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Exonuclease I of *Saccharomyces cerevisiae* Functions in Mitotic Recombination In Vivo and In Vitro

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We previously described a 5′-3′ exonuclease required for recombination in vitro between linear DNA molecules with overlapping homologous ends. This exonuclease, referred to as exonuclease I (Exo I), has been purified more than 300-fold from vegetatively grown cells and copurifies with a 42-kDa polypeptide. The activity is nonprocessive and acts preferentially on double-stranded DNA. The biochemical properties are quite similar to those of *Schizosaccharomyces pombe* Exo I. Extracts prepared from cells containing a mutation of the *Saccharomyces cerevisiae EXO1* gene, a homolog of *S. pombe exo1*, had decreased in vitro recombination activity and when fractionated were found to lack the peak of activity corresponding to the 5′-3′ exonuclease. The role of *EXO1* on recombination in vivo was determined by measuring the rate of recombination in an *exo1* strain containing a direct duplication of mutant *ade2* genes and was reduced sixfold. These results indicate that *EXO1* is required for recombination in vivo and in vitro in addition to its previously identified role in mismatch repair.

The currently favored models for homologous recombination predict a requirement for nucleases at several steps in the recombination reaction (18, 37, 55). Endonucleases are envisioned to function as initiators of recombination and in the resolution of crossed-strand intermediates. Exonucleases are thought to process break sites to generate single-stranded DNA, the substrate for binding by homologous pairing proteins. Late steps in the reaction, such as the repair of heteroduplex DNA (mismatch correction), also require the activity of exonucleases. The importance of exonucleases in recombination has been clearly demonstrated in *Escherichia coli*, in which 5′-to-3′ exonucleases are required for all homologous recombination pathways (30). In addition, the single-stranded exonucleases RecJ (5′), exonuclease VII (Exo VII) (5′ and 3′), and Exo I (3′) are required for mismatch repair in vitro (8).

In vivo studies of double-strand break (DSB) repair in *Saccharomyces cerevisiae* provide further support for the role of exonucleases in recombination. After HO endonuclease cleavage at the *MAT* locus during mating-type switching, the DNA is resected to produce a 3′ tail on the distal side of the HO cut site (64). This single-strand tail is believed to invade the donor locus, thus initiating the transfer of information. Formation of the 3′ single-stranded tail is thought to result from the activity of a double-stranded 5′-3′ exonuclease, but it could arise from the combined activities of a helicase and single-stranded endonuclease or exonuclease (35). Although mutation of several known recombination genes prevents mating-type switching, none appears to block the exonucleolytic processing step (49, 64). However, the degradation of the 5′ strand is slower in *rad50* and *xrs2* mutants, suggesting that these genes may encode or regulate the activity of a nuclease (21). Most meiotic recombination hot spots are cleaved by endonucleases during meiosis, and the resulting DSBs are processed to leave 3′ single-strand tails (5, 50). An allele of the *RAD50* gene called *rad50S* prevents this degradation and also prevents further progression through meiosis (1). Thus, the 3′ tails generated by the processing of DSB sites are essential intermediates in meiotic recombination. The failure of meiotic DSBs to be processed in *rad50S* mutants appears to result from the covalent attachment of a protein to the 5′ ends at the break site (10, 25, 32).

Several 5′-3′ exonuclease activities have been identified in extracts of *S. cerevisiae* (3, 4, 7, 9, 12, 62). The genes encoding two of these activities, *NUC1* and *SEP1*, have been cloned (58, 65). Nuc1 is a mitochondrial protein that is tightly associated with the inner membrane (9). Sep1 is an abundant protein that catalyzes DNA strand exchange in vitro (29), is a potent 5′-3′ exoribonuclease, and displays weak exo-DNase activity (23, 48). Although Sep1 has the expected in vitro activity for a protein that could be involved in processing DSB sites and utilizing the 3′ tail in a strand exchange reaction, *sep1* mutants are proficient at mating-type switching and undergo exonucleolytic processing of meiotic DSBs (2, 59). These results suggest that another exonuclease is responsible for the processing observed at DSB sites in vivo or that there is functional redundancy for this activity. The Rad2 protein, which acts endonucleolytically during nucleotide excision repair, has a weak 5′-3′ exonuclease activity (14). However, the phenotypes of *rad2* mutants, such as resistance to ionizing radiation and normal levels of recombination, are inconsistent with this nuclease playing a major role in recombinational repair. A protein with homology to Rad2, identified through the chromosome *XI* sequencing project (22) and subsequently called Rad27, Rth1, or Erc11 (40, 47, 61), also has 5′-3′ exonuclease activity (17). For simplicity, this protein is referred to as Rad27 in this report. The mammalian homolog of Rad27 was identified as an exonuclease required to complete lagging-strand DNA synthesis in vitro (13, 26, 60, 63) and as the structure-specific endonuclease FEN-1 (16, 17). Rad27 is proposed to be

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TABLE 1. Characteristics of yeast strains used in this study

Strain	Genotype[a]	Source or reference
W303-1A	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
W303-1B	*MAT*α *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
W838-19C	*MAT*α *rad1::LEU2 rad52::TRP1*	R. Rothstein
LSY346-3A	*MAT***a** *nuc1::LEU2 pep4::LEU2 sep1::URA3*	20
LSY346-5A	*MAT*α *nuc1::LEU2 pep4::LEU2 sep1::URA3*	20
LSY380	*MAT*α *rad2::TRP1*	This study
LSY381-3A	*MAT*α *rad2::TRP1 nuc1::LEU2 pep4::LEU2 sep1::URA3*	This study
LSY381-13C	*MAT***a** *rad2::TRP1 nuc1::LEU2 pep4::LEU2 sep1::URA3*	This study
LSY485	*MAT***a** *yen1::HIS3*/*MAT*α *YEN1*	This study
LSY485-2C	*MAT***a** *yen1::HIS3*	This study
LSY495-14A	*MAT***a** *yen1::HIS3 nuc1::LEU2 pep4::LEU2 sep1::URA3*	This study
LSY486-2A	*MAT*α *rad27::URA3*	This study
LSY494-5C	*MAT***a** *nuc1::LEU2 pep4::LEU2 sep1::URA3 rad27::URA3*	This study
RKY2321	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200*	57
RKY2662	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 exo1::HIS3*	57
LSY492	*MAT*α *nuc1::LEU2 pep4::LEU2 sep1::URA3 exo1::HIS3*	This study
LSY496-10D	*MAT***a** *exo1::HIS3*	This study
LSY496-20A	*MAT*α *exo1::HIS3*	This study
YKH10a	*MAT***a** *ADE2*	19
YKH19a	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ*	This study
LSY496-16D	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ exo1::HIS3*	This study
LSY497-25B	*MAT*α *ade2-3′Δ-URA3-ade2-5′Δ rad1::LEU2*	This study
LSY497-6D	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ exo1::HIS3 rad1::LEU2*	This study
LSY497-7D	*MAT*α *ade2-3′Δ-URA3-ade2-5′Δ rad52::TRP1*	This study
LSY497-17C	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ rad52::TRP1 exo1::HIS3*	This study
LSY497-9C	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ rad1::LEU2 rad52::TRP1*	This study
LSY497-11D	*MAT***a** *ade2-3′Δ-URA3-ade2-5′Δ exo1::HIS3 rad1::LEU2 rad52::TRP1*	This study
LSY509	*MAT***a** *exo1::HIS3 din7::LEU2*	This study
LSY511-42A	*MAT***a** *din7::LEU2 nuc1::LEU2 pep4::LEU2 sep1::URA3*	This study

