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The *rec8* Gene of *Schizosaccharomyces pombe* Is Involved in Linear Element Formation, Chromosome Pairing and Sister-Chromatid Cohesion During Meiosis

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* does not form tripartite synaptonemal complexes during meiotic prophase, but axial core-like structures (linear elements). To probe the relationship between meiotic recombination and the structure, pairing, and segregation of meiotic chromosomes, we genetically and cytologically characterized the *rec8–110* mutant, which is partially deficient in meiotic recombination. The pattern of spore viability indicates that chromosome segregation is affected in the mutant. A detailed segregational analysis in the *rec8–110* mutant revealed more spores disomic for chromosome *III* than in a wild-type strain. Aberrant segregations are caused by precocious segregation of sister chromatids at meiosis I, rather than by nondisjunction as a consequence of lack of crossovers. *In situ* hybridization further showed that the sister chromatids are separated prematurely during meiotic prophase. Moreover, the mutant forms aberrant linear elements and shows a shortened meiotic prophase. Meiotic chromosome pairing in interstitial and centromeric regions is strongly impaired in *rec8–110*, whereas the chromosome ends are less deficient in pairing. We propose that the *rec8* gene encodes a protein required for linear element formation and that the different phenotypes of *rec8–110* reflect direct and indirect consequences of the absence of regular linear elements.

IN sexually reproducing organisms, meiosis halves the ploidy by two successive nuclear divisions. During the first (reductional) division, homologous chromosomes pair and undergo high levels of recombination before they segregate from each other. Besides the generation of genetic diversity, meiotic recombination is required for proper segregation of chromosomes during meiosis I (for review see BAKER *et al.* 1976). The second (equational) division corresponds to a mitotic division.

In most organisms, pairing of homologous chromosomes during meiotic prophase is accompanied by the formation of a meiosis-specific structure, the synaptonemal complex (SC) (for review see VON WETTSTEIN *et al.* 1984; GIROUX 1988). In early prophase, after premeiotic DNA synthesis, axial cores (SC precursors) appear along the chromosomes and connect the sister chromatids. Chromosome pairing and full-length SC formation culminate in the pachytene stage of meiotic prophase. By this time, the formation of the tripartite SC structure is complete: two axial elements (now called lateral elements) are synapsed at a distance of ~100 nm with a central component between them. The role of the SC and its function in chromosome pairing and meiotic recombination is not well understood.

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The fission yeast *Schizosaccharomyces pombe* is a haploid organism and normally enters meiosis immediately after mating (zygotic meiosis). However, meiosis can also be induced from diploid cells (azygotic meiosis) (EGEL 1973; EGEL and EGEL-MITANI 1974). Interestingly, *S. pombe* does not form tripartite SCs, but recombination still occurs at a high frequency in meiosis (for review see KOHLI and BAEHLER 1994). In meiotic prophase, filamentous structures (linear elements) appear that strongly resemble the axial cores of other eukaryotes (OLSON *et al.* 1978; HIRATA and TANAKA 1982; BAEHLER *et al.* 1993). It has been suggested that these linear elements represent minimal structures required for proper chromosome function during meiosis I (KOHLI 1994; KOHLI and BAEHLER 1994; SCHERTHAN *et al.* 1994). Recently, the organization and pairing of meiotic chromosomes have been analyzed directly by *in situ* hybridization (CHIKASHIGE *et al.* 1994; SCHERTHAN *et al.* 1994).

Because *S. pombe* has only three chromosomes (KOHLI *et al.* 1977), some spores are expected to be viable even when the chromosomes segregate at random. The surviving spores can then be assayed for recombination. This property has allowed the isolation of recessive mutations in 16 different genes with a meiosis-specific three- to 1000-fold reduction in recombination frequencies (PONTICELLI and SMITH 1989; DE VEAUX *et al.* 1992). The genes were grouped into different classes depending on the severity with which mutations affect meiotic recombination (DE VEAUX *et al.* 1992). Four of these genes have been sequenced and shown to be

TABLE 1

Strains

Strain	Genotype	Origin
1	*h^-* (972, wild type)	Bern collection
2	*h^+* (SA21, wild type)	HEIM (1990)
3	*rec8-110 ade6-M375 h^+*	PONTICELLI and SMITH (1989)
4	*rec8-110 lys1-131 h^-*	This study
5	*rec8-110 lys1-131 ade6-M210 h^+*	This study
6	*rec8-110 his5-330 ade6-M216 h^-*	This study
JB8	*ade6-M216/ade6-149 h^+/h^-* (diploid)	BAEHLER et al. (1993)
7	*ade6-M216/ade6-M210 h^+/h^-* (diploid)	This study
8	*ade6-M375 h^+*	This study
9	*lys1-131 h^-*	Bern collection
10	*lys1-131 ade6-M210 h^+*	This study
11	*his5-330 ade6-M216 h^-*	This study

expressed specifically before, or early in meiotic prophase (LIN *et al.* 1992; LIN and SMITH 1994). The genes show no homology to known genes, although a number of proteins involved in meiotic recombination in other organisms, particularly the budding yeast *Saccharomyces cerevisiae* are known (for review see PETES *et al.* 1991).

So far little has been published on the analysis of meiotic chromosome segregation in fission yeast (NIWA and YANAGIDA 1985; MOLNAR and SIPICZKI 1993). We further characterized the mutant *rec8-110* that strongly reduces recombination at the *ade6* locus, which is centrally located on chromosome *III*. The *rec8* gene has a coding capacity for 393 amino acids and is specifically transcribed at about the time of premeiotic DNA synthesis (LIN *et al.* 1992). Recently, it has been shown that the *rec8-110* mutant reduces intra-and intergenic meiotic recombination more than 100-fold in a 2-Mb region on chromosome *III*, whereas reduction of recombination is much less pronounced in other regions of the genome (DE VEAUX and SMITH 1994). The present study describes other phenotypes resulting from the *rec8-110* point mutation. By a combination of genetical and cell biological approaches, we demonstrate that sister chromatids are often separated precociously leading to reduced spore viability. Moreover, meiotic prophase is shortened and normal linear elements are not formed. Meiotic pairing of homologs is most defective in centromeric regions. These data are discussed with respect to possible functions of the *rec8* protein in meiotic prophase and suggest specific roles of linear elements in pairing, recombination, and segregation of chromosomes.

MATERIALS AND METHODS

Media, strains, and centromere markers: For cytological and genetic studies, media were as reported by BAEHLER *et al.* (1993) and by SIPICZKI and FERENCZY (1977), respectively. Strains were constructed by standard genetic methods described by GUTZ *et al.* (1974). The strains used in all experiments are listed and numbered in Table 1. The starting strains used for constructions are from the Bern collection, with exception of the strains carrying *rec8-110*, which is probably a point mutation. They are the gift of G. SMITH (Seattle) and have been described by PONTICELLI and SMITH (1989). After isolation of an h^{90} segregant from the strain *rec8-110 ade6-M26 h^+* (PONTICELLI and SMITH 1989), the low spore viability was confirmed. For the following strain constructions, we used the low spore viability to assay for presence of the *rec8-110* mutation. Protoplasts from the *rec8-110 ade6-M26 h^{90}* strain and a *lys1-131 h^-* strain were fused (SIPICZKI and FERENCZY 1977) and a *rec8-110 lys1-131 h^-* segregant (strain 4) was isolated by tetrad analysis. The strains *rec8-110 lys1-131 h^-* (strain 4) and *rec8-110 ade6-M375 h^+* (strain 3) were used for cytological experiments and to study spore viability in detail. The strains *ade6-M375 h^+* (strain 8) and *lys1-131 h^-* (strain 9) were used as *$rec8^+$* controls for the spore viability analysis. Strain *ade6-M375 h^+* (strain 8) was isolated from the cross of *rec8-110 ade6-M375 h^+* (strain 3) with the wild-type strain 972 h^- (strain 1). For segregation analysis *rec8-110 lys1-131 ade6-M210 h^-* (5) was constructed by crossing of *rec8-110 lys1-131 h^-* (strain 4) with *ade6-M210 h^+*. From crosses of strain 3 with *his5-303 h^-* and strain 4 with *ade6-M216 h^+*, respectively, the two strains *rec8-110 his5-303 h^-* and *rec8-110 ade6-M216 h^+* were isolated. These strains were crossed to obtain *rec8-110 his5-303 ade6-M216 h^-* (strain 6). The *$rec8^+$* strains *lys1-131 ade6-M210 h^+* (strain 10) and *his5-303 ade6-M216 h^-* (strain 11) were generated from the *rec8-110* strains 5 and 6 by crosses to 972 h^- (strain 1) and SA21 h^+ (strain 2), respectively. The diploid strain *ade6-M216/ade6-M210 h^+/h^-* (strain 7) is a derivative of the adenine-dependent haploid strains *ade6-M210 h^-* and *ade6-M216 h^+*. The diploid strains 7 and JB8 are adenine independent and form white colonies due to interallelic complementation between the mutations *M210* and *M216*. In haploids both mutations lead to adenine dependence for growth, but colonies show different pigmentation on media with limiting adenine concentration: dark red *(M210)* and pink *(M216)*.

