Organization and pairing of meiotic chromosomes in the ciliate *Tetrahymena thermophila*

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Summary

During meiotic prophase in the ciliate *Tetrahymena thermophila* micronuclei dramatically elongate and form thread-like crescents. The arrangement of the chromosomes within the crescent as well as the timing of chromosome pairing and recombination with respect to the elongation process have been subjects of ongoing debate. Here, we addressed these issues by means of fluorescence in situ hybridization, labeling of individual chromosomes by BrdU (BrdU-painting) and by immunostaining of the recombination protein, Rad51. BrdU-painting indicated that chromosomes are arranged as parallel bundles within the crescent, and telomere-directed fluorescent in situ hybridization (FISH) revealed that most if not all telomeres are assembled near one end of the developing crescent. Prior to full crescent formation, Rad51 localizes to chromatin as numerous foci. Locus-specific FISH demonstrated that close pairing of homologues only occurs in the full crescent. Meiotic DNA double-strand break formation and the initiation of recombination thus seem to precede close pairing. A synaptonemal complex was not detected. We conclude that the chromosomes adopt a polarized arrangement within the crescent, probably resembling the classical bouquet arrangement. Furthermore, we propose that the elongated shape of meiotic micronuclei promotes the parallel arrangement of chromosomes and supports the juxtaposition of homologous regions in the absence of a synaptonemal complex. Several pieces of evidence indicate the presence of one to four chiasmata per bivalent, which would call for crossover interference to explain regular bivalent formation in spite of this low mean number. *Tetrahymena* might, therefore, pose a case of interference in the absence of a synaptonemal complex.

Key words: Chromosome pairing, Meiosis, Recombination, Rad51, Protists

Introduction

The two homologous chromosome sets of diploid cells must separate prior to sexual reproduction in order to allow cells with a single set of chromosomes to fuse and to restore the diploid complement in the zygote. The separation of homologues during meiosis requires their pairing and, in most cases, the reciprocal exchange of chromosomal portions in a process called crossing over. Crossing over results in the formation of physical bridges, known as chiasmata, between homologues, which ensure their faithful segregation. In addition, it causes the rearrangement of alleles within chromosomes and contributes to the genetic diversity of offspring.

One of the mysteries in meiosis is how homologous chromosomes recognize each other and move towards each other to form pairs. Meiotic chromosome pairing probably employs sophisticated mechanisms for an efficient chromosome-wide homology screen, which excludes ectopic homologies from subsequent recombination. In most organisms homologous contacts first occur during meiotic prophase and it is possible that different organisms employ different strategies to facilitate these contacts (see Loidl, 1990; Loidl, 1994; Dorninger et al., 1995). In many organisms, all of the chromosome ends assemble within a limited area at the nuclear periphery. This leads to the so-called bouquet arrangement of chromosomes. The physical proximity of corresponding chromosome regions within the bouquet or the dynamics involved in bouquet formation could promote homologous contacts (for review, see Zickler and Kleckner, 1998; Cowan et al., 2001; Scherthan, 2001; Bass, 2003). A very similar phenomenon was observed in the fission yeast, *Schizosaccharomyces pombe*. During meiotic prophase, the nucleus assumes an oblong shape (‘horsetail’) and performs oscillatory movements within the cell. The telomeres of all six chromosomes are attached to the spindle pole body in the nuclear membrane, which directs the movement. In the course of the movements, chromosomes are arranged in parallel and homologous pairs are sorted according to their lengths, which promotes contact between homologous loci (Chikashige et al., 1994; Chikashige et al., 1997; Ding et al., 1998; Yamamoto and Hiraoka, 2001; Chikashige and Hiraoka, 2001).

The freshwater ciliate *Tetrahymena thermophila* [formerly known as variety 1 or syngen 1 of *T. pyriformis* (Nanney and McCoy, 1976)], possesses a polyploid macronucleus (MAC) and a micronucleus (MIC), which represent the soma and the germline, respectively. For sexual reproduction, starving cells of different mating types conjugate and the MICs of the conjugating partners undergo meiosis. Haploid products of the conjugation process then fuse to form new diploid cells.
meiosis fuse to form zygote nuclei and these divide and differentiate into new MACs and MICs, while the old MACs are eliminated (see Martindale et al., 1982; Cole et al., 1997; Karrer, 2000).

