Evidence that the DNA Mismatch Repair System Removes 1-Nucleotide Okazaki Fragment Flaps**

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**Background:** The DNA mismatch repair (MMR) system protects humans from cancer.
**Results:** Combining an MMR system defect (msh2Δ) with rad27Δ causes a strong synergistic increase in the rate of 1-bp insertions, and a reconstituted MMR system removes 1-nt flaps.
**Conclusion:** The MMR system removes 1-nt Okazaki fragment flaps.
**Significance:** A new function of the MMR system was identified.

The DNA mismatch repair (MMR) system plays a major role in promoting genome stability and suppressing carcinogenesis. In this work, we investigated whether the MMR system is involved in Okazaki fragment maturation. We found that in the yeast *Saccharomyces cerevisiae*, the MMR system and the flap endonuclease Rad27 act in overlapping pathways that protect the nuclear genome from 1-bp insertions. In addition, we determined that purified yeast and human MutSα proteins recognize 1-nucleotide DNA and RNA flaps. In reconstituted human systems, MutSα, proliferating cell nuclear antigen, and replication factor C activate MutLα endonuclease to remove the flaps. ATPase and endonuclease mutants of MutLα are defective in the flap removal. These results suggest that the MMR system contributes to the removal of 1-nucleotide Okazaki fragment flaps.

* Genome stability is essential for maintaining life and preventing numerous genetic disorders. The MMR system promotes genome stability by correcting replicative DNA polymerase errors, removing mismatches formed during homologous recombination, impeding homologous recombination, and participating in DNA damage response (1–5). Genetic or epigenetic inactivation of the MMR system strongly predisposes humans to several types of cancers (6). MMR has been extensively studied in *Escherichia coli* and eukaryotes (7, 8).

**MutLoa (MLH1–PMS2 heterodimer in humans and MLH1–PMS1 heterodimer in yeast), MutSα (MSH2–MSH6 heterodimer), MutSβ (MSH2–MSH3 heterodimer), EXO1, PCNA, and RFC are the key eukaryotic MMR factors (9–23).** Eukaryotic MMR occurs both on the leading and lagging strands, but mismatches on the lagging strands are corrected more efficiently than those on the leading strands (24). The first step in eukaryotic MMR is recognition of the mismatch by the MutS homolog MutSα or MutSβ (11, 12, 15, 19). After mismatch recognition, MutSα or MutSβ and loaded PCNA activate MutLoa to incise the discontinuous daughter strand in the vicinity of the mismatch (25–28). The endonuclease activity of MutLoa depends on the integrity of its ATPase sites and the DQHA(Δ3E)(Δ3E) motif (25, 26). A strand break generated by MutLoa 5’ to the mismatch serves as the entry site for MutSα–activated exonuclease 1 to degrade a mismatch-containing segment of the daughter strand in a 5’ → 3’-excision reaction (21, 25). The resulting gap is repaired by DNA polymerase δ holoenzyme (29). The loss of exonuclease 1 causes only a modest MMR defect in yeast and mice (18, 22). Consistent with these observations, a reconstituted system lacking exonuclease 1 is proficient in MMR (30). The reconstituted system bypasses the requirement for exonuclease 1 in the mismatch removal by relying on the strand-displacement activity of DNA polymerase δ holoenzyme.

In addition to mismatches, several other aberrant structures with significant mutagenic potential are formed during DNA replication. Among them are Okazaki fragment flaps (31, 32). Okazaki fragment maturation is a process that removes the flaps and joins the trimmed ends together producing continuous strands (33, 34). Genetic evidence indicates that defective removal of Okazaki fragment flaps causes genome instability (31, 32). In eukaryotes, Rad27/FEN1 endonuclease, Dna2 helicase/nuclease, and the 3’ → 5’-exonuclease activity of DNA polymerase δ remove Okazaki fragment flaps (32–36). PCNA interacts with Rad27, and this interaction strongly stimulates the flap endonuclease activity of Rad27 (37). An important question is whether there are additional proteins that contribute to the removal of Okazaki fragment flaps.

The MMR system corrects DNA polymerase errors on newly replicated DNA (38–41). It has been unknown whether the MMR system plays a direct role in DNA replication. In this report, we describe genetic and biochemical experiments that indicate that the MMR system removes 1-nt Okazaki fragment flaps.

**Experimental Procedures**

**Yeast Strains and Genetic Methods—*Saccharomyces cerevisiae* wild-type haploid strains used in this study were as follows:**
TABLE 1
The sequences of oligonucleotides described in this report

| Oligonucleotide | Oligonucleotide sequence |
|-----------------|-------------------------|
| 1               | 5'-ACACTCAATTCTGGAATGTTCTCTTCAGATTCGAAA-3' |
| 2               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 3               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 4               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 5               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 6               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 7               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |
| 8               | 5'-TTCTCTAGATGCAAAAAGAATGCGATTTCTCAGATTCGAAA-3' |

