Behavior of Centromeres in Univalents and Centric Misdivision in Wheat

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Abstract

Centromeres are responsible for the proper behavior of chromosomes in cell divisions. In meiosis the process is more complicated than in mitosis, as each chromosome in a bivalent has 2 sister centromeres and their behavior has to be strictly coordinated. Here, the behavior of sister centromeres in univalents in wheat is examined, showing that by metaphase I they often lose their coordination. This loss accelerates with the progression of anaphase I, leading to stable bipolar attachment and frequent separation of sister chromatids or to misdivision. Depending on the orientation of a univalent and its sister centromeres, misdivision may occur across the centromere region or across the pericentric chromatin. Chromosome fragments consisting of only the centromere region did not survive to the next generation. Midget chromosomes composed of the centromeres and parts of the pericentric chromatin did survive, but their transmission rates were low and appeared related to the amount of pericentric chromatin, probably because only the pericentric chromatin provides sister chromatid cohesion. As the cohesion of sister chromatids appears to be a function of the proximity to the kinetochore region, the definition of the centromere need not include pericentric regions.

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Standard mitotic division of the nucleus, while a complex and carefully choreographed process, appears simple by comparison to the meiotic division. Simply put, in the mitotic cycle chromosomes replicate and separate the copies on a karyokinetic spindle into 2 daughter nuclei. Each chromosome is built of 2 sister chromatids, each chromatid has its centromere, and the karyokinetic spindle has 2 poles towards which the chromatids pull themselves. Meiosis is more complicated in this regard, because in the first anaphase (AI) the division is not from single replicated chromosomes but from pairs of chromosomes (or other configuration if the number of associating chromosomes is different from 2) and each of these paired chromosomes is fully replicated. Hence, each chromosome has 2 functional centromeres. A bivalent, the structure formed by 2 chromosomes usually connected by chiasmata, has therefore 4 centromeres, but the division still occurs on a bipolar spindle and into 2 daughter nuclei. For this process to proceed smoothly, the functions of sister centromeres, i.e. centromeres of sister chromatids, have to be tightly coordinated. This is to preclude any possibility of sister centromeres interacting with the opposing poles of the karyokinetic spindle in the first meiotic division. For the second division, this coordination has to be broken down and steps taken to assure that at this point the 2 sister centromeres do exactly the opposite: they interact with the opposing poles of the spindle.

It was noted early in the history of cytogenetics [Darlington, 1939] that univalents in meiosis have a tendency

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to misdivide (break) across their centromeres producing telocentrics. In wheat this process was described with considerable detail originally by Sears [1952] and Steinitz-Sears [1966] and recently by Friebe et al. [2005] and used to generate extensive sets of cytogenetic stocks such as telocentrics and isochromosomes [Sears and Sears, 1978]. Univalent misdivision was also used to generate and to manipulate translocations of alien chromosome arms into wheat [Lukaszewski, 1993, 1997a]. The most common alien introgression in wheat, translocation 1RS.1BL, is a result of centric misdivision and fusion of the misdivision products.

Centric breakage of univalents in wheat may take place in the first or second meiotic anaphase, with several different types of breakage in each [Sears, 1952]. In the first division the breakage may involve separation of the arms (breakage across both sister chromatids and both sister chromatids of one arm travelling toward one pole while the other pair segregates into the other pole) or separation of a single chromatid of one arm from the remainder of the chromosome. Separation of sister chromatids in the first division may lead to breakage across the centromere in the second division, producing telocentric chromosomes. It is not entirely clear when fusion of the misdivision products takes place, either to form isochromosomes or centric translocations. Friebe et al. [2005] found 2 instances, both in the anaphase II/telophase II transition, of what appeared to be newly-formed centric translocations. It is unclear whether any chromosome repair in wheat takes place in meiosis itself. Telomeres are not restored to newly broken wheat chromosome ends until early embryo divisions [Lukaszewski, 1995; Friebe et al., 2001]. It is therefore quite likely that in most cases chromosomes with broken centromeres persist without telomeric repeats through the gametophyte divisions. On the other hand, chromosome fusions are likely generated by DNA repair mechanisms which identify and fuse chromosome ends not protected by telomeres. Since gametophyte development involves rounds of DNA replication and nuclear division, it is possible that isochromosomes are generated in the gametophyte and delivered to the embryo as stable chromosomes, while centric translocations and stable telocentrics are generated only in the embryo.

This study does not attempt to define once again all possible types of univalent behavior in meiosis in wheat, as this has been done before [Sears, 1952; Friebe et al., 2005]. Instead, it takes advantage of chromosomes with marked centromeres and concentrates on the observation of the centromeres in the process of univalent misdivision and then attempts to reconcile cytological observations with the current knowledge on the molecular aspects of chromosome behavior. The chromosomes used here are a wheat chromosome with an introgression of a centromere from rye and the donor rye chromosome. The centromere introgression itself was produced by repeated cycles of centric misdivision of univalents [Zhang et al., 2001]. While this process likely has altered the structure of the centromere relative to its native state in the donor chromosome, the chromosome in question is perfectly stable both in mitosis and meiosis and is transmitted through generations without any apparent handicap. Given the opportunity, this chromosome pairs normally with its homologue and homoeologues which have normal (native) centromeres [Corredor et al., 2007]; hence, it appears suitable for the type of study described herein.

The definition of the centromere has evolved over the years, from the 'kinetochore', understood as a part of the chromosome responsible for the anaphase movement, to the 'centromere' as a part of the chromosome responsible for its proper behavior in cell divisions, including movement and timing. This issue will be addressed in the discussion section; until then, the 'centromere' will be understood as the part of a chromosome interacting with the karyokinetic spindle.

Materials and Methods

The material for this study were plants of hexaploid wheat, *Triticum aestivum* L, cv. Pavon 76, double monosomic for reconstructed wheat chromosome 2B and rye chromosome 2R. Reconstructed 2B ($2B_{rec}$) was produced by misdivision of 2B with 2R to generate the centric translocations 2BS.2RL and 2RS.2BL, followed by misdivision of the 2 translocations and recovery of reconstructed 2B and 2R. As far as the limits of in situ probing with specific DNA probes permit to establish, this $2B_{rec}$ chromosome contains the entire centromere from 2R [Zhang et al., 2001]. The double monosomic plants ($20' + 2B_{rec}' + 2R'$) were produced by intercrossing a line of Pavon 76 with a substitution of a reconstructed chromosome $2B_{rec}$ for a normal chromosome 2B with a line of Pavon 76 with a 2R(2B) substitution. Lines disomic for $1B_{rec}$ and $2B_{rec}$ of Pavon 76 were used as controls for the centromere appearance and behavior in bivalents and were treated in the same manner as the double monosomics. Chromosome $1B_{rec}$ was produced in the same way as $2B_{rec}$, by repeated centric misdivision, and appears to have about 3/4 of its centromere from rye chromosome 1R [Zhang et al., 2001].

