A General Method for Identifying Recessive Diploid-Specific Mutations in *Saccharomyces cerevisiae*, Its Application to the Isolation of Mutants Blocked at Intermediate Stages of Meiotic Prophase and Characterization of a New Gene *SAE2*

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ABSTRACT

We describe a general new approach for identifying recessive mutations that affect diploid strains of yeast *Saccharomyces cerevisiae* and the application of this method to the identification of mutations that confer an intermediate block in meiotic prophase chromosome metabolism. The method uses a temperature-sensitive conjugation mutation *ste7-1* in combination with homothallism. The mutations of interest confer a defect in spore formation that is dependent upon a gene required for initiation of meiotic recombination and development of meiosis-specific chromosome structure (*SPO11*). Identified in this screen were null mutations of the *DMC1* gene, nonnull mutations of *RAD50* (*rad50S*), and mutations in three new genes designated *SAE1*, *SAE2* and *SAE3* (Sporulation in the Absence of Spo Eleven). Molecular characterization of the *SAE2* gene and characterization of meiotic and mitotic phenotypes of *sae2* mutants are also presented. The phenotypes conferred by a *sae2* null mutation are virtually indistinguishable from those conferred by the previously identified nonnull mutations of *RAD50* (*rad50S*). Most notably, both mutations confer only weak sensitivity to the radiomimetic agent methyl methane sulfonate (MMS) but completely block resection and turnover of meiosis-specific double-strand breaks. These observations provide further evidence that this constellation of phenotypes identifies a specific molecular function.

SEXUALLY reproducing organisms must reduce their genome complement by half when producing gametes so that the succeeding generation, formed by the fusion of two gametes, is of the same ploidy as the current generation. In general this reduction in ploidy is achieved by meiosis, a specialized type of division where a single round of DNA synthesis is followed by two rounds of chromosome separation producing four daughter nuclei. In the first round (meiosis I, MI) homologous chromosomes are disjoined, and in the second round (meiosis II, MII) sister chromatids separate in a manner that resembles mitotic anaphase. In yeast, as in most other organisms, proper disjunction of homologs in MI requires that they be connected. The requisite connection is provided by one or more crossovers in combination with connections between sister chromatids (and possibly other features) (CARPENTER 1994; KLECKNER 1996). Correspondingly, recombination between homologs is induced in meiotic cells. In *Saccharomyces cerevisiae* the frequency of meiotic recombination is about four orders of magnitude higher than the per generation rate of spontaneous mitotic recombination (PETES *et al.* 1991; see below).

In yeast meiotic recombination is initiated by the creation of a double-strand break (DSB) in one (or more) of the four chromatids of a bivalent (ROEDER 1995; GOLDMAN and LICHTEN 1996). Following the cut, the two 5′ chains of the broken chromosome are resected to produce a single-stranded tail. This tail invades DNA of a homologous chromosome and ultimately yields a double Holliday junction. It is generally assumed that double Holliday junctions are resolved into both crossover and noncrossover recombination products (SCHWACHA and KLECKNER 1995 and references therein).

Meiotic prophase also involves the development of an axial structure along each chromosome, *i.e.*, each pair of sister chromatids, and polymerization of synaptonemal complex (SC), which joins the structural axes of each pair of homologs. Recombination and SC formation are functionally related events. Many mutants coordinately affect both processes (*e.g.*, ROEDER 1990; KLECKNER *et al.* 1991), and, in some organisms, there is a 1:1 correspondence between the sites of crossover recombination events and SC initiation sites (ZICKLER *et al.* 1992; MAGUIRE and RIESS 1994). The weight of current evidence suggests that normal execution of recombination is required for normal SC formation, rather than the converse (ROEDER 1990; KLECKNER *et al.* 1991; HAWLEY and ARBEL 1993; ROEDER 1995; KLECKNER 1996). It is unclear whether the SC plays any part

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in recombination. For instance, the recombination defect in strains mutant for *ZIP1* could be independent of the structural role Zip1p serves in the SC (for discussion, see KLECKNER 1996; STORLAZZI *et al.* 1996).

Earlier work in yeast has revealed that an intermediate block to meiotic prophase chromosome metabolism can cause a defect in spore formation (MALONE 1983; ALANI *et al.* 1990; BISHOP *et al.* 1992). In contrast mutations that block prophase at early stages do not confer such a block and are epistatic to intermediate block mutations for this phenotype (MALONE 1983; BISHOP *et al.* 1992). In general the spore formation defect accompanies, and is likely largely a reflection of, a defect in progression of meiosis beyond the pachytene stage. Specifically, all intermediate block mutations confer either permanent arrest or transient delay at the end of prophase as assayed by analysis of either meiosis I spindle pole body separation and/or telophase I; early block mutations do not cause any delay in these processes (*e.g.*, XU *et al.* 1997).

These two phenotypes are illustrated by mutations in the *RAD50* gene. During meiosis, *RAD50* is required for the formation of meiosis-specific DSBs; it is also required for normal axial element formation and for formation of tripartite SC (ALANI *et al.* 1990). Correspondingly, a *rad50* null mutation does not impede formation of the two meiotic divisions or spore formation (*e.g.*, ALANI *et al.* 1990). Thus, a *rad50* null mutation can be considered to confer an early block in prophase chromosome metabolism.

In contrast, certain nonnull alleles of the *RAD50* gene, termed *rad50S*, cause meiosis-specific DSBs to accumulate in an unresected form and permit normal axial element formation but still cause a specific delay and defect in formation of tripartite SC. Moreover, *rad50S* mutants exhibit a 4-hr delay in the onset of meiosis I (telophase) and a defect in spore formation (ALANI *et al.* 1990; XU *et al.* 1997). Furthermore, the defect conferred by a *rad50S* mutation is alleviated by an upstream block in prophase chromosome metabolism that eliminates both DSBs and formation of both axial elements and tripartite SC, *i.e.*, a null mutation in the *SPO11* gene (ALANI *et al.* 1990). Thus, a *rad50S* mutation can be considered to be an "intermediate block" mutation (KLECKNER *et al.* 1991; XU *et al.* 1997).

At the time the current work was initiated, the only mutations known or recognized as intermediate block mutations were *rad50S* (ALANI *et al.* 1990) and *rad52* (MALONE 1983). We reasoned by analogy that additional mutations of this type could be identified as mutations that block spore formation in an otherwise wild-type strain but not in a strain carrying an early block mutation such as a null mutation in the *SPO11* gene. In the interim, null mutations in at least three other genes have been identified in which mutations confer an intermediate block phenotype. These three genes are *DMC1*, which encodes a meiosis-specific RecA homolog; *RAD51*, which encodes a cousin of *DMC1* that is found in all types of cells; and *ZIP1*, which encodes a structural component of the SC and also is required for meiotic recombination independent of any potential for SC formation (BISHOP *et al.* 1992; SHINOHARA *et al.* 1992; SYM *et al.* 1993; SYM and ROEDER 1994; STORLAZZI *et al.* 1996). These and other findings point to the likelihood that meiotic cells have a checkpoint regulatory system that monitors the progress of prophase chromosome metabolism. It appears that this system monitors the status of interhomolog recombination complexes in their normal meiotic chromosome context (LYDALL *et al.* 1996; XU *et al.* 1997).

The current work was initiated with the goal of identifying additional intermediate block mutations. To this end a new method of identifying mutants defective in spore formation was developed and numerous mutants were isolated. A large number of such mutants were then examined further for their ability to make spores after *SPO11* function was eliminated. The results presented describe the identification of eight new intermediate block mutations, named *SAE*, for Sporulation in the Absence of Spo Eleven. Three of these mutations identify three new genes. *SAE1* and *SAE3* have meiosis-specific phenotypes and are described in MCKEE and KLECKNER (1997). *SAE2*, which is not meiosis-specific, is described here. The *sae2* null mutant phenotypes are virtually identical to the previously identified phenotypes of *rad50S* nonnull mutant in both mitotic and meiotic cells, as described below. The identification of this gene provides a strong indication that the "S" function of *RAD50* corresponds to a specific function at the molecular level, the nature of which remains to be determined. The *SAE2* gene has also been identified and named *COM1* by PRINZ *et al.* (1997), using an approach conceptually similar to that of this report.

The genetic approach developed for this work is applicable more generally to the isolation of a number of other types of mutants that specifically affect the viability or behavior of vegetatively growing diploid cells as compared to haploids.

MATERIALS AND METHODS

Media, strains and plasmids: All yeast strains used in this work were derived from the rapidly sporulating strain SK1 (KANE and ROTH 1974; ALANI *et al.* 1990), with the exception of the *mre11::hisG* strain (KJ5-A2, provided by K. JOHZUKA) that was used in complementation tests (Table 1). Media preparation and genetic procedures generally followed SHERMAN *et al.* (1986). Specific media used were as follows: SPM, 1% potassium acetate, 0.1% glucose, 0.125% Difco yeast extract, supplemented with 100 mg/liter adenine sulfate; MYPD, 0.3% malt extract, 0.3% yeast extract, 0.5% bacto peptone and 1.0% glucose; SC is SD supplemented with 100 mg/liter of each of the 20 amino acids, 50 mg/liter of both uracil and adenine, and 10 mg/liter of para-amino benzoic acid; omission media is SC minus particular amino acids or bases; SC-lactate is SC that contains 1% potassium lactate pH 6.0 rather than 2% glucose. The plasmid pNKY1159 is based on

TABLE 1

Yeast strains

Strain	Genotype
NKY278	*MATa lys2 ura3 ho::LYS2* *MATα lys2 ura3 ho::LYS2*
NKY487	*MATa lys2 ura3Δ::hisG leu2::hisG ho::hisG trp1::hisG*
NKY536	*MATα lys2 ura3 ho::hisG trp1::hisG*
NKY551	*MATa lys2 ura3 ho::LYS2 rad50Δ::hisG* *MATα lys2 ura3 ho::LYS2 rad50Δ::hisG*
NKY648	*MATa lys2 ura3 spo11Δ::hisG-URA3-hisG ho::LYS2* *MATα lys2 ura3 spo11Δ::hisG-URA3-hisG ho::LYS2*
NKY730	*MATa leu2::hisG lys2 ura3Δ::hisG HO* *MATα leu2::hisG lys2 ura3Δ::hisG HO*
NKY819	*MATa leu2::hisG his4XLEU2 ho::LYS2 lys2 ura3*
NKY895	*MATa leu2::hisG lys2 ura3Δ::hisG HO ste7-1 ade2::LK* *MATα leu2::hisG lys2 ura3Δ::hisG HO ste7-1 ade2::LK*
NKY1063	*MATα ho::LYS2 lys2 ura3 leu2::hisG ade2::LK*
NKY1113	*MATa leu2::hisG his4XLEU2-URA3 ho::LYS2 lys2 ura3 arg4-nsp* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 arg4-bgl*
NKY1245	*MATa leu2::hisG his4XLEU2 ho::LYS2 lys2 ura3 arg4-nsp rad50Δ::hisG* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 arg4-bgl rad50Δ::hisG*
NKY1276	*MATa leu2::hisG spo11Δ::hisG-URA3-hisG spo13::LEU2 ura3 lys2 ade2::LK ste7-1 HO* pNKY1159 *MATα leu2::hisG SPO11 SPO13 ura3 lys2 ade2::LK ste7-1 HO (SPO11 ADE2)*
NKY1370	*MATa ura3 lys2 ho::LYS2 leu2::hisG his4B rad50Δ::hisG*
NKY1408	*MATa leu2::hisG his4XLEU2(Bam)-URA3 ho::LYS2 lys2 ura3 rad50-K181::ura3* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 rad50-K181::ura3*
NKY1409	Same as NKY1408
NKY1459	*MATa ura3 lys2 ho::LYS2 leu2::hisG arg4-nsp dmc1Δ::LEU2*
NKY1552	*MATa leu2::hisG his4XLEU2(Bam)-URA3 ho::LYS2 lys2 ura3 arg4-nsp* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 arg4-bgl*
NKY1408	*MATa leu2::hisG his4XLEU2(Bam)-URA3 ho::LYS2 lys2 ura3 rad50-K181::URA3* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 rad50-K181::URA3*
NKY2601	*MATa leu2::hisG HO ste7-1 ade2::LK ura3Δ::hisG rad50Δ::hisG-URA3-hisG* *MATα leu2::hisG HO ste7-1 ade2::LK ura3Δ::hisG RAD50*
NKY2629	*MATa leu2::hisG his4XLEU2(Bam)-URA3 HO ste7-1 trp1::hisG lys2 ura3 sae2-1 ade2::LK* *MATα leu2::hisG his4BLEU2 HO ste7-1 TRP1 lys2 ura3 sae2-1 ADE2*
NKY2640	*MATa leu2::hisG his4XLEU2-URA3 ho::LYS2 lys2 ura3 arg4-nsp sae2Δ::hisG-URA3-hisG ade2::LK* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 arg4-bgl sae2Δ::hisG-URA3-hisG ADE2*
NKY2641	*MATa leu2::hisG his4XLEU2(Bam)-URA3 ho::LYS2 lys2 ura3 arg4-nsp sae2Δ::hisG-URA3-hisG ade2::LK* *MATα leu2::hisG his4BLEU2 ho::LYS2 lys2 ura3 arg4-bgl sae2Δ::hisG-URA3-hisG ADE2*
NKY2666	*MATa leu2::hisG his4BLEU2 lys2 ura3 spo11Δ::hisG-URA3-hisG sae2-1 HO ste7-1* *MATα leu2::hisG his4BLEU2 lys2 ura3 spo11Δ::hisG-URA3-hisG sae2-1 HO ste7-1*
NKY2728	*MATa leu2::hisG lys2 ura3 ste7-1 HO sae2-1* *MATα leu2::hisG lys2 ura3 ste7-1 HO sae2-1*
NKY2731	*MATa lys2 ura3 ade2::LK spo11Δ::hisG spo13::LEU2 HO ste7-1 rad50-KI81::URA3* *MATα lys2 ura3 ade2::LK spo11Δ::hisG spo13::LEU2 HO ste7-1 rad50-KI81::URA3* pNKY1159 (SPO11 ADE2)
KJ5-A2	*MATa ho::LYS2 leu2 his4-4 ura3 trp1 lys2 ade2 mre11::hisG*

