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Reconstitution of 5′-Directed Human Mismatch Repair in a Purified System

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Summary

This paper reports reconstitution of 5′-nick-directed mismatch repair using purified human proteins. The reconstituted system includes MutSα or MutSβ, MutLα, RPA, EXO1, HMGB1, PCNA, RFC, polymerase δ, and ligase I. In this system, MutSβ plays a limited role in repair of base-base mismatches, but it processes insertion/deletion mispairs much more efficiently than MutSα, which efficiently corrects both types of heteroduplexes. MutLα reduces the processivity of EXO1 and terminates EXO1-catalyzed excision upon mismatch removal. In the absence of MutLα, mismatch-provoked excision by EXO1 occurs extensively. RPA and HMGB1 play similar but complementary roles in stimulating MutSα-activated, EXO1-catalyzed excision in the presence of a mismatch, but RPA has a distinct role in facilitating MutLα-mediated excision termination past mismatch. Evidence is provided that efficient repair of a single mismatch requires multiple molecules of MutSα-MutLα complex. These data suggest a model for human mismatch repair involving coordinated initiation and termination of mismatch-provoked excision.

Introduction

Hereditary deficiency in mismatch repair (MMR) causes an increased rate of gene mutations and susceptibility to certain types of cancer, including hereditary nonpolyposis colorectal cancer (Kolodner and Marsischky, 1999; Kunkel and Erie, 2005; Modrich and Lahue, 1996). MMR promotes genomic stability by correcting base-base and small insertion/deletion (ID) mispairs that arise during DNA replication (Kolodner and Marsischky, 1999; Modrich and Lahue, 1996; Schofield and Hsieh, 2003) and by blocking homeologous recombination (Harfe and Jinks-Robertson, 2000; Myung et al., 2001). Recent evidence also suggests that MMR plays a role in DNA-damage response (Li, 1999; Stojic et al., 2004).

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The methyl-directed MMR pathway in *Escherichia coli* is well characterized and provides an excellent model for eukaryotic MMR. In *E. coli*, 11 proteins carry out the repair reaction in three stages: initiation, excision, and repair DNA synthesis. MutS, MutL, and MutH recognize mismatches and incise the newly synthesized unmethylated DNA strand (initiation). One of four exonucleases (Exo1, Exo VII, Exo X, or RecJ) carries out 5′ or 3′ excision from the DNA-strand break in conjunction with UvrD helicase (excision). Finally, DNA polymerase III holoenzyme catalyzes repair DNA synthesis, and repair is completed by DNA ligase (for a review, see Kunkel and Erie, 2005).

MMR is a highly conserved pathway, such that MMR in eukaryotic cells is mechanistically similar to bacterial methyl-directed MMR and involves similar protein components. Since the strand-discrimination signal in eukaryotic cells has not been defined, how the eukaryotic MMR machinery specifically targets the newly synthesized DNA strand for repair is unknown. However, the strand-specific MMR reaction can be directed by a preexisting nick in vitro (Modrich and Lahue, 1996). Although the nick-directed reaction may actually be a subreaction of MMR, repair of a nick-containing heteroduplex requires at least MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3), MutLα (MLH1-PMS2), RPA, PCNA, EXO1, HMGB1, RFC, and DNA polymerase δ (for a review, see Kunkel and Erie, 2005).

Whereas the initiation, resynthesis, and ligation reactions in human cells are poorly understood, the excision step of base-base MMR in the human system was recently reconstituted using MutSα as a recognition protein and EXO1 as a nuclease (Dzantiev et al., 2004; Genschel and Modrich, 2003). Interestingly, although eukaryotic EXO1s were originally identified as a 5′→3′ exonuclease (Lee and Wilson, 1999; Szankasi and Smith, 1995; Tishkoff et al., 1997), the reconstituted excision reaction carried out both 5′→3′ and 3′→5′ excision (Dzantiev et al., 2004; Genschel and Modrich, 2003). Similarly, extracts from *Exo1*$^{-/-}$ mouse cells are partially defective in both 5′- and 3′-nick-directed MMR (Wei et al., 2003). One possibility is that EXO1 possesses a cryptic 3′→5′ hydrolytic activity (Genschel et al., 2002); alternatively, it could be attributed to EXO1's ability to stabilize MMR protein complexes by playing both structural and enzymatic roles (Amin et al., 2001), the presence of which may reveal the cryptic 3′→5′ nuclease activity by another component (or components) in the reaction. Nevertheless, the cryptic 3′→5′ nuclease activity associated with EXO1 requires further investigation. It is obvious that EXO1 is not the only nuclease involved in MMR, as *Exo1* null mutants in yeast and mice confer only a partial MMR defect in vivo (Amin et al., 2001; Wei et al., 2003).

Despite the reconstitution of the excision step, how strand-specific excision is initiated is still quite controversial. Three distinct models have been proposed, and the major differences among these models are (1) both the translocation model (Allen et al., 1997) and the sliding clamp model (Gradia et al., 1999) propose that MutS

or its homologs leave the mismatch in search of the strand-discrimination signal, but the DNA bending-mismatch verification model (Junop et al., 2001) suggests that MutS-like proteins stay at the mismatch during the repair process, and (2) the sliding model suggests that several MutS molecules need to be accumulated between the mismatch and the discrimination signal, whereas the other two models believe that a single MutS complex (dimer or tetramer) is sufficient for a heteroduplex repair. Clearly, these models require further evaluation. In addition, how mismatch-provoked excision is properly terminated is unknown. Although the requirement for RPA in MMR has been established (Lin et al., 1998; Ramilo et al., 2002), its precise role in the reaction is not yet defined. Whereas RPA was initially reported to stimulate mismatch-provoked excision (Genschel and Modrich, 2003; Ramilo et al., 2002), recent studies suggest that it negatively regulates the EXO1 processivity (Genschel and Modrich, 2003). Therefore, there are still many fundamental questions about eukaryotic MMR to be answered.

This study reports complete in vitro reconstitution of human MMR using purified proteins and a 5′-nicked heteroduplex DNA substrate. The reconstituted MMR system containing MutSα efficiently repairs both base-base and ID mismatches. In contrast, the reconstituted system using MutSβ has a limited role in repair of base-base mismatches but repairs ID mispairs much more efficiently than the MutSα system. HMGB1 and RPA stimulate MutSα- or MutSβ-activated, mismatch-provoked excision by EXO1. We show that MutLα regulates mismatch-provoked excision by EXO1 and terminates the excision upon mismatch removal. Titration experiments revealed that multiple MutSα-MutLα complexes are required for efficient repair of a single mismatch. These data provide novel insight into the initiation and termination of mismatch-provoked excision in human cells, and the reconstitution assays presented here offer ideal opportunities to investigate the mechanisms of MMR and the consequences of cancer-associated mutations in a variety of MMR genes.

Results

Reconstitution of 5′-Nick-Directed Repair of Base-Base Mismatches

Human MMR proteins were purified to homogeneity (see Figure S1 in the Supplemental Data available with this article online), including MutSα, MutSβ, MutLα, RPA, EXO1, HMGB1, PCNA, RFC, and DNA polymerase δ (pol δ). MMR reactions were performed in the MutSα system (including all MMR components except MutSβ) or the MutSβ system (including all MMR components except MutSα). The MutSα system was tested initially with a G-T heteroduplex DNA substrate containing a strand break 128 bp 5′ to the mismatch (Figure 1A). With the complete MutSα system, >50% of the G-T heteroduplex was repaired in 10 min (Figures 1B and 1C, reaction 1). The repair level in the reconstituted system is equivalent to the level repaired by 50 μg of human extracts for 15 min (Parsons et al., 1993).