All plants were grown in a greenhouse at the University of California, Riverside during 3 growing seasons in 2005–2007. At meiosis individual tillers were cut, spikes dissected, and sampled for the stage of their pollen mother cell (PMC) development. One anther from a flower was live-squashed in a drop of acetocarmine and observed under a microscope. If a desired stage of meiosis was present, the remaining 2 anthers were fixed in a 3:1 mixture of

absolute ethanol:glacial acetic acid for a week at 37°C, stained for 2 h in 1% acetocarmine in 45% glacial acetic acid and frozen at –20°C until needed.

In situ probing with labeled DNA was done according to the protocol of Dr. T. Endo, Kyoto University, Japan [Masoudi-Nejad et al., 2002] kindly demonstrated by him to the author. To be used as probes, total genomic DNA of rye and the rye centromere-specific probe pAWRC1 of Francki [2001] were labeled with digoxigenin and detected with anti-digoxigenin-FITC using standard kits and protocols from Roche Applied Science (USA). The pAWRC1 probe was kindly provided for this study by Dr. B. Friebe of the Kansas State University. The 2 probes were used alone or combined together in various proportions and mixed with blocking wheat DNA, prepared according to Masoudi-Nejad et al. [2002], usually in the probe to block ratio of ca. 1:100. All counterstaining was done with 1.5 μg/ml propidium iodide (PI) in the Vectashield antifade solution (Vector Laboratories).

Observations were made with a Zeiss Axioscope 20 equipped with epi-fluorescence, recorded with a SPOT RT Color digital camera (Diagnostic Instruments Inc.), and processed using the SPOT Advanced and Adobe Photoshop CS software. All images presented here were manipulated to enhance contrast, decrease background distortion, and provide visibility of as much detail as possible given the small size of reproduction.

Terminology used in relation to the centromere itself will be discussed later. Until then the 'centromere' is the part of the chromosome painted by the probes used in this study and interacting with the karyokinetic spindle apparatus. Centromeres on sister chromatids in a replicated chromosome will be called 'sister centromeres'. 'Modified mitoses' will be understood as mitoses observed after standard cytogenetic handling of material, with inhibition of anaphases here by a cold-water treatment. Material collected without any pre-treatment will be referred to as 'untreated mitoses'.

Results

The rye centromere in $2B_{rec}$ reacts to the rye total genomic DNA probe with a similar intensity and physical location as to the Francki probe [Francki, 2001]. Labeling with rye total genomic DNA visualized $2B_{rec}$ as a wheat chromosome with a labeled centromere while 2R was completely painted by the probe (fig. 1a). Labeling with the Francki probe alone visualized 2 chromosomes with rye centromeres; only the size gave indications for the identity of the chromosomes, which was not always reliable. Simultaneous labeling with both probes, with the total genomic rye probe at ca. 1/4 of its regular concentration and the Francki probe at a normal strength, identified both chromosomes: chromosome 2R was labeled yellow-green with a bright green centromere and $2B_{rec}$ as a red chromosome with a green centromere. These probing systems were used liberally and not necessarily consistently in all stages of meiosis.
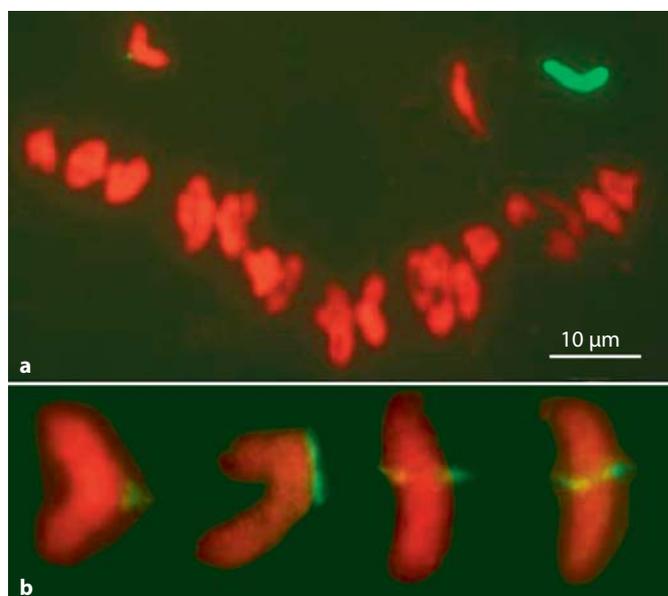
Fig. 1. a Metaphase I with 20 bivalents and $2B_{rec}$ (left) and 2R (green, right) univalents, labeled with total genomic rye DNA. The $2B_{rec}$ univalent has sister centromeres fused in a monopolar attachment to the karyokinetic spindle. **b** $2B_{rec}$ univalents. Two on the left with fused sister centromeres in monopolar and bipolar attachment, 2 on the right with sister centromeres separated in bipolar attachment.