FKY688 (MATa ade5-1 lys2::InsE-A14 trpl-1 289 his7-2 leu2-3,112 ura3-52 V29617::URA3) (42); E134 (MATa ade5-1 lys2::InsE-A16 trpl-1 289 his7-2 leu2-3,112 ura3-52) (43); E35 (MATa ade5-1 lys2::InsE-A8 trpl-1 89 his7-2 leu2-3,112 ura3-52) (43); BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0); and 1B-D770 (MATa ade5-1 lys2::InsE-A14 trpl-1 3 Ts15 trpl-1 289 his7-2 leu2-3,112 ura3-4) (44). The wild-type diploid strain FKY1037 was prepared by crossing the E134 and 1B-D770 strains. Gene replacements were generated by transforming yeast haploid or diploid cells with disruption cassettes in the presence of lithium acetate/PEG4000/DMSO. The PMS1 gene located in its natural chromosomal location was mutated to the pms1-E707K allele using the “delitto perfetto” technique (45). Spontaneous mutation rates were measured, and mutation spectra were determined as described previously (42).

Oligonucleotides—Oligonucleotides used in this work were synthesized by IDT (Coralville, IA). The sequences of the indicated oligonucleotides are shown in Table 1.

Proteins—Human MutSα, MutLα, MutLα-D699N, MutLα-E705K, MutLα-EA, PCNA, RFC, RPA, CAF-1, histone H3-H4 complex, and FEN1 were isolated in nearly homogeneous forms as described previously (23, 25, 30, 46). Yeast MutSα containing the FLAG tag at the N terminus of its Msh6 subunit was expressed in and purified from insect Sf9 cells. The protein that was used in the DNA-binding reactions was more than 95% pure.

Gel Mobility Shift Assays—Gel mobility shift assays that used the oligonucleotide-based substrates (Fig. 2) were carried out as described below. The oligonucleotide-based substrates were produced using oligonucleotides 1–8 (Table 1). Each of the substrates contained oligonucleotide 1, which was labeled with 32P at the 5’ end with T4 polynucleotide kinase. In addition, the homoduplex, 1-nt insertion, dynamic 1-nt DNA flap, static 1-nt 3’ DNA flap, static 1-nt 5’ DNA flap, and nicked substrates contained oligonucleotides 2, 3, 4 and 5, 6 and 7, 4 and 8, and 4 and 7, respectively. To make the DNA substrates, the indicated oligonucleotides were mixed and annealed. The annealing was carried out in a buffer containing 20 mM HEPES-NaOH, pH 7.4, and 100 mM KCl at 40 °C for 4 h, followed by incubation of the mixtures at 20 °C for 30 min. After annealing, the resulting duplex DNAs were separated on native 6% polyacrylamide gels and then purified from the gels. The gel-purified DNAs were used as substrates in the DNA-binding reactions. The DNA-binding reactions were carried out in 20-μl mixtures each containing 20 mM HEPES-NaOH, pH 7.4, 120 mM KCl, 5 mM MgCl2, 0.2 mM ATP, 0.2 mg/ml BSA, 2 mM DTT, 1.9 nM (50 ng) of the indicated circular 2-kb DNA, 50 nM of the competitor 40-bp DNA, and human MutSα (5, 10, 20, 40, 100, 200, 400, 550, 800, 1200, and 1600 nM). Human MutSα concentration in the mixtures was in the range of 5–800 nM (the actual concentrations used were 5, 10, 20, 40, 100, 200, 400, 550, 800, and 1200 nM). The competitor 40-bp DNA was prepared by annealing two complementary phosphorylated 40-mer oligonucleotides 9 and 10. Reaction mixtures containing yeast MutSα were incubated for 10 min at 30 °C, and reaction mixtures containing human MutSα were incubated for 5 min at 37 °C. The reaction products were immediately subjected to electrophoresis on 6% polyacrylamide gels in the 0.5× Tris borate/EDTA running buffer at 4 °C. The gels were dried, and 32P-labeled DNAs were visualized with a Typhoon phosphorimager (GE Healthcare). Each experiment was repeated at least twice. After quantification of the images with ImageQuant software (GE Healthcare), the apparent Kd values were determined using GraphPad Prism 6 software. The data were fit into the equation of nonlinear regression curve with Hill slope (Y = Bmax*X^h/(Kd + X^h)). In this equation, Y is the concentration of MutSα-DNA complexes; Bmax is the maximum concentration of MutSα-DNA complexes; X is the concentration of MutSα; Kd is the apparent dissociation constant, and h is the Hill coefficient.

Gel mobility shift assays that used 2-kb circular DNA substrates (Fig. 3) were performed as detailed below. The substrates were prepared using the pSYAH1A plasmid DNA containing a 36-nt gap (47). The gap was generated according to a described protocol (47). The no-flap, G-T, 1-nt DNA flap, and 1-nt RNA flap substrates were prepared by annealing the gapped pSYAH1A DNA with oligonucleotides 11, 12, 13, and 14, respectively. The G-T and no-flap substrates each contain two ligatable nicks that are 36 nt apart. Cleavage with restriction endonucleases HindIII and HpyCH4III was utilized to determine what fraction of each of the substrates contains the annealed oligonucleotide. These restriction endonucleases do not cleave DNA within a gap due to the destruction of their sites by the gap. Based on this approach, we determined that ∼95% of each of the circular substrates contained the annealed oligonucleotide.