The MIC undergoes a remarkable change in shape during meiotic prophase (Ray, 1956; Sugai and Hiwatashi, 1974; Wolfe et al., 1976; Martindale et al., 1982) and six stages of meiotic prophase development of the MIC (Fig. 1) have been discriminated (Sugai and Hiwatashi, 1974; Martindale et al., 1982). Soon after the pairing of cells of different mating types, the MIC migrates away from the pocket in the side of the macronucleus (MAC), where it resides during interphase. In the process, it changes from spherical (stage I) to drop- or egg-shaped (early stage II). Next, it assumes the shape of a spindle (late stage II) and later of a torch (stage III). During stages I to III Sugai and Hiwatashi (Sugai and Hiwatashi, 1974) noted that the distribution of chromatin within the MIC is polarized. There is a dense mass near one end of the elongating MIC, from which thin chromonemata extend towards the opposite pole. In stage III nuclei the region on the side of the dense chromatin is elongated and narrowed (the ‘neck’) with a thickening at its end (the ‘head’), whereas the ‘trunk’ contains less densely packed parallel chromatin threads (Fig. 1a). This stretching of the MIC is followed by further, extreme elongation to form the thread-like so-called crescent (stage IV). MIC elongation is promoted by intranuclear microtubules (Davidson and LaFountain, 1975; Wolfe et al., 1976; Gaertig and Fleury, 1992). During stages V and VI, the MIC shortens again, which finally leads to the formation of five bivalents at diakinesis/metaphase I.

Establishing a temporal correlation between the stages of MIC development and the key events of meiosis, namely chromosome pairing and crossing over, is not easy as the chromosomes within the MIC cannot be clearly resolved. In addition, a synaptonemal complex (the meiotic pairing structure) has not been detected in Tetrahymena (Wolfe et al., 1976). Early interpretations of stage I MICs as corresponding to the pairing stage were inspired by the observation in related ciliate species of a ‘parachute’ stage [see p. 200 of Raikov (Raikov, 1982)] in which the chromatin arrangement resembled the bouquet, commonly believed to correspond to zygotene. The parachute/presumptive bouquet precedes the elongation of the MIC in these species and hence, it was inferred that the formation of the elongated MIC/crescent followed upon homologue pairing.

There are, however, several lines of evidence suggesting that recombination takes place in crescents prior to their maximal elongation. First, labeling RNA with [³H]uridine only produces grains on MICs from stages II to IV, suggesting the occurrence of RNA synthesis related to chromosome pairing activity during these stages (Sugai and Hiwatashi, 1974; Martindale et al., 1985). Second, DNA synthesis in micronuclei, which could be interpreted as repair synthesis occurring in the course of meiotic recombination, does take place prior to full crescent formation (Allis et al., 1987). Third, the thickness of Giemsa-stained chromatin threads in stage II and III MICs led Sugai and Hiwatashi (Sugai and Hiwatashi, 1974) to conclude that the former corresponds to leptotene and the latter to zygotene. The interpretation that the critical stages of chromosome pairing do occur prior to full crescent formation is supported by the observation that the microtubule poison nocodazole prevents bivalent formation at metaphase I when it is applied after the initiation of but prior to full MIC elongation (Kaczanowski et al., 1985).

Ray (Ray, 1956) and Sugai and Hiwatashi (Sugai and Hiwatashi, 1974) proposed that the crescent contains chromosomes or bivalents in a tandem arrangement, rather than bundles of chromosomes in parallel arrangement. This interpretation was based on the observation that long crescents sometimes appear longitudinally split into two.

Fig. 1. (a) Schematic diagram of MIC development according to Sugai and Hiwatashi (Sugai and Hiwatashi, 1974), and Martindale et al. (Martindale et al., 1982). (b) Changing morphology and divisions of the MIC throughout conjugation as visualized by DAPI staining. Bar, 10 µm.
Materials and Methods

Strains and culture conditions

Strains B2086.1 (wild type) mating type II and CU428.2 mppl-1/imprl-1 (6-methylpurine resistant) MAC mp sensitive mating type VII were kindly provided by Arno Tiedke (University of Münster, Germany). Culture was performed according to the method of Orias (1975) and by Karrer (Karrer, 1985), several strands of chromatin can be discriminated. Moreover, Bruns et al. (Bruns et al., 1982) found in nullisomic strains, in which some chromosomes are missing from the MICs, that chromosomes are arranged side-by-side within the crescent and that the pairing of chromosome arms of similar lengths is promoted by the spatial constraints within the crescent in a similar manner to S. pombe horsetails.

We have examined which stages of meiotic prophase correspond to the various conformations of the MIC and how the chromosomes are arranged within it. Fluorescence in situ hybridization (FISH) was used to determine the stage at which chromosomes pair, individual chromosomes with incorporated BrdU were traced inside the MIC, and the temporal pattern of the localization of the recombination protein, Rad51, was studied by immunostaining. Finally, we discuss how the observed disposition of chromosomes can help to bring homologues into juxtaposition.

Preparation and immunostaining

The preparation protocol was modified from Cole et al. (Cole et al., 1997). In short, a suspension of conjugating cells was fixed by the addition of formaldehyde and Triton X-100 (final concentrations of 4% and 0.5%, respectively). After careful mixing the cells were left for 30 minutes at room temperature, then centrifuged and the pellet resuspended in 1:10 volume of 4% formaldehyde + 3.4% sucrose. Eighty microliters of this mixture were spread on a clean slide and air-dried. These slides can be used for immunostaining right away or stored for several days (up to several weeks in the freezer). The sucrose forms a viscous layer that prevents the denaturation of antigens.

