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Exo1 Roles for Repair of DNA Double-Strand Breaks and Meiotic Crossing Over in *Saccharomyces cerevisiae*

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The *MRE11*, *RAD50*, and *XRS2* genes of *Saccharomyces cerevisiae* are involved in the repair of DNA double-strand breaks (DSBs) produced by ionizing radiation and by radiomimetic chemicals such as methyl methanesulfonate (MMS). In these mutants, single-strand DNA degradation in a 5′ to 3′ direction from DSB ends is reduced. Multiple copies of the *EXO1* gene, encoding a 5′ to 3′ double-strand DNA exonuclease, were found to suppress the high MMS sensitivity of these mutants. The *exo1* single mutant shows weak MMS sensitivity. When an *exo1* mutation is combined with an *mre11* mutation, both repair of MMS-induced damage and processing of DSBs are more severely reduced than in either single mutant, suggesting that Exo1 and Mre11 function independently in DSB processing. During meiosis, transcription of the *EXO1* gene is highly induced. In meiotic cells, the *exo1* mutation reduces the processing of DSBs and the frequency of crossing over, but not the frequency of gene conversion. These results suggest that Exo1 functions in the processing of DSB ends and in meiotic crossing over.

INTRODUCTION

In mating-type switching and meiotic recombination in budding yeast, the formation of double-strand breaks (DSBs) is the initiating event. The DSB ends are subjected to 5′ to 3′ directed exonucleolytic processing to leave 3′-tailed single-strand (ss) DNA (Stahl, 1996). These ssDNA tails can be detected in vivo as intermediates during mating-type switching (White and Haber, 1990) and during meiotic recombination (Sun *et al.*, 1989, 1991; Cao *et al.*, 1990). Intermediates with ssDNA tails accumulate if the strand invasion step is blocked by a mutation in the *RAD51*, *-52*, *-54*, *-55*, or *-57* gene during mating-type switching and also in *DMC1* during meiotic recombination (Bishop *et al.*, 1992; Shinohara *et al.*, 1992; Sugawara and Haber, 1992; Ogawa *et al.*, 1993; Sugawara *et al.*, 1995). Several of these genes, *RAD51*, *-55*, *-57*, and *DMC1*, encode homologues of the *Escherichia coli* RecA protein.

One candidate for the enzyme involved in the production of 3′-tailed ssDNA is the Mre11/Rad50/Xrs2 protein complex. A lack of any one of these proteins makes cells highly sensitive to DSBs (Game and Mortimer, 1974; Ivanov *et al.*, 1992; Ajimura *et al.*, 1993) and reduces processing of DSB ends produced by the HO endonuclease during mating-type switching (Ivanov *et al.*, 1994; Lee *et al.*, 1998; Tsubouchi and Ogawa, 1998). As a consequence, the kinetics of recombinant formation is slow, but the amount of recombinant DNA ultimately formed is the same as in wild type (Ivanov *et al.*, 1994; Tsubouchi and Ogawa, 1998).

The Mre11/Rad50/Xrs2 complex is implicated in several aspects of DNA metabolism, including mitotic recombination, telomere maintenance, and nonhomologous end joining (Haber, 1998). Recent biochemical studies show that Mre11 and its mammalian homologues have double-strand (ds) DNA 3′ to 5′ exonuclease activity and ssDNA endonuclease activity (Furuse *et al.*, 1998; Paull and Gellert, 1998; Trujillo *et al.*, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999; reviewed by Haber, 1998). These activities are not directly involved in the formation of 3′-tailed ssDNA at DSB ends, because *mre11* mutants that lack them still show the wild-type level of DSB processing in vivo (Moreau *et al.*, 1999).

The Exo1 protein of fission and budding yeasts has been isolated as a 5′ to 3′ dsDNA exonuclease (Szankasi and Smith, 1995; Fiorentini *et al.*, 1997). This protein is conserved through higher eukaryotes and is implicated in recombination and mismatch correction in vivo (Digilio *et al.*, 1996; Tishkoff *et al.*, 1997, 1998; Qiu *et al.*, 1999). In accord with the mismatch correction function, budding yeast Exo1 interacts with the Msh2 protein (Tishkoff *et al.*, 1997). A homology search of the complete genome of budding yeast reveals four predicted proteins that share sequence similarity with Exo1: Rad2, Rad27, Din7, and Yen1 (Mieczkowski *et al.*, 1997; Tishkoff *et al.*, 1997). There could be functional redundancy among these proteins.

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Proper segregation of homologous chromosomes at the reductional division of meiosis requires crossing over to establish chiasmata, which physically connect homologues after disassembly of the synaptonemal complex. Gene conversion has no significant role in segregation (Roeder, 1997). A mutation in any one of a group of genes (*ZIP1*, *ZIP2*, *MSH4*, *MSH5*, *MLH1*, *MLH3*, and *MER3*) reduces crossing over (Ross and Roeder, 1994; Sym and Roeder, 1994; Hollingsworth *et al.*, 1995; Hunter and Borts, 1997; Chua and Roeder, 1998; Nakagawa and Ogawa, 1999; Wang *et al.*, 1999). Among these, *MSH4*, *MSH5*, *MLH1*, and *MLH3* are homologues of the mismatch repair proteins of *E. coli*, suggesting a mechanistic relationship between mismatch repair and crossing over (Nakagawa *et al.*, 1999).

In this study, we isolated the *EXO1* gene as a high-copy suppressor of the methyl methanesulfonate (MMS) sensitivity of *mre11* mutants. The *exo1* mutant itself is moderately sensitive to MMS. The *exo1 mre11* double mutant, however, is more sensitive to MMS and shows a greater reduction in DSB processing efficiency during mating-type switching than either single mutant. During meiosis, the *exo1* mutation reduces both DSB processing and crossover frequency and causes nondisjunction of homologous chromosomes. We suggest that Exo1, as well as the Mre11 complex, is involved in the processing of DSB ends and that Exo1 is also involved in crossing over to promote the accurate segregation of chromosomes during meiosis.

MATERIALS AND METHODS

Plasmids

Plasmids were constructed by standard procedures. A 4.4-kilobase (kb) *Sph*I fragment containing *EXO1* was cloned into the *Sph*I site of YEplac195 (Gietz and Sugino, 1988) to make pHT131. An *Eco*RI fragment in the *EXO1* coding sequence was replaced with a 1.1-kb *Eco*RI fragment containing *URA3* from YEp24 to make pHT133 or with a 2.2-kb *Hpa*I fragment containing *LEU2* from YEp13 to make pHT256. Plasmids pHT133 and pHT254 were used for *EXO1* disruption experiments. pHT59 is a pUC118-based plasmid carrying a 3.4-kb *Nsi*I–*Bam*HI fragment of *XRS2* on which a *Xba*I–*Bgl*II fragment was replaced with a 3.8-kb *hisGURA3hisG* fragment from pNKY51 (Alani *et al.*, 1987). pHT127 carries a 4.3-kb *Bam*HI fragment containing *MRE11* on YEplac195. pNKY291 contains sequences downstream of *HIS4*. pYtel is based on pBluescriptII SK− and carries a 1.0-kb *Xho*I fragment of a yeast chromosomal end, which contains 120-base pair telomere repeats and part of the Y′ subtelomeric repeat (a gift from F. Ishikawa, Tokyo Institute of Technology, Tokyo, Japan). pYA301 carries a 3.4-kb *Bam*HI–*Eco*RI fragment that includes the yeast actin gene cloned into the *Bam*HI–*Eco*RI sites of pBR322 (Gallwitz and Seidel, 1980). pBTM116 (Chien *et al.*, 1991) was used to measure plasmid end joining (Boulton and Jackson, 1996b)

Yeast Strains and Media

Strains used in this study are listed in Table 1. All gene targeting was carried out by the one-step gene disruption method (Rothstein, 1983). KJC010 contains an *mre11-1* allele and is MMS sensitive at 34°C (Ajimura *et al.*, 1993). HTY836 was made by replacing a *Bgl*II–*Bgl*II region of the *THR4* coding sequence of the R167 strain (a gift from J.E. Haber, Brandeis University, Waltham, MA) with a *hisGlacZ* insertion. HTY525, -1076, -1104, -1106, -1214, -1215, -1330, and -1336 are SK1 derivatives that enter the meiotic cell cycle in a highly synchronous manner (Fast, 1973). HTY1212, -1213, -1326, -1328, -1434, -1436, -1442, and -1444 are congenic to SK1. HTY1212, -1213, -1214, and -1215 are identical to a pair of haploids consisting of MY257 and a pair of haploids consisting of MY261, respectively (Sym and Roeder, 1994), and are gifts from G.S. Roeder (Yale University, New Haven, CT). The *EXO1* gene was disrupted by transforming strains with pHT133 or pHT256 after *Sph*I digestion. The *XRS2* gene was disrupted by transforming strains with pHT59 after *Sph*I and *Bam*HI double digestion. Disruption of *MRE11*, *RAD50*, *RAD52*, and *DMC1* was carried out as described previously (Alani *et al.*, 1989; Bishop *et al.*, 1992; Johzuka and Ogawa, 1995; Tsubouchi and Ogawa, 1998).

