The Mitotic DNA Damage Checkpoint Proteins Rad17 and Rad24 Are Required for Repair of Double-Strand Breaks During Meiosis in Yeast

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ABSTRACT

We show here that deletion of the DNA damage checkpoint genes *RAD17* and *RAD24* in *Saccharomyces cerevisiae* delays repair of meiotic double-strand breaks (DSBs) and results in an altered ratio of crossover-to-noncrossover products. These mutations also decrease the colocalization of immunostaining foci of the RecA homologs Rad51 and Dmc1 and cause a delay in the disappearance of Rad51 foci, but not of Dmc1. These observations imply that *RAD17* and *RAD24* promote efficient repair of meiotic DSBs by facilitating proper assembly of the meiotic recombination complex containing Rad51. Consistent with this proposal, extra copies of *RAD51* and *RAD54* substantially suppress not only the spore inviability of the *rad24* mutant, but also the γ-ray sensitivity of the mutant. Unexpectedly, the entry into meiosis I (metaphase I) is delayed in the checkpoint single mutants compared to wild type. The control of the cell cycle in response to meiotic DSBs is also discussed.

MEIOSIS generates gametes by halving the diploid genome. This process is accomplished by two successive rounds of chromosome segregation, which follow a single round of DNA replication. Reciprocal crossover recombination, together with sister chromatid cohesion, provides physical connections that facilitate proper segregation of homologous chromosomes at the first meiotic division.

In the budding yeast *Saccharomyces cerevisiae*, the DNA events of meiotic recombination have been defined in some detail. Recombination is initiated by double-strand breaks (DSBs), the ends of which are resected to produce 3′-single-strand tails (Roeder 1997; Keeney 2001). Intermediates are differentiated into two types: those that will ultimately form crossovers and those that will not, *i.e.*, noncrossovers. The crossover/noncrossover differentiation process is thought to occur at a very early stage, as one end of a processed DSB becomes engaged with homologous sequences on a nonsister chromosome in a nascent joint molecule intermediate. Extensive strand-exchange ensues to form a displacement-loop intermediate known as a single-end invasion (SEI; Hunter and Kleckner 2001). Then, new DNA synthesis and interaction of the second DSB end forms a joint molecule structure called a double-Holliday junction (dHJ; Schwacha and Kleckner 1994, 1995). Both SEIs and dHJs are thought to be specific to the crossover pathway (Allers and Lichten 2001; Hunter and Kleckner 2001). Noncrossovers presumably arise via intermediates that are less readily detectable, *e.g.*, are less stable and/or more transient (Allers and Lichten 2001; Hunter and Kleckner 2001).

In yeast, meiotic recombination involves many different proteins (Roeder 1997). Two RecA homologs, Rad51 and Dmc1, play a critical role in strand invasion and exchange of single-strand DNA (ssDNA) with the homologous double-strand DNA (dsDNA; Bishop *et al.* 1992; Shinohara *et al.* 1992). Rad51 is necessary for both mitotic and meiotic recombination, but Dmc1 is specific to meiosis. Rad51 and Dmc1 occur together on meiotic chromosomes, and the complex can be seen by immunostaining (Bishop 1994; Dresser *et al.* 1997; Shinohara *et al.* 2000). Rad51 and Dmc1 cooperate both in the formation of crossovers and in the control of recombination (Shinohara *et al.* 2003).

In mitosis, checkpoint proteins sense DNA damage and link repair with cell cycle progression (Weinert 1998; Zhou and Elledge 2000). These proteins can detect one or a few DSBs in the genome (Sandell and Zakian 1993; Lee *et al.* 1998). A number of the proteins have been identified in budding yeast, and they are highly conserved from yeast to human. For instance, in yeast, Rad24 forms a complex with RFC2/3/4/5 and recruits a PCNA-like complex, Rad17-Mec3-Ddc1, onto chromatin (Zhou and Elledge 2000). This recruitment activates a key protein kinase, Mec1/Esr1, which binds directly to the site of DNA damage (Kondo *et al.* 2001; Melo *et al.* 2001). Activated Mec1/Esr1 triggers a kinase cascade, which delays the cell cycle and induces a transcriptional response to DNA damage.

In meiosis, the checkpoint proteins are also required.

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TABLE 1

Strain and plasmid list

	Genotype	Reference
	Strains	
NKY1551	*MAT***a**/α *ho::LYS2/″ ura3/″ leu2::hisG/″ lys2/″ his4X-LEU2-BamHI-URA3/his4B-LEU2 arg4-nsp/arg4-bgl*	Storlazzi *et al.* (1996)
MSY717	A derivative of NKY1551 with *rad24::LEU2/″*	This study
MSY587	A derivative of NKY1551 with *rad17::hisG/″*	This study
MSY833	*MAT***a** *ho::LYS2 ura3 leu2::hisG trp1::hisG lys2*	This study
MSY966	*MAT***a** *ho::LYS2 ura3 leu2::hisG trp1::hisG lys2 rad24::LEU2*	This study
MSY968	*MAT*α *ho::LYS2 ura3 leu2::hisG trp1::hisG lys2 rad24::LEU2*	This study
KSY170	*MAT***a**/α *ho::LYS2/″ ura3/″ leu2::hisG/″ lys2/″ his4X-LEU2-BamHI-URA3/his4B-LEU2 arg4-nsp/arg4-bgl trp1::hisG/″ spo11-Y135F::kanMX4/″*	Cha *et al.* (2000)
	Plasmids	
pRS424	2μ *TRP1*	Christianson *et al.* (1992)
pWL5	pRS416 with *RAD24 URA3 ARS-CEN*	T. Weinert
pMS48	YEplac195 with *RAD51*	This study
pMS117	YEplac195 with *RAD54*	This study
pMS180	pRS424 with *RED1*	This study
pMS181	pRS424 with *MEK1/MRE4*	This study
pMS300	YEplac195 with *TID1/RDH54*	This study

Some checkpoint mutations suppress the meiotic prophase arrest induced by abnormal recombination and chromosome synapsis in mutants such as *dmc1*, *hop2*, and *zip1* (Lydall *et al.* 1996; Leu *et al.* 1998; San-Segundo and Roeder 1999; Hong and Roeder 2002). These studies have established the concept of the pachytene checkpoint, which is believed to coordinate recombination, and possibly chromosome synapsis, with progression of meiosis (Roeder and Bailis 2000).