Metaphase I

Each PMC analyzed at MI had 2 clearly identifiable univalents: 2R and $2B_{rec}$. In a few isolated cases, additional unlabeled (wheat) univalents were present. Among 694 PMCs at MI analyzed there was no homoeologous pairing of the 2 chromosomes. The 2 univalents were scattered randomly in the cell, with no specific pattern. In a majority of univalents (89.6%) sister centromeres were fused into a single unit, usually on one side of the chromosome (fig. 1b). These fused sister centromeres were capable of monopolar or bipolar attachment to the spindle apparatus (fig. 1b), with the former accounting for 82.8% and the latter for 13.2% of the total 622 univalents scored for this feature. In a minority of cases (10.6%) sister centromeres of a univalent were separated into distinct units one on each side of the chromosome and giving the univalent a mitotic appearance (fig. 1b). In most cases of separated sister centromeres, each one was apparently capable of independent interaction with the spindle apparatus resulting in a bipolar attachment and placing the univalent on the metaphase plate. Occasionally, one of the non-fused sister centromeres was in a bipolar attachment to the spindle (fig. 2e). There was no
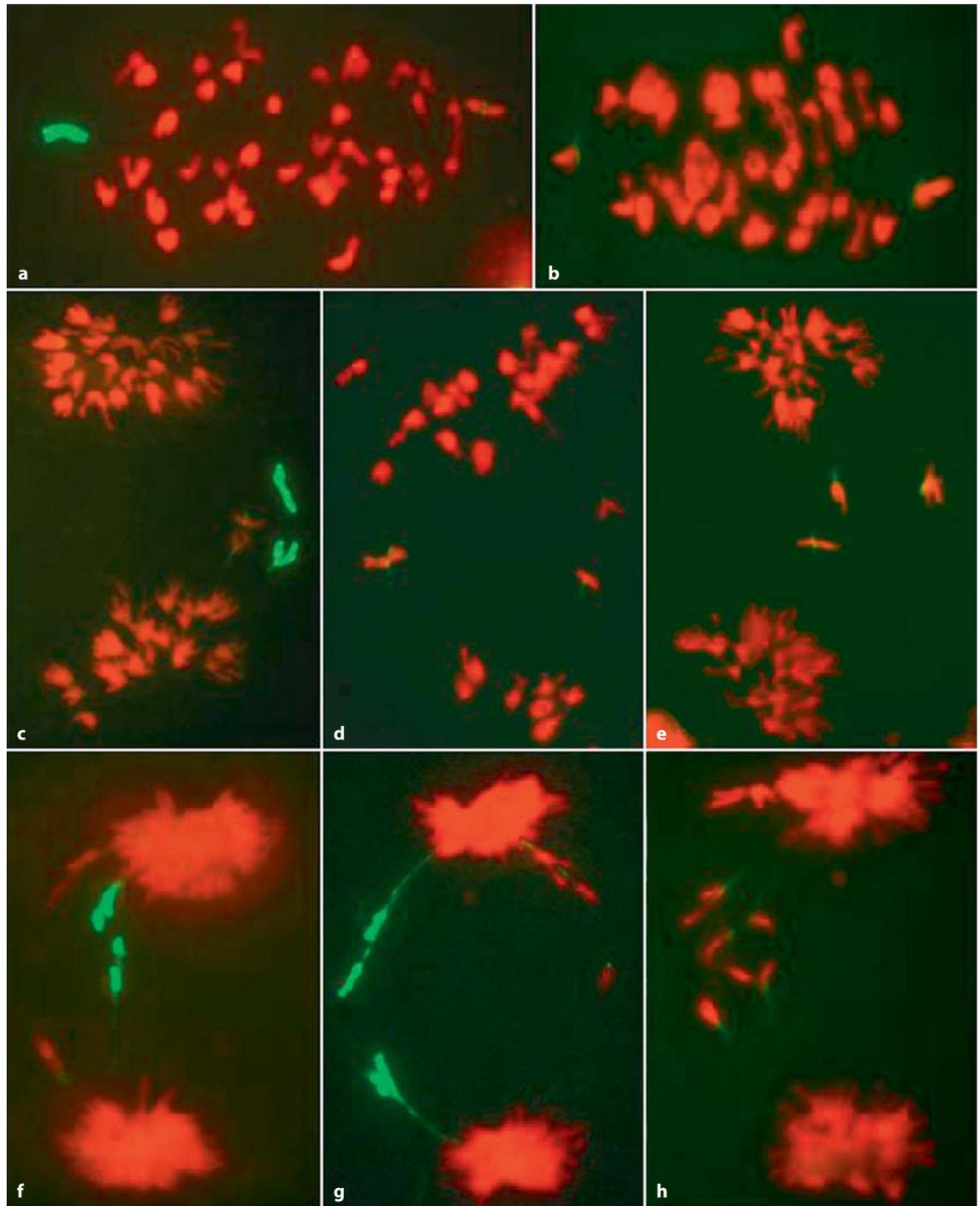
Fig. 2. Behavior of $2B_{rec}$ and 2R univalents in anaphase I in wheat. Chromosomes are labeled in green with total genomic DNA of rye and the rye centromeric probe. **a**, **b** Early AI. **a** Both univalents in bipolar attachment with separated sister kinetochores. **b** Both univalents in a bipolar attachment of fused sister centromeres. **c**–**e** Mid 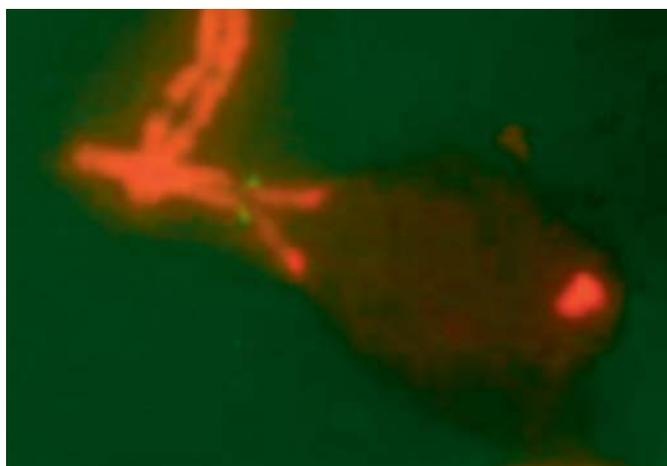AI. **c** Separation of sister chromatids in $2B_{rec}$ and 2R. **d** Separation of sister chromatids in $2B_{rec}$ and breakage across the centromere in 2R. **e** Separation of sister chromatids of $2B_{rec}$, with one of them in bipolar attachment. **f**–**h** Late AI. **f** Separation of sister chromatids. **g** Separation of a one single chromatid arm from $2B_{rec}$. **h** Breakage of one of the 2 chromosomes into independent telocentrics.

synchrony between the 2 types of the centromere organization (fused and non-fused sister centromeres) in the 2 univalents present per PMC, and there were no indications that the wheat or the rye univalent was more prone to one or the other type of behavior.

In all MI bivalents of $1B_{rec}$ and $2B_{rec}$ observed (364 PMCs), both sister centromeres were fused into single units, and all such fused sister centromeres were in proper monopolar attachment to the spindle apparatus. Therefore, separation of sister centromeres and their ability to act independently are features of univalents and do not appear to occur in normally paired chromosomes. When bivalents of $1B_{rec}$ and $2B_{rec}$ were observed in stages preceding MI (diplotene – diakinesis), sister centromeres were always fused and in most cases positioned toward the outside of the bivalent as if to facilitate proper future interactions with the spindle apparatus (fig. 3).

Fig. 3. Wheat $1B_{rec}$ bivalent in late diplotene with sister centromeres (green) fused and oriented outward.

Anaphase I

As AI is relatively short, only 213 PMCs were observed at this stage. The univalents either intact migrated to the poles, separated their sister chromatids which then migrated to the opposing poles, or broke across their centromeres with some or all parts migrating to the poles (fig. 2). In a few cells one or both univalents appeared left on the metaphase plate in a bipolar attachment giving no indication as to their later fate. Since these univalents were not in the process of sister chromatid separation or breakage, they were included in the class of intact (migrating) univalents. Hence, among 426 univalents counted, 32% were intact, 49% separated sister chromatids, and 19% broke across the centromere, with breakage either separating the arms of both chromatids or a single arm of one chromatid. In the last case, 5 anaphases are included where a major portion of the centromere itself was removed from a chromatid and migrated independently to the pole, leaving one or both single chromatid arms on the metaphase plate (fig. 4). In all univalents separating sister chromatids, sister centromeres were separated. There was a clear difference in the frequency of sister chromatid separation between early and late AI, as judged by the position of chromosomes separating from bivalents. Among 50 PMCs judged to be early AI, 21 univalents separated sister chromatids; among 21 PMCs judged as late AI, 19 univalents did. This is a highly significant difference in proportions (χ^2 = 12.2261, p < 0.01) indicating that the proportion of univalents in bipolar attachment increases over time and that most of this increase is by separation of the originally fused sister centromeres. Hence, the probability of sister chromatid separation in AI increases with the time univalents spend on the metaphase plate.