The genes *lys1* on chromosome *I* and *ade6* on chromosome *III* map 4 and 13 cM from their respective centromeres (KOHLI *et al.* 1977; MUNZ *et al.* 1989). DE VEAUX and SMITH (1994) have shown that *rec8-110* reduces intergenic recombination ~100-fold in the central part of chromosome *III*. Thus, it is justified to use *lys1* and *ade6* as centromere markers in crosses homozygous for *rec8-110*.

Spore viability and segregation analysis: Determination of spore viability and segregation studies were based on classical genetic methods (interrupted mating for construction of diploid strains, random spore analysis and dissection of asci; GUTZ *et al.* 1974). For analysis of random spore viability, spores

from crosses of strains 3 and 4 (*rec8*) as well as 8 and 9 (wild type, see Table 1) were counted in a haemocytometer. In each case, >1000 spores were then plated followed by scoring of colony formation. Mean values of three (mutant) and two (wild type) experiments were determined. For segregation analysis diploids were generated from strains 5 and 6 (mutant) as well as 10 and 11 (wild type). To study segregation in random spores, ~400 colonies were examined for both mutant and wild type. Sporulation-proficient diploid colonies (h^+/h^-) were identified by iodine treatment on sporulation medium (GUTZ *et al.* 1974). Their full genotype was determined by analysis of the secondary spore clones on diagnostic plates. The nonsporulating colonies were checked microscopically for cell size to distinguish nonsporulating diploids (h^-/h^- or h^+/h^+) from haploids. The full genotype of nonsporulating diploids was deduced from crosses to tester strains. The remaining colonies (haploids or disomics for chromosome *III*) were streaked on YEA to check for the two different versions of chromosome *III*, as revealed by the dark and light colors of *ade6-M210* and *ade6-M216* colonies, respectively. The full genotype, including mating type, was then determined on diagnostic plates and by crosses. The same procedure was applied for segregants derived from tetrad analyses.

Cytological procedures: For cytology, a diploid strain was constructed from strains 3 and 4 (*rec8–110*), and the diploid strains JB8 or 7 were used as *rec8*$^+$ controls (Table 1). The protocol for meiotic time courses, the procedures for nuclear spreading and silver staining, measurement of commitment to meiosis, as well as DAPI staining of DNA were performed as described previously (BAEHLER *et al.* 1993). Sporulation was followed by phase-contrast microscopy.

Fluorescence *in situ* hybridization (FISH): *In situ* hybridization was performed on spread nuclei as described by SCHERTHAN *et al.* (1994). Plasmids and cosmids for probing centromeres, telomeres, and different regions of chromosome *II* were the same as those used by SCHERTHAN *et al.* (1994). In addition a cosmid covering the *ade6* region of chromosome *III* was used. This cosmid (23E4) was kindly provided by E. MAIER and H. LEHRACH (MAIER *et al.* 1992; HOHEISEL *et al.* 1993). Probes were labeled with biotin or digoxigenin and detected with FITC- and TRITC-conjugated antibodies, respectively, as described (SCHERTHAN *et al.* 1994). The signals were evaluated in an epifluorescence microscope (Zeiss Axiovert). The same criteria for clustered and paired signals were used as defined by SCHERTHAN *et al.* (1994).

RESULTS

Unusual ascospore formation and reduced spore viability in *rec8–110*: Because recombination is essential for proper chromosome segregation during the first meiotic division, mutants defective in meiotic recombination are expected to yield irregular segregation patterns and reduced spore viability. PONTICELLI and SMITH (1989) have shown that the *rec8–110* point mutation impairs meiotic recombination and has a spore viability of 12–19%. To gain more information, we have examined in detail spore formation and viability, as well as chromosome segregation.

The morphology of *rec8–110* asci and spores is frequently aberrant (Figure 1), but four spores were formed in the great majority of asci. Sizes of spores vary considerably, their arrangement in the asci is often irregular, and the spore walls mature to different stages. DNA staining with DAPI revealed unequal distribution of the genetic material. Some spores showed no DAPI-stained material.

To test the effect of the mutation on spore viability, the products of zygotic meiosis (cross of haploids) and azygotic meiosis (sporulation of a diploid formed from the same haploids) were analyzed. The *rec8–110* strains 3 and 4 were compared with the *rec*$^+$ strains 8 and 9 by plating of random spores. The *rec8* mutants produced 19.2% viable spores in zygotic and 19.6% in azygotic meiosis, while the control strains displayed 75.8% and 77.7% viability in zygotic and azygotic meiosis, respectively. Spore viability in these controls is lower than in tetrad analysis (Figure 2). Most likely this is caused by the glusulase treatment used to digest vegetative cells to obtain pure spore suspensions from mixed suspensions. The same crosses and diploid sporulations were also subjected to tetrad analysis (Figure 2). The spore viability patterns were strikingly different between *rec*$^+$ and *rec8–110*. The abundance of asci with less than four viable spores was much higher in *rec8–110*. The quantitative difference in the patterns for zygotic and azygotic meiosis in Figure 2 may be due to a technical problem (biased visual selection of asci during micromanipulation). The frequent occurrence in *rec8–110* of asci with one viable spore suggests that nondisjunction at meiosis I is not the only segregation problem, because this would result in asci with no or two viable spores in *S. pombe* (see below).

Disomics for chromosome *III* are frequently formed in *rec8–110* meiosis: Most aneuploid cells of *S. pombe* are not able to form colonies. Only disomics for chromosome *III* form slowly growing colonies and they frequently segregate haploid sectors (NIWA and YANAGIDA 1985). A first approach to analyze meiotic missegregation consisted of plating random spores on full medium. After growth, the resulting colonies were inspected for their morphology. Regular morphology of colonies correlated well with euploid genotypes of spores. Cells disomic for chromosome *III* were rather unstable. Breakdown of the disomic state during mitotic divisions frequently resulted in small, sectored colonies. Therefore, aneuploid colonies could be detected with high fidelity by simple visual inspection. The cross homozygous for *rec8–110* produced a great variety of colony morphologies with regard to size and shape, whereas the control cross yielded uniformly sized and regularly shaped colonies.

After inspection of colony morphology, diploids were identified by their ability to sporulate (h^+/h^- diploids), or by the large cell size (h^+/h^- and h^-/h^- diploids). Their genotype at all segregating genetic loci was determined as described in MATERIALS AND METHODS. The crosses were heterozygous for two complementing auxotrophic mutations in the *ade6* gene on chromosome *III* (*ade6-M210:* dark red; *ade6-M216*: light-red colony pigment; heteroallelic diploid: white colonies and prototrophic). Nondiploid colonies containing cells