All media used in this study were detailed previously (Tsubouchi and Ogawa, 1998). YPLac contains 1% yeast extract, 2% bacto peptone, and 2% sodium lactate (pH 5.5), and YPGal is identical except that it contains 2% galactose instead of lactate.

Isolation of High-Copy Suppressors for MMS Sensitivity of mre11

KJC010 was transformed with a yeast genomic library constructed in YEp24 (Botstein *et al.*, 1979), plated on SD-URA (synthetic complete medium lacking uracil) plates, and incubated at 34°C for 3 d. Transformants were replica-plated onto YPD plates containing 0.01% MMS and incubated for 3 d at 34°C. MMS-resistant Ura+ clones were streaked on 5-FOA plates to isolate plasmid segregants, and MMS sensitivity of *mre11-1* strains with and without the suppressor candidate plasmids was compared. The plasmids that make the *mre11-1* strain resistant to MMS were isolated and sequenced from both sides of the insert fragment. The primers used are Pri 1 (cagtcctgctcgcttcgc) and Pri 2 (atgtcggcgatataggcg).

Plasmid End-joining Assay

This assay was performed as described previously (Boulton and Jackson, 1996b). In brief, HTY987, -989, and -991 were transformed with supercoiled, or linear, pBTM116 (Chien *et al.*, 1991) digested with *Bam*HI. One hundred nanograms of each DNA was used to transform each strain. The ratio of Ura+ Trp+ transformants obtained with cut or uncut DNA was determined for each strain. The average value of two independent assays for each strain is shown.

Detection of Mating-type Switching

Induction of HO break and detection of mating-type switch process by Southern blot analysis have been described (Tsubouchi and Ogawa, 1998). Genomic DNA samples obtained at indicated times were cut with *Sty*I (*Eco*130I), separated on 0.7% agarose gels, and subjected to Southern blot analysis with a 1.0-kb *Nde*I–*Hin*dIII fragment of pHT46 as a probe.

Detection of Telomeres

Genomic DNA was isolated from overnight cultures of the strains indicated (see Figure 7), cut with *Xho*I, separated on 0.7% agarose gels, and subjected to Southern blot analysis with a 1-kb *Xho*I–*Bam*HI fragment of pYtel as a probe.

Return-to-Growth Experiment and Meiotic DSB Detection

Synchronous entry of cells into meiosis, return-to-growth experiments, and detection of meiosis-specific DSBs were performed as described previously (Cao *et al.*, 1990; Johzuka and Ogawa, 1995). Genomic DNA samples at different times during meiosis were cut with *Pst*I, separated on 0.7% agarose gels, and subjected to Southern blot analysis with a 1.5-kb *Pst*I–*Bgl*II fragment of pNKY291 as a probe (Cao *et al.*, 1990).

Table 1. Strains used

Strains	Genotypes
CG379	*MATα ura3-52 leu2-3, 112 trp1-289 ade5-1 his7-2*
KJC010	*MATα can1 ura3 ade2 ade6 cyh2 trp1 mre11-1*
HTY525	*MAT***a** *ho*::*LYS2 lys2 ura3 leu2*::*hisG his4B*::*LEU2 arg4-bgl* / *MATα ho*::*LYS2 lys2 ura3 leu2*::*hisG his4X*::*LEU2 arg4-nsp*
HTY836	*matΔ::LEU2 hmr-3Δ mal2 trp1 leu2 ura3 thr4::hisGlacZ*
HTY837	HTY836 but *mre11::hisG*
HTY838	HTY836 but *rad50::hisG*
HTY839	HTY836 but *xrs2::hisG*
HTY975	HTY836 but *rad52::hisG*
HTY987	HTY837 carrying YEplac195
HTY989	HTY837 carrying pHT131
HTY991	HTY837 carrying pHT127
HTY1013	HTY836 but *exo1::URA3*
HTY1015	HTY836 but *mre11::hisG* and *exo1::URA3*
HTY1017	HTY836 but *rad50::hisG* and *exo1::URA3*
HTY1076	HTY525 but homozygous for *exo1::URA3*
HTY1104	*MAT***a** *ho*::*LYS2 lys2 ura3 leu2*::*hisG his4B*::*LEU2 arg4-bgl dmc1*::*LEU2* / *MATα ho*::*LYS2 lys2 ura3 leu2*::*hisG his4X*::*LEU2 arg4-bgl dmc1*::*LEU2*
HTY1106	*MAT***a** *ho*::*LYS2 lys2 ura3 leu2*::*hisG his4B*::*LEU2 arg4-bgl dmc1*::*LEU2 exo1*::*URA3* / *MATα ho*::*LYS2 lys2 ura3 leu2*::*hisG his4X*::*LEU2 arg4-nsp dmc1*::*LEU2 exo1*::*URA3*
HTY1212	*MAT***a** *ho*::*LYS2 lys2 URA3 HOM3 TRP2 leu2*::*hisG can1*
HTY1213	*MATα ho*::*LYS2 lys2 ura3 hom3-10 trp2 leu2*::*hisG CAN1*
HTY1214	*MAT***a** *ho*::*LYS2 lys2 ura3 leu2*::*hisG trp1-H3 CEN3-URA3*
HTY1215	*MATα ho*::*LYS2 lys2 ura3 leu2*::*hisG trp1-H3 CEN3-TRP1 his4B*::*LEU2*
HTY1326	HTY1212 but *exo1*::*LEU2*
HTY1328	HTY1213 but *exo1*::*LEU2*
HTY1330	HTY1214 but *exo1*::*LEU2*
HTY1332	CG379 with pHT51
HTY1333	HTY1332 but *mre11::hisGURA3hisG*
HTY1334	HTY1332 but *exo1::URA3*
HTY1335	HTY1332 but *mre11::hisG exo1::URA3*
HTY1336	HTY1215 but *exo1*::*LEU2*
HTY1434	HTY1213 but *msh4::URA3*
HTY1436	HTY1212 but *msh4::URA3*
HTY1442	HTY1213 but *exo1*::*LEU2 msh4::URA3*
HTY1444	HTY1212 but *exo1*::*LEU2 msh4::URA3*

Detection of the EXO1 *Transcript during Meiosis*

Total RNA was extracted from meiotically dividing cells of HTY525, a diploid SK1 derivative, as reported previously (Johzuka and Ogawa, 1995), and subjected to Northern blot analysis. The amounts of RNA loaded to keep the amount of the *ACT1* transcript nearly constant through meiosis are as follows: 10 μg for 0 h, 20 μg for 2 and 4 h, 40 μg for 6 h, and 60 μg for 8 and 10 h. As probes, a 0.9-kb *Eco*RI fragment of pHT130 and a 0.6-kb *Cla*I fragment of pYA301 were used to detect transcripts of *EXO1* and *ACT1*, respectively.