For immunostaining, slides were incubated in 1× phosphate buffered saline (PBS) and 1× PBS + 0.05% Triton X-100 for 10 minutes each. Primary antibodies (1:50 mouse monoclonal antibody against recombinant Rad51 protein, Clone 51RAD01, NeoMarkers, Fremont, CA or 1:500 rabbit anti-phosphorylated H3(Ser10), Upstate Biotechnology, Charlottesville, VA, USA) were applied separately or together under a coverslip for 3 hours to overnight at room temperature. The coverslip was removed and the preparations were rinsed with 1× PBS, postfixed with ice-cold 96% ethanol for 30 seconds and incubated in 1× PBS + 0.05% Triton X-100 for 10 minutes. FITC-conjugated anti-mouse antibody and/or Cy3-conjugated anti-rabbit antibody was applied under a coverslip for ~2 hours at room temperature. Finally, the slides were incubated twice for 10 minutes in 1× PBS and mounted under a coverslip in Vectashield anti-fading agent (Vector Laboratories Inc., Burlingame, CA, USA) supplemented with 1 ng/ml DAPI (4′,6-diamidino-2-phenylindole) as a DNA-specific counterstain.

BrdU incorporation and preparation for BrdU staining

5-Bromo-2′-deoxyuridine (BrdU) was added to a final concentration of 2×10⁻⁷ M to exponentially growing cultures of vegetative cells. All manipulations with cultures exposed to BrdU were performed under red darkroom illumination. After 75 minutes exposure, unincorporated BrdU was removed by washing the cells twice with an excess of growth medium. The cells were then appropriately diluted in fresh medium and grown for 4-5 mitotic cycles to a density of ~2×10⁶ cells/ml. At a generation time of ~2.5 hours this density was reached after 10-13 hours. Cells were then starved and cultures of different mating types were mixed as described above. Those MICs that have not completed S phase in rich medium will do so after the transfer to starvation medium, such that by the initiation of meiosis most nuclei have gone through five replication cycles in the absence of BrdU and contain on average less than one BrdU-labeled chromosome (see below). Cells were harvested and prepared for cytology at 2.5 hours, 3.5 hours, 4.5 hours and 5.5 hours after mixing.

Five milliliters of conjugating cells were spun for 4 minutes at 1500 rpm, the supernatant was gently removed and the pellet was resuspended in 500 ml of Carnoy’s fixative (methanol-chloroform-acetic acid, 6:3:2). Fixation was performed for 1 hour at room temperature; for longer storage, the cells were transferred to −20°C. Prior to preparation, the Carnoy’s fixative was replaced with 100 µl of 70% ethanol. A 10 µl drop of cell suspension in ethanol was put on a slide and most of the liquid allowed to evaporate. A drop of 45% acetic acid was added and the material was squashed under a coverslip. The coverslip was then removed in liquid nitrogen and the preparation air-dried.

For the immunodetection of incorporated BrdU, slides were incubated in 70% formamide for 2 minutes at 65°C to induce DNA denaturation and to expose the bases to the antibody, Abcam (Cambridge, UK) ab6326 rat anti-BrdU antibody (1:40) was used as primary antibody and FITC- or Cy3-conjugated anti-rabbit antibody as secondary antibody. The staining regimen was the same as described above for Rad51 and phosphoH3 immunostaining.

Calculation of the frequency of occurrence of a single BrdU-painted chromosome per nucleus

Assuming that intact sister chromatids segregate independently into daughter cells, the probability P₀ that all chromosomes are unlabeled after a given number of generations (g) post BrdU pulse is $P_{0} = (1 - 1/2^{x})^{y}$, where x is the diploid chromosome number (i.e. 10 in Tetrahymena). The probability P₁ that exactly one chromosome is labeled after g generations is $P_{1} = x(1/2^{y} - 1)(1 - 1/2^{x} - 1)$. From the observed fraction of unlabeled nuclei (P₀), the mean number of replication cycles undergone after withdrawal of BrdU can be determined. The expected frequency P₁ of one nucleus with more than one labeled chromosome is $P_{1} = 1 - P_{0} - P_{1}$.

Fluorescence in situ hybridization (FISH)

Two single-copy DNA probes, Teth1 and Teth2 were amplified from Tetrahymena genomic DNA using the following primers: T1.3 forward CATCATCCATATACCGAGC and T1.2 reverse ACTC-TGAGACATCATACGG (Teth1) and: T2.3 fwd CTTCTATTA-
TGCTATAGCCA and T2.2 rv AAAGGAATGCTTGAAGGCTC (Teh2). The primers were designed from the Tetrahymena MAC genomic sequence available from The Institute for Genomic Research website at http://www.tigr.org. Each reaction yielded a PCR product of approx. 4 kb. Teh1 and Teh2 are located on the same contig and separated by 167 kb of sequence. The PCR products were labeled with digoxigenin or biotin using the respective Nick Translation kit according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany).

