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Reconstitution of *Saccharomyces cerevisiae* DNA polymerase ε-dependent mismatch repair with purified proteins

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Mammalian and *Saccharomyces cerevisiae* mismatch repair (MMR) proteins catalyze two MMR reactions in vitro. In one, mispair binding by either the MutS homolog 2 (Msh2)–MutS homolog 6 (Msh6) or the Msh2–MutS homolog 3 (Msh3) stimulates 5′ to 3′ excision by exonuclease 1 (Exo1) from a single-strand break 5′ to the mispair, excising the mispair. In the other, Msh2–Msh6 or Msh2–Msh3 activate the MutL homolog 1 (Mlh1)–postmeiotic segregation 1 (Pms1) endonuclease in the presence of a mispair and a nick 3′ to the mispair, to make nicks 5′ to the mispair, allowing Exo1 to excise the mispair. DNA polymerase δ (Pol δ) is thought to catalyze DNA synthesis to fill in the gaps resulting from mispair excision. However, colocalization of the *S. cerevisiae* mispair recognition proteins with the replicative DNA polymerases during DNA replication has suggested that DNA polymerase ε (Pol ε) may also play a role in MMR. Here we describe the reconstitution of Pol ε-dependent MMR using *S. cerevisiae* proteins. A mixture of Msh2–Msh6 (or Msh2–Msh3), Exo1, RPA, RFC-Δ1N, PCNA, and Pol ε was found to catalyze both short-patch and long-patch 5′ nick-directed MMR of a substrate containing a +1 (+T) mispair. When the substrate contained a nick 3′ to the mispair, a mixture of Msh2–Msh6 (or Msh2–Msh3), Exo1, RPA, RFC-Δ1N, PCNA, and Pol ε was found to catalyze an MMR reaction that required Mlh1–Pms1. These results demonstrate that Pol ε can act in eukaryotic MMR in vitro.

mutator phenotype | genome instability | DNA replication fidelity | DNA excision | DNA repair

DNA mismatch repair (MMR) is a key pathway in DNA metabolism that acts on mispaired bases that occur in replication and recombination intermediates, and as the result of some types of chemical damage to DNA (1–10). MMR also plays an important role in some types of cellular DNA damage responses (11–14). Because defects in MMR genes result in inherited cancer susceptibility, underlie a significant number of sporadic cancers of different tissues, and result in resistance to some chemotherapeutic agents, there has been considerable interest in understanding the mechanisms of eukaryotic MMR (15–18). A number of types of studies have contributed to our current understanding of MMR mechanisms (for reviews, see refs. 1–4, 8, 10, 11, and 19). Genetic studies have identified many MMR genes, defined different types of MMR defects, and provided pathway models for guiding mechanistic studies of MMR proteins. Cell biology studies have facilitated visualization of MMR proteins and the coupling of MMR to DNA replication as well as facilitating the study of the role of MMR proteins in cellular responses to DNA damage. And finally biochemical studies have provided numerous insights into MMR proteins and the biochemical reactions they promote.

The development of cell-free systems from *Saccharomyces cerevisiae*, *Xenopus*, *Drosophila*, human, and mouse cells that could catalyze repair of mispair-containing DNAs provided a foundation for the biochemical fractionation and reconstitution of eukaryotic MMR (20–24). Two types of mispair-dependent excision/repair reactions have been reconstituted with human and *S. cerevisiae* proteins. In the first type of reaction, a combination of one of the mispair recognition factors MutS homolog 2 (Msh2)–MutS homolog 6 (Msh6) or Msh2–MutS homolog 3 (Msh3), exonuclease 1 (Exo1), DNA polymerase δ (Pol δ), the single-stranded DNA binding protein replication protein A (RPA), proliferating cell nuclear antigen (PCNA), and the PCNA loading factor replication factor C (RFC) catalyze the repair of a circular mispaired substrate containing a single-strand break (referred to as a nick) on the 5′ side of the mispair (25–27). In this reaction, the mispair recognition factors stimulate excision by Exo1 from the nick past the mispair to produce a gap that is filled in by Pol δ, PCNA, and RFC repairing the mispair (25, 28). In the human protein reactions, the mismatched DNA substrates contain a 5′ nick that is 128 bp from the mispair, resulting in excision tracts that are relatively short, and typically there is only one repair event per molecule of Exo1 (26–28). In contrast, the repair tracts of the *S. cerevisiae* protein reactions can be up to ∼3 kb long and there may be up to 10–12 repair events per molecule of Exo1 (25). In a second type of reaction, a combination of Msh2–Msh6 or Msh2–Msh3, MutL homolog 1 (Mlh1)–postmeiotic segregation 1 (Pms1) (called Mlh1–Pms2 in humans), Exo1, Pol δ, RPA, PCNA, and RFC promotes the repair of a circular mispaired substrate containing a nick on the 3′ side of the mispair (25, 26). In this reaction, the Mlh1–Pms1