To determine apparent Kd values for binding of human MutSα to the circular DNAs, the reactions were carried out in 20-μl mixtures each containing 20 mM HEPES-NaOH, pH 7.4, 120 mM KCl, 5 mM MgCl2, 0.2 mM ATP, 0.2 mg/ml BSA, 2 mM DTT, 1.9 nM (50 ng) of the indicated circular 2-kb DNA, 50 nM of the competitor 40-bp DNA, and human MutSα (5, 10, 20, 40, 100, 200, 400, 550, or 800 nM). After a 5-min incubation at 37 °C, each reaction mixture was mixed with 3 μl of loading buffer (1× TAE, 40% glycerol, and 0.02% bromphenol blue), and the reaction products were immediately subjected to electrophoresis on 1.2% agarose gels in 1× TAE at 4 °C, followed by ethidium bromide staining of the gels. The separated DNAs were transferred onto nylon membranes and hybridized with 32P-labeled oligonucleotide 15. The labeled DNAs were visual-
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TABLE 2
Impact of deletion of MSH2 and RAD27 on rates of his7-2 mutations

| Genotype                   | Absolute mutation rate (×10⁻⁸) | Relative rate | 1-bp insertions in the A₁ run (A₁→A₈) | Complex mutations* | Other +1 frameshifts |
|----------------------------|--------------------------------|---------------|----------------------------------------|--------------------|---------------------|
| Wild type<sup>a</sup> (n = 42) | 0.6 (0.5–1.2)                  | 1             | 0.5 [1]                               | 0.03               | 0.06                |
| msh2Δ (n = 41)             | 120 (88–150)                   | 200           | 120 [240]                              | <3                  | <3                  |
| rad27Δ (n = 39)            | 82 (66–110)                    | 140           | 48 [96]                               | 27                  | 6                   |
| msh2Δ rad27Δ (n = 41)      | 6700 (5900–9400)               | 11,000        | 6700 [13,400]                          | 1/160              | 1/160               |

<sup>a</sup> Each of the complex mutations consisted of an insertion and four or more other genetic alterations, all located within an ~20-bp DNA segment.

<sup>b</sup> The wild-type data are from a previous report (42).

The wild-type strains are isogenic to E134 (wild type) and were obtained by dissection of tetrads of mutant strains that both the MMR system and Rad27 are necessary for the suppression of mutations in the +1 frameshift reporter his7-2 (32, 38–40, 44). +1 frameshifts that occur in a 51-bp his7-2 sequence containing an A₁ run revert the phenotype of the cells to His<sup>+</sup> (42, 44). To study whether there is a functional overlap between the MMR system and the Rad27 flap endonuclease, we determined the his7-2 mutation rates in the haploid and diploid yeast strains shown in Tables 2 and 3. The his7-2 mutation rate in the haploid double mutant msh2Δ rad27Δ (6700 × 10⁻⁸) was 33 times higher than the sum of the his7-2 mutation rates in the haploid single mutants msh2Δ and rad27Δ (i.e. combining msh2Δ with rad27Δ resulted in a 33-fold synergistic increase in the his7-2 mutation rate) (Table 2). Likewise, the his7-2 mutation rate for the diploid double mutant msh2Δ/msh2Δ rad27Δ/ rad27Δ (13,000 × 10⁻⁸) was increased 36 times relative to the sum of the his7-2 mutation rates for the diploid single mutants msh2Δ/msh2Δ RAD27/RAD27 and MSH2/MSH2 rad27Δ/ rad27Δ (Table 3). These findings indicate that there is a functional overlap between the MMR system and Rad27 in haploid and diploid yeast S. cerevisiae.

lys2::InsE-A₈ is a yeast +1 frameshift reporter (43). +1 frameshifts that are formed within a 71-bp lys2::InsE-A₈ region, including an A₈ run, produce Lys<sup>+</sup> cells (43). To ascertain that the above findings (Tables 2 and 3) were not reporter-specific, we measured the lys2::InsE-A₈ mutation rates in the msh2Δ, rad27Δ, and msh2Δ rad27Δ mutants (Table 4). Analysis of the data demonstrated that the lys2::InsE-A₈ mutation rate in the msh2Δ rad27Δ double mutant (21,000 × 10⁻⁸) was 24 times higher than the sum of the lys2::InsE-A₈ mutation rates in the msh2Δ and rad27Δ single mutants. Thus, the use of the lys2::InsE-A₈ mutation assay provided additional evidence that a genetic stabilization function of the MMR system overlaps with a genetic stabilization function of the Rad27 flap endonuclease. Collectively, these genetic experiments suggest that an MMR system-dependent mechanism and a different mechanism dependent on the Rad27 flap endonuclease repair the same or related types of pre-mutagenic intermediates which, if left unrepaired, give rise to +1 frameshifts.