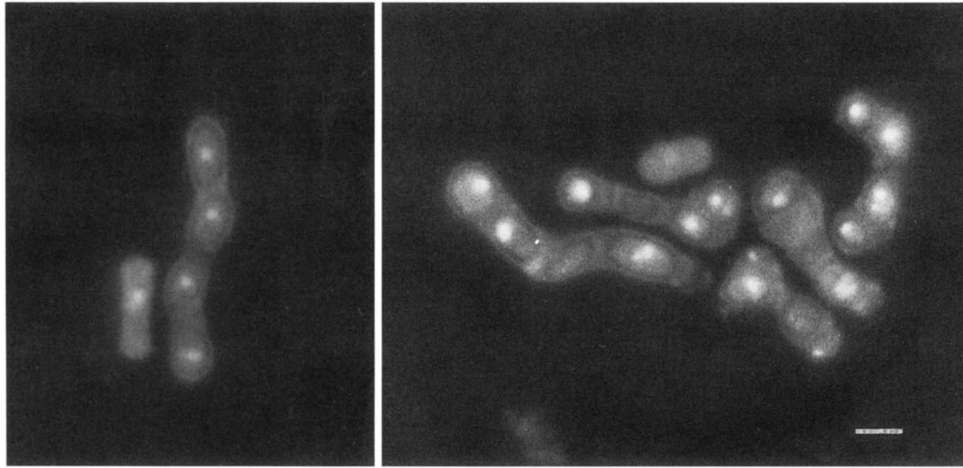


Figure 1.—DAPI staining of zygotic asci of *rec*$^+$ (left) and *rec8–110* (right) strains. Note the irregular spore shapes and locations in the mutant. While DNA staining is regular in the *rec*$^+$ strain, mutant spores show widely differing amounts and unequal placing of DNA. Empty spores are generally surrounded by thinner walls. Bar, 5 μm.

with the two different alleles of *ade6* were identified (Materials and Methods). These colonies obviously originated from aneuploid spores carrying both versions of chromosome *III.*

The classification of colonies resulting from the crosses of strains 5 and 6 (*rec8–110)*, and strains 10 and 11 (*rec*$^+$) are shown in Table 2. Four classes of colonies deriving from segregation errors were detected in *rec8–110* while the analysis of a similar number of *rec8*$^+$ colonies revealed only one type of missegregation. Most pronounced was the 14-fold higher frequency of chromosome *III* disomy in *rec8–110* (19%) than in *rec8*$^+$ (1.3%). The other remarkable finding was the frequent occurrence of diploid spores in *rec8–110* (8%). The majority of them (6%) showed heterozygosity for the centromere-linked markers. Because the chromosome configuration can only be deduced for spores that grow to colonies (because they are equipped with a chromosome set allowing survival), this analysis underestimates the overall frequency of missegregation.

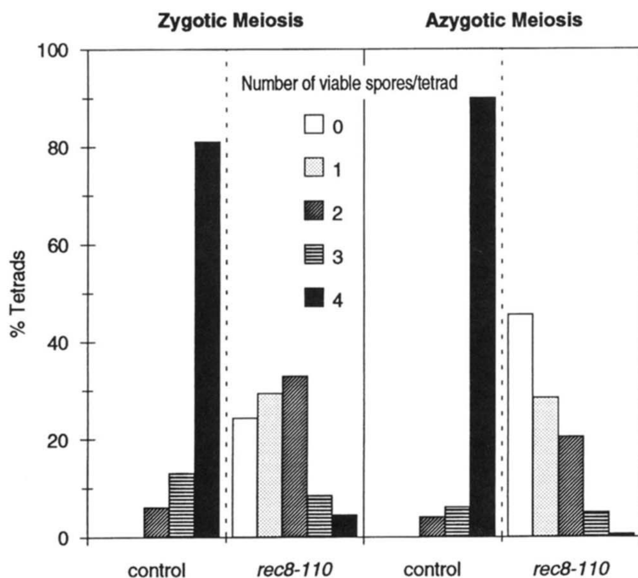


Figure 2.—Spore viability in tetrads homozygous for *rec*$^+$ or *rec8–110.* The tetrads were obtained from crosses of haploids (zygotic) or from sporulation of diploids (azygotic). Two hundred tetrads from *rec8–110* and 100 from *rec*$^+$ were dissected and classified by the number of viable spores.

Precocious separation of sister chromatids in *rec8–110*: The nature of the segregation defect in *rec8–110* can be examined directly by following the segregation of chromosome *III* markers in tetrads. Figure 3 illustrates the different segregation types. While nondisjunction at meiosis I and precocious separation of sister chromatids can be identified with this assay, nondisjunction at meiosis II and simple chromosome loss (lack of attachment of chromosomes to spindles) cannot be identified. In addition, precocious separation of sister chromatids in meiosis I may occur in both homologs and lead to segregation defects in meiosis II. For this study, 156 tetrads were dissected from a cross of *rec8–110* strains 5 and 6 and analyzed for segregation of chromosome *III* and ploidy of spores. Of these tetrads, 86 were also analyzed for segregation of the other markers: mating type, *his5*, and the centromere-linked *lys1.* Fifty asci from this cross did not contain colony-forming spores, and 38 carried only one (not studied further, because they are not informative concerning the nature of the missegregation event). The deduced spore genotypes of 68 tetrads containing at least two viable spores are given in Table 3.

Comparison of these results with those from random spore analysis (Table 2) shows a lower level of aneuploid spores. A bias toward selection of asci with well-developed spore morphology during micromanipulation may contribute to this difference as already mentioned above in connection with the data shown in Figure 2. Moreover, the exclusion of tetrads with only one viable spore may contribute to the lower number of observed segregation defects.

TABLE 2

Segregation analysis with random spores derived from crosses homozygous for *rec8-110* or *rec*⁺

Segregation of markers				Cell size	Deduced genotype and origin of spore	No. of colonies	
lys1[a]	*his5*[b]	*mat1*[b]	*ade6*[a]			*rec8-110*	*rec8*⁺
+ or −	+ or −	h^+ or h^-	*M210* or *M216*	Haploid	Haploid	306	367
+ or −	+ or −	h^+ or h^-	*M210/M216*	Haploid	Disomic chromosome *III*	78	5
+/−	+/−	h^+/h^-	*M210/M216*	Diploid	Diploid, heterozygous for centromere markers	25	0
+/+ or −/−	+/+ or −/−	h^+/h^+ *or* h^-/h^-	*M210/M210* or *M216/M216*	Diploid	Diploid, homozygous for centromere markers	2	0
+/+ or −/−	+/+ or −/−	h^+/h^+ *or* h^-/h^-	*M210/M216*	Diploid	Diploid, mixed segregation pattern for centromere markers	6	0

[a] Location of centromere markers: *lys1* maps 4 cM from the centromere on chromosome *I*, and *ade6* 13 cM from the centromere on chromosome *III* in *rec8*⁺ strains.
[b] *mat1* and *his5* are located far from the centromere on chromosome *II*. Recombinant colonies with respect to these markers are included without distinction.

Three tetrads (class 4 in Table 3) showed evidence for precocious separation of sister chromatids. One of the three cases was shown to correspond exactly to the scheme shown in Figure 3C by determination of *lys1* segregation. The segregation of *lys1* on chromosome *I* was not determined in the two other tetrads. No tetrads diagnostic for simple nondisjunction at meiosis I were detected (two viable spores disomic for chromosome *III*, see also Figure 3B). The rare tetrad classes 5 and 10 correspond well with the diploid spores observed in random spore analysis (Table 2) and the irregular distribution of nuclear material discussed above and shown in Figure 1. Tetrad classes 5 and 10 may have originated from precocious separation of the sister chromatids of all three chromosomes. The single tetrad in class 2 may have arisen from a rare triploid meiosis, but spontaneous endodiploidization in *rec8–110* carrying strains is not higher than in the wild type (data not shown).

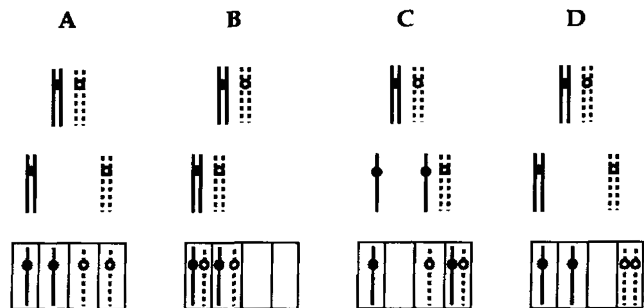


FIGURE 3.—Types of chromosome segregation. The emphasis is on chromosome *III* that carries different alleles of *ade6* in the crosses: *M210* shows a dark-red and *M216* a light-red color of colonies. The top row shows the pairing of homologs in meiotic prophase, the middle row the content of the daughter nuclei after meiosis I, and the bottom row the genotypes of the four nuclei resulting from meiosis II. (A) Regular meiotic divisions. (B) Meiosis I nondisjunction of homologs results in two viable, disomic sister spores. (C) Precocious separation of sister chromatids of one homologous chromosome in prophase I leads to a tetrad with three viable spores. The single disomic spore carries different copies of the marked chromosome *III* and is the sister of one of the haploid spores. Not shown is the case when both homologs undergo precocious separation of sister chromatids. Then tetrads may result with the disomic spore not being the sister of a haploid spore. The two different types of tetrads can be distinguished by following the segregation of centromere markers (see text). (D) Meiosis II nondisjunction. The disomic spore of the tetrad with three viable spores carries identical copies of chromosome *III*. This class can not be detected with our assay.