An interesting feature of the observed anaphases was an apparent coordination of the fate of both univalents. Among 213 PMCS studied, both univalents met the same fate (intact migration, separation of sister chromatids, or breakage) in 174 cells (82%) and different fates in the remaining 18% of cells.

Metaphase II

This stage was not studied in much detail. Due to technical difficulties in making preparations from material fixed for in situ probing by the Endo technique, pairs of dyads separated during squashing, frustrating attempts to determine the fate of all arms of the 2 univalents. A cursory examination of 83 MII cells did reveal the presence of intact $2B_{rec}$ and 2R as well as wheat and rye telocentrics with proportions that appeared to correspond to the frequencies of different univalent fates in AI. Among 26 MII nuclei with more than one clearly identifiable broken centromere, no evidence of fusion of the broken chromosome ends into wheat-rye translocation chromosomes was observed. No isochromosomes were present, and given the arm ratios of both chromosomes studied, these would have been easily identified.
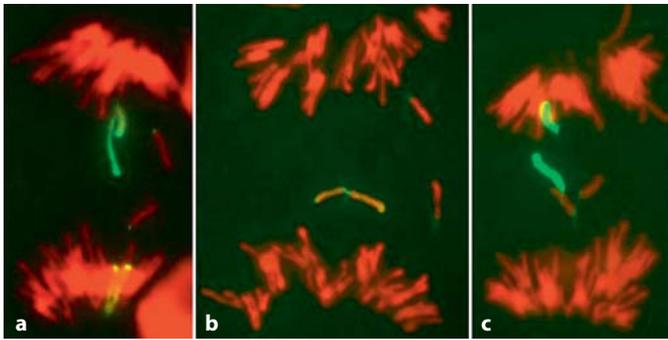
Anaphase II

The same technical problem as with MII prevented an analysis of fates of each chromosome in AII. However, the general pattern of chromosome behavior was clear: whenever a dyad received one or both intact univalents follow-
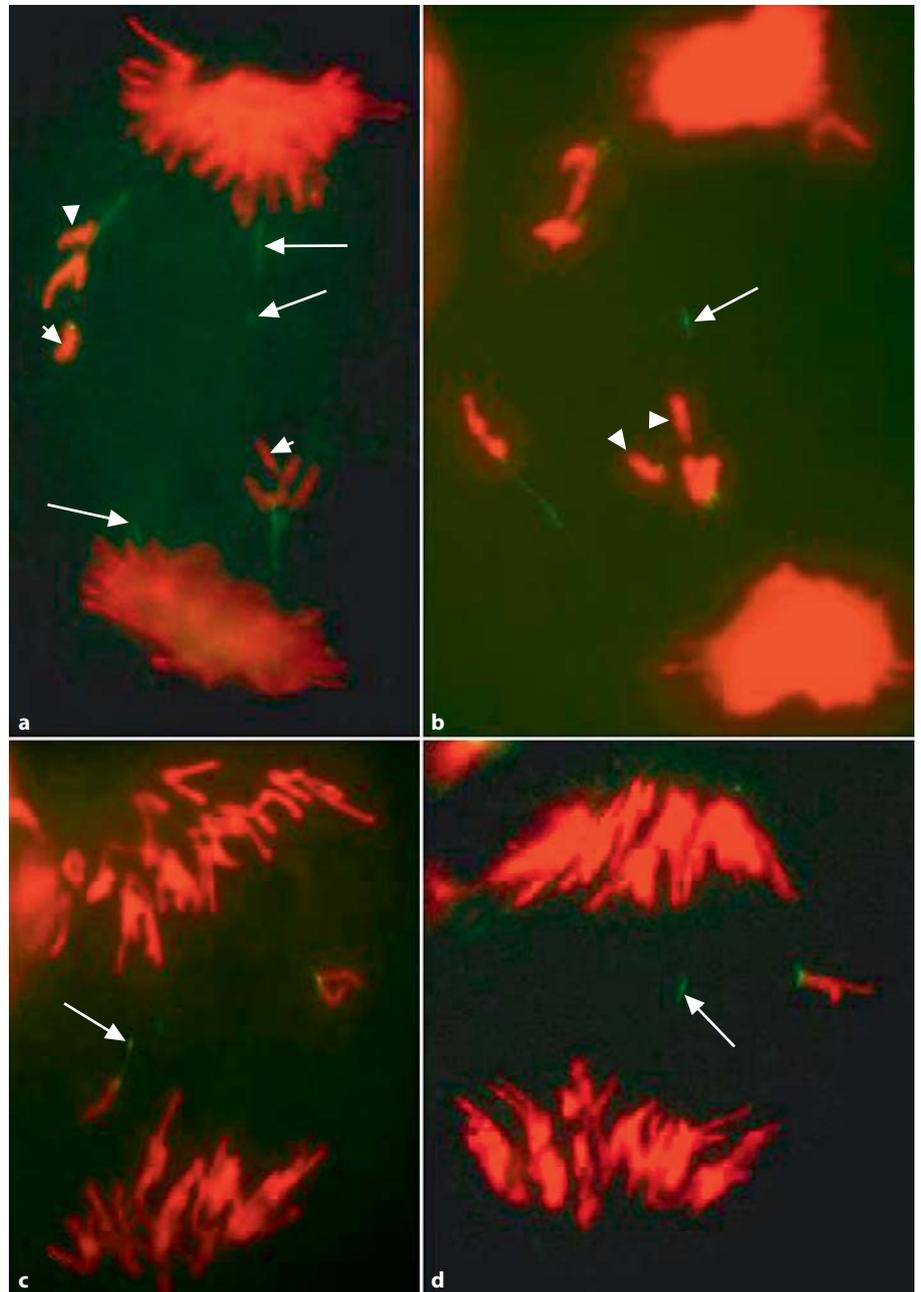
Fig. 4. Separation of the chromatin region underlying the kinetochore from univalents $2B_{rec}$ and 2R in anaphase I (**a**, **b**) and anaphase II (**c**, **d**). The rye centromere probe is labeled in green. **a**–**c** Detached kinetochore regions (arrowed). **d** A fragment of the kinetochore region separates from a telocentric chromosome (arrowed). In AI the chromatids that provided the kinetochore regions can still be identified (arrowheads). In AII single chromatid chromosomes are in a bipolar attachment to the spindle.

