DMC1 Functions in a *Saccharomyces cerevisiae* Meiotic Pathway That Is Largely Independent of the *RAD51* Pathway

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ABSTRACT

Meiotic recombination in the yeast *Saccharomyces cerevisiae* requires two similar recA-like proteins, Dmc1p and Rad51p. A screen for dominant meiotic mutants provided *DMC1-G126D,* a dominant allele mutated in the conserved ATP-binding site (specifically, the A-loop motif) that confers a null phenotype. A recessive null allele, *dmc1-K69E,* was isolated as an intragenic suppressor of *DMC1-G126D.* Dmc1-K69Ep, unlike Dmc1p, does not interact homotypically in a two-hybrid assay, although it does interact with other fusion proteins identified by two-hybrid screen with Dmc1p. Dmc1p, unlike Rad51p, does not interact in the two-hybrid assay with Rad52p or Rad54p. However, Dmc1p does interact with Tid1p, a Rad54p homologue, with Tid4p, a Rad16p homologue, and with other fusion proteins that do not interact with Rad51p, suggesting that Dmc1p and Rad51p function in separate, though possibly overlapping, recombinational repair complexes. Epistasis analysis suggests that *DMC1* and *RAD51* function in separate pathways responsible for meiotic recombination. Taken together, our results are consistent with a requirement for *DMC1* for meiosis-specific entry of DNA double-strand break ends into chromatin. Interestingly, the pattern on CHEF gels of chromosome fragments that result from meiotic DNA double-strand break formation is different in *DMC1* mutant strains from that seen in *rad50S* strains.

THE initiation of meiotic recombination in the yeast *Saccharomyces cerevisiae* involves the formation of 100–200 DNA double-strand breaks (DSBs) per nucleus (SUN *et al.* 1989; CAO *et al.* 1990; WU and LICHTEN 1994), producing a stringent requirement for recombinational repair mechanisms. The successful completion of meiosis requires not only that the DSBs be repaired without loss of genetic information but also that repair results in the formation of chiasmata, attachments that hold homologous chromosomes together until they are properly oriented for disjunction during the first meiotic division (BAKER *et al.* 1976). Functions required for recombination repair throughout the life cycle are required in meiotic recombination (PETES *et al.* 1991) but must be supplemented by functions that channel and regulate repair to produce outcomes required specifically for meiosis.

Multiple genes with homology to *recA* are required for wild-type DNA transactions in yeast. The *recA* homologues *DMC1* and *RAD51* (BISHOP *et al.* 1992; SHINOHARA *et al.* 1992; STORY *et al.* 1993) are required early in meiotic recombination and colocalize cytologically (BISHOP 1994). Strains that are deleted for either gene can complete some level of recombination and sporulation (BISHOP *et al.* 1992; SHINOHARA *et al.* 1992; ROCKMILL and ROEDER 1994; ROCKMILL *et al.* 1995). In the case of *dmc1*Δ, completion of sporulation requires suppression or absence of a meiotic block (LYDALL *et al.* 1996). *RAD51* functions as part of a complex that includes proteins with DNA binding activity (*RAD52;* MORTENSEN *et al.* 1996) and ATPase/helicase homology (*RAD54;* JIANG *et al.* 1996), as well as two other proteins with *recA* similarities, *RAD55* and *RAD57* (DONOVAN *et al.* 1994; HAYS *et al.* 1995).

Based on the cytological evidence for colocalization, it has been suggested that *DMC1* interacts with *RAD51* to promote recombination, perhaps being involved in determining whether crossovers are formed (BISHOP 1994) or in facilitating interactions specifically between nonsister chromatids (ROCKMILL *et al.* 1995). Consistent with the two genes functioning in a single pathway, *dmc1*Δ and *rad51*Δ strains show similar reductions in pairing and delays in synapsis (ROCKMILL *et al.* 1995), and a *dmc1*Δ *rad51*Δ strain pairs its chromosomes as well as (or more effectively than) *dmc1*Δ alone (WEINER and KLECKNER 1994, *rad51*Δ alone not reported). The evidence reported here indicates that *DMC1* and *RAD51* function in different though perhaps analogous complexes that have unique roles in the early stages of meiotic DNA recombination repair.

MATERIALS AND METHODS

Strain and plasmid construction: Diploid strains (Table 1) were constructed by mating isogenic haploids MDY431 and MDY433 or derivatives made by transformation gene replace-

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TABLE 1

Yeast strains

Strain	Genotype
MDY431	*MAT***a** *ade2 ADE5 CAN1^S cyh2^R his7-1 leu1-c lys2-2 met13-c trp1-63 tyr1-2 ura3-1*
MDY433	*MATα ADE2 ade5 can1^R CYH2^S his7-2 leu1-d lys2-1 met13-d trp1-63 tyr1-1 ura3-13*
MDY507	MDY433, except *MAT***a**
MDY508	MDY431, except *MATα*
MDD1	*MAT***a** *ade2 ADE5 CAN1^S cyh2^R his7-1 leu1-c lys2-2 met13-c trp1-63 tyr1-2 ura3-1* / *MATα ADE2 ade5 can1^R CYH2^S his7-2 leu1-d lys2-1 met13-d trp1-63 tyr1-1 ura3-13*
MDD2	MDD1, except *DMC1-G126D/dmc1Δ::TRP1*
MDD3	MDD1, except *dmc1-K69E/dmc1Δ::TRP1*
MDD4	MDD1, except *dmc1-K69E/dmc1-K69E*
MDD5	MDD1, except *dmc1Δ::TRP1/dmc1Δ::TRP1*
MDD6	MDD1, except *rad51Δ/rad51Δ*
MDD7	MDD1, except *dmc1Δ::TRP1/dmc1Δ::TRP1 rad51Δ/rad51Δ*
MDD8	MDD1, except *DMC1-G126D/DMC1* and heterozygous for inversion of chromosome VII segment from bases 210, 230 to 478,952[a]
MDD9	MDD1, except *DMC1-G126D/DMC1-HA*
MDD10	MDD1, except *rad50S-KI81/rad50S-KI81*
MDD11	MDD1, except *dmc1Δ::TRP1/dmc1Δ::TRP1 rad50S-KI81/rad50S-KI81*
MDD12	MDD1, except *DMC1-G126D/dmc1Δ:::hisG*
MDD13	MDD1, except *dmc1Δ::hisG/dmc1Δ:::hisG*
Y187	*MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, 112 GAL → lacZ*, provided by S. J. ELLEDGE
Y190	*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL → lacZ LYS2::GAL → HIS3 cyh^R*, provided by S. J. ELLEDGE.