Next, we used DNA sequencing to identify +1 frameshifts that reverted his7-2 in the msh2Δ, rad27Δ, and msh2Δ rad27Δ mutants (Table 2). The results revealed that all of the his7-2 reversions in the msh2Δ and msh2Δ rad27Δ spectra and a majority of the reversions in the rad27Δ spectrum were 1-bp insertions, each of which extended the A₁ run into an A₈ run (Table 2). In addition, we found that combining msh2Δ with rad27Δ led to a 40-fold synergistic increase in the rate of 1-bp insertions (Table 2). This finding implies that one or several related types of pre-mutagenic intermediates producing 1-bp insertions are repaired by both an MMR system-dependent mechanism and a Rad27-dependent mechanism.
TABLE 3
Effect of deletion of MSH2 and RAD27 on his7-2 mutation rate in the diploid S. cerevisiae

The mutant diploid strains are derivatives of FYK1037 (wild type) and were prepared using the lithium acetate/PEG/DMSO transformation method. CI, confidence interval.

| Genotype                  | Absolute rate (×10⁻⁶) | 95% CI   | Relative rate |
|---------------------------|------------------------|----------|---------------|
| Wild type                 | 0.9                    | 0.7–1.3  | 1             |
| RAD27/rad27Δ msh2/msh2Δ   | 1.1                    | 0.9–1.5  | 1             |
| RAD27/rad27Δ msh2/msh2Δ   | 160                    | 140–210  | 180           |
| rad27Δ/rad27Δ msh2/msh2   | 200                    | 150–230  | 220           |
| RAD27/rad27Δ msh2/msh2Δ   | 150                    | 30–190   | 160           |
| rad27Δ/rad27Δ msh2/msh2Δ  | 610                    | 320–860  | 680           |
| his7-2 mutation rate      | 13,000                 | 11,000–16,000 | 14,000       |

The MMR system contains two mismatch recognition complexes, MutSα and MutSβ. As shown in Table 5, the his7-2 mutation rate in the msh3Δ msh6Δ mutant was indistinguishable from that in the msh2Δ mutant but 23 times higher than the sum of those in the msh3Δ and msh6Δ mutants. This result indicates that the partially overlapping activities of MutSα and MutSβ (19, 50–52) are engaged in the suppression of +1 frameshifts in his7-2. To study whether an MMR system-dependent function overlapping with a Rad27 function involves MutSα and/or MutSβ, we determined the his7-2 mutation rates for the msh2Δ, rad27Δ, msh2Δ rad27Δ, msh3Δ msh6Δ rad27Δ, msh3Δ rad27Δ, msh6Δ rad27Δ, and msh2Δ rad27Δ mutants (Table 5).

We found that the his7-2 mutation rate for the msh3Δ msh6Δ rad27Δ mutant did not differ from the his7-2 mutation rate for the msh2Δ rad27Δ mutant, but it was ~12 or ~70 times higher than the rate for the msh3Δ rad27Δ or msh3Δ rad27Δ mutants, respectively. These data indicate that both MutSα and MutSβ participate in an MMR system-dependent function that overlaps with a Rad27 function. We also found that the his7-2 mutation rate in msh6Δ rad27Δ exceeded that in msh3Δ rad27Δ by 6-fold (Table 5). This result is consistent with the view that compared with MutSβ, MutSα plays a more important role in an MMR system-dependent function that overlaps with a Rad27 function.

MutLα endonuclease is a key component of the eukaryotic MMR system (9, 13, 14, 25, 26, 38, 39). The endonuclease activity of yMutLα depends on the integrity of the Pms1 DQHA(X)_2E(X)_4E motif, which is part of the putative active site of the endonuclease (25, 26, 53, 54). The E70K substitution, which replaces the first glutamate residue in the DQHA(X)_2E(X)_4E motif of yMutLα, inactivates the yeast MMR system (26). We found that combining rad27Δ with msh1Δ, pms1Δ, or pms1Δ-E707K resulted in a 20–26 times synergistic increase in the his7-2 mutation rate (Table 5). Nevertheless, the his7-2 mutation rate in the pms1Δ-E707K rad27Δ, pms1Δ rad27Δ, or msh1Δ rad27Δ strain was half that in the msh2Δ rad27Δ strain (Table 5). Taken together, these data suggest that an MMR system-dependent function overlapping with a Rad27 function often involves the endonuclease activity of MutLα.

The results described above were obtained using the his7-2 and lys2::Ine-A_Aγ reversion assays that only allow scoring of +1 frameshifts. Unlike the his7-2 and lys2::Ine-A_Aγ reversion assays, the CAN1 forward mutation assay allows scoring of many different types of genetic alterations, including 1-bp insertions, base substitutions, and 1-bp deletions. The CAN1 forward mutation assay takes advantage of the fact that mutational inactivation of the CAN1 gene encoding arginine permease makes the yeast cell resistant to canavanine, a structural analog of arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine. In this assay, Canr cells are selected on a minimal medium lacking arginine.
in the msh2Δ and rad27Δ mutants. This information supports the view that one or several related types of pre-mutagenic intermediates causing 1-nt insertions are removed by both an MMR system-dependent mechanism and a Rad27-dependent mechanism.