A compound probe against the telomere (G_{4}T_{3})_{n} repeat (approximately 2.0-3.4 kb per average telomere) and the subtelomere (G_{4}T_{3})_{n} repeat (corresponding to 0.6-1.0 kb per average telomere) (Kirk and Blackburn, 1995) was used. 5’ biotinylated 24-mer oligonucleotides homologous to the telomere and subtelomere sequences were synthesized (Invitrogen GmbH, Karlsruhe, Germany). To enhance the telomere repeat signal oligos were 3’-tailed with biotin-11-dUTP as described previously (Scherthan, 2002).

FISH was performed on slides prepared either by formaldehyde fixation (as for immunostaining) or by Carnoy fixation (as for BrdU detection) as described above. Preparations were hydrated in H_{2}O followed by treatment with 1 M sodium thiosulfate at 90°C for 15 minutes. Denaturation was performed in 70% formamide, 2× SSC, pH 7.1 for 2 minutes at 68°C. The slides were then rinsed in H_{2}O and air-dried. DNA probes were dissolved in hybridization solution (50% formamide, 10% dextran sulphate, 2× SSC) at 50 ng/μl and unlabeled Tetrahymena DNA was added to 125 ng/μl to suppress unspecific binding of the probe. Telomere probes, either G-strand probes only or a mixture of all oligonucleotides, were diluted to 0.5 ng/μl in hybridization solution containing 1 μg/μl of E. coli carrier DNA. The hybridization mixtures were denatured at 95°C for 3 minutes. The PCR product-containing mixture was incubated at 37°C for 25 minutes. Probes were sealed with rubber cement (Marabu, Hamm, Germany) on the denatured slides under a coverslip. Hybridization was allowed to proceed for 48 hours at 37°C in a moist incubator. After hybridization, slides were washed three times in 0.05× SSC at 37°C and biotin was detected using avidin-FITC (Sigma) as described previously (Scherthan, 2002). Hybrid molecules were visualized by rhodamine-conjugated anti-digoxigenin Fab fragments (Roche) and/or Extravidin-FITC (Sigma) for biotin molecules (for details, see Scherthan, 2002). Finally, slides were embedded in Vectashield supplemented with DAPI.

Microscopy and evaluation
Preparations were evaluated using an epifluorescence microscope equipped with single-band-pass filters for the excitation of blue (DAPI), green (FITC) and red (rhodamine and Cy3) fluorescence. Pictures were taken with a cooled CCD camera of sufficiently flattened cells or, in some instances, a series of focal planes was recorded, deconvolved and projected in two dimensions using Zeiss Axiovision and ImageJ (Wayne Rasband, N.I.H.; http://rsb.info.nih.gov/ij/) software.

Whole-mount spreading and silver staining of nuclei
The method for the detection of silver-positive structures within nuclei was similar to protocols that have been used previously for visualizing SCs of a variety of plant, fungal and animal species (e.g. Loidl et al., 1998). For the spreading of nuclei, 20 μl of a dense suspension of conjugating cells (4×10^{6} cells/ml) in 10 mM Tris (pH 7.4) were mixed with 50 μl detergent (0.5% LipoL laboratory cleaning agent) on a slide, which caused the cells to swell and burst. After a few seconds, 80 μl of fixative (4% paraformaldehyde + 3.4% sucrose in distilled water) was added. The slides were air-dried in a chemical hood overnight. Staining with silver nitrate (a 50% solution of AgNO_{3} in distilled water) and transfer of stained material to electron microscopic grids was performed as described previously (Loidl et al., 1998).

Results
The recombination protein Rad51 is abundant in meiotic micronuclei
Conjugating cells were immunostained for the conserved Rad51 protein. Rad51 is a recombinase that is involved in the homologous DNA pairing and strand exchange reaction in the course of recombinational DNA repair, and it is generally believed to associate with meiotic double-strand DNA breaks (DSBs) (Shinohara et al., 1992) (for reviews, see Paques and Haber, 1999; Sung et al., 2003). MICs up to early stage II contained no or only traces of Rad51 (Fig. 2a,b). Homogenous Rad51 labeling was observed in spindle-shaped MICs in late stage II (Fig. 2c). To establish a temporal correlation between incipient chromosome condensation in MICs and the deposition of Rad51, double immunolabeling with Rad51 and phosphorylated histone H3 (phosH3) was performed (Fig. 2e-l). Phosphorylation of H3 has been found to correlate with mitotic and meiotic chromosome condensation in Tetrahymena (Wei et al., 1998).

Crescents in stages III to V showed strong Rad51 labeling along their entire lengths (Fig. 2d-h). High magnification and image deblurring revealed that the label consisted of numerous individual spots (Fig. 2d). From a few particularly well-flattened crescents it was estimated that they contain over 100 spots. To rule out the possibility that the grainy appearance of the Rad51 staining merely reflects the overall staining of inhomogeneously distributed chromatin, parallel immunostaining of phosH3 was performed. Rad51 and phosH3 spots did not colocalize (Fig. 2f), thus validating the genuineness of the Rad51 foci. That Rad51 was most abundant in the crescent stage agrees with the report that RAD51 transcription is strongest around 4 hours after conjugation (Marsh et al., 2000). Rad51 was still present in stage V nuclei (Fig. 2h), but much of the staining fell outside the DAPI-positive areas and hence probably represents Rad51 that has detached from chromatin. Only during metaphase I and anaphase I, did Rad51 staining become progressively weaker and disappeared completely during the second meiotic division (Fig. 2i-l). In post-meiotic MICs, Rad51 reappeared, probably in conjunction with DNA replication (data not shown).