[a] Numbers from Stanford Genome Database; see DRESSER *et al.* (1994).

ment (ROTHSTEIN 1983) that were confirmed by Southern blots and by standard genetic complementation tests. MDY508 and MDY507 were made by transformation of MDY431 and MDY433, respectively, with pGAL-HO and brief induction of *HO* by growth on galactose-containing medium (HERSKOWITZ and JENSEN 1991) to create isogenic pairs of haploids of opposite mating type. Double mutants were made by crossing appropriate single mutants (for example, MDY431 with MDY508 derivatives) and dissecting tetrads to identify further haploids in isogenic backgrounds. *rad50S-KI81* was introduced by transformation with pNKY349 (ALANI *et al.* 1990). *rad51Δ* was introduced by transformation with pOL163 (SHINOHARA *et al.* 1992). The *DMC1* gene was identified by complementation (see below) and a ~3-kb *Xba*I fragment containing the gene was subcloned into pRS316. A PCR reaction with DNA from a λ*gt11 S. cerevisiae* meiotic cDNA library (E. PERKINS and M. DRESSER, unpublished data) as template and oligonucleotide primers that flank the *DMC1* intron provided a *Bgl*II-*Avr*II fragment to replace in the *DMC1* coding sequence, removing the intron. A ~630-bp *Hin*dIII-*Bgl*II fragment flanking the start site was subcloned and used to introduce a *Sca*I site between the second and third codons by PCR mutagenesis using primers 5′-CAAATATGAGTACTA CAGGAACTGAGATCG-3′ and 5′-GTAGTACTCATATTTGT TCAAATGC-3′ (JONES and HOWARD 1991). A blunt-ended HA epitope cassette (KOLODZIEJ and YOUNG 1991) was inserted at the *Sca*I site, then the *Hin*dIII-*Bgl*II fragment was moved into pRS306 to create pMDE376, which was cut with *Pac*I to target replacement into the genome. The same *Hin*dIII-*Bgl*II fragment was combined with the intronless *DMC1* coding sequence, and a blunt-ended 6-HIS cassette (HOCHULI *et al.* 1988) was cloned into the *Sca*I site. This 6-HIS version was cloned into pET-11c to create pMDE367, which was used to produce and purify Dmc1p from *Escherichia coli* using a Ni^{2+} column and standard procedures (HOCHULI *et al.* 1988; Novagen). Single-step *DMC1* deletion constructs, *dmc1Δ::hisG-URA3-hisG* and *dmc1Δ::TRP1* were made by replacing the *DMC1* coding sequence from the *Bgl*II site to the *Sna*BI sites with the *URA3* cassette from pNKY51 (ALANI *et al.* 1987) and from the engineered *Sca*I site to the *Sna*BI site with *TRP1* to make pMDE339 and pMDE379, respectively.

Mutant isolation: To screen for dominant meiotic mutants, haploid cells of strain MDY431 were treated with EMS at a level that induced 95% inviability, plated on YPDA to grow to small colonies and cross-stamped with lawns of MDY433 on YPDA to allow mating. Diploids were selected by replica-plating to minus-adenine medium and were then replica-plated to sporulation medium. After 5 days at 30°, colonies were replica-plated to medium containing canavanine and cycloheximide to screen for haploidization. Among ~49,500 mutagenized isolates examined for sporulation or spore viability defects we identified 1245 non-maters, 496 autodiploids that mate to give triploids that have greatly reduced spore viability, 18 *MAT***a** gene mutants that mate but fail to sporulate, two temperature-conditional dominant mutants, and two nonconditional dominant mutants. Meiotic development stopped at the pachytene stage in one of the nonconditional mutants. This haploid isolate was further mutagenized by treatment with UV and revertants, which allow sporulation when crossed to the wild-type tester strain MDY433, were selected on medium containing canavanine and cycloheximide. One of the revertants provided spores that, when intercrossed, revealed a recessive meiotic defect (data not shown). The involved gene was identified as *DMC1* by complementation of the meiotic defect using a wild-type plasmid library (ROSE *et al.* 1987), subcloning and sequencing. The mutant allele of *DMC1* was recovered from the twice-mutated strain by gap-retrieval (ROTHSTEIN 1991) and sequenced, revealing two point mutations that result in amino acid substitutions at residues 69 (LYS replaced by GLU; the *dmc1-K69E* allele) and

126 (GLY replaced by ASP; the *DMC1-G126D* allele). These mutations were separated and placed singly into the wild-type gene by subcloning, then the mutated versions were used to replace *DMC1* in the genome of appropriate strains.

Meiotic recombination and DNA double-strand break assays: A stationary phase YPDA culture was used to inoculate YPA medium for growth overnight at 30°, 300 rpm. Cells were harvested at 3–5 × 10^7cells + buds/ml, washed twice with sterile, distilled water, washed once with SP2-CS medium (1% potassium acetate supplemented with essential amino acids), resuspended in SP2-CS at half the original volume and sporulated at 30° while shaking at 300 rpm (DRESSER *et al.* 1994). Meiotic induction of gene conversion was measured by plating sporulating cells on complete synthetic medium to monitor viability and on appropriate dropout media at time-points after the shift into sporulation medium. To assay DNA fragments formed by double-strand breaks, 5-ml aliquots were processed (GERRING *et al.* 1991) to produce sample plugs in 0.8% InCert agarose. Samples were run on a Bio-Rad CHEF II in 1% FastLane agarose, 0.25× TBE at 12°, 6 V/cm using a 20–40 sec ramp over 24 hr. Southern transfer was performed using alkaline capillary transfer (CHOMCZYNSKI 1992) onto Zeta Probe membrane for 1.25 hr. Radiolabeled probes (FEINBERG and VOGELSTEIN 1983) represent DNA segments from near-telomeric regions of chromosome III. The chromosome IIIL probe was generated from a 980-bp fragment centered at chromsome III base number 22,322 (Stanford Genome Database), comprised of an *Eco*RI to *Sal*I segment delineated as 2* by BUTTON and ASTELL (1986). The chromosome IIIR probe, centered at chromosome III base number 296,706 (Stanford Genome Database), was the PCR product of 24-mer primers encompassing a unique 2550-bp portion of the right arm. Results of hybridization (PLUTA and ZAKIAN 1989) were imaged and quantified using a Molecular Dynamics PhosphorImager 425.

Two-hybrid assays and screen: Interaction assays using the two-hybrid system were performed using *S. cerevisiae* host strain Y190 (DURFEE *et al.* 1993) and filter lifts of colonies using standard techniques. Interactions were quantified using crude protein extracts from 5 ml log-phase cultures grown in synthetic medium lacking leucine and tryptophan, standardized against total protein (BRADFORD 1976; MILLER 1976). The *DMC1* and *dmc1-K69E* bait and prey constructs were made in pAS1/CYH2 and pACTII (DURFEE *et al.* 1993) and encode fusions with amino acids 3–334 of Dmc1p (pMDE422 and pMDE467, respectively) and dmc1-K69Ep (pMDE486 and pMDE501, respectively). To screen for new interacting gene products, a random-sheared yeast genomic two-hybrid library was prepared from DNA extracted from the *gal4*Δ strain YJ0 (provided by the S. JOHNSTON lab), using the system developed by Elledge (DURFEE *et al.* 1993). Of 1.6 × 10^7 original clones, ~40% have *Bgl*II-releasable inserts (ranging from 800 bp to 3 kb in length), indicating that, on average, the library represents a breakpoint in both directions at every ~5 bases in the *S. cerevisiae* genome. Approximately 5.5 × 10^6 transformants were screened for interaction with *DMC1*, and 316 positives were isolated. These were winnowed to 76 interacting clones by plasmid loss followed by mating to strain Y187 containing control-negative bait constructs pAS1-lamin, pAS1-SNF1 and pAS1-p53. The plasmids were retrieved into *E. coli* and many apparent duplicates were screened out by Southern blot analyses. The inserts of 40 clones were sequenced from the amino-terminal side of the putative fusion proteins, revealing long in-frame fusions for 26 plasmids to 10 separate open reading frames (Table 3). *GAL4* binding and activation domain fusions for two-hybrid assays of interaction with *RAD50, RAD51, RAD52, RAD54* and *RPA1* were made in standard vectors (JIANG *et al.* 1996).