pBR322 and contains *SPO11* and *ADE2* genes and *CEN3* (McKee 1996).

Cloning of the *ste7-1* allele and introduction into SK1 strains: The wild-type *STE7* gene was provided on plasmid pSTE7.4, by D. Chaleff (Chaleff and Tatchell 1985). The *ste7-1* allele was cloned using the gap repair technique of Rothstein (1991) from strain 43a (Hartwell 1980). The specifics are as follows: the *STE7* gene was subcloned from pSTE7.4 on a 7.3-kilobase (kb) *Bam*HI fragment into *Bam*HI-cut YCp50 to make pNKY1154. The coding sequence of *STE7* was deleted from this plasmid by cutting with *Sac*I to remove a 5.5-kb internal fragment and ligated closed to make pNKY1155. This plasmid was cut with *Sac*I prior to transformation of a *ura3* version of strain 43a (made by selection on 5-FOA; Boeke *et al.* 1984) to recover the *ste7-1* allele. This created pNKY1215, identical to pNKY1154 except it carries the gap rescued *ste7-1* allele. To integrate the temperature-sensitive allele into the SK1 strain background the *ste7-1* allele was subcloned on a 7.3-kb *Bam*HI fragment into the *Bam*HI site of YIp5 to make pNKY516. This plasmid was cut with *Xba*I to target it to the *STE7* locus upon transformation. The plasmid was integrated at the *STE7* locus of a heterothallic SK1 haploid strain; deletions of the wild-type allele and vector sequences were then selected as 5-FOA-resistant clones (Orr-Weaver *et al.* 1983; Boeke *et al.* 1984) and deletion strains carrying the *ste7-1* allele were identified by temperature-sensitive mating in the frequency of mating test (below).

Frequency of mating test: Haploid strains were tested for their ability to mate by mixing cells from each of two separate strains on a YEPD plate and then determining the frequency of diploids among total cells. Haploid strains carried complementing markers; diploids are selected as prototrophs. Diploid frequency was measured by titering the mixture on complete medium and on medium selective for the prototrophic diploids and calculating the ratio of the two titers. Details of the mating procedure are as follows: individual colonies of haploid strains to be tested were grown at either 34° (for *ste7-1*) or 30° (for wild type) on YEPD, unless otherwise noted. First, approximately 10^6 cells (1 mm^3) of one strain were transferred onto a sterile flat toothpick that was then touched onto a YEPD plate preheated to the desired testing temperature. For assays of mating at 34°, the actual manipulations were carried out in a 37° room to prevent the preheated plate from cooling. Then, approximately 10^6 cells of a second strain were similarly picked and spread into cells of the first strain; the mixture was then spread over a 1-cm^2 area of the plate. The plates were incubated for 18 hr, and then the entire patch (usually 10^8 cells) was scraped off the plate, resuspended in sterile water and dilutions were titered as described above. In the experiment where a frozen culture of homothallic *ste7-1* haploids was used, cells were grown into a patch at 34°, a portion of which was used for the mating test.

Fluorescence assay for sporulation: Located in the outermost layers of the ascospore wall is a dityrosine-containing peptide that fluoresces blue when it is illuminated with long-wave UV light (Briza *et al.* 1986). In the efficiently sporulating yeast SK1 it is unnecessary to treat the ascospores with either glusulase or ammonia as in previous reports (Briza *et al.* 1990a,b); the fluorescence is easily detected in a patch of cells on sporulation medium. Yeast colonies were tested for their ability to make spores by patching approximately 10^6 cells from a colony using a sterile toothpick onto SPM agar, incubating at 30° for 4 days, and then illuminating the patch with UV light and examining for blue fluorescence. The details are as follows: a sheet of gridded nitrocellulose (S&S brand, BA85/20, 0.45 μm) was placed on the surface of the agar using sterilized tweezers and the surface of the membrane was sterilized by exposure to 25 J of 256 nm UV light. This was done by removing the lid of the plate and placing a 4-W hand-held mercury lamp just above the surface of the agar for 30 sec. The room lights were left on during the UV exposure. The nitrocellulose is necessary to block out background fluorescence of the sporulation medium when the patches of yeast are illuminated with UV light. Cells from individual yeast colonies were picked up on the wide end of a flat toothpick and spread onto squares of the grid. The dish was incubated at 30° for 4 days by which time all sporulation was complete. Fluorescence was tested by illuminating the plate with a hand-held UV lamp (310 nm). Plates were photographed with black and white film through a Wratten 47B filter.

EMS mutagenesis: The diploid yeast strain NKY1276 was recovered from a frozen culture and grown on synthetic complete medium minus adenine. A single colony was picked, spread on YP glycerol medium and following an overnight incubation the cells from this patch were used to inoculate liquid SC medium lacking adenine. These were grown to late log phase, approximately 10^8 cells/ml. The cells were washed and resuspended in an equal volume of 10 mM sodium phosphate buffer, pH 7.0. Thirteen aliquots of 1.0 ml were made into sterile 1.5-ml screw-cap microfuge tubes. One vial was kept as a no mutagen control, and to the rest EMS (Sigma) was added to 3%. The EMS was dispersed into the solution by vigorous shaking, and the tubes were incubated at 22° on a rocking table. At 40 min, four of the aliquots were quenched into four separate 5-ml tubes of 5% Na-thiosulfate. These were centrifuged, washed in an additional 5 ml of 5% Na-thiosulfate and then centrifuged and resuspended in 1 ml of sterile water. Fifty aliquots of 10 μl of this cell suspension were spotted onto SPM agar. Each one of these spots was considered to be an independent pool of mutants for the subsequent screen. Four aliquots were incubated with EMS for 60 min and another four for 80 min, and these were treated by the same procedure. The SPM plates were incubated at 30° for 2 days, by which time sporulation of the treated cells was essentially complete. The independent patches of ascospores were scraped off the plates and frozen in individual tubes in 1 ml of 15% glycerol, 0.5% 2-mercaptoethanol at −70°.

Random spore preparation: Sporulated pools from the mutagenesis were used for random spore preparations. A tube containing a pool of $\sim 5 \times 10^7$ ascospores in 1.0 ml volume was thawed from −70°. To this was added 20 μl of zymolase (0.5 mg/ml zymolase 100T (ICN Biochemicals) in 10 mM $NaPO_4$, pH 7.0, 50 mM KCl, 4% glucose, 5 mM DTT) and then it was incubated at 37° for 30 min. The tube was vortexed and an additional 25 μl of zymolase solution was added and incubated for a further 30 min. Following the zymolase treatment the ascospore preparation was sonicated for three 30-sec bursts, with cooling on ice between treatments. The preparation was examined at the microscope and if less than 80% of the spores had been separated from their siblings further sonication was performed.

Assessment of mutagenesis: The extent of EMS mutagenesis was assayed by measuring the frequency of newly created canavanine-resistant mutants among random spores prepared from pools of each of the four EMS exposure time points. Random spores were titered for colony formation on synthetic complete medium lacking arginine and on synthetic complete medium lacking arginine and containing 25 μg/ml canavanine sulfate (Sigma). The frequency of canavanine-resistant spores derived from the 80-min EMS exposure was 3.11×10^{-3} (1/322). The 40- and 60-min EMS exposures resulted in lower frequencies. The frequency of survival of the spores in the 80-min EMS exposure was 26 of 52 that had been micromanipulated onto YEPD. Spores from cells untreated

with EMS give 50 colonies from 52 spores. The 80-min EMS exposure pools were used to screen for mutants.

Isolation of meiotic mutants, primary screen: Pools of random spores from EMS-treated strain NKY1276 were germinated at 34° on SC minus both adenine and leucine medium at ~50 colony-forming units (CFU) per 83-mm plate. These were incubated for 2 days and then colonies were picked on sterile flat toothpicks and touched to the following types of agar media in this order: YEPD adenine, YEP glycerol and the remainder of the colony mixed with patches containing $\sim 5 \times 10^6$ cells of the wild-type haploid strain NKY1063 on YEPD adenine. The YEPD adenine plate was used for diploidization of the mutant haploids prior to testing for sporulation (described below). YEP glycerol tests the cells for oxidative metabolism. The purpose of mixing the cells with NKY1063 on the final set of plates is to save the new mutations in a heterozygous diploid. These plates were incubated at 18° for 2 days, and then replica stamped to media selective for crossed diploids and incubated at 30° for 2 days. These plates were stored at 4° until potential mutants were identified. The YEPD adenine plate was used to allow the *ste7-1* HO haploids to self-cross to form homothallic diploids. These were incubated at 18° for 2 days, replica stamped to fresh YEPD adenine medium and incubated at 18° for 2 more days. They were then replica stamped to SC lactate (adenine omission) media for selection of pNKY1159 and presporulation growth and incubated at 30° for 1 day. The cells were then transferred by toothpick to SPM plus 100 mg/liter adenine sulfate that had been overlaid with nitrocellulose for the sporulation fluorescence assay (see above). A sporulation defective strain NKY2731 that contains a homozygous *rad50-KI81* mutation (ALANI *et al.* 1990) was included on the sporulation plates as a negative control for comparison purposes. The cells that appeared to be sporulation defective were retested. This was done by returning to the SC-lactate presporulation plate and streaking cells from the corresponding patch on a fresh SC-lactate plate. For each potential mutant five individual colonies were then tested for sporulation using the fluorescence assay.

Isolation of meiotic mutants, secondary screen: Sporulation defective mutants were cured of pNKY1159 to make them *spo11* by nonselective growth on YEPD adenine media. The YEPD adenine medium diploidization plates of the initial screen served this purpose. From these patches, cells were streaked for single colonies on MYPD media. This resulted in a mixture of red *ade2* and tan *ADE2* colonies. Five *ade2* and five *ADE2* colonies from each candidate were touched to SC-lactate agar that contained and lacked 100 mg/liter adenine, respectively, and were incubated at 30° for 1 day. These 10 colonies, five with *SPO11* plasmid and five without, were then tested side by side for sporulation using the fluorescence assay.

