Yeast exonuclease 1 (Exo1) is induced during meiosis and plays an important role in DNA homologous recombination and mismatch correction pathways. The human homolog, an 803-amino acid protein, shares 55% similarity to the yeast Exo1. In this report, we show that the enzyme functionally complements Saccharomyces cerevisiae Exo1 in recombination of direct repeat DNA fragments, UV resistance, and mutation avoidance by in vivo assays. Furthermore, the human enzyme suppresses the conditional lethality of a rad27Δ mutant, symptomatic of defective RNA primer removal. The purified recombinant enzyme not only displays 5′-3′ double strand DNA exonuclease activity, but also shows an RNase H activity. This result indicates a back-up function of exonuclease 1 to flap endonuclease-1 in RNA primer removal during lagging strand DNA synthesis.

Mutations cause genomic instability and gene dysfunction, many of which affect cell growth and lead to tumorigenesis. Fortunately, in normal cells, mutation rate is low due to the existence of different DNA repair systems such as mismatch, excision, and recombinational DNA repair pathways. In humans, DNA mutation accumulation is a critical step in carcinogenesis. Dysfunctions of mutations of DNA mismatch repair genes such as MSH2, MLH1, and PMS2, are the main cause of the hereditary non-polyposis colorectal cancer (1–6). A portion of sporadic cancers is due to acquired mutations in mismatch repair genes as well (7). Mutations of genes encoding nucleotide excision repair proteins including XPG nuclease are linked to xeroderma pigmentosum (8–17).

Nucleases play important roles in several pathways including DNA replication, repair, and recombination. DNA fragments containing a lesion are removed by the combined efforts of a helicase and a nuclease. For instance, in the DNA mismatch repair of a helicase and a nuclease. For instance, in the DNA mis-

repair genes as well (7). Mutations of genes encoding nucleotide

In eukaryotes, DNA polymerases lack an intrinsic 5′-nuclease. Removal of RNA primers is carried out by an independent enzyme, Rad27/FEN-1 nuclease, with both flap endonuclease and 5′-3′exonuclease activities (28–38). Disruption of the flap endonuclease gene RAD27 in the yeast Saccharomyces cerevisiae resulted in DNA replication defective symptom including the conditional lethality (39–42). Survival of the null mutant at 30 °C suggests that other enzymes with 5′-3′ exonuclease activity could back up the function of Rad27/FEN-1 in DNA replication (29, 43).

A 5′-3′ exonuclease, called exonuclease 1 (Exo1) in Schizo-
sacccharomyces pombe and S. cerevisiae and Tosc a in Drosophila melanogaster, has recently been identified and partially characteriz ed (44–48). The enzyme is a non-processive double-stranded DNA nuclease (44). Both the messenger RNA level and enzyme activity were dramatically induced during meiosis in S. pombe (44, 45). In D. melanogaster, the gene is specifically expressed in the early embryogenesis and female germline (46).

The S. cerevisiae and human exonucleases 1 interact with mismatch repair protein Msh2 as demonstrated by the two-
system and immuno-coprecipitation (48–50). Disrup-
tion of EXO1 increased mutation rate in both S. pombe and S. cerevisiae cells, indicating that EXO1 is a mutator gene (45, 48).

Detailed analysis of the mutational spectrum of the exo1Δ cells suggested a role for the encoded protein in mismatch correction, most likely during homologous recombination. In addition, deletion of yEXO1 significantly decreased both meiotic and mitotic recombination rates (45, 47, 48). One speculation is that the nuclease plays a role creating 3′ single-stranded complementary tails, thereby promoting joint molecule formation. Moreover, the null mutant displays a minor UV sensitivity unlikely due to a nucleotide excision repair deficiency but may be due to a defective DNA replication by-pass pathway (51). These findings indicate that the Exo1 homologs function as 5′-3′ exonucleases in mutation prevention via multiple DNA metabolic pathways. More recently, human homolog of the exonuclease 1 has been identified and the gene was named HEX1 (52). It is specifically expressed in fetal liver and adult bone marrow, suggesting that the enzyme may operate prominently in processes specific to hematopoietic stem cell development. The gene has been mapped to 1q43, a region lost in some cases of acute leukemia and in several solid tumors (49, 52).