Significance

By performing reconstitution studies with purified *Saccharomyces cerevisiae* proteins, this study provides the first demonstration of eukaryotic mismatch repair (MMR) reactions dependent on DNA polymerase ε (Pol ε) in addition to the previously described DNA Pol δ (Pol δ)-dependent MMR reactions. The MutS homolog 2 (Msh2)–MutS homolog (or Msh2–MutS homolog 3)-dependent MMR reactions that Pol ε was found to promote in vitro include both short-patch and long-patch MutL homolog 1 (Mlh1)–postmeiotic segregation 1 (Pms1)-independent, exonuclease 1 (Exo1)-dependent MMR and Mlh1–Pms1 endonuclease-dependent, Exo1-dependent MMR. The availability of these reconstituted MMR reactions provides a unique foundation for biochemical reconstitution studies whose ultimate goal is to reconstitute leading-strand and lagging-strand DNA replication-coupled MMR using purified proteins.

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The authors declare no conflict of interest.

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See Commentary on page 3552.

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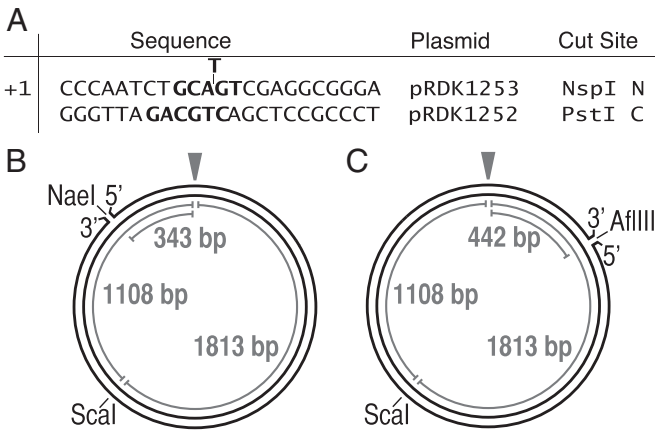

Fig. 1. pBluescript-based substrates for detecting MMR in vitro. (*A*) Sequence of the polylinker region between the ApaI and BamHI sites of different substrates indicating the mispair, the restriction sites in each strand, and the plasmid from which each strand was derived. N, nicked strand; C, continuous strand. (*B*) Map of the 5′ NaeI-nicked +T substrate and (*C*) map of the 3′ AflIII-nicked +T substrate showing the positions of the various features used in the assays and the relevant distances between key sites. The mispair is indicated by the arrowhead.

endonuclease is activated in a mispair-dependent fashion by a combination of Msh2–Msh6 or Msh2–Msh3, PCNA, and RFC to generate DNA nicks 5′ to the mispair (29–33). Once the 5′ nicks are formed, repair appears to occur as observed in the 5′ nick-directed MMR reactions. MMR can also occur in the absence of Exo1 (24, 34, 35); however, our knowledge of Exo1-independent MMR mechanisms is not as well developed as it is for Exo1-dependent MMR (19, 36, 37).

The requirement for Msh2–Msh6, Msh2–Msh3, Mlh1–Pms1 (hMlh1–Pms2), Exo1, PCNA, and RFC in MMR is well substantiated by genetic studies (1, 2, 10, 19, 34, 35, 38–42). Mutations in the genes encoding each of these proteins or protein complexes are well known to cause MMR defects in vivo. In addition, hypomorphic mutations causing different types of MMR defects have been extensively used in mechanistic studies of many of these proteins (31, 35, 36, 39, 40, 43–45). Genetic studies have suggested that the Mlh1–MutL homolog 2 (Mlh2) and Mlh1–MutL homolog 3 (Mlh3) complexes also play a role in MMR (46–48); however, the possible biochemical roles of these two complexes in reconstituted MMR reactions have not been well studied. A role for RPA in MMR in vitro is not surprising, given the rather ubiquitous role of RPA in different aspects of DNA metabolism; however, a role for RPA is at present only supported by biochemical studies as no mutations affecting RPA that cause MMR defects have yet been reported (25–27, 31, 49). A role for Pol δ is primarily supported by fractionation studies in which MMR proficiency was restored to a depleted extract by the addition of a single protein, either fractions containing Pol δ or purified Pol δ (50). Importantly, Pol δ is sufficient to support in reconstituted MMR reactions (25–27, 31). Purified DNA polymerase α (Pol α) could not substitute for Pol δ in these studies, suggesting that Pol α cannot not act by itself in MMR (50). Because the depleted extracts used in the assays used in the fractionation experiments appeared to contain low levels of Pol α and Pol ε, it was not possible to rule out the possibility that these DNA polymerases might act in conjunction with DNA Pol δ in MMR (50). However, inhibitor studies demonstrated that Pol α is not required for MMR in vitro (50) and reconstitution studies showed that neither Pol α or Pol ε were required for Pol δ to support MMR in vitro (26), supporting the idea that neither Pol α or Pol ε were required for Pol δ-dependent MMR in the fractionation studies implicating Pol δ (50). Mutations inactivating the gene encoding the Pol32 subunit of DNA Pol δ cause defects in Exo1-independent but not in Exo1-dependent MMR in vivo, possibly suggesting an important role for Pol δ in Exo1-independent MMR (35, 37). However, because Pol32 is a subunit of more than one DNA polymerase, further analysis is required to understand the role of Pol32 in MMR (51, 52). Finally, one study has suggested that the editing exonuclease functions of Pol δ and Pol ε could play a role in the excision step of MMR (53), but this has not been definitively established. In the present study, we have further investigated a possible role for Pol ε in MMR.