The protein requirements for the MMR reaction were defined by systematically omitting one component of the reaction at a time. These assays showed that MutSα, EXO1, and pol δ are indispensable for repair (Figures 1B and 1C, reactions 2, 3, and 8 and data not shown). In contrast, omission of RPA or HMGB1 reduces repair efficiency slightly (Figures 1B and 1C, reactions 5 and 6), but omission of both RPA and HMGB1 reduces repair efficiency dramatically (Figures 1B and 1C, reaction 7). This suggests that HMGB1 and RPA play similar roles in MMR. Surprisingly, addition of MutLα appears to reduce the repair efficiency (Figures 1B and 1C, compare reaction 4 with reaction 1). Omission of PCNA or RFC from the reconstituted MMR assay reduces repair by ~17% (Figures 1B and 1C, reaction 9), suggesting that, while PCNA and RFC facilitate pol δ-catalyzed DNA synthesis, pol δ alone is able to fill short gaps under the experimental conditions. The marginal dependence of the reconstituted reaction on RFC and PCNA seems to be in conflict with in vitro DNA-synthesis studies, where RFC and PCNA greatly stimulate pol δ processivity (Podust et al., 1998; Xie et al., 2002). This discrepancy was found to be caused by different assay conditions. In particular, the repair assay in this study contained 110 mM KCl, but no monovalent salt was used in the in vitro synthesis assay (Podust et al., 1998; Xie et al., 2002). Under the later conditions, RFC and PCNA, along with RPA, dramatically enhance pol δ-catalyzed DNA synthesis on a primed 6.4 kb f1MR1 ssDNA substrate (data not shown). However, these conditions do not support in vitro-reconstituted MMR (data not shown).

The MutSβ system was also tested with the 5′-nicked G-T heteroduplex. The MutSα-to-MutSβ ratio in the cell is ~6–10:1 (Drummond et al., 1997), so MutSβ was added at an 8-fold lower concentration than MutSα. Under these conditions, MutSβ repaired ~10% of the G-T substrate (Figure 1C), and no additional repair was observed at 8-fold higher concentration of MutSβ (data not shown). These data suggest that MutSβ does not play a major role in repair of base-base mismatches in human MMR.

Reconstitution of 5′-Nick-Directed Repair of ID Mispairs

ID mispairs are a major class of DNA-replication error that are generated by strand slippage in tracts of simple repetitive DNA (Kunkel, 1993). The process by which ID mispairs are repaired by MMR is less well understood than the process by which base-base mismatches are repaired by MMR. Here, a 3 nt unpaired ID heteroduplex (Figure 1A) was tested for repair in the MutSα and MutSβ reconstituted systems. The result showed that the ID DNA substrate is efficiently processed by both the MutSα and MutSβ systems (Figures 2A–2C), even though an 8-fold lower concentration of MutSβ was used in the MutSβ system. Furthermore, when MutSα and MutSβ were titrated in the assay for ID-mispair excision, the MutSβ system was much more active than the MutSα system. Under the same conditions, 100 fmol MutSβ efficiently promoted the removal of the ID mispair in nearly 60% of heteroduplexes, but 100 fmol MutSα could only mediate ~25% of the DNA substrate for excision (Figure 2D). These results suggest that ID heteroduplexes are likely to be preferen-

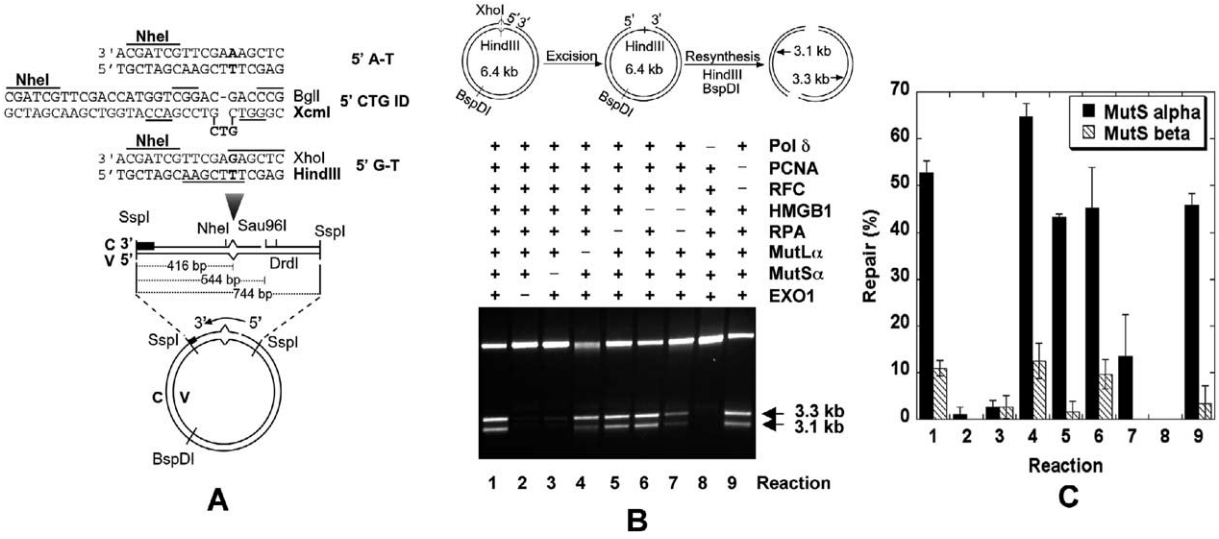

Figure 1. Reconstitution of 5′-Directed Base-Base MMR

(A) DNA substrates. DNA substrates were made from f1MR phage series as described (Parsons et al., 1993). Each substrate contained a nick 128 bp 5′ to the mismatch. The mismatch is located within the overlapping recognition sites of two restriction enzymes so that repair of the mismatch can be scored with these enzymes. Since human MMR is nick directed, HindIII or XcmI was used to score for repair. The SspI fragment, 744 bp in length, was employed to analyze excision intermediates by Southern hybridization (see Figures 4–6) using a ^{32}P-labeled probe, which anneals to the nicked strand at the solid bar position.

(B) MMR reconstitution assays. Repair assays were performed in 20 μl reactions containing 24 fmol of the 5′ G-T heteroduplex, 5 fmol EXO1, 400 fmol MutSα, 260 fmol MutLα, 800 fmol RPA, 1.2 pmol HMGB1, 190 fmol RFC, 290 fmol homotrimer of PCNA, and 600 fmol pol δ as described in Experimental Procedures. Arrows indicate the repair products.

(C) Quantification of the 5′ G-T repair by the MutSα and MutSβ systems. Reaction numbers in (C) correspond to those in (B). Error bars show standard deviation of three independent repair assays for each reaction.

tially repaired by MutSβ in vivo, consistent with yeast genetic studies showing that the dependence of ID-heteroduplex repair on MSH3 increases as the size of the ID increases (Marsischky et al., 1996; Sia et al., 1997). In addition, these data are consistent with the idea that the relatively low physiological concentration of MutSβ is sufficient to promote efficient repair of ID mismatches, while a high in vivo concentration of MutSβ could have deleterious effects on repair of base-base mismatches by reducing the in vivo MutSα concentration (Drummond et al., 1997; Marra et al., 1998). We found that the repair of the ID mispair requires MutSα or MutSβ, EXO1, RPA, HMGB1, and pol δ (Figures 2A–2C and data not shown).

Multiple Molecules of MutS Homologs Are Required for Efficient Repair of a Single Mismatch

The data shown in Figure 2D also indicate that excess amounts of MutSα or MutSβ are required for efficient repair of the 24 fmol ID substrate. Similar analysis was performed for the 5′ G-T substrate with various MutSα-to-DNA ratios (from 0.1 to 16). At a fixed DNA concentration (24 fmol), increasing the MutSα-to-DNA ratio resulted in increased excision (Figure 2E). Little excision was detected when the MutSα-to-DNA ratio was at 2 or less. A sharp increase in excision was observed when the ratio increased from 2 to 4 (Figure 2E), suggesting that a minimum of 4-fold excess MutSα is necessary to carry out efficient repair of the DNA heteroduplex used. Similar results were obtained by fixing the MutSα concentration (96 fmol) and changing the DNA concentration (Figure 2F). Reactions with a MutSα-to-DNA ratio ≤1 (lanes 10–12) generated little MMR-specific excision products. The signal from reactions with no proteins may be derived from incomplete digestion or minor impurity of the DNA substrate. These results strongly suggest that multiple molecules of MutSα are required for efficient repair of a single molecule of mismatch.