At the pachytene checkpoint, Rad24-RFC and Rad17-Mec3-Ddc1 complexes recognize incomplete recombination and activate the Mec1/Esr1 kinase, as in mitosis (Bailis and Roeder 2000; Hong and Roeder 2002). This phosphorylates a meiosis-specific kinase, Mek1/Mre4. Activated Mek1/Mre4 in turn promotes the phosphorylation of a meiosis-specific chromosomal protein, Red1 (Bailis and Roeder 2000; Hong and Roeder 2002). Dephosphorylation of Red1 by Glc7 plays a critical role in the exit from pachytene (Bailis and Roeder 2000), although the role of Glc7 itself in meiosis is controversial (Tachikawa *et al.* 2001). In mitosis, Mec1/Esr1 also phosphorylates the Rad53 kinase, which is homologous to Mek1/Mre4, but Rad53 itself is not required for meiosis (Roeder and Bailis 2000). Interestingly, in mitosis, a few DSBs are sufficient to delay or arrest the cell cycle. In meiosis >200 DSBs are formed per nucleus, but the checkpoints have not yet been studied in detail.

In meiosis, the DNA damage checkpoint proteins also determine gamete viability. This is inferred because spore viability is reduced in some checkpoint single mutants of budding yeast (Lydall *et al.* 1996). In fruit flies, a mutant of the *mei-41* gene, the homolog of yeast Mec1/Esr1, also reduces crossover frequencies (Baker and Carpenter 1972; Carpenter 1979). Similarly, the *mec1/esr1* mutation decreases the frequencies of crossovers when assayed by forcing the meiotic mutant cells into mitotic growth (Kato and Ogawa 1994). Furthermore, some checkpoint single mutants show increased ectopic recombination, which is an exchange between nonallelic sites on nonsister chromosomes (Grushcow *et al.* 1999). The checkpoint mutations also increase meiotic recombination between sister chromosomes (Thompson and Stahl 1999), but the exact role of these proteins in meiosis remains unclear.

In this report, we show that the *rad17* and *rad24* checkpoint mutants are defective in the repair of meiotic DSBs. These mutants also affect formation of Rad51 and Dmc1 complexes on chromosomes. We also found a genetic interaction between *RAD51* and *RAD24* in both meiosis and mitosis. These findings suggest that Rad24 and Rad17 are involved in the strand invasion and exchange steps of meiotic recombination. In addition, we unexpectedly found that in the mutants meiosis proceeds more slowly than usual. The control of the cell cycle in response to DSBs is also discussed.

MATERIALS AND METHODS

Strains: All strains were derivatives of rapidly sporulating yeast SK-1 and are listed in Table 1. The *spo11-Y135F* mutant strains are generous gifts from Scott Keeney.

Plasmids: See Table 1. To construct a high-copy *RAD51* plasmid, pMS48, a 3.7-kb *Bam*HI fragment containing the *RAD51* gene was inserted into the *Bam*HI site of YEplac195 (Gietz and Sugino 1988). For pMS117, a *Pst*I-*Eco*RI fragment containing the *RAD54* gene was inserted into the *Pst*I and *Eco*RI sites of YEplac195. pMS181 was constructed by inserting

a *Bam*HI-*Pst*I fragment containing the *MRE4/MEK1* gene into the *Bam*HI and *Pst*I sites of pRS424 (Christianson *et al.* 1992). pMS155 was made by inserting a *Xho*I-*Xba*I fragment containing *DMC1* into the *Xho*I and *Xba*I sites of pRS314. pMS300 was constructed by cloning a PCR-amplified fragment containing *TID1/RDH54* (nos. 382564–386130 of chromosome *II* in the YPD database) into the *Bam*HI and *Xba*I site of YEplac195. pMS180 was made by inserting a PCR-amplified fragment (nos. 669869–672932 of chromosome *XII*) containing the *RED1* gene into the *Bam*HI and *Eco*RI sites of pRS424. The sequences of oligonucleotides for PCR amplification are available upon request.

Physical analysis of genomic DNAs: Meiotic time course experiments were carried out as described (Cao *et al.* 1990; M. Shinohara *et al.* 1997). Presporulation culture was carried out in special presporulation medium for 16 hr. The cells were collected, washed, and resuspended in sporulation medium (SPM) to initiate meiosis. Aliquots of cells were withdrawn and genomic DNAs were prepared as described (M. Shinohara *et al.* 1997). For DSB analysis, genomic DNAs were digested with *Pst*I and subjected to electrophoresis in a 0.8% agarose gel for 24 hr at 10 V/cm. For crossover analysis, DNAs were cut with *Xho*I and analyzed on a 0.6% agarose gel for 48 hr. For crossover/noncrossover and heteroduplex analysis, DNAs were digested with *Xho*I and *Mlu*I and with *Xho*I, *Bam*HI, and *Mlu*I, respectively, and analyzed on a 0.6% agarose gel for 48 hr. DNAs were transferred onto a nylon membrane (Hybond N; Amersham, Buckinghamshire, UK) by capillary transfer and analyzed by Southern hybridization. Blots were visualized and quantified using a phosphorimager, BAS2000 (Fuji). Probes were pNKY155 (Storlazzi *et al.* 1995) for crossover/noncrossover and heteroduplex assays and pNKY291 for DSB and crossover assays. The amounts of crossover and noncrossover heteroduplexes were <0.5% of total DNA, which made it difficult to accurately compare absolute amounts of the products. The ratio of crossover-to-noncrossover heteroduplexes is a more accurate measurement for the comparison, since the amount of total DNA could be ignored.

Cytology: To determine the frequency and kinetics of meiotic division, cells were fixed with 70% ethanol and frozen at −20°, stained with 4′,6-diamidino-2-phenylindole (DAPI), and then observed and counted under a fluorescent microscope. At least 200 nuclei were assessed. Spindles were examined by staining with rat antitubulin (YOL1/57; Sera Lab) as described previously (Kaiser *et al.* 1994).

Meiotic chromosome spreads were prepared as described (Bishop 1994; Shinohara *et al.* 2000). Meiotic cells were spheroplasted and surface spread on glass slides in the presence of detergent (lipsol) and fixative (4% paraformaldehyde). After drying, nuclei were immunostained as described previously (Shinohara *et al.* 2000). The slides were incubated simultaneously with guinea pig anti-Rad51 and rabbit anti-Dmc1 overnight at 4°, followed by incubation with secondary antibodies for 2 hr at 4°. Epifluorescence microscopy was carried out using Olympus BX51 or Zeiss Axiovert 135M. Images were captured with a cooled charge-couple device digital camera (Cool Snap; Photometrix) and processed using IP lab (Solution Systems) and Photoshop (Adobe) software. For triple staining, filter exchange was carried out using an automatic filter wheel (Roper Japan). The absence of offset between each filter was assessed using 500-nm microsphere beads with multiple colors (TetraSpeck microspheres; Molecular Probes, Eugene, OR). There was little offset of images by exchanging filters. Foci were scored, and colocalization frequency was determined as previously described (Padmore *et al.* 1991; Shinohara *et al.* 2000).