RESULTS

The EXO1 *Gene on a Multicopy Plasmid Suppresses the Repair Defect of an* mre11 *Mutant*

In an *mre11*, *rad50*, or *xrs2* null mutant, the kinetics of recombination during mating-type switching is slow because of inefficient DSB processing. However, the wild-type amount of recombinant is formed eventually, suggesting that there are additional proteins involved in this process. To identify a gene involved in the process, we screened for high-copy suppressors of the high MMS sensitivity of the *mre11-1* missense mutant, which shows temperature-dependent MMS sensitivity (Ajimura *et al.*, 1993). The mutant was transformed with a genomic DNA library constructed in a 2-μm vector, YEp24. From transformants that grew on YPD plates containing 0.01% MMS at 34°C, plasmids were retrieved and DNA sequences from both ends of the inserts were determined. Seventeen clones were found to contain the *MRE11* gene itself, and eight contained the *EXO1* gene. The 4.4-kb *Sph*I–*Sph*I fragment containing the *EXO1* gene was cloned into YEplac195, a 2-μm vector, and this plasmid (pHT131) was used for further analysis.

To determine if multiple copies of the *EXO1* gene can suppress the defect of the *mre11* null mutant, the mutant was transformed with vector alone (YEplac195), a plasmid containing *EXO1* (pHT131), or a plasmid containing *MRE11* (pHT127). Suppression of MMS sensitivity was clearly observed for the mutant with a high dosage of the *EXO1* gene (Figure 1). Because the phenotypes of *mre11*, *rad50*, and *xrs2* mutants are indistinguishable (Alani *et al.*, 1990; Ivanov *et al.*, 1992; Johzuka and Ogawa, 1995) and the Mre11 protein forms a complex with the Rad50 and Xrs2 proteins (Johzuka

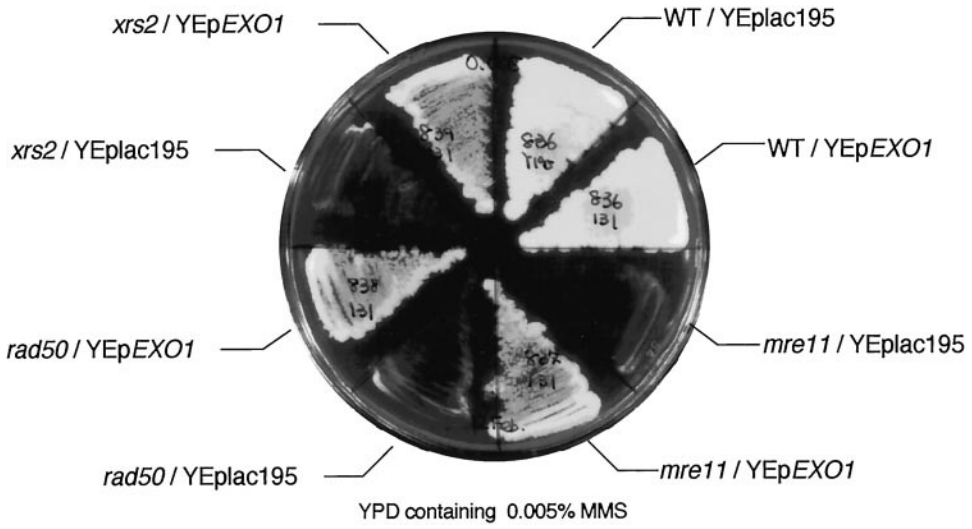

Figure 1. Suppression of the MMS sensitivity of the *mre11* null mutant by high-copy *EXO1*. Wild-type (HTY836), *mre11* (HTY837), *rad50* (HTY838), and *xrs2* (HTY839) null mutants containing YEplac195 or YEp*EXO1* (pHT131) were streaked on YPD containing 0.005% MMS.

and Ogawa, 1995; Usui *et al.*, 1998), the *EXO1* gene was tested for its ability to suppress the MMS sensitivity of *rad50* and *xrs2* mutants as well. As expected, suppression was observed in these null mutants to the same extent as in the *mre11* mutant (Figure 1). High dosage of *EXO1* increased resistance to MMS >1000-fold at 0.005% MMS (Figure 2 A). However, *EXO1* overexpression did not suppress the repair defect of the *rad52* mutant (Figure 2 A), indicating that the suppression effect is specific to the *mre11*, *rad50*, and *xrs2* mutants.

Mating-type Switching Is Extremely Retarded in the Absence of Both Exo1 and Mre11

To test whether *EXO1* is involved in DSB repair, the phenotype of an *EXO1* deletion mutant was examined. The mutant is more sensitive to MMS than wild type, particularly in the presence of >0.0125% MMS (Figure 2B). Furthermore, *exo1 mre11* and *exo1 rad50* double mutants demonstrate more pronounced repair defects than the *mre11* and *rad50* single mutants (Figure 2C). Therefore, the *EXO1* gene is involved

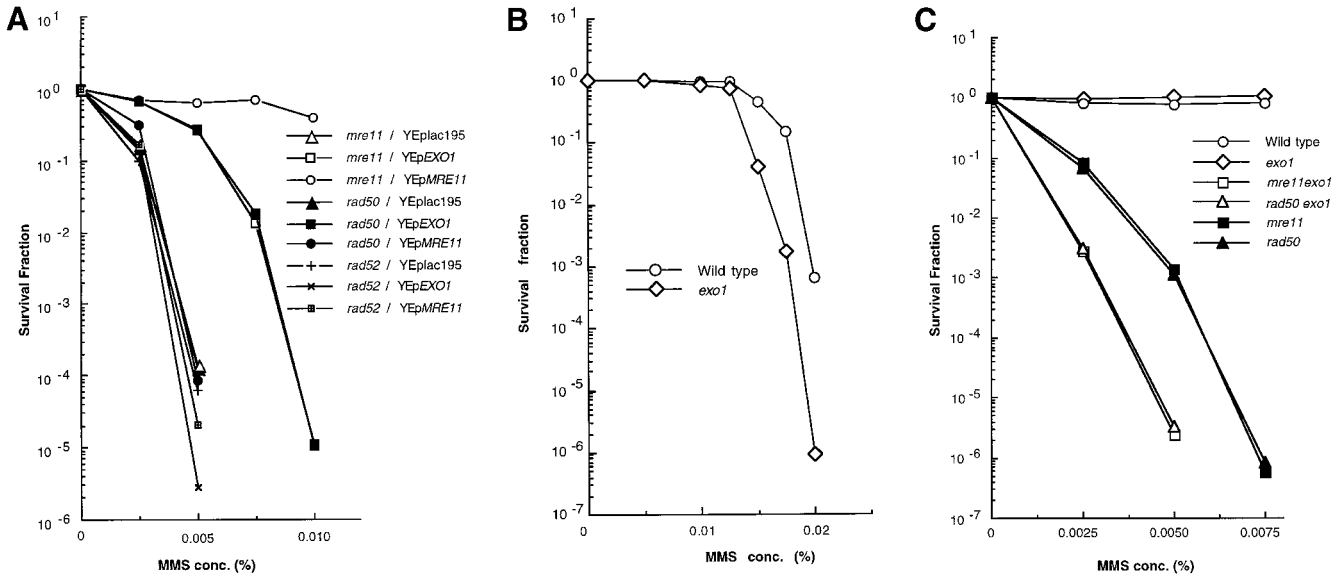

Figure 2. (A) Quantitative analysis of the suppression efficiency of a high dosage of *EXO1*. Mutant *mre11* (HTY837), *rad50* (HTY838), and *rad52* (HTY975) strains containing YEplac195, YEp*EXO1* (pHT131), or YEp*MRE11* (pHT127) were cultured and plated on SD-URA plates containing various concentrations of MMS. Colonies were counted after 5 d at 30°C and normalized to the number of colonies formed in the absence of MMS. (B) MMS sensitivity of the *exo1* null mutant. (C) MMS sensitivity of double mutants. (B and C) Wild-type (HTY836), *exo1* (HTY1013), *mre11* (HTY837), *rad50* (HTY838), *mre11 exo1* (HTY1015), and *rad50 exo1* (HTY1017) were cultured and plated on YPD plates containing various concentrations of MMS. Survival fractions were calculated as in A. Each experiment was performed at least twice for each strain; the results of single representative experiments are shown here.

in a damage repair pathway that is independent of *MRE11*, *RAD50*, and *XRS2*. However, its contribution to repair is not as great as that of *MRE11/RAD50/XRS2*.