Role of DNA Ligase I in MMR

A DNA ligase activity is required for the final step of MMR, but the human enzyme carrying out this reaction has not been identified. Given that DNA ligase I participates in nucleotide excision repair and interacts with PCNA (Aboussekhra et al., 1995; Levin et al., 2000), this ligase might also play a role in MMR. Indeed, when DNA ligase I was incubated with the MutSα system and a G-T heteroduplex with a 36 nt gap 5′ to the mismatch, ~40% of the DNA substrates were converted to covalently closed supercoiled DNA (Figure 3A, reaction 2). PCNA and RFC seem to have little impact on the ligation efficiency under the experimental conditions (see reaction 3). To determine if ligation products were repaired molecules, the supercoiled DNA was recovered from the gel and examined by restriction digestion with the repair scoring enzyme HindIII. The result showed that ~60% of the supercoiled DNA was sensitive to HindIII (Figure 3B, reaction 3). This result indicates that DNA ligase I likely catalyzed the last step (ligation reac-

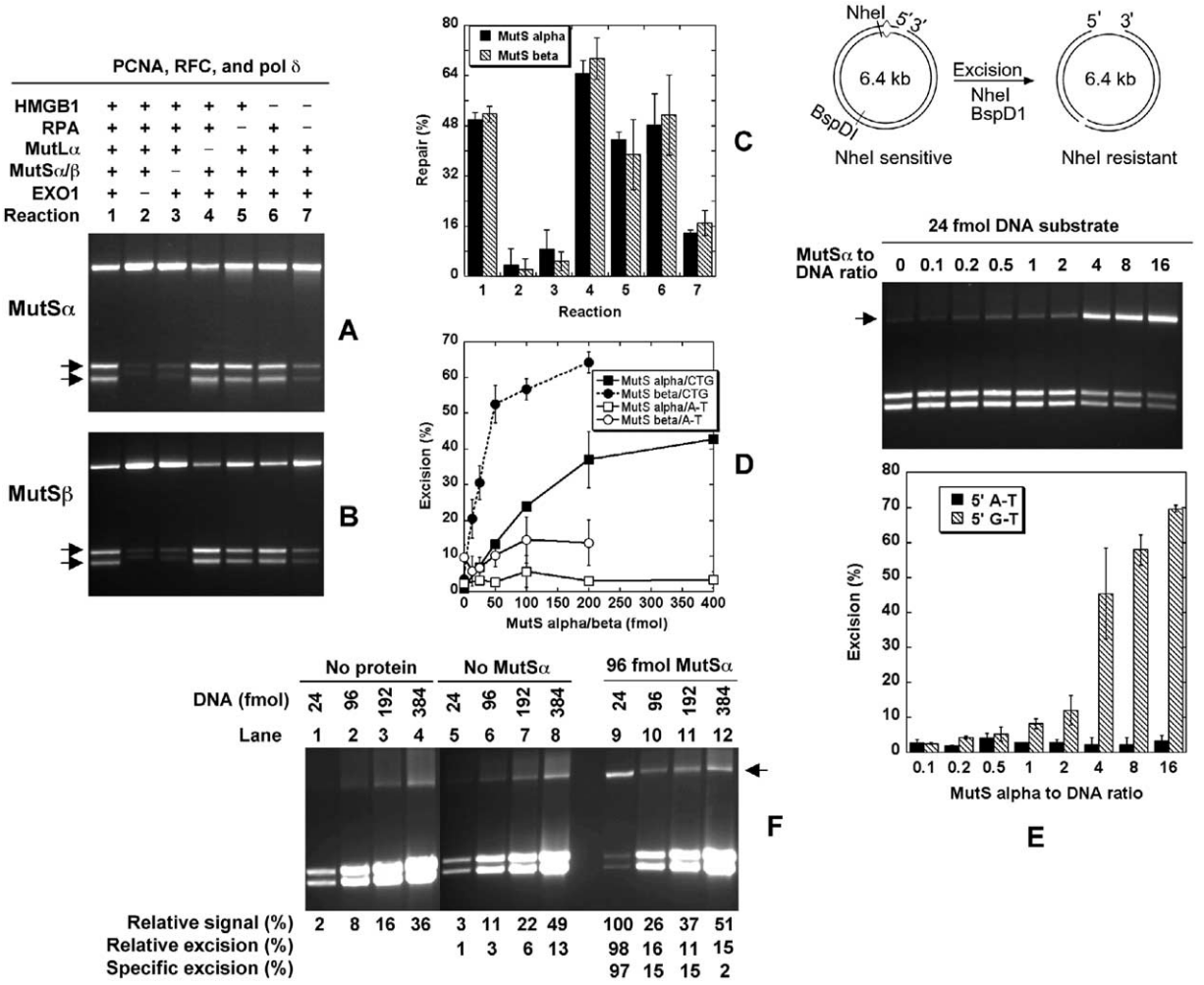

Figure 2. Repair of 5′ ID Mispairs and Importance of the MutSα-to-DNA Ratio in MMR

(A and B) Repair of a CTG ID substrate by the MutSα and MutSβ systems, respectively. Repair assays were performed in essentially the same manner as in Figure 1B, except that the ID heteroduplex was used. The MutSβ concentration was 50 fmol. Repair products are indicated by arrows.

(C) Quantification of the CTG ID-mispair repair by the MutSα and MutSβ systems.

(D) Comparison of the ID-mispair-provoked excision by the MutSα and MutSβ systems.

(E and F) MutSα-to-DNA ratio is critical in MMR. Excision assays were performed similarly as described for the repair assay in Figure 1B, except that RFC, PCNA, pol δ, and dNTPs were omitted and different amounts of MutSα (E) or 5′ G-T substrate (F) were used. The excision products (indicated by an arrow) were scored by NheI digestion (see diagram at the top of [E] and Guo et al., 2004). Relative signal was determined by comparing the signal in individual reactions with that in lane 9. Relative excision was obtained by subtracting the corresponding nonspecific signal shown in reactions with no proteins. Specific excision was obtained by subtracting the corresponding signal shown in the reaction with no MutSα. Error bars indicate standard deviation of three independent experiments.

tion) of the MMR reaction in the reconstituted MutSα system. Similar experiments were performed using the MutSβ system and the nicked 5′ CTG ID substrate (Figure 3A, reactions 10–12). Although close to 60% of DNA substrates became supercoiled (i.e., ligated; see Figure 3A, reactions 11 and 12), only ~10% of the supercoiled DNA molecules were repaired products (Figure 3B, reaction 12), suggesting that the nicked heteroduplex was efficiently ligated by DNA ligase I before repair. A similar result was also seen when ligation assays were performed using the MutSα system and the nicked G-T or ID heteroduplex (Figures 3A and 3B, reactions 6 and 9). Nevertheless, these results indicate that DNA ligase I is competent to perform the ligation step in MMR.