Formation of linear elements is defective in *rec8–110* meiosis: Meiotic chromosome pairing and recombination are not accompanied by formation of tripartite SCs in fission yeast (see Introduction). Instead, linear elements appear whose behavior defines different stages of meiotic prophase (BAEHLER *et al.* 1993). It was proposed that the linear elements play essential roles for meiotic chromosome structure and function (BAEHLER *et al.* 1993; KOHLI 1994; KOHLI and BAEHLER 1994). Moreover, if the linear elements are related to axial cores of other eukaryotes, they are expected to connect sister chromatids before the first meiotic division (BAEHLER *et al.* 1993). It is therefore of interest whether the *rec8–110* mutant is impaired in linear element morphology.

Spreading and silver staining of azygotic meiotic nuclei of *rec8–110* cells revealed clear differences in linear element morphology when compared with wild-type nuclei (Figure 4). The time course of meiotic events was studied as well (Figure 5). Early in meiotic prophase, short pieces of elements appeared that resemble those seen in wild-type stage I. However, this stage was less pronounced than in wild type, as both its duration and the length of the elements were shorter (class A nuclei, see Figures 4C and 5). A small number of short and thick "elements" were observed later, and they represent the major and most striking phenotype (class B, see Figures 4, D and E, and 5, B and C). Their morphology

TABLE 3

Segregation of chromosome *III* studied by tetrad analysis of a cross homozygous for *rec8-110*

Tetrad class	Genotypes of colony-forming spores[a]				No. of tetrads
1	*M210*	*M210*	*M216*	*M216*	4
2	*M216*	*M216*	*M210/M216*	Diploid	1
3	*M210* or *M216*	*M210*	*M216*	0	10
4[b]	*M210/M216*	*M210*	*M216*	0	3
5	*M210*	*M216*	Diploid	0	1
6	*M210*	*M210*	0	0	4
7	*M216*	*M216*	0	0	2
8	*M210*	*M216*	0	0	39
9	*M210* or *M216*	*M210/M216*	0	0	2
10	*M210* or *M216*	Diploid	0	0	2

0, spores forming no colony.

[a] The genotypes were deduced from the genetic configuration of chromosome *III* and the size of cells in the colony. *M210/M216* indicates heterozygous disomy for chromosome *III*. Homozygous disomy for chromosome *III* cannot be detected reliably. Thus all others are classified as haploids with exception of the rare diploids that were found to be heterozygous for the markers on all chromosomes (second division segregation pattern).

[b] It was shown for one of these tetrads that the segregation pattern corresponds exactly to Figure 3C. For further explanations, see text.

suggests that these structures are an unspecific aggregation of linear element material rather than linear elements regularly organized into chromosomes. Finally, the aberrant structures decreased in number and became longer (class C, see Figure 4, F and G). Shortly thereafter they were degraded, and the first meiotic division followed immediately (Figure 5B). Four independent time-course experiments with qualitatively similar results were performed with the *rec8–110* diploid strains. Moreover, the same classes of spread nuclei were observed during prophase of zygotic *rec8–110* meiosis (crosses of strains 3 and 4 in Table 1, results not shown).

Meiotic prophase is shortened in *rec8–110* meiosis: A significantly shorter prophase is another characteristic feature of *rec8–110* meiosis. This was shown by DAPI staining of meiotic nuclei. In the mutant the changes of nuclear shape typical for horse-tail nuclei (elongation and other deformations) (see Robinow 1977; Baehler *et al.* 1993; Chikashige *et al.* 1994) during meiotic prophase were most abundant at 3h (Figure 5B), whereas in wild type the peak was observed 2 hr later (Figure 5A). Moreover, the first meiotic division occurred significantly earlier, suggesting a shortened meiotic prophase (compare Figure 5, A and B). This was confirmed by comparison of the commitment to meiosis, which occurs at premeiotic S-phase in wild type (Baehler *et al.* 1993). No difference in timing of the commitment to meiosis could be detected between mutant and wild type (data not shown). Thus, the *rec8* mutation seems to influence specifically the duration of meiotic prophase I. The *rec8* mutant sporulated as efficiently as wild-type cells in these experiments (80–95%).

Meiotic chromosome pairing is impaired in *rec8–110* meiosis: We were interested in whether the reduced recombination frequencies and aberrant linear element formation observed in *rec8–110* meiosis are accompanied by a defect in meiotic chromosome pairing. Clustering of telomeres and centromeres may facilitate meiotic chromosome pairing. It has recently been shown that all centromeres are clustered in vegetative interphase and during meiotic prophase. All telomeres specifically cluster near the spindle pole body upon sexual differentiation and maintain this configuration throughout meiotic prophase (Chikashige *et al.* 1994; Scherthan *et al.* 1994). We therefore visualized centromeres and telomeres in wild type and *rec8–110* strains by fluorescence *in situ* hybridization (FISH, see Materials and Methods) (Scherthan *et al.* 1994). The centromere probe contains a 6.4 kb repeated sequence present at all three centromeres of fission yeast. The telomere probe contains 8 kb of subtelomeric repeated sequences. Compared with the control, centromere and telomere clustering were not significantly reduced in *rec8–110* mutants (Figure 6A). Very similar results were obtained in a second independent experiment (data not shown).

It has been shown by painting of whole chromosomes with composite probes that homologous chromosomes occupy joint nuclear territories which are distinct from the territories of other, nonhomologous chromosomes (Scherthan *et al.* 1994). Painting of chromosomes *I* and *II* in *rec8–110* nuclei did not reveal any difference in overall territorial arrangement of chromosomes compared with wild type, both in vegetative and meiotic nuclei (data not shown).

Homologous chromosome pairing in azygotic meiosis was then studied in detail at six defined chromosomal regions, using cosmids with unique sequences as probes for FISH (Figure 6E) (Hoheisel *et al.* 1993).