Determination of γ-ray sensitivity: Three individual transformants were analyzed for their ability to repair γ-ray damage. The strains were pregrown in synthetic medium lacking tryptophan or uracil (SC-Trp or SC-Ura) overnight. After sixfold dilution, the cells were grown for 3 hr and irradiated with γ-ray using a SHIMADZU Isotron RTGS-21 (Shimadzu, Tokyo). After serial dilution, cells were plated on SC-Trp or SC-Ura plates and incubated for 4 days. The numbers of colonies on the plates were counted.

RESULTS

Meiotic cell cycle progression of *rad17* and *rad24* mutants: In mutants such as *dmc1*, *zip1*, and *hop2*, abnormal meiotic recombination arrests the cell cycle, but mutations in the DNA damage checkpoint genes can suppress this delay (Lydall *et al.* 1996; Leu and Roeder 1999; Bailis and Roeder 2000). However, the effect on meiosis of mutations in individual checkpoint genes has not been analyzed in detail. We therefore studied the timing of meiotic divisions I (MI) and II (MII) in mutants such as *rad17* and *rad24*, since the mutants show the worst spore viability among checkpoint mutants (Lydall *et al.* 1996; Leu *et al.* 1998; San-Segundo and Roeder 1999; Hong and Roeder 2002), and compared each of them to wild-type cells. To assess cell cycle progression, cells were stained with DAPI, and DAPI-staining spots were counted. In this study, MI and MII are defined as cells with more than two (two, three, and four) and more than three (three and four) DAPI-staining spots, respectively, and thus score cells after anaphase I and anaphase II. We also studied the *spo11-Y135F* mutant, which is defective in the formation of meiotic DSBs but does not affect the progression of premeiotic S-phase (Cha *et al.* 2000).

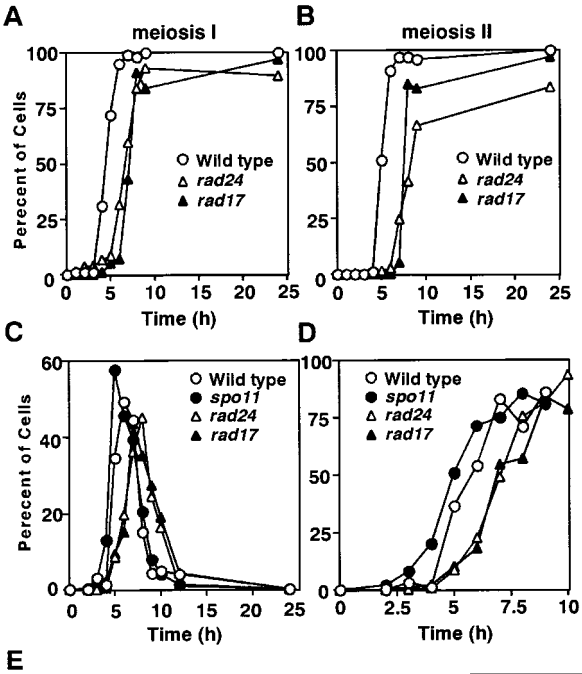
As reported, *spo11-Y135F* mutant cells enter MI 1 hr earlier than wild type (data not shown). Surprisingly, the *rad24* and *rad17* mutants show a substantial delay (0.9 and 1.0 hr, respectively; Student's *t*-test, $P < 0.001$) in entry into MI (Figure 1, A and E). In addition, in the checkpoint mutants, ~20–30% of the cells did not enter MII (Figure 1B). The delay in the *rad17* mutant was reported previously, although neither mentioned nor analyzed statistically (Grushcow *et al.* 1999). These results are somewhat unexpected, considering the role of *RAD17* and *RAD24*: if checkpoint proteins monitor meiotic DSBs and delay cell cycle as in mitosis, then mutants would be predicted to enter divisions earlier than wild-type cells.

We confirmed the delay by analyzing the timing of spindle elongation (Figure 1, C–E). Meiotic cells were stained with antitubulin antibody and cells containing short spindles were counted. When cells enter the metaphase of MI, short spindles are formed, and these spindles elongate slightly before the nuclear division. Compared to wild type, in the *spo11-Y135F* mutant, elongation occurs 0.7 hr earlier, while in the *rad17* and *rad24* mutants, elongation occurs ~1 hr later. These findings confirm that meiotic prophase progression is slower in *rad17* and *rad24* than in wild type.

The delay of cell cycle progression seen in *rad17* and *rad24* is not due to a prolonged premeiotic S-phase. Analysis of DNA contents by fluorescence-activated cell sorter indicates little delay (K. Sakai and A. Shinohara, unpublished results). Consistent with this, the checkpoint mutant with the *spo11-Y135F* mutation shows kinetics of meiosis I and II similar to those of the *spo11-Y135F* mutant (K. Sakai and A. Shinohara, unpublished results), implying that the defect in the delay is dependent on *SPO11* function, and possibly on DSB formation. Furthermore, the meiotic DSBs form at the same time as they do in the wild type (see below), suggesting normal S-phase progression.

Repair of meiotic DSBs is impaired in the *rad17* and *rad24* mutants: The spore inviability cannot be explained by the known role of the checkpoint genes in meiosis. We therefore used Southern blotting to analyze the formation and repair of meiotic DSBs at a well-known recombination hot spot, *HIS4-LEU2* (Cao *et al.* 1990). After a 3-hr incubation in SPM, wild-type cells gave two bands at this locus, implying the formation of a break. The bands disappeared after a 7-hr incubation (Figure 2, B and C), indicating that the break had been turned over. In the *rad17* and *rad24* mutants, the bands formed at the same time as they did in wild type, but their disappearance was delayed (compare blots at 6 and 7 hr in mutants and wild type). In addition, the bands in the mutants are much more heterogeneous than those in wild type. Lydall *et al.* (1996) showed the formation of 3′-OH ssDNA of DSB ends. The defect is common to mutants that are defective in strand exchange, *e.g.*, *dmc1*, *rad51* (Bishop *et al.* 1992; A. Shinohara *et al.* 1992, 1997; Schwacha and Kleckner 1997). Furthermore, *rad50S rad24* double mutants accumulate an amount of DSBs similar to that of the *rad50S* single mutant (A. Shinohara and M. Shinohara, unpublished results), indicating that the checkpoint mutations do not affect the formation of meiotic DSBs. These results suggest that the *rad17* and *rad24* mutants are defective in the conversion of meiotic DSBs into the next recombination intermediate.