Cytology: Sporulating cells were assayed for entry into the meiotic divisions by fluorescence microscopy of DAPI-stained nuclei. Meiotic nuclei were prepared for immunocytology using a standard spreading procedure (DRESSER and GIROUX 1988). Antibodies against Dmc1p were prepared from serum from a rabbit immunized with purified 6-HIS-Dmc1p by preabsorption with meiotic protein extracted from MDD13 (*dmc1*Δ*::hisG*). Serial absorption of 0.1 ml of serum, diluted to 10 ml in Tris-buffered saline/4% nonfat dried milk/0.1% Tween 20, by three meiotic protein-saturated 82-mm nitrocellulose filters was required to provide staining of Western blots of wild-type meiotic protein that revealed only the expected Dmc1p band (at a further dilution of 1/100) and immunocytological preparations of MDD5 that showed no background labeling of spots (at further dilutions of 1/2 or 1/10). 12CA5 monoclonal antibody (Babco) was used to visualize HA-tagged Dmc1p and gave no background signal in control samples without the HA epitope. Rad51p was visualized using mouse serum prepared by immunization with purified Rad51p (JIANG *et al.* 1996); this serum gave no background labeling in *rad51*Δ nuclei (data not shown). Labeling times with mixed primary and then secondary antibodies were typically 2 hr at room temperature. Images for colocalization were taken using an intensified CCD camera (Hamamatsu) and an Image-1/AT system (Universal Imaging Corporation). Single-wavelength exposures for DAPI (DNA staining), FITC (anti-rabbit) and CY3 (anti-mouse) signals were aligned electronically against a simultaneous exposure made using a triple-wavelength filter cube (Omega #XF56) before being compared to one another.

Sensitivity to γ-irradiation: Cells were grown in YPDA at 30° with shaking at 300 rpm and were harvested at late stationary phase or at log phase, as defined by reaching one-tenth the cell density reached at late stationary phase in prior control experiments. Log phase samples were concentrated 10-fold in their growth medium before irradiation with a cesium-137 source at a rate of 1.1 kilorad per min. At appropriate dosages, aliquots were diluted serially in sterile distilled water, plated on YPDA and incubated at 30°; colonies counted to determine survival. Growth phase and ploidy of the samples irradiated were verified by flow cytometry of ethanol-fixed cells treated with 1 mg/ml ribonuclease/PBS (phosphate-buffered saline) and stained with propidium iodide in 0.1% Triton X-100/PBS (CRISSMAN and STEINKAMP 1973; FREID *et al.* 1978).

RESULTS

The dominant *DMC1-G126D* and recessive *dmc1-K69E* mutants are phenotypically null: Meiotic induction of gene conversion was tested in strains carrying each of the two point mutations (hemizygous with *dmc1*Δ) and compared with the homozygous *dmc1*Δ strain. The three are indistinguishable for conversion of heteroalleles at *HIS7* (Figure 1), *LEU1* and *TYR1* (data not shown; *LYS2* and *MET13* heteroalleles not tested). In each of the mutant strains, cells block uniformly in meiotic prophase, failing to enter the first meiotic division (data not shown). While held at the block, *DMC1-G126D/DMC1* heterozygotes produce no visible crossover products as measured by using a heterozygous inversion assay (DRESSER *et al.* 1994), as expected from the phenotype reported for *dmc1*Δ (BISHOP *et al.* 1992 and data not shown). A small fraction of cells (~10^{-5}) do complete sporulation, having undergone homozygosis for the wild-type allele during vegetative growth

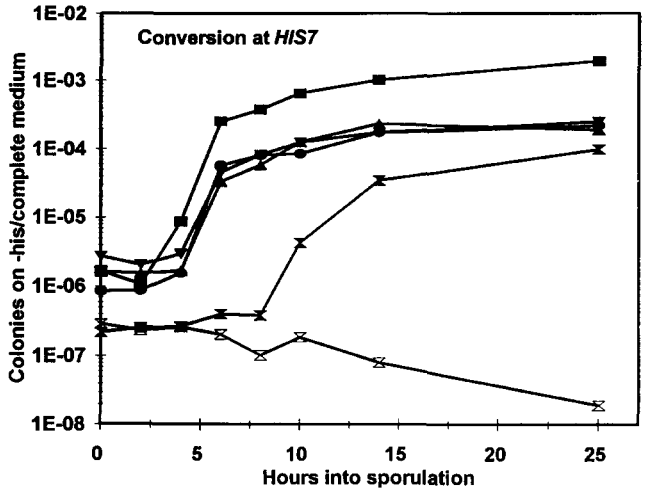

FIGURE 1.—Meiotic induction of gene conversion at *HIS7*. Samples of sporulating cultures are washed, diluted and plated on medium lacking histidine to select for conversion at *HIS7* and on complete synthetic medium to correct for viability. Values are from one of two independent experiments with essentially identical results. Wild type (MDD1), squares; *DMC1-G126D/dmc1Δ* (MDD2), triangles; *dmc1-K69E/dmc1Δ* (MDD3), circles; *dmc1Δ/dmc1Δ* (MDD5), inverted triangles; *rad51Δ/rad51Δ* (MDD6), filled hourglasses; *dmc1Δ/dmc1Δ rad51Δ/rad51Δ* (MDD7), hollow hourglasses.

(data not shown); otherwise, *DMC1-G126D/DMC1* heterozygotes are similar to *DMC1-G126D/dmc1Δ*. The levels of accumulated protein during meiosis are similar for wild-type, recessive and dominant proteins as assayed by Western blots (data not shown), demonstrating that the mutant effects are not due to protein turnover. Immunocytology reveals that the recessive and dominant proteins, and the wild-type protein in *DMC1-G126D/DMC1* cells still localize to the nucleus but that the accumulation in cells at the block can be considerably less discrete than for wild-type protein in wild-type cells (Figure 2).

Wild-type *DMC1* but not *dmc1-K69E* protein interacts homotypically: Dmc1p is similar to recA protein that binds DNA cooperatively (ROCA and COX 1990; KOWALCZYKOWSKI 1991; RADDING 1991; WEST 1992). In the two-hybrid assay, *DMC1* fusion proteins interact homotypically, but the *dmc1-K69E* fusion proteins interact less well with wild-type fusions and not at all with one another (Table 2) even though the accumulation of fusion protein as assayed by Western blots is higher for the recessive mutant fusions than for wild type (Figure 3). Given the failure of the recessive fusion proteins to interact with one another it is unlikely that the homotypic interaction of the wild-type protein is an artifact from, *e.g.*, making normally internal parts of Dmc1p available for interaction (see BARTEL *et al.* 1996). In addition, even though the *dmc1-K69E* bait fusion protein fails to interact with the *dmc1-K69E* prey fusion, it nevertheless does interact with prey fusions representing other proteins (see below; *TID1-4*, *PDC1*, and *PDC5* were tested). Plasmids carrying the dominant *DMC1-G126D* protein fusions gave no stable transformants in yeast, presumably because the dominant protein is lethal to vegetative cells when present at high levels.