Omission of MutLα Leads to Extensive Excision

The role of MutLα in human MMR is still unclear. A previous study suggested that MutLα is not required for 5′-directed heteroduplex excision, but it promotes repair specificity by suppressing excision on homoduplex DNA in a MutSα-dependent manner (Genschel and Modrich, 2003). As shown above, omission of MutLα has little effect on the efficiency of the MutSα reconstituted system (Figure 1), but it does result in lower yield of the 3.1 kb product (Figure 1B, reaction 4 versus reac-

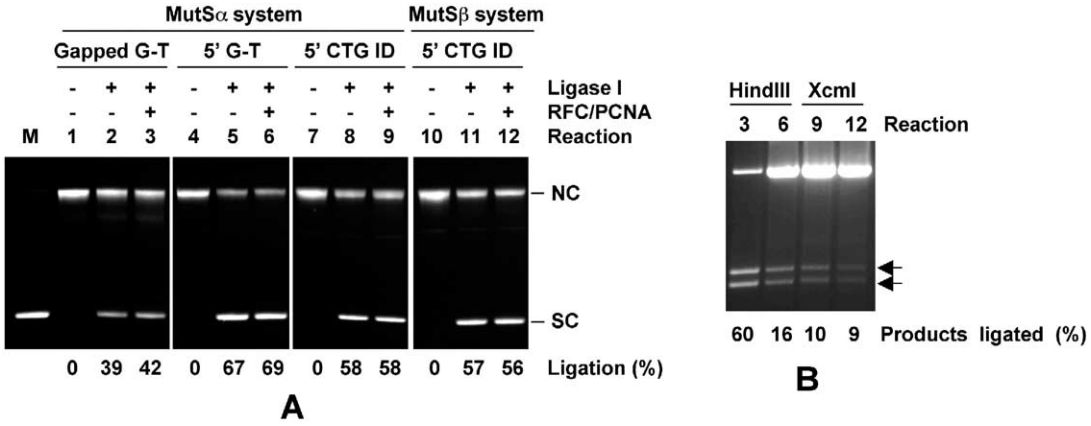

Figure 3. Ligation of MMR Products by DNA Ligase I

(A) Conversion of nicked circular (NC) DNA substrate to supercoiled (SC) DNA by DNA ligase I (0.7 pmol). MMR assays were assembled similarly as described in Figure 1B using indicated DNA substrates and proteins. DNA ligase I was purified as described (Levin et al., 2000). Reactions were performed in the presence of ethidium bromide to introduce supercoiled DNA during ligation as described (Ramilo et al., 2002). 5′ G-T and 5′ CTG ID are nicked heteroduplexes (see Figure 1A), while gapped G-T heteroduplex is identical to the 5′ G-T except that the 5′ nick was replaced by a 5′ gap with 36 nt, which was created by annealing Sau96I-DrdI-digested f1MR3 dsDNA (see Figure 1A) with f1MR1 ssDNA. f1MR3 replicative-form DNA (M) was used as a control for supercoiled DNA.

(B) Determination of ligated repair products. Repair reactions 3, 6, 9, and 12 in (A) were performed on a large scale (5× for reaction 3 and 10× for reactions 6, 9, and 12), and supercoiled DNA in each reaction was isolated from agarose gel and digested with HindIII-BspDI (for both G-T substrates) or XcmI-BspDI (for the ID substrate).

tions 1, 5, and 6). This could indicate that the 3.1 kb reaction product has more single-strandedness in reactions lacking MutLα than in reactions with MutLα. Single-stranded regions stain poorly with ethidium bromide and could result from less-efficient DNA resynthesis and/or more-vigorous DNA excision by EXO1. The total amount of DNA recovered in reactions lacking MutLα was also significantly lower than in reactions with MutLα (Figure 1B, reaction 4 versus reactions 1, 5, and 6), which is also consistent with more-vigorous DNA excision in the absence of MutLα.

The extent of DNA excision in the reconstituted MutSα system was assessed in the absence of PCNA, RFC, and pol δ, as they are not essential for excision (Figure 4A, reaction 1 and Genschel and Modrich, 2003). Figure 4C shows that, in reactions containing MutLα, reaction products were either unreacted (starting at the nick) or were excised to a position past the nick and/or the mismatch site (Figure 4C, reactions 1–3 and 5–7; bracket). In contrast, in the reaction lacking MutLα, few excision products were detected (Figure 4C, reaction 4), even though the amount of unreacted DNA substrate in this reaction was similar to that of the complete reaction (reaction 1). This result suggests that excision without MutLα was so extensive that the probe annealing site (solid bar in Figure 4C) was degraded, implying that MutLα may regulate the extent of mismatch-provoked excision by EXO1 during MMR.

Figure 4 also shows that HMGB1 and RPA regulate mismatch-provoked excision. When both HMGB1 and RPA were omitted from the reaction, the extent of excision was greatly reduced, and only ~10% of the DNA substrate participated in excision (Figures 4A and 4C, reaction 7). In reactions containing HMGB1 but no RPA, extensive excision was detected (Figure 4C, reaction 5). In reactions containing RPA but no HMGB1, little unreacted DNA substrate remained, and a significant number of molecules were excised to just beyond the mismatch. Thus, the reaction products are similar in the complete reaction and in the reaction lacking only HMGB1 (reactions 1 and 6). These results suggest that RPA may both stimulate and suppress mismatch-provoked excision. Although HMGB1 and RPA independently facilitate MutSα-activated excision, evidence from this study and previous studies suggests that they play complementary roles in regulating excision during MMR (see below for details).

MutLα Negatively Regulates Mismatch-Provoked Excision and Terminates the Excision upon Mismatch Removal

The above results indicate that MutLα is required for excision endpoints beyond the mismatch (Figure 4C). To further elucidate the role of MutLα in mismatch-provoked excision, excision endpoint was determined as a function of the concentration of MutLα. As shown in Figure 4D, supplementation of MutLα to the reaction resulted in two prominent excision endpoints: one that is ~25 nt (site I) and another that is 40–60 nt (site II) beyond the mismatch. As the concentration of MutLα was increased, more excision products at sites I and II were observed (Figures 4D and 4E). It is noted that efficient termination requires 260 fmol or more MutLα, indicating that, like the requirement for multiple MutSα complexes in repair of a single molecule of heteroduplex, multiple molecules of MutLα are required for terminating excision on a single molecule of heteroduplex. Additional minor termination sites (bracket in Figure 4D) were also observed in variable amounts in the presence of MutLα; these products were most abundant in the reaction with 130 fmol MutLα but were