[a] All strains except RKY2321 and RKY2662 are derivatives of W303-1A or W303-1B. Only differences in the genotype from the parental strains are indicated.

the functional and structural homolog of the 5′-3′ exonuclease domain of *E. coli* DNA polymerase I (40, 47). Analysis of the completed DNA sequence of *S. cerevisiae* identified three other open reading frames (ORFs) encoding proteins with homology to Rad2. These include *DHS1* (ORF YOR033C, chromosome *XI*), *DIN7* (ORF YDR263C, chromosome *IV*), and *YEN1* (ORF YER041W, chromosome *V*). The *DHS1* gene was originally identified by genetic complementation of a drug-hypersensitive mutant (31). However, the protein sequence of Dhs1 (GenBank accession number S69545) is 219 amino acids shorter than the ORF identified by the *S. cerevisiae* genome sequencing project, and most of the conserved nuclease motifs are found within these 219 amino acids. Dhs1 was also identified as a protein that interacts with the mismatch recognition protein Msh2 (57), and Dhs1 shows higher homology to *Schizosaccharomyces pombe* Exo I (see below) than to Rad2 or Rad27. Because of the high homology between the complete Dhs1 sequence and *S. pombe* Exo I, the Dhs1 protein has been renamed *S. cerevisiae* Exo1 (57).

Two 5′-3′ exonucleases have been purified and characterized from *S. pombe*. Exo I is double-strand specific and is induced during meiosis (52). *exo1* mutants display increased rates of spontaneous mutation and increased meiotic intragenic recombination between close markers, two phenotypes consistent with a role in mismatch correction (53). The sequence of Exo I shows considerable homology to both Rad2 and Rad27 of *S. cerevisiae*, indicating that Exo I is part of the superfamily of eukaryotic proteins with homology to the 5′-3′ exonuclease domain of prokaryotic Pol I proteins, T4 RNase H, T5 exonuclease D15, T7 gene 6 exonuclease, and *E. coli* RecJ (33, 38, 53). A *Drosophila* homolog of *S. pombe* Exo I was recently identified as a gene that is specifically expressed in the female germ line (11). Exo II of *S. pombe* acts preferentially on single-stranded DNA and appears to be a homolog of the *S. cerevisiae* Sep1 protein (24, 54).

In previous work aimed at developing an in vitro system for recombination in *S. cerevisiae*, we identified a 5′-3′ exonuclease that was required for an end-joining reaction and for the formation of joint molecules between two linear duplexes that had a region of overlapping terminal homology (20, 51). Because this activity was undiminished in cell extracts prepared from a strain containing mutations in the *NUC1* and *SEP1* genes, we concluded that it was distinct from these previously characterized exonucleases. In this report, we describe the purification and properties of this exonuclease and provide evidence that it is the product of the *EXO1* gene, a homolog of *S. pombe* Exo I. Furthermore, we show that recombination between repeated sequences is reduced in *exo1* mutants.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used in this study are derivatives of W303-1A or W303-1B (Table 1). Strains LSY346-3A and LSY364-5A, containing disruptions of the *SEP1*, *NUC1*, and *PEP4* genes, have been described previously (20). Strain LSY380 was constructed by one-step replacement (41) of W303-1B with a PCR fragment to generate a deletion-disruption allele of *RAD2*. PCR was performed on a *TRP1*-containing template (pRS404) with the primers 5′-GAAGGTTCTACACGTCATCCATGAAGAAAAGCATT TTCGGGAGAAggtcgaaaaaagaaaaggaga-3′ and 5′-TCATACCTCACCGACG CAAAGATACCAAAAGGACCGTATATATCTatgcttgcttttcaaaaggcc-3′. Uppercase bases represent *RAD2* sequences, and lowercase bases correspond to *TRP1* sequences. Trp^+ transformants that showed sensitivity to UV light were analyzed by Southern hybridization to verify disruption of the *RAD2* gene. The resulting *rad2::TRP1* allele has had the entire *RAD2* ORF replaced by the *TRP1* gene. The diploid that resulted from crossing LSY380 to LSY346-3A was sporulated, and tetrads were dissected to identify haploid segregants (LSY381-3A and LSY381-13C) that contained the *rad2*, *sep1*, *pep4*, and *nuc1* deletion alleles. Strain LSY486-2A was generated by transformation of W303-1B with a DNA

fragment containing *rad27::URA3*, derived from plasmid pMR*rad27*Δ*::URA3* (40). Ura$^+$ transformants that showed a temperature-sensitive growth defect and sensitivity to methyl methanesulfonate were analyzed by Southern blotting to verify disruption of the *RAD27* gene and then were backcrossed to W303-1A. Strain LSY486-2A was crossed to strain LSY346-3A, and segregants that contained the *rad27::URA3* allele, in addition to *sep1*, *pep4*, and *nuc1* mutations, were identified among the haploid progeny generated by sporulation and tetrad analysis. Strain LSY485 was constructed by one-step replacement of a W303 diploid strain with a PCR fragment to generate a deletion-disruption allele of *YEN1*. PCR was performed on a *HIS3*-containing template DNA (pRS403) with the primers 5′-GGTCCATCCAATGAAATGACAGTTCTATTGCATTTTAC CTACTTGTATgtgagcgctaggagtcactg-3′ and 5′-CAACTGTGGTGGCGGATT TTTTGACGCTGTGCCCGTTAACTCATTCAAggaaagcgcgcctcgttcag-3′. Uppercase bases represent *YEN1* sequences, and lowercase bases represent *HIS3* sequences. His$^+$ transformants were screened by PCR analysis of genomic DNA with primers complementary to sequences within the *HIS3* gene and 3′ to the disruption of the *YEN1* locus. The resulting *yen1::HIS3* allele has had the entire *YEN1* coding region replaced by the *HIS3* gene. This diploid was sporulated, and tetrads were dissected to obtain haploid segregants containing the *yen1::HIS3* allele. Strain LSY485-2C was crossed to LSY346-5A, and the resulting diploid was sporulated to obtain haploid segregants that contained the *yen1::HIS3*, *sep1::URA3*, *nuc1::LEU2*, and *pep4::LEU2* alleles (LSY495-14A). Strain LSY492, containing a disruption of the *EXO1* gene, was made in two steps as follows. First, an *exo1* deletion strain (RKY2662) was made by transformation of RKY2321 with a PCR-amplified DNA fragment containing *EXO1* gene sequences bordering the *HIS3* gene. This fragment was constructed by amplification of the *HIS3* gene present in pPS729 (P. Silver, Dana-Farber Cancer Institute) by PCR with primers 22244 (5′-AAAGGAGCTCGAAAAAACTGAAAG GCGTAGAAAGGAATGGGTATCCAAGGTggcctcctctagtacactc) and 21964 (5′-CCTCCGATATGAAACGTGCAGTACTTAACTTTTATTTACCTTTAT AAACAAATTGGGgcgcgcctcgttcagaat). Uppercase bases represent *EXO1* sequences, and lowercase bases represent *HIS3* sequences. In this strain, residues 6 to 695 of the *EXO1* ORF were replaced with the *HIS3* gene. This disruption was then introduced into LSY346-5A by transformation with a DNA fragment containing the *HIS3* gene bordered by several hundred base pairs of *EXO1* sequence; this fragment was generated by PCR with the primers 22388 (5′-CC GGCCCGAGAAGGAGAAGTA) and 22237 (5′-TGCGGAGAATAAAAGGT TGTGACG) and genomic RKY2662 DNA as the template. The correct integration of the *exo1::HIS3* allele in strain LSY492 was verified by PCR analysis of genomic DNA preparations with primers complementary to sequences in *EXO1* and *HIS3*. Strain LSY492 was crossed to YKH19a to generate haploid progeny containing the *exo1::HIS3* allele (LSY496-10D and LSY496-20A). Strain LSY509 was constructed by one-step replacement of strain LSY496-10D with a PCR fragment to generate a deletion-disruption allele of *DIN7*. A *LEU2*-containing DNA template (pRS405) was used for PCR with the primers 5′-ATTCGATAG GAATGGGAATACCTGGCTTACTGCCTCAATTctcgaggagaacttctagta-3′ and 5′-GGTACGGTGCCTGAGATCGCTGCTGGTGAGGTCATATAAAtcg actacgtcgtaaggccg-3′. Uppercase bases represent *DIN7* sequences, and lowercase bases represent *LEU2* sequences. Leu$^+$ transformants were screened by PCR analysis of genomic DNA with primers complementary to sequences internal to the *LEU2* gene and to sequences downstream of the disruption at the *DIN7* locus. The disruption alleles of *YEN1* and *DIN7* made by the PCR method were also verified by Southern hybridization analysis. The resulting *din7::LEU2* allele has had residues 11 to 413 of the 430-residue ORF replaced by the *LEU2* gene. Strain LSY509 was crossed to strain LSY346-5A, the resulting diploid was sporulated, and the tetrads were dissected to obtain haploid segregants that contained the *din7::LEU2*, *sep1::URA3*, *nuc1::LEU2*, and *pep4::LEU2* alleles.