The *rad17* and *rad24* mutants reduce the formation of crossover and noncrossover intermediates: Since DSB turnover is delayed, we were interested in looking at the formation of crossover and noncrossover heteroduplexes at the *HIS4-LEU2* recombination hotspot (Figure 2A). The analytical method was originally developed by Kleckner and her colleagues (Storlazzi *et al.* 1995). Parental chromosomes contain a unique restriction site (*Mlu*I or *Bam*HI) near the site of the DSB (DSB I). In addition, a polymorphism at *Xho*I restriction sites can distinguish crossover molecules from parental molecules. Thus, after the digestion of genomic DNAs with



E

	wild type	*spo11-Y135F*	*rad17*	*rad24*
(DAPI staining)				
Time of entry into MI	5.89	5.03	6.93	6.80
Confidence intervals	0.09	0.20	0.16	0.11
No. of cultures	18	5	17	13
P-value	-	<0.001	<0.001	<0.001
(Anti-tubulin staining)				
Time of entry into MI	5.64	4.87	6.79	6.53
Confidence intervals	0.20	0.09	0.32	0.26
No. of cultures	3	3	3	3
P-value	-	0.026	0.039	0.049

Figure 1.—Meiotic cell cycle progression of mutants in single checkpoint genes. (A and B) Cells incubated with sporulation medium were collected at different time points and scored for meiotic division by staining with DAPI. Nuclei with more than two and three or four DAPI-staining bodies are plotted in A and B, respectively. More than 200 cells were counted for each time point. Time "0" is the time when cells were transferred into SPM. ○, wild type (NKY1551); △, *rad24* (MSY717); ▲, *rad17* (MSY587). (C and D) Cells were stained with antitubulin antibody, cells with elongated spindles were counted, and the percentages of the cells were plotted (C). More than 200 cells were counted for each time point. Cumulative curves (D) were calculated from noncumulative curves in C, as described in Padmore *et al.* (1991). ○, wild type (NKY1551); ●, *spo11-Y135F* (KSY170); △, *rad24* (MSY717); ▲, *rad17* (MSY587). (E) Time of entry into MI (anaphase I), when 50% of cells enter into MI, was determined from several independent experiments of DAPI and antitubulin staining as described above (A and D). Confidence intervals indicate ± values for 95% confidence in the mean values given. One-way Student's *t*-tests were carried out to obtain *P* values for each mutant against wild type. Similar results were obtained by a one-way ANOVA test (data not shown).

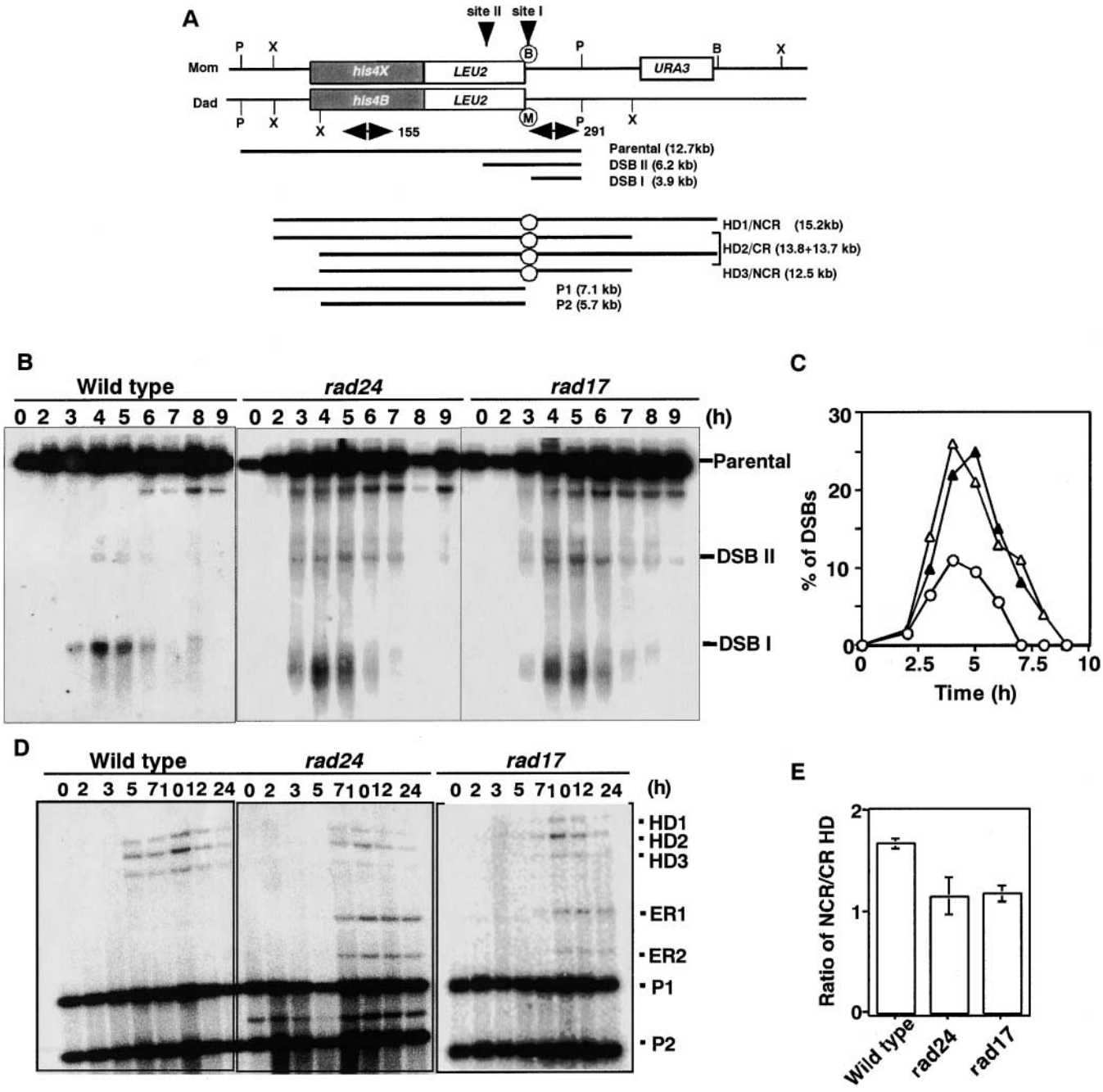


FIGURE 2.—DSB repair and recombinant formation in checkpoint mutants. (A) Schematic drawing of the *HIS4-LEU2* recombination hotspot. B, *Bam*HI; M, *Mlu*I; P, *Pst*I; X, *Xho*I. (B and C) DSB and its repair in the checkpoint mutants. Genomic DNAs from cells harvested at different times after the induction of meiosis were analyzed for DSB (B) and quantified (C). (C) ○, wild type (NKY1551); △, *rad24* (MSY717); ▲, *rad17* (MSY587). (D) The formation of heteroduplex in various mutants. Genomic DNAs from cells harvested at different times after the induction of meiosis were analyzed for heteroduplex. P1 and P2 are parental DNAs. HD1 and HD3 are noncrossover structures while HD2 (contains two bands) is a crossover structure. ER1 and ER2 are products of ectopic recombination. Wild type, NKY1551; *rad24*, MSY717; *rad17*, MSY587. (E) The ratios of amounts of noncrossover heteroduplexes (HD1 plus HD3) to that of crossover HD (HD2) at 10 hr were calculated. We show the ratios of the products, which give more accurate measurement than the amounts, since the amount of each product is <0.5% of total DNAs. The average ratios for three independent experiments are shown.