ing the migration of intact chromosomes in AI, they separated their sister chromatids in a regular way. Whenever a dyad received a single chromatid chromosome following separation of sister chromatids in AI, such chromosome either migrated intact to one of the poles or broke across the centromere following a bipolar attachment to the spindle apparatus (fig. 5). Given that in 49% of cases sister chromatids separated in AI, most dyads had single chromatid chromosomes, and these chromatids often

misdivided into telocentrics in AII (fig. 4c, d). In all cells analyzed, telocentrics produced by misdivision across both sister chromatids in AI separated them in AII without any apparent handicap (fig. 5). As in M II, despite the frequent presence of 2 broken centromeres, not a single case of centric fusion was observed and no isochromosomes were present. In 2 cases, separation and independent migration of a fragment of the Francki probe-labeled chromatin was observed (fig. 4).
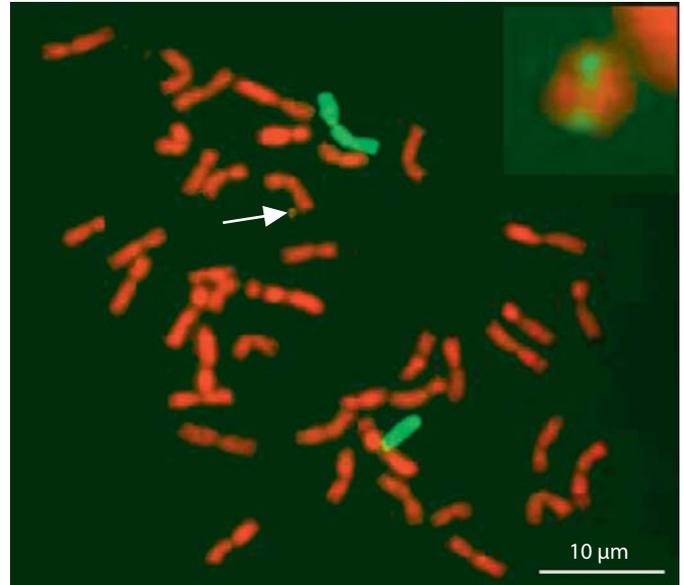
Fig. 5. Anaphase II, labeled with total rye genomic DNA (1/3 standard concentration in the middle photo) and rye centromere probe (both green). **a** Separation of sister chromatids in 2R and in a telocentric of $2B_{rec}$. **b** Bipolar attachment of a single chromatid 2R initiating breakage across the centromere and separation of sister chromatids of a $2B_{rec}$ telocentric. **c** Breakage across single chromatid 2R and $2B_{rec}$.

Fig. 6. A centromeric midget chromosome (arrow) in a progeny plant (43 chromosomes, 2R +2RL), probed with total genomic rye DNA. Inset: the same midget chromosome enlarged ca. $30\times$.

Progeny

Double monosomics $20' + 2B_{rec}' + 2R'$ were self pollinated and the resulting progeny were screened by a combination of in situ probing with total genomic rye DNA and C-banding to identify the misdivision products. Among 300 plants screened, there were 82 centric translocations in each of the 4 possible arm combinations, 121 telocentrics, 12 isochromosomes, 5 midget chromosomes, and 3 unusual chromosome rearrangements that could not be explained by centric misdivision. Of the 300 progeny analyzed, 181 (60.3%) had at least one misdivision product; the average number of such products per progeny plant was 0.74.

No concerted effort was made to recover various classes of the misdivision products, except for the midget chromosomes. Of the 5 midgets identified in the first generation after misdivision, 2 were of wheat origin (fig. 6) and 3 of rye, as judged by the pattern of in situ probing with both probes. The transmission rates of all midgets were low and they appeared related to their size. The shortest midget, of wheat, was never recovered in the subsequent generation; the remaining wheat midget was transmitted with the overall frequency of ca. 4%. Of the 3 rye midgets, the smallest one was recovered in one plant out of 25 screened in the first generation and was not recovered among 159 screened progeny of the following generation; the other 2 midgets, considerably larger, were recovered with frequencies of 5–6%. The surviving wheat midget appears to be somatically unstable, being present in the interphase nuclei only in some sectors of root-tip squashes, but at times in greatly increased numbers. Where the individual probe signals could be counted, up to 24 copies were accumulated, but nuclei with what appeared to be even larger but undetermined numbers were observed. However, no progeny with multiple midgets was ever recovered, and their ability to pair in meiosis could not be observed. In unmodified somatic metaphases and anaphases, midget chromosomes, where present, appeared to behave properly, separating sister chromatids together with normal chromosomes. In meiosis with single midgets present, they behaved as univalents, but there was no evidence of misdivision perhaps because of a small number of anaphases observed (37). Their tendency to separate sister chromatids in AI was similar to that of the univalents $2B_{rec}$ and 2R.

Discussion

The observed frequencies of centric misdivision in AI and AII correspond only broadly to the frequencies of misdivision products recovered among progeny. Given that some selection may be taking place among gametes with missing or incomplete chromosomes especially on the male side, the absence of direct correspondence is not

surprising. In AI, separation of sister chromatids was the most common fate of univalents (49% of all cases observed), and about a third of AII PMCs with single chromatids present have undergone breakage. Therefore, the overall rate of breakage among all PMCs with both anaphases combined was about 35%, and either one of the gametes or both were likely capable of contributing a broken chromosome to the offspring. The frequencies of recovered misdivision products observed here were similar to those observed by Sears [1952] for wheat univalents, by Lukaszewski [1993, 1997a] in the reconstruction, by centric misdivision, of chromosomes 1B and 1R, and by Friebe et al. [2005] for a wheat and *Elymus trachycaulus* univalents.

The extensive stretching of the centromeric signal in AI and AII was surprising. Threads of the Francki probe-positive centromeric chromatin stretching about half the distance from the metaphase plate to the pole were common (fig. 2, 4), but in extreme cases they could reach almost to the pole. Such stretched segments regularly showed uneven thickness, reminiscent of the kinetochore stretching experiments of Zinkowski et al. [1991]. This stretching did not appear to translate into a higher tendency for misdivision. As a matter of fact, more often than not, misdivision products observed in AI had compact centromere signals while instances such as that illustrated in figure 2h cannot be interpreted as definitely leading to arm separation.

The Francki probe used in this study hybridized only to primary constrictions of the chromosomes tested, labeling only the parts of chromosomes interacting with the spindle apparatus. Given the overall stretching of the centromeres detected in AI and AII, some stretching of unlabeled parts of the chromosomes was occasionally visible, but non-labeled chromatin never appeared to interact directly with the spindle fibers. Therefore, given the resolution level of light microscopy, in the chromosomes studied here DNA labeled by the Francki probe underlies the entire kinetochore regions both in $2B_{rec}$ and in 2R.