Human and S. cerevisiae exonuclease sequences have high similarity. This similarity leads us to predict that functions of these two enzymes are also similar and the human gene may complement the Exo1 functions in yeast. In addition, when
Szankasi and Smith (45) identified the S. pombe gene (SpEXO1), they noticed that the encoded protein displays significant similarity to the proteins of rad13 (XPG/Rad2) and rad2 (FEN-1/Rad27) nuclease families. However, the similarities were limited to regions of N-terminal putative nuclease domains and the major part at C terminus was largely divergent (45). We hypothesize that the human exonuclease 1 may further complement the Rad27 nuclease functions in RNA primer removal during lagging strand DNA synthesis based on the observations of the sequence conservation between these two proteins, their enzymological properties, and phenotypical characteristics (i.e., conditional lethality) of the rad27 null mutants. Our in vitro and in vivo data demonstrate that the human exonuclease 1 can functionally complement the yeast homologues (yExo1 and yRad27) in recombination, UV resistance, RNA primer removal during the Okazaki fragment processing, and mutation avoidance.

**EXPERIMENTAL PROCEDURES**

**Materials**—A cDNA clone (number 843301) harboring a putative human exonuclease 1 was obtained from ATCC (Manassas, VA). Oligonucleotides used in this study were synthesized in the City of Hope Cancer Center core facility. The vector pET28a and E. coli strain BL21 and BL21 (DE3) were from Novagen (Madison, WI) and E. coli strain XL2 blue and pBSK vector were from Stratagene (La Jolla, CA). Yeast expression vector pDB20 was a gift from D. Becker, California Institute of Technology. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Yeast culture media including YPD, synthetic complete (SC), minimal sporulation, and synthetic dextrose minimal (SD) media were prepared according to Sherman et al. (53).

### TABLE I

| Strain | Deletion | Genotype | Source |
|--------|----------|----------|--------|
| W1021–7c | a, ade2–1, can1–100, his3–11, 17, leu2–3, ura3–1 | PRS314-ADH1 | R. Rothstein |
| W1089–6c | a, ade2–1, can1–100, his3–11, 17, trp1–1, ura3–1 | PRS314-ADH1 | R. Rothstein |
| AB15a | a, ade2–1, can1–100, his3–11, 17, leu2–1, trp1–1, ura3–1 | This work |
| AB15b* | Genotype of AB15B + PDB | This work |
| AB15b* | Genotype of AB15B + PDB-ScEXO1 | This work |
| AB15c* | Genotype of AB15B + PDB-ScEXO1 | This work |
| HUWTa | a, his3-3-his3:URA3; his3+; exol-hisG | This work |
| HUWTb* | Genotype of HUWT + PRS314-ADH1 | This work |
| HUWTc* | Genotype of HUWT + PRS314-ADH1-eexo1 | This work |
| AB15da | EXO1 | a, exol1:URA3 | This work |
| AB15da | EXO1 | Genotype of AB15D + PRS314-ADH1 | This work |
| AB15db | EXO1 | Genotype of AB15D + PRS314-ADH1-eexo1 | This work |
| AB15dc | EXO1 | Genotype of AB15D + PRS314-ADH1-eexo1 | This work |
| HUWT1a | EXO1 | Genotype of HUWT1 + PRS314-ADH1 | This work |
| HUWT1b | EXO1 | Genotype of HUWT1 + PRS314-ADH1-eexo1 | This work |
| HUWT1c | EXO1 | Genotype of HUWT1 + PRS314-ADH1-eexo1 | This work |
| FDERa | EXO1&RAD51 | a, exol1:URA3, rad51–1:LEU2 | This work |
| FDERb | EXO1&RAD51 | Genotype of FDER + PRS314-ADH1 | This work |
| FDERb | EXO1&RAD51 | Genotype of FDER + PRS314-ADH1-ScEXO1 | This work |
| IC2–1a | RAD27 | a, rad27–1:LEU2 | This work |
| IC2–1a* | RAD27 | Genotype of IC1–2 + PDB | This work |
| IC2–1b* | RAD27 | Genotype of IC1–2 + PDB-ScEXO1 | This work |
| IC2–1c* | RAD27 | Genotype of IC1–2 + PDB-ScEXO1 | This work |
| U687a | RAD51 | a, rad51–1:LEU2 | A. Bailis |
| U687b | RAD51 | Genotype of U687 + PRS314-ADH1 | This work |
| U687c | RAD51 | Genotype of U687 + PRS314-ADH1-ScEXO1 | This work |
| RKY2672a | a, ade2–1, ade8, his3–10, his3–200, 17, leu2–1, lys2ΔBgl, trp1–3, ura3–52 | R. D. Kolodner |
| RKY2672a | Genotype of RKY2672 + PRS314-ADH1 | This work |
| RKY2672b | Genotype of RKY2672 + PRS314-ADH1-ScEXO1 | This work |
| RKY2672c | Genotype of RKY2672 + PRS314-ADH1-eexo1 | This work |
| MEXO1a | EXO1 | a, exol1:LEU2 | This work |
| MEXO1a | EXO1 | Genotype of MEXO1 + PRS314-ADH1 | This work |
| MEXO1b | EXO1 | Genotype of MEXO1 + PRS314-ADH1-ScEXO1 | This work |
| MEXO1c | EXO1 | Genotype of MEXO1 + PRS314-ADH1-eexo1 | This work |