*Bam*HI, *Mlu*I, and *Xho*I, DNA molecules containing a heteroduplex migrate more slowly than parental molecules. Furthermore, crossover and noncrossover molecules containing the heteroduplex exhibit a unique mobility.

In wild type, as shown previously, both crossover and noncrossover heteroduplexes were formed simultaneously (Figure 2D). They started to appear at 5 hr and accumulated during further incubation. However, in *rad17* and *rad24* mutants, crossover and noncrossover

heteroduplexes appeared 2 hr later (compare blots at 5 and 7 hr in mutants and wild type). Furthermore, the mutants showed extra bands, which are consistent with intrachromosomal ectopic recombination between *LEU2* of the *HIS4-LEU2* locus and the *leu2::hisG* locus (Grushcow *et al.* 1999). The mutants also showed lower amounts of crossover and noncrossover heteroduplexes relative to wild type, with noncrossover heteroduplexes reduced more severely than crossover heteroduplexes. The ratio of noncrossover-to-crossover heteroduplex in wild type, *rad17*, and *rad24* mutants is 1.6, 1.1, and 1.1, respectively (Figure 2E).

In parallel, we analyzed the formation of crossover and noncrossover products by digesting DNAs with *Mlu*I and *Xho*I, which distinguish crossover and noncrossover irrespective of the presence or absence of heteroduplex. The amount of crossover recombinants in the mutants was reduced to 70% in wild type (data not shown). The results were similar to those discussed above. In summary, in the *rad17* and *rad24* mutants: (1) recombinants with or without heteroduplex form later (Grushcow *et al.* 1999); (2) there are fewer total recombinants (Grushcow *et al.* 1999); (3) the ratio of crossover to noncrossover is altered (Figure 2E) because noncrossovers are preferentially reduced; and (4) there is a concomitant increase in ectopic recombination (Grushcow *et al.* 1999).

Formation of Rad51 and Dmc1 foci and their colocalization in the *rad17* and *rad24* mutants: The accumulation of DSBs is reminiscent of the phenotypes of mutants defective in strand exchange, such as *rad51*. It would therefore be interesting to know whether the checkpoint mutants also affect the assembly and disassembly of Rad51 and Dmc1 complexes. Rad51 and Dmc1 colocalize on meiotic chromosomes as punctate staining called foci, and the colocalization of the two proteins is genetically controlled (Bishop 1994; Shinohara *et al.* 2000). We therefore prepared meiotic chromosome spreads, stained them with anti-Rad51 and anti-Dmc1 antibodies simultaneously, and counted foci under an epifluorescence microscope (Figure 3).

First, we analyzed the distribution of Rad51 and Dmc1 separately. We counted nuclei containing Rad51 foci and nuclei with Dmc1 foci, to define the assembly and disassembly phases of each type of molecule (Figure 3, B and C). In wild type, as reported previously (Shinohara *et al.* 2000), both focus-positive nuclei show very similar kinetics, indicating that both proteins load onto chromosomes at the same time. However, the coordination is compromised in *rad17* and *rad24*. The life span of Dmc1-positive nuclei is slightly longer (0.2–0.4 hr), but the life span of Rad51-positive nuclei is extended by 1.5 hr (Table 2). Consistent with this, we found nuclei with only Rad51 foci (Figure 3A, xv), which are not detected in wild type. Thus, in the checkpoint mutants, Rad51 foci outnumber Dmc1 foci later in meiosis.

Next, we analyzed colocalization of Rad51 and Dmc1 foci after a 3-hr incubation, when most of the *rad17* and *rad24* mutant cells are in the assembly phase. In wild type, 76% of the foci contained both Rad51 and Dmc1, as previously reported (Shinohara *et al.* 2000), whereas in the *rad17* and *rad24* mutants, only 52 and 43% of the foci contained both molecules (Table 2). Interestingly, the checkpoint mutants show more nuclei with a side-by-side configuration of Rad51-Dmc1 (Figures 3A, xiii and xiv), which is rarely found in wild type. This shows that the *rad17* and *rad24* mutants are partially defective for the colocalization of Rad51 and Dmc1. In addition, the total number of foci containing either Rad51 or Dmc1 in the mutants is the same or slightly lower than in wild type (Table 2 and M. Shinohara, unpublished results). This suggests that assembly of Rad51 and/or Dmc1 is slightly defective in the mutants, given that the turnover of the foci is delayed in the mutants; *e.g.*, the number of the foci should be higher in the mutants than in wild type, as in the *tid1/rdh54* mutant (Shinohara *et al.* 2000). Thus, both *RAD17* and *RAD24* are required for the proper assembly/disassembly of the RecA homologs, particularly Rad51, on meiotic chromosomes.

A high copy of *RAD51* and *RAD54* partially suppresses spore inviability of the *rad24* mutant: The results described above suggest that Rad24 acts after DSB formation. We therefore studied genetic interactions between *RAD24* and various recombination genes, particularly ones engaged in strand invasion and exchange. Extra copies of *RAD51*, *RAD54*, and *TID1/RDH54* were introduced into the *rad24* mutant cells to check whether they could restore spore viability. A substantial increase in spore viability was observed when *RAD51* was overexpressed (from 26.2% with vector alone to 40.5%; Table 3). *RAD51* also increased the fraction of asci containing four viable spores from 7.3 to 18%. These differences are statistically significant (Mann-Whitney's *U*-test, $P < 0.001$). The overexpression of *RAD54* also significantly increases spore viabilities of the *rad24* and fractions of four-viable spores per tetrad. In addition, a high copy of *TID1/RDH54* substantially decreases the spore inviability of the *rad24* mutant. We also tested a high copy number of *RED1* and *MEK1/MRE4*, which are downstream targets of Rad24 in the pachytene checkpoint (Bailis and Roeder 2000; Hong and Roeder 2002), but neither could suppress the inviability of *rad24*. These results support the idea that *RAD24* works with *RAD51*, *RAD54*, and *TID1/RDH54* during meiotic recombination.