The collected data suggest some coordination in the fates of 2 univalents in the first meiotic division. In a majority of PMCs both univalents behaved the same: either both migrated intact to the poles together with the chromosomes segregating from bivalents, separated their sister chromatids, or broke across their centromeres; however, concerted breakage was the least frequent (29 PMCs out of 213 analyzed). This apparent coordination between seemingly independent chromosomes may, however, be superficial. As the division progressed from metaphase I

to late anaphase I, the proportion of univalents with separated sister centromeres increased, and all univalents still left on the metaphase plate by late anaphase I were in a bipolar attachment and orientation. The increase in sister centromere separation and bipolar attachment was clearly evident with the progression of AI. Whether there was any change of this nature in MI is unclear, as MI could not be reliably timed. However, the change in AI suggests that separation of sister centromeres, bipolar attachment, and a tendency to separate sister chromatids in meiosis I may be a consequence of longer exposures to separase, an enzyme which is responsible for the dissolution of sister chromatid cohesion and which is controlled by securin [Nasmyth, 2001; Cleveland et al., 2003; Craig and Choo, 2005]. The pattern of chromosome separation, first from bivalents and then from univalents lagging on the metaphase I plate, implies localized action of separase or other enzymes controlling separase's action within a meiocyte. In this sense, concerted separation of sister chromatids by both univalents may not be indicative of any specific system coordinating their fate. Rather it may reflect a general condition increasing chances of bipolar attachment and consequent placement of univalents on the metaphase plate where they have to remain either until they break or their sister chromatid cohesion is released. Signals that stabilize securin, hence preventing separase from releasing sister chromatid cohesion, are emitted by unattached centromeres [Craig and Choo, 2005]. Even if this stabilization of securin by univalents is localized, when they eventually assume a stable bipolar attachment, nothing can prevent separase from acting, and even the most lagging univalents safely separate their sister chromatids (if they did not break earlier). Experience with generation of telocentric chromosomes via centric misdivision dating back to Darlington [1939] indicates that as in mice the presence of an unpaired centromere in meiosis does no cause division arrest [Mee et al., 2003]. Here, PMCs appeared to proceed at a normal pace and produce viable products. Whether all these products were capable of converting into viable and functional gametes is a different issue.

Coordinated behavior of sister chromatids is a prerequisite for a safe passage of a chromosome through meiosis I. This coordination involves 2 aspects: coordination of sister centromeres, acting in unison and interacting together with only one pole of the karyokinetic spindle as illustrated by Dawe [1998], and cohesion of sister chromatids along the entire chromosome length that maintains chiasmata and, therefore, assures proper alignment of bivalents on the MI plate. Such fusion of sister centromeres

was described in maize [Yu and Dawe, 2000] and studied in detail in grasshoppers [Paliulis and Nicklas, 2005], where, when labeled with specific probes, centromeres on sister chromatids produced single signals in the first division and by MII reoriented into 2 separate signals, one on each sister chromatid. Even a causal observation of wheat bivalents in MI and chromosomes in MII, such as disomics $1B_{rec}$ and $2B_{rec}$ analyzed as controls here, indicates that the process and its timing are the same as in grasshoppers. In all paired chromosomes observed, sister centromeres were fused; this fused state persisted through AI, but by MII all sister centromeres were separated. As chromosomes in bivalents in pre-MI stages, i.e. before the karyokinetic spindle formation, already had their sister centromeres fused into single signals and in most cases positioned on the outside of bivalents (fig. 3), specific positioning of sister centromeres cannot be imposed by interactions with the spindle fibers originating from one pole. Instead it is generated in advance to facilitate future proper monopolar interaction of each chromosome in a bivalent. Univalents did not follow this pattern. Already in MI about 10% of them had sister centromeres separated and in a bipolar attachment to the spindle; the process of sister centromere re-orientations accelerated into AI as by late AI about half of them did, leading to mitotic-like separation of sister chromatids in the first division. In an extreme case, an F_1 hybrid of tetraploid wheat with diploid rye was observed in which all univalents (19 to 21 per PMC) regularly oriented on the MI plate in a standard mitotic configuration (bipolar attachment) and separated sister chromatids in AI; the phenomenon is probably responsible for the restitution of the first meiotic division and an almost complete fertility of this hybrid [Lukaszewski, unpublished]. It was the separation of sister centromeres in univalents here that promoted their normal bipolar attachment and separation of sister chromatids in AI. The rate of sister centromere separation increased from ca. 10% in MI to ca. 80% in late AII (of univalents left on the metaphase I plate; it does not account for earlier migration of intact chromosomes and misdivision). It would be of interest to determine the mechanism that fuses 2 sister centromeres in a single unit in meiotic prophase and the mechanism that separates them. While the frequency of sister centromere separation increases dramatically with the time spent on the metaphase plate, removal of sister chromatid cohesion cannot be entirely responsible, as already in MI about 10% of univalents show independence of their sister.

Separation of sister chromatids in AI requires a change in the standard meiotic pattern of dissolution of sister chromatid cohesion. Rieder and Cole [1999] postulated 2 different types of sister chromatid cohesion in meiosis: one specific to the centromere regions and persisting into MII-AII transition and another along the length of the chromosomes, specific to the first meiotic division, that makes chiasma maintenance possible. When the latter is removed at the onset of AI, chiasmata are released and the poleward movement of previously paired homologues is permitted. Nasmyth [2001] discussed meiosis-specific cohesins that may be responsible for the difference between mitotic and meiotic patterns of sister chromatid cohesions. Such 2 different mechanisms for sister chromatid cohesion also imply 2 different mechanisms of its removal. However, a frequent loss of centromeric cohesion of sister chromatids in univalents lingering on the metaphase plate in AI suggests that perhaps the difference between centromeric and non-centromeric sister chromatid cohesion is more a matter of degree than state. Cohesion along the chromosome length is removed more rapidly, and chromosomes with sister chromatids still attached at the centromeres are removed from the area of separase activity by the spindle tension before centromeric sister chromatid cohesion is affected. Univalents lingering on the metaphase I plate are exposed to separase for a longer time, and their centromeric sister chromatid cohesion eventually dissolves as well. This may not even require a longer action of separase but may reflect only a later start of the process. In most cases a univalent can assume a stable bipolar attachment only after the sister centromere fusion is removed; only then the signal preventing sister chromatid separation is no longer released. Even if this signal has a limited range and may not prevent a cell passing the checkpoint [Mee et al., 2003], it ought to be able to cover the chromosome whose kinetochore emits it. The fact remains: univalents can separate their sister chromatids already in the first division just as efficiently as in the second even with a delay relative to paired chromosomes and with up to 100% success in some genotypes, as illustrated by the meiotic restitution in a wheat × rye hybrid.

If this interpretation of strictly cytological observations makes any sense, the action of separase or enzymes controlling it may be localized, with a high concentration on the metaphase plate and decreasing or disappearing toward the poles. Chromosomes in bivalents, especially those with terminal chiasmata, may already keep their centromere regions in the lower enzyme activity/concentration area and then quickly escape the danger of the dissolution of their centromeric sister chromatid cohesion by a movement away from the metaphase plate.
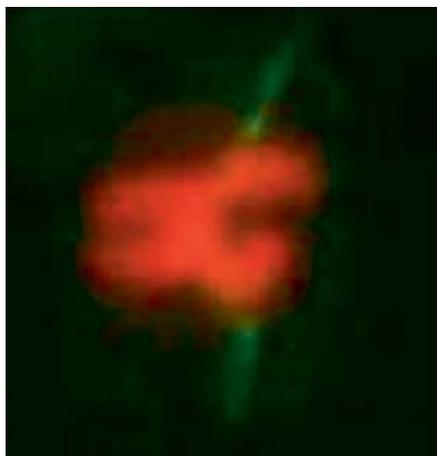
Fig. 7. Univalent $2B_{rec}$ lagging in anaphase I with separated sister centromeres in bipolar orientation. There is tension between the spindle attachment forces at the centromeres and sister chromatid cohesion in the long arms. This arrangement may produce acrocentric 2BS chromosomes.