Duplications are formed at a high rate in rad27Δ/H9004 mutants (31, 32). These duplications have been suggested to be the products of unprocessed Okazaki fragment flaps (31, 49). Strikingly, 6–14-bp duplications were produced at a rate of 630/H11003108 in CAN1 in the msh2Δ rad27Δ strain, but they were absent in the can1 spectra of the rad27Δ and msh2Δ mutants (Fig. 1B). These data suggest that one or several related types of pre-mutagenic intermediates triggering 6–14-bp duplications are removed by both an MMR-dependent mechanism and a Rad27-dependent mechanism.

The Dna2 helicase/nuclease is an essential enzyme that participates in the removal of flaps during Okazaki fragment maturation (33–36). Yeast strains carrying a dna2 allele, dna2-1, are temperature-sensitive (55, 56) and show a weak defect in the maintenance of dinucleotide repeats (56). We established that the his7-2 mutation rate in the dna2-1 strain was increased 10-fold relative to that in the wild-type strain (Table 6). We then studied the effect of combining dna2-1 with msh2Δ on the his7-2 mutation rate in the dna2-1 msh2Δ double mutant was two times higher than the sum of those in the single mutants. This observation is consistent with the idea that one or several related types of pre-mutagenic intermediates causing +1 frameshifts are repaired by both an MMR system-dependent mechanism and a Dna2-dependent mechanism.

**Recognition of 1-nt DNA Flaps by MutSα**—We considered two models to explain the observation that combining msh2Δ with rad27Δ leads to the strong synergistic increases in the rates of spontaneous 1-bp insertions (Table 2 and Fig. 1B). In the first model, DNA polymerase α errors are corrected not only by MMR (57) but also by a Rad27-dependent mechanism, and DNA polymerase α errors that escape both MMR and the Rad27-dependent mechanism produce mutations, including 1-bp insertions. However, this model is not supported by the observation that the deletion of RAD27 in the msh2Δ strain does not significantly increase the rate of base substitutions (Fig. 1B), which are the most common products of DNA polymerase α errors (57, 58). Thus, it is unlikely that a considerable fraction of 1-bp insertions formed in msh2Δ rad27Δ mutants

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**TABLE 6**

| Genotype         | 1-bp insertions a | Base substitutions | 1-bp deletions | Duplications (6-14 bp) | Duplications (17-416 bp) | Other mutations | Total |
|------------------|-------------------|--------------------|----------------|------------------------|--------------------------|-----------------|-------|
| Wild type (n=43) | 1.4 (1)           | 17 (1)             | 1.4 (1)        | < 0.5                  | < 0.5                    | 0.5             | 20    |
| rad27Δ (n=47)    | 115 (82)          | 77 (5)             | 38 (27)        | < 38                   | 1,500                    | 77              | 1,600 |
| msh2Δ (n=48)     | 51 (36)           | 210 (12)           | 360 (260)      | < 13                   | < 13                     | < 13            | 620   |
| msh2Δ rad27Δ (n=49) | 2,000 (1,400)    | 380 (22)           | 630 (450)      | 630                    | 2,400                    | < 130           | 6,200 |

* a The mutation rates were measured at 25 °C. 95% confidence intervals are in parentheses.
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The second model is based on the knowledge that the key function of the 5′ flap endonuclease Rad27 is the removal of short Okazaki fragment flaps (33, 34). In this model, 1-nt Okazaki fragment flaps are removed by both a Rad27-dependent mechanism and an MMR system-dependent mechanism, and the unprocessed flaps are converted by misalignment and ligation into 1-bp insertions. Thus, this model suggests that the majority of 1-bp insertions produced in msh2 rad27 mutants are formed from 1-nt Okazaki fragment flaps. Because Okazaki fragment flaps do not cause base substitutions, the second model is consistent with our genetic data (Table 2 and Fig. 1B).

The second model postulates that the MMR system removes 1-nt Okazaki fragment flaps. To determine whether there is evidence for this, we carried out the biochemical experiments described below. We first examined whether yeast MutSα recognizes 1-nt DNA flaps present on the 32P-labeled oligonucleotide-based substrates (Fig. 2). The data revealed that yeast MutSα bound the substrate containing the dynamic 1-nt flap with an apparent $K_d$ of $38 \pm 2$ nM (Fig. 2, A and B). The control experiments indicated that yeast MutSα bound the 1-nt insertion-containing DNA, nicked DNA, and homoduplex DNA with apparent $K_d$ values of $25 \pm 1$, $180 \pm 10$, and $200 \pm 8$ nM, respectively (Fig. 2, A and B). Therefore, these experiments demonstrate that yeast MutSα recognizes the dynamic 1-nt flap nearly as efficiently as the 1-nt insertion. We then investigated whether yeast MutSα recognizes static 1-nt 3′ and 5′ flaps. The experiments showed that yeast MutSα bound the static 1-nt 3′ and 5′ flaps with apparent $K_d$ values of $60 \pm 2$ and $55 \pm 3$ nM, respectively. Thus, yeast MutSα recognizes the static 1-nt 3′