The process of mating-type switching is well characterized and provides a good model system for analyzing the effect of the *exo1* mutation on homologous recombination. DSBs were formed at the *MAT* locus by the HO endonuclease, whose expression was controlled by a galactose-inducible promoter. When wild-type cells are incubated in medium containing galactose for 1 h, a 1.8-kb *MAT*α band, which contains an HO target site, is reduced in amount and a 0.7-kb fragment corresponding to an HO-cut fragment appears (Figure 3, A, B, F, and G). When cells are shifted to glucose medium for 1 h, most of the 0.7-kb HO-cut fragment disappears and a 0.9-kb *MAT***a** band, the product of mating-type switching, appears. The 0.7-kb band completely disappears with an additional 1 h of incubation (Figure 3, B, F, and G). In the *exo1* mutant, the kinetics of MAT switching is indistinguishable from that of wild type (Figure 3, D, F, and G). In the *mre11* mutant, however, the 0.7-kb band persists after 3 h in glucose medium, and the appearance of the 0.9-kb *MAT***a** band is delayed about 2 h (Tsubouchi and Ogawa, 1998) (Figure 3, C, F, and G). In the *exo1 mre11* double mutant, ~10% of the 0.7-kb HO-cut fragment still remains at 9 h, and the appearance of the 0.9-kb *MAT***a** band is further delayed compared with the *mre11* single mutant (Figure 3, E, F, and G). The relative intensity of HO-cut fragments in the *mre11 exo1* double mutant increases even after shutting off the expression of HO, presumably because the remaining HO endonuclease is active while DSB processing is very slow, resulting in further accumulation of HO-cut fragments. These results indicate that recombination is retarded, but still occurs, even in the absence of both Exo1 and Mre11.

EXO1 *Does Not Suppress the Reduction in Nonhomologous End Joining in* mre11

MRE11 is involved in the nonhomologous end joining (NHEJ) pathway as well as the homologous recombination pathway of DSB repair. To test whether *EXO1* can suppress the *mre11* defect in NHEJ, a plasmid recircularization assay was used (see MATERIALS AND METHODS). The *mre11* null mutant containing vector alone (HTY987), a plasmid carrying *EXO1* (HTY989), or a plasmid carrying *MRE11* (HTY991) was transformed with pBTM116 linearized by *Bam*HI or with a supercoiled plasmid control. Because the linearized plasmid must be recircularized to be propagated, the ratio of the number of transformants obtained with the linear plasmid tested relative to the number obtained with the supercoiled plasmid reflects the ability of the strains to repair DSBs. In pBTM116, there is no yeast-derived sequence around the restriction enzyme cleavage site; therefore, repair occurs predominantly by NHEJ. The proportion of transformants recovered with cut plasmid relative to uncut plasmid in *mre11* carrying a vector alone is almost identical to that of *mre11* carrying multicopy *EXO1* (2.5 and 1.9%, respectively), and both of these values are ~10-fold lower than the value obtained with *mre11* carrying the *MRE11* gene (18.8%) (Figure 4). We also tested the recovery of a plasmid linearized with a restriction enzyme that creates blunt ends; there was no difference in the plasmid recircularization ratio regardless of whether or not *mre11* carried multicopy *EXO1* (our unpublished result). These results indicate that *EXO1* does not suppress the NHEJ defect of *mre11*.

EXO1 *Suppresses Neither Telomere Shortening nor Spore Inviability in* mre11

Telomeres become shortened in the *mre11*, *rad50*, and *xrs2* mutants (Kironmai and Muniyappa, 1997; Boulton and Jackson, 1998; Nugent *et al.*, 1998). Because a high dosage of the *EXO1* gene suppresses the DSB repair defect of the *mre11* mutant, we examined the effects of *EXO1* on telomere shortening in *mre11*. In the *exo1* mutant, telomeres are not shortened (Figure 5, lane 3), and shortening is not enhanced in the *exo1 mre11* double mutant compared with *mre11* alone (Figure 5, lane 4). Furthermore, high dosage of the *EXO1* gene does not suppress the telomere shortening of *mre11* (Figure 5, lane 6). Thus, unlike Mre11, Exo1 does not appear to be involved in telomere maintenance.

mre11 produces inviable spores during meiosis. To determine if a high dosage of *EXO1* suppresses this defect, spore viability was compared between an *mre11* strain carrying multicopy *EXO1* and a control *mre11* strain carrying vector alone. In both cases, no viable spores were generated (44 tetrads dissected for each).

Meiotic Phenotypes of the exo1 *Mutant*

Transcription of* EXO1 *Is Induced during Meiosis The *EXO1* gene of the fission yeast was reported to be induced during meiotic prophase and implicated to have functions in mismatch correction (Szankasi and Smith, 1995). The Exo1 homologue of *Drosophila*, named Tosca, is also induced during meiosis (Digilio *et al.*, 1996). We tried to determine if transcription of the *EXO1* gene of budding yeast is induced during meiosis. Cells progressing synchronously through meiosis were harvested, and total RNA was extracted and subjected to Northern blot analysis. The amount of RNA loaded at each time point was normalized to the amount of *ACT1* mRNA. In the strain used, premeiotic DNA synthesis is completed by 4 h, the DSB level reaches a maximum at around 5 h, and the fraction of cells that are binucleate is maximal at around 6 h. *EXO1* transcripts are present at a low level during mitosis but increase drastically between 4 and 6 h after entry into meiosis, as has been shown before (Chu *et al.*, 1998a,b) (Figure 6). The maximum increase of 36-fold is reached by 6 h; by 10 h, transcript levels begin to decline, suggesting its role by meiosis I.

DSB Processing Is Impaired in the* exo1 *Mutant Almost all meiotic recombination is initiated by DSBs formed at recombination hot spots (Haber, 1997). The effect of the *exo1* mutation on meiotic recombination was monitored at one of these hot spots, the *HIS4::LEU2* locus (Cao *et al.*, 1990) (Figure 7A). DSB products appeared at 3 h and reached a maximal level at 5 h in both wild type and *exo1*, but their disappearance was delayed by 1–2 h in *exo1* (Figure 7B). Progression through the meiotic cell cycle was monitored at the same time. In *exo1*, the fraction of cells that had passed the first meiotic division reached a maximum at 7 h after