Complementation tests: Newly isolated *sae* mutants were tested for complementation by *rad50Δ*, *dmc1Δ* and *mre11::hisG*, and by each other. The newly created *sae* mutations were maintained in a heterozygous state. Strains contained (relevant markers only) *HO/ho::LYS2, STE7/ste7-1, SAE/sae, SPO11/spo11Δ::hisG-URA3-hisG, ura3/ura3, lys2/lys2.* To cross a *sae* mutant by another mutant, the heterozygote was sporulated, 13 tetrads were dissected and the spores were germinated and grown at 34°, the restrictive temperature for *ste7-1*. The resulting colonies of the tetrad dissection were mixed with *STE7* haploid strains that contained either *rad50Δ*, *dmc1Δ* or *mre11::hisG* (strains NKY1370, NKY1459 and KJ5-A2, respectively) on YEPD medium and were incubated at 22° for 20 hr to allow conjugation. These were then transferred to medium that would select for crossed diploids using complementing nutritional markers in the two parental strains. Simultaneously, to determine the location of *sae* mutants in the tetrads, an aliquot of cells from the spore colonies was placed on YEPD and grown into patches at 22° for 2 days. The *HO ste7-1 SPO11* spore segregants (identified by nutritional markers) were then tested for sporulation to locate the *sae* (sporulation defective) mutants. The diploids that had been created by crossing the *sae* mutant clones by *rad50Δ*, *dmc1Δ* and *mre11::hisG* were tested for sporulation. Having thus identified the *dmc1* and *rad50* mutants amongst the group of *sae* mutants (no *mre11* mutants were found), the remainder were tested for complementation among themselves by a similar procedure.

Mitotic recombination frequency: Spontaneous mitotic recombination was determined for two sets of heteroalleles, one at the *HIS4* locus and one at the *ARG4* locus. Strains to be tested were recovered from frozen cultures and incubated on YP lactate plates at 30° for 24 hr and then were streaked for single colonies on YEPD supplemented with 100 μg/ml histidine and arginine. This is the period of growth for which recombination rates were measured. Following 2 days incubation at 30° (3 days for *rad50Δ*) 15 entire colonies were removed from the YEPD plate and the frequency of arginine and histidine prototrophs in each was determined by titering on synthetic complete medium and on medium lacking histidine or arginine. Determination of mutation rates requires that all colonies have the same population so that the prototroph frequencies can be compared to each other. In this set of 15 colonies the population varied over a two- to threefold range. To correct for this the 15 prototroph frequencies obtained were normalized to the average colony population and the "method of the median" was used to determine an approximate recombination rate (LEA and COULSON 1948). Normalization of recombination frequencies introduces an error in the recombination rate per generation, since each new generation contributes fresh mutants to the population and thus increases the mutant frequency. The approximate mutation rate obtained by the method of the median was used to correct the mutation frequencies as they were normalized to average colony population (MCKEE 1996). Those colonies that had a higher than average population had their mutation frequencies lowered by this procedure, and vice versa for smaller than average colonies. These corrected mutation frequencies were then used to calculate the mutation rate by the "maximum likelihood method" (LEA and COULSON 1948).

Sequencing and sequence analysis: Double-stranded plasmids were sequenced using ^{32}P-labeled primers and a BRL dsDNA cycle sequencing kit according to the manufacturer's instructions. DNA sequence analysis was performed using the following E-mail servers: BLAST@NCBI.NLM.NIH.GOV (ALTSCHUL *et al.* 1990), FASTA@EBI.AC.UK, BLITZ@EMBL-heidelberg.DE and BLOCKS@howard.fhcrc.org (HEINKOFF and HEINKOFF 1991).

Meiotic time course: Yeast cells were prepared for meiotic time course experiments according to XU and KLECKNER (1995) with the following changes. Cells were recovered from a frozen culture on YP lactate and incubated at 30° for ~20 hr. All subsequent incubations were at 30°. From this patch of cells single colonies were isolated on YEPD plus adenine and incubated for 2 days. A single colony was used to inoculate 5 ml of liquid YEPD + adenine and this was incubated on a roller drum for 18 hr. The presporulation YP acetate medium was supplemented with 50 mg/liter adenine and where strains had amino acid auxotrophies the particular amino acid was supplemented at 100 mg/liter. Preheated SPM, supplemented with amino acids and bases in the same concentrations as in the YP acetate, was used to wash the cells prior to and for the sporulation.

Genomic DNA for Southern blot analysis was prepared

from 10-ml aliquots of sporulating culture. Cells were resuspended in 0.5 × TE pH 8.0, 50% ethanol and stored at −20° until the remainder of the purification procedure was performed. To extract genomic DNA, the cells were resuspended in 0.5 ml of spheroplast buffer (20% sorbitol, 10 mM $NaPO_4$, 10 mM EDTA, pH 7.0 + 3 μl of 2-mercaptoethanol) and 5 μl of 5 mg/ml zymolase 100T (ICN) were added. Cells were incubated at 37° for 30 min and were mixed by inversion following the first 15 min. Cells were lysed by the addition of 100 ml of 250 mM EDTA, 500 mM Tris-Cl, 2.5% SDS, pH 7.5. Proteinase K was added (10 μl of 20 mg/ml), the tubes were mixed by inversion and then incubated at 65° for 60 to 120 min, with mixing by inversion approximately every 20 min. A precipitation step was performed where 100 μl of 5 M potassium acetate were added followed by an incubation on ice for 20 min, and 15 min of centrifugation at 14,000 × *g*. The supernatant was collected and the nucleic acids precipitated by the addition of 1.5 to 2 volumes of 100% ethanol. The ethanol pellet was partially resuspended in 500 ml of TE, pH 8.0, 20 μg/ml RNase A, and was incubated at 37° for 30 minutes. The DNA was reprecipitated with the addition of 50 μl of 20% sodium acetate and 500 μl of isopropanol. To the isopropanol pellet was added 100 μl of TE, pH 8.0 and the DNA was allowed to dissolve at 4° for ≥18 hr.

For analysis of meiotic DSBs $^1/_{10}$ of the purified genomic DNA (in 10 μl) was cut to completion with *Xho*I in a total volume of 20 μl and was applied to a 0.6% agarose gel cast in TAE buffer. Electrophoresis was carried out at 1.3 V/cm for 48 hr at 4° with recirculated buffer. Southern transfer of the DNA to Hybond N+ membrane was done according to manufacturer's instructions.

Construction of *SAE2* null allele: A null allele of *SAE2* was constructed *in vitro* using the KUNKEL method (SAMBROOK *et al.* 1989). A deletion that removed every codon of the open reading frame was made using the synthetic oligonucleotide 5′-GTGTTTGTATGTGAGCTGCAGTAAACGCCAGCGATC-3′. This oligo hybridizes just outside of each end of the *SAE2* ORF and creates a *Pst*I site at its center. To make the deletion a 1.9-kb *Sal*I to *Sac*II DNA fragment containing *SAE2* was put into pBluescript II KS+ and a small (701 base pair) deletion was made by cutting, filling in and ligating between the *Bsi*WI and *Aat*II sites located in the *SAE2* ORF to make pNKY501. Single-stranded DNA of this plasmid was made by infecting *Escherichia coli* with M13rv1. Once the null mutation was created the *Pst*I site was cut, filled in and ligated to a *Bam*HI linker. At this *Bam*HI site a *hisG-URA3-hisG* cassette was integrated (ALANI *et al.* 1987).

The GenBank accession number for the *SAE2/COM1* gene nucleotide sequence is U49447.

RESULTS

A general genetic method for identification of diploid-specific mutations: A protocol has been developed specifically to identify recessive meiotic mutations whose phenotypes must be observed in diploid cells (Figure 1). The outline of this technique is as follows. Diploid cells bearing the *HO* homothallism gene and a temperature-sensitive conjugation mutation that blocks mating at nonpermissive temperature are mutagenized and sporulated to produce haploid spores. These spores are germinated at the restrictive temperature and grown into individual spore clone colonies. Due to the absence of conjugation, the cells in these colonies are all haploids. Due to the presence of the *HO* gene, mating type switching occurs constitutively in these cells. Thus, the resultant colonies contain a mixture of *MAT***a** and *MATα* haploid cells.

A portion of each such colony is then subcloned at permissive temperature, under which condition the component cells undergo conjugation and grow further. The resulting colony consists primarily of homozygous diploid cells, which can then be tested for any desired phenotype(s).

The remainder of the colony is then crossed by cells of a suitable wild-type strain. The resulting diploid cells will be heterozygous for any mutation of interest present in the original mutagenized spore clone and other relevant markers and can thus be used for genetic analysis of the mutation. A specific application of this approach to the identification of meiotic mutants is described in detail below.

The conditional conjugation mutation used in this method is *ste7-1* (HARTWELL 1980); this mutation confers temperature-sensitive mating, even when one of the cells in a cross is wild type. To introduce *ste7-1* into SK1 yeast the allele was cloned from strain 43a (HARTWELL 1980) by plasmid gap repair using flanking sequences from the cloned wild-type gene (CHALEFF and TATCHELL 1985). The cloned *ste7-1* allele was used to replace the SK1 *STE7* gene (MATERIALS AND METHODS).

To confirm that the *ste7-1* mutation could be used in the desired ways, the mating and sporulation characteristics of heterothallic and homothallic *ste7-1* derivatives of SK1 (KANE and ROTH 1974; ALANI *et al.* 1990) were analyzed (MCKEE 1996). Heterothallic *ste7-1* strains were found to mate with near wild-type efficiency at 18° and to be completely blocked for mating at 34° ($<10^{-5}$ cells are capable of conjugation). Spores from a homothallic *ste7-1* strain germinated and grown to colonies at 34° can then be crossed by a wild-type haploid strain at 24° (Table 2).

Furthermore, homothallic *ste7-1* spore clone colonies grown at permissive temperature were found to form ascospores with wild-type efficiency (Table 3) from which it can be concluded that both mating and spore formation occurred efficiently in such colonies. Also, spore viability is normal for such strains (>95%).

Cultures containing *HO ste7-1 MAT*a and *MATα* haploid cells can be stored at −70° for subsequent use: To facilitate introduction of the *HO* gene into new strain backgrounds we have found it convenient to be able to grow and then store mixtures of *HO MAT***a** and *MATα* haploid cells for future use. Such mixtures can be propagated and retain their capacity to mate efficiently with haploids of either mating type as documented by the following tests. A spore clone of the *HO ste7-1* diploid strain NKY895 was germinated at 34°, the clone picked and subcultured as a 1-cm^2 patch on solid YPD medium for 12 hr at 34°, and a cell aliquot was then frozen in 15% glycerol at −70°. After storage cells were scraped