Chromosome transplantation experiments between meiosis I and meiosis II in grasshoppers [Paliulis and Nicklas, 2005] lend some basis for these speculations.

In most cases, centric misdivision occurred across the area of the 2 univalents delimited by the Francki probe; i.e. across the kinetochore regions (understood here as the segments of chromatin underlying the kinetochore). However, in some cases, including all midget chromosomes recovered, breakage occurred outside kinetochore regions not labeled by the probe. The exact position of the point of chromosome breakage depends on 2 counter forces involved: (a) the force exerted by tension generated by the attachment of the spindle fibers to the kinetochores of one or both of the 2 sister centromeres, and (b) the force generated by cohesion of sister chromatids. When sister centromeres are fused and in a bipolar attachment to the spindle apparatus, the break, if any, will be across the kinetochore region either of one or both chromatids. This generates telocentrics, each one with a fragment of the original kinetochore region (see fig. 2d). The exact point of breakage will depend on specific points of attachment; hence, the proportion of the original centromere present in each of the resulting telocentrics may vary. This type of breakage appears to be the most common and reflects the most frequent arrangement of the sister centromeres. All telocentric chromosomes analyzed in rice [Cheng et al., 2002] and maize [Jin et al., 2005] broke across the kinetochore regions, i.e. regions

interacting directly with the spindle apparatus. Most breakage products recovered among the progeny in this study also were located across the centromere regions. If sister centromeres are not fused and act independently, bipolar attachment of one of them may tear out a single chromatid telocentric with breakage again across the kinetochore region. On the other hand, bipolar attachment of separated sister centromeres generates tension between the kinetochore regions and the sites of sister chromatid cohesion. If this tension is strong enough, breakage may occur outside the kinetochore region and before or inside the region of the chromosome providing sister chromatid cohesion (fig. 7). This will generate an acrocentric chromosome and, less frequently, centromeric midgets such as those recovered here and on numerous other occasions when centric misdivision was used to generate wheat-alien translocations [Lukaszewski, unpublished].

This study cannot answer the question why univalents are prone to misdivision while paired chromosomes are not. Chiasma formation in itself does not protect a chromosome from misdivision: isochromosomes which frequently form ring univalents do misdivide with considerable frequencies and served as a steady source of telocentrics in wheat [Sears, 1952]. The ability to misdivide is clearly related to a redundancy and the repetitive nature of centromeres in higher organisms. Repeated misdivision of the same centromeres is possible, even without being followed by fusion with other centromeres. This fragments original centromeres into smaller and smaller segments. In maize such repeated misdivision reduced the centromere complexity [Kaszas and Birchler, 1996]. In wheat fragmentation of original centromeres into smaller segments reduced their ability to misdivide further but did not eliminate it completely [Lukaszewski, 1997b]. In theory, misdivision without fusion could be repeated to the point where only point centromeres remain, each capable of interaction with only one microtubule fiber and thereby incapable of bipolar spindle attachment and further misdivision.

In several cases a bipolar univalent attachment to the spindle apparatus dissected the kinetochore regions from the chromosome, producing stand-alone centromeric fragments. Five such events were observed among 213 AI PMCs analyzed (fig. 4a, b) and 2 among the AII PMCs (fig. 4d), demonstrating this as a relatively frequent phenomenon. However, not one midget chromosome composed only of the Francki probe-labeled chromatin was recovered among progeny. All 5 recovered midget chromosomes had observable amounts of non-labeled pericentric chromatin flanking the kinetochore region

(fig. 6). Interestingly, formation of such midgets has not been directly observed in this study. Presumably, the presence of the pericentric chromatin was a prerequisite for the passage of the chromosome to subsequent generations. The molecular sizes of the recovered midgets can only be guessed based on their proportions relative to the total chromosome lengths in wheat and its genome size. In mice, a 4.5-Mb mini chromosome composed of various mouse and human repeats and chromosome fragments was regularly transmitted via eggs and reasonably well via sperm. A mini chromosome in maize was estimated at 15–30 Mb in size [Ananiev et al., 2009]. In *Arabidopsis thaliana*, a ca. 7.5-Mb mini chromosome was recovered and passed on quite regularly to next generations [Murata et al., 2006]. This mini chromosome contained ca. 1/3 of the centromere sequences of the chromosome from which it was derived. The smallest midget recovered here was probably in the same order of size as the *A. thaliana* mini chromosome; the biggest one perhaps twice as large. The low transmission rate of these midgets may be related not to their small overall sizes but to proportionately low content of the pericentromeric chromatin. If indeed they originated by tension forces of the kinetochore region against sister chromatid cohesion, they very likely contain complete kinetochore regions of their mother chromosomes ($2B_{rec}$ and 2R).

Meiotic behavior of these midget chromosomes has not been studied in any detail. Their pairing in MI could not be ascertained for the simple reason that not a single progeny plant was recovered with a pair of such chromosomes. However, given the inability of the proximal regions of rye chromosome 1R [Lukaszewski, 2008] and of 2 wheat chromosome arms 2BS and 4AL tested so far to form chiasmata, is it highly unlikely that they would be able to form chiasmate associations with each other or with their donor chromosomes. In a low number of the MI PMCs observed, the largest of the midgets recovered here appeared to behave in a manner similar to univalents: it did interact with the spindle apparatus and often separated sister chromatids in AI, but no misdivision events were observed. Some mini chromosomes of maize were capable of pairing with each other, but this ability was not necessarily related to their length [Han et al., 2007].

In the classical cytogenetic sense, the centromere was synonymous with the kinetochore: the part of a chromosome responsible for its movement in anaphases. Later, the term 'the centromere' encompassed all functions necessary for a proper behavior of a chromosome in cell divisions, such as movement and timing. In recent literature