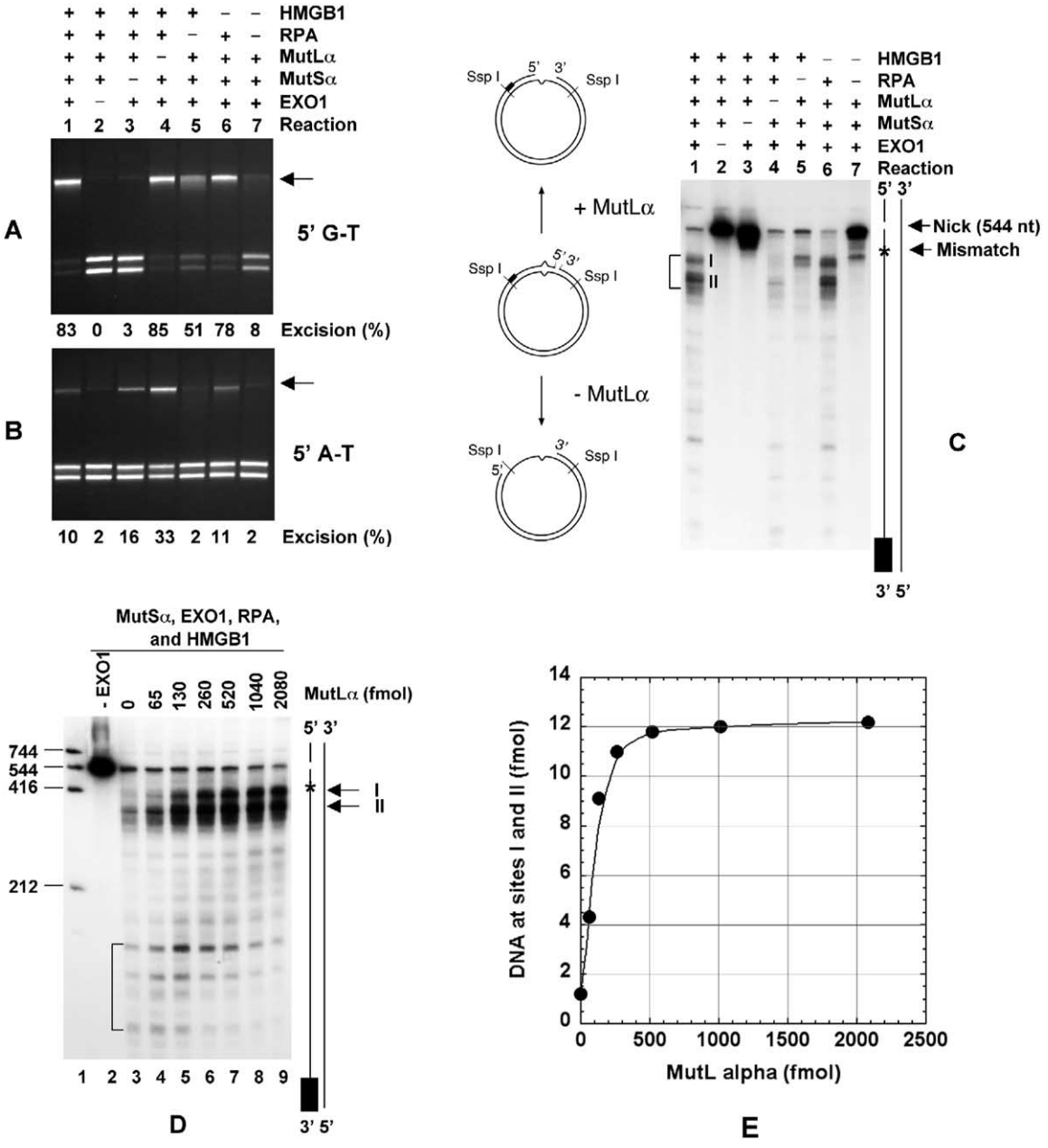

Figure 4. Deregulation of Mismatch-Provoked Excision in the Absence of MutLα

(A and B) Agarose-gel analysis of heteroduplex and homoduplex excision products generated by the MutSα system, respectively. Excision assays were performed as described for the repair assay in Figure 1B in the absence of RFC, PCNA, pol δ, and dNTPs. The percentage of the excision (species resistant to NheI) is shown at the bottom of the gel.

(C) Southern hybridization analysis of excision products by the MutSα system. Excision assays were performed identically to those described in (A), and the excision products were digested with SspI and subjected to Southern blot analysis as described in Experimental Procedures. Schematic representation of the 5′ G-T substrate after SspI digestion is shown on the right side of the gel. Positions of the nick and the mismatch (asterisk) are indicated by arrows. The solid bar indicates where the ^{32}P-labeled probe anneals. A bracket emphasizes the region where differences were identified in reactions with and without MutLα.

(D) MutLα mediates termination of mismatch-provoked excision. Excision assays were performed essentially as described in (C) with increasing MutLα. Reaction 2 contained no EXO1 and MutLα. Arrows point to major termination sites (I and II). The position of the mismatch is indicated by an asterisk (the 416 nt marker). Molecular-size markers in nt, which were derived from f1MR1 dsDNA by restriction-enzyme digestions, are shown on the left.

(E) Quantification of DNA terminated at sites I and II shown in (D). The excision-termination level was determined by dividing the amount of DNA at sites I and II in each reaction by the amount of DNA in reaction 2 and multiplying by 24 fmol.

barely detected in the reaction with 2080 fmol MutLα. These observations suggest that excision in these molecules extends beyond the probe site in the absence of MutLα, but it tends to terminate at minor termination sites when the concentration of MutLα is low and at major termination sites (I and II) when the concentration of MutLα is high. Taken together, these data strongly suggest that MutLα plays a major role in terminating mismatch-provoked excision shortly after the mismatch is removed.

Termination of Mismatch-Provoked Excision Involves MutLα and RPA

Recent studies show that RPA also plays a role in suppressing mismatch-provoked excision after mismatch removal (Genschel and Modrich, 2003). Thus, MutLα and RPA could function together or independently in this step of the MMR reaction. These possibilities were tested by varying the amount of RPA in the MutSα system in the presence or absence of MutLα. Since RPA alone stimulates mismatch-provoked excision essentially as efficiently as RPA plus HMGB1 (Figures 4A and 4C), HMGB1 was initially eliminated from this experiment to simplify the reaction. In the absence of MutLα, mismatch-provoked excision increased significantly with increasing RPA concentrations, as judged by the reduction of the 544 nt unreacted substrate (Figure 5A). At 0.8 pmol RPA (reaction 5), unreacted substrate reached a minimum, but little excision product was detected. This suggests that excision proceeds beyond the probe site under these conditions. In the presence of MutLα and 0.8 pmol RPA, a large number of reaction products terminated at sites I and II (Figure 5A, reaction 11). At 1.6 pmol RPA, excision tended to stop at sites I and II in the absence of MutLα (reaction 6) but occurred much more frequently at sites I and II in the presence of MutLα (reaction 12). These results again suggest that MutLα plays a key role in terminating mismatch-provoked excision when it is stimulated by RPA.

Similar experiments were performed in the presence of HMGB1. When HMGB1 was included in reactions lacking RPA and MutLα, some reaction products were observed at site I (Figure 5B, reaction 7). These products decreased in abundance with increasing amounts of RPA (from 0.1–0.8 pmol; reactions 8–11). In the presence of 1.6 pmol RPA, few reaction products were located at sites I and II. These results are consistent with the fact that RPA stimulates EXO1-catalyzed excision at a low concentration and suppresses EXO1-catalyzed excision at a high concentration. In the presence of MutLα and RPA, excision termination at sites I and II was greatly enhanced (see Figure 5B, reactions 2–6). Since effective accumulation of excision products at the major sites requires both MutLα and appropriate amount of RPA, it is likely that the termination of mismatch-provoked excision past mismatch is conducted by a concert interaction of several MMR proteins, including MutLα and RPA (see Discussion below).

It is noteworthy that EXO1-catalyzed excision was very robust in the presence of HMGB1 (compare Figures 5A and 5B), especially in the absence of MutLα (Figure 5B, reactions 7–12). These results suggest that HMGB1 enhances RPA-mediated, EXO1-catalyzed excision (also see Figure S2).

HMGB1 Stimulates Mismatch-Provoked Excision

HMGB1 plays a role in eukaryotic MMR, but its precise role has not been defined (Yuan et al., 2004). To determine if HMGB1 stimulates excision, we analyzed the excision intermediates generated by the MutSα system in the absence of RPA. As shown in Figure 6A, in the absence of MutLα, increasing HMGB1 resulted in increased heteroduplex excision. Analysis of the excision products by Southern blotting revealed that almost no excision products were detectable beyond the mismatch site in the presence of high concentrations of HMGB1 (see Figure 6B, reaction 6). These results indicate that, in the absence of MutLα, HMGB1 stimulates mismatch-provoked excision in a concentration-dependent way such that most excision products no longer contain the probe site. However, supplementation of these reactions with MutLα resulted in more-condensed excision products (compare Figures 6C and 6A) and termination of excision beyond the mismatch site (Figure 6D, reactions 4–6). Approximately 18% of DNA samples were detected at termination sites I and II (Figure 6D, reactions 5 and 6), and they are directly related to MutLα since the presence of these products is independent of HMGB1 concentrations (compare reactions 4–6) and was not detected in the same reactions without MutLα (Figure 6B, reaction 5 and 6). Furthermore, a small amount (~5% of total DNA) of excision endpoints beyond site II (see bracket in Figure 6D) were associated with high concentrations of HMGB1, suggesting that HMGB1 may have some limited role in suppressing excision in a MutLα-dependent manner. However, given the fact that no significant differences in amount of DNA and excision pattern beyond the mismatch were observed between reactions with and without HMGB1 (see Figure 4C, reactions 1 and 6), the limited termination function of HMGB1 may be insignificant when RPA is present. Therefore, HMGB1 is likely to function as an excision stimulator in MMR.