A high copy number of *RAD51* and *RAD54* substantially suppresses the *rad24* mutant's sensitivity to γ-rays in mitosis: The above result prompted us to test whether a high copy of *RAD51* and *RAD54* could suppress the defects of checkpoint mutants in mitosis. Compared to wild-type cells, the *rad24* mutant is sensitive to ionizing radiation, presumably because it is unable to repair damage. We therefore created *rad24* cells containing various high-copy-number plasmids, irradiated them with various doses of γ-rays, and measured cell survival. As shown

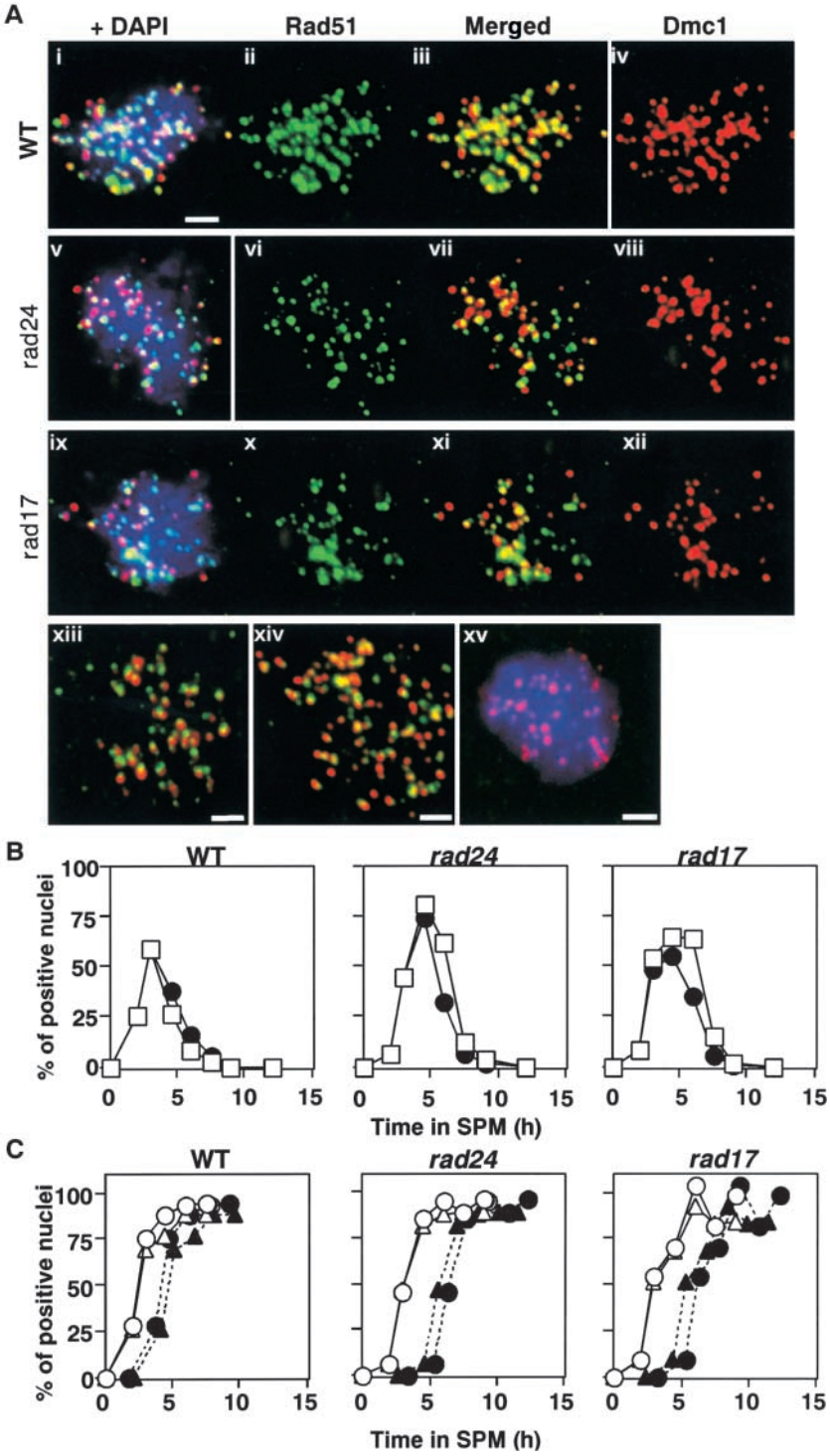


FIGURE 3.—Colocalization of Rad51 and Dmc1 on meiotic chromosomes in *rad17* and *rad24* mutants. (A) Immunolocalization: nuclear spreads of wild type (NKY1551), *rad17* (MSY587), and *rad24* mutants (MSY717) were stained with anti-Rad51 and anti-Dmc1 and then with the secondary antibodies. (i–iv) Wild type; (v–viii) *rad24*; (ix–xii) *rad17*. Rad51 (green, ii, vi, and x) and Dmc1 (red, iv, viii, and xii) were pseudocolored. Nuclei with a side-by-side configuration of Rad51 and Dmc1 foci in *rad17* (xiii) and *rad24* (xiv) mutants are shown. Nuclei with only Rad51 foci in the *rad24* are shown in xv. Bar, 2 μm. (B) Noncumulative analysis of Rad51- and Dmc1-focus positive stages. The fractions of nuclei containing more than five Rad51 or more than five Dmc1 foci were counted at each time. At least 100 nuclei were counted at each point. □, Rad51-focus positive nuclei; ●, Dmc1-focus positive nuclei. (C) Cumulative analysis of Rad51- and Dmc1-focus positive stage, based on the previous figure and analyzed as described in SHINOHARA *et al.* (2000). ○, entry of Rad51-focus positive nuclei; ●, exit of Rad51-focus positive nuclei; △, entry of Dmc1 focus-positive nuclei; ▲, exit of Dmc1-focus positive nuclei.

in Figure 4A, a high copy of both *RAD51* and *RAD54* partially suppresses sensitivity to radiation, while a vector alone has no effect. This indicates that an increased dosage of *RAD51* and *RAD54* suppresses *rad24*'s effect. The overexpression of *RAD51* or *RAD54* does not increase the resistance of wild-type haploid cells (Figure 4B). In addition, the overexpression of *TID1/RDH54* has an opposite effect: it increases γ-ray sensitivity of the *rad24* mutant significantly and that of the wild type slightly. Thus, the positive effect of *TID1/RDH54* overexpression on the *rad24* is specific to meiosis, consistent with a much more critical role of the gene in meiosis than in mitosis (M. SHINOHARA *et al.* 1997).

DISCUSSION

***RAD17* and *RAD24* are required for normal meiotic recombination:** Previous genetic analyses suggest that DNA damage checkpoint proteins identified in mitosis are also involved in meiotic recombination (see Intro-

TABLE 2

Double-staining analysis of Rad51 and Dmc1 foci in the DNA damage checkpoint mutants

	Focus analysis: No. of foci per nucleus at 3 hr[b]				Stage analysis			
					Time of entry[c] (hr)		Life span[d] (hr)	
Strains[a]	RD co-foci	R foci	D foci	% of RD co-foci	R^+ stage	D^+ stage	R^+ stage	D^+ stage
Wild type	67	10	11	76 (10)	2.3	2.3	1.8	2.2
rad24	43	22	17	52 (8)	2.6	2.6	3.3	2.6
rad17	29	26	13	43	2.5	2.5	3.3	2.4

[a] The wild type, *rad17*, and *rad24* mutants were NKY1551, MSY587, and MSY717, respectively.