* The strains are derivatives of W1021–7c and W1089–6c.

1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).
used to confirm the deletion of RAD27 gene by PCR: RAD27F (AAAC-GGACCGTGAATACCG) and RAD27R (ATATGGCAAGGTGAAGGAGG-CC). Null mutant strains of RAD51 and RAD51/EXO1 were from laboratory stock (51).

Complementation of SceEXO1 and HEX1 in S. cerevisiae—Two vectors, pDB20 or pES314, were used for the expression of EXO1 in S. cerevisiae. Vector pDB20 was a URA3 and ADH1 promoter-based yeast expression vector. Two plasmids, PDB-Sc and PDB-HE, were constructed to express Sc- and h-exonuclease 1 in S. cerevisiae. PDB-Sc was an insertion of S. cerevisiae EXO1 (ScEXO1) at the HindIII site of pDB20 while PDB-HE has an insertion of human EXO1 (hEXO1) at the NotI site of pDB20. pHS314 is a yeast two-hybrid cloning vector using TRP 1 as a selection marker. PRS-Sc and PRS-HE were constructed to complement the exonuclease 1 function in the rad27Δ:URA3 background. PRS-Sc has an insertion of padh-ScEXO1-Tadh (Padh, ADH1 promoter; Tadh, ADH1 terminator) at SacI and ApaI sites. PRS-HE has an insertion of padh-hEXO1-Tadh at BamHI site. The plasmids were transformed into different S. cerevisiae strains for exonuclease 1 functional complementation as listed in Table 1.