Rad51 foci are present in macronuclei
As can be seen in many of the figures showing Rad51 immunostaining (Fig. 2), the MACs also often display granular or filamentous Rad51 signals. In fact, all of the 200 MACs in conjugating cells that were specifically inspected for this feature, exhibited Rad51 signals. To determine if this was specific to nuclei in conjugating cells, both exponentially growing and starving cells (that had been kept for 72 hours on 10 mM Tris) were scored. In the former, 77% (n=300) of non-dividing MACs, and in the latter all 300 MACs scored showed Rad51 signals. Similarly, detectable RAD51 message has been found to be present in cells at 0 hours of mating (Marsh et al., 2000). Notably, Rad51 signals were absent in amitotiically dividing MACs and in the new macronuclear precursors (anlagen) after fertilization. Thus, while it is clear from these preliminary observations that the occurrence of Rad51 spots in MACs is not limited to meiotic cells, their nature has to remain the subject of future studies.
Chromosomes span much of the length of the crescent

Two reports in the past (Ray, 1956; Sugai and Hiwatashi, 1974) have proposed that chromosomes are arranged in tandem within the crescent. The basis for this interpretation was that the chromatin in the crescents sometimes appears segmented and, in addition, is longitudinally split in half. While this observation (Fig. 3a) may appear to imply the tandem arrangement of bipartite structures, either replicated chromosomes or bivalents, this interpretation cannot be reconciled with the occasional observation of crescents that seem to contain bundles of DAPI-positive chromatin fibers (Fig. 3b) [see also Fig. 1 of Davidson and LaFountain (Davidson and LaFountain, 1975) and Fig. 1b of Karrer (Karrer, 1985)].

To study the organization of chromosomes within the crescent in more detail, we applied the method of replication labeling (see Zink et al., 1998). With this method, individual chromosomes are differentially stained through the incorporation of BrdU instead of thymidine during S phase. At the end of S phase in the presence of BrdU, both chromatids of every chromosome have incorporated BrdU in one half-strand and daughter cells obtain BrdU chromatids. During subsequent S phases in the absence of BrdU, the newly synthesized half-strand will incorporate thymidine. During the following mitosis, chromatids are segregated randomly, such that only some (on average 50%) of the chromosomes of a daughter nucleus are BrdU labeled. During each subsequent cell cycle following the withdrawal of BrdU, the number of BrdU-labeled chromosomes of a nucleus is further reduced, and after a certain number of divisions (which depends on the number of chromosomes), on average, only a single chromosome will be labeled with BrdU. For Tetrahymena MICs in which 2n=10, the probability of a nucleus carrying a single labeled chromosome after five generations post BrdU pulse is ~24%. The probability that two or more chromosomes are labeled is ~3%; i.e., in seven out of eight nuclei carrying a signal, it stems from a single chromosome (see Materials and Methods). If MICs are then induced to perform meiosis and BrdU is detected by immunostaining, the spatial organization of individual chromosomes can be studied.

After the described pre-treatment, MICs at stages II, III and IV always contained elongated BrdU signals, which extended for most of or the entire length of the nuclei (Fig. 4). If chromosomes were arranged in tandem, labeling would be expected only in a sector of the MIC. Although we cannot exclude that a minority of the nuclei contained two or more BrdU-stained chromosomes, the sole presence of thread-shaped signals strongly suggests that the chromosomes within the crescents are arranged in parallel and that they extend from one end of a conjugated MIC to the other. BrdU threads occasionally formed a U-turn in stage II and III MICs (Fig. 4b), which is consistent with the presence of DAPI-stained chromatin loops in the trunk (Fig. 3d). BrdU-labeled chromosomes were often observed not to extend along the entire length of crescents (Fig. 4e), which could be explained by sister chromatid exchanges (SCEs) between labeled and unlabeled chromatids during one of the mitotic S phases prior to conjugation. We have obtained circumstantial evidence for the existence of such SCEs (data not shown).
While most nuclei exhibited a single BrdU-positive thread, some contained double threads (Fig. 4d,e). The latter could represent nuclei containing more than one BrdU-labeled chromosome or chromosome portion. Alternatively, double threads could result from loop formation by a single BrdU-painted chromosome, which is consistent with a bouquet arrangement of chromosomes. In contrast to stage II to IV MICs, late stage V and stage VI nuclei showed one or several thick spots of BrdU (Fig. 4f-h), which suggests that the chromosomes that initially fill the space from one end of the MIC to the other, shorten prior to diplotene.