FIGURE 2. Human and yeast MutSα proteins recognize 1-nt DNA flaps. The gel mobility shift assays with the oligonucleotide-based DNA substrates and calculations of the apparent $K_d$ values were performed as described under “Experimental Procedures.” All six substrates had the same bottom strand. The DNA sequences of the homoduplex and nicked DNA substrates were identical to each other and to the his7-2 sequence, in which the majority of +1 frameshifts are formed. Compared with the top strand of the homoduplex or nicked substrate, the top strands of the flapped and 1-nt insertion substrates each contained an extra nucleotide residue, which was necessary to produce the 1-nt flap or 1-nt insertion. A, representative images showing binding of yeast MutSα to the different DNA substrates. Each DNA-binding reaction was carried out in the mixture containing the indicated concentration of yeast MutSα and the indicated DNA substrate (2 nM). B and C, apparent $K_d$ values for binding of yeast MutSα (B) and human MutSα (C) to the indicated DNA substrates. The apparent $K_d$ values were calculated using the data that were obtained by quantification of images, including those shown in A. The numbers above the bars are the apparent $K_d$ values.
and 5' flaps with the same affinity. Surprisingly, yeast MutSα detected the static 1-nt 3' and 5' flaps somewhat less efficiently than the dynamic 1-nt flap (Fig. 2, A and B). Because a dAMP residue forms the flap in the dynamic substrate and a dCMP residue produces the flaps in the static substrates, it is possible that yeast MutSα recognizes a flapped dCMP residue less efficiently than a flapped dAMP residue.

We also studied whether human MutSα recognizes the dynamic 1-nt flap (Fig. 2C). Our experiments indicated that human MutSα bound the dynamic 1-nt flap with an apparent $K_d$ of 30 ± 1 nM. An apparent $K_d$ value for binding of human MutSα to the 1-nt insertion is 30 ± 6 nM. These $K_d$ values are 7–12 times lower than those for binding of human MutSα to the nicked and homoduplex DNAs (Fig. 2C). Thus, human MutSα efficiently recognizes the dynamic 1-nt flap. Collectively, these findings support the view that the ability to recognize 1-nt DNA flaps is conserved in eukaryotic MutSα proteins.

We also analyzed whether human MutSα recognizes a dynamic 1-nt flap present on a circular 2-kb DNA (Fig. 3). Each of the substrates contained a 1-nt DNA flap, a 1-nt RNA flap, no flap, or a G-T mispair (Fig. 3A). The results revealed that MutSα bound the 1-nt DNA and RNA flap-containing DNAs with $K_d$ values of 119 ± 3 and 115 ± 10 nM, respectively (Fig. 3B). These $K_d$ values are half of that of 254 ± 35 nM for the binding of MutSα to the control no-flap DNA. Thus, MutSα detects that the circular DNA carries a 1-nt flap, which may be a deoxyribonucleotide or ribonucleotide residue.

**MutLα Endonuclease-dependent Removal of 1-nt Flaps**

Having shown that MutSα recognizes the 1-nt DNA and RNA flaps on the circular DNA, we carried out and analyzed the reconstituted reactions to determine whether these flaps activate human MutLα endonuclease to incise the discontinuous strand in the presence of human MutSα, PCNA, RFC, and RPA (Fig. 4). The circular DNAs were used as substrates in these reactions because loaded PCNA, required for the activation of MutLα endonuclease (25–27), slides off of linear DNA. The reactions were performed under conditions that were very similar to those used for the identification of the MutSα-, PCNA-, RFC-, mismatch-, and ATP-dependent endonuclease activity of human MutLα (25). Analysis of the reactions (Fig. 4, A and B) led to the following observations. First, 34 ± 5% of the discontinuous strand of the 1-nt DNA flap-containing substrate was incised by MutLα, whereas the endonuclease cleaved only 10 ± 2% of the discontinuous strand of the control flap-free substrate. Second, MutLα incised 30 ± 1% of the discontinuous strand of the 1-nt RNA flap. Third, an endonuclease-deficient MutLα variant, MutLα-E705K (25), did not incise the discontinuous strands of the tested substrates. Together, these observations indicate that 1-nt flaps activate MutLα endonuclease to incise the discontinuous strand in the presence of MutSα, PCNA, RFC, and RPA.

To determine whether incision of the discontinuous strand by MutLα results in the removal of flaps, we performed experiments summarized in Fig. 5. As shown in lane 2 of Fig. 5A, the incubation of MutLα, MutSα, PCNA, RFC, and RPA with the 1-nt DNA flap-containing circular substrate led to incision of the $^32$P-labeled 37-nt fragment at several sites. The most abundant product of the incision reaction had an apparent length of 5 nt, indicating that the incision occurred at a site that is four nucleotides 3' to the flap. The incision products were not formed when MutSα, MutLα, RFC, or PCNA was omitted from the reaction mixture, but the omission of RPA did not have a significant effect on the incision (Fig. 5, A, lanes 3, 4, 6, and 7, and B). These results indicate that MutSα, MutLα, RFC, and PCNA are required for the incision, but RPA is not. The time course experiments demonstrated that the incision reaction produced the 5-nt fragment in a time-dependent manner (Fig. 5C). The efficiency of the incision of the site located 4 nt downstream from a 1-nt flap was three times higher than that of the same site on the control flap-free substrate (Fig. 5, A, lanes 2 and 10, and B and C). Thus, the flap dependence of the MutLα incision was 3-fold. Changing the incubation temperature from 37 to 25 °C decreased the flap dependence of the MutLα incision from 3- to 2-fold (data not shown). MutLα, MutSα, PCNA, and RFC were also required for the incision of the 1-nt RNA flap-containing substrate (Fig. 5, A, lanes 18–20, 22, and 23, and B). Consistent with a previous study (59), the 5-nt incision product containing the 5'-ribonucleotide residue migrated in the gel slightly slower than the 5-nt incision product lacking a ribonucleotide residue (Fig. 5A, lanes 2 and 18).