Results

Properties of the DNA Substrate Used to Detect MMR. In the present study, we used two mutant phagemids that differ from each other by a single nucleotide insertion to construct two different mispair-containing substrates that allow detection of nick-directed MMR in vitro (25, 31, 49). The substrates each contain a +1 (+T) insertion in the nicked strand. This single T insertion disrupts the PstI restriction endonuclease cleavage site whose sequence is present in the continuous strand and creates a sequence for an NspI restriction endonuclease cleavage site in the nicked strand. One substrate, called the NaeI substrate, contains a nick 343 bp 5′ from the mispair and the other substrate, called the AflIII substrate, contains a nick 442 bp 3′ from the mispair. Nick-directed MMR excision and resynthesis of these substrates excises the T-containing strand, converting the PstI-resistant substrate to a PstI-sensitive product by changing the sequence in the nicked strand to the complement of the sequence present in the continuous strand. This process allows nick-directed MMR to be monitored by cleaving the product DNA with ScaI and PstI to produce a diagnostic pair of repair-specific 1.1-kb and 1.8-kb fragments. The +1 (+T) insertion mispair allows the same substrate to be used to study both Msh2–Msh6- and Msh2–Msh3-dependent MMR (25, 42). Previous studies have shown that these substrates are efficiently repaired in reconstituted DNA polymerase δ-dependent MMR reactions (25, 31). The key details of these substrates are illustrated in Fig. 1.

DNA Pol ε Supports Efficient Short- and Long-Patch 5′ to 3′ Excision Repair of a +T Insertion Mispair Substrate. We previously demonstrated that a combination of Msh2–Msh6 (or Msh2–Msh3), Exo1, RPA, RFC-Δ1n (or RFC), PCNA, and Pol δ would promote efficient nick-directed, mispair-dependent short-patch repair of the NaeI substrate (25). In this reaction, mispair

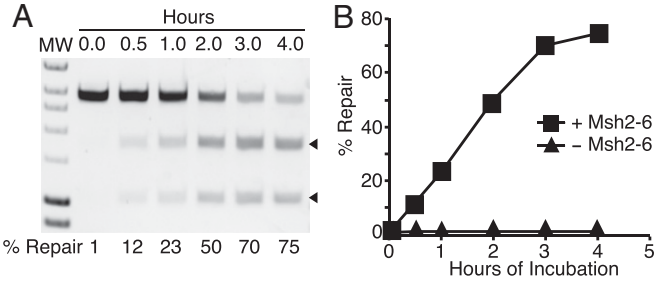

Fig. 2. Time course of DNA polymerase ε-dependent MMR of the NaeI-nicked +1 (+T) substrate. Assays of 5′ nick-directed repair of the +1 (+T) substrate containing a nick at the NaeI site were performed for the indicated times as described in *Materials and Methods*. The presence/absence of Msh2–Msh6 is as indicated. (*A*) Repair was detected by digestion with PstI and ScaI, and the repair products were visualized after agarose gel electrophoresis, and (*B*) the repair products seen on the gels were quantified as described in *Materials and Methods*. MW, molecular weight markers; arrows, markers for repair products. One hundred percent repair is repair of 100 ng or 52.75 fmol of substrate.