Most if not all telomeres assemble near one pole of the early crescent

Sugai and Hiwatashi (Sugai and Hiwatashi, 1974) observed chromatin threads projecting from a densely-packed chromatin mass near one pole of the MIC. Our DAPI staining (Fig. 3d) and BrdU-painting (Fig. 4b) suggest that these chromatin threads may form loops within the trunk, at least in stage II and stage III MICs. To decide whether chromosomes within crescents are arranged in such a way that one end points to one end of the crescent and the other end to the opposite end, or if chromosomes form loops, we performed FISH to highlight chromosome ends. While FISH signals were too weak to be detected on individual telomeres, possibly owing to the small size of the target sequences (no signals were observed in diakinesis bivalents or on anaphase I chromosomes), the chromatin-dense pole of stage II MICs and the head of stage III MICs regularly carried a signal, which presumably resulted from the assembly of several if not all of the telomeres in this region (Fig. 5a-c). Furthermore, in the strongly elongated stage IV crescents, the former head end (as identified by the adjacent DAPI-dull neck) carried strong telomere FISH signals (Fig. 5d). This suggests that at the beginning of MIC elongation, telomeres congregate near the head end of the MIC and that during MIC elongation the arrangement of chromosomes resembles the bouquet (Scherthan, 2001; Bass, 2003). Condensed chromosomes in late stage V and stage VI can assume positions anywhere along the spindle-shaped MIC (see Fig. 4f-h), which implies that telomeres have detached by these stages.
Homologous loci become paired in stage IV micronuclei

To determine the onset and progress of meiotic pairing, we performed FISH with a 4 kb probe (Teth2 – see Materials and Methods) which was derived from the random sequences of the approx. 270 micronuclear ‘chromosomes’ (http://www.tigr.org). Since these ‘scaffolds’ have not been anchored to micronuclear chromosomes, the precise position of this probe remains to be elucidated. However, the probe created two distinct signal spots, thus providing us with the means of monitoring the pairing of homologous loci in the MIC.

FISH signals were mostly clearly separated in stage I to stage III MICs (Fig. 5e-h) and appeared as single or as associated (touching) double dots in stage IV crescents (Fig. 5i). There was little evidence of progressive alignment prior to stage IV (Table 1). From the point at which two opposite poles (DAPI-bright and DAPI-dull) could be discriminated in stage I nuclei, signals were found to localize to the DAPI-bright area (next to what is later to become the ‘neck’) (Fig. 5e-h). In stage IV crescents whose two poles could be discriminated, the position of Teth2 FISH signals was determined. In the 30 nuclei scored, the average position of the signal was at 28±5% of the distance from the head (=telomere pole) to the opposite end.

In another series of experiments, two chromosomal loci (Teth1 and Teth2) separated by 167 kb were labeled with different colors (Fig. 5l). In all 20 stage IV crescents where the two colors could be detected simultaneously, the Teth1 probe was closer to the head. This indicates that the orientation of the chromosomes within the crescent is straight and not meandering. The fact that the probed chromosomal locus virtually always occupied the head-proximal portion of the crescent, coupled to the fact that the order of red and green signals was always the same, indicates that the probe-tagged chromosome arm was positionally fixed and suggests that it is anchored with its telomere in the head region, which carries the telomere cluster. If this is the case for one chromosome, it is reasonable to assume that all of the chromosome arms behave similarly and hence, that chromosomes are arranged in a bouquet configuration.

Table 1. Pairing of homologous loci at different meiotic stages as indicated by the presence of separate, closely associated (double) and fused (single) FISH signals

| Stage    | Separate dots | Early stage II | Late stage II | Stage III | Stage IV |
|----------|---------------|----------------|---------------|-----------|----------|
|          | 83%           | 84%            | 86%           | 77%       | 16%      |
| Double dots | 9%            | 11%            | 6%            | 17%       | 38%      |
| Single dot | 8%            | 5%             | 8%            | 6%        | 46%      |

100 nuclei from each stage were scored.
The search for a synaptonemal complex (SC)

The SC is a proteinaceous structure, which has been described in most organisms that perform canonical meiosis and is of similar appearance across phylogenetic groups. The SC has been implicated both in the stabilization of homologous pairing and in the conversion of crossovers into chiasmata (see Zickler and Kleckner, 1999). While previously it had also been attributed a role in crossover interference (the phenomenon in which only a subset of recombination intermediates is transformed into crossovers and that there is a reduced probability of neighboring intermediates becoming crossovers), this has been challenged recently (see Discussion).

As Wolfe et al. (Wolfe et al., 1976) failed to detect an SC by electron microscopy of thin-sectioned crescents, we applied a spreading method in an attempt to observe SCs in silver-stained whole-mount nuclei. Neither structures resembling SCs or SC precursors nor even SC-related structures, such as the linear elements of S. pombe (Bähler et al., 1993), were observed (data not shown). Moreover, a preliminary bioinformatic search of the Tetrahymena genome has not recovered sequences with an obvious homology to genes for SC structural proteins.