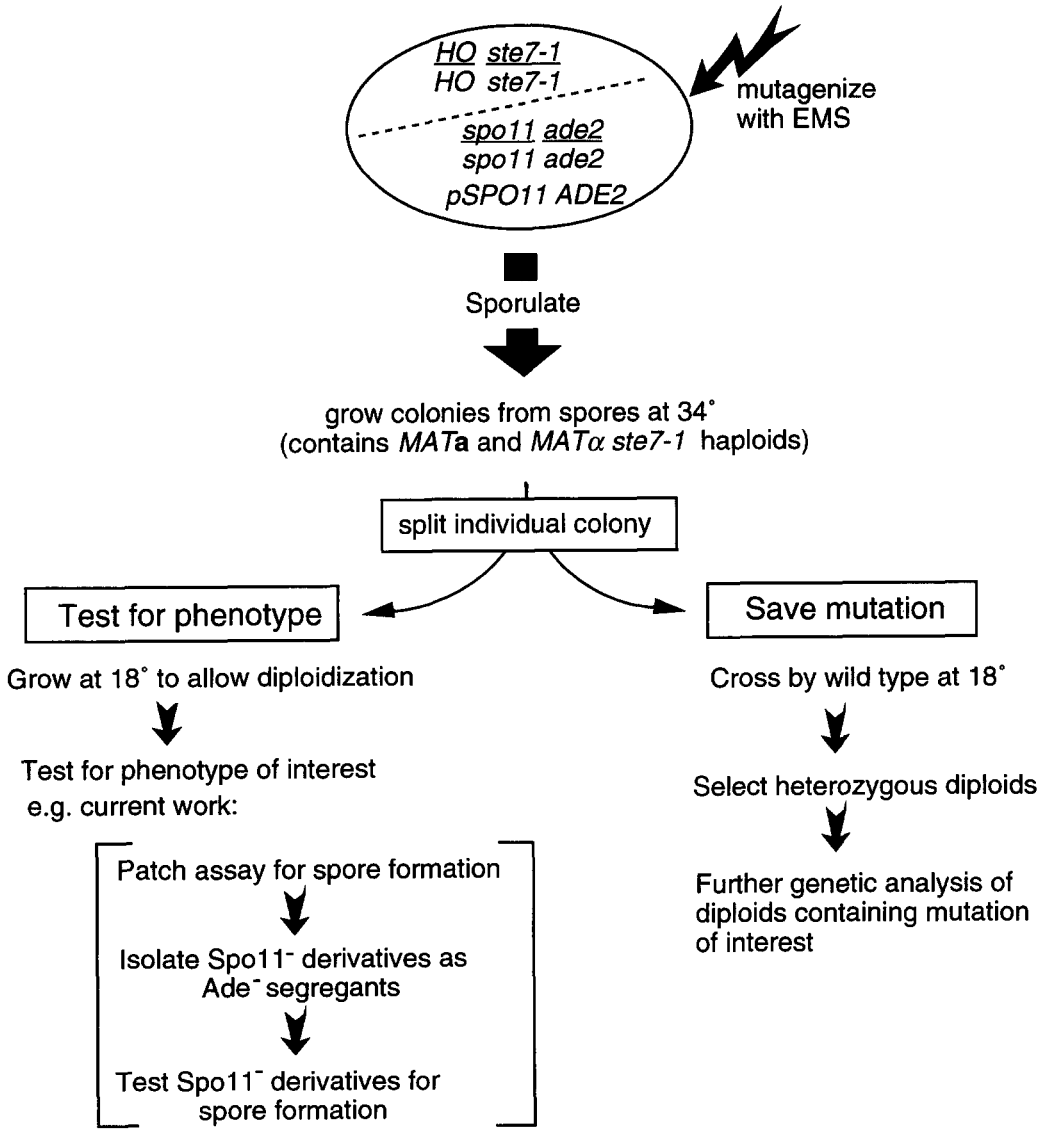


FIGURE 1.—Outline of approach to isolate mutants. Identification of recessive mutations whose phenotypes can be observed only in diploids requires that strains homozygous for these mutations be made and analyzed. In the case of mutations that preclude development of viable haploids via sporulation and tetrad dissection, further genetic analysis of these mutations is difficult. To create strains containing new mutations that are homozygous diploids suitable for phenotypic analysis, and at the same time place these mutations in a genetic background tractable for further genetic analysis, a method using homothallic temperature-sensitive sterile yeast strains was devised. The *ste7-1* mutation confers temperature-sensitive mating. Homothallic spore clones containing mutations induced by mutagen treatment either before or after their parental strain was sporulated are germinated and grown to colonies at 34°, nonpermissive for the *ste7-1* mutation. These colonies contain a mixture of *MAT***a** and *MATα* haploids. Fractions of this colony can be subcultured at the *ste7-1* permissive temperature, either in the presence of wild-type haploid cells to achieve a genetic cross, or alone, to diploidize the new mutation. The diploidized strains can be analyzed for the desired phenotype, *e.g.*, sporulation defective mutants, or mutants unable to survive as diploids. Once the mutant phenotype is found, segregation and complementation analysis can easily be done using the corresponding strain that was produced when the mutant *ste7-1* haploid was crossed by wild type. In the current study diploid strain NKY1276 (Table 1; relevant markers in circle at top) was treated with EMS to create mutations in its genome. From this strain random spores were made, and Ade$^+$ (*SPO11*) colonies were germinated and raised at 34°. Each colony was split, part used for self-diploidization to make homozygous diploid mutants, the remainder crossed by wild-type NKY1063. The self-diploidized strains were tested for two phenotypes. First, mutants that were unable to produce spores when incubated on sporulation medium were identified. Second, this set of spore formation defective mutant strains were made *spo11*Δ by loss of the *SPO11 ADE2* plasmid and were retested for spore production. The desired phenotype at this time was return of spore production competency. Both tests were done using a spore fluorescence assay (MATERIALS AND METHODS). Candidate *sae* mutations thus identified were further analyzed in derivatives of the diploid made earlier by the cross of the mutant strain by NKY1063.

TABLE 2

Mating of a homothallic spore colony with heterothallic cells

Cross[a]	Germination temperature	Mating temperature	Frequency of diploids
HO ste7-1 × *ho STE7*	34°	24°	0.57
	34°	34°	1.4×10^{-4}
	24°	24°	4.8×10^{-4}
	24°	34°	$<1 \times 10^{-5}$
HO STE7 × *ho STE7*	34°	24°	6×10^{-2}
	34°	34°	8×10^{-4}
	24°	24°	1×10^{-2}
	24°	34°	3×10^{-4}

Tetrads from homothallic diploids were dissected and germinated into single colonies at either 24° or 34°. The cells from these colonies were then tested for their ability to mate to wild-type tester strain NKY819 at either 24° or 34° using the "frequency of mating test" (MATERIALS AND METHODS). The residual mating of the *STE7* spore clones at both high and low temperature and the mating of the *ste7-1* spore colonies germinated and mated at 24° may be due to low level of spontaneous conversion to form *MATα/MATα* diploids, which will mate with the tester strain to make triploids.

[a] Homothallic spore clones were derived from NKY730 (for *HO STE7*) and NKY895 (for *HO ste7-1*).

off the surface of the frozen culture and applied to YPD agar at 34°. Following 12 hr incubation these cells were crossed by representative *MAT***a** and *MATα* haploid strains NKY487 and NKY536, respectively, and a frequency of mating test was performed (MATERIALS AND METHODS). The frequency of diploids observed in the two cases was 52 and 11%, respectively.

Isolation of *SAE* mutants

We were particularly interested in using the method described above to identify recessive meiotic mutations that confer an intermediate block to meiotic prophase as defined above (see Introduction). Such mutations should cause a defect to spore formation when present in an otherwise wild-type background but this defect should be alleviated when *SPO11* gene function is eliminated.

Such mutants were sought in a two-step screen (Figure 1). First, mutants incapable of making ascospores were identified. Second, this group of mutants was screened to identify strains in which ascospore production was restored when meiosis occurred in the absence of *SPO11* gene function. To permit easy identification mutations were generated in an *ade2* mutant strain containing a *URA3* marked deletion of the *SPO11* gene with the wild-type alleles of these genes provided on a *SPO11 ADE2* plasmid. In this situation *spo11* derivatives will arise spontaneously at a high frequency due to loss of the plasmid and can thus be detected as Ade^- segregants on appropriate indicator plates (see below).

TABLE 3

Diploidization and sporulation of cells from homothallic *ste7-1* spore colonies sporulated at 30°

Genotype	Germination temperature	Frequency of asci
HO STE7	21°	0.94
HO STE7	24°	0.95
HO STE7	30°	0.94
HO STE7	34°	0.96
HO ste7-1	21°	0.94
HO ste7-1	24°	0.94
HO ste7-1	30°	0.01
HO ste7-1	34°	0.04

Four sets of tetrads were dissected, followed by incubation at the temperatures indicated in the table. When the colony population reached 10^7, cells were transferred to SPM agar and incubated for 2 days. Frequencies represent the average of four colonies, where 250–300 cells were counted for each colony. Strains used were NKY730 (for *HO STE7*) and NKY895 (for *HO ste7-1*).

Mutants were isolated in NKY1276. A culture of this strain was mutagenized with EMS, sporulated and random spores germinated and grown to colonies at 34°, the restrictive condition for *ste7-1* (Figure 1). One portion of the cells from each colony was crossed by an appropriate wild-type strain NKY1063 at 18°, a temperature permissive for conjugation. The remaining cells of each colony were transferred to a patch on YEPD medium and were also incubated at the permissive temperature 18°. Control experiments demonstrate that the resulting patch contains mainly homozygous diploids (data not shown).

Patches of diploidized cells were taken through meiosis by replica plating onto sporulation medium. The patches of sporulated cells were then screened for the presence of ascospores using a modification of the dityrosine fluorescence assay (BRIZA *et al.* 1990a). Figure 2 shows the fluorescence of a patch of cells of a wild-type strain NKY278 that had been incubated on sporulation medium for 4 days. Also shown in Figure 2 are cells from a sporulation defective mutant *rad50S-KI81*. The patch of wild-type cells is white, indicating fluorescence and thus the presence of spores. The mutant has no background fluorescence. These results demonstrate that this assay can be used to identify mutants defective in spore formation.

For the actual mutant screen, cells from mutagenized, diploidized spore clone colonies were touched to a piece of gridded nitrocellulose that had been laid overtop of SPM and were then incubated at 30° for 4 days. Approximately 150 colonies per plate were analyzed in this way. A photograph of a typical screening plate appears in Figure 2. Again, patches containing spores are white, and those where the cells fail to sporulate, appear as empty spaces.

A total of 11,469 colonies were screened for sporulation proficiency in this way. Each of these colonies was

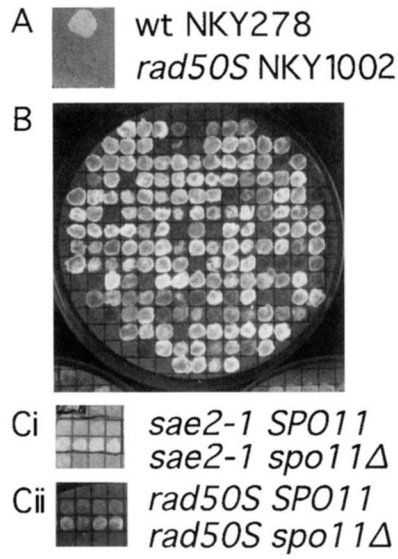


FIGURE 2.—(A) Sporulated cells fluoresce when irradiated with long wave UV light. Equal amounts of wild-type and *rad50S-KI81* mutant cells were incubated on nitrocellulose-covered sporulation agar, and then photographed with UV illumination. Sporulated wild-type cells (NKY278) appear bright: spore formation defective mutant *rad50S-KI81* exhibits no fluorescence over background. (B) Screen for sporulation defective mutants. Approximately 190 colonies can be analyzed on a single SPM plate overlaid with gridded nitrocellulose. Patches of sporulation proficient cells appear bright, while patches of sporulation defective cells appear as dark squares. (C) Screen for mutant strains where ascospore production is restored by the absence of the *SPO11* gene function. A comparison of spore formation in *SPO11* and *spo11Δ* derivatives of new mutant strains was done by sporulating several colonies of each *SPO11* genotype side by side and comparing relative fluorescence. In Ci, a row of four *sae2-1 SPO11* cells is sporulated next to four *sae2-1 spo11Δ* cells. Cii exhibits the same for a *rad50S* mutant.

also tested in parallel for aerobic metabolism, necessary for sporulation, by their ability to grow on YP glycerol medium. A total of 1361 colonies scored negative for spores and were aerobically competent. Among the aerobically competent colonies some will be defective in spore formation for reasons unrelated to meiosis. One such class are nonconditional sterile mutants, which will fail to make spores because they fail to form diploid cells. Twenty-nine of the 1361 spore clones were identified to be sterile by their inability to make heterozygous diploids when mixed with wild-type haploid NKY1063. Formation of diploid cells also requires mating type switching, but switching-defective mutants are excluded in this screened by the fact that they do not make viable spore clone colonies at the diploidization stage (see below).

The remaining 1332 candidates were carried into the second stage screen, where restoration of sporulation in the absence of *SPO11* gene function is the desired phenotype. To identify segregants lacking the *SPO11 ADE2* plasmid, homozygous diploid cells were patched onto medium nonselective for *ADE2* (YEPD plus adenine). Cells from such patches were then streaked for individual colonies on MYPD medium where Ade^- and Ade^+ cells can be distinguished by inspection. To test for sporulation when the *SPO11* gene is absent, cells from five different Ade^- colonies then were picked from the MYPD plate for each mutant candidate. These were grown overnight on SC-lactate medium supplemented with appropriate amino acids and bases and then transferred to SPM. As a control sample five *SPO11* (Ade^+) spore clones were also taken from the MYPD plates, grown overnight on SC-lactate supplemented with amino acids in parallel with the *ade2* clones and placed beside the *spo11* cells on the SPM plate. Sporulation was then assessed using the fluorescence assay. Such analysis is presented in Figure 2 for two mutants: one containing a *rad50S* allele and one containing a new mutation *sae2-1*. The difference in fluorescence between the Ade^+ and Ade^- clones for both mutants indicates that they both have the desired phenotype. Eighty-seven mutant candidates passed this test.