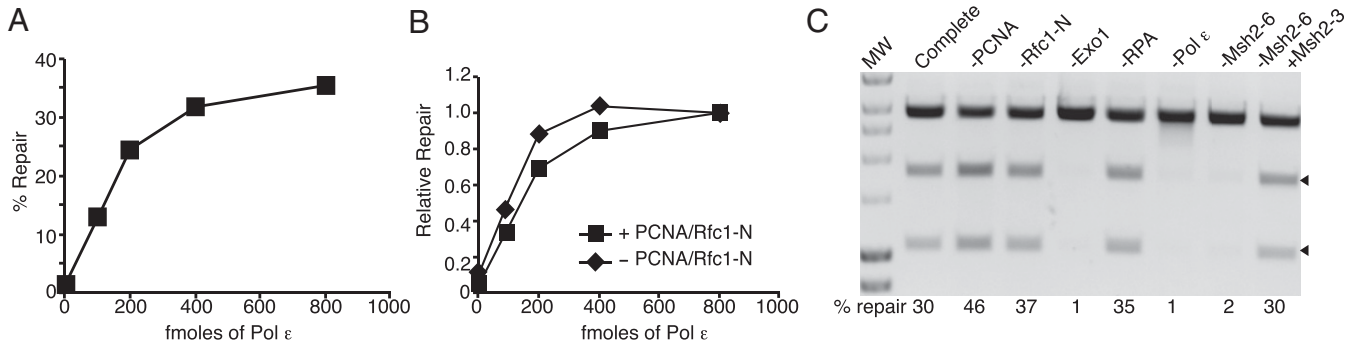

Fig. 3. Protein requirements for 5′ nick-directed MMR of the NaeI-nicked +T substrate. (*A*) Assays of 5′ nick-directed repair of the +1 (+T) substrate containing a nick at the NaeI site containing the indicated amounts of DNA Pol ε. (*B*) Assays of 5′ nick-directed repair of the +1 (+T) substrate containing the indicated amounts of Pol ε as in *A*, with or without PCNA and RFC-Δ1N as indicated. Relative repair of 1.0 was the amount of repair observed at 800 fmol of Pol ε. (*C*) Assays of 5′ nick-directed repair of the +1 (+T) substrate containing a nick at the NaeI site in which different proteins were omitted or substituted as indicated. MW, molecular weight markers; arrows, markers for repair products.

recognition by Msh2–Msh6 stimulated 5′ excision of the mispair by Exo1, followed by filling in of the resulting single-stranded gap by a combination of RFC-Δ1n (or RFC), PCNA, and Pol δ. To determine whether Pol ε could support MMR in vitro, we substituted Pol ε for Pol δ in this reconstituted MMR system. In the presence of Pol ε, a robust MMR reaction was observed that was linear for 3 h, with repair approaching 75% (Fig. 2). Omitting Msh2–Msh6 completely eliminated MMR (Fig. 2). The level of repair seen at 3 h is in the range of 107 repair events per Exo1 molecule, which is significantly greater than previously seen in reactions with Pol δ (25); we think it is likely that this enhanced activity reflects the greater activity of the Exo1 preparations used in the present studies compared with that used in previous studies (25). Overall, these results were comparable to those observed previously with reactions containing Pol δ, with the exception that the reactions with Pol ε had a lower background of Msh2–Msh6-independent repair than generally observed in previously published reactions with Pol δ (25).

To further evaluate the Pol ε-dependent MMR of the NaeI substrate, the requirements for the various proteins were examined (Fig. 3). Maximum levels of MMR were observed at 400 fmoles of Pol ε regardless of whether the reactions did or did not contain PCNA and RFC-Δ1N. There was an absolute dependence of these MMR reactions on Msh2–Msh6, and Msh2–Msh3 could fully substitute for Msh2–Msh6. Omitting Exo1 or DNA Pol ε eliminated repair. Omitting RFC-Δ1n or PCNA did not reduce repair in contrast to the previously observed complete dependence of Pol δ-dependent repair on RFC-Δ1n or PCNA (25). This result is consistent with prior observations that extensive DNA synthesis by Pol δ requires PCNA and its loading factor RFC, whereas extensive DNA synthesis by Pol ε is not absolutely dependent on PCNA or RFC (54). Finally, omitting RPA did not reduce the amount of repair observed.