Dmc1p and Rad51p interact with different sets of proteins: In the two-hybrid assay, Rad51p also interacts homotypically, but Dmc1p and Rad51p fusions do not interact (Table 2). Rad51p interacts with Rad52p and with Rad54p but not with Rad50p, Rad57p or Rpa1p (JIANG *et al.* 1996). Dmc1p does not interact with any of these fusions as bait or as prey. These results suggested that Dmc1p either acts alone or by associations with previously undefined proteins. A two-hybrid screen using Dmc1p as bait identified 10 open reading frames by clones representing from one to seven different fusion breakpoints (Table 3). Previously unnamed open reading frames were assigned the provisional designation *TID* for two-hybrid interaction with Dmc1p.

Accumulations of Dmc1p and Rad51p do not invariably colocalize immunocytologically: Immunocytological experiments using the yeast SK-1 background have shown that (1) Dmc1p and Rad51p colocalize, (2) Rad51p accumulates to wild-type levels in a *dmc1Δ* strain, and (3) Dmc1p does not accumulate to wild-type levels in a *rad51Δ* strain (BISHOP 1994). We have made similar observations in our strains with the exception that we do not see as high an incidence of colocalization of these proteins in wild-type cells as reported for the SK-1 strain background (Figure 4 and Table 4). The level of coincidence is little changed by scoring only the brighter spots to reduce the possibility of interference from nonspecific or background signals, or by segregating the data from early prophase *vs.* pachytene stage nuclei. Only 42–53% of the Rad51p spots coincide with Dmc1p spots and, conversely, 21–40% of the Dmc1p spots coincide with Rad51p spots.

The phenotype of the double mutant *dmc1Δ rad51Δ* is unlike either single mutant: Cells with *dmc1Δ* fail to repair meiotic DSBs as measured by accumulation of chromosome III fragments on CHEF gels (Figure 5A). Meiotic induction of gene conversion, as measured by return-to-growth, begins after a slight delay but fails to reach wild-type levels (Figure 1). Cells with *dmc1Δ* block in meiotic prophase maintain almost wild-type viability for ~24 hr (Figure 6) and fail to complete sporulation even after 5 days in sporulation medium (as reported for the SK-1 background, BISHOP *et al.* 1992).

Cells with *rad51Δ* turn over some but not all meiotic DSBs (Figure 5A) and show induced gene conversion after a marked delay, also failing to reach wild-type levels (Figure 1). Cells with *rad51Δ* also sporulate slowly to ~39% final asci and exhibit a decrease in viability during sporulation (Figure 6; SHINOHARA *et al.* 1992). There is a delay in the accumulation of DSB fragments in strains with *rad51Δ* by comparison with the *rad50S* and *dmc1Δ* single mutant strains; the slower vegetative growth of *rad51Δ* strains may make the entry into meio-

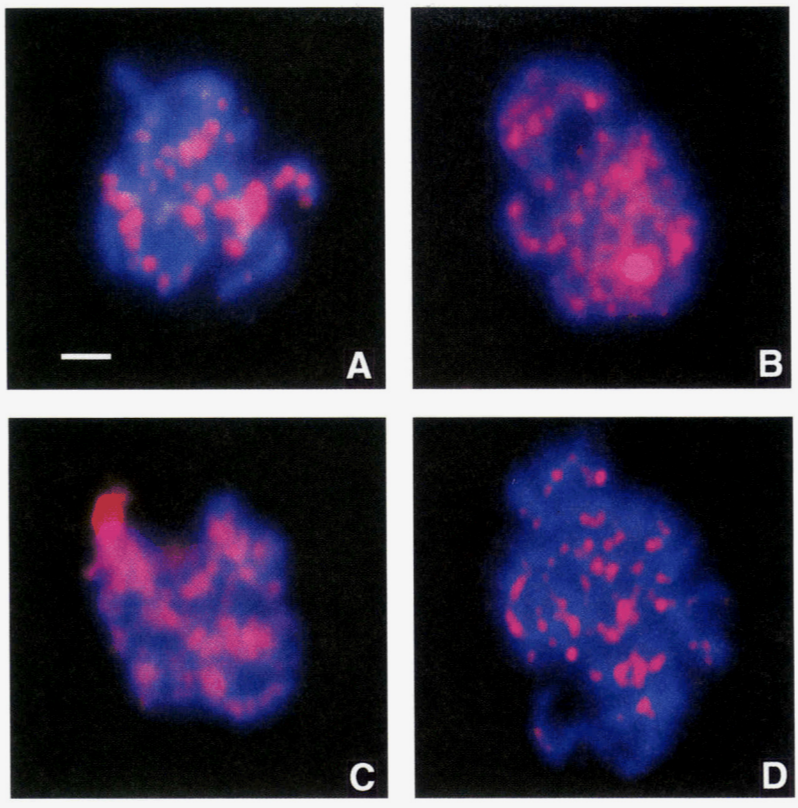

FIGURE 2.—Immunofluorescence micrographs of *DMC1* wild-type and mutant proteins (red signal) in meiotic prophase nuclei labeled with polyclonal antibodies directed against Dmc1p (A–C) and with monoclonal antibody directed against the HA epitope (D). DNA (blue signal) is stained with DAPI. Mutant protein (B and C) and wild-type protein in the dominant mutant background (D) accumulate in nuclei blocked from progression through meiotic prophase. (A) Wild-type protein in wild-type (MDD1) nucleus. (B) *dmc1-K69E* protein in *dmc1-K69E/dmc1-K69E* (MDD4) nucleus. (C) *DMC1-G126D* protein in a *DMC1-G126D/dmc1*Δ (MDD12) nucleus. (D) *DMC1-HA* protein in a *DMC1-HA/DMC1-G126D* (MDD9) nucleus. Magnification bar, 2 μm.

sis or meiotic prophase less synchronous in these strains, or there may be a more direct, meiotic cause for the delay. The delay in DSB accumulation is consistent with the delay in appearance of induction of gene conversion (but see *Speculation,* below).

Cells with *dmc1*Δ *rad51*Δ fail to repair meiotic DSBs (Figure 5, A and B) and fail to complete sporulation like *dmc1*Δ but lose viability during sporulation like *rad51*Δ (Figure 6). The higher final levels of DSB fragments in *dmc1*Δ *rad51*Δ as compared with *rad50S* (Figure 5A) probably result from nonspecific breaks that accumulate as cells lose viability, consistent with a heavier background in lanes from later timepoints (Figure 5B). Unlike either single mutant, meiotic induction of gene conversion is apparently absent in *dmc1*Δ *rad51*Δ (Figure 1). Spontaneous levels of gene conversion during vegetative growth are similar in *rad51*Δ and *dmc1*Δ *rad51*Δ cells (Figure 1, initial timepoint, and data not shown). Viability following γ-irradiation was used to assess DNA DSB repair capability of the strains in nonmeiotic cells. In log-phase growth, radiation sensitivity of cells with *dmc1*Δ is similar to that of wild type (Figure 7; BISHOP *et al.* 1992), and cells with *rad51*Δ are considerably more sensitive (SHINOHARA *et al.* 1992). In late stationary phase, *dmc1*Δ partially suppresses the radiation sensitivity of *rad51*Δ (Figure 7). This suppression develops as the cells continue shaking at 30° for several days after reaching stationary phase (data not shown). The strain background used here does not sporulate under these conditions (unlike other strain backgrounds, for example SK-1) but this suppression nevertheless could represent a "partial entry" into meiosis.