These 87 potential mutants were then analyzed to determine if the mutant phenotype segregated as a single locus in a cross with a wild-type strain using the corresponding heterozygous diploids made in the previous stage. For each mutant a segregant of the heterozygous diploid that had lost the *SPO11 ADE2* plasmid was identified, sporulated and 26 tetrads were dissected. Spores were germinated at a temperature permissive for *ste7-1*, thus allowing the homothallic spore clones to diploidize during colony formation. Cells from these colonies were then patched on SPM and tested for spore formation by the dityrosine fluorescence assay.

The *HO* gene, the *SPO11* gene and any new mutation(s) all assort in these tetrads. For any mutation of the desired type, the resultant sporulation defect can be detected only in *HO SPO11* spore clones, which are identified specifically by the presence of nutritional markers, *lys2* and *ura3*, respectively. If the spore formation defect segregates as a single locus, it will occur in half of the *HO SPO11* spore clones. In addition since the phenotype of interest is *SPO11*-dependent absence of spore formation, all of the *HO spo11* clones should be sporulation proficient irrespective of whether they contain the new mutation. Eight of the 87 potential mutants met both of these criteria.

To determine if any of these eight mutations were in new genes, complementation tests were performed between these mutations and relevant known mutations. Mutations in the *dmc1* gene and *rad50S* mutations are known to confer *SPO11*-dependent sporulation defects. Another candidate for a gene that could yield such mutations is *MRE11*, as a *mre11* null mutant has phenotypes similar to *rad50* null mutants (AJIMURA *et al.* 1993; JOHZUKA and OGAWA 1995), and "*mre11S*" alleles analogous to *rad50S* alleles have recently been identified (CHEPURNAYA *et al.* 1995; F. KLEIN, personal communication; H. OGAWA, personal communication).

Consequently we assayed for complementation between each new mutation and null mutations in the *DMC1*, *RAD50* and *MRE11* genes. Homothallic *ste7-1* haploid mutant spore clones were crossed with *dmc1*Δ, *rad50*Δ or *mre11*Δ haploids and the resulting diploids were tested for the ability to make spores (MATERIALS AND METHODS). All eight mutations are complemented by a *mre11*Δ, indicating that none is a nonnull allele of *MRE11*. Two mutations of the set of eight fail to complement a *dmc1*Δ mutation for spore formation, indicating that they are new alleles of *DMC1*. Three mutations fail to be complemented by a *rad50*Δ mutation, indicating that they are new nonnull alleles of *RAD50*, presumably of the *rad50S* type. The three remaining mutants were tested against each other and found to represent three separate complementation groups designated *SAE1*, *SAE2* and *SAE3*.

Molecular analysis of *SAE2*

Cloning of *SAE2*: The *SAE2* gene was cloned from a YCp50 genomic library (ROSE *et al.* 1987) on the basis of complementation of the spore formation defect. A diploid *sae2-1* mutant NKY2728 (backcrossed twice from the original mutant) was transformed by electroporation (BECKER and GUARENTE 1991), and transformants were plated on uracil omission medium at a density that yielded ~75 Ura^+ colonies/83-mm plate. When colonies had reached 2-mm diameters they were replica stamped to SPM + 50 mg/liter adenine. The replicas were incubated at 30° for 4 days and then screened for fluorescent colonies. Four were found, and plasmid DNA was recovered from the corresponding colonies of the master transformation plates. To confirm that it was the plasmids that conferred the ability to make spores, rather than a suppressor mutation in strain NKY2728, each plasmid was reintroduced into NKY2728 by transformation and a number of transformants of each plasmid were tested for their ability to make ascospores. All colonies tested were sporulation proficient, and the spores had a frequency of viability indistinguishable from wild type (data not shown).

Restriction enzyme mapping of the four complementing plasmids revealed inserts that varied in size from 8 to 14 kb, with a common 8-kb region. Subclone analysis narrowed down the minimum complementing region to a *Sal*I-*Sac*II fragment of just under 2 kb in length.

Sequence of *SAE2* gene: The minimal complementing *Sal*I to *Sac*II fragment was sequenced in its entirety and agrees without mismatch with that produced by the genome sequencing project. Analysis of this sequence reveals a single large open reading frame of 345 codons entirely contained on the fragment (Figure 3). There are no splicing signals in the sequence (RYMOND and ROSBASH 1992). Analysis of the predicted protein sequence using the programs TBLASTN (ALTSCHUL *et al.* 1990), FASTA, BLITZ and Blocks (HEINKOFF and HEINKOFF 1991) failed to find any entries in sequence databases that have a statistically significant similarity to Sae2 protein. The codon adaption index is a measure of comparative use of synonymous codons. For this protein the index is very low, 0.133, which predicts that this gene is likely to have a low level of translation (SHARP and LI 1987).

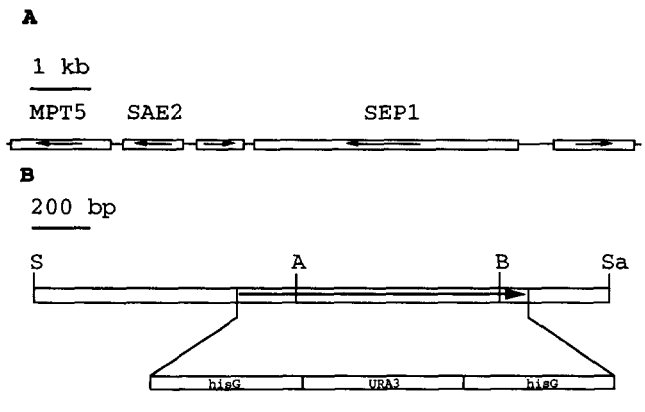
Construction of null allele: A *SAE2* deletion allele that precisely deletes every codon of the corresponding open reading frame was made using site-directed mutagenesis. This deletion allele was introduced into the genome of a wild-type diploid strain by a one-step replacement method (ROTHSTEIN 1991) and its correct integration into one chromosome was verified by Southern blot. Tetrads dissected from the resulting diploid gave four viable spores, indicating that *SAE2* is not an essential gene. When a haploid strain carrying the deletion allele was crossed by a *sae2-1* haploid, the resulting diploid failed to sporulate, thus demonstrating that the cloned gene is in fact *SAE2*. In all assays used, both meiotic and mitotic, the phenotypes conferred by the deletion allele and the original EMS-induced allele are the same.

***SAE2* is on the left arm of chromosome VII:** The location of the *SAE2* gene was determined by probing a blot of overlapping lambda and cosmid clones obtained from the ATCC. A positive signal was obtained for two overlapping lambda clones from the left arm of chromosome VII, ATCC no. 70391 and ATCC no. 70353. The genomic sequencing effort of chromosome VII has revealed that *SAE2* is located between *SEP1* and *MPT5* (BERTANI *et al.* 1995; Figure 3). Comparison of sequences has revealed that *SAE2* is the same gene as *COM1*, isolated by Prinz *et al.* (1997). Hereafter the wild-type gene and protein will be refered to by both names.

Phenotypic analysis of strains mutant in the *SAE2/COM1* gene

MI and MII divisions of meiosis are delayed in *sae2* mutants: In a synchronous meiosis of a wild-type SK1 yeast culture, 50% of cells have undergone the first meiotic division by ~6 hr after transfer of cells to sporulation medium, with the second division following shortly thereafter (PADMORE *et al.* 1991; XU *et al.* 1997; Figure 4). In *sae2* deletion mutants the first division is delayed by ~1.5 hr (Figure 4). The second division is further delayed by 1–2 hr, though many *sae2*Δ nuclei disintegrate at later time points and thus cannot be scored for this event (Figure 4). The same is true for *sae2-1* (data not shown). The synchrony of the divisions is only slightly reduced for both the point and null mutants, however, as compared to wild type.

Failure to form mature spores: The *sae2* mutant executes MI with reasonable efficiency but fails to make



C

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  61  I  L  C  H  E  K  N  A  P  Q  Q  S  S  Q  T  S  A  G  P  G

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  81  E  Q  D  S  E  D  F  I  L  T  Q  F  D  E  D  I  K  K  E  S

 301  gcagaggttcactatcgtaatgaaaataaacacactgtgcagttaccgctggttactatg
 101  A  E  V  H  Y  R  N  E  N  K  H  T  V  Q  L  P  L  V  T  M

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 121  P  P  N  R  H  K  R  K  I  S  E  F  S  S  P  L  N  G  L  N

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 321  E  Q  L  N  Q  I  V  D  D  G  C  F  F  W  S  D  K  L  L  Q

1021  atatatgctagatgttaaacgccagcgatcatcattctttattcattacttcaaatacat
 341  I  Y  A  R  C