The probable absence of an SC raises the question of whether its lack in Tetrahymena is an ancient property of meiosis, because the ciliates are phylogenetically widely separated from animals, plants and fungi (Frankel, 2000; Stechmann and Cavalier-Smith, 2003) in which SC have been found. However, because the ciliates are phylogenetically widely separated from animals, plants and fungi (Frankel, 2000; Stechmann and Cavalier-Smith, 2003) in which SC have been found, the probable occurrence of an SC in other members of the ciliates Cavalier-Smith, 2003) (in which SC have been found). This has been challenged recently (see Discussion).

A polarized chromosome arrangement prevails in meiotic MICs

Since an SC has not been detected in Tetrahymena, correlating the stages of MIC development to the classical meiotic prophase stages of leptotene, zygotene and pachytene is difficult, as these stages are normally defined by SC development and the progression of synapsis. In most organisms studied to date, the Rad51 protein, which plays a role in the recombinational repair of meiotic DSBs (Paques and Haber, 1999; Brush, 2002), first appears as foci around leptotene/early zygotene and disappears soon after the initiation of SC formation, at which time the intimate pairing of homologues is accomplished (Alpi et al., 2003; Colalíaçovo et al., 2003) (also references cited in Alpi et al.). Accordingly, the period between early and late stage II of Tetrahymena meiosis, might roughly correspond to zygotene.

It was proposed that Rad51 plays a role in the homology search in addition to its role in recombination (Pawlowski et al., 2003). In Tetrahymena, Rad51 is still abundantly present in stage IV crescents, which might suggest that the processing of DSBs (and homology search) continues up to this stage. In fact, close pairing as visualized by the association of homologous FISH signals, only occurs in stage IV. However, considerable amounts of Rad51 persist until metaphase I (when the presence of chiasmata indicates the successful completion of crossing over) and beyond, and it is conceivable that Rad51 may be retained within nuclei in an inactive state.

In addition to Rad51 foci in meiotic MICs, we also detected Rad51 foci in the MAC precursors (anlagen) of exconjugants (but not of conjugants!), in MACs of exponentially growing cells except during amitosis, as well as in MACs of conjugating and starving cells. The probable explanation for the presence of Rad51 in anlagen and in the MACs of dividing cells is the involvement of Rad51 in repair processes during replication (Marsh et al., 2000) as well as in chromosome fragmentation, sequence elimination, telomere addition and recombination processes during macronuclear development (Deak and Doerder, 1998). Furthermore, transcription-associated recombination may take place and may require the activity of Rad51 (Garcia-Rubio et al., 2003). A possible explanation for the presence of Rad51 in starving cells could be its involvement in apoptosis-like DNA degradation in starved cells (Maercker et al., 1999).

Discussion

Rad51-dependent recombination initiates in stage II MICs

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A polarized chromosome arrangement prevails in meiotic MICs

The organization of chromosomes in the crescent stage of Tetrahymena has so far remained elusive (see Karrer, 1985). We have now shown that after BrdU-painting, individual labeled chromosomes in stage II to stages IV-V MICs occupy most of or the entire length of the crescent. Moreover, telomere FISH revealed that most if not all chromosome ends are clustered near one pole of the nucleus, and finally, chromosomal loci highlighted by FISH always occupied a fixed position within the polarized MIC. This combined evidence suggests that chromosomes are arranged either in a classical bouquet or in a one-sided bouquet [the polarization of only one telomere per chromosome – cf. fig. 1c in Scherthan (Scherthan, 2001)] in stage II to stage IV MICs.

The bouquet is a widely conserved arrangement of chromosomes in meiotic prophase which is characterized by the congression of telomeres at the inner surface of the nuclear membrane (Zickler and Kleckner, 1998; Scherthan, 2001). It is not yet clear if and how the bouquet supports the mutual recognition and alignment of homologues. Recently, it was shown that homologous chromosomes in Sordaria are presynaptically aligned before the bouquet is formed (Storlazzi et al., 2003). It is possible that the contact of chromosomes with a specialized region of the nuclear periphery facilitates SC initiation (Moens et al., 1989; Alsheimer et al., 1999). However, simultaneous pairing and initiation of synopsis of chromosomes in the bouquet was convincingly demonstrated by Scherthan et al. (Scherthan et al., 1996) and Bass et al. (Bass et al., 2000).