Strain YKH19a, containing a direct repeat of mutant *ade2* genes, was used to measure recombination rates. One repeat contains a 3′ truncation of the *ade2* gene, and the other contains a truncation of the 5′ end. The repeats are separated by pRS306 vector sequences, including the *URA3* gene. This strain was made by transformation of strain YKH10a (19) with plasmid pKH11C, which had been digested with the restriction endonuclease *Bgl*II, and selection for Ura$^+$ transformants. The structure of the resulting Ura$^+$ Ade$^-$ transformants was confirmed by Southern analysis. Strain YKH19a was crossed to LSY492, and after tetrad analysis of the resulting diploids, haploid segregants containing the *ade2* recombination reporter and the *exo1::HIS3* allele were identified (LSY496-16D and LSY496-19B). LSY496-16D was crossed to a *rad1 rad52* strain (W838-19C), and haploid segregants containing the *ade2* recombination reporter and combinations of the *exo1*, *rad1*, and *rad52* alleles were identified by phenotype.

Transformation of yeast cells and sporulation and dissection of diploid strains were performed as described previously (45). *E. coli* DH5α was used for propagation of plasmids, and strain JM109 was used for propagation of M13 derivatives.

Plasmids. The plasmid used to generate the direct repeat of mutant *ade2* genes was constructed as follows. First, pKH5, which contains a 3.6-kb *Bgl*II fragment containing *ADE2* cloned into the *Bam*HI site of pRS314, was digested with *Aat*II and *Pst*I. The resulting overhangs were removed by digestion with T4 DNA polymerase, and the plasmid was then circularized by ligation. The resulting plasmid was digested with *Nde*I and *Eag*I, and the resulting 3′ recessed ends were filled with the Klenow fragment of DNA polymerase and then ligated. The resulting plasmid, pKH10, had the 947-bp internal fragment of the *ADE2* gene within the polylinker region of pRS314. pKH11C was constructed by insertion of the 960-bp *Sal*I/*Sac*I fragment from pKH10 into *Sal*I/*Sac*I-digested pRS306.

Media and growth conditions. Rich medium (yeast extract-peptone-dextrose [YEPD]) and synthetic complete (SC) medium with bases and amino acids omitted as specified were used as described previously (45). Yeast strains were grown at 30°C unless otherwise indicated. Strains with *RAD27* deleted were grown at 23°C.

Enzymes. Restriction endonucleases, the *E. coli* DNA polymerase I large fragment, calf intestinal phosphatase, and T4 polynucleotide kinase were obtained from New England Biolabs or GIBCO BRL and used according to the manufacturer's recommendations. Reagents for PCR were purchased from Boehringer Mannheim.

Exonuclease assays. Uniformly labeled ^{3}H-P22 DNA (6) digested with *Rsa*I was used to detect exonuclease activity. Reaction mixtures contained 1 nmol of substrate, 5 μl of each fraction, 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) in a 50-μl reaction volume and were incubated at 30°C for 1 h. The reactions were terminated by the addition of 100 μl of "stop" solution (20 mM EDTA, 25 mM sodium pyrophosphate, 30 μg of sonicated salmon sperm DNA per ml). Trichloroacetic acid (TCA) was added to a final concentration of 5%, and the mixtures were held on ice for 10 min. After centrifugation, aliquots (0.2 ml) were removed, and radioactivity was measured by scintillation counting. One unit is defined as the amount of enzyme required to digest 1 nmol of ^{3}H-P22/*Rsa*I DNA in 30 min at 30°C. 3′-end-labeled DNA was prepared with [α-^{32}P]dATP and Klenow DNA polymerase by using pUC18 DNA cut with *Eco*RI. 5′-end-labeled DNA was prepared with [γ-^{32}P]ATP and T4 polynucleotide kinase by using dephosphorylated linear pUC18. Reactions with end-labeled substrates were carried out in a final volume of 50 μl. For each reaction, 0.1 pmol (ends) of substrate was incubated with 5 μl of each fraction (or the amount indicated in Fig. 3) for 15 min at 30°C and then treated as described above.

The overlap recombination assay with substrates pLS89 digested with *Sal*I and M13BS digested with *Bam*HI and crude whole-cell extracts was performed as described previously (20). Reaction products were separated by electrophoresis through 0.8% agarose, followed by staining with ethidium bromide. For reactions with crude extracts, the reaction products were detected by Southern hybridization. The amount of product present on hybridized filters was quantitated with a PhosphorImager (Molecular Dynamics).