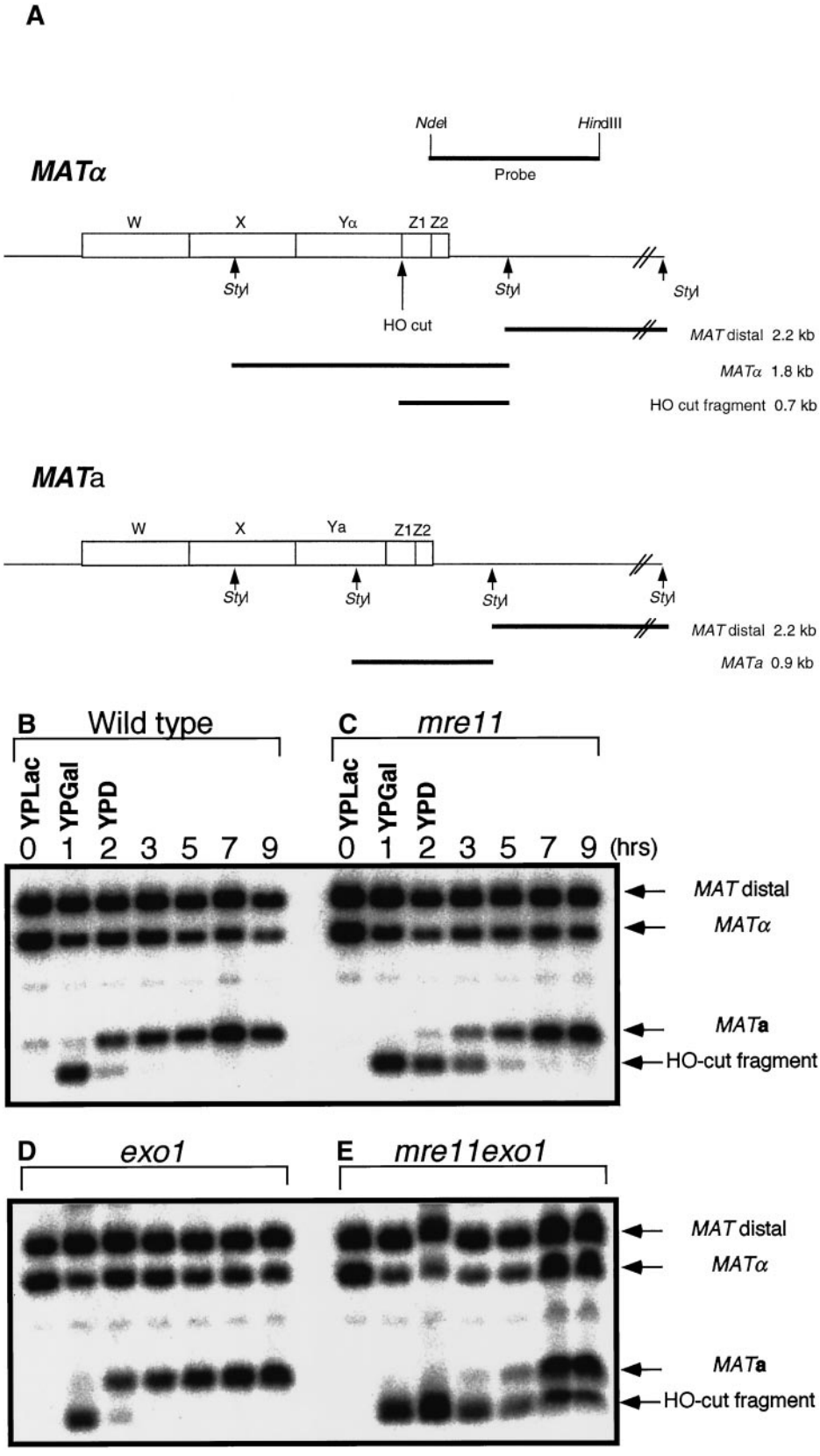

Figure 3. Mating-type switching. (A) Physical map of the *MAT* locus. (B–E) Cells were preincubated in YPLac, then transferred into YPGal for 1 h, to induce a DSB at the Y/Z junction by HO endonuclease. Transcription was then repressed by changing the culture medium to YPD. Cells were harvested at the indicated times, and genomic DNA was extracted and subjected to Southern blot analysis with a probe shown in A. (B) Wild type (HTY1332); (C) *mre11* (HTY1333); (D) *exo1* (HTY1334); (E) *mre11 exo1* (HTY1335). (F and G) Densitometric analyses of HO-cut fragments (F) and recombinant fragments (G) in Southern blot data from B–E. The band intensities of HO-cut fragments (in F) or recombinant fragments (in G) were divided by the combined intensities of bands corresponding to *MAT* distal, *MATα*, *MAT***a**, and HO cut fragment to obtain the relative intensities.

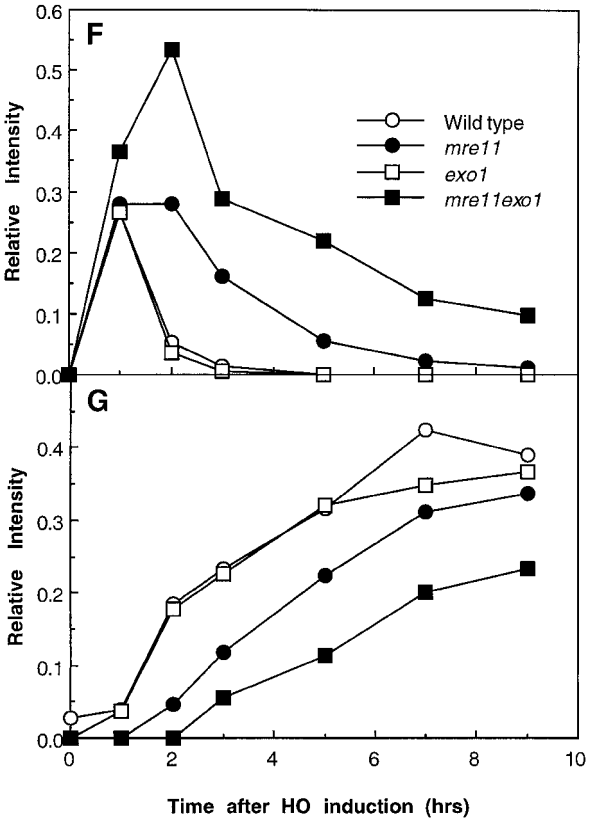

Figure 3 (cont).

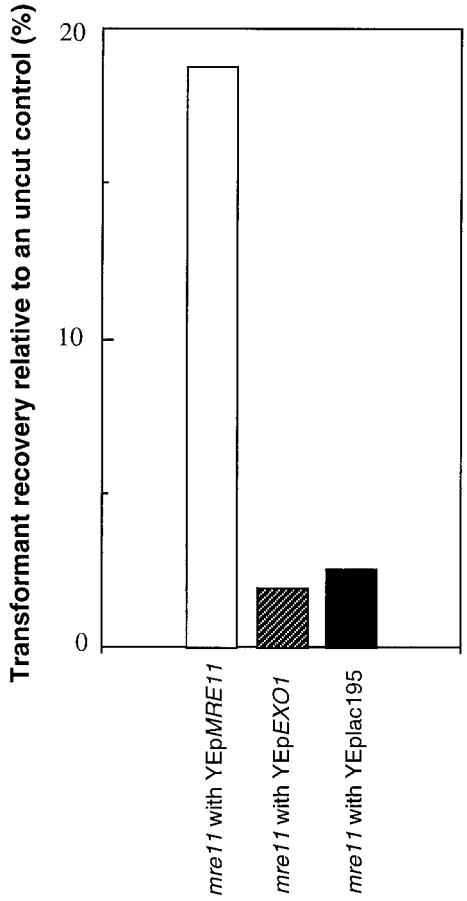

Figure 4. Multicopy *EXO1* does not suppress the reduced NHEJ efficiency of *mre11*. The NHEJ assay is described in MATERIALS AND METHODS.

entry into meiosis, which is 1 h later than in wild type (our unpublished result). To understand the effect of the *exo1* mutation on DSB processing more clearly, we took advantage of the *dmc1* mutant, in which the recombination reaction is blocked at the strand-transfer stage and DSBs with ssDNA tails accumulate (Bishop *et al.*, 1992). In the *exo1 dmc1* double mutant, meiotic DSBs were formed as in a wild-type time course, but the DNA fragment appeared more discrete (Figure 7C), indicating a defect in exonucleolytic processing to expose ssDNA tails. This reduction in processing was first evident at 3 h and became more pronounced at later times (Figure 7C).