Meiotic DNA double-strand break fragments accumulate differently in *rad50S* vs. *DMC1* mutant strains: Persistence of DNA double-strand breaks in *rad50S* strains results in the accumulation of chromosome fragments that can be assayed on blots of CHEF gels (GAME 1992; ZENVIRTH *et al.* 1992). The locations and relative abundance of the bands in *rad50S* are different from that seen for the *dmc1*Δ and *DMC1-G126D* strains, and *rad50S* is epistatic to *dmc1*Δ for band position and intensity (Figure 8) as it is for DSB processing at a specific DSB hot-spot (BISHOP *et al.* 1992). Although there is some variation in the appearance of the bands in different blots representing a given mutant (compare *rad50S* in Figure 8, A and B), the differences between mutants, in the relative positions as well as in the relative densities of bands, are consistent.

TABLE 2

Levels of 2-hybrid interactions

	Activation domain fusion		
	DMC1	*dmc1-K69E*	*RAD51*
Binding domain fusion			
DMC1	106.2 ± 60.9[a]	8.0 ± 1.4	0.9 ± 0.3
dmc1-K69E	18.7 ± 1.9	0.7 ± 1.0	ND
RAD51	0.2 ± 0.3	ND	25.6 ± 2.4

[a] Miller units of activity.

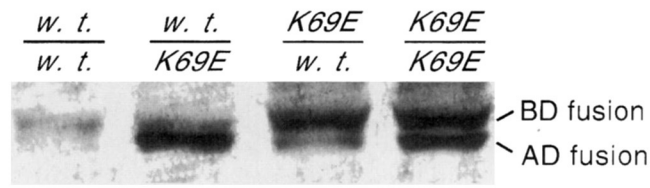

FIGURE 3.—Western blot of two-hybrid DNA-binding and activation-domain fusion proteins in Y190, encoded by *DMC1* constructs pMDE422 and pMDE467, and *dmc1-K69E* constructs pMDE486 and pMDE501 (see MATERIALS AND METHODS), labeled with polyclonal antibodies directed against Dmc1p. The *dmc1-K69E* fusion proteins accumulate to higher levels than the *DMC1* fusion proteins.

DISCUSSION

Homotypic interaction and the nucleotide binding site are important for *DMC1* function: Sequence and predicted structural similarities between *recA* and the two yeast genes *DMC1* and *RAD51* suggest similar functions for the three proteins (STORY *et al.* 1993). Biochemical analysis has indicated that yeast Rad51p, like RecAp, is a DNA-dependent ATPase that coats DNA to form a helical filament and facilitates strand exchange, although in a polarity opposite to that of RecAp (SUNG and ROBBERSON 1995). Similar *in vitro* activities have been difficult to demonstrate for Dmc1p (our unpublished results) but we do have genetic evidence that two activities important for *recA* function, self-association and binding of ATP (ROCA and COX 1990; KOWALCZYKOWSKI 1991; RADDING 1991; WEST 1992), are also required for *DMC1* function.

The *dmc1-K69E* mutation simultaneously eliminates mutant protein homotypic interaction and *in vivo* function. The observation that the *dmc1-K69E* bait fusion protein still interacts with other two-hybrid prey fusions that interact with wild-type Dmc1p bait supports the conclusion that this mutation specifically eliminates the ability for self-association. Further, the mutation does not completely eliminate interaction between *DMC1* and *dmc1-K69E* proteins, suggesting that one pairwise interaction is still available when wild-type protein is available and that the interaction depends on two differ-

TABLE 3

Genes identified by 2-hybrid interaction with *GBD-DMC1*

	Location					Interaction with		
Gene	Chromosome	ORF (start. . .stop)	Residues	Hits	Fusion starts	*DMC1*	*RAD51*	Comments
DMC1	V	548417. . .549513[a]	334	1	548646	++	−	
				2	548712	++	+/−	
TID1	II	383065. . .385941	958	2	383187	+	+/−	*YBR073W; RDH54*
TID2	XII	398319. . .395758	853	1	396916	+	−	*YLR127C*
				1	396634	+	−	
				1	396565	++	+	
				1	396481	+	−	
				3	396379	++	+	
				1	396364	++	+	
				1	396358	++	++	
TID3	IX	78074. . .80149	691	1	78680	++	−	*YIL144W*
				1	78760	++	−	
TID4	XV	692473. . .6973312	1619	1	695001	++	−	*YOR191W; RAD16* homology; zinc finger
SWI5	IV	750780. . .7486501	709	1	750401	++	−	Transcriptional activator
				1	750379	++	−	
PET309	XII	270710. . .267813	965	2	270495	++	−	Nuclear gene; mitochrondrial RNA stability
ACC1	XIV	661373. . .654672	2233	1	655161	++	−	Acetyl CoA carboxylase
PDC1	XII	234081. . .232390	562	1	233608	++	−	Pyruvate decarboxylase
				1	233701	++	−	
PDC5	XII	410723. . .412414	563	2	411184	++	−	Pyruvate decarboxylase

[a] Base numbers from Stanford Genome Database.

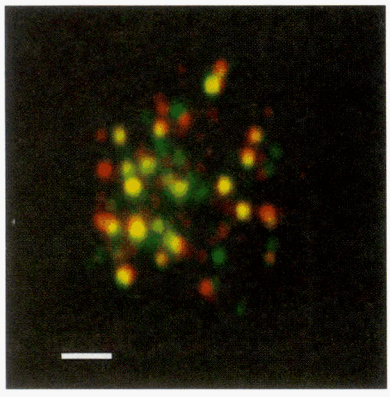

FIGURE 4.—Immunofluorescence micrograph of *DMC1* (red) and *RAD51* (green) proteins in a wild-type (MDD1) meiotic prophase nucleus. Colocalization of *DMC1* and *RAD51* proteins results in overlap of the signals to give yellow. In many spots only one signal is apparent, or the two signals lie side-by-side. Magnification bar, 2 μm.

ent sites with little or no overlap. These observations are in good accord with what is known of *recA* protein interactions (YARRANTON and SEDGWICK 1982; STORY *et al.* 1992; and see JIANG *et al.* 1993) and with conservation between Dmc1p and recAp (STORY *et al.* 1993). Dmc1p residue 69 maps to one of the two sites on recAp that appear at the interface between adjacent subunits in the recAp polymer, at the N-terminus between/at α-helix "A" and β-sheet "O," (Figure 9; see STORY *et al.* 1993 and Discussion in CHANET *et al.* 1996). The surrounding segment is reasonably well-conserved among *DMC1* and *RAD51* homologues but K69 in *DMC1* is the first of three residues that show nonconservative changes in the *DMC1 vs.* the *RAD51* families (Figure 9). The differences in these residues may account for the specificity of Dmc1p-Dmc1p interactions, in particular with the failure of Dmc1p and Rad51p to interact directly (see below). Further two-hybrid analysis coupled with *in vitro* mutagenesis will help to map functional sites where Dmc1p interacts with itself and with other proteins.