Functional Complementation of Human Exonuclease 1 in Yeast

Expression of HEX1 Restores the Recombination Rate in an exo1Δ Strain—ScEXO1 is involved in spontaneous mitotic and chromosomal recombination events. Cloning the ScEXO1 gene into the yeast expression vector pDB-Sce exposed the importance of ScEXO1 in genomic stability. When ScEXO1 was expressed in the rad27Δ strain in conjunction with a suitable h-exonuclease 1, the recombination rate was restored to wild-type levels. This suggests that exo1Δ strains are sensitive to genomic instability because the expression of ScEXO1 is sufficient to restore the recombination rate to wild-type levels.
meiotic recombination between direct repeats (45, 47). Complementation of this function during *S. cerevisiae* mitotic growth by *HEX1* was determined by measuring the rate of recombination between nontandem direct repeats of the *HIS3* genes (Fig. 2). The two *HIS3* alleles are truncated at their 3′ and 5′ ends, respectively, and are separated by plasmid vector sequences including the *URA3* gene. The end repeats of the *HIS3* gene in this system are 413 bp in length. Strains containing this duplication form can grow on the SD medium without uracil. Recombination events that excise the DNA between the repeats and restore the *HIS3* gene are visualized as the recombinant cells can grow on the medium without histidine. Recombination rate in a strain containing this direct repeat and a disruption of the *EXO1* gene were 58% lower than that of the wild type strain hosting the *HIS3* gene construct as a control. Expression of either *ScEXO1* or *HEX1* restored the recombination rate to the wild type level as shown in Table II.

Expression of HEX1 Ablates the UV Sensitivity of an *exo1*Δ Strain—Deletion of the *EXO1* gene in *S. cerevisiae* causes a minor UV sensitivity (51). Epistatic analysis indicated that the observed phenotype was not due to the defective *RAD2*-dependent nucleotide excision repair or loss of its known function in *RAD51*-dependent double strand break-induced recombination pathway or mismatch repair pathway. It is most likely due to its involvement in a DNA replication by-pass pathway. Double deletion of *EXO1* and *RAD51* made the strain very sensitive to UV treatment. Introduction of the human and yeast *EXO1* expression plasmids into this double deletion mutant strain recovered the UV sensitivity close to the level of the *rad51*Δ mutant (Fig. 3).

*Purified hExo1 Efficiently Removes RNA Primers from Okazaki Fragment Model Substrates—*Full-length human exo-nucleases-1 cDNA was cloned into the pET28a vector and expressed in *E. coli* using sorbitol medium as described previously (54, 58). PCR primers were designed to add both N- and C-terminal His tags onto the recombinant protein. The final products of fast protein liquid chromatography purification using a nickel affinity column contained two protein bands (approximately 93 and 45 kDa). Microsequencing of both polypeptides revealed the expected N-terminal sequence of the human exonuclease 1 deduced from its cDNA sequence (MGIQGLL). Therefore, the smaller polypeptide is a C-terminal His tag.
indicated that the expression of both medium at 37 °C to determine the growth curve. The results (Fig. 6). All of these strains were grown in the liquid YPD primers in yeast as the transformants formed smaller colonies though the human gene has less capacity to remove RNA rad27 null mutant promotes the colony formation at 37 °C even ScEXO1 cells (data not shown). Expression of EXO1 and HEX1 gene made the rad27 null mutant grow more closely to the wild type even though the strain harboring the human EXO1 gene grew more slowly than the rad27Δ mutant with the ScEXO1 (Fig. 7).

Expression of Both ScEXO1 and HEX1 Reversed the Mutator Phenotypes—Due to the functions of the EXO1 gene in recombinational mismatch correction and probably in RNA primer removal, deletion of the gene increased yeast mutation rates 29-fold in the cycloheximide resistance assay (Table III). The rate increased about 6-fold in the CANr and HOM assays (59). Expression of both ScEXO1 and HEX1 reversed the mutator phenotypes of an exon1 null mutant and the mutation rate was reduced to the wild type level. These data indicate that the human gene is a functional homolog of the yeast gene and can play a role in mutation avoidance in heterogeneous cellular environment.
DISCUSSION