Purification of Exo I. *S. cerevisiae* LSY346-5A was grown at 30°C with vigorous shaking in YEPD medium. Cells were collected at mid-log phase, resuspended in water (20 ml of water/liter of culture volume), and irradiated with 50 kilorads from a Gammacell-220 irradiator containing ^{60}Co (Atomic Energy of Canada). Cells were then transferred to fresh YEPD medium (equivalent to the original volume) and grown for an additional 90 min at 30°C. The cells were collected by centrifugation, washed once with water, and then resuspended in a volume of cell breakage buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 5 mM EDTA, 300 mM NaCl, 10% glycerol [vol/vol], 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, 0.5 μg of leupeptin per ml) equivalent in milliliters to the wet weight of the cells. The resuspended cells were quickly frozen and stored at −80°C until use. All of the purification steps were performed at 4°C, and protease inhibitors were added to all of the buffers at the same concentration as in the breakage buffer immediately prior to use. One hundred grams of cells was thawed in a mixture of ice and water and broken by eight 45-s pulses with a Beadbeater (Biospec Products). The cell debris was removed by centrifugation at 13,000 rpm in a Sorvall GSA rotor for 45 min. Nucleic acids were removed from the resulting supernatant by precipitation with polyethylene glycol 8000 (PEG) to a final concentration of 8%. The PEG precipitate was collected by centrifugation at 13,000 rpm in a Sorvall GSA rotor for 30 min, and the clear supernatant was carefully removed (fraction I). Fraction I was immediately applied to a column of ceramic hydroxylapatite (Bio-Rad Laboratories CHT type I; 5 cm^2 by 6 cm) that had been equilibrated with 10 mM potassium phosphate (KPO_4 [pH 7.2])–10% glycerol–1 mM DTT plus protease inhibitors. The column was developed with a 300-ml linear gradient of 10 mM KPO_4 at pH 7.2 to 400 mM KPO_4 at pH 6.8 (including glycerol, DTT, and protease inhibitors at the same concentrations as those used above), and fractions were assayed with [^{3}H]P22 substrate DNA. Approximately 30% of the nuclease activity present in fraction I did not bind to the column. Two minor peaks and one major peak of activity were eluted. The major peak that eluted at 210 to 260 mM KPO_4 (fraction II) was dialyzed against buffer H (20 mM HEPES [pH 7.6], 10% glycerol, 1 mM EDTA, 1 mM DTT, and protease inhibitors) containing 50 mM NaCl. After dialysis, fraction II was applied to a 1-ml SP-Sepharose column (Pharmacia LKB; Hitrap columns) that had been equilibrated with buffer H containing 50 mM NaCl. The column was developed with a 20-ml linear gradient of 50 to 500 mM NaCl, and 0.4-ml fractions were collected. One major peak of activity was recovered that eluted at 280 to 350 mM NaCl (fraction III). Fraction III was diluted with water to a conductivity equivalent to that of buffer H plus 40 mM NaCl and immediately applied to a 1-ml column of Q-Sepharose (Pharmacia LKB; Hitrap columns) equilibrated with buffer H plus 40 mM NaCl. Most of the activity present in fraction III failed to bind to Q-Sepharose (fraction IV), and this was applied directly to a 1-ml column of heparin agarose (Pharmacia LKB; Hitrap columns) equilibrated with buffer H plus 40 mM NaCl. The heparin agarose column was developed with a 15-ml linear gradient of 40 to 800 mM NaCl, and 0.4-ml fractions were collected. A

TABLE 2. Purification of *S. cerevisiae* Exo I

Fraction	Total amt of protein (mg)	Vol (ml)	Total activity (U)	Sp act (U/mg)	Fold purification (total/step)	% Yield (total/step)
I (PEG supernatant)	1,407	210	1,014	0.72	1	100
II (hydroxylapatite)	21.6	60	413	19.12	26.5	40.7
III (SP-Sepharose)	2.0	3.33	133	66.5	92.4/3.5	13.1/32
IV (Q-Sepharose)	0.66	22	115	174.2	243/2.6	11/86
V (heparin agarose)	0.4	1.6	91.43	229	318/1.3	9/79

single peak of activity eluted at 460 to 525 mM NaCl (fraction V). Fraction V (0.25 ml) was applied to a 24-ml Superdex 75 gel filtration column (Pharmacia LKB) equilibrated with buffer H plus 300 mM NaCl. Activity was recovered in fractions 30 and 31, corresponding to the fractions in which ovalbumin eluted.

For analysis of mutant extracts, 4 liters of each culture was grown to mid-log phase in YEPD medium and irradiated as described above. Cells were broken by four 50-s pulses in the small chamber of a Beadbeater and then processed as described above. One hundred milligrams of fraction I from each extract was applied to a 5-ml hydroxylapatite cartridge (Bio-Rad; CHT) and developed with a 50-ml gradient of 10 mM KPO_4 at pH 7.2 to 400 mM KPO_4 at pH 6.8 (including glycerol, DTT, and protease inhibitors at the same concentrations described above).

Determination of mitotic recombination rates. Cells containing the recombination reporter recombine to adenine prototrophy with the concomitant loss of the *URA3* gene and plasmid sequences. Thus, cultures to be used for measurement of recombination rates were grown in medium lacking uracil to select for maintenance of the recombination reporter. Cells were grown to an approximate density of 10^7 cells per ml and washed with water, and dilutions were plated on rich medium and on medium lacking adenine to determine the recombination frequency. Since division of Ade^+ recombinants does not occur in the medium lacking uracil, the frequency of Ade^+ cells is a measure of the recombination rate. The median value was derived from seven independent cultures of each strain. This was repeated two more times, and the average rate from the three trials is given in Table 4.

RESULTS

Purification of Exo I. We previously identified and partially purified a 5′-3′ exonuclease required for recombination of two linear DNA molecules with terminal overlapping homology (20). A typical purification of this activity, now called *S. cerevisiae* Exo I, is summarized in Table 2. The purification began with the supernatant obtained after PEG precipitation of DNA from a crude whole-cell extract. The PEG precipitation step removed contaminating DNA from the extract that would otherwise interfere with quantitation of exonuclease activity and removed about 50% of the total cellular protein. Approximately 30% of the nuclease activity present in fraction I failed to bind to hydroxylapatite, and two small peaks of nuclease activity eluted before the major peak of Exo I. Although a single peak of nuclease activity was resolved by fractionation of fraction II on SP-Sepharose, this fraction appeared to contain four nuclease activities (data not shown). The major activity did not bind to Q-Sepharose under the conditions employed; it was subsequently purified by heparin agarose and gel filtration chromatography. Two polypeptides with sizes of 44 and 42 kDa coeluted with the single peak of activity recovered by heparin agarose chromatography. In subsequent fractionation by gel filtration chromatography, a single peak of nuclease activity eluted at the same elution volume as ovalbumin (Fig. 1A). Analysis of the protein profile by sodium dodecyl sulfate (SDS) gel electrophoresis indicated that the 42-kDa polypeptide coeluted with the nuclease activity (Fig. 1B, fractions 30 and 31). The elution profile obtained by gel filtration chromatography indicates that the exonuclease is about 45 kDa in size and is monomeric.

Cells treated with ionizing radiation were used for the preparation described in Table 2. The specific activity of Exo I (fraction II) from untreated cells was 2.5-fold less (data not shown). The exonuclease had been purified more than 300-fold through the heparin agarose step. This is likely to be an underestimate, because there are several nuclease activities present in fraction I and additional weak activities that are removed by chromatography on Q-Sepharose. Because the protein concentration was too low for accurate measurement, we were unable to estimate the purification obtained from the Superdex 75 step. Based on the amount of protein present in fraction V and a 9% yield, we estimate that there are 2,500 molecules of Exo I per induced cell.