It is worth mentioning that HMGB1-stimulated excision requires MutSα or MutSβ (Figure 4A, reaction 3 and data not shown) and does not occur on a homoduplex substrate (Figures 6C and 6D, reactions 8–12). The latter characteristic distinguishes the effects of HMGB1 and RPA, as RPA also promotes homoduplex excision in a concentration-dependent manner (Figure 4B, Figure S2B, and Genschel and Modrich, 2003). The specific interaction between HMGB1 and MutSα (Yuan et al., 2004) could account for the HMGB1's specificity to heteroduplexes.

Discussion

This study reports in vitro reconstitution of 5′-nick-directed MMR using purified recombinant human proteins. This reconstituted system carries out all steps of nick-directed MMR, including the initiation and termination of excision, resynthesis, and ligation. This study also demonstrates that MutLα promotes termination of mismatch-provoked, EXO1-catalyzed DNA excision upon mismatch removal. A complex complementary role for RPA and HMGB1 was identified: both proteins stimulate EXO1-catalyzed DNA excision in the presence of a mismatch, but RPA also plays a role in

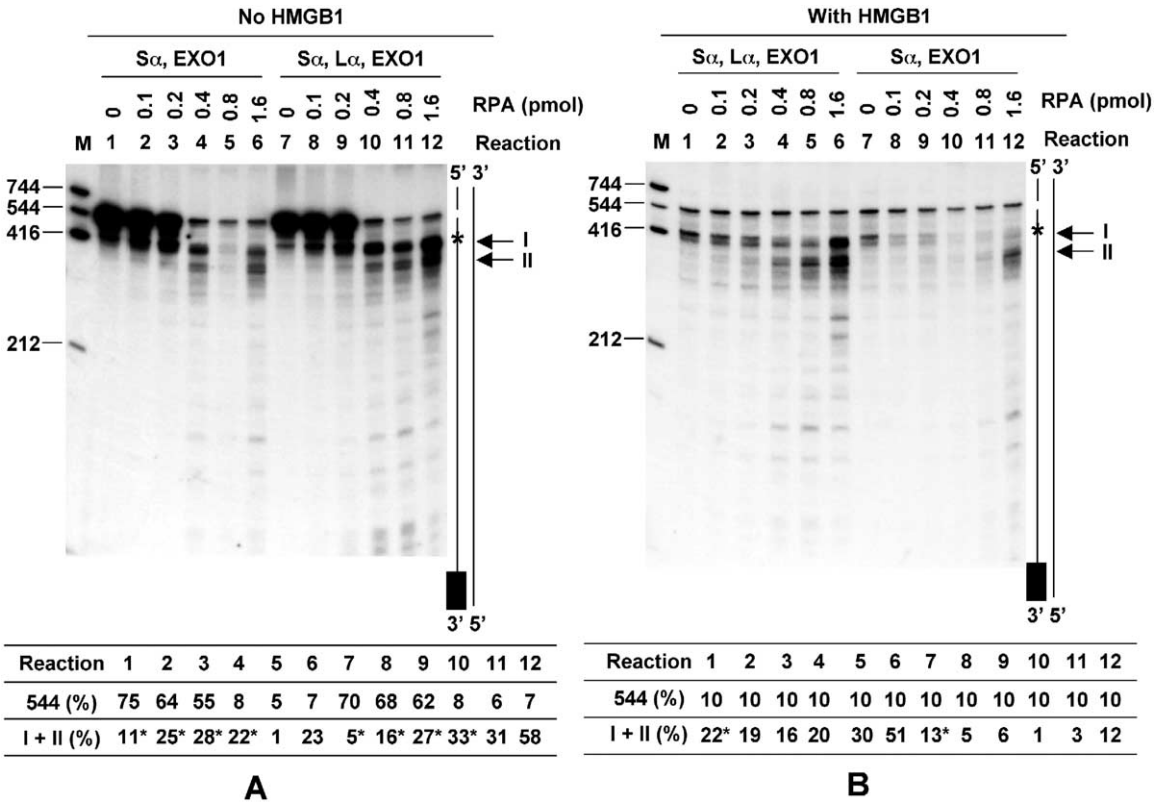

Figure 5. Cooperative Role for RPA and MutLα in Terminating EXO1-Catalyzed Excision

Excision assays and Southern blot analysis were performed essentially the same as in Figure 4 using the indicated proteins.
(A and B) Effect of RPA on excision termination in the absence and presence of HMGB1, respectively. The SspI fragment, which contains the mismatch (see *, the 416 nt marker), strand break (the 544 nt marker), and the probe site (solid bar), is shown on the right of each gel. Arrows point to major termination sites I and II. The percentage of excision products at sites I and II (I + II) and unreacted DNA substrates (544) is given below each gel. Numbers with an asterisk (*) may represent not the amounts of excision termination but excision intermediates. Sα and Lα represent MutSα and MutLα, respectively.

suppressing the excision past the mismatch in a MutLα-dependent manner. We also show that repair of a single molecule of heteroduplex requires multiple MutSα-MutLα complexes. These data represent a significant advance in understanding the molecular mechanisms of human MMR, especially with regard to how mismatch-provoked excision is regulated.

The in vitro MMR system described here specifically catalyzes 5′-nick-directed MMR but not a 3′-nick-directed reaction (data not shown). It should be noted that 3′-directed excision was recently reconstituted using a six-component system containing MutSα, MutLα, RPA, EXO1, PCNA, and RFC (Dzantiev et al., 2004). The reason for the difference in specificity of the two systems is unknown, but it could be due to the fact that this study used recombinant RFC, which was purified to near homogeneity (see Figure S1), while the previous study used native RFC, which was purified to ~60% homogeneity from HeLa cell extracts (Dzantiev et al., 2004). Therefore, it is possible that native but not recombinant RFC stimulates a cryptic EXO1-catalyzed 3′-to-5′ DNA excision activity (Dzantiev et al., 2004) or that a component copurified with the native RFC requires EXO1 to promote 3′-directed excision, which is consistent with a structural role for EXO1 in MMR (Amin et al., 2001). Further investigation is needed to resolve this question.

To reconstitute human MMR in vitro, each protein component was titrated for optimum activity, or its concentration was based on the concentration of that protein in 50 μg of a HeLa cell nuclear extract (see Experimental Procedures; Dzantiev et al., 2004). Titration experiments showed that excision and repair were very inefficient when MutSα and MutSβ were equimolar with the DNA substrate. Efficient excision or repair occurs only when a higher ratio of MutSα to DNA is present (Figures 2E and 2F). MutSβ was also required in molar excess over the DNA substrate, but it was effective at a lower molar excess to DNA than MutSα (Figure 2D). The effect of MutLα on the in vitro repair efficiency was not concentration dependent (Figure 4D and data not shown), but a 10-fold excess of MutLα over the DNA substrate is required for efficient termination of excision past the mismatch (Figure 4D). These results are consistent with the assumption that the molar ratio of MutSα to MutLα is likely 1:1 and also suggest that more than one ternary complex of MutSα-MutLα or MutSβ-MutLα is required for efficient repair of a single heteroduplex, supporting the sliding clamp model proposed for MMR catalyzed by MutS-like and MutL-like proteins