1081  ttttttggtatggttgaaagggctttttcctcgttggcatatgggaaaaactgaaaaa

1141  taaaaaaaggcaatgagatgaacaacaatggaaaacaaaaaagggcgaataaataagcag

1201  cagttggtaaagcagaaaggggaagttaacggacgttaaacaaatgacacctaacttacc

1261  agggttttattatgatagagaacgtcgacctcgagggggggcccggtacc
```

mature ascospores. When observed with phase-contrast optics at the 24-hr time point the cells have not arrested with a uniform appearance as in *CDC* mutants. Some cells contain what appear to be multiple rudimentary spores, others contain a single dense body, which may be a vacuole or a developing spore, and others lack dense material (data not shown).

The *spo11*Δ mutation is epistatic to *sae2-1* for spore formation and cell viability: In liquid sporulation cultures wild-type SK1 strains and *spo11*Δ mutant strains typically form spores with high efficiency (>80%; ALANI *et al.* 1990; PADMORE *et al.* 1991; somewhat lower in the experiment presented in Table 4). A *sae2-1* mutant strain makes fewer than 1% ascospores, yet a *sae2-1 spo11*Δ double mutant produces >100-fold more ascospores, a frequency similar to that of the *spo11*Δ mutant alone (Table 4). This confirms the dependence of the *sae2* spore formation defect on *SPO11* function inferred from the original patch tests (above). A *spo11*Δ mutation is similarly epistatic to the *sae2-1* mutation with respect to cell survival after induction of meiosis (Table 4). Finally, since timely spore formation likely requires timely occurrence of the two divisions, the *spo11*Δ mutation presumably alleviates the division delays of the *sae2* mutant, although this has not been examined directly.

Commitment to heteroallelic recombination: When yeast cells that have begun meiosis are returned to rich growth medium at a sufficiently early stage, prior to the first meiotic division, they leave meiosis and resume vegetative growth (SHERMAN and ROMAN 1963). In such cells, meiotic levels of recombination can be observed and are thus said to have become "committed" to meiotic recombination. *sae2* mutants have been analyzed in return to growth experiments for cell viability and for commitment of meiotic recombination at two loci, *HIS4* and *ARG4*. Two differences from wild type are observed. First, whereas wild-type cells do not lose viability following induction of meiosis in such an experiment (SHERMAN and ROMAN 1963; CAO *et al.* 1990), a *sae2* mutant exhibits a rapid loss of viability, down to

FIGURE 3.—(A) Map location of *SAE2/COM1* gene. *SAE2/COM1* is located on the left arm of chromosome VII between the genes *MPT5* and *SEP1*. (B) Extent of deletion allele. The *SAE2/COM1* gene is located on a 1975-bp *Sal*I to *Sac*II DNA fragment, which is capable of complementing the *sae2-1* mutation. A deletion allele that precisely deletes the entire open reading frame of *SAE2/COM1* was made using site-directed mutagenesis. The open reading frame was replaced by a *URA3* gene flanked by direct repeats of Salmonella *hisG* DNA (ALANI *et al.* 1987). (C) DNA sequence of the *SAE2/COM1* gene. Analysis of the sequence of a 1975-bp *Sal*I-*Sac*II DNA fragment that complements the sporulation defect of the *sae2-1* mutation revealed a single large open reading frame of 345 codons. Numbering of the DNA begins at the first nucleotide of the first codon of the *SAE2/COM1* open reading frame. The predicted protein sequence is not homologous to any other in the GenBank or SwissProt databases nor does it have any recognizable motifs.

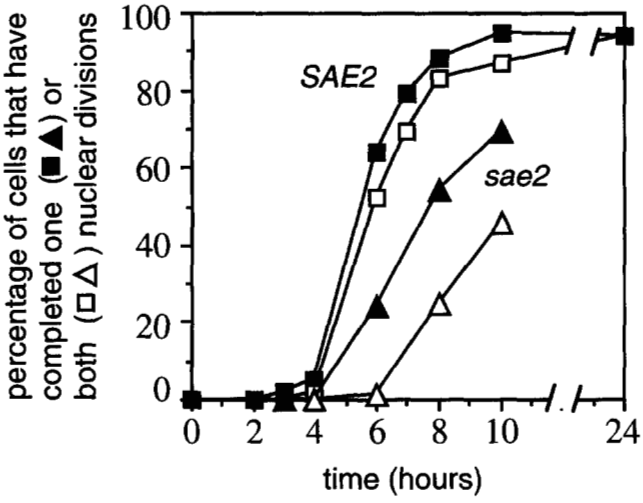

FIGURE 4.—Meiosis is delayed in *sae2* mutants. In a meiotic time course, a comparison of wild-type cells (NKY1552, indicated by boxes) and *sae2*Δ mutant cells (NKY2641, indicated by triangles) reveals that meiosis I (filled symbols) is delayed in *sae2* mutants. Assuming that approximately 80% of cells ultimately carry out MI, the divisions in those cells occur ~1.5 hr later than normal. There is an additional delay in the occurrence of meiosis II (open symbols), again of 1–2 hr. Accurate assessment of divisions at late time points is precluded by degeneration of nuclei. Diploids were prepared for a meiotic time course as described in MATERIALS AND METHODS. At the indicated times aliquots of cells were fixed in 50% ethanol, 1× TE, pH 8.0. To visualize DNA, 10-μl aliquots of fixed cells were stained by mixing with an equal volume of 1 μg/ml DAPI in 50% ethanol, 1× TE. These were scored for meiosis I (two DAPI-staining bodies per cell) and meiosis II (four DAPI-staining bodies).

~5% the starting level by $t = 6$ hr (Figure 5). Second, in wild-type meiosis, the frequency of prototrophic recombinants per viable cell at the two test loci is usually induced from $\sim 10^{-4}$ at $t = 0$ to $\sim 10^{-2}$ (His^+) or $\sim 4 \times 10^{-2}$ (Arg^+) by $t = 5$ hr, with little or no increase thereafter (*e.g.*, MCKEE and KLECKNER 1997); for a *sae2* mutant, in contrast, the frequency of prototrophic recombinants per viable cell rises slightly at early times but plateaus at $^1/_{20}$ to $^1/_{50}$ the wild-type level, $2–6 \times 10^{-4}$ (Figure 5). Interestingly, at very late times, *i.e.*, between t = 10 and t = 24 hr, the frequency of colony forming units drops only two- to threefold while the frequency of prototrophs per viable colony forming unit increases ~20-fold. Since DSBs have presumably all formed long before $t = 10$ hr, this late increase in commitment to

TABLE 4

Meiotic viability and spore formation of *sae2-1* and *sae2-1 spo11*Δ double mutants

Mutant	CFU per ml ($\times 10^{-7}$) $t = 0$	CFU per ml ($\times 10^{-7}$) $t = 24$	Survival at 24 hr (%)	Asci at 24 hr (%)	Strain
Wild type	1.46	0.66	46.0	67	NKY1551
*spo11*Δ	0.92	0.0099	1.1	55	NKY648
sae2-1	1.83	0.0070	0.4	0.3	NKY2629
*sae2-1 spo11*Δ	1.04	0.0603	3.1	26	NKY2666

Cell titers represent the averages of two separate dilutions on YEPD from the same culture. Survival at 24 hr represents CFU at 24 hr divided by the number at zero hours, represented as a percent. Percent asci was determined by counting the number of asci among 300 cells. Presporulation and sporulation were done as in Time Course Analysis, described in MATERIALS AND METHODS. CFU, colony-forming unit.

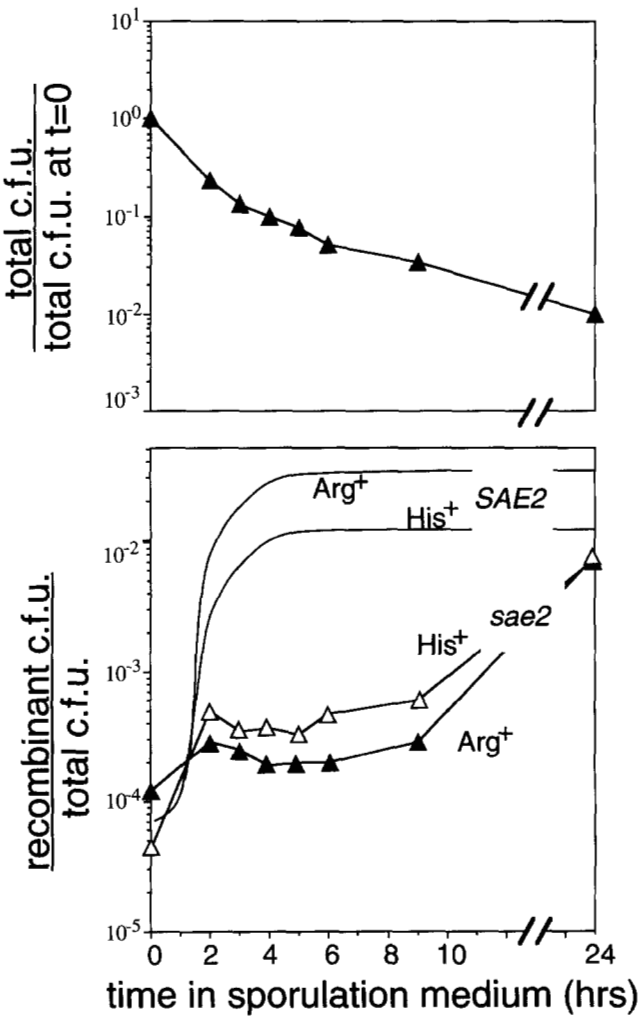

FIGURE 5.—Rapid loss in viability as *sae2* mutants enter meiosis. A deletion strain of *sae2* (NKY2640) rapidly loses viability in return to growth analysis when it enters synchronous meiosis. Survival decreases continuously throughout the time course to a low of 1% at 24 hr. Wild-type cells usually maintain full viability throughout sporulation. Histidine (△) and arginine (▲) prototrophs created by recombination between heteroalleles of, respectively, *HIS4* and *ARG4* genes, arise after incubation in sporulation medium. Induction in wild-type cultures typically reach a maximum at 4 hr after transfer to sporulation medium, with approximately 1% of cells His^+ and 3% Arg^+. Among surviving cells at each time point, *sae2*Δ mutant strains exhibit induction of prototrophs, albeit at 2–5% of the wild-type level. The increase in frequency of prototrophs at 10- and 24-hr samplings may be due to a small fraction of cells that repair the DNA damage made earlier in meiosis. At 24 hr only 0.9% of cells survive: a fraction of these may have repaired meiotic DSBs, the remainder may never have entered meiosis.

recombination may suggest that in cells that have survived to a late time point, DSBs often progress to the point required for commitment to be observed.

Physical analysis of meiotic DNA in *sae2* mutants reveals unresected DSBs and no recombinants: Physical analysis of recombination was carried out in wild-type and *sae2* mutants at the *HIS4LEU2* locus in strains carrying suitable restriction site differences at this locus (CAO *et al.* 1990). Meiotic DSBs occur at *HIS4LEU2* at two hot spots, referred to as site I and site II. Occurrence of DSBs at these sites can be monitored by the appearance of a diagnostic signal on a Southern blot in restriction enzyme-digested DNA. Additionally, crossovers between homologs yield specific diagnostic fragments that are intermediate in size between the two parental fragments (see map in Figure 6). For analysis of these events, a culture of cells is taken through a synchronous meiosis and genomic DNA is extracted from aliquots removed at appropriate time points. The DNA is then cut with enzyme *Xho*I, separated by gel electrophoresis and analyzed by Southern blot using a radioactive probe.

In wild-type cells (Figure 6) DSBs appear transiently during prophase, reaching a maximum at 3 to 4 hr after the transfer of cells to sporulation medium and diminishing thereafter. The diagnostic signal of the breaks appears "fuzzy" on the Southern blot due to the varying extent of resection at the 5′ DNA strand termini of the break. Crossover products are first detectable at approximately $t = 5$ hr and reach a maximal level by $\sim t = 7$ hr.

The *sae2* mutant differs from wild type in several respects. First, DSBs accumulate to a high level and persist throughout the time course, indicating that DSB turnover is blocked. Second, the DSB signal on the Southern blot remains sharp, indicating that the 5′ strand termini of the breaks are not resected. Third, DSBs appear slightly earlier than normal, a feature expected from the absence of turnover. Fourth, no fragments representative of crossovers appear, even as late as 24 hr after the cells are transferred to sporulation medium (Figure 6). This constellation of phenotypes is essentially identical to that observed in strains carrying an "S" allele of the *RAD50* gene (ALANI *et al.* 1990).

In *rad50S* strains, since DSBs are not resected, the appearance of the diagnostic fragment can be used to evaluate the total number of breaks that have occurred. Such experiments have revealed that two slightly different *HIS4LEU2* alleles, differing only in the particular sequence at site I, give significantly different levels of DSBs. The original allele, *Mlu*I, gives fewer DSBs than an altered allele, *Mlu*I::*Bam*HI, which contains an additional 36 bases relative to the original; furthermore, a chromosome that harbors the *Mlu*I::*Bam*HI allele exhibits a lower level of DSBs at the adjacent hot spot, site II, than a chromosome carrying the *Mlu*I allele (XU and KLECKNER 1995). Both of these effects are seen in both *sae2-1* and *sae2*Δ mutants as shown by analysis of an *Mlu*I/*Mlu*I homozygote and an *Mlu*I/*Mlu*I::*Bam*HI heterozygote (Table 5)

