precociously.

Next, we analyzed for sister chromatid cohesion by MC-specific PAC FISH, which creates minute FISH signals and allows to discriminate chromatid signals on MCs. Approximately 66% (mean; range 59–78%; n = 55 MII nuclei) and 74% (mean; range 66–82%; n = 44 MII nuclei) of the MCs in MII of respectively three monosomic and two disomic mice displayed two sister chromatid FISH signals (Fig. 6, C and D), which suggests that the MCs segregate to the same

Figure 5. MC/XY body associations as revealed by 3D imaging. (A) Disomic pachytene nucleus (2MC in 6) shows the XY body (as defined by XMR IF staining, green) associated with two separated MCs (red signals, α-satellite FISH; arrowheads). The image represents the projection of 10 optical sections encompassing the XY body as obtained by 3D imaging. Bar, 2 μm. (Bi) Deconvoluted 3D volume image from image planes encompassing an XY body from another disomic nucleus. The two paired MCs (one large red signal) are partially embedded (indicated by the shadowed part of the MC signal) in the XY body chromatin (green). (Bii) Shows a projection of the images before volume imaging. (C) Volume reconstruction of a γ-H2ax–stained XY body (red) and associated MCs (green). The paired MCs appear partially embedded in the XY chromatin. (D) Disomic spermatocyte (DNA blue; partial detail) showing the MC bivalent signal distant from the γ-H2ax–stained XY body (red). The paired MCs lack γ-H2ax signals.

Figure 6. MCs in MI and MII. (A–D) FISH with an MC chromatid-specific PAC probe (red; see Materials and methods) on MI (A and B) and MII (C and D) spreads of a disomic mouse. (Ai) An MC bivalent, remote from mouse centromeres, displays split FISH signals (arrowheads), indicating maintenance of cohesion at sister centromeres. (Bi) Two MCs showing split chromatid signals (arrowheads) are located separate from each other, but each are associated with a mouse pericentric DAPI-bright heterochromatin block (black in ii). Bar, 5 μm. (Ci) An MC displaying sister chromatid signals (arrowhead) in an MII spermatocyte. Bar, 10 μm. (Di) An MII spermatocyte with one MC with two chromatid signals (arrowhead) and a second MC that is present as a single sister chromatid (arrow) due to loss of sister chromatid cohesion during MI. All DAPI images (Aii–Dii) are shown in grayscale. (Ei) IF staining for STAG3 (red) combined with MC FISH (green) on a meiotic MI showing a STAG3 signal at the MC (arrowhead). (Eii) STAG3 channel only.
pole during anaphase I. In agreement with maintenance of sister centromere cohesion at MI, staining for the meiotic cohesin STAG3 revealed a dotlike signal at the MCs of both monosomic and disomic MI spermatocytes (Fig. 6 E).

**MCs can trigger the spindle checkpoint**

Homologous centromeres that fail to attach to opposite spindle poles, and hence the lack of tension during MI, may trigger the spindle assembly checkpoint in MI (Rieder et al., 1995; Woods et al., 1999; Shonn et al., 2000; Nicklas et al., 2001). Thus, we determined that in the behavior of MCs during mouse MI and MII in stage XII tubules of monosomic, disomic, and wild-type testes sections by TUNEL labeling and anti-phosphohistone H3 (pH3; a metaphase marker) immunofluorescence, meiotic divisions occur only at this stage of mouse spermatogenesis (Oakberg, 1956). In-