In Tetrahymena, it is not yet possible to say with certainty whether the chromosomes adopt a classical bouquet arrangement or a ‘one-sided bouquet’ arrangement, with only one end of each chromosome anchored to the head pole of the MIC and the other pointing towards the opposite pole (trunk). In late stage II nuclei, DAPI-positive chromatin strands and BrdU-painted chromosomes sometimes exhibit loops (Fig. 3d, Fig. 4b), which suggests a classical bouquet arrangement with U-shaped chromosomes. This arrangement is not, however, necessarily maintained until stage IV. The observation of telomere clustering at the head-pole does not help in discerning between a classical bouquet and a one-sided bouquet, as the free ends of chromosomes, if scattered across the crescent, might escape detection. The fixed position of loci (as detected by FISH) near the head region, strongly suggests that the telomere of the corresponding chromosome arm is invariably anchored in the
The crescent shape of the MIC may promote the juxtaposition of homologous loci

The change in shape of the MIC invites the interpretation that spatial constraints are imposed on the chromosomes, which promote and stabilize the alignment of homologues. This could be analogous to the situation in the so-called horsetail nuclei of S. pombe where the nucleus becomes elongated and moves within the cell. This is believed to facilitate homologous chromosome pairing by aligning homologous telomere-bundled chromosomes (e.g. Yamamoto and Hiraoka, 2001). In organisms that develop an SC, the bouquet is transient and the SC helps to stabilize the physical contact of homologous regions beyond the bouquet stage even when chromosomes disperse within a normally spherical nucleus. In S. pombe, which does not form an SC, the bouquet is maintained until crossing over is achieved (Ding et al., 2004). In Tetrahymena, in which an SC has not yet been detected (Wolfe et al., 1976) (this study), chromosomes might be kept in register until completion of the crossing over process by the prolonged maintenance of telomere attachment and their confinement within the narrow tube of the crescent.

Does chiasma interference occur in the absence of an SC?

Chiasma/crossover interference is defined as the mutual suppression of chiasma or crossovers in adjacent regions of chromosomes (for review see Roeder, 1997). Another expression of chiasma interference is that the distribution of chiasma number per bivalent is narrower than the Poisson distribution predicts (Haldane, 1931; Jones, 1984). As organisms lacking an SC do not exhibit interference (Egel-Mitani et al., 1982; Maguire, 1988; Kohli and Bähler, 1994) and in mutants incapable of synopsis, interference is reduced or missing (Sym and Roeder, 1994), it has been proposed that the SC mediates crossover interference by transporting a suppressor signal (Chua and Roeder, 1997; Tung and Roeder, 1998; Novak et al., 2001).

The failure of our and previous attempts (Wolfe et al., 1976) to cytologically detect an SC in Tetrahymena prompts the question of the existence of crossover/chiasma interference in this organism. Limited cytological evidence suggests that Tetrahymena features morphologically regular bivalents with one or two chiasmata [see figures of bivalents in Ray (Ray, 1956) and Karrer (Karrer, 2000)]. With a genome size of ~210 megabases (Karrer, 2000), a recombination frequency of 200 kb/cM [estimated from RAPD polymorphisms (Wickert and Orias, 2000)] would translate into ~21 chiasma per chromosome complement and meiosis or ~4 chiasma per bivalent. The presence of such a low number of chiasmata (estimated to be 1-2 or ~4 from cytological and genetic assays, respectively) would suggest that chiasma formation is under some form of control. If random chiasma formation resulted in an average of one or two chiasmata per bivalent, a high percentage of bivalents would run the risk of not receiving a single chiasma. According to the Poisson distribution \( P(0) = e^{-\lambda} \) (where \( P(0) \) is the probability of receiving zero chiasmata, \( e=2.718 \), and \( \lambda \) is the average number of chiasmata for a pair of homologues), homologous pairs with an average number of 2 or 4 chiasmata would remain univalent in over 10% or ~2% of cases, respectively. For Tetrahymena with five pairs of homologues, this would mean that ~50% or ~9% of metaphase I cells, respectively, would contain at least one pair of univalents prone to nondisjunction. (In order to reduce the risk for a pair of homologues receiving no chiasma to less than 0.1%, an average seven crossovers per bivalent would have to occur. In this case, 99.5% of cells would form only bivalents.) In the presence of interference, a large number of recombination events would be initiated (as is in fact suggested by the presence of numerous Rad51 spots) but not result in crossovers except for the 2-4 chiasma per bivalent that are believed to occur.

An alternative possibility, which remains to be tested, is that a minimum number of chiasmata is ensured by the existence of strong crossover hotspots, leading to localized chiasmata. It is also possible that Tetrahymena has an SC whose components are uncommon, in that they are refractory to the conventionally used contrasting and staining procedures. Future searches of the sequenced Tetrahymena genome will reveal whether orthologues of genes encoding known SC components are present in Tetrahymena.

Evidence is, however, accumulating that the SC is dispensable for crossover interference. Page and Hawley (Page and Hawley, 2001) showed in Drosophila mutants that complete SC formation in oocytes is not required for crossover interference. In budding yeast, Fung et al. (Fung et al., 2004) observed interference between Zip3 foci, which are considered to mark the sites of crossing over and appear prior to or in the absence of the SC. However, these authors did not preclude the existence of an additional interference mechanism that depends on the SC. Similarly, Börner et al. (Börner et al., 2004) compared the timing of DNA events of recombination and SC formation in yeast and showed that the crossover/non-crossover (gene conversion) decision is made before or during the appearance of single-end invasions, which is long before the appearance of the SC. In the light of these recent findings, it is possible that Tetrahymena presents yet another example of crossover/chiasma interference in the absence of an SC.

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