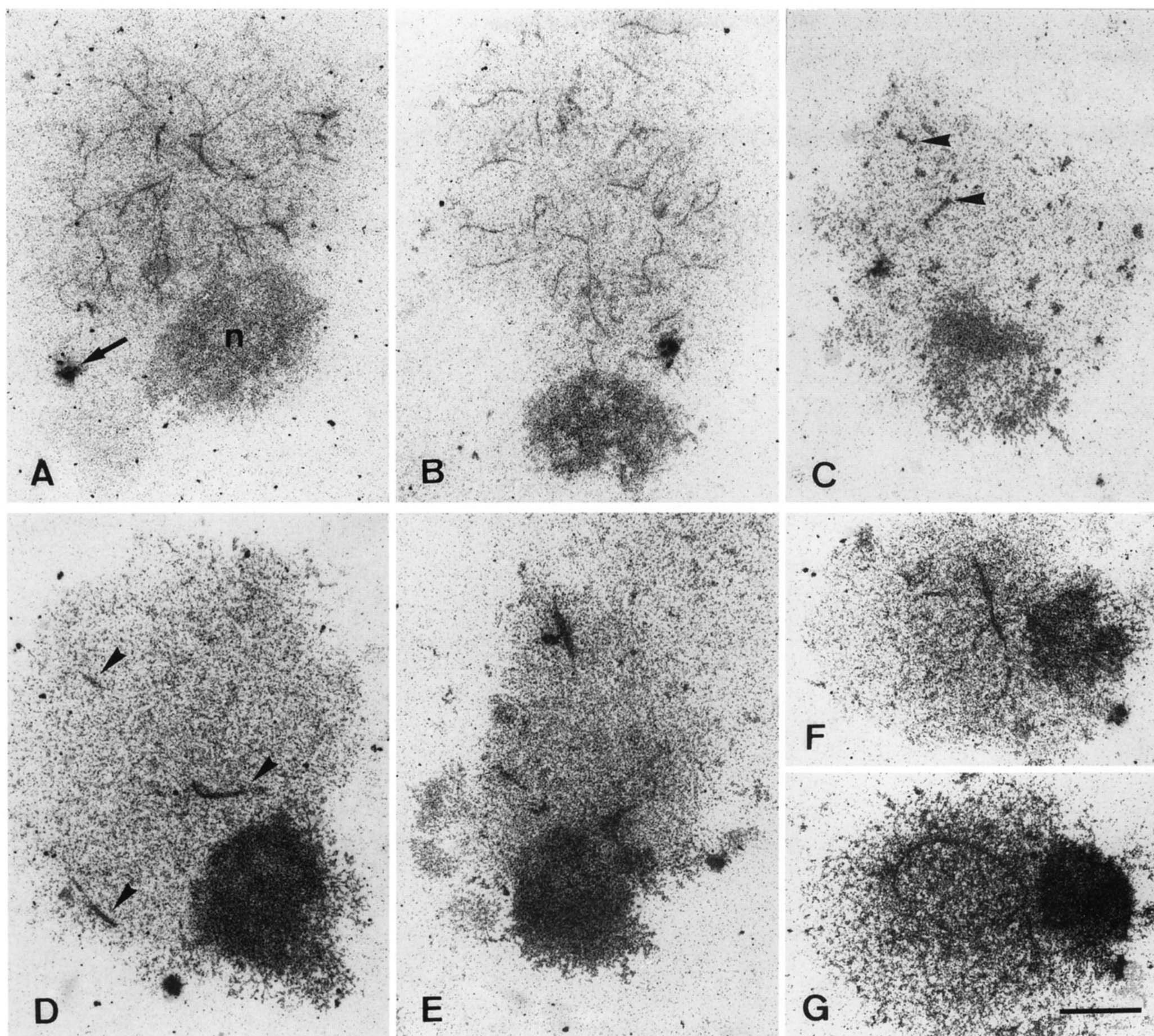


FIGURE 4.—Meiotic nuclei from wild-type and *rec8–110* carrying strains. Nuclei were spread and stained with silver for electron microscopy according to BAEHLER *et al.* (1993). (A) A network of linear elements typical for a wild-type class II nucleus. The arrow points to the spindle pole body. The nucleolus is marked with n. (B) Isolated long elements are shown. They are typical for wild-type class III nuclei. (C) Short, single elements (arrowheads) represent the first meiotic prophase stage in the *rec8–110* mutant (class A nucleus). (D and E) The most frequently seen type of aberrant elements observed in the mutant. Usually two or three short and abnormally thick elements were found (class B nuclei). (F and G) Single, longer aberrant elements appeared in a late stage (class C nuclei). Bar, 1 μm.

The cosmids from chromosome *II* map close to both ends (regions 1 and 5), to interstitial regions (2 and 4), and to a centromere-adjacent region (3). The same probes have been used before to compare meiotic pairing at different chromosomal sites (SCHERTHAN *et al.* 1994). Because meiotic recombination in *rec8–110* is most strongly reduced in the *ade6* region of chromosome *III* (DE VEAUX and SMITH 1994), we also studied the pairing behavior in that region (6).

Chromosome pairing at the six chromosomal regions was compared between wild type and *rec8–110* (Figure 6, B–D). As shown before (SCHERTHAN *et al.* 1994), homologous pairing in the control strain showed a basic level in vegetative nuclei (0h), increased after induction of meiosis, and was maximal in late meiotic prophase. An exception was the centromeric region 3, which was frequently paired also in vegetative cells. Cells in late meiotic prophase were abundant at 6h in the control strain (Figure 5A) and already at 4h in the mutant (Figure 5B). Thus, the same time points cannot be compared directly between wild type and mutant. In *rec8–110*, the regions near the ends of chromosome *II* showed a lower increase of pairing during meiotic prophase (Figure 6B). Compared with the control strain,

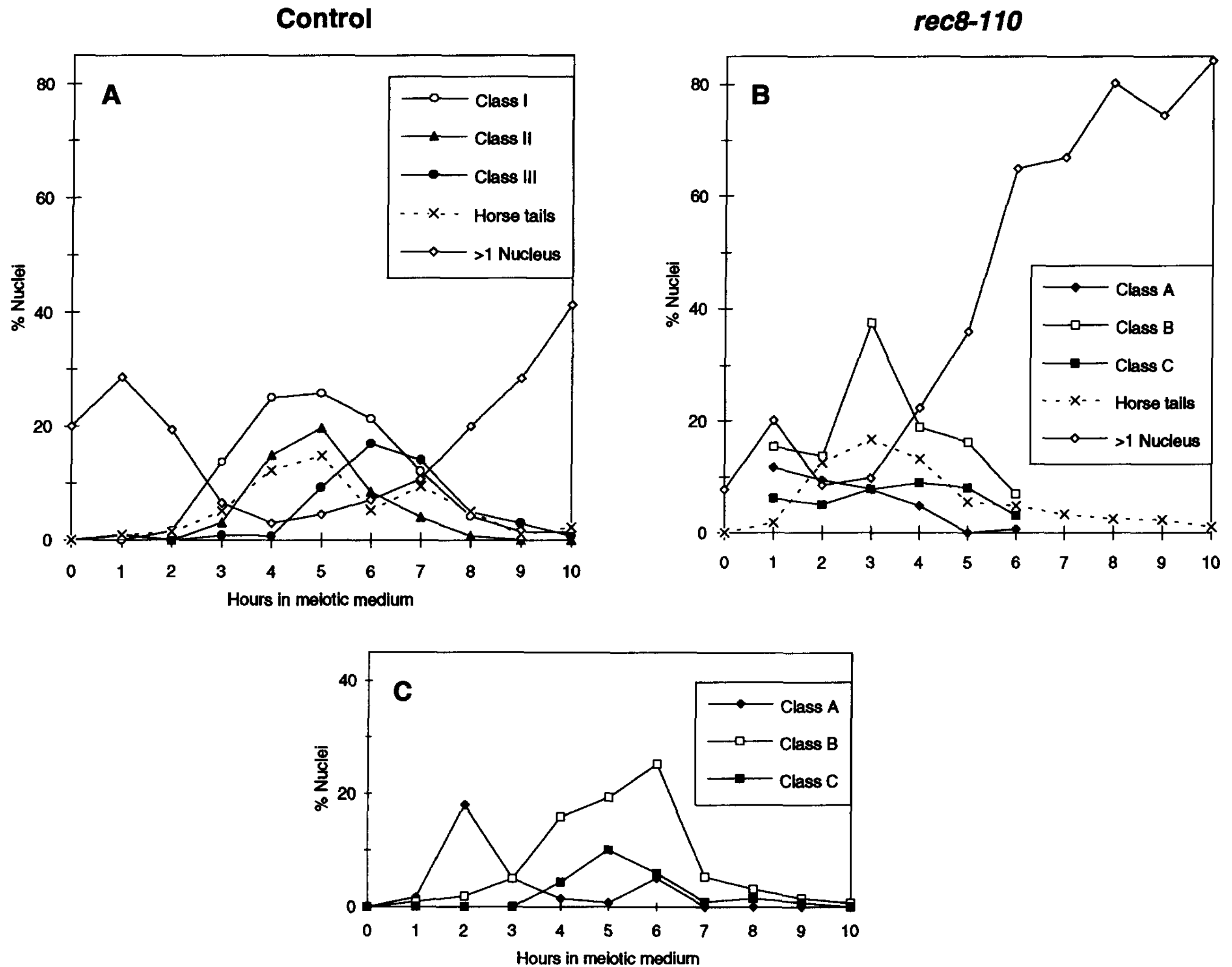


FIGURE 5.—Meiotic time-course experiments. Cells were analyzed by nuclear spreading and by DAPI staining at different times after induction of meiosis (0h) in a diploid wild-type strain (A) and a diploid strain homozygous for *rec8–110* (B and C). The spread nuclei were classified according to BAEHLER *et al.* (1993) and Figure 4. More than 120 cells were examined at each time point. (A) In wild-type cells, nuclei with isolated and short linear elements (class I) are prominent during the first (formation) and the last (degradation) prophase stages which display linear elements (BAEHLER *et al.* 1993). The second stage shows networks of linear elements (class II nuclei, see Figure 4A). Characteristic for the third stage are class III nuclei with isolated linear elements of maximal length (see Figure 4B). Horse-tail nuclei (ROBINOW 1977) are of elongated or irregular shape when observed by staining of DNA in whole cells and are indicative of nuclear movement (CHIKASHIGE *et al.* 1994). Cells with more than one nucleus after DAPI staining have either passed meiosis I or have not entered prophase yet. Many cells have two nuclei at 1h after meiotic induction, because they were in G2 phase at 0h and perform another mitotic division before entering premeiotic S phase from G1. At late times the meiotic divisions lead to accumulation of cells with two and finally four nuclei. The final sporulation frequency was 67% in this experiment. (B) Three classes of spread nuclei were distinguished in the *rec8* mutant (see Figure 4). The accumulation of horse-tail nuclei and the meiotic divisions start earlier than in wild type. The final sporulation frequency was 95% in this experiment. (C) An independent experiment with mutant cells giving another example for the timing and abundance of the three classes of mutant linear elements in spread nuclei. The final sporulation frequency was 80% in this experiment.