We previously demonstrated that at fourfold higher concentrations of Exo1, a combination of Msh2–Msh6 (or Msh2–Msh3), Exo1, RPA, RFC-Δ1n (or RFC), PCNA, and Pol δ would promote efficient nick-directed, mispair-dependent long-patch repair of the AflIII substrate in a reaction that involved 5′-excision tracts from the nick at the AflIII site that extended at least 2.9 kb (25). To determine whether Pol ε would also support long-patch MMR in vitro, we substituted Pol ε for Pol δ in MMR reactions containing the AflIII substrate and fourfold higher levels of Exo1. In the presence of Pol ε, a robust MMR reaction was observed that was linear for up to 4 h with total repair approaching 70% (Fig. 4). Omitting Msh2–Msh6 completely eliminated MMR (Fig. 4). The level of repair seen at 4 h is in the range of 25 repair events per Exo1 molecule, which is greater than previously seen in reactions with Pol δ (25). We then evaluated the protein requirements of this MMR reaction (Fig. 4). Omitting Exo1 or Pol ε eliminated repair, whereas omitting RFC-Δ1n or PCNA did not reduce repair. Interestingly, omitting RPA resulted in a partial reduction of repair. Msh2–Msh3 did not completely substitute for Msh2–Msh6, as previously seen for Pol δ-dependent nick-directed, mispair-dependent long-patch repair of the AflIII substrate (25). These results are consistent with the conclusion that Pol ε also supports long-patch 5′-excision repair of the AflIII substrate as observed for short-patch 5′-excision repair of the NaeI substrate.

DNA Pol ε Supports Mlh1–Pms1 Endonuclease-Dependent Repair of a +T Insertion Mispair Substrate. We previously demonstrated that a combination of Mlh1–Pms1, Msh2–Msh6 (or Msh2–Msh3), Exo1

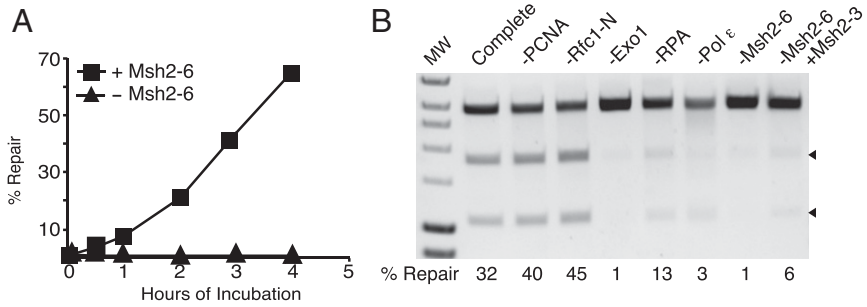

Fig. 4. Time course and protein requirements for 5′ nick-directed repair of the AflIII-nicked +T substrate. (*A*) The 5′ nick-directed repair reactions with the +1 (+T) substrate containing a nick at the AflIII site were performed for the indicated times with the presence/absence of Msh2–Msh6 as indicated. (*B*) The 5′ nick-directed repair reactions with the +1 (+T) substrate containing a nick at the AflIII site in which different proteins were omitted or substituted as indicated. MW, molecular weight markers; arrows, markers for repair products.

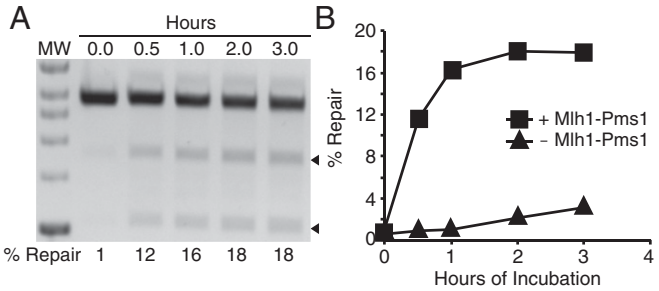

Fig. 5. Time course of DNA polymerase ε- and Mlh1–Pms1-dependent MMR of the AflIII-nicked +T substrate. Two-stage repair reactions with the +1 (+T) substrate containing a 3′ nick at the AflIII site were performed for the indicated times as described in *Materials and Methods*. (*A*) Repair was detected by digestion with PstI and ScaI, and the repair products were visualized after agarose gel electrophoresis, and (*B*) the repair products seen on the gels were quantified as described in *Materials and Methods*. MW, molecular weight markers; arrows, markers for repair products.