To ensure that the recombination activity of the purified exonuclease was similar to the activity identified in crude nuclear extracts (20), the overlap recombination assay was performed with pLS89 and M13BS substrates with fraction V. Incubation of the substrates with fraction V resulted in the formation of two products (Fig. 2). The 11.5-kb product (P1) corresponds in size to the expected overlap recombination

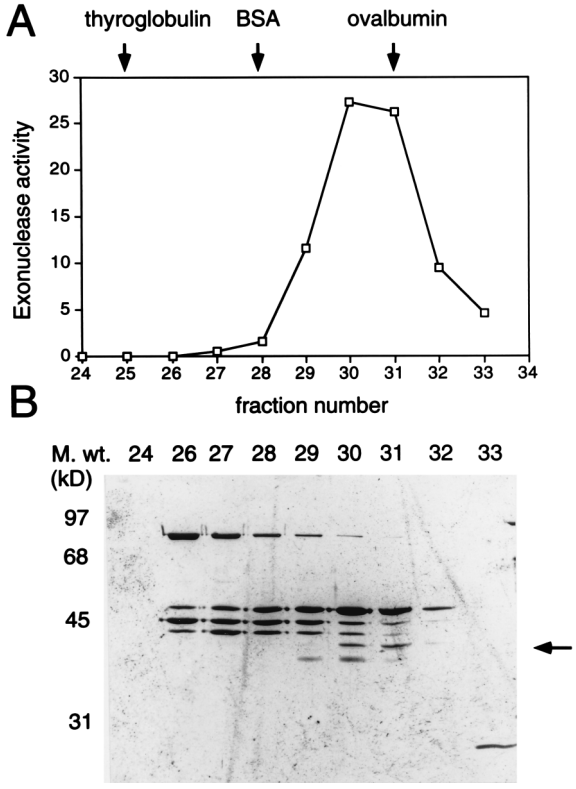

FIG. 1. Purification of *S. cerevisiae* Exo I. (A) Fractions from the Superdex 75 column were assayed for exonuclease activity as described in the text. Exonuclease activity refers to picomoles of TCA-soluble nucleotides released with the ^{3}H-labeled P22 substrate. The fractions in which molecular weight standards eluted are marked by arrows. BSA, bovine serum albumin. (B) Fractions from the Superdex 75 column displayed on a 10% polyacrylamide–SDS gel stained with silver. The arrow to the right indicates the 42-kDa polypeptide that coelutes with the peak of exonuclease activity. M.wt., molecular mass.

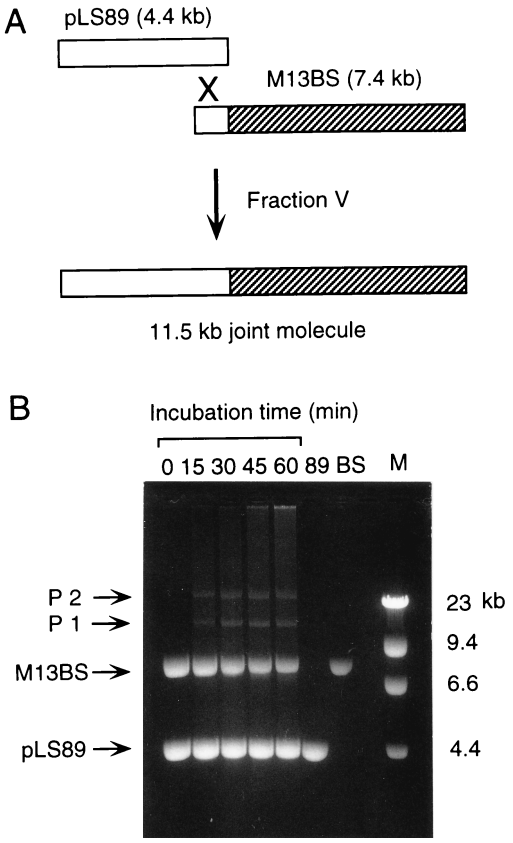

FIG. 2. Exo I promotes recombination between linear DNA molecules with overlapping terminal homology. (A) The substrates for the reaction are pLS89 digested with *Sal*I and M13BS digested with *Bam*HI to reveal 262 bp of terminal homology. Recombination produces an 11.5-kb joint molecule. (B) Time course of the incubation of pLS89 and M13BS with fraction V. The lanes designated 89 and BS refer to the two unreacted substrates pLS89 and M13BS, respectively, and M refers to size markers of lambda DNA digested with *Hin*dIII. The two major products formed during the course of the reaction are marked by the arrows labeled P1 and P2.

product. The high-molecular-weight product (P2) has been observed previously with crude extracts and has a branched structure, as determined by two-dimensional agarose gel electrophoresis (20, 51). The kinetics of product formation observed with the purified exonuclease were similar to those observed with crude nuclear extracts.

Exo I is a nonprocessive 5′-3′ double-stranded exonuclease. The degree of processivity was determined by challenging Exo I with a threefold molar excess of unlabeled P22 during the course of the reaction with ^{3}H-labeled P22 DNA (Fig. 3A). The addition of excess unlabeled DNA resulted in the immediate cessation of activity on the labeled substrate, indicating that Exo I is nonprocessive. The polarity of degradation was determined by incubation of subsaturating concentrations of fraction V (for ends) with either 3′- or 5′-end-labeled substrates. Removal of 60% of the terminal nucleotide from the 5′-end-labeled substrate occurred within 40 min of incubation, whereas little loss of label occurred from the 3′-end-labeled substrate, even after a 40-min incubation (Fig. 3B). To compare the activities of Exo I on single- and double-stranded DNA, reactions were carried out with native or heat-denatured ^{3}H-labeled P22 DNA. As shown in Fig. 3C, the activity of Exo I was about two times greater on double-stranded DNA than