region 1 was ~1.2-fold reduced in meiotic pairing at late prophase in the mutant. Region 5 is more distant from the telomere than region 1 (Figure 6E) and showed a >1.5-fold reduction of late prophase pairing in the mutant. The pairing of interstitial chromosomal regions seemed to be affected more strongly by the *rec8–110* mutation (Figure 6C). The mutant showed almost no increase of pairing of regions 2 and 4 after induction of meiosis. The *rec8* mutant is ~1.6- and 2.3-fold reduced in meiotic prophase pairing of regions 2 and 4, respectively. The centromeric regions 3 and 6 showed a significant decrease of pairing in the mutant right after induction of meiosis (Figure 6D). The reduction in meiotic prophase pairing was ~1.5- and 2.2-fold for regions 3 and 6, respectively. These reductions in pairing may be underestimates, because both regions map near centromeres (Figure 6E) that did cluster in the mutant (Figure 6A). This may lead to coalescence of centromere-adjacent regions in spread nuclei independent of homologous pairing. The presented results

were confirmed in an independent time-course experiment (data not shown).

Sister chromatid separation during prophase I of *rec8–110* meiosis: The pairing analysis resulted in another interesting observation. In ~60% of the nuclei from wild-type cells at all time points, the sister chromatids could not be distinguished as distinct signals. There were either two signals (if regions were separated; Figure 7B) or only one signal (if regions were paired; Figure 7C). In the residual nuclei, sister chromatids were visible as distinct signals, but mostly appeared as closely associated double signals both in vegetative and meiotic cells (upper signals in Figure 7A). This indicates that sister chromatids are normally closely associated (see also SCHERTHAN *et al.* 1994). The strain homozygous for *rec8–110* showed wild-type arrangement of sister-chromatid signals in vegetative nuclei (0h). After meiotic induction (2h–6h), however, ~70% of the nuclei revealed four distinct signals. These signals were widely separated in >20% of these nuclei (Figure 7, D–F). In wild-type nuclei, <5% of sister signals were widely separated. These observations are in accordance with the genetic results described above that indicated precocious separation of sister chromatids in meiosis I of *rec8–110.* They further show that sister chromatids often fail to be associated during meiotic prophase, except at centromeres and telomeres whose clustering is not significantly affected by *rec8–110.*

DISCUSSION

The fission yeast *S. pombe* shows two peculiarities of meiosis in comparison with most other eukaryotes. There is no formation of a tripartite synaptonemal complex, but linear elements resembling axial cores of other organisms are formed (reviewed by KOHLI and BAEHLER 1994). In addition, crossover interference is not observed (MUNZ 1994). Obviously tripartite SC formation is not required for recombination, because *S. pombe* has on average 45 crossovers per meiosis (MUNZ *et al.* 1989). EGEL (1978) has first proposed that the synaptonemal complex may be responsible for generation of crossover interference. This causal relation has been supported by recent experiments with the *zip1* mutant of *S. cerevisiae* that abolishes SC formation, but not crossovers: interference is also abolished in *zip1* (SYM and ROEDER 1994). Although tripartite SC and interference may be required in meiosis of eukaryotes with many chromosomes and large genomes, it is conceivable that they were not part of the minimal structures and processes that promoted meiotic pairing, recombination and segregation of chromosomes in early eukaryotes. If this is correct, the investigation of *S. pombe* will contribute to the understanding of the basic structures and processes of meiosis.

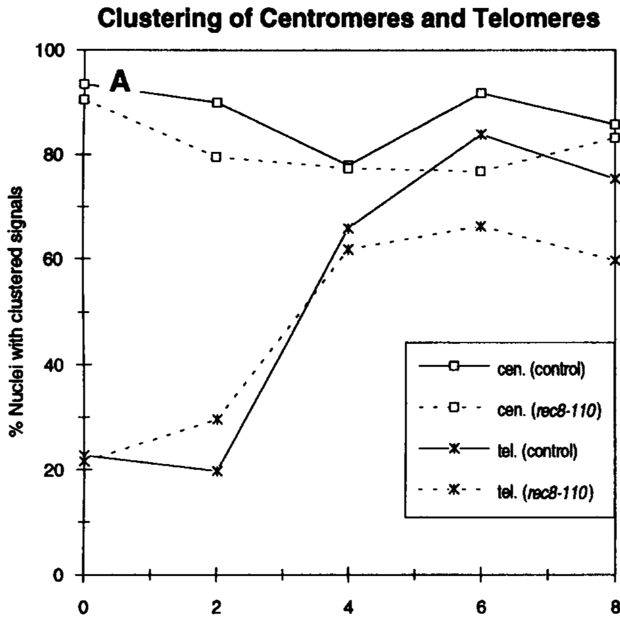
In this context, we have carried out a genetic and cytological analysis of chromosome structure, pairing and segregation in strains with *rec8–110.* This mutation has been shown to reduce meiotic intragenic recombination essentially to the background mitotic level at the *ade6* locus near the centromere of chromosome *III.* Intergenic recombination is also affected (PONTICELLI and SMITH 1989; DE VEAUX *et al.* 1994). Interestingly this reduction of recombination frequency is strong for a 2-Mb region around the *ade6* gene, but much less pronounced at one end of chromosome *III* and in several regions on the other two chromosomes (DE VEAUX and SMITH 1994).

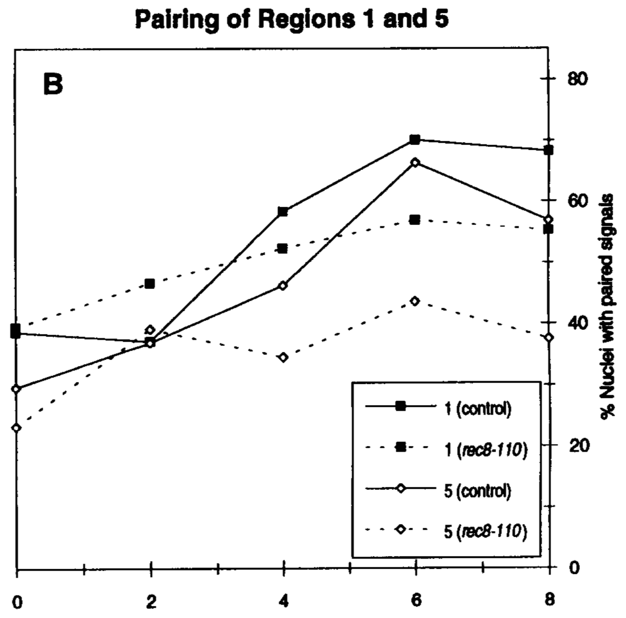
Absence of crossovers is expected to lead to nondisjunction, one form of irregular chromosome segregation during the meiotic divisions (for review, see HAWLEY 1988). In addition, it has been shown that lack of sister chromatid cohesion also contributes to irregular chromosome segregation (for review, see MIYAZAKI and ORR-WEAVER 1994). As a result many of the meiotic products are inviable. Low spore viablility of *rec8–110* was demonstrated by PONTICELLI and SMITH (1989) and our own results (Figure 2).