(at the reduced levels used for 5′ nick-directed short-patch repair of the NaeI-nicked substrate), RPA, RFC-Δ1n (or RFC), PCNA, and Pol δ would promote efficient Mlh1–Pms1 endonuclease-dependent, mispair-dependent repair of the AflIII substrate provided that the MMR reactions contained both Mg^{2+} and Mn^{2+} (31). In this reaction, mispair recognition by Msh2–Msh6 (or Msh2–Msh3) recruits Mlh1–Pms1, which, in a reaction that requires PCNA and RFC, makes nicks 5′ to the mispair on the strand that contains the 3′ nick. These nicks then direct 5′ excision of the mispair by Exo1 followed by filling in of the resulting single-stranded gap by DNA synthesis by a combination of RFC-Δ1n (or RFC), PCNA, and Pol δ (26, 29, 31, 33). Optimal repair was observed if minimally Mlh1–Pms1 was preincubated with the substrate DNA followed by addition of the other required proteins (called two-stage reactions). To determine whether Pol ε would support Mlh1–Pms1-dependent MMR in vitro, we substituted Pol ε for Pol δ in two-stage reconstituted MMR reactions. In the presence of Pol ε, a robust MMR reaction was observed that was linear for up to 1 h with repair approaching 18% in the experiment shown (Fig. 5). Omitting Mlh1–Pms1 almost completely eliminated MMR (Fig. 5); the low amount of Mlh1–Pms1-independent MMR observed late in the reaction is most likely 5′ nick-directed long-patch MMR from the AflIII site nick. These results were comparable to those observed previously with reactions containing DNA Pol δ (31).

To further evaluate the Mlh1–Pms1-dependent, Pol ε-dependent MMR of the AflIII substrate, the requirements for the various proteins were examined (Fig. 6). Omitting Msh2–Msh6 or Mlh1–Pms1 strongly reduced repair, and Msh2–Msh3 could fully substitute for Msh2–Msh6. In contrast to that observed for 5′ nick-directed MMR, omitting RFC-Δ1n or PCNA significantly reduced repair. This observation is most likely because activation of the Mlh1–Pms1 endonuclease requires RFC and PCNA (29–31, 33, 36). The residual repair seen in the absence of RFC-Δ1n or PCNA is likely the result of Mlh1–Pms1-independent 5′ nick-directed long-patch MMR from the AflIII site nick that also occurs under these reaction conditions and does not require RFC and PCNA (Fig. 4 and ref. 25), but could also suggest that Pol ε is able to activate the Mlh1–Pms1 endonuclease at low levels. We did not evaluate a requirement for Pol ε, Exo1, or RPA as these proteins have been well established to act in the excision and resynthesis steps that occur once a 5′ nick is introduced by Mlh1–Pms1 (Figs. 3 and 4 and ref. 25).

Discussion

Previous studies established that Pol δ is required for human MMR in vitro (50) and can support MMR reactions that have been reconstituted in vitro using purified human and *S. cerevisiae* proteins (25–27, 31, 50). We recently showed that MMR is coupled to DNA replication and that the mispair recognition proteins Msh2–Msh6 and Msh2–Msh3 colocalize with the replicative DNA polymerases during DNA replication (55, 56). This latter observation led us to investigate whether Pol ε might also function in MMR in vitro. The results presented here clearly show that Pol ε can robustly support the gap-filling reactions required for both the 5′ and 3′ nick-directed MMR reactions that have been reconstituted in vitro. These include the 5′ nick-directed Exo1-dependent mispair excision reaction that is coupled to gap filling and 3′ nick-directed MMR that involves activation of the Mlh1–Pms1 endonuclease to make nicks 5′ to the mispair that are required for MMR to occur. In addition, Pol ε was able to support filling of both short ~350-bp gaps and ~2.9-kb gaps that are excised during short-patch and long-patch 5′ nick-directed Exo1-dependent mispair excision, respectively (25). We did not investigate a possible role for Pol α as previous studies showed that it cannot substitute for Pol δ in the types of MMR reactions studied here (50). Our observation that Pol ε and Pol δ both function robustly in reconstituted MMR reactions suggests that MMR mechanisms may be more diverse than previously appreciated.

The results reported here are different from the results of previous studies of human MMR, which concluded that Pol δ supports MMR in vitro (50) and, in conjunction with reconstitution studies (26, 29, 37), did not identify a role for Pol ε in MMR in vitro. There are a number of possible explanations for this difference. First, it is possible that there are differences between *S. cerevisiae* and human MMR reactions in vitro and that Pol ε does not function in human MMR in vitro. Second, it is possible that Pol ε was inactivated by the fractionation procedure used in the previous human MMR reconstitution studies. And finally, it is possible that Pol ε was present in the human cell extracts at levels that were too low to detect in the fractionation experiments, especially because Pol ε was required at higher levels than Pol δ in the experiments reported here. This latter observation raises the possibility that the previous assays used were not sensitive enough to detect human DNA Pol ε. Based on the results reported here, it will be important to determine whether Pol ε can substitute for Pol δ in reconstituted human MMR in vitro.