The* exo1 *Mutation Reduces Meiotic Crossing Over To examine the effect of the *exo1* mutation on recombination, gene conversion frequencies were measured with two sets of heteroalleles, *his4X/his4B* and *arg4-bgl/arg4-nsp* (Sherman and Roman, 1963; Esposito and Esposito, 1974). At both of these loci, recombination was induced almost to the same extent as in the wild type at 24 h after entry into meiosis (Table 2). Crossing over was measured in four intervals (*CEN3-HIS4*, *CAN1-URA3*, *URA3-HOM3*, and *HOM3-TRP2*) by tetrad analysis (Sym and Roeder, 1994). In each interval, map distance was reduced 1.5- to 2.0-fold in the *exo1* mutant (Table 3); this reduction is statistically significant (*CEN3-HIS4*, $p < 0.02$; *CAN1-URA3*, $p \ll 0.0001$; *URA3-HOM3*, $p \ll 0.0001$; *HOM3-TRP2*, $p < 0.004$). There is a modest but significant reduction of spore viability in the *exo1* mutant (84%, 216 tetrads dissected, $p \ll 0.0001$), as reported previously (Fiorentini *et al.*, 1997). Furthermore, the number of viable spores per ascus is not random: fractions of asci that contain zero, two, or four viable spores per ascus (7, 12, and 74%, respectively) are more frequent than asci containing one or three viable spores per ascus (2 and 4%, respectively) (Figure 8). This suggests that a large fraction of the nondisjunction events is due to pairs of homologues segregating to the same pole at the first meiotic division. To examine the segregation of homologous chromosomes directly, a diploid strain was used in which the centromere on one chromosome III was marked with *TRP1* and the other with *URA3* (Sym and Roeder, 1994). In a cross between *CENIII-TRP1* and *CENIII-URA3* strains, spores that are disomic for chromosome III result from nondisjunction at the first meiotic division and can be identified as both Ura+ and Trp+. Among 63 two-spore–viable tetrads, 8 resulted from nondisjunction of chromosome III. Among them, no recombinant was found in *CEN3-HIS4*. In 45 of the remaining 55 tetrads containing two viable spores, the two spores were sisters

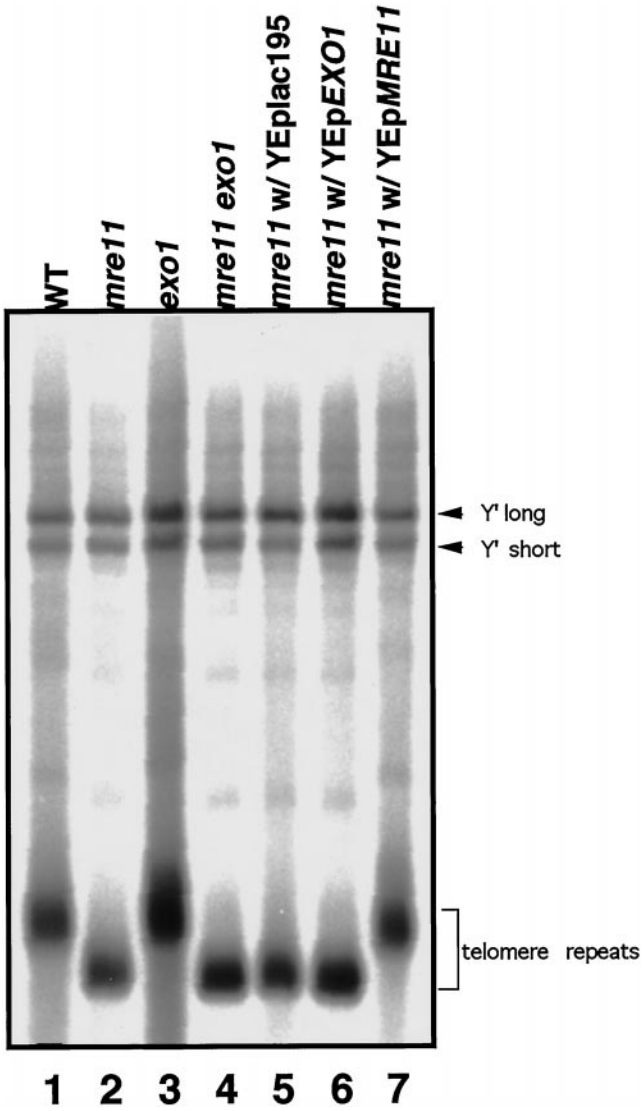

Figure 5. Telomere length in mutant strains. Genomic DNAs were extracted, cut with *Xho*I, and subjected to electrophoresis, followed by Southern blot analysis to detect telomere sequences (see MATERIALS AND METHODS). Lane 1, wild-type (HTY836); lane 2, *mre11* (HTY837); lane 3, *exo1* (HTY1013); lane 4, *mre11 exo1* (HTY1015); lane 5, *mre11* with YEplac195 (HTY987); lane 6, *mre11* with YEp*EXO1* (HTY989); lane 7, *mre11* with YEp*MRE11* (HTY991).

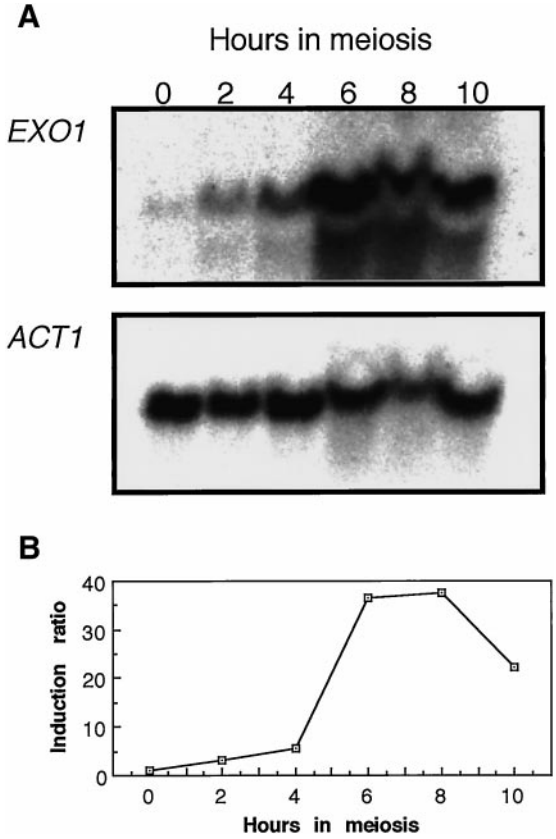

Figure 6. Meiotic induction of *EXO1* transcription. Total RNA from a wild-type diploid (HTY525) was isolated at the indicated times during meiosis, fractionated on formaldehyde agarose gels (0.7%), and subjected to Northern blot analysis. (A) Transcription of the *EXO1* and *ACT1* genes was monitored with *EXO1* (0.9-kb *Eco*RI fragment from pHT130) and *ACT1* (0.6-kb *Cla*I fragment from pYA301) coding sequences as probes. The *ACT1* transcript, which is expressed at a constant level during meiosis, was used as an internal control (McKee and Kleckner, 1997). (B) Relative intensity of the *EXO1* transcript compared with that of *ACT1*. At each time, the intensities of the *EXO1* and *ACT1* bands were measured and the amount of *EXO1* transcript was divided by the amount of *ACT1* transcript.

(i.e., both spores were Ura+ or Trp+), suggesting that they resulted from meiosis nondisjunction of another pair of chromosomes. These results indicate that crossing over is reduced and nondisjunction is increased in *exo1*.

It was shown previously that the meiosis-specific mismatch repair homologues *MSH4* and *MSH5* are involved in meiotic crossing over (Ross and Roeder, 1994; Hollingsworth *et al.*, 1995). These two gene products form a complex and act in the same pathway. In the *msh4* mutant, crossing over is less than (in *CAN1-HOM3*, $p \ll 0.0001$) or similar to (in *HOM3-TRP2*, the difference of the two values at this interval is statistically insignificant [$p > 0.4$]) that in the *exo1* mutant (Table 3). To understand the relationship between the *MSH4*-dependent pathway and *EXO1*, a *msh4 exo1* double mutant was constructed and crossing over was measured in the *CAN1-HOM3* and *HOM3-TRP2* intervals. The reduction of crossing over in *msh4* was not much affected by the additional *exo1* mutation (Table 3) (the differences of genetic distances in each interval are statistically insignificant [$p > 0.5$ and $p > 0.9$, respectively]). Unexpectedly, however, spore viability in *msh4 exo1* (28%, 870 tetrads dissected) is lower than that in *msh4* (43%, 570 tetrads dissected). The difference is statistically significant ($p < 0.0001$)