A *sae2* mutation has no effect on mitotic recombination: Since *sae2*Δ shares meiotic phenotypes with *rad50S* alleles, and the *RAD50* gene plays a role in mitotic cells, we examined *sae2* mutants for two mitotic phenotypes. First, spontaneous mitotic recombination was assayed. Compared to wild type, null mutations of *rad50* have a high level of spontaneous mitotic recombination (MALONE *et al.* 1990), and the *rad50-KI81* allele (a *rad50S* type allele) is reported to have a slightly elevated level over wild type (ALANI *et al.* 1990). The rates of spontaneous mitotic recombination between *his4* heteroalleles and between *arg4* heteroalleles were determined by fluctuation tests on sets of 15 cultures using the method of maximum likelihood (LEA and COULSON 1948) for wild type, *sae2*Δ, *rad50*Δ and *rad50-KI81* mutants (see MATERIALS AND METHODS). The rates are not significantly different in wild-type, *sae2*Δ mutants and *rad50-KI81* mutants. The spontaneous mitotic recombination rate in a *rad50* null mutant is elevated 3.8- and 12.4-fold over wild type at the *HIS4* and *ARG4* loci, respectively (Table 6). Second, the colony growth size of wild-type, *sae2*Δ and *rad50S* mutants are all similar, in contrast to that of *rad50*Δ, which is somewhat smaller (Table 7).

A *sae2* mutation confers very weak sensitivity to MMS: MMS is a DNA-damaging agent to which *rad50* mutants are highly sensitive. The efficiency of plating of a *rad50*Δ mutant for colonies on YEPD medium containing 0.006% MMS is just 0.03 at 30° and 6×10^{-5} at 18°, a slightly more stringent condition (ALANI *et al.* 1990). A *rad50-KI81* mutant is only slightly sensitive to MMS, with efficiency of plating close to 1.0 but with a slightly reduced colony size. This effect is more pronounced at 18° than at 30°. The *sae2*Δ mutant is identical to *rad50-KI81* in both respects (Table 7).

***RAD50* is required for efficient mating type switching:** In the course of developing the assay for recessive diploid-specific mutants, an *HO ste7-1* strain heterozygous for a *rad50*Δ mutation was constructed. When this strain is sporulated and spores are germinated at temperatures permissive for the *ste7-1* mutation, *RAD50* spore clone colonies are large while *rad50*Δ spore clone colonies are of medium size (Figure 7), as expected from known effects of a *rad50*Δ mutation on vegetative cell growth (*e.g.*, FARNET *et al.* 1988). If spores are germinated at temperatures nonpermissive for the *ste7-1* mutation, however, the *RAD50* spore clone colonies are again large while the *rad50*Δ colonies are extremely small (Figure 7).

HO ste7-1 spore clone colonies arising at higher temperatures comprise haploid cells whereas those arising at lower temperatures comprise diploid cells (above); DSB-mediated mating type switching is repressed in diploid cells and is active in haploid cells (HERSKOWITZ *et*

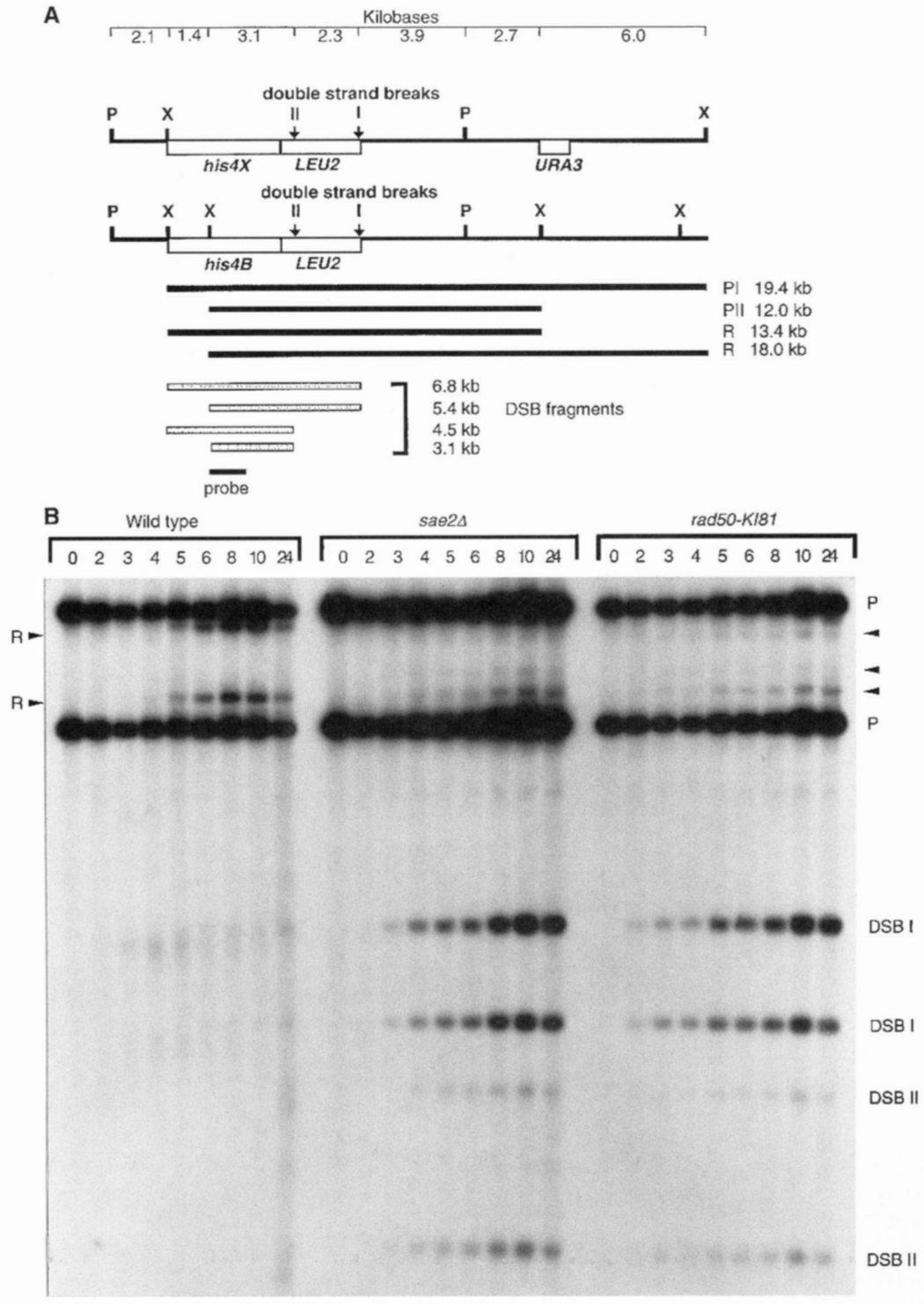

FIGURE 6.—(A) Map of chromosomes used in physical analysis of meiotic DSBs. Restriction site polymorphisms and ectopic DNA were inserted at the *HIS4* locus on the left arm of chromosome III (CAO *et al.* 1990). Two hot spots for meiotic DSBs are located in and near the introduced *LEU2* gene, indicated as site I and site II. DSBs at these sites and crossover recombination in this region can be detected by the appearance of new fragments in DNA cut with *Xho*I and probed with a *HIS4* fragment pNKY155, as indicated in the figure. (B) Physical analysis of DNA at the *HIS4* locus in a meiotic time course in wild-type, *sae2*Δ and *rad50S* mutants. Genomic DNA was digested with enzyme *Xho*I and probed with pNKY155 (Figure 6). Two locations in the *HIS4LEU2* locus are hot spots for meiosis-specific DSBs. In wild type the breaks are transient: the steady-state level of breaks peak at the 3- and 4-hr time points. The signal appears blurred due to the varying extent of exonucleolytic excision on chromosomes in different cells. At 5 hr mature recombinants that are of intermediate size between the two parental signals are visible. The *sae2* mutant strain makes a DSB signal that appears with the same kinetics as wild type, but it remains sharp; there is no resection from the break. Signals appear in both the *sae2* and *rad50S* mutants (marked with arrowheads) that are intermediate in molecular weight to the two parental fragments. These are likely to be additional DSB sites rather than recombinants, as their location on the gel does not correspond exactly to that of the recombinant signals. Strains used in this experiment: wild type, NKY1551; *sae2*Δ, NKY2641; *rad50S,* NKY2559.

TABLE 5

Distribution of meiotic double strand breaks at the *his4LEU2-MluI* and *his4LEU2-MluI::BamHI* alleles in *sae2-1* and *sae2Δ* strains

Strain and allele configuration at *his4LEU2*	Chromosomes broken (%)	
	Site I	Site II
NKY2629 *sae2-1*		
MluI::BamHI	17.1	1.0
MluI	7.5	3.1
NKY2640 *sae2Δ*		
MluI	9.8	2.2
MluI	10.4	2.4
NKY2641 *sae2Δ*		
MluI::BamHI	21.4	1.6
MluI	11.9	3.9

Yeast cells containing the *sae2* mutation were treated according to the meiotic time course procedure. Aliquots of culture were used to prepare genomic DNA at 0, 2, 3, 4, 6, 10 and 24 hr following induction of meiosis. The DNA was cut with the enzyme *Xho*I, which gives fragments that distinguish the two homologous chromosomes (see Figure 6), separated by agarose gel electrophoresis and subjected to Southern blot analysis using a probe made from pNKY155 (Figure 6). The amount of DNA in intact and broken chromosomes was quantitated using a Fuji phosphoimager. The values presented are calculated for the 4-hr time point, where DSB frequencies are maximal, except for NKY2641, which is presented for the 3-hr time point.

TABLE 6

Mitotic gene conversion rates of *his4*, *arg4* heteroalleles in wild type, *sae2Δ*, *rad50Δ* and *rad50S*

	Rate of formation of prototrophs between heteroalleles per cell division	
	his4 locus ($\times 10^6$)	*arg4* locus ($\times 10^6$)
Wild type	3.8 ± 0.4	4.0 ± 0.3
sae2Δ	3.1 ± 0.3	6.3 ± 0.5
rad50-KI81	2.7 ± 0.2	ND
rad50Δ	14.3 ± 1.2	49.4 ± 3.2

Recombination rates are calculated as described in MATERIALS AND METHODS. Confidence range reported is 1 SD of the normally distributed variable. Strains used were wild type, NKY1113; *sae2Δ*, NKY2640; *rad50-KI81*, NKY1409; and *rad50Δ*, NKY1245. ND, not done.

al. 1992); and *RAD50* is a member of the DSB repair epistasis group (HAYNES and KUNZ 1981). Thus, it seemed likely that the *HO ste7-1 rad50Δ* spore clone colonies grew poorly at nonpermissive temperatures because, under these conditions, mating type switching was being initiated in many or all cells but was aberrant due to aberrant DSB processing; the consequence of such a defect would be a delay and/or arrest of the cell cycle. At permissive temperature, in contrast, one or a few rounds of mating type switching might suffice to give cells of opposite mating type which, upon conjugation, give stable diploids in which HO-mediated DSBs no longer occurred; such diploids would grow (relatively) normally.

This possibility was examined directly by physical analysis of HO-mediated DSBs at the *MAT* locus. In colonies from spores germinated at permissive temperature, where switching should not be occurring, DNA fragments diagnostic of DSBs at *MAT* cannot be detected for either the *MAT***a** allele or the *MATα* allele (Figure 7, lanes 1 and 2). In contrast, DSB fragments for both alleles are detectable in all four colonies from spores of a single tetrad germinated at nonpermissive temperature; additionally, however, the steady state level of DSBs is much higher in the *rad50Δ* spore clones than in the *RAD50* spore clones (Figure 7C, lanes 5 and 6 *vs.* lanes 3 and 4, respectively).

These observations show that *RAD50* is required for efficient processing of DSBs. Dependence of HO-promoted recombination on *RAD50* function has also been demonstrated in a *MAT*-switching system where HO is under control of galactose induction (IVANOV *et al.* 1994). In that work, no delay in the cell cycle was observed as a consequence of the slow processing of the HO cut. The slow growth of the haploid *rad50Δ ste7-1* colonies in this work represent the accumulated effect of small delays on several generations, and thus is a more sensitive test of a cell cycle delay.

DISCUSSION

A new genetic method for identifying recessive mutations that confer diploid specific phenotypes: *Approach:* We have developed a new approach to the isolation of recessive mutations that specifically affect diploid strains of yeast *S. cerevisiae.* The essential new feature of this method is utilization of both a temperature-sensitive conjugation mutation *ste7-1* and the homothallism gene *HO* in combination. The *HO* gene makes possible efficient diploidization of haploid cells containing recessive mutation(s) to yield diploids homozygous for such mutation(s), as in previous studies (ESPOSITO and ESPOSITO 1969; ROCKMILL and ROEDER 1988). The *ste7-1* mutation permits, in addition, efficient rescue of recessive mutations into a heterozygous diploid condition; this feature greatly facilitates subsequent genetic analysis since these diploids can be examined directly for 2:2 segregation of any phenotype of interest.

It is notable that use of the *ste7-1* mutation should not limit the types of phenotypes recoverable. The *ste7-1* mutation does not affect meiosis or sporulation. More generally, the effect of this mutation is confined rather specifically to conjugation. *STE7* encodes a protein that is homologous to a kinase. It functions in a signaling pathway that controls cell cycle arrest in response to mating pheromone and cell and nuclear fusion immediately following conjugation (TEAGUE *et al.* 1986;

TABLE 7

Mitotic phenotype of *sae2Δ*: colony diameter on MMS media

Temperature	Strain	Concentration of MMS			
		0 (μg/ml)	60 (μg/ml)	170 (μg/ml)	510 (μg/ml)
30°	Wild type	4.0 ± 0.2	3.9 ± 0.3	2.8 ± 0.2	0
30°	*rad50Δ*	2.4 ± 0.8	0	0	0
30°	*rad50-KI81*	3.3 ± 0.2	3.2 ± 0.5	1.7 ± 0.4	0
30°	*sae2Δ*	3.5 ± 0.2	4.2 ± 0.5	1.2 ± 0.3	0
18°	Wild type	2.9 ± 0.2	1.8 ± 0.4	0.5	0
18°	*rad50Δ*	1.7 ± 0.8	0	0	0
18°	*rad50-KI81*	1.1 ± 0.2	0.5 ± 0.1	0.2	0
18°	*sae2Δ*	1.4 ± 0.2	0.6 ± 0.2	0	0

Isogenic SK1 derivatives containing the indicated mutations were grown in a patch on YP glycerol plates for 20 hr and then streaked for individual colonies on YEPD plates. Colonies were picked in triplicate and diluted in water and then plated on fresh (<24 hr old) YEPD plates that contained MMS at the indicated concentrations. Colony diameter was measured after 2 days incubation at 30° and 4 days incubation at 18°. Only colonies that were well separated from others were measured, generally only on plates that contained <50 colonies (using 83-mm petri dishes). Values are the means ± SD of the diameters (in mm) of at least 16 and frequently >25 measured colonies. Strains used were wild type, NKY278; *sae2Δ*, NKY2640; *rad50Δ*, NKY551; and *rad50-KI81*, NKY1408.