We also studied whether the endonuclease activity of MutLα is necessary for the incision of the discontinuous strand at a 1-nt flap (Fig. 5, A and B). The replacement of the wild-type MMR System and Okazaki Fragment Maturation
MutLα with the endonuclease-deficient MutLα-E705K led to the disappearance of the incision products indicating that the endonuclease activity of MutLα is responsible for the incisions (Fig. 5, A, lanes 5 and 21). Further analysis revealed that the presence of a 1-nt flap did not activate the MutLα endonuclease to incise the discontinuous strand immediately upstream from the flap (data not shown). Taken together, these experiments demonstrate that MutSα, RFC, and PCNA activate MutLα endonuclease to incise the discontinuous strand 4 nt downstream from a 1-nt DNA or RNA flap. Because the incision is so close to the flaps, it triggers their dissociation from the substrates.

Newly replicated DNA is rapidly assembled into nucleosomes by a mechanism that depends on the histone H3-H4 chaperone CAF-1 (60–62). The first step in CAF-1-dependent nucleosome assembly is the deposition of histone H3-H4 tetramers. CAF-1-dependent nucleosome assembly probably impacts many processes that take place on the nascent DNA. Consistent with this idea, CAF-1-dependent nucleosome assembly modulates MMR (46, 63). Because the MMR system-dependent flap removal (Fig. 5) is likely to occur during CAF-1-dependent nucleosome assembly, we studied whether histone H3-H4 deposition by CAF-1 affects the flap-removing activity of the MMR system. We determined that CAF-1-dependent histone H3-H4 deposition stimulated the flap-removing activity of the MMR system by 2-fold (Fig. 6, A, lanes 11 and 12, and B) and increased the flap dependence of the incision from 3- to 6-fold (Fig. 6 C). The efficiency of the flap removal was not changed when MutSα and MutLα were added to the reaction mixtures that were incubated with CAF-1, the histone
The H3-H4 complex, PCNA, RFC, and RPA for 15 min suggesting that the MMR system efficiently removes 1-nt DNA flaps in the presence of pre-loaded H3-H4 tetramers (data not shown). The omission of CAF-1 significantly decreased both the efficiency and flap dependence of the incision (Fig. 6C).

Control experiments revealed that the flap removal occurring in the presence of CAF-1-dependent histone H3-H4 deposition required both MutSα/H9251 and MutLα/H9251 (Fig. 6A and B). An endonuclease-deficient MutLα variant, MutLα-D699N (25), as well as a MutLα ATPase mutant, MutLα-EA (64), could not substitute for the wild-type MutLα in the incision reaction. Thus, these experiments demonstrate that the CAF-1-dependent histone H3-H4 deposition promotes the removal of 1-nt DNA flaps by the activated MutLα endonuclease.

We then studied how CAF-1 and the histone H3-H4 complex affect the incision of the discontinuous strand at sites that are distant from the 1-nt flap (Fig. 7). Strikingly, the presence of CAF-1 and the histone H3-H4 complex suppressed the MutLα endonuclease-dependent incision of the discontinuous strand at the remote sites (Fig. 7, A, lanes 9 and 14–16, and B). A similar suppression of the MutLα endonuclease-dependent incision of the discontinuous strand was observed in the six-protein system containing the histone H3-H4 complex but not CAF-1 (Fig. 7, A, lanes 9–12, and B). These findings imply that
both CAF-1-dependent histone H3-H4 deposition onto the DNA and nonspecific binding of the histone H3-H4 complex to the DNA protect the remote sites from the incision by the activated MutLα endonuclease.

Next, we performed experiments to study whether the reconstituted MMR system is able to remove flaps in the presence of FEN1 (Fig. 8). The data showed that increasing the FEN1 concentration decreased the yield of the product of MutLα endonuclease-dependent flap removal and increased the yield of the product of FEN1-dependent flap removal (Fig. 8, A–C). In addition, the data indicated that one or several proteins present in the eight-protein system suppressed the flap endonuclease activity of FEN1 (Fig. 8, A, lanes 3–10, and C). These experiments provide evidence that the MMR system removes flaps in the presence of FEN1 and sug-
gest that the flap endonuclease activities of FEN1 and the MMR system compete with each other.