DISCUSSION

Exo1 and Mre11 Are Involved in DSB Processing

We isolated the *EXO1* gene as a high-copy suppressor of the MMS sensitivity of the *mre11-1* mutant. The *EXO1* gene also

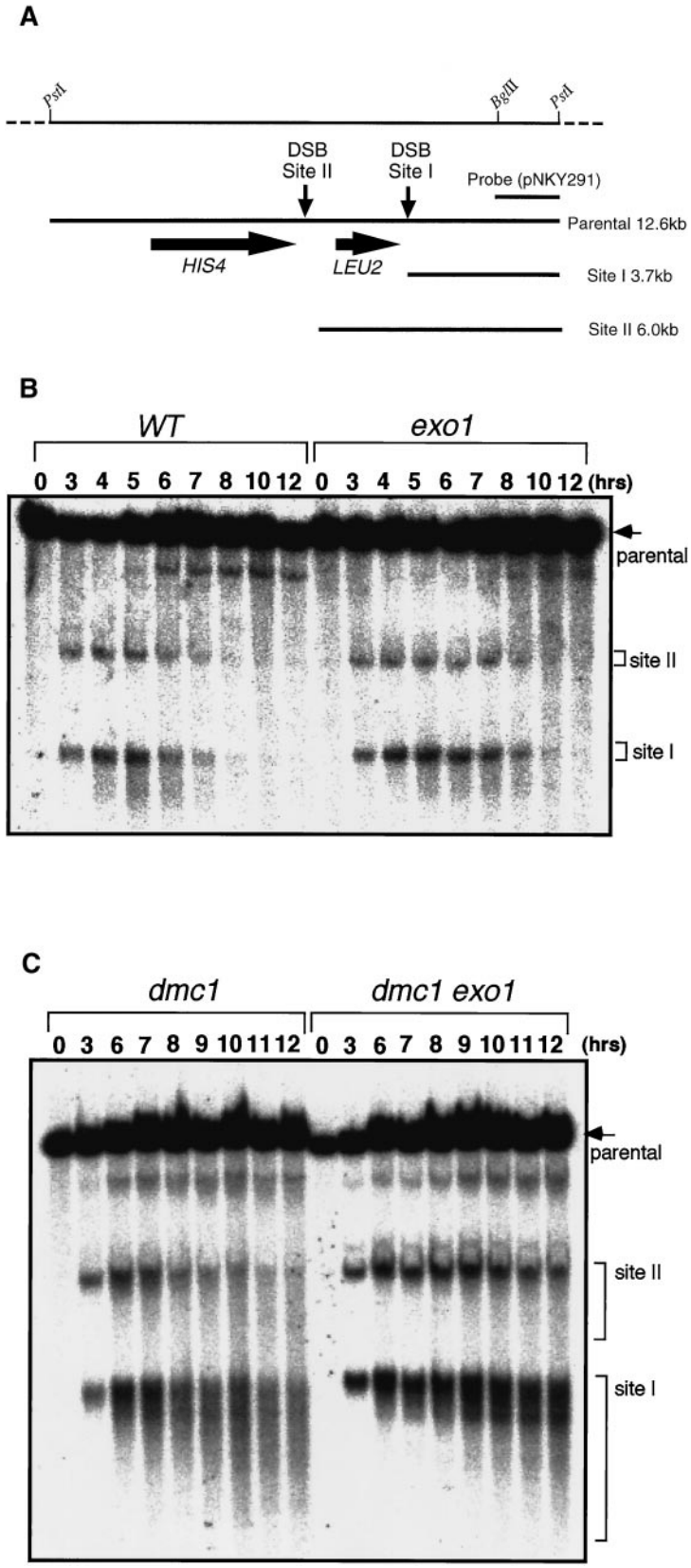

Figure 7. Processing of meiotic DSBs is reduced in *exo1*. (A) Restriction map of the *HIS4::LEU2* construct. DSBs are formed at sites I and II. Fragments diagnostic of DSBs at sites I and II and the unbroken parental fragment (parental) are shown. (B) Detection of meiotic DSBs in wild type (HTY525) and *exo1* (HTY1076). (C) Detection of meiotic DSBs in *dmc1* (HTY1104) and *dmc1 exo1* (HTY1106). DNA was extracted from cultures of each strain at the indicated times during meiosis. After digestion with *Pst*I and electrophoresis, DSBs were detected by Southern blot analysis with an appropriate probe (1.5-kb *Pst*I–*Eco*RI band from pNKY291).

Table 2. Gene conversion

Locus	Wild type (HTY525)	*exo1* (HTY1076)
his4X::LEU2/*his4B::LEU2*	0.013 ± 0.007	0.012 ± 0.009
arg4-bgl/*arg4-nsp*	0.040 ± 0.012	0.034 ± 0.012

The meiotic prototroph frequencies at *HIS4* and *ARG4* loci at 24 h after entry into meiosis are shown. Values are the averages of four independent cultures for each strain (mean ± SD).

suppresses the MMS sensitivity of the *mre11*, *rad50*, and *xrs2* null mutants but not the *rad52* mutant. These results suggest that the function of Exo1 can compensate for the defect of the *mre11*, *rad50*, and *xrs2* mutants in MMS-induced DNA damage repair. The *exo1* mutant shows moderate MMS sensitivity, and in combination with the *mre11* mutation, MMS sensitivity is more pronounced. These results suggest that Exo1 and the Mre11/Rad50/Xrs2 complex act independently in MMS-induced DNA damage repair.

MMS, a radiomimetic agent, is assumed to cause DSBs, and the MMS sensitivity of the *mre11* mutant is attributed to its inability to repair DSBs (Tavassoli *et al.*, 1995; Bressan *et al.*, 1998). DSBs are predominantly repaired by homologous recombination in yeast (Friedberg *et al.*, 1995), and the process of mating-type switching serves as a model system for analyzing the recombination process (Haber, 1995). In this system, the *mre11* and *rad50* null mutations reduce 5′ to 3′ exonucleolytic processing from DSB ends, which delays the formation of mature recombinants (Sugawara and Haber, 1992; Lee *et al.*, 1998; Tsubouchi and Ogawa, 1998). Although DSB processing at the *MAT* locus appears normal in the *exo1* mutant, processing in the *exo1 mre11* double mutant is delayed compared with that in the *mre11* single mutant.

Because *EXO1* encodes a dsDNA 5′ to 3′ exonuclease (Fiorentini *et al.*, 1997; Tishkoff *et al.*, 1997), promotion of ssDNA formation at DSB ends by *EXO1* overexpression could explain the suppression of the repair defect of *mre11*. Although *MRE11* is involved in the NHEJ pathway as well, *EXO1* overproduction does not suppress the reduction of NHEJ, suggesting that *EXO1* suppression is independent of the NHEJ pathway.

These results argue that Exo1 and Mre11 are involved in DSB processing to create 3′-tailed ssDNA. It is likely that Exo1 is involved directly in this process, whereas recent biochemical studies have demonstrated that Mre11 (and human Mre11 complex, consisting of hMre11, hRad50, and p95) displays 3′ to 5′ dsDNA exonuclease activity as well as ssDNA endonuclease activity in vitro, and that these activities are not important for the creation of 3′-tailed ssDNA in vivo (Furuse *et al.*, 1998; Paull and Gellert, 1998; Trujillo *et al.*, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999). Thus, it is not likely that Mre11 plays a major role in DSB processing as a nuclease. A second possibility is that the role of the Mre11 complex in DSB processing is to facilitate ssDNA formation indirectly, e.g., the complex may recruit other enzyme(s) and help them to process DSB ends.