McCaffrey *et al.* 1987; Truehart *et al.* 1987; Chang and Herskowitz 1990; Elion *et al.* 1990; Meluh and Rose 1990; Gartner *et al.* 1992; Stevenson *et al.* 1992; Zhou *et al.* 1993). Ste7p is also required for filamentous and invasive growth (Liu *et al.* 1993; Roberts and Fink 1994), which occurs under specific nutritional conditions and is somewhat strain specific (Gimeno *et al.* 1992). To our knowledge, no other defects have been reported for haploid or diploid cells containing either conditional or deletion alleles of STE7.

Potential applications: We demonstrate above that our genetic method can be used to identify recessive mutations that block spore formation and have used the method to identify a specialized subclass of such mutations. We note that this method has certain advantages over other genetic approaches. The *ste7-1* feature makes it more tractable than methods that employ homothallism alone (*e.g.*, Esposito and Esposito 1969). Also, meiotic mutations can be identified using haploid cells in special circumstances (*e.g.*, Roth and Fogel 1971), but haploid meiosis differs from diploid meiosis in important ways (*e.g.*, De Massy *et al.* 1994).

In addition, however, this method can be used to identify mutations that affect either the viability of diploid cells specifically, as opposed to haploid cell viability, or mutations that affect behaviors that occur specifically in diploid cells. Mutations of the first type are not known but would be an interesting class to look for. The method could also be used to identify mutations that affect diploid formation, *e.g.*, mating type switching, conjugation or karyogamy; such mutants will be among those defective in spore formation.

Identification of meiotic intermediate block mutations: The current analysis sought to identify mutations that caused an intermediate block in prophase chromosome metabolism. This screen ultimately yielded eight mutations that had the correct meiotic phenotype and exhibited 2:2 segregation. Many more mutations appeared to have the correct phenotype but did not exhibit 2:2 segregation. This finding emphasizes the utility of a genetic system in which segregation tests can be carried out at a relatively early stage in the analysis.

This screen for additional intermediate block mutants is far from saturating, as alleles of three new genes were each found only once. On the other hand, a significant fraction of genes in which mutations could confer an intermediate block phenotype have likely been identified, as multiple alleles of two previously known types were found, two mutations in the *DMC1* gene and three nonnull "S" alleles of the *RAD50* gene. Mutations in another intermediate block gene *ZIP1* were not identified. This is expected, as a *zip1* null mutation confers no reduction in spore formation in the SK1 strain background (Sym and Roeder 1994; Xu *et al.* 1997) and does not score as spore-minus in the dityrosine fluorescence assay (K. Haack, unpublished results).

The *sae2* null mutant phenotype: The *sae2-1* and *sae2Δ* mutations confer indistinguishable phenotypes in every comparison performed thus far. We conclude that *sae2-1* allele is a null allele.

Neither *sae2* mutation has a discernible effect on the sizes of colonies growing on standard media; furthermore, neither mutation discernibly alters the frequency of spontaneous mitotic recombination. These observations suggest that the *SAE2/COM1* gene plays little if any role in normally growing mitotic cells. The *sae2-1* and *sae2Δ* mutations do, however, confer a subtle sensitivity to MMS in vegetative cells. This effect shows

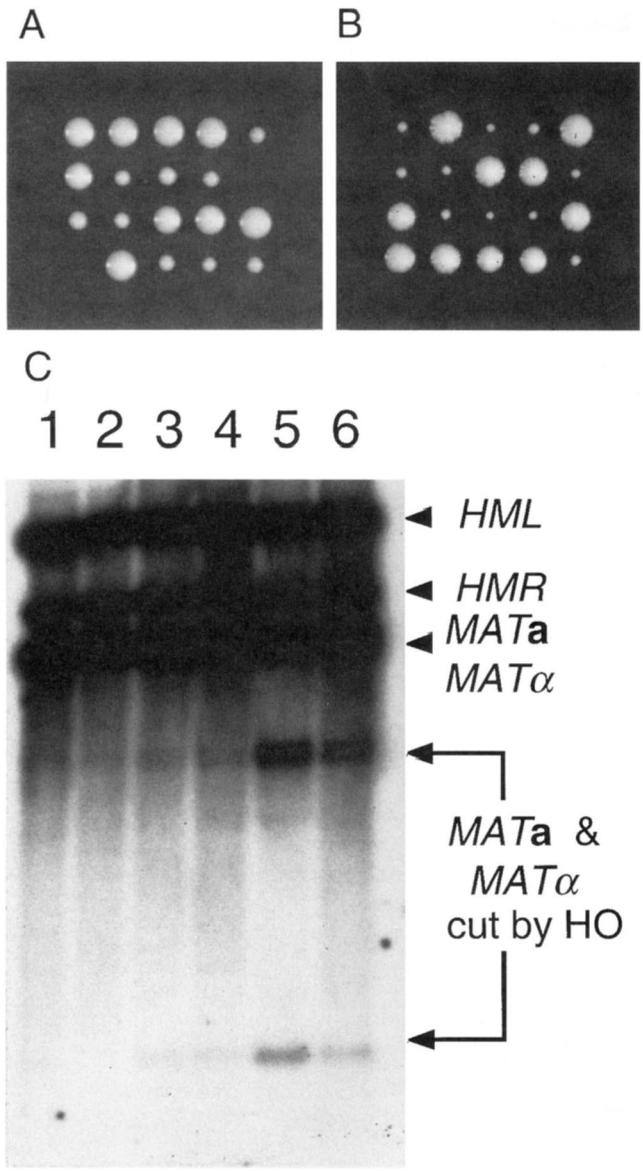

FIGURE 7.—Effects of a *rad50Δ* mutation on *HO ste7-1* spore clone colonies. Tetrads from a homothallic sterile strain that is heterozygous for *rad50Δ::hisG-URA3-hisG* (NKY2601) were dissected on YEPD. Five tetrads were germinated at 24° (A) and five germinated at 34° (B). The two small colonies in each tetrad are *rad50Δ*. The 24° plate exhibits the usual slow growth phenotype of *rad50Δ* observed in heterothallic cells (FARNET *et al.* 1988). An additional reduction in growth rate is evident at 34°. (C) Steady state level of breaks at the HO cut site in the *MAT* locus in exponentially growing diploid and haploid cells. Lanes 1–6 are *Hin*dIII cut genomic DNA probed with *MAT* sequences. Fragments hybridizing are: *HML*, *HMR*, *MAT***a** and *MATα*. HO endonuclease cuts *MAT***a** and *MATα* into 3.3- and 3.4-kb fragments, respectively, and a common 1.1-kb fragment. DNA in lanes 1 and 2 is from *RAD50* spore clones of NKY2601 grown at 24°. DNA in lanes 3–6 is from spore clones germinated and grown at 34°. Lanes 3 and 4 represent *RAD50*, and lanes 5 and 6 represent *rad50Δ*: the four constitute a complete tetrad of NKY2601. A signal indicative of HO-induced breaks at the *MAT* locus appears in all four lanes. The steady-state level of breaks in the *rad50Δ* clones is elevated relative to that of the *RAD50* spore clones.

that *SAE2/COM1* must be expressed in mitotic cells before or during MMS exposure. Repeated attempts to detect an RNA transcript from the *SAE2/COM1* gene in mitotic or meiotic cells failed, despite the clear presence of a control actin transcript (data not shown). The *SAE2/COM1* transcript may be present at a very low level in these cell types. Mitotic cells exposed to MMS were not examined for *SAE2/COM1* transcripts.

In meiotic cells, the *sae2* mutations confer a strong defect in commitment to meiotic levels of heteroallelic recombination as assayed genetically and in the formation of crossovers as determined by physical analysis. Meiosis-specific DSBs occur at normal levels but 5′ terminal resection is blocked; DSB turnover is also blocked, presumably as a consequence of the block at the resection stage.

The *sae2* mutations also cause delays in the occurrence of each of the two meiotic divisions, deterioration in the morphological integrity of the chromosome complement in most cells at late times and a severe defect in spore formation. Deterioration of chromosome complement is most simply attributed to the large number of unrepaired DSBs. Failure of meiosis I to occur on time is likely also attributable to the recombination defect (XU *et al.* 1997). The basis for the later defects is unknown. Defective spore formation could be a consequence of aberrant SPB behavior during the divisions (ALANI *et al.* 1990). Alternatively, chromosome degradation and/or chromosome loss could result in loss of genes whose expression late in meiosis is required for spore formation. A precedent for such genes is provided by the *dit1* and *dit2* mutations (BRIZA *et al.* 1990a); strains heterozygous for one of these mutations produce two normal and two affected spores, suggesting that the corresponding genes are normally expressed after the second meiotic division.

The *sae2* phenotype is indistinguishable from the *rad50S* phenotype: confirmation of a distinct meiosis-specific recombination function: In all of the tests performed thus far, the phenotypes conferred by the *sae2* null mutations are indistinguishable from those conferred by *rad50S* mutations, which are alterations of function alleles. The finding that the same array of meiotic phenotypes, most notably the defect in DSB processing, can be conferred by both types of mutations further supports that these phenotypes identify a discrete function, presumably one involved directly in the recombination process.

Rad50 protein is known to bind DNA in an ATP-dependent reaction (RAYMOND and KLECKNER 1993). Also, Rad50p interacts physically with functionally related proteins Mre11p and via Mre11p with Xrs2p (JOHZUKA and OGAWA 1995). Purified Mre11 protein has DNA-binding activity and has been shown to colocalize with Rad50 protein to meiotic chromosomes in a *rad50S* mutant (H. OGAWA, personal communication). Rad50p and Mre11p share homology with the *E. coli* proteins

SbcCp and SbcDp, respectively, and these two proteins exist as a complex (SHARPLES and LEACH 1995). Thus, Rad50p, Mre11p and Xrs2p could all be components of a multiprotein complex that promotes recombination reactions, by recruiting other functions and/or as direct participants in the chemical steps. Sae2/Com1 protein could well be a component of such a complex as well.

In wild-type meiotic cells, formation of a DSB is followed rapidly by resection of 5′ strands: the steady-state level of unresected breaks is below the level of physical detection, <1% per chromosome. *sae2* and *rad50S* mutations both uncouple cleavage of DNA strands from subsequent exonucleolytic resection. Thus, most simply, Sae2/Com1 protein and the "S" function of Rad50 protein could act within the recombination complex to promote resection. These two proteins could be components of the exonuclease itself. This possibility is supported by the fact that the *E. coli* sbcCD protein, to which Rad50p and Mrellp appear to be analogous, has double-strand exonuclease activity (CONNELLY and LEACH 1996). Alternatively, Sae2/Com1 and/or Rad50 "S" function could be positive factors that enable the exonuclease to assemble into the recombination function or to function once it is there.

Although *sae2*Δ and *rad50S* mutants are unable to convert meiotic DSBs into mature recombinants, these mutants are able to process certain other types of breaks. *HO* endonuclease-induced breaks are processed normally in both mitotic and meiotic *rad50S* cells (IVANOV *et al.* 1994; MALKOVA *et al.* 1996; MALKOVA and HABER, cited in KEENEY and KLECKNER 1995); correspondingly, *MAT* switching can occur in *sae2* mutants. Furthermore, mitotic cells of both *rad50S* and *sae2*Δ mutants are only very mildly defective in repairing damage caused by MMS, a radiomimetic compound presumed to induce DSBs.

One qualitative difference between meiotic DSBs and both *HO* and chemically induced breaks is that meiotic DSBs carry the Spo11 protein covalently attached at the end of the DNA chain on the 5′ strand (DE MASSY *et al.* 1995; KEENEY and KLECKNER 1995; LIU *et al.* 1995; KEENEY *et al.* 1997). Thus, the specific requirement for Sae2/Com1 protein and the Rad50S function of the Rad50 protein during meiosis may reflect the special need to remove the 5′ terminal protein. In fact, protein removal and nucleolytic resection could be one and the same process (KEENEY *et al.* 1997).

The weak MMS sensitivity of *sae2* and *rad50S* mutants remains to be explained. One possibility raised by the considerations above is that a small fraction of MMS-induced DSBs breaks are converted into a substrate that contains a protein or an unusual chemical group at the 5′ termini and thus requires the Sae2p/Com1p and the Rad50S function of Rad50p to be (rapidly) resected.

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