Discussion

High fidelity DNA replication is required for the maintenance of genome integrity and the suppression of human diseases (65). The MMR system improves the fidelity of DNA replication by correcting the errors of DNA polymerization (3, 4, 7). We have used genetic analysis and reconstituted systems to study whether the MMR system contributes to the removal of Okazaki fragment flaps. The major findings described in this report are: 1) combining rad27Δ with msh2Δ produces strong synergistic increases in the rates of 1-bp insertions in his7-2 and can1 (Table 2 and Fig. 1B); 2) combining rad27Δ with msh2Δ, pms1Δ, or pms1-E707K causes a 20–26 times synergistic increase in the rate of +1 frameshifts in his7-2 (Table 5); 3) purified yeast and human MutSα proteins recognize 1-nt flaps (Figs. 2 and 3); 4) MutLα endonuclease activated by MutSα, RFC, and PCNA removes 1-nt flaps (Fig. 5); 5) the flap-removing activity of the reconstituted MMR system is stimulated by CAF-1-dependent histone H3-H4 deposition (Fig. 6); and 6) the reconstituted MMR system removes 1-nt flaps in the presence of FEN1 (Fig. 8).

These findings indicate that the eukaryotic MMR system removes a subset of 1-nt Okazaki fragment flaps and support a model illustrated in Fig. 9. This model suggests that MutSα,

![Figure 7](https://example.com/f7.png)
MutLα, PCNA, and RFC provide the minimal set of activities required for the removal of 1-nt Okazaki fragment flaps by the MMR system. According to this model, the mechanism of the removal of a 1-nt Okazaki fragment flap by the MMR system can be divided into three key steps as follows: recognition of the flap by MutSα; activation of MutLα endonuclease by MutSα, PCNA, and RFC; and the removal of the flap by the activated MutLα endonuclease. Our genetic results also suggest that there is an Msh2-dependent, MutLα-independent mechanism of removal of 1-nt Okazaki fragment flaps (Table 5). In addition, our genetic results are compatible with another model. In this model, misalignment and ligation converts some 1-nt Okazaki fragment flaps into 1-nt loops, which are then removed by the strand-specific MMR (8, 11, 19, 28, 66). However, it has not yet been demonstrated that a replicative DNA ligase is able to convert 1-nt flaps into 1-nt loops in the presence of Rad27/FEN1 and/or the MMR system.

The absolute his7-2 mutation rate in the rad27Δ/rad27Δ msh2Δ/msh2Δ diploid (Table 3) is half that of the previously described strong mutator diploid pol3-01/pol3-01 msh2Δ/msh2Δ (40). (The pol3-01 mutation inactivates the proofreading activity of DNA polymerase δ.) This observation reveals that the MMR system is nearly as important for the removal of +1 frameshift intermediates in rad27Δ strains as for the repair of +1 frameshift intermediates in pol3-01 strains. Genetic interactions between the MMR system and Rad27 have been investigated in the past (31, 48, 49), but none of the previous studies utilized a +1 frameshift assay or determined can1 mutation spectrum in a strain that lacks an MMR gene and RAD27. Nevertheless, Johnson et al. (48) reported that the relative CAN1 mutation rate in the msh2Δ mutant is in a weak synergistic relationship with that in the rad27Δ mutant. Thus, the results of the measurements of the relative CAN1 mutation rates in the msh2Δ, rad27Δ, and msh2Δ rad27Δ mutants obtained in this work (Fig. 1A) and the study of Johnson et al. (48) are consistent with each other.

MutSα was initially identified as an MMR factor that detects single base-base mismatches (11, 12). Subsequent work established that MutSα recognizes 1–12-nt insertion/deletion loops (15, 19) and damaged base pairs (67). We have described in this report that MutSα recognizes 1-nt DNA/RNA flaps (Figs. 2 and 3). This finding extends the range of potentially mutagenic DNA structures recognized by MutSα. Our genetic experiments support the idea that MutSβ plays a role in the MMR system-dependent removal of 1-nt Okazaki fragment flaps (Table 5). Thus, it is possible that MutSβ, like MutSα, recog-
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Assembled into nucleosomes by the histone chaperone CAF-1 (60). Our analysis demonstrates that the CAF-1-dependent histone H3–H4 deposition increases the efficiency and specificity of the flap removal by MutLα and protects the discontinuous strand from MutLα incision at the remote sites (Figs. 6 and 7). The mechanism behind these effects is not known. We speculate that the loaded histones H3–H4 tetramers trap the MutLα-containing incision complex at the flap-containing site where it was assembled, and as a result the MutLα is not able to incise the discontinuous strand at the remote sites and instead removes the flap.

Previous research demonstrated that during eukaryotic Okazaki fragment maturation, the strand displacement activity of DNA polymerase δ (30, 33, 74) produces flaps that are removed by the Rad27/FEN1 endonuclease (33, 34), the 3′–5′-exonuclease activity of DNA polymerase δ (32), and the nuclease/helicase Dna2 (36). In this report, we have described evidence that the eukaryotic MMR system contributes to the removal of Okazaki fragment flaps.

Author Contributions—F. A. K. and L. Y. K. designed experiments. L. Y. K., B. K. D., and F. A. K. performed experiments and analyzed data. F. A. K. and L. Y. K. wrote the paper.

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