[b] For each strain, at least 100 nuclei unselected meiotic nuclei at 3 hr were counted. In the case of wild type and the *rad24* mutant, three independent experiments were carried out and the averages are shown. Standard deviation is indicated in parentheses. RD co-foci, Rad51-Dmc1 co-foci; R foci, Rad51-only foci; D foci, Dmc1-only foci.

[c] Time of entry at which 50% of nuclei had entered the focus-positive stage was determined from the cumulative curves in Figure 3C. R^+ stage, Rad51 focus-positive stage; D^+stage, Dmc1 focus-positive stage.

[d] Life spans of focus-positive stages are determined from the noncumulative curves in Figure 3B.

duction). However, it was not clear which biochemical process these checkpoint proteins perform. Here, we report that mutants of two DNA damage checkpoint genes, *RAD17* and *RAD24*, are partially defective in the repair of meiotic DSBs, as well as in the formation of crossover and noncrossover products. The mutants are also deficient in coordinating Rad51 and Dmc1 complex formation and share some similarity of meiotic phenotypes with mutants of *TID1/RDH54* and *RAD51*, which play a direct role in meiotic recombination, *e.g.*, strand invasion/exchange. The *rad51* and *tid1/rdh54* mutants accumulate DSBs with more resected ends and form reduced amounts of crossover (A. Shinohara *et al.* 1997; M. Shinohara *et al.* 1997). The *tid1/rdh54* mutant reduces colocalization of Rad51 and Dmc1 (Shinohara *et al.* 2000). In addition, similar to checkpoint mutants, the *tid1/rdh54* mutant shows increased ectopic recombination (M. Shinohara and A. Shinohara, unpublished results). Taken together, these results strongly suggest that Rad17 and Rad24 are involved in strand invasion and exchange (see below).

The *rad17* and *rad24* mutations affect not only the coordination of Rad51 and Dmc1 complexes on DNA, but also the disassembly of Rad51 complexes, suggesting that Rad17 and Rad24 are likely to work together with Rad51 during meiosis. This idea is further strengthened by the fact that an increased dosage of *RAD51* and *RAD54* substantially improves spore viability of the *rad24* mutant. Interestingly, a high copy of the *TID1/RDH54* also increases spore viability of the *rad24* mutant, but its effect is specific to meiosis. Tid1/Rdh54, which interacts with both Rad51 and Dmc1, plays a more critical role

TABLE 3

Spore viability of the *rad24* mutant containing plasmids with various genes involved in recombination in meiosis and mitosis

		Viable spores per ascus[b]					
Plasmid	Overall spore viability[a] (%)	4	3	2	1	0	*P* value
Vector	26.2	19	22	43	46	130	—
RAD24[c]	86.5*	189	36	16	4	15	$P < 0.001$
RAD51	40.5*	48	31	46	44	91	$P < 0.001$
RAD54	40.8*	51	22	60	34	93	$P < 0.001$
TID1/RDH54	35.3*	37	27	53	32	111	0.013
RED1	26.9	21	24	50	24	141	0.86
MEK1/MRE4	26.1	24	22	44	21	149	0.51

*Values were significantly different from that for the vector control; in all of these cases $P < 0.001$.

[a] The *rad24* haploid (MSY966) with various plasmids was mated with MSY968 for 6 hr and then transferred onto a sporulation plate. After a 48-hr incubation, 260 asci were dissected, and spore viability and distribution of tetrad types were measured. For viability comparisons, raw data of each transformant were compared to those for the vector plasmid control alone to obtain *P* values (chi-square test).

[b] The numbers of asci with 4, 3, 2, 1, and 0 viable spores are shown. Total 260 asci were dissected. The distribution of the number of each class in the transformant was compared to that in the strain with the vector alone to obtain *P* values (Mann-Whitney's *U*-test).

[c] A low copy number of *RAD24*.

in meiosis than in mitosis (M. Shinohara *et al.* 1997). We propose that during meiosis, the Rad24-RFC complex and the PCNA-like complex containing Rad17 collaborate with Rad51 (and Rad54 and Tid1/Rdh54) and, possibly, facilitate Rad51-filament formation to promote proper DSB repair.

The high-copy suppression of low spore viability of the *rad24* mutant by the recombination genes instead could be explained by the overexpression of recombination genes activating a second recombination pathway, which is independent of *RAD24* function. The overexpression of *RAD54* suppresses the defects in the *dmc1* null mutant by bypassing to a second recombination pathway, which does not occur in wild type (Bishop *et al.* 1999).

Although checkpoint proteins are likely to play a direct role in meiotic recombination, we cannot eliminate indirect pathways. For example, a downstream target protein belonging to a checkpoint pathway that depends upon Rad17 and Rad24 appears to modulate meiotic recombination. The downstream target(s) could be proteins involved in the repair of DSBs. Obvious candidates are Rad51 or Rad51-accessory proteins. In mitosis, one of the Rad51-binding proteins, Rad55, is phosphorylated in response to DNA damage (Bashkirov *et al.* 2000). Rad55 forms a complex with Rad57 and promotes proper assembly of Rad51 filaments on ssDNA (Sung 1997). Rad55 may also be involved in meiosis, as the *rad55* mutant partially delays DSB repair, decreases crossover formation, and is defective in the formation of Rad51 foci (Schwacha and Kleckner 1997; Gasior *et al.* 1998). However, Rad55 phosphorylation depends on Rad53 and Dun1 kinases, neither of which is required during meiosis (Roeder and Bailis 2000).

During meiosis, Rad17 and Rad24 activate a meiosis-specific kinase, Mek1/Mre4, which in turn appears to phosphorylate Red1, a meiosis-specific chromosome component (Bailis and Roeder 1998; de los Santos and Hollingsworth 1999). The Mek1/Mre4-Red1 pathway is proposed to mediate the pachytene checkpoint (Roeder and Bailis 2000). In the mitotic DNA damage checkpoint, the overexpression of Rad53, a paralogue of Mek1/Mre4 (Bailis and Roeder 2000; Roeder and Bailis 2000), can suppress the mitotic defect in mutants of upstream checkpoint genes (Sanchez *et al.* 1996; Sugimoto *et al.* 1996). On the other hand, a high copy number of Mek1/Mre4 does not suppress meiotic defects in the *rad24* mutant (Table 3). This argues against a function for the checkpoint proteins in signaling during meiosis.

The roles of checkpoint proteins in meiotic recombination: Given the structural analogy between the RFC-PCNA complex and the Rad24-Rad17 complex, Rad17 and Rad24 are likely to bind to a D-loop structure such as SEI formed during meiotic recombination and to stabilize it by preventing the 3′-OH strand from dissociating from the duplex.