on the subject [Birchler et al., 2009; Kanizay and Dawe, 2009] there appears a trend to revert to the original meaning of the centromere as a chromosome part responsible for chromosome movement. Hence, what is here understood as the 'kinetochore region', the stretch of chromatin visualized by the Francki probe that interacts with the spindle apparatus, corresponds to what Birchler et al. [2009] and Kanizay and Dawe [2009] understand as the 'centromere'. In this sense the centromere has ill-defined borders. If the centromere is the chromosome region responsible for the motoric function, the 'kinetochore region' and the 'centromere' are equivalent. If the centromere is also responsible for timing and coordination of the chromosome movement, its limits (borders) become fuzzy, especially that the centromeric functions themselves are not well understood. Proper timing and coordination of chromosome movement in anaphases require sister chromatid cohesion. In the material studied here, there was no evidence of interactions between microtubule fibers and non-labeled parts of the chromosome; no non-labeled part of the centromere was ever stretched out from univalents by the pulling forces of microtubules. It therefore appears likely that the Francki probe, a DNA sequence, underlies the entire kinetochore area of the chromosomes studied. These kinetochore regions did not provide sister chromatid cohesion, and while such regions detached from the chromosomes in AI in a noticeable frequency (ca. 2.5%), they were not transmitted to progeny. This is in contrast to detached kinetochores of Zinkowski et al. [1991] capable of performing many typical mitotic steps. In unmodified metaphases, where live material is fixed directly without spindle inhibition and where normal spindle apparatuses are present and operational exerting considerable forces on bipolarly attached chromosomes, the signals of sister kinetochore regions are clearly separated (fig. 8), illustrating the absence of sister chromatid cohesion throughout their lengths. This is line with the AuroraB/Shogushin model of the kinetochore function and the stabilization of the microtubule attachment in metaphase [see Craig and Choo, 2005]. In the chromosomes studied here, cohesion of sister chromatids is provided by pericentric chromatin flanking the kinetochore region. These stretches must be of considerable length; the actual amount of pericentric chromatin may be genotype-dependent, as illustrated by differences in the transmission rate of the same minichromosome in different genetic backgrounds of *A. thaliana* [Murata et al., 2006].

Cohesion of sister chromatids is critical for normal chromosome behavior during divisions. If this cohesion
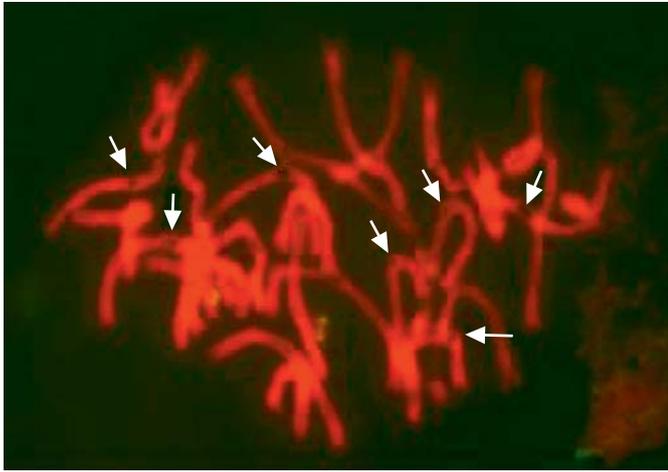
Fig. 8. Untreated mitotic metaphase showing absence of sister chromatid cohesion in the kinetochore regions of 2 midget (with green centromeres) and most normal chromosomes of wheat (some are arrowed). The midget chromosome in the center shows separation of the kinetochore region signals (green) and cohesion of non-labeled chromatin.

is provided by pericentric chromatin, it is fair to consider if this pericentric chromatin is therefore an integral part of the centromere and if sister chromatid cohesion is an intrinsic characteristic of pericentric chromatin. If so, there will be problems in defining the physical borders of the centromere.

The positions of non-centric breaks, such as in acrocentrics and midgets recovered here, are a measure of the expanse of pericentric chromatin. An acrocentric chromosome is probably a less reliable measure as to how much pericentric chromatin is required for a proper chromosome behavior: it has one normal arm with its standard amount of pericentric chromatin to provide all necessary cohesion. Therefore, acrocentrics only show how far sister chromatid cohesions must have reached or how close to the centromere it must have been removed when the break occurred. Midget chromosomes, on the other hand, provide some measure of the minimum requirement for pericentric chromatin but not of its expansion in a normal chromosome. That extent may be considerable, in wheat perhaps as much as 10% of the average length of a chromosome arm. Somatic instability of the midget chromosomes, which probably accounts for their low recovery rates among progeny, provides some measure of the minimum length of pericentric chromatin required for a normal behavior of a chromosome. In wheat, midget telocentrics consisting of about 12–15% of the en-

tire donor chromosome (1B) were somatically stable and were normally transmitted to progeny [Lukaszewski, 1997b]. Their disomics have been produced and they are true breeding, suggesting normal meiotic behavior [Lukaszewski, unpublished]. On the other hand, a midget consisting of perhaps only 2% of the original 1B [Lukaszewski, 1997b] and all midgets recovered here, are somatically unstable and are transmitted to progeny only infrequently, if at all.

Mini chromosomes of maize offer some measure of the relationship between chromosome size and proper sister chromatid cohesion [Han et al., 2007]. It remains to be seen if there is some absolute minimum value for plants or eukaryotes. Wheat chromosomes are about twice as large as those of maize, and rye chromosomes are still larger. Wheat and rye midgets observed here, while of a similar physical size to those of maize, had serious transmission problems, suggesting that wheat/rye chromosomes require longer stretches of pericentric chromatin for proper sister chromatid cohesion. Ignoring for the moment any possible differences in length of the kinetochore regions within and between genomes, on the one hand this implies that the minimum size of a chromosome in any given species may remain in some proportion to the average chromosome length in that species. On the other hand, extremely asymmetrical karyotypes of many birds [Mayr et al., 1990] imply that if any such relationship exists, it may not be generally applicable to all organisms or all chromosomes.

While inadequate cohesion of sister chromatids in mitosis appears to be a logical explanation for somatic instability and low transmission of midgets recovered here, the mechanism by which very large numbers of some of them can accumulate in some nuclei remains a mystery. This mechanism is quite intriguing, especially so that at times, a rapid increase in the numbers of midgets in a small fraction of the observed nuclei appeared to be associated with a change in their structure.

The question that remains to be addressed here is if cohesion of sister chromatids is an intrinsic characteristic of specific chromosome segments, defined as pericentric chromatin, or if it is a facultative function. Chromosome inversions, such as reverse tandem duplications with breakpoints in the vicinity of the kinetochore regions that now form the telomeric regions, paracentric inversions 1RL in rye [Lukaszewski, 2008], the In(2Rh)PL inversion in *Drosophila melanogaster* [Chlamydas et al., 2009], and translocation of the kinetochore regions to intercalary positions on wheat chromosomes [Lukaszewski, 1997b] suggest that sister chromatid cohesion is a

facultative characteristic of a chromosome segment. If a pericentric region is translocated away from the kinetochore region, it loses its sister chromatid cohesion and behaves as any other chromosome segment, both in mitosis and meiosis. If a chromosome region which normally does not provide sister chromatid cohesion is translocated to the vicinity of the kinetochore region, it gains sister chromatid adhesion. The signal for chromatid cohesion must, therefore, emanate from the kinetochore region itself, as discussed by Topp and Dawe [2006]. Therefore, it is the kinetochore region of a chromosome that dictates all functions necessary for proper behavior of a chromosome in cell divisions: chromosome movement and its timing. In this sense, the borders of the centromere can be defined precisely as the segment of chromatin responsible for the formation of the kinetochore.

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