The dominant *DMC1-G126D* mutation lies in the putative ATP-binding site of Dmc1p at a very well-conserved residue corresponding to the GLY at residue 71 in *recA* (in the A-site motif; see STORY *et al.* 1993; Figure 9). *RecA* protein binds DNA tightly in the presence of ATP and must hydrolyze the ATP to dissociate from the complex (SHIBATA *et al.* 1979; WEINSTOCK *et al.* 1981; MENETSKI and KOWALCZYKOWSKI 1985). Substituting glycine 126 by the relatively bulky and charged aspartate is likely to interfere with ATP binding but we assume that the *DMC1*-protein still engages the recombination machinery to exert its dominant effect. Presumably, addition of the second, *K69E,* mutation prevents the dominant effect by eliminating engagement of the doubly mutant protein in the putative Dmc1p polymer/recombination complex even though it does not prevent accumulation of protein in the nucleus (Figure 3). The homologous mutation in *RAD51, RAD51-G190D,* is similarly null for activity. In addition, *RAD51-G190D* is dominant (and nearly null) for reductions in viability and recombination following treatment with γ-irradiation in vegetative cells (CHANET *et al.* 1996). Surprisingly, the *RAD51-G190D/RAD51* heterozygote shows only a relatively mild reduction in spore viability, from 98% in wild type to 69% in the heterozygote (*vs.* 10% in *rad51Δ;* CHANET *et al.* 1996).

***DMC1* protein does not interact directly with *RAD51* or other *RAD52* epistasis group proteins:** The possibility that Dmc1p and Rad51p might interact directly is suggested by their homologies and by their immunocytological colocalization (Figure 4; BISHOP 1994). Nevertheless, in a two-hybrid assay using essentially full-length fusions, Dmc1p does not interact with Rad51p, even though each interacts homotypically. Furthermore, Dmc1p does not interact with two other proteins in the *RAD52* epistasis group for which interaction with

TABLE 4

Coincidence of Dmc1p and Rad51p spots

Stages of nuclei	No. of nuclei	Dmc1p	Rad51p	Coincident
Mixed, all spots	66	65 ± 14	49 ± 17	21 ± 11
Mixed, bright spots	37	28 ± 10	18 ± 8	9 ± 5
Early prophase	25	24 ± 15	12 ± 11	5 ± 6
Pachytene	14	20 ± 8	15 ± 7	8 ± 3

Photographs were made of nuclei in samples taken at 4, 5 and 6 hr after the shift into sporulation, where 0/22, 8/24 and 6/20 nuclei were judged from their DAPI-stained fluorescence appearance to be in pachytene (DRESSER and GIROUX 1988). Numbers reported are total numbers of spots per nucleus ±1 SD. The following are scored: (1) all visible spots in all nuclei ("Mixed, all spots"), (2) the brighter spots in all nuclei ("Mixed, bright spots," where the "Offset" in the Image-1/AT image analysis program was increased to a level such that only 43% of the Dmc1p spots and 38% of the Rad51p spots remained visible), (3) the brighter spots in early prophase nuclei ("Early prophase," where only nuclei from the four hour time-point with homogeneous chromatin are scored), and (4) the brighter spots in pachytene nuclei ("Pachytene"). Spots are scored as "Coincident" if their signals touch or overlap with the offset set at the "all spots" level (increasing the offset reduces the area covered by each spot).

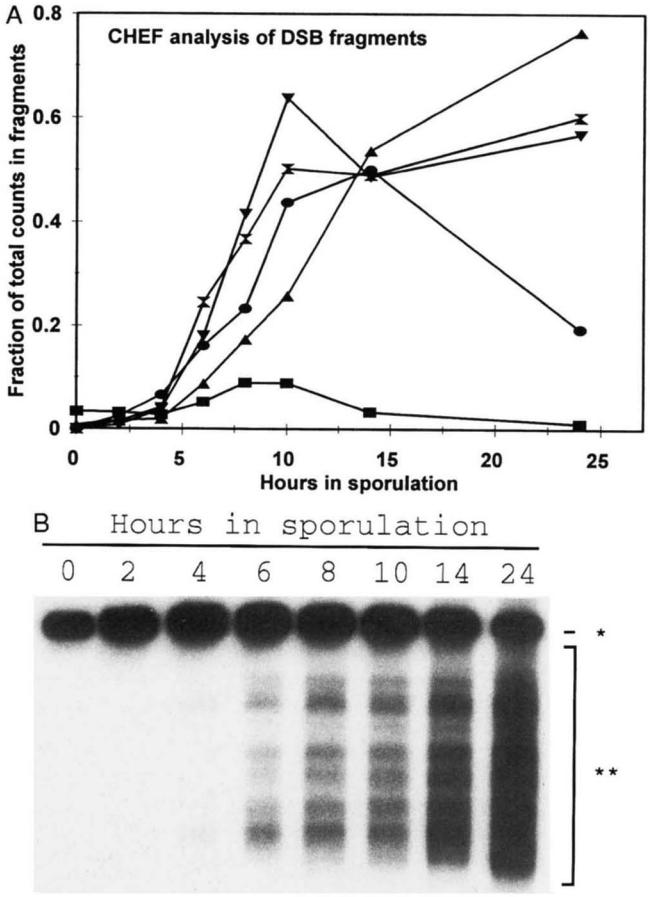

FIGURE 5.—Quantification of chromosome III double-strand break fragment accumulation during meiotic prophase (A) with one example of the primary data shown in (B). (A) Phosphoimager quantification of fragments from cells taken at different time-points during sporulation, representing the signal over the fragments (** in B) divided by the total signal over unbroken chromosome III (* in B) plus the fragments. The chromosome IIIL probe was used for all experiments. Wild type (MDD1), squares; *dmc1Δ/dmc1Δ* (MDD5), inverted triangles; *dmc1Δ/dmc1Δ rad51Δ/rad51Δ* (MDD7), triangles; *rad51Δ/rad51Δ* (MDD6), circles; *rad50S/rad50S* (MDD10), filled hourglasses. (B) Autoradiograph of Southern blot of a CHEF gel of fragments from a *dmc1Δ/dmc1Δ rad51Δ/rad51Δ* strain (MDD7; quantification plotted in A).

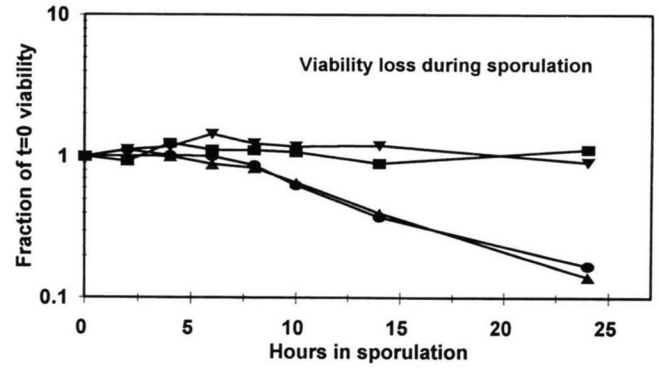

FIGURE 6.—Viability of sporulating cells. Samples of sporulating populations of cells were washed, diluted and plated on rich medium for colony counts and were compared with the colony counts at the time (=0 hr) of shift into sporulation medium. Wild type (MDD1), squares; *dmc1Δ/dmc1Δ* (MDD5), inverted triangles; *dmc1Δ/dmc1Δ rad51Δ/rad51Δ* (MDD7), triangles; *rad51Δ/rad51Δ* (MDD6), circles.