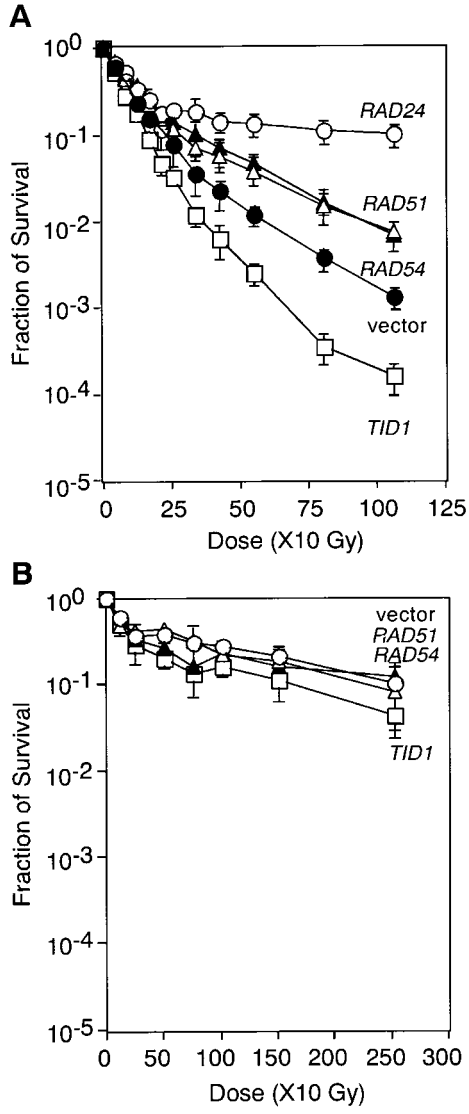


Figure 4.—Radiation sensitivity of *rad24* with increased dosage of various recombination genes. (A) *rad24* mutant haploids (MSY966) transformed with various high-copy-number plasmids were irradiated with various doses of γ-ray and plated on SC plates lacking tryptophan or uracil. After a 4-day incubation, colonies were counted. The data are an average of three independent experiments. Error bars indicate 95% confidence intervals. ○, *RAD24* (a low-copy plasmid); ●, vector alone; △, *RAD51*; ▲, *RAD54*; □, *TID1/RDH54*. (B) Wild-type cells (MSY833) transformed with various high-copy-number plasmids were irradiated with various doses of γ-rays. ○, vector alone; △, *RAD51*; ▲, *RAD54*; □, *TID1/RDH54*.

Alternatively, although not exclusively, the checkpoint proteins might prevent the invading end in the SEI from being extended by DNA polymerase(s) before the second end interacts with the same dsDNA. In this scenario, checkpoint proteins might monitor recombination events and cause both ends of a DSB to interact with the same dsDNA. This idea is similar to Grushcow *et al.*'s (1999) original proposal: in checkpoint mutants,

the coordination of DSB ends is disrupted. This is consistent with our observation that the colocalization of Rad51 and Dmc1 is disrupted in the *rad17* and *rad24* mutants. We previously suggested that Rad51 forms a complex on one end of the DSB, and Dmc1 forms an independent complex on the other end of the DSB (Shinohara *et al.* 2000). The checkpoint proteins might coordinate assembly of recombination complexes on the DSB ends.

What couples meiotic recombination with meiotic cell cycle progression? The pachytene checkpoint is proposed to inhibit meiotic cell cycle progression in response to incomplete meiotic recombination and chromosome synapsis (Roeder and Bailis 2000). This has been inferred from the analysis of abnormal meiosis induced by a class of mutants, *e.g.*, *dmc1*, *zip1*, and *hop2*. These mutants arrest or delay meiotic prophase, but this arrest is alleviated by mutations in some mitotic DNA damage checkpoint genes, suggesting that the checkpoint genes might also act in meiosis as in mitosis. However, meiosis involves at least 200 intrinsic DSBs and the cell cycle control might be quite different. Here, we suggest that in meiosis DSBs are not monitored solely by the mitosis checkpoint proteins Rad17 and Rad24, as *rad17* or *rad24* delay the entry into MI relative to wild type. If these genes were involved in the checkpoint control of meiosis, the mutations should abolish the checkpoint and cause either no delay or, possibly, acceleration. Alternatively, in meiosis, the defect in these mutants might trigger some other checkpoint response controlling the cell cycle. Previous analyses of meiotic prophase arrest by *rad50S* have shown that a second checkpoint response operates to monitor unprocessed DSBs. This arrest requires the Mec1/Esr1 homolog, Tel1, and Rad9 (Usui *et al.* 2001). However, Tel1 is unlikely to play a role in the delay seen in the checkpoint mutants studied here, since the *tel1 rad24* double mutant shows meiotic cell cycle progression similar to the *rad24* single mutant (A. Shinohara and M. Shinohara, unpublished results). Furthermore, the Tel1-dependent *rad50S* checkpoint still requires *RAD24* function (Usui *et al.* 2001).

Although meiotic cells certainly monitor DSBs, the DNA damage checkpoint genes identified in mitosis may not be involved. The link between DNA damage and control of timing in meiosis is complex. Mutants such as *spo11* and *rec104*, which are deficient in the formation of meiotic DSBs, enter MI earlier than wild-type cells, arguing for monitoring. On the other hand, the *mei4* mutant, which is also deficient in the formation of the DSBs, enters MI at the same time as wild type, which is different from the other early recombination mutants (Galbraith *et al.* 1997; Jiao *et al.* 1999). Furthermore, the induction of a single DSB into a *rec104* mutant does not delay the first division (Jiao *et al.* 1999), suggesting that the cell cycle is normally slow enough that DSBs can be repaired. Here, we showed that single checkpoint mutants enter MI later than wild type. If the DNA damage checkpoint proteins normally monitor 200 meiotic DSBs, checkpoint mutants should enter into MI earlier than wild type, similar to the *spo11* mutant, but clearly this does not happen. In mitosis, checkpoint proteins are usually very sensitive to strand breaks. One irreparable DSB is sufficient to delay the cell cycle, and two irreparable DSBs are sufficient to arrest it (Sandell and Zakian 1993; Lee *et al.* 1998). Thus, these suggest that DNA damage checkpoint genes are not required for the meiotic cell cycle control at least in wild-type meiosis.

In yeasts as well as in fruit flies and nematodes, the combination of a mitotic checkpoint mutant with meiotic recombination-deficient mutants accumulating abnormal recombination intermediates has been used to analyze the checkpoint in meiosis (Ghabrial and Schüpbach 1999; Gartner *et al.* 2000; Shimada *et al.* 2002). However, as described in this article, the *SPO11*-dependent delay in entry into meiosis I is independent of mitotic DNA damage checkpoint proteins in wild-type cells. Therefore, the concept of the pachytene checkpoint in wild-type meiosis should be treated cautiously.

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