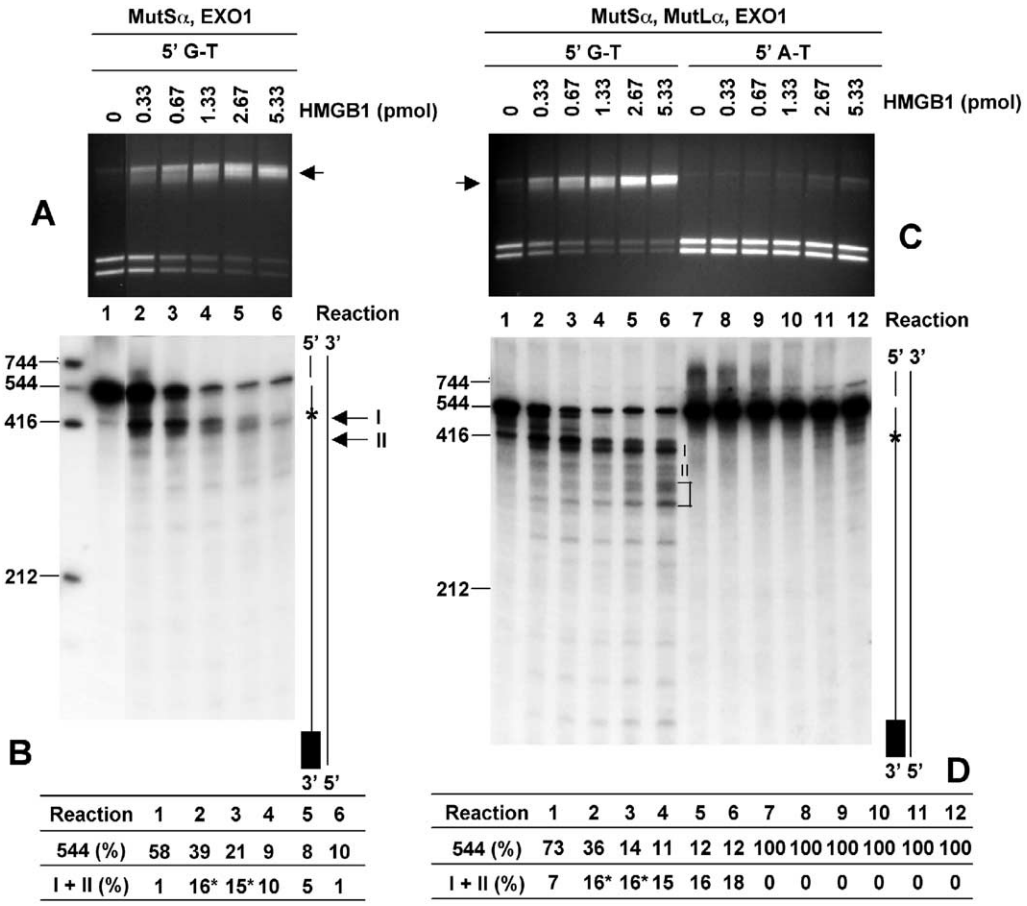

Figure 6. Role of HMGB1 in Mismatch-Provoked Excision

HMGB1-mediated excision was performed essentially the same as in Figure 4 using the indicated DNA substrates and proteins, and the excision products were analyzed by agarose gels (A and C) and Southern blotting (B and D). The SspI fragment is shown on the right of each Southern blot. Bracket indicates excision tracts enhanced by HMGB1. I and II in (D) represent major termination sites. The percentage of unreacted DNA substrate (544) or excision products at sites I and II (I + II) is shown below each gel. Numbers with an asterisk (*) may represent not the amounts of excision termination but excision intermediates.

from bacterial and eukaryotic species (Acharya et al., 2003; Gradia et al., 1999; Mendillo et al., 2005). In contrast, EXO1 is effective in the reconstituted system at a 1:5 molar ratio with the DNA substrate (i.e., 5-fold excess of DNA substrate to EXO1). Under these conditions, more than 70% of the DNA substrate is utilized as an EXO1 substrate (Figure 4), implying that EXO1 executes multiple catalytic cycles in the reconstituted system. Because EXO1 physically interacts with MutSα (Amin et al., 2001; Schmutte et al., 1998; Tishkoff et al., 1997) and MutSα is required for EXO1 excision (Figures 4A and 4C, Genschel and Modrich, 2003), we speculate that MutSα might play a role in loading EXO1 onto DNA. In contrast, MutLα is not required for EXO1 excision (Figures 4A and 4C) and therefore would not be involved in loading EXO1 onto DNA, although it also interacts with EXO1 (Amin et al., 2001; Nielsen et al., 2004; Schmutte et al., 2001; Tran et al., 2001). However, the excision-termination role of MutLα identified here may result from its physical interaction with EXO1 (see below).

RPA is essential for MMR in a cell-free system (Lin et al., 1998; Ramilo et al., 2002), presumably due to its ability to stimulate mismatch-provoked excision and protect ssDNA template (Genschel and Modrich, 2003; Ramilo et al., 2002). However, RPA was also reported to be a potent inhibitor of EXO1 excision for gapped heteroduplexes and can suppress EXO1-catalyzed excision past mismatch (Genschel and Modrich, 2003). Our data in this study show that RPA vigorously stimulates EXO1-catalyzed excision as long as the mismatch is present, and its ability to suppress excision past mismatch absolutely requires MutLα when it is at a low concentration, e.g., 0.8 pmol (Figure 5). In the absence of MutLα, RPA at a high concentration (1.6 pmol) may promote some excision termination, but its role is very limited, especially in the presence of HMGB1 (Figure 5B), which is one of the most abundant nuclear proteins. Therefore, RPA promotes excision termination in a MutLα-dependent manner.

We show that RPA and HMGB1 can independently stimulate mismatch-provoked excision in vitro (Figures 5 and 6). However, previous studies indicate that these two proteins cannot substitute for each other in a cell-

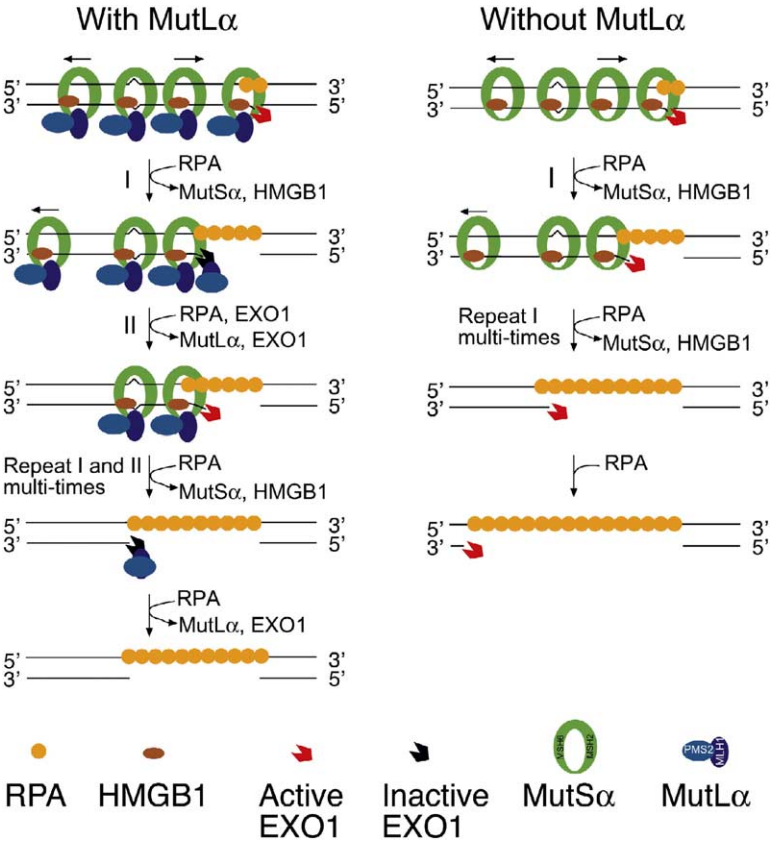

Figure 7. Model of Mismatch-Provoked Excision

See Discussion for explanation. Horizontal arrows indicate sliding directions of MutSα or the MutSα-MutLα complex.

free extract system, as the in vitro MMR is blocked at the excision step in HMGB1-depleted extracts (Yuan et al., 2004) and is blocked prior to the DNA-synthesis step in RPA-depleted extracts because the ssDNA template is degraded by nucleases in the absence of RPA (Ramilo et al., 2002). These observations suggest that RPA and HMGB1 have a complementary role in MMR. Despite the fact that the absence of other nuclear proteins (e.g., nucleases) in the in vitro system makes either RPA or HMGB1 expendable in MMR, we indeed find a mutual enhancement effect for RPA and HMGB1 on EXO1-catalyzed excision (Figure 5, Figure S2). We propose that the stimulation of the exonucleolytic activity of EXO1 by RPA could be attributed to its strong ssDNA binding ability, which not only protects the template strand from degradation but also displaces DNA bound proteins such as MutSα. Like RPA, HMGB1 can bind to ssDNA (Isackson et al., 1979). In addition, it has a local DNA-unwinding activity (Javaherian et al., 1979). Thus, these two proteins can act together to stimulate mismatch-provoked excision first through the HMGB1 unwinding of the heteroduplex at the nick site and then through the binding of HMGB1 to the nicked strand and RPA to the continuous strand, thereby preventing the locally separated DNA strands from rewinding and providing EXO1 an easy access for its substrate.