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**Figure 7. Metaphase checkpoint response in monosomic and disomic MC° spermatogenesis.** (A and B) IF staining for phosphohistone H3 (pH3, red signal) combined with TUNEL labeling (green) on frozen testes sections of a disomic mouse. XII denotes a part of a seminiferous tubule at stage XII. (Ai) An apoptotic metaphase positive for TUNEL (green) and pH3 labeling (red); (ii) green channel, (iii) red channel, (iv) color inverted, grayscale DAPI image. (C) Table of the mean number of apoptotic nuclei detected in stage XII tubules of two WT, four monosomic (1MC°-64, -66, -73 and -7), and four disomic testes (2MC°-4, -6, -13 and -16). Apoptotic cell death of metaphase cells (>70% of all apoptotic cells) was increased in disomic as well as monosomic MC° spermatogenesis. (Di) TUNEL-labeled (green) apoptotic MI that displays a precociously separated MC bivalent (red, arrowheads). One homologue has lost sister cohesion (bottom arrowheads). (Ei) TUNEL-positive apoptotic MI (red) with an MC (arrowhead) off the MI plate. (Ai; Bi, Di, and Ei) Corresponding, color-inverted, grayscale DAPI images.
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vestigation of >10 stage XII tubule cross sections per testis of each of four monosomic, four disomic, and two wild-type testes revealed a 2–10-fold increase in the mean number of apoptotic cells in monosomic and disomic MC-containing stage XII tubules when compared with wild-type stage XII tubules (Fig. 7 C), with ≥70% of the apoptotic cells being metaphases (Fig. 7, A and B). Furthermore, apoptotic MIs generally revealed one or more MC signals off the MI plate (Fig. 7, D and E), which suggests that precocious separation of MC bivalents and/or noncongressed MCs triggered the spindle checkpoint. In agreement, it was found that MC kinetochores in prometaphase I and II spermatocytes displayed distinct signals of MAD2 (Chen et al., 1996) and BUBR1 (Chan et al., 1998; Fig. 8, A–D), proteins that are central to the spindle checkpoint (Cleveland et al., 2003).

Figure 8.  MAD2 and BUBR1 staining of (pro-)metaphases I and II.  (Ai) IF staining for MAD2 (red) combined with MC-FISH (green) on a meiotic prometaphase I reveals presence of MAD2 at the MC kinetochores. (Bi) Meiotic prometaphase II with MAD2 present at the MC kinetochores. (Ci) Presence of BUBR1 signals (red) and MC-FISH signals in a prometaphase I. (Di) Meiotic prometaphase II with BUBR1 detected at the MC kinetochores. Arrowheads denote the MC FISH signals (i) and their respective kinetochore signals (ii). (Ai–Di) Red channels only. (Ai–Di) Corresponding color-inverted, grayscale DAPI images.

MCs associate with pericentric heterochromatin at MI

However, the question remained how the majority of mono- and disomic MCs bypass the meiotic spindle checkpoint and are transmitted to the offspring. Univalent human mini- or microchromosomes have been reported to pass through the germ line and show an inherent tendency for centromeric associations (Felbor et al., 2002). Because heterochromatin associations have the capacity to perpetuate the segregation of nonexchange chromosomes in some organisms (for review see McKim and Hawley, 1995; Bernard and Allshire, 2002), we determined whether our MC(s) undergo associations with mouse pericentromeric heterochromatin by MC-specific α-satellite and mouse major satellite DNA FISH to MIs. About 60% of the MCs of monosomic mice showed associations between the MC and the pericentric heterochromatin of MI bivalents (Fig. 9 A). A high frequency of MC–heterochromatin association was also seen in disomic MC spermatocytes (Fig. 9 B). Thus, it may be assumed that centromeric associations, possibly instigated by the general stickiness of pericentric heterochromatin earlier during mouse prophase I (Hsu et al., 1971; Scherthan et al., 1996), may contribute to transmission of MCs through the mammalian germ line.

Discussion

We have investigated the transmission of one or more circular MC during mammalian spermatogenesis. These MCs contain human α-satellite and unique DNA sequences, and assemble functional centromeres that contain kinetochores that are associated with spindle checkpoint proteins like MAD2 (Chen et al., 1996) and BUBR1 (Chan et al., 1998), whereas MCs lack telomere sequences (Voet et al., 2001) and associated proteins.
Telomere-independent homologue pairing of MCs.

The unique MC⁺ karyotype allowed for the first time to test the requirement of telomeres for participating in meiotic telomere clustering (bouquet formation) and its contribution to the meiotic homologue pairing process in mammals. MCs without telomeres generally located remote from the telomere cluster in the majority of monosomic and disomic bouquet nuclei. The occasional association of a circular MC at the bouquet basis could be a consequence of MC–heterochromatin associations during mouse prophase I. Thus, our data provide evidence that telomeres are required for participation in the mammalian bouquet stage. They contrast with the association of a plant ring chromosome with the telomere cluster in the maize bouquet (Carlton and Cande, 2002), and suggest that the latter is mediated by a significant amount of interstitial telomere sequences still present at the bouquet basis. The occasional association of a circular MC at the bouquet basis could be a consequence of MC–heterochromatin associations during mouse prophase I. Thus, our data provide evidence that telomeres are required for participation in the mammalian bouquet stage. They contrast with the association of a plant ring chromosome with the telomere cluster in the maize bouquet (Carlton and Cande, 2002), and suggest that the latter is mediated by a significant amount of interstitial telomere sequences still present at the fusion site of the maize ring chromosome. The findings in mammalian MC⁺ meiosis are in agreement with those in Schizosaccharomyces pombe meiosis, where functional telomeres are required for bouquet formation and the tethering of an MC to the bouquet base (for review see Yamamoto and Hiraoka, 2001). Thus, it will be interesting to investigate the behavior of telomere-bearing MCs in mammalian meiosis.