Sequence and in Vitro Functional Conservation of Eukaryotic 5'-Exonuclease 1—The enzyme has so far been identified in S. pombe, S. cerevisiae, D. melanogaster, and human (44–52). Sequence similarity of Exo1s from these eukaryotic organisms indicates evolutionary and functional conservation of this important group of 5'-exonucleases. The biochemical properties of hExo1 are similar to that of ScExo1. The enzyme is a non-processive 5'-3' double-stranded and single-stranded DNA exonuclease. In S. cerevisiae, the 5'-3' exonuclease activity is 2-fold more on double-stranded DNA than on single-stranded DNA. The purified protein from S. pombe, however, has very low 5'-3' single-stranded DNA exonuclease activity. It is more specific for the double-stranded DNA. It is possible that the truncated protein purified from S. pombe lacks a structure responsible for 5'-3' single-stranded DNA exonuclease activity. Resembling the ScExo1, human exonuclease 1 has both activities but prefers a double-stranded DNA substrate to single-stranded DNA. However, it does not discriminate between RNA and DNA (Fig. 4 and see below). Because the observed sequence conservation of the putative nuclease domains between the exonuclease-1 and flap endonuclease-1 (see Refs. 28, 29, 60, and 62 for additional information on FEN-1 nuclease), we tested the flap endonuclease activities of human exonuclease-1 with a typical flap DNA substrate with DNA or RNA as the single-stranded portion and human flap endonuclease-1 as a positive control. The result indicates that the enzyme does not possess a specific flap endonuclease activity. A detailed description of the human exonuclease 1 substrate specificity will be published elsewhere.

Human Exonuclease 1 Functionally Complements the Yeast DNA Recombination and UV Resistance and Mutation Avoidance—Expression of human exonuclease 1 in yeast complements several characterized phenotypes in the exo1Δ mutants. hExo1 complements the function of ScExo1 in recombination (Table II). The recombinational rate decreases 2-fold when the Exo1Δ gene was deleted in our assay system, where the repeat fragments were about 400 bp. 7-fold decrease was reported when the repeats were extended to 900 bp (45). It was also demonstrated in vitro that the enzyme significantly promotes the recombination of the two DNA fragments with end overlapping sequence. As revealed in S. pombe, mRNA level and enzyme activity was induced during meiosis (44, 45) and in D. melanogaster, it is only expressed in the early embryogenesis of the female germline (46). We speculate that the functional 5'-exonuclease 1 may be involved in recombinational mismatch correction during meiosis in mammalian cells as no such eukaryotic enzyme has been identified to date.

In S. cerevisiae, it has been shown that Exo1 is involved in UV DNA damage repair, which is distinct from nucleotide excision repair pathway (49). In this study, we revealed that hExo1 complements the function of ScExo1 in UV DNA damage repair, indicating that hExo1 could be involved in UV DNA damage repair in human cells as well. Among the different UV
implies that hExo1 could play a role in the mutation avoidance mechanism in human cells. The result is also consistent with the role of exo1 in mismatch correction in S. pombe (44). The protein interacts with Msh2 (48, 49) and double knockout of EXO1 and MSH2 showed the similar mutation rate as the single knockout of MSH2. Altogether, the evidence available indicates that these two proteins may work together in a mutation avoidance mechanism.

**Human Exonuclease 1 Backs Up the Function of Flap Endonuclease 1 in DNA Replication RNA Primer Removal**—Fig. 4 shows a time-dependent ribonuclease activity of hExo1 on RNA/DNA hybrid duplexed to a DNA template regardless of multiple or monoribonucleotide preceding the DNA portion. This activity may indicate the involvement of Exo1 in RNA primer removal during lagging strand DNA synthesis. In prokaryotes, the role of RNA primer removal during DNA lagging strand synthesis is played by the 5’-3’ exonuclease activity of DNA polymerase I (24–27). In eukaryotes, the function of removing RNA primers of the lagging DNA strand is performed by the FEN-1/Rad27 nuclease in two alternative pathways. One pathway is that RNase H removes all ribonucleotides except for the last one adjunct to DNA fragments; then, the last ribonucleotide is removed by FEN-1/Rad27 nuclease. The second pathway is performed by FEN-1/Rad27 nuclease via its flap endonuclease activity independent of RNase H. If there are only these two pathways involved in the removal of the RNA primers, disruption of the FEN-1/Rad27 gene in yeast should completely block the RNA primer removal pathways and the null mutant may be lethal. However, disruption of SxRad27 caused a conditional lethality phenotype (39–42); the null mutant could still grow at the normal temperature (30 °C) but become lethal at 37 °C. For this reason, a third pathway for removing RNA primers has been proposed (29) and Exo1 is one of the best candidates for this pathway due to its 5’-3’ exonuclease activity. The hExo1 was as efficient in removal of ribonucleotides as deoxyribonucleotides, resembling the function of Rad27 in RNA primer removal pathway in our experiments. As we have also shown, the overexpression of HEX1 as well as ScExo1 in yeast S. cerevisiae cells suppressed the temperature-sensitive phenotype of rad27/fen-1 null mutant at 37 °C. This result indicates that the Exo1 enzyme is involved in removal of RNA primers during lagging strand DNA synthesis.