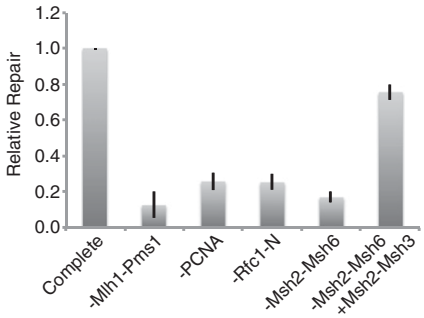

Fig. 6. Protein requirements for DNA polymerase ε- and Mlh1–Pms1-dependent repair of the AflIII-nicked +1 (+T) substrate. Two-stage repair reactions with the +1 (+T) substrate containing a 3′ nick at the AflIII site were performed in which different proteins were omitted or substituted as indicated. The amount of repair was quantified as described in *Materials and Methods* and normalized to the amount of repair seen in complete reactions. The average of at least three independent experiments is presented; the error bars indicate the SE.

DNA replication is a highly coordinated process with leading-strand DNA synthesis being performed by Pol ε coupled to the cell division cycle 45 (Cdc45)/mini-chromosome maintenance proteins 2–7 (Mem2–7)/Sld5-Psf1–3 (GINS) DNA helicase complex (also called the CMG complex), and other proteins, and lagging-strand DNA synthesis primarily being performed by Pol δ and its accessory factor PCNA following initiation of lagging-strand synthesis by DNA Pol α/primase (52, 54). MMR appears to be coupled to the replication fork as evidenced by the timing of MMR and the colocalization of the Msh2–Msh6 mispair recognition complex with replication fork proteins during S phase (55, 56). Previous studies suggesting that only Pol δ acts in MMR (50) could be interpreted as implying that the selection of the DNA polymerase used for the gap-filling step of MMR is not directly coupled to DNA replication because Pol δ does not act in leading-strand DNA synthesis. However, the results presented here that both Pol δ and Pol ε can act in MMR raise the possibility that there could be strand-specific coupling of MMR to the DNA replication machinery, even at the level of selection of the DNA polymerase that acts in MMR. Because there is no direct evidence supporting this idea, this hypothesis suggests that reconstituting replication-coupled MMR may provide additional insights into MMR mechanisms.

Materials and Methods

Protein Purification. Msh2–Msh6, Msh2–Msh3, Mlh1–Pms1, Exo1, RPA, RFC-Δ1n, PCNA, and DNA Pol δ were all overproduced and purified as previously described (25) and in most cases, are the same batches that were used in previously published experiments (25, 31). All of the protein preparations were confirmed to be greater than 95% pure as judged by SDS/PAGE followed by staining the resulting gels with Coomassie Blue. The only modification made to our published procedures was that Nonidet P-40 was added to the Exo1 storage buffer at a final concentration of 0.02%, which resulted in increased activity and helped prevent nonspecific binding of Exo1 to tubes during dilution. The optimal requirement for each protein in the experiments reported was determined for each batch of protein prepared.

DNA Pol ε was purified from *S. cerevisiae* cells using published overexpression plasmids/strains, buffers, and methods (54), with the following modifications: protease inhibitor cocktail (PIC) D and PIC W protease inhibitor mixtures were added to all buffers (25). Cells were grown to an OD_{600} of 1.0 and protein expression was induced for 16 h before harvesting the cells and lysing them with seven passes through a Microfluidizer (Microfluidics). The clarified supernatant was batch bound with 2 mL of anti-FLAG M2 affinity resin (Sigma) by intermittent mixing on ice for 1 h. Then the resin was packed into a column, washed, and the bound proteins were eluted with 0.4 mg/mL of 3× FLAG peptide (Biomatik). The FLAG column fractions were pooled, loaded onto a 1-mL MonoS column, and the bound proteins were eluted with a 10-mL gradient from 0.1 M to 1 M KCl. MonoS fractions were selected, frozen in liquid N_2, and stored at –80 C.

DNA Substrates. The DNA substrates containing a +1 insertion mispair due to the presence of an inserted T in the nicked strand of the substrate were constructed following previously described methods using the mutant derivatives of pBluescript SK+, pRDK1252, and pRDK1253 (25). One of the substrates contained a single-strand break at the NaeI site 5′ to the mispair and the other substrate contained a single-strand break at the AflIII site 3′ to the mispair.