Due to the above features, we expected that the MC homologues would fail to pair. Surprisingly, a high number of the disomic pachytene nuclei displayed paired MCs that were connected by short SCP1-positive structures, which indicates a telomere-independent pathway to homologue pairing—at least for small mammalian supernumerary chromosomes. Surprisingly, in both monosomic and disomic spermatocytes, the MC(s) located to the periphery of the XY body. Notwithstanding other possibilities, it seems likely that passive movements of MCs, induced by the general nuclear and chromosome movements that occur during leptotene/zygotene of mammalian prophase I (Parvinen and Soderstrom, 1976; Scherthan et al., 1996), may have led to the association of the MCs with each other and the XY body.

MCs pass the pachytene checkpoint.

Analyses in knockout mice and yeasts have provided evidence for tight control of meiotic prophase progression (Roeder and Bailis, 2000). Pachytene checkpoint control likely monitors timely completion of DSB repair and chromosome synapsis, and elicits apoptotic cell death in male mice if triggered by the presence of unpaired axial cores (Odorisio et al., 1998; Cohen and Pollard, 2001). Surprisingly, similar amounts of apoptotic cell death in stage I–XI testes tubules and similar testes weights of MC⁺ and wild-type mice indicate that the MCs bypass these surveillance mechanisms at prophase I. This is likely mediated by assembly of a small meiotic chromosome core on the MC that contains meiosis-specific cohesins SCP3 and SCP1. The presence of SCP1 on univalent MCs suggests that these undergo self-synthesis. Self-synthesis occurs at univalent X chromosomes in female XO mice (Speed, 1986) and at unpaired XY axes (Eaker et al., 2001), and perpetuates meiosis in the haploid condition (Levan, 1942; Loidl et al., 1991). In the disomic condition, short SCP1-staining cores connected MC homologues, suggesting an authentic mode of synopsis.

Furthermore, the association of MC(s) with the XY body may mask any unpaired AEs from surveillance mechanisms because it tolerates the presence of unpaired chromosome axes (Jablonka and Lamb, 1988). The association of monosomic and disomic MCs with the sex body may thus be a crucial feature for their transmission through the male germ line and may explain the absence of fertility problems in the MC⁺ mice. In agreement, passage of a supernumerary chromosome 21 up to MI was most likely mediated by XY body association (Johannisson et al., 1983).

MCs and the spindle checkpoint.

The spindle checkpoint in male mammalian meiosis I (LeMaire-Adkins et al., 1997) is triggered by kinetochores that are not under tension (Eaker et al., 2001; Skoufias et al.,
C57Bl/6 and NMRI genetic background. The F3 and F4 inbreeds were
dependent MC homologue–pairing mechanism and the ex-
checkpoint in male meiosis.

largely of satellite DNA, a building block of heterochroma-
(Felbor et al., 2002). Therefore, and because MCs consist
at metaphase have been transmitted through the germ line
microchromosomes that showed centromeric associations
chromatin of mouse MI chromosomes. Moreover, human

in 2-methyl-butate at
somal vector (MC) by standard blastocyst injection of MC
al., 2001). In brief, we generated two male chimeras containing a chromo-

Materials and methods

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During meiosis I, chiasmata contribute to the forma-
tion of tension at bivalents (Rieder et al., 1995; Shonn et al.,
2000; Nicklas et al., 2001; for review see Petronczki et
al., 2003). Mammalian univalent chromosomes have been
found to trigger the spindle assembly checkpoint leading to
apoptotic cell death of MI spermatocytes (Eaker et al., 2001,
2002). MAD2 and BUBR1, proteins whose turnover is cru-
cial for spindle checkpoint function (Skoulas et al., 2001;
Cleveland et al., 2003), both were present at MC kineto-
chores, suggesting that MCs are subject to checkpoint con-
trol. In agreement, disomic MCs that had separated preco-
ciously triggered apoptosis of metaphase cells. Similarly,
univalent (monosomic) MCs were also able to trigger the
spindle checkpoint. However, most univalent MCs main-
tained centromere cohesion and segregated randomly in the
MI division, thus undergoing a mode of segregation that has
been observed for univalent chromosomes in grasshopper
meiosis (Rebollo and Arana, 1998).