**Table III**

| Strains   | Genotype               | Mutation frequency (× 10⁻⁶) | Relative rate |
|-----------|------------------------|----------------------------|---------------|
| AB15Be    | Wild type/pRS314       | 0.22 ± 0.05                | 1             |
| AB1Ba     | exo1/pRS314            | 6.21 ± 0.81                | 29            |
| AB1Bb     | exo1/PR8-ScE           | 0.28 ± 0.09                | 1.3           |
| AB1Bc     | exo1/PR8-He            | 1.12 ± 0.21                | 5.1           |

DNA damage repair pathways, the XPG/Rad2 nucleotide excision repair pathway is a major pathway. In humans, dysfunction of this repair pathway results in xeroderma pigmentosum syndrome. Epistatic analysis in yeast showed that the UV DNA damage repair involving Exo1 is independent of XPG/Rad2 nucleotide excision repair and other UV DNA damage repair pathways (51). The minor UV sensitivity is due to the failure of DNA replication-driven bypass compensation mechanism.

Failure of mismatch correction during DNA recombinational repair increases mutation rate. Deletion of ScEXO1 caused a 29-fold increase in cycloheximide resistance in S. cerevisiae. The assay is based on the fact that mutation in the yeast gene CYH2 can lead to resistance to cycloheximide, an inhibitor of eukaryotic protein synthesis. The gene product of CYH2 is ribosomal protein L29, a component of the 60 S ribosomal subunit. In most cases, resistance to cycloheximide is due to a transversion mutation resulting in replacement of a glutamine by a glutamic acid in position 37 of Leu-29 (57). Mutation rates such as transversions; 2) the HOM3 assay is sensitive to single base deletions and additions, which happens frequently in mismatch repair defective cells. This is a reversion assay. The mutant strain causes at a high rate the deletion of a single T in a run of 7 Ts. Otherwise, the HOM3 protein does not tolerate much sequence variation elsewhere. Table III also shows that HEX1 complements the function of ScExo1 and reverses the mutator phenotype in exo1Δ mutants. This result implies that hExo1 could play a role in the mutation avoidance
erator RNA removal is supported by the fact that overexpression of EXO1 suppresses the temperature-sensitive phenotype of rad27/fen-1 null mutant at 37 °C and the lethality of the double knockout of RAD27 and EXO1 (48).

Recently, however, it has also been proposed that the unligated Okazaki fragments of the rad27 null mutant can also be repaired by the recombinational double strand break repair pathway (43). This hypothesis is based on the fact the disruption of FEN-1/RAD27 can elevate recombination rate by 20–30-fold (43). In addition, the double knockout of RAD27 and RAD51 or RAD52 (the key protein components of double strand break repair) is lethal. The double strand break repair pathway requires 5’-3’ exonuclease to generate the single-stranded 3’-overhangs for strand invasion. EXO1 is a good candidate for this process as well. Meanwhile, if EXO1 is the only enzyme so far available, which possibly generates the single-stranded 3’-overhangs for this process as well. Meanwhile, if EXO1 is the only enzyme so far available, which possibly generates the single-stranded 3’-overhangs for strand invasion. EXO1 is a good candidate for this process as well. Meanwhile, if EXO1 is the only enzyme so far available, which possibly generates the single-stranded 3’-overhangs for strand invasion. EXO1 is a good candidate for this process as well.

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