Repair Assays. MMR assays for short- and long-patch 5′ excision-mediated MMR contained Msh2–Msh6 or Msh2–Msh3, Exo1, PCNA, RFC-Δ1N, RPA, and Pol ε and were analyzed using a modification of our previously published procedures (25) as follows: Proteins were diluted if necessary with 7.5 mM Hepes, pH 7.5, 10% (vol/vol) glycerol, 200 mM KCl, 1 mM DTT, and 0.5 mg/mL BSA. For the short-patch 5′ excision-mediated MMR reaction, 0.37 fmol of Exo1, 390 fmol of Mlh1–Pms1, 390 fmol Msh2–Msh6, 290 fmol of PCNA (PCNA trimers), 400 fmol of Pol ε, 220 fmol of RFC-Δ1N, and 1,800 fmol of RPA were combined into 4 μL and mixed with 1 μL of 100 ng/μL of 5′ NaeI-nicked substrate DNA and 5 μL of 33 mM Tris pH 7.6, 75 mM KCl, 2.5 mM ATP, 1.66 mM glutathione, 8.3 mM $MgCl_2$, 80 μg/mL of BSA, and 200 μM each of the dNTPs. The reactions, containing a final concentration of 118 mM KCL, were then incubated at 30 °C for 3 h and then processed as described below. For the long-patch 5′ excision-mediated MMR reaction, 3′ AflIII-nicked substrate DNA was substituted for the 5′ NaeI-nicked substrate DNA and the amount of Exo1 was increased to 1.48 fmol. The key modifications of our previously published reaction conditions (25) were that the reaction volumes were reduced to 10 μL, the amount of substrate DNA was reduced to 100 ng (52.75 nmol), and the reduced levels of Exo1 that were used due to the increased activity of Exo1 preparations containing Nonidet P-40 compared with our previous preparations of Exo1 (25). The presence or absence of individual proteins and modified incubation times were as indicated in individual experiments.

Assays for Mlh1–Pms1 endonuclease-dependent MMR were performed in 10-μL volumes and analyzed using the two-stage assay procedure using a modification of our previously published procedures (31) as follows: In the first stage, 195 fmol of Msh2–Msh6, 145 fmol Mlh1–Pms1, 110 fmol of RFC-Δ1N, and 145 fmol PCNA (PCNA trimers) were incubated for 10 min at 30 °C with 100 ng of 3′ AflIII-nicked substrate DNA in a final volume of 5 μL. The proteins and DNA were combined in 2.5 μL and mixed with 2.5 μL of a master reaction buffer mix containing 33 mM Tris pH 7.6, 75 mM KCl, 2.5 mM ATP, 1.66 mM glutathione, 8.3 mM $MgCl_2$, 80 μg/mL BSA, 200 μM dNTPs, and 1 mM $MnSO_4$. Following the 10-min incubation period at 30 °C, 195 fmol of Msh2–Msh6, 145 fmol Mlh1–Pms1, 110 fmol of RFC-Δ1N, 145 fmol PCNA (PCNA trimers), 400 fmol of Pol ε, 0.19 fmol Exo1, and 900 fmol RPA were added to the initial 5 μL along with H_2O as required and 2.5 μL of a modified version of the above master reaction buffer mix lacking $MnSO_4$, bringing the final reaction volume to 10 μL followed by a 2-h incubation period at 30 °C. The first-stage reaction contained final concentrations of 0.5 mM $MnSO_4$ and 4.2 mM $MgCl_2$ and the second-stage reaction contained final concentrations of 0.25 mM $MnSO_4$ and 4.2 mM $MgCl_2$; we did not add additional $MnSO_4$ to the second stage reaction because the $MnSO_4$ present in the first stage reaction was sufficient to support full activity. Note that because KCl was present in the different protein dilutions, the final KCl concentration in the repair reaction was 100 mM. The key modification of our previously published reaction conditions (31) was the reduced levels of Exo1 that were used due to the increased activity of Exo1 preparations containing Nonidet P-40 compared with our previous preparations of Exo1 (25). The presence or absence of individual proteins and modified incubation times were as indicated in individual experiments.

The percent repair was determined as previously published (25) as described briefly here. The reactions were terminated by the addition of 500 mM EDTA to a concentration of 20 mM followed by the addition of 20 μL of 360 μg/mL of proteinase K and 0.4 mg/mL of glycogen. Reactions were then incubated at 55 °C for 30 min. The DNA products were then purified by phenol extraction and ethanol precipitation and digested with 2.5 units each of PstI and ScaI for 1 h at 37 °C. The DNA products were then separated by electrophoresis on a 0.8% agarose gel run in Tris-acetate-EDTA buffer (BioRad) containing 0.6 μg/mL of ethidium bromide, and the gels were photographed using a BioRad ChemiDoc XP imaging system and Image Lab software, version 4.1. The amounts of DNA in each band were quantified and the amount of DNA present in the repair-specific 1.1-kb and 1.8-kb fragments was then expressed as the percent of total DNA present in the repair-specific 1.1-kb and 1.8-kb fragments and the 2.9-kb substrate fragment. In all assays, 100% repair is repair of 100 ng or 52.75 fmol of substrate.

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