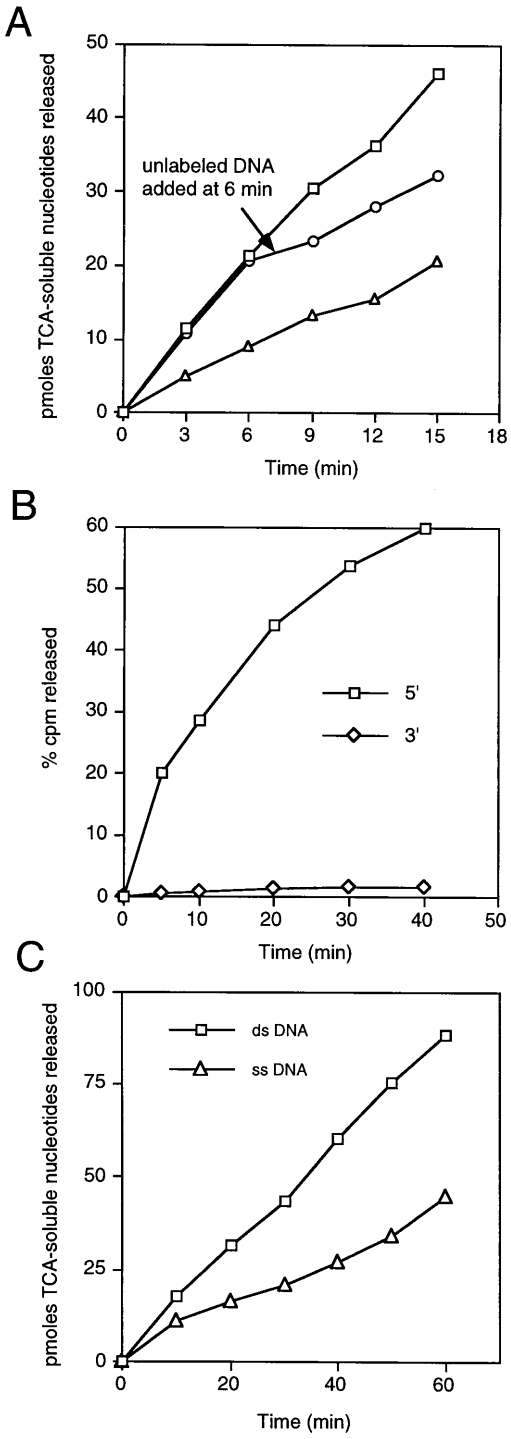

FIG. 3. Characterization of Exo I. (A) The processivity was determined by addition of a threefold excess (ends) of unlabeled DNA to a reaction mixture containing labeled DNA and subsaturating amounts (for ends) of fraction V (0.1 U). The substrates for the reaction were 3 nmol of ^{3}H-labeled P22 DNA digested with *Sna*BI and 0.56 nmol of P22 DNA digested with *Rsa*I. □, reaction with ^{3}H-labeled P22 DNA alone; ○, reaction in which unlabeled P22 DNA was added at the 6-min time point; △, reaction in which unlabeled P22 DNA was added at 0 min. (B) Activity on 3′- and 5′-end-labeled substrates. End-labeled pUC18 DNA (0.1-pmol ends) was incubated with 0.025 pmol of Exo I (0.05 U of fraction V), and the percentage of end-labeled nucleotides released is presented. (C) Activity on double-stranded (ds) and single-stranded (ss) DNA was determined by incubation of 0.05 U of fraction V with 1 nmol of ^{3}H-labeled P22 DNA that was native or heat denatured.

TABLE 3. Reaction requirements

Reaction conditions	% Activity
Standard reaction mixture	100
+1 mM $CaCl_2$	49
+5 mM $CaCl_2$	27
+0.1 mM $ZnCl_2$	64
+50 mM NaCl	96
+100 mM NaCl	87
+150 mM NaCl	50
−$MgCl_2$	2
−$MgCl_2$ + 5 mM $MnCl_2$	84
−DTT	99
20 mM Tris-OAc[a] (pH 8.0), 5 mM $Mg(OAc)_2$, 1 mM DTT	81

[a] OAc, acetate.

on single-stranded DNA, and the reaction on both substrates was linear for at least 1 h.

Cofactor requirements. The activity of the exonuclease was determined under a variety of reaction conditions (Table 3). The pH optimum was found to be maximal between pH 7.5 and 8, but considerable activity (greater than 50%) was detected at pH 6 and 9 (data not shown). A divalent cation was essential for activity. Mg^{2+} at 5 to 10 mM provided optimal activity, but considerable activity was observed in the presence of 5 mM Mn^{2+} (84%). The addition of Ca^{2+} or Zn^{2+} to the standard reaction mixture was inhibitory. The activity was not stimulated by the addition of ATP, bovine serum albumin (data not shown), or DTT. The activity was unaffected by the addition of 50 mM NaCl, and 50% activity was still detected in the presence of 150 mM NaCl. The substitution of Tris-OAc and $Mg(OAc)_2$ for Tris-HCl and $MgCl_2$ in the standard reaction mixture had no stimulatory effect.

The 5′-3′ exonuclease is absent from *exo1* mutants. The characteristics of the exonuclease described here, such as low processivity, 5′-3′ specificity, and cofactor requirements, are quite similar to those described for *S. pombe* Exo I. The *S. cerevisiae* homolog of *S. pombe* Exo I was recently identified in a screening for proteins that interact with the mismatch binding protein Msh2 (57). The *S. cerevisiae EXO1* gene encodes an 80-kDa protein, and the overproduced and purified *EXO1* gene product has enzymatic properties similar to those of the exonuclease reported here (3a). Exo I is a member of a large family of endo- and exonucleases that have homology to the 5′-3′ exonuclease domain of *E. coli* DNA polymerase I, T4 RNase H, T5 exonuclease D15, T7 gene 6 exonuclease, and *E. coli* RecJ (33, 38, 53). In *S. cerevisiae*, there are four other members of this family of nucleases, Rad2, Rad27, Din7, and Yen1. To test whether the exonuclease described here is the product of the *S. cerevisiae RAD2*, *RAD27*, *DIN7*, *EXO1*, or *YEN1* genes, extracts were prepared from isogenic strains containing deletion alleles of these genes. Extracts were prepared from induced cells by PEG precipitation, followed by hydroxylapatite chromatography of fraction I. From the wild-type extract, three peaks of nuclease activity were resolved. The largest peak, which eluted at 200 to 260 mM potassium phosphate, corresponds to the 42-kDa 5′-3′ exonuclease described in this report. This peak of activity was undiminished in fractionated extracts from the *rad2*, *rad27*, *din7*, and *yen1* strains, but it was completely absent from the *exo1* strain (Fig. 4). This result, together with the biochemical characterization, is strong evidence that the 5′-3′ exonuclease is the product of the *S. cerevisiae EXO1* gene. Because the polypeptide we have purified is substantially smaller than that predicted from the *EXO1* ORF, it is likely to correspond to a proteolytic degradation product.

We originally identified Exo I through its activity in the overlap recombination assay. Fractionation of wild-type extracts revealed that Exo I was the major activity responsible for this in vitro reaction (20a). To determine whether *exo1* mutant extracts were defective in this assay, we compared the activities of *EXO1* and *exo1* extracts prepared from isogenic strains by using the overlap recombination substrates (Fig. 5). The overlap recombination product was clearly observed with the *EXO1* extract, even at low protein concentrations. However, the amount of product was greatly reduced when the *exo1* strain was used as a source of extract, consistent with the view that Exo I is the major activity that catalyzes overlap recombination in this in vitro system. As described previously, a product due to end joining was observed when pLS89 was incubated alone in the extract.

***EXO1* is involved in spontaneous mitotic recombination between direct repeats.** The role of Exo I in mitotic recombination in vivo was determined by measuring the rate of recombination between nontandem direct repeats of the *ade2* gene (Fig. 6). The two *ade2* alleles are truncated at their 3′ and 5′ ends, respectively, and are separated by plasmid vector sequences including the *URA3* gene. Strains containing this duplication form red colonies on agar plates. Recombination events that excise the DNA between the repeats and restore a wild-type copy of the *ADE2* gene are visualized as white sectors within the red colony. The rate of recombination is determined from the frequency of Ade^+ prototrophs within a population of cells. Strains containing this direct repeat and a disruption of