Rad51p has been demonstrated, Rad52p and Rad54p (SHINOHARA *et al.* 1992; DONOVAN *et al.* 1994; HAYS *et al.* 1995; JIANG *et al.* 1996). These results suggest that Dmc1p may form a complex (see below) that largely is separate from the Rad51p complex and further suggests that Dmc1p and Rad51p colocalize either because of common or neighboring substrates or because some bridging protein(s) connects the complexes.

***DMC1* protein interacts with a unique set of proteins:** Fusions representing 10 different open reading frames were identified as interacting with Dmc1p in a screen of a randomly sheared yeast genomic DNA library (Table 3). Two of the genes have homology to known DNA repair genes, *TID1* being most similar to *RAD54* (BLAST score 8.0E-120, see ALTSCHUL *et al.* 1990; recently named *RDH54* and implicated in vegetative repair, H. KLEIN, personal communication), and *TID4* being most similar to *RAD16* (BLAST score 2.3e-72). The importance of the *DMC1-TID1* interaction is suggested both by the similar *RAD51-RAD54* interaction and by epistasis analyses that place *RAD51* and *RAD54* in a narrowly defined common pathway (see LIEFSHITZ *et al.* 1995). In addition, we have observed that *TID1* is required in meiosis and that epistasis places it in the *DMC1,* not the *RAD51,* pathway (M. DRESSER and D. EWING, unpublished results). Finally, both *TID1* and *RAD54* appear to have homologues in *Schizosaccharomyces pombe* (Swiss-Prot protein sequence database accession Q09772 and gene described in MURIS *et al.* 1996, respectively), suggesting conservation of separate complexes as postulated here.

Whether any of the other two-hybrid interactions are biologically significant remains to be seen, as some detritus is to be expected from the two-hybrid screen. The pattern of interactions of *TID2* with *DMC1* and *RAD51* might reflect a role for the *TID2* protein in physically coupling or perhaps in regulating the respective activities of the Dmc1p- and Rad51p-containing complexes. Interaction with *SWI5,* which is required for normal exit from mitosis (TOYN *et al.* 1997), could possibly play a regulatory role in the cell cycle block occasioned by deletion of *DMC1.* The importance of Dmc1p interactions with any of the gene products identified by the screen can be determined by assessing the phenotypes (if any) caused by mutations in *DMC1* that eliminate selected interactions.

***DMC1* and *RAD51* function in largely separate pathways:** Our observations indicate the extent to which some function(s) of the *DMC1* and *RAD51* proteins are not interchangeable. *DMC1* and *RAD51* both are required for normal meiotic DNA DSB metabolism and their deletion phenotypes are different (BISHOP *et al.* 1992; SHINOHARA *et al.* 1992; BISHOP 1994; ROCKMILL

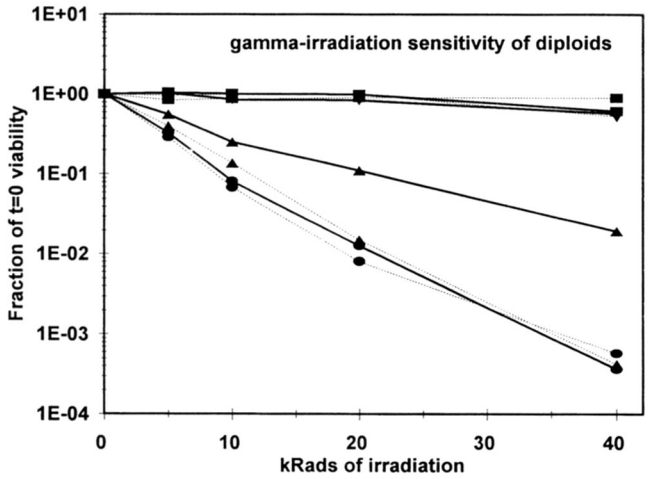

FIGURE 7.—Sensitivity to γ-irradiation. The loss of viability following successive doses of γ-rays from a ^{137}Cs source was measured for diploids grown to stationary phase (—) or in log phase (- - -) by plating dilutions on rich medium and dividing the number of colonies at each dose by the number from the untreated population. Wild type (MDD1), squares; *dmc1*Δ/*dmc1*Δ (MDD5), inverted triangles; *dmc1*Δ/*dmc1*Δ *rad51*Δ/*rad51*Δ (MDD7), triangles; *rad51*Δ/*rad51*Δ (MDD6), circles.

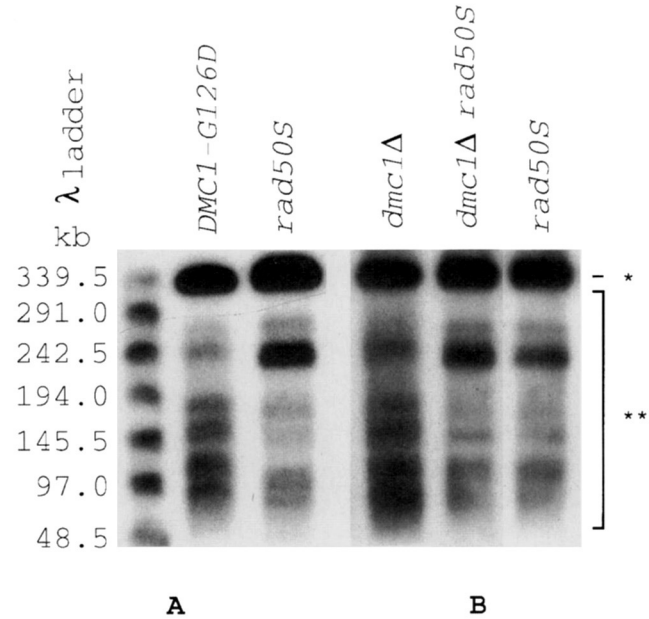

FIGURE 8.—Comparison of DNA double-strand break fragment accumulation in *rad50S vs. DMC1* mutant strains. (A) Comparison of *DMC1-G126D*/*dmc1*Δ (MDD2) and *rad50S*/*rad50S* (MDD10) strains, demonstrating differences in band intensities and positions. (B) Comparison of bands from *dmc1*Δ/*dmc1*Δ (MDD5), *dmc1*Δ/*dmc1*Δ *rad50S*/*rad50S* (MDD11) and *rad50S*/*rad50S* (MDD10) strains, demonstrating that *rad50S* is epistatic to *dmc1*Δ for band intensities and positions. Samples were taken at 14 hr into sporulation.

and ROEDER 1994; ROCKMILL *et al.* 1995). Furthermore, contrary to indicating a simple epistasis relationship, the double *dmc1*Δ *rad51*Δ mutant phenotype is not completely like that of either of the single mutants. Instead, *dmc1*Δ *rad51*Δ cells accumulate meiotic DSBs and block in meiotic prophase as in *dmc1*Δ but lose viability during sporulation as in *rad51*Δ and, uniquely, show no induction of gene conversion. In the return-to-growth assay, the induction of gene conversion in meiosis can depend on mitotic repair for completion, and it is conceivable that *DMC1* might have a previously unrecognized role in recombination repair of DSBs in mitotic cells that accounts for the absence of viable gene convertants. If so, then by comparison with *rad51*Δ, the *dmc1*Δ *rad51*Δ strain might (1) lose viability more rapidly or completely during sporulation (as measured by return to vegetative growth), (2) show a decrease in spontaneous conversion during vegetative growth, or (3) be considerably more sensitive to γ-irradiation-induced DNA damage. None of these are observed (Figure 6; Figure 1, $t = 0$ timepoint and unpublished observations; Figure 7, respectively).