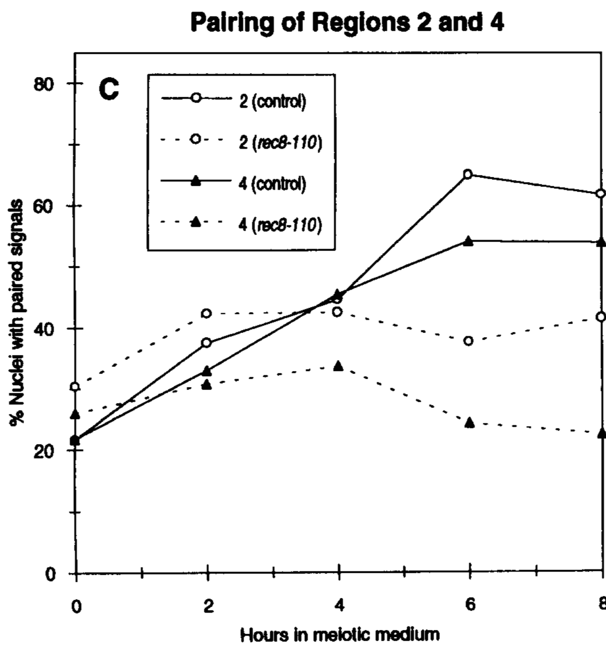
Genetic analysis of chromosome segregation in *rec8–110:* A system for genetic analysis was developed to get a better understanding of the nature of chromosome segregation in *rec8–110.* Because *S. pombe* has only three chromosomes, a considerable amount of viable spores is expected, even when chromosomes segregate at random. This characteristic of *S. pombe* explains the (compared to *S. cerevisiae*) high frequency of viable spores in mutants strongly deficient in meiotic recombination (PONTICELLI and SMITH 1989). A problem for the analysis of chromosome segregation in fission yeast is the inviability of most aneuploid spores. They may germinate and undergo a few divisions, but fail to form colonies. Only disomy for chromosome *III* is tolerated (NIWA and YANAGIDA 1985), and thus forms the basis of our system for segregation analysis (Figure 3). Tetrad dissection of crosses heterozygous for markers on chromosome *III* allow the detection of two types of missegregation: nondisjunction at meiosis I and precocious separation of sister chromatids. Nondisjunction at meiosis II and chromosome loss (failure of attachment to spindle) are not detectable by this approach.

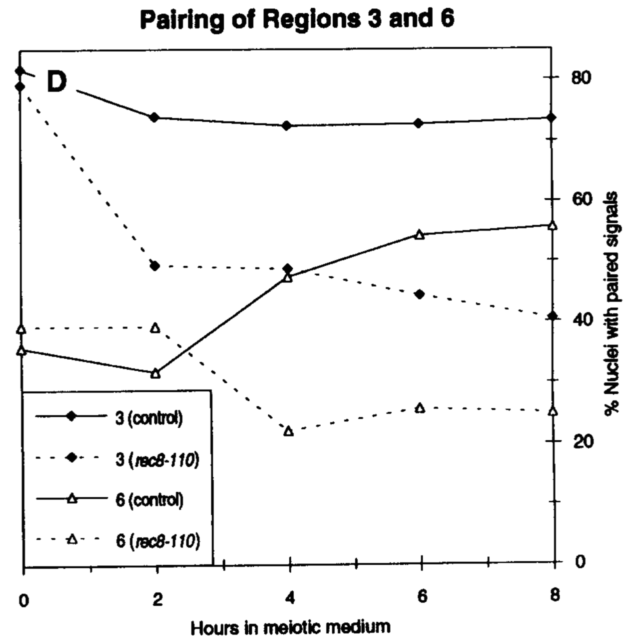
Compared with wild type, *rec8–110* shows a 14-fold increase of spores that are disomic and heterozygous for chromosome *III* (Table 2). Tetrad analysis has identified precocious sister chromatid segregation as one cause for the occurrence of these disomics (Table 3). The frequent occurrence of diploid spores (8%, Table 2) in random spore analysis, and especially the abundance of diploids heterozygous for both centromere markers (6%), may be explained by precocious separation of sister chromatids followed by random segregation. Alternatively, they may be explained by nondisjunction of all three bivalents at meiosis I, but tetrads indicating formation of chromosome *III* disomics by nondisjuntion I of bivalent *III* alone have not been

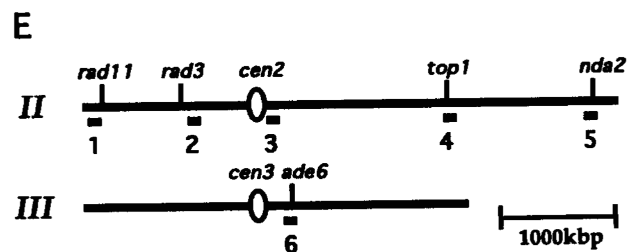
found. A quantitative estimate of the different types of segregation errors is not possible, since most products of *rec8–110* segregation are inviable and cannot be studied. It should be born in mind that *rec8–110* is a point mutation and may retain some residual activity. Nondisjuntion at meiosis II cannot be assessed directly with our genetic detection system. Chromosome loss might also occur and reduce spore viability. However, examination of 20 living meiotic *rec8–110* cells by time-lapse microscopy revealed that all genetic material segre-











gated to daughter nuclei, suggesting that chromosome loss is not a frequent event in this mutant (J. BÄHLER and Y. HIRAOKA, unpublished observations).

Our favored interpretation of the genetic data on *rec8–110* is that segregation problems arise mainly at meiosis I, as a consequence of precocious separation of sister chromatids. Additional segregation errors at meiosis II cannot be excluded. The study of nuclear divisions in living *rec8–110* cells showed that the genetic material is often distributed to daughter nuclei in unequal amounts, both in meiosis I and II (J. BÄHLER and Y. HIRAOKA, unpublished results). The observed asymmetrical second divisions can be explained by precocious separation of sister chromatids. Even if sister chromatids were evenly segregated by chance in the first division, a random segregation of the unattached sisters can still occur in the second division (see also ROCKMILL and ROEDER 1994).

Chromosome structure and pairing in *rec8–110*: If it is assumed that the linear elements are required for meiotic recombination, mutants defective in recombination may be impaired in linear element formation. In fact, this was found in *rec8–110*. Time-course experiments revealed that linear element formation starts similarly to that in wild type. The following stages of meiotic prophase are characterized by striking aberrations of element morphology in the mutant. Spread meiotic *rec8–110* nuclei show abnormally thick linear structures of variable length (Figure 4, D–G). Such linear element morphology has not been observed in wild-type nuclei. This apparently unspecific aggregation of linear element material may be compared with polycomplex formation observed in wild-type and mutant meiosis of other organisms (for review, see VON WETTSTEIN *et al.* 1984; LOIDL *et al.* 1994).

Appropriate organization of meiotic chromosomes along linear elements may facilitate the pairing process and consequently the initiation of recombination. In addition, pairing of interstitial chromosome regions may be initiated from the clustered telomeres and centromeres (SCHERTHAN *et al.* 1994). Therefore it is of interest whether clustering and pairing are affected by the *rec8* mutation. This can be studied in spread nuclei by FISH (SCHERTHAN *et al.* 1994). The *rec8–110* mutation does not interfere with the low-level chromosome pairing in vegetative cells that are about to be shifted to meiosis-inducing conditions, nor does it strongly affect the clustering of centromeres and telomeres throughout prophase of azygotic meiosis (Figure 6). Meiotic chromosome pairing, however, is clearly impaired. The effect on meiotic pairing in the mutant depends on the chromosomal region investigated. The ends of chromosome *II* show reduced meiosis-specific pairing, interstitial regions on the two arms of chromosome *II* remain at about the level of pairing in vegetative nuclei, whereas regions near the centromeres of chromosomes *II* and *III* display a decrease of pairing after entry into meiosis. Thus, the more distant a region is located from the chromosome ends, the more it seems to be defective in meiotic chromosome pairing.

In this context, findings in living cells obtained by time-lapse microscopy are noteworthy. It has recently been shown that the elongated nuclei of prophase show dramatic movements that are led by the spindle pole body and the associated telomere cluster (CHIKASHIGE *et al.* 1994). In *rec8–110* the leading ends of nuclei move as in wild type, but the bulk of the nuclear mass does not follow and mostly remains in a central position within the cell (J. BÄHLER and Y. HIRAOKA, unpublished results). Thus, only the chromosome ends are pulled out and move alternatingly to both cell poles. This may facilitate the pairing of regions near telomeres. Linear elements may provide a scaffolding structure for the chromosomes that is necessary for movement of the whole nucleus and for efficient pairing of internal chromosomal regions.