DSB Processing and DSB Repair

We have observed a correlation between MMS sensitivity and the efficiency of DSB processing during mating-type

Table 3. Crossing over

Interval	PD	TT	NPD	Total	cM	Decrease (fold)
Wild type						
CEN3-HIS4	47	87	2	136	36.4	
CAN1-URA3	62	177	9	248	46.6	
URA3-HOM3	80	160	7	247	40.9	
HOM3-TRP2	183	58	2	243	14.4	
exo1						
CEN3-HIS4	199	122	7	328	25	1.5
CAN1-URA3	270	161	6	437	22.5	2.1
URA3-HOM3	265	170	7	442	24.0	1.7
CAN1-HOM3	155	254	31	440	44.2	–
HOM3-TRP2	371	61	0	432	7.1	2.0
msh4						
CAN1-HOM3	61	42	2	105	25.7	
HOM3-TRP2	81	20	0	101	9.9	
exo1 msh4						
CAN1-HOM3	52	39	3	94	30.3	
HOM3-TRP2	73	19	0	92	10.3	

To examine the *CEN3-HIS4* interval, HTY1214 and HTY1215 (wild type) and HTY1330 and HTY1336 (*exo1*) were dissected after mating. In the *CAN1-URA3, URA3-HOM3, CAN1-HOM3,* and *HOM3-TRP2* intervals, HTY1212 and HTY1213 (wild type), HTY1326 and HTY1328 (*exo1*), HTY1434 and HTY1436 (*msh4*), and HTY1442 and HTY1444 (*msh4 exo1*) were dissected after mating. Only four-spore–viable tetrads that did not show gene conversion of the markers indicated were used to calculate map distances. Three classes of tetrads were scored as follows: PD, parental ditype; TT, tetratype; NPD; nonparental ditype. Map distances are expressed in terms of centimorgans (cM), which were calculated from the following formula: cM = 100/2(TT + 6NPD)/(PD + TT + NPD).

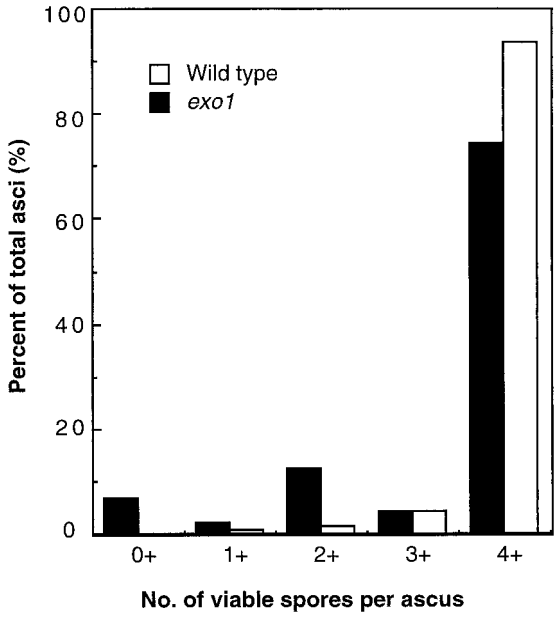

Figure 8. Distribution of tetrad types. The distribution of 216 asci with four (4+), three (3+), two (2+), one (1+), or no (0+) viable spores is shown for the wild type (HTY525) and the *exo1* strain (HTY1076).

switching: no reduction in DSB processing and moderate MMS sensitivity in *exo1*; reduced processing and more severe MMS sensitivity in *mre11*; and even greater reduction in processing and sensitivity to MMS in the *mre11 exo1* double mutant. The fact that the *exo1* mutation does not affect mating-type switching, although the mutant is slightly sensitive to MMS, might be due to a difference in the number of DSBs induced. In mating-type switching, only one DSB is created per cell, whereas multiple DSBs are produced at high doses of MMS.

Functional Redundancy in DSB Processing

Although delayed, recombination still occurs in the absence of both Mre11 and Exo1, suggesting the existence of other protein(s) with exonuclease activity. There are four genes (*RAD2*, *RAD27*, *DIN7*, and *YEN1*) that encode putative proteins sharing homology with Exo1. *DIN7* was recently shown to function specifically in mitochondria (Fikus *et al.*, 2000), so it is unlikely that *DIN7* and *EXO1* function redundantly. In particular, *RAD27* may share common functions with *EXO1*, because *exo1 rad27* double mutants exhibit a synthetic lethal phenotype, and the *EXO1* gene is a high-copy suppressor of the temperature-sensitive and mutator phenotypes of a *rad27* mutant (Tishkoff *et al.*, 1997). There may be other enzymes with exonuclease activity in vivo.

Mre11 and Regulation of Telomere Length

Although *MRE11/RAD50* are implicated in the telomerase-mediated pathway for telomere replication, it remains unclear how they function (Haber, 1998; Nugent *et al.*, 1998). We have confirmed that telomere shortening occurs in the *mre11* null mutant, as expected from the earlier report for the *rad50* mutant (Kironmai and Muniyappa, 1997; Boulton and Jackson, 1998; Nugent *et al.*, 1998). The *exo1* null mutation, however, does not affect the telomere length, and high copies of the *EXO1* gene do not affect the telomere shortening seen in the *mre11* null mutant. Therefore, the reduction in DSB processing observed in the *mre11* mutant is not likely to be related to telomere shortening. Mutations in yeast Ku homologues abolish NHEJ and reduce telomere length (Boulton and Jackson, 1996a,b, 1998; Milne *et al.*, 1996; Porter *et al.*, 1996). Both yeast Ku homologues and *MRE11*, *RAD50*, and *XRS2* are implicated in the same pathway of NHEJ (Milne *et al.*, 1996; Boulton and Jackson, 1998); therefore, the NHEJ activity of the Mre11 complex could contribute to telomere maintenance. This is consistent with our finding that high-copy *EXO1* does not suppress the NHEJ defect of the *mre11* mutant.

Exo1 and Crossing Over during Meiosis

In the present investigation, we observed increased fractions of tetrads exhibiting zero, two, and four viable spores in *exo1* mutants, reminiscent of conditions that cause defects in meiosis I. Crossing over is reduced to ~50% of the wild-type level and increased levels of chromosome nondisjunction are observed, suggesting that Exo1 is important for homologous chromosome segregation by ensuring crossing over. Consistent with this view, none of the eight chromosome III disomes was recombinant. In the absence of Mlh1, Mlh3, Msh4, Msh5, Zip1, Zip2, and Mer3, crossing over is reduced twofold to threefold during meiosis, leading to abnormal reductional division (Ross and Roeder, 1994; Sym and Roeder, 1994; Hollingsworth *et al.*, 1995; Hunter and Borts, 1997; Chua and Roeder, 1998; Nakagawa and Ogawa, 1999; Wang *et al.*, 1999). Mlh1, Mlh3, Msh4, and Msh5 are homologues of mismatch repair proteins, and both Mlh1 and Mlh3, like Exo1, are also required for mismatch correction; thus, a close mechanistic correlation between mismatch repair and crossing over is suggested. Our genetic data show that crossing over in the *exo1 msh4* double mutant is no more reduced than in *msh4*, arguing that Exo1 and Msh4 work in a common pathway. There is a strong interaction between *EXO1* and *MSH2*; *MSH2* encodes a homologue of the MutS protein of *E. coli* and plays a central part in recognition of mismatch base pairing (Tishkoff *et al.*, 1997), but it does not play a role in crossing over (Hunter and Borts, 1997). Exo1 physically interacts with Msh2, and *EXO1* acts in the *MSH2*-dependent mismatch repair pathway. Because Msh2 and Msh4 are two homologues of the bacterial MutS protein, Exo1 function in mismatch repair may be mechanistically related to its role in crossing over.

Although the rate of meiotic crossing over in *msh4* is not affected by the *exo1* mutation, fewer spores are viable in the *exo1 msh4* double mutant than in the *msh4* single mutant, suggesting that Exo1 and Msh4 have additional roles during meiosis other than crossing over. Crossing over interference is severely reduced in *msh4* strains, and the distribution of crossing over among chromosomes is random (Roeder, 1997). The greater spore viability of *exo1* strains, despite a similar reduction in crossing over, suggests that interference is unaffected by the *exo1* mutation. On the other hand, we have observed a reduction in the processing of meiosis DSBs in *exo1*. The greater reduction in spore viability in *msh4 exo1* strains, compared with the corresponding single mutants,

might be due to the combination of a defect in interference and a failure to repair some DSBs.

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