MCs associate with pericentric heterochromatin

Despite spindle checkpoint activation in some MC+ meta-
phase spermatocytes, a large number of MCs were present
in mature sperm, suggesting a bypass to spindle check-
point control. Heterochromatin associations have the po-
tential to compensate for absence of chiasmata and perpetu-
ate the segregation of nonexchange chromosomes in flies
(Dernburg et al., 1996). In MC+ MIs, we noted a frequent
association of the MCs with the pericentromeric hetero-
chromatin of mouse MI chromosomes. Moreover, human
microchromosomes that showed centromeric associations
at metaphase have been transmitted through the germ line
(Felbor et al., 2002). Therefore, and because MCs consist
largely of satellite DNA, a building block of heterochroma-
tin, it may be speculated that such associations could pro-
vide for a physical tether that compensates for the absence
of a homologue, thereby allowing for bypass of the spindle
checkpoint in male meiosis.

Altogether, our current data disclose a telomere-inde-
pendent MC homologue–pairing mechanism and the ex-
istence of important escape routes to meiotic checkpoint
control, both in prophase I and meiotic divisions. Such by-
passes may underlie the efficient germ line transmission of
human MCs and the generation of aneuploid sperm in
healthy human males.

Materials and methods

Mice

Mice containing the MC were generated as described previously (Voet et
al., 2001). In brief, we generated two male chimeras containing a chromo-
somal vector (MC) by standard blastocyst injection of MC+ embryonic
stem cells. These chimeras were mated with C57Bl/6 wild-type mice. Start-
ing from monosomic F1–1MC+ mice, the MC was further bred into the
C57Bl/6 and NMRI genetic background. The F3 and F4 inbreeds were
used for meiotic analyses. For the generation of disomic mice, monosomic
and disomic mice were respectively mated with each other.

Testicular specimens

Adult and 12-d post partum male mice were killed by cervical disloca-
tion, and testes were immediately resected and processed. For generating
frozen testes sections and IF staining, testes were shock frozen for 5 min
in 2-methyl-butate at −70°C and stored at −70°C until further use (Scher-
than, 2002).

Testicular preparations

Preparations for simultaneous IF staining and FISH were prepared by gen-
erating 14-μm frozen testes sections or by a modified squash procedure
(Page et al., 1998). In brief, frozen testicular tissue was minced in hypo-
tonic solution (0.075 M KCl) at RT. A drop of the suspension was placed
on clean amniolyse-coated glass slides and immediately mixed with two
drops of fixative (3.7% formaldehyde, pH 7.4). A coverslip was placed on
top of the suspension and pressure was applied. Slides with coverslip were
submerged in liquid nitrogen. After freezing, the coverslip was removed
and preparations were subjected to IF staining as described later in Materia-
ls and methods. Meiotic chromosome spreads were prepared as de-
scribed by Evans (1979).

Detergent spreading of spermatocytes was performed as described previ-
ously (Peters et al., 1997; Scherthan et al., 2000) with modifications as fol-
lows: ∼10 μl of a testicular suspension was placed on a glass slide and
mixed gently with 80 μl iodic detergent solution 1% LipsoL (LIP Equipment).
After 5 min, cells were mixed with 1% PFA, 5 mM NaBH3, pH 9.2, and
0.15% Triton X-100, and slowly dried in a humid chamber for 2 h, washed
3× with 0.01% Agepon (Agfa), and stored at −70°C until further use.

DNA probes

The 5×-satellite DNA probe was generated by low stringency PCR using
primers (5′-AGTAGTTTCTGGGCTCTC-3′; 5′-CAGAGGTTTTC-
CAAACTACTCTATG-3′) and as template DNA of an MC+ hamster cell line
(Voet et al., 1998). The mouse major satellite DNA probe was prepared by
PCR using mouse genomic DNA as template and primers (5′-CTCTG-
GAAATGCGCAGAAAA-3′; 5′-TCTCATTTTCAATCAGTCAC-3′; Scher-
than et al., 1996). The probe for the repeat cluster near the centromere of
the X chromosome (DXWas70; Distech and Adler, 1990) was a gift of C.
Distech (University of Washington, Seattle, WA). P1 artificial chromo-
somes (RP5–837O21 and RP5–573H3) were used as probes for detecting
human chromosome 1p sequences on the MC.