The FISH analysis has provided support for another proposed function of the linear elements: sister chromatid cohesion during prophase I (BAEHLER *et al.* 1993). It has been proposed for other organisms that sister attachment prevents precocious separation of sister chromatids and thus stabilizes chiasmata. Mutations abolishing sister attachment are expected to yield frequent errors of segregation corresponding to the genetically observed precocious separation of sister chromatids (MIYAZAKI and ORR-WEAVER 1994). A recent

FIGURE 6.—*In situ* hybridization analysis of centromere and telomere clustering, and of homologous chromosome pairing. Meiotic time-course experiments were performed with a diploid wild-type strain (control; same experiment as in Figure 5A) and a diploid strain homozygous for *rec8–110* (same experiment as in Figure 5B). Before (0h) and every 2 hr after induction of meiosis (2h-8h), nuclear spreads were hybridized (FISH) as described in MATERIALS AND METHODS. At each time point ≥80 nuclei were examined. (A) Centromeres and telomeres were visualized with plasmid probes containing specific repeated sequences (see text). The percentages of nuclei with clustering of all centromere and telomere signals were determined as described before (SCHERTHAN *et al.* 1994). (B–D) Study of the extent of homologous pairing in the six chromosomal regions illustrated in E. A region was defined as being paired if the corresponding signals from the homologous chromosomes touched or overlapped each other (see Figure 7C) (SCHERTHAN *et al.* 1994). (B) Meiotic pairing behavior of regions 1 and 5 located near the ends of chromosome *II*. (C) Meiotic pairing behavior of regions 2 and 4 located interstitially in the short and long arms of chromosome *II*, respectively. (D) Meiotic pairing behavior of regions 3 and 6 located near the centromere of chromosome *II* and in the *ade6* region of chromosome *III*, respectively. (E) Schematic representation of chromosomes *II* (4.6 Mb) and *III* (3.5 Mb) showing the chromosomal regions studied in the pairing analysis. Six cosmids (numbered 1–6) with ~35 kb of unique sequences from the selected regions were used as probes. The positions of probes and markers are indicated approximately according to the map presented by HOHEISEL *et al.* (1993). The centromeres are marked with ellipses.

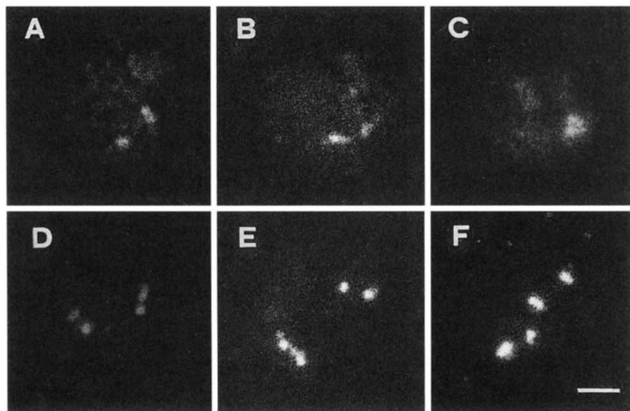


FIGURE 7.—Lack of sister chromatid cohesion in *rec8–110* prophase. The FISH analysis has been described in the legend to Figure 6. (A–C) show wild-type meiosis and (D–F) *rec8–110* meiosis, respectively. (A) Region 2 (see Figure 6E) was labeled with digoxigenin and detected with TRITC. Two separated signals corresponding to the homologous chromosomes are visible. The upper one is split into a double signal, reflecting the two associated sister chromatids. (B) Region 6 was labeled with biotin and detected by FITC. Two separated signals are visible. The background results from DAPI staining to visualize the chromatin area (double exposure). (C) The labeling was as in B, but now region 6 is paired, *i.e.*, the two signals are fused to one signal. (D) The labeling was as in A, but four distinct signals for region 2 were visualized, reflecting the four sister chromatids. (E and F) The experiments for region 6 were performed as in B, but now four distinct signals are visible which are widely separated in F. Bar, 2 μm.

example is the *med1–1* mutant in *S. cerevisiae* that has been renamed *dmc1–1* after discovery of its allelism with the *recA* homolog *DMC1* (BISHOP *et al.* 1992; ROCKMILL and ROEDER 1994; B. ROCKMILL, personal communication). In *rec8–110* nuclei, four distinct hybridization signals for the four homologous chromatids were frequently seen after induction of meiosis (see RESULTS), indicating that meiotic sister chromatid attachment is significantly weakened. This observation is in accordance with the results of the segregation analysis and shows that sister chromatids are frequently separated already during meiotic prophase. This early defect in sister attachment can be explained by the lack of linear elements. It is likely that linear elements correspond to axial elements of other eukaryotes (BAEHLER *et al.* 1993). Recent findings indicate that components of axial elements are maintained on the chromosomes and at the centromeres to stabilize chiasmata and to keep sister centromeres attached until anaphase II (DOBSON *et al.* 1994 and references cited therein). Similarly, linear element proteins may normally contribute to sister chromatid and centromere attachment not only during prophase I, but also after the cytologically visible elements have been degraded.

Conclusions and possible roles of the Rec8 protein: Linear elements have been suggested to function in establishing the proper chromosome architecture in meiotic prophase, especially also sister chromatid cohesion, as a prerequisite for chromosome pairing, recombination and proper chromosome segregation (BAEHLER *et al.* 1993; KOHLI and BAEHLER 1994). The reported results on *rec8–110* are consistent with the proposed model. In addition, the linear elements possibly contribute to the establishment of the prophase chromosome structure that is necessary for normal movement of nuclei during prophase. This movement has been proposed to facilitate efficient pairing of the homologs as well (CHIKASHIGE *et al.* 1994; KOHLI 1994). The above model also suggests that meiosis-specific homolog pairing begins at the clustered telomeres and proceeds toward the centromeres with help of the linear elements. This is consistent with the observation of strong reduction of chromosome pairing near the centromeres of chromosomes *II* and *III*, while pairing at the ends of chromosome *II* is only mildly affected in *rec8–110*. Thus, linear elements may facilitate chromosome pairing directly, and/or indirectly by contributing to nuclear movement. Because *rec8–110* does not affect clustering of telomeres and centromeres, it is likely that the mechanism for clustering is different from the mechanism responsible for pairing of unique chromosome sequences and attachment of sister chromatids.

Homologous pairing starting at telomeres and proceeding toward centromeres may contribute to the explanation of the region-specificity of recombination frequency reduction in *rec8–110* (DE VEAUX and SMITH 1994). On chromosome *III* this reduction is strong at *ade6* close to the centromere (300-fold). Toward the chromosome ends the effect becomes progressively weaker and is only 10-fold at *ura4*, which is located close to the left end of the unique sequences on chromosome *III*. Chromosome *III* is unique in comparison with the other chromosomes: it carries repeated sequences coding for ribosomal RNA at both ends (HOHEISEL *et al.* 1993). This may lead to a different behavior of chromosome *III* in comparison with chromosomes *I* and *II*, which are larger and have not been studied as thoroughly with respect to region-specific recombination reduction by *rec8–110*. So far no strong reduction was found on chromosomes *I* and *II* (DE VEAUX and SMITH 1994). Experiments with a *rec8* deletion are needed to settle several questions on region-specific reduction of recombination and other phenotypes of *rec8–110*.

How can the different phenotypes of *rec8–110* be explained by loss of function of its gene product? The *rec8* sequence has no homology to any other known gene or protein (LIN *et al.* 1992). It is particularly interesting that *rec8–110* has a shortened meiotic prophase. The Rec8 protein may be a regulator responsible for the development of linear elements. If so, the loss of normal element formation may cause the other phenotypes: region-specific defects in chromosome pairing and recombination, as well as precocious separation of sister chromatids and consequently errors in chromosome segregation in the meiotic divisions. Prophase

shortening would then be due to omission of the whole program dependent on proper function of linear elements. Alternatively, the regulator may affect different meiotic processes in parallel, independent of linear element formation. It is also possible that the Rec8 protein is a structural protein or enzyme participating in the establishment of proper linear element structure and, in consequence, function. In this case all the other phenotypes would be a direct or indirect consequence of lack of proper linear element formation.

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