Another interesting finding from this study is that MutLα is not required for initiating excision but is required for terminating excision during MMR. In contrast, *E. coli* MutL activates MutH and UvrD and is required for initiating mismatch excision (Guarne et al., 2004; Schofield and Hsieh, 2003). It is not clear why or how the functions of human MutLα and *E. coli* MutL have diverged to this extent. However, the fact that in vitro human MMR requires a preexisting strand break may at least in part determine the distinct roles of human MutLα and *E. coli* MutL. Based on our results, we propose that MutL may also function as a terminator in suppressing mismatch-provoked excision past mismatch in *E. coli* MMR. These data and previously published data on human MMR support the idea that mismatch-provoked excision and its termination are regulated in a concerted manner by MutSα/MutSβ, MutLα, RPA, HMGB1, and EXO1, with MutLα acting as a negative regulator and MutSα/MutSβ as a positive regulator of excision. A working model for mismatch-provoked excision in human cells is proposed in Figure 7.

In the proposed model, MutSα and MutLα load onto the mismatched DNA in a bidirectional manner (Acharya et al., 2003; Mendillo et al., 2005). At the same time, RPA binds to the strand break (S. Guo and G.-M.L., unpublished data), where it meets the MutSα-MutLα complex, preventing the latter from further sliding. Once it reaches the strand break, MutSα stops its loading onto DNA. HMGB1 is then recruited to the protein-DNA complex by specifically interacting with MutSα (Yuan et al., 2004) and unwinds the duplex DNA at the strand break. RPA and HMGB1 bind to the relaxed DNA strands to prevent rewinding. Loading of EXO1 at the relaxed

nicked strand, likely through its interaction with MutSα (Amin et al., 2001; Schmutte et al., 1998; Tishkoff et al., 1997), initiates processive excising in a 5′-to-3′ orientation. The resulting ssDNA gap is filled by RPA, which displaces MutSα and HMGB1 from the DNA and promotes a physical interaction between EXO1 and MutLα (Amin et al., 2001; Schmutte et al., 2001; Tran et al., 2001). When a MutLα-EXO1 complex forms, EXO1 is inactivated. The MutLα-EXO1 complex separates from the DNA substrate and then dissociates, which reactivates EXO1. As mentioned above, EXO1 can participate in multiple rounds of binding to DNA, excision, dissociation from DNA, and reassociation/reactivation. Once the mismatch is removed, EXO1 again is released from DNA upon its interaction with MutLα. However, because of unavailability of a MutSα complex immediately 3′ to the mismatch due to sliding away of the previously loaded MutSα complexes from the mismatch, EXO1 cannot be reloaded to DNA, and therefore excision is terminated permanently.

This model explains why extensive excision occurs in the absence of MutLα. In this case, even though RPA displaces MutSα and HMGB1 at the mismatch site, EXO1 continues to excise in a processive manner. This is because (1) EXO1 acts processively on homoduplex DNA in the absence of MutSα (Genschel and Modrich, 2003) and (2) EXO1 may be further stimulated through interaction with MutSα bound in the vicinity of the mismatch. In the absence of MutLα, a high concentration of RPA alone may be sufficient to inhibit excision beyond the mismatch (Figure 5A, reaction 6) by tightly binding to both DNA strands to prevent either EXO1 from loading or the DNA from undergoing hydrolysis by EXO1.

Alternatively, a "MutSα staying with the mismatch" model that is similar to the one proposed by Junop et al. (2001) but with multiple MutSα-MutLα complexes ($\geq$4 with the substrate used in this study) could also explain the major excision-termination sites. However, this model cannot explain the minor termination sites shown in Figure 4D, which are ~200 bp away 3′ to the mismatch. The products in these minor termination sites are likely derived from excision that escapes termination at the major sites and is eventually stopped by MutSα-MutLα complexes that have migrated ~200 bp away from the mismatch.

This study shows that MutLα is required for termination of excision beyond the mismatch but has no other detectable role in MMR in the in vitro-reconstituted system. This result explains why nuclear extracts derived from several tumor cell lines with silent expression of *MLH1* are proficient in 5′-nick-directed MMR (Drummond et al., 1996, G.-M.L. and P. Modrich, unpublished data). It also explains a puzzling genetic phenomenon in yeast that *mlh1* mutants have a smaller impact on homeologous recombination than *msh2* mutants (Nicholson et al., 2000; Sugawara et al., 2004). This result suggests that suppression of homeologous recombination either may not require excision or may be insensitive to uncontrolled excision. However, it seems to be difficult to reconcile the present results with the fact that mutations in human *MLH1* lead to genomic instability and predisposition to cancer. One simple explanation is that 5′-nick-directed MMR may not have functionally uncovered the in vivo role of MutLα. The nick-directed reaction is likely only a subreaction of the in vivo system. It is not known yet how the nick is generated in vivo, especially for the leading strand, and whether or not the nick generation requires MutLα. Finally, in vitro competition between excision and DNA polymerization may nonetheless yield repair products in the absence of proper termination of excision, but proper excision termination could be very critical in vivo. Thus, the properties of the in vitro-reconstituted system described here are not in conflict with the fact that *MLH1* plays an important role in maintaining genomic stability in vivo.

Experimental Procedures

Repair and Excision Assays

Unless otherwise mentioned, reconstituted MMR assays were performed in 20 μl reactions containing indicated proteins, 24 fmol heteroduplex, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1.5 mM ATP, 0.1 mM dNTPs, and 110 mM KCl. The amount of each protein used, except for EXO1 and DNA ligase I, was essentially based on its amount in 50 μg of HeLa nuclear extracts (Dzantiev et al., 2004). Titration experiments were used to determine the concentrations used for EXO1 and ligase I, at which optimal or minimal excision/repair was obtained for heteroduplexes or homoduplexes, respectively. MMR reactions were assembled on ice, incubated at 37°C for 10 min, and terminated by Proteinase K digestion. Repair was scored by restriction-enzyme digestions as described (Parsons et al., 1993). Mismatch-provoked-excision assays were conducted essentially the same as for the repair assay, except pol δ, PCNA, RFC, and dNTPs were omitted. Excision was scored by the conversion of double-stranded substrates to gapped molecules using restriction enzyme NheI as described (Guo et al., 2004).

Southern Blot Analysis

Excision assays were performed as described above. DNA-excision intermediates were visualized using Southern blotting as described (Guo et al., 2004). Briefly, excision products were digested with SspI, separated on 6% denaturing polyacrylamide gels, and transferred onto nylon membranes. Membranes were blotted with a ^{32}P-labeled oligonucleotide probe 5′-ATTGTTCTGGATATTACC-3′. Reaction products were visualized by autoradiography.

Expression and Purification of Recombinant MMR Proteins

Among proteins used in this study, RPA, PCNA, HMGB1, and DNA ligase I were expressed in *E. coli* cells, and MutSα, MutSβ, MutLα, EXO1, RFC, and pol δ were expressed in insect cells through the baculovirus system. All proteins contained the native sequence only, with the exception of the following, which also contained a hexahistidine tag: HMGB1, the PMS2 subunit of MutLα, the p38 and p140 subunits of RFC, and the p66 subunit of pol δ. The recombinant proteins were purified to near homogeneity (see Figure S1). Protein concentrations were determined by a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, California).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and two figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/5/693/DC1/.

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