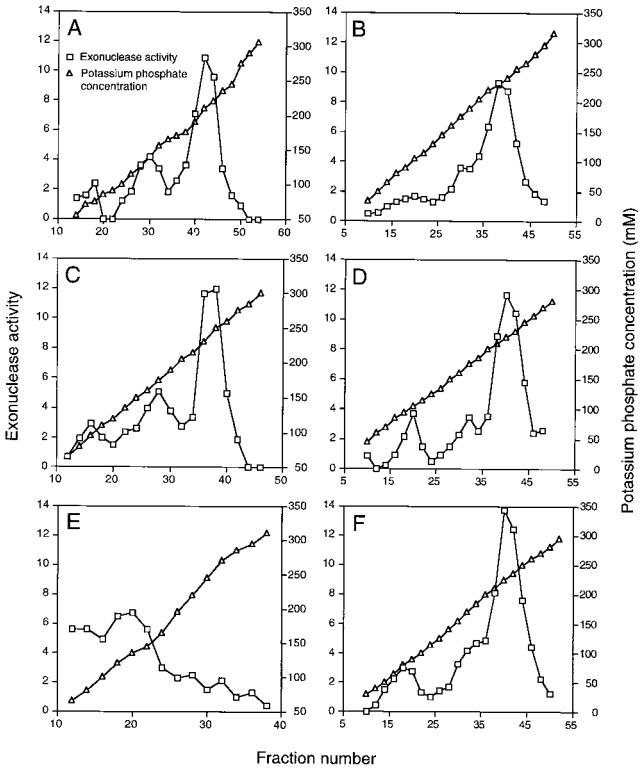

FIG. 4. The 5′-3′ exonuclease is absent from *exo1* extracts. Extracts were prepared from wild-type (A), *rad2* (B), *rad27* (C), *din7* (D), *exo1* (E), and *yen1* (F) strains by the PEG precipitation procedure followed by chromatography on hydroxylapatite. Exonuclease activity refers to the picomoles of TCA-soluble nucleotides released with the ^{3}H-labeled P22 substrate.

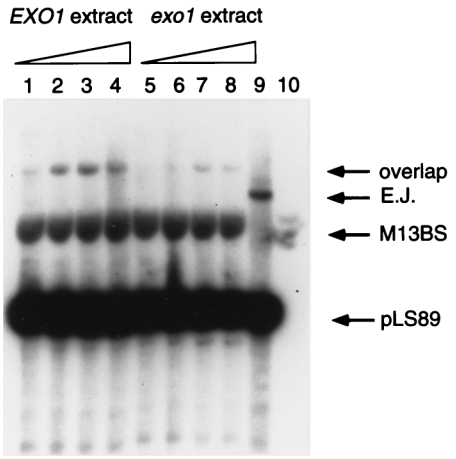

FIG. 5. In vitro recombination activity is diminished in *exo1* extracts. Lanes 1 to 4 are pLS89 and M13BS incubated with crude whole-cell extract prepared from an *EXO1* strain, and lanes 5 to 8 are incubated with the *exo1* extract. Lanes 9 and 10 are pLS89 and M13BS incubated separately with the *EXO1* extract. Protein concentrations (in milligrams per milliliter) are as follows: lane 1, 0.42; lane 2, 0.84; lane 3, 1.68; lane 4, 2.52; lane 5, 0.43; lane 6, 0.86; lane 7, 1.72; lane 8, 2.58; lane 9, 2.52; and lane 10, 2.52. The arrows to the right indicate the substrates pLS89 and M13BS, overlap refers to the joint molecules produced by overlap recombination, and E.J. refers to the product of end joining. Reaction products were separated on a 0.8% agarose gel which was then Southern blotted. The probe used hybridizes to the pLS89 substrate and the overlap product. Some nonspecific hybridization to M13BS is also observed.

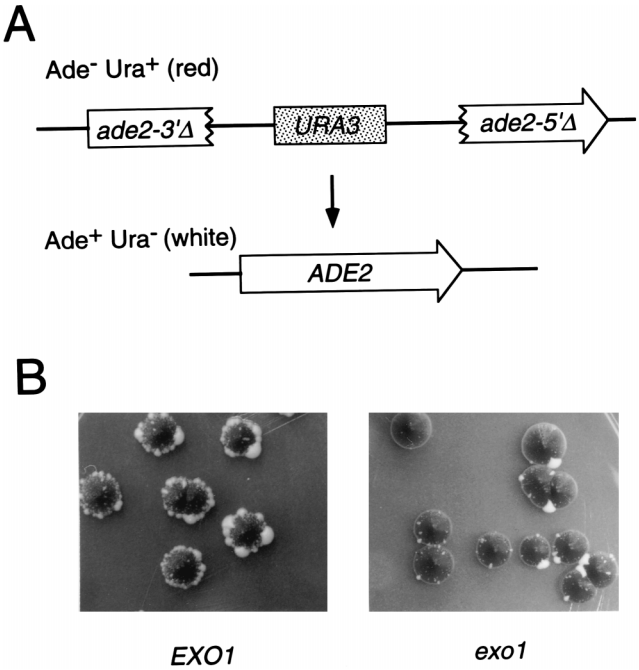

FIG. 6. Mitotic recombination between direct repeats is reduced in *exo1* mutants. (A) The substrate consists of two nontandem direct repeats of truncated alleles of the *ade2* gene separated by vector sequences, including the *URA3* gene. Recombination to restore the *ADE2* gene results in loss of the intervening sequences. (B) Recombination events are visualized as white sectors and papillae within a red colony.

the *EXO1* gene were found to have reduced sectoring (Fig. 6) and showed a sixfold lower rate of recombination than wild-type cells (Table 4).

Recombination between direct repeats is reduced synergistically by mutation of the *RAD1* and *RAD52* genes (27, 43, 56). Since the mismatch repair genes *MSH2* and *MSH3* have been shown to act in the Rad1 pathway for direct repeat recombination (42), it was of interest to determine the effect of an *exo1* mutation on recombination in either a *rad1* or *rad52* background. Mutation of *RAD1* or *RAD52* was shown to reduce recombination 9- or 14-fold, respectively (Table 4). As has been demonstrated previously, the *rad1* and *rad52* double mutant showed a synergistic reduction in the frequency of recombination. However, the *exo1* mutation had no effect in either the *rad1* or *rad52* background, and the *exo1 rad1 rad52* triple mutant showed the same frequency of recombination as the *rad1 rad52* double mutant.

To determine whether Exo1 is involved in the repair of DSBs in vivo, *exo1* strains were also tested for gamma-ray sensitivity, mating-type switching, and sporulation. *exo1* mutants showed wild-type resistance to ionizing radiation and were proficient at mating-type switching (data not shown). Homozygous *exo1* diploids showed reduced sporulation (44% unsporulated cells compared with 14% for an isogenic wild-type diploid), and spore viability was slightly reduced (78% for *exo1* strains compared with 98% for the wild-type diploid).

DISCUSSION

We previously described a 5′-3′ exonuclease that catalyzed recombination between two linear DNA molecules with terminal overlapping homology (20). The exonuclease exposed 3′ single-stranded complementary tails that could be annealed to form a joint molecule. Here, we describe the purification of this exonuclease and provide evidence that it is the product of the *EXO1* gene.

The exonuclease has been purified more than 300-fold from mitotic cells treated with ionizing radiation. The increased activity observed by treatment of cells with ionizing radiation could be due to damage inducibility and/or cell cycle-regulated expression. There is an *Mlu*I site, or *Mlu*I cell cycle box (MCB) (36), located 253 nucleotides upstream of the ATG which could be involved in cell cycle-regulated transcription of the *EXO1* gene. Transcription of the *RAD27* gene has been shown to be both cell cycle regulated and inducible by methyl methanesulfonate (61). *RAD2* transcript levels are also elevated after exposure of cells to DNA-damaging agents (46).