Immunoctytological colocalization of the *DMC1* and *RAD51* proteins (BISHOP 1994 and RESULTS) suggests that these proteins are required for different aspects of single recombination events as demonstrated genetically. The absence of a cytologically detectable coincidence in every case, in our strains, does not rule out the possibility that each protein is required at each event. *DMC1* clearly functions in a *rad51*Δ strain in the absence of an immunocytological signal that is wild type in magnitude, indicating that the accumulation of protein required to make cytologically bright spots is not required for some *DMC1* function. This observation is consistent with the suggestion that turnover of Dmc1p at many or most sites may account for the shortfall of Dmc1p spots with respect to the number expected if Dmc1p were to be present at all recombinational interactions (BISHOP 1994). Rad51p accumulation may be similarly transient, a possibility that would account for the relatively low incidence of colocalization if, for example, the processing of double-strand breaks is less synchronous within a single nucleus in our strain background as compared with SK-1. Mutations in components in the two pathways, or in separate regulatory pathways, will help to address these issues and the timing of the cytological and physical events.

***rad50S* is epistatic to *DMC1* mutations for the appearance of DNA double-strand break fragments in CHEF gels:** Meiotic recombination in yeast follows the formation of DSBs (GAME *et al.* 1989; SUN *et al.* 1989; CAO *et al.* 1990) and a general correlation between frequencies of DSBs and levels of crossing over at defined sites in a *rad50S* diploid are consistent with a cause-effect relationship (LICHTEN and GOLDMAN 1995). At the *his4::LEU2* artificial recombination hot-spot, DSBs accumulate in *rad50S* and in *dmc1*Δ strains (ALANI *et al.* 1990; CAO *et al.* 1990; BISHOP *et al.* 1992). It is possible that DSB accumulation is higher with *rad50S dmc1*Δ than with *dmc1*Δ alone (LYDALL *et al.* 1996), though our results indicate instead that the amount of label

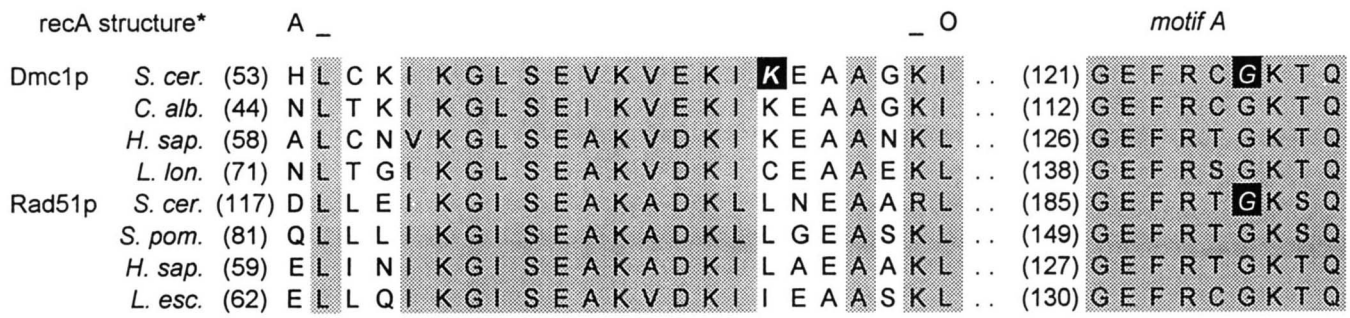

FIGURE 9.—Comparison of the amino acid sequences of selected members of the *DMC1* (*S. cerevisiae, C. albicans, H. sapiens, L. longiflorum*) and *RAD51* (*S. cerevisiae, S. pombe, H. sapiens, L. esculentum*) families, in the regions surrounding the K69E and G126D mutant substitutions. Well conserved residues are shaded gray; residues changed by mutations discussed here are shaded black. The number of the first residue in each sequence is in parentheses. *The *recA* structures are from STORY *et al.* (1993).

representing the sum of the broken fragments is similar in different mutant backgrounds (Figure 5A). One possibility is that fragments are lost to recovery following the step blocked by *rad50S* but are replaced by new fragments in different locations (*i.e.*, new DSBs) before the step blocked by *dmc1*Δ. If so, the total number of DSBs presumably is regulated even in the mutants so that the final levels are similar. Another possibility is that some anomaly of migration of the fragments in the gel is responsible, perhaps because one end of the *rad50S* fragments is blunt and covalently attached to a polypeptide (DE MASSY *et al.* 1995; KEENEY and KLECKNER 1995; LIU *et al.* 1995) while one end of the *DMC1* mutant fragments has a long 3′ single-strand tail (BISHOP *et al.* 1992; the other end in the present experiments presumably is telomeric and may not be broken; see KLEIN *et al.* 1996). Experiments to resolve these possibilities are underway.

Speculation: Our observations emphasize the extent to which *DMC1* functions independently of *RAD51* in meiotic DSB repair, in a complex that includes *TID1* (M. DRESSER and D. EWING, unpublished results) and probably other genes listed in Table 3. Nevertheless, the similarities between *DMC1* and *RAD51* and their associated proteins suggest a similarity of function that for *DMC1* presumably is adapted specifically to meiotic prophase. *RAD51* is required for DSB repair in specific chromatin contexts (SUGAWARA *et al.* 1995). Similarly, *DMC1* may be required for DSB repair in chromatin with a meiosis-specific organization that is inaccessible to the *RAD51* pathway acting alone. In the absence of *DMC1*, a *RED1*-dependent process arrests meiosis in the SK-1 background (XU *et al.* 1997) and in the strain background employed here (M. DRESSER and D. EWING, unpublished results). Abrogation of the arrest by non-null mutation in *RED1* (M. DRESSER and D. EWING, unpublished results) or in the BR strain background (ROCKMILL and ROEDER 1994) allows completion of sporulation but with defects in recombination and chromosome segregation. The proposed activity for Dmc1p could promote synapsis and foster crossing over in the context of appropriate chromatid and homologue associations to make chiasmata, a role that has been suggested for *DMC1* (ROCKMILL and ROEDER 1994).

Our observations could be explained as follows:

1. In cells with *dmc1*Δ, the *RAD51* pathway fosters entry into chromatin and recombinogenic repair of meiotic DSBs but only following events that are prevented by the *RED1*-mediated block or following return to growth. The observed reductions in recombination (ROCKMILL and ROEDER 1994; M. DRESSER and D. EWING, unpublished results) suggest that in these strains some fraction of the DSBs that in *DMC1* would give rise to interhomologue recombination and chiasmate associations are in *dmc1*Δ repaired non-recombinogenically, perhaps via sister-sister interaction, the pathway that is preferred in vegetative cells (KADYK and HARTWELL 1992) and is fostered by *RAD51* in the absence of *DMC1*.

2. In cells with *rad51*Δ, the *DMC1* pathway repairs DSBs, though inefficiently and only after some delay and, potentially (given the low spore viability), without orienting the chromosomes for disjunction.

3. In cells with *dmc1*Δ *rad51*Δ, neither pathway is available for DSB repair, and the cells either are killed by irreparable DSBs or survive by repair that does not lead to gene conversion, presumably by sister-sister recombinational repair, by non-homologous end-joining repair (MOORE and HABER 1996), or by an as yet undefined mechanism.

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