Antisera

Antisera to SC proteins SCP3 (Lammers et al., 1994) and SCP1 (Meuwis-
sen et al., 1992) were gifts of C. Heyting (Wageningen University,
Wageningen, Netherlands); anti-STAG3 (Pezzi et al., 2000) was a gift of J.
Barbero (Universidad Autónoma de Madrid, Madrid, Spain); anti-MAD2
(Chen et al., 1996) was a gift of R.H. Chen (Cornell University, Ithaca,
NY); anti-hBUBR1 (Chan et al., 1998) was a gift from T.J. Yen (Fox Chase
Cancer Center, Philadelphia, PA); anti-XMR (Calenda et al., 1994) was a
gift from H. Garchon (INSERM U25, Paris, France); and anti-CENP-C was
a gift from W.C. Earnshaw (University of Edinburgh, Edinburgh, UK).
Anti-
M31 was from Serotec; anti-phosphorylated histone H3 was from New
England Biolabs, Inc.; and anti-MIL1 was from BD Biosciences; anti-
γ-H2ax was from Upstate Biotechnology. Anti-TRF1 and anti-hRAP1 anti-
odies (Broccoli et al., 1997; Li et al., 2000) were gifts of T. de Lange (The
Rockefeller University, NY). CREST antiserum was as described previ-
ously (Scherthan, 1995).

Immunofluorescence

Frozen testes sections were cut on a cryostat (Microm) and immediately
fixed with 1% PFA/PBS for 10 min. After two 10-min washes in PBS, 0.2%
Triton X-100/PBS/0.5% blocking reagent (Roche) were applied (Scher-
than, 1995). Next, the slides were washed 3× and detection was
performed with a fluorescently labeled secondary antibody (Vector
Laboratories). After three 10-min washes in PBS, the signal was fixed with
1% PFA/PBS for 5 min followed by a PBS wash and FISH. A similar protoco-
lar was used for IF staining on the squashed cells, leaving out the first fixa-
tion step. Spread meiocytes were obtained, FISHed, and immunoaassaayed as
described previously (Scherthan, 2002).

FISH

Probes were labeled with digoxigenin-11-dUTP using the DIG-Nick
Translation Mix (Roche), or with either biotin-16-dUTP, fluoresceine-12-
dUTP (Invitrogen), or lissamine-5-dUTP (Dupont) using the Nick Transla-
tion System (Invitrogen). The FISH procedure and fluorescent detection
was performed as described previously (Voet et al., 2001). FISH with a
FITC-(C3TA2)3 PNA probe was performed as described previously (Ver-
meesch et al., 1998). In some experiments, a conventional telomere re-
peat probe was applied (Scherthan, 2002). Finally, DNA was counter-
stained with DAPI and the slides mounted in Vectashield® (Vector
Laboratories). FISH on sperm was performed as described by Kobayashi
et al. (1999).
Apoptosis assay
Apoptotic cells in testes tissue sections and suspensions were labeled using a TUNEL assay (Roche) according to instructions of the supplier.

Light microscopic evaluation and image recording
Preparations were evaluated using an epifluorescence microscope (Leica). Digital images were recorded with a CCD camera (Photometrics) using the QuipsFISH system (Vysis) or the ISIS fluorescence image analysis system (MetaSystems).

3D evaluation of protein axes and MC colocalization was performed using an epifluorescence microscope (Axioskop; Carl Zeiss MicroImaging, Inc.) equipped with a 100× plan-neofluar oil-immersion lens (NA 1.35; Carl Zeiss MicroImaging, Inc.) attached to a PIFOC z-SCAN (Physik Instrumente), and a 12-bit CCD digital camera (SensiCam; PCO) controlled by TILLvision v4.0 software. Fluorochromes were excited using a poly-chrome IV monochromator (T.I.L.L. Photonics) in combination with a quadruple band pass beam splitter and barrier filter (Chroma Technology Corp.) allowing subsequent recording of blue (DAPI), red (Cy3), green (FITC), and infrared (Cy5) fluorescence for the same focal plane. Spatial relationship of protein signals and MC signal was also determined by interactively scanning through the image stacks. Deconvolution was performed using the cMLE algorithm of Huygens 2.1.4.1 (Scientific Volume Imaging) running under Windows NT. Rendering of 3D data was done using the Surpass module of Imaris 3.3.2 (Bitplane).

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