The exonuclease activity was shown to copurify with a 42-kDa polypeptide and appears to be monomeric in solution. The biochemical properties, such as polarity, nonprocessivity, and cofactor requirements, are quite similar to those described for *S. pombe* Exo I (52) and for the purified, overproduced *S. cerevisiae EXO1* gene product (3a). However, the *S. pombe* and *S. cerevisiae* activities do differ in their ability to degrade single-

TABLE 4. Frequency of Ade^+ recombinants in strains containing the *ade2Δ3′::URA3::ade2Δ5′* duplication

Relevant genotype	Frequency of recombinants/10^4 viable cells	% Relative frequency
Wild type	27.9 ± 13.9	100
exo1	4.38 ± 0.62	16
rad1	3.05 ± 1.65	11
exo1 rad1	3.72 ± 1.28	13
rad52	1.95 ± 0.65	7
exo1 rad52	1.59 ± 0.11	6
rad1 rad52	0.035 ± 0.02	0.13
exo1 rad1 rad52	0.036 ± 0.01	0.13

stranded DNA. Exo I of *S. pombe* is quite specific for double-stranded DNA, whereas the exonuclease described here shows only a twofold preference for double-stranded over single-stranded DNA substrates. Because of the similarity between the two exonucleases, it seemed quite likely that the exonuclease described here is the homolog of *S. pombe* Exo I or one of the other members of the Rad2 family. The Rad2 family of proteins have significant homology over two regions designated the N and I boxes. These two regions have motifs that are also conserved in prokaryotic DNA polymerase I, T4 RNase H, T5 exonuclease D15, T7 gene 6 exonuclease, and *E. coli* RecJ (33, 38). Within these regions are several highly conserved acidic residues that constitute the active site of T4 RNase H. In *S. cerevisiae*, there are five members of this family: Rad2, Rad27, Exo1/Dhs1, Din7, and Yen1. Rad2 and Rad27 have demonstrated endonuclease and exonuclease activities (14, 15, 17), whereas Din7 and Yen1 are predicted to be members of this family based on sequence homology. The predicted Din7 protein is highly homologous to Exo1 (56% identity and 75% similarity over the N-terminal 367 amino acids). Although Yen1 is the least-conserved member of this family, it does contain most of the acidic residues that form the T4 RNase H active site.

When fractionated by PEG precipitation and hydroxylapatite chromatography, the *exo1* extracts were found to lack the major peak of exonuclease activity corresponding to the 42-kDa exonuclease described here, whereas fractionated extracts from *rad2*, *rad27*, *din7*, and *yen1* strains retained this activity (Fig. 4). Extracts prepared from *exo1* mutant strains were deficient in the overlap recombination assay, consistent with Exo I being the major nuclease that promotes this in vitro reaction (Fig. 5). The residual activity observed in the *exo1* extracts could be due to one or more of the other nucleases or putative nucleases described above. The purified exonuclease was shown to promote joint molecule formation in the overlap recombination assay with kinetics similar to those observed with crude nuclear extracts. Together, these results provide strong evidence that the 42-kDa exonuclease is the product of the *EXO1* gene.

The predicted Exo1 protein is much larger than the 42-kDa protein described above. The N and I box regions which contain the nuclease domain are found within the N-terminal half of the protein. This region of the protein is predicted to be contained within a 33-kDa polypeptide. Thus, it seems plausible that proteolysis of Exo1 could generate a polypeptide with a size of 42 kDa that retains nuclease activity. Since *S. pombe* Exo I was also purified as a proteolytic degradation product (52, 53), it seems quite possible that this part of both proteins is particularly susceptible to proteolysis.

The *S. cerevisiae EXO1* gene was identified in a screening for proteins that interact with the MutS homolog, Msh2 (57). Mutation of *EXO1* in *S. cerevisiae*, or of *exo1* in *S. pombe*, results in a weak mutator phenotype consistent with a role in mismatch repair (53, 57). Although *exo1* mutants of *S. pombe* and *S. cerevisiae* show no apparent defect in meiotic recombination (53), the *S. cerevisiae exo1* strain is deficient in mitotic recombination between direct repeats. The assay used in this study measures deletion formation between truncated repeats of the *ADE2* gene. This type of recombination assay was used because it most closely resembles the in vitro assay originally used to identify Exo I. Deletions are thought to arise by a variety of mechanisms, including intrachromatid crossing over, single-strand annealing, sister strand mispairing, replication slippage, one-ended invasion, sister chromatid exchange, and sister chromatid conversion (28, 39). Because there are many alternate pathways for deletion formation, mutation of any one gene rarely results in a strong reduction in the rate of recombination between directly repeated sequences. The reduction observed in the *exo1* strain is of a magnitude similar to that observed with mutation of the *RAD1*, *RAD10*, or *RAD52* genes (Table 4) (27, 43, 44, 56).

It has recently been demonstrated that mutation of *MSH2* or *MSH3* results in reduced rates of recombination between repeated sequences (42). However, *pms1* and *mlh1* mutants do not show a reduction in the rate of direct repeat recombination, suggesting that mitotic recombination deficiency is not a general phenotype of mismatch repair mutants (42). The effect of the *exo1* mutation on direct repeat recombination is very similar to that observed with *msh2* and *msh3* mutations. Epistasis analysis has shown that Msh2 and Msh3 act in the Rad1 pathway of direct repeat recombination. As shown in Table 4, no synergism was observed for the *exo1 rad52* double mutant, indicating that its effect is distinct from that of *msh2* or *msh3*. One interpretation of this observation is that Exo1 acts early in the recombination reaction to produce a substrate that can be utilized in either of the Rad1 or Rad52 pathways. Exonucleases are predicted to be involved in early steps of recombination to generate regions of single-stranded DNA that are the substrate for binding by homologous pairing proteins. In this capacity, 5′-3′ exonucleases could act at double-strand ends to produce 3′ single-stranded tails or could act to enlarge a nick to a single-stranded gap. We suggest that there is redundancy for this step to explain the weak phenotype in recombination and radiation resistance of the *exo1* mutant. In this respect, the phenotypes of *exo1* strains may be compared with those of *E. coli recJ* mutants. RecJ is a component of the methyl-directed mismatch repair pathway, yet *recJ* mutants show a very modest mutator phenotype due to functional redundancy for the exonuclease processing step. Furthermore, *recJ* mutants show recombination deficiency and UV sensitivity only in the absence of RecBCD (34). An exonuclease could also act at later steps in the recombination reaction by processing recombination intermediates. Since the *ade2* alleles employed in this study are truncation alleles, we would not expect to generate heteroduplex intermediates containing mismatches that would then be subject to repair by the mismatch repair system. Thus, it is unlikely that the defect in direct repeat recombination observed in *exo1* mutants results from failure to repair heteroduplex DNA. It is also unlikely that the reduction in the rate of Ade^+ prototroph formation is due to the accumulation of mutations within the *ade2* repeats, because the rate of recombination is several orders of magnitude greater than the rate of spontaneous mutation in *exo1* strains.

In summary, we have purified and characterized a 5′-3′ exonuclease from *S. cerevisiae* which has biochemical properties very similar to those of *S. pombe* Exo I. This activity is absent from extracts prepared from an *S. cerevisiae exo1* mutant strain, indicating that it is the homolog of *S. pombe* Exo I. We have shown that extracts prepared from the *exo1* mutant are defective in the in vitro recombination assay that was originally used to identify the exonuclease. Finally, the *exo1* mutant strain was also shown to have a reduced rate of recombination between direct repeats, suggesting a role in recombination in vivo.

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