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A Defined Human System that Supports Bidirectional Mismatch-Provoked Excision

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Summary

Mismatch-provoked excision directed by a strand break located 3′ or 5′ to the mispair has been reconstituted using purified human proteins. While MutSα, EXOI, and RPA are sufficient to support hydrolysis directed by a 5′ strand break, 3′ directed excision also requires MutLα, PCNA, and RFC. EXOI interacts with PCNA. RFC and PCNA suppress EXOI-mediated 5′ to 3′ hydrolysis when the nick that directs excision is located 3′ to the mispair and activate 3′ to 5′ excision, which is dependent on loaded PCNA and apparently mediated by a cryptic EXOI 3′ to 5′ hydrolytic function. By contrast, RFC and PCNA have only a limited effect on 5′ to 3′ excision directed by a 5′ strand break.

Introduction

Genome stabilization functions of mismatch repair include the correction of DNA biosynthetic errors, suppression of illegitimate recombination, and participation in the cellular response to certain types of DNA damage (Kolodner, 1996; Modrich and Lahue, 1996; Buermeyer et al., 1999; Li, 1999; Bellacosa, 2001; Schofield and Hsieh, 2003). The best understood of these functions is the role of mismatch repair in replication fidelity. Function in this manner relies on secondary signals in the helix that direct repair to the newly synthesized strand.

Basic features of the reaction responsible for replication error correction are conserved from bacteria to mammalian cells, and the *Escherichia coli* reaction has been reconstituted with purified components (Modrich and Lahue, 1996; Burdett et al., 2001; Viswanathan et al., 2001). In the bacterial system, mismatch recognition is mediated by MutS, which recruits MutL to the heteroduplex in an ATP-dependent fashion. Assembly of the MutL•MutS•heteroduplex complex activates the MutH endonuclease, which incises the unmethylated strand at a hemimethylated d(GATC) site in newly replicated DNA. The resulting strand break serves as the signal that directs repair to the new DNA strand. MutS and MutL also activate the excision system, which is comprised of DNA helicase II and several single-strand-specific exonucleases. The strand break that directs repair can be located either 3′ or 5′ to the mismatch, and MutS and MutL coordinate recognition of the two DNA sites in a manner that permits orientation-dependent loading of the helicase at the strand break so that unwinding proceeds back toward the mispair (Dao and Modrich, 1998). The helicase-displaced strand is degraded by one of several single-strand exonucleases. In the presence of SSB, DNA polymerase III holoenzyme is sufficient to repair the ensuing gap.

A single-strand break is sufficient to direct the reaction in human cell extracts (Holmes et al., 1990; Thomas et al., 1991). As in the bacterial reaction, the strand break that directs repair may reside 3′ or 5′ to the mispair, and excision removes that portion of the incised strand spanning the nick and the mismatch (Fang and Modrich, 1993; Wang and Hays, 2002). Two mismatch recognition activities have been identified in human cells, the MSH2•MSH6 heterodimer MutSα and the MSH2•MSH3 heterodimer MutSβ (Kolodner, 1996; Modrich and Lahue, 1996), but the former activity appears to mediate most mismatch recognition events in mammalian cells (Genschel et al., 1998; de Wind et al., 1999; Edelmann et al., 2000). Multiple MutL activities have also been identified in human cells, but the MLH1•PMS2 heterodimer MutLα probably plays the major role in mutation avoidance (Buermeyer et al., 1999; Bellacosa, 2001).

Analysis of nick-directed mismatch repair in mammalian cell extracts has implicated several activities in addition to MutSα, MutSβ, and MutLα. DNA polymerase δ and the replication clamp PCNA participate in repair DNA synthesis (Longley et al., 1997; Gu et al., 1998), but PCNA has also been implicated at an early step in the reaction (Umar et al., 1996). However, the requirement for PCNA in initiation of mismatch repair is orientation dependent. While PCNA is required for mismatch-provoked excision directed by a 3′ strand break in HeLa nuclear extracts, it is not essential for excision directed by a 5′ nick (Genschel and Modrich, 2003; Guo et al., 2004). RPA, the eukaryotic single-strand DNA binding protein has been shown to enhance excision and stabilize excision intermediates in crude fractions (Lin et al., 1998; Ramilo et al., 2002). Genetic evidence implicating yeast EXOI in mismatch repair (Schofield and Hsieh, 2003) led to the demonstration that the mammalian EXOI homolog is involved in repair of both 5′ and 3′ heteroduplexes in extracts of mammalian cells (Genschel et al., 2002; Wei et al., 2003). Participation of this double strand exonuclease in mismatch repair directed by a 5′ strand break is consistent with the known 5′ to 3′ polarity of the human activity (Lee et al., 2002), and 5′ directed, mismatch-provoked excision that terminates upon mismatch removal has been reconstituted in a purified system comprised of human MutSα, MutLα, EXOI, and RPA (Genschel and Modrich, 2003).

Given the 5′ to 3′ polarity of EXOI, the finding that this activity is also required for 3′ directed excision in cell-free extracts was unexpected. Yeast genetic studies have led to the suggestion that in addition to an

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excision function, EXOI may play a structural role in assembly of multiprotein repair complexes (Amin et al., 2001). It has also been suggested that EXOI may harbor a cryptic 3′ to 5′ hydrolytic function that is activated on 3′ heteroduplexes by other components of the repair system (Genschel et al., 2002). As noted above, MutSα, MutLα, EXOI, and RPA support a mismatch-provoked excision reaction on a 5′ heteroduplex that is similar to that which occurs in nuclear extracts (Genschel and Modrich, 2003). However, incubation of a 3′ heteroduplex with this set of proteins triggers an excision reaction that proceeds from the strand break with incorrect 5′ to 3′ polarity away from the mismatch. Since EXOI-dependent excision on a 3′ heteroduplex in nuclear extracts proceeds with apparent 3′ to 5′ polarity (Genschel et al., 2002), it is clear that the EXOI-dependent excision must be modulated by other activities in accord with the orientation of the strand break and the mismatch.

We show here that RFC and PCNA play key roles in controlling polarity of mismatch-provoked excision. A purified system comprised of RFC, PCNA, MutSα, MutLα, EXOI, and RPA supports bidirectional excision in a reaction that depends on integrity of the EXOI active site, i.e., 5′ to 3′ hydrolysis directed by a 5′ strand break and 3′ to 5′ excision directed by a 3′ nick.

Results

In the Presence of RFC and PCNA, MutSα, MutLα, EXOI, and RPA Support 3′ to 5′ Excision on a 3′ Heteroduplex

MutSα, MutLα, EXOI, and RPA support a 5′ to 3′ excision reaction on a 5′ heteroduplex that terminates upon mismatch removal (Genschel and Modrich, 2003). However, this set of four proteins supports an anomalous reaction when the strand break that directs repair is located 3′ to the mismatch. In contrast to the nuclear extract reaction where excision on a 3′ heteroduplex occurs from the strand break toward the mispair with apparent 3′ to 5′ polarity (Fang and Modrich, 1993; Wang and Hays, 2002; Figure 1A, lane 6), excision on a 3′ substrate in this four protein, purified system proceeds away from the mismatch with incorrect 5′ to 3′ polarity (Figure 1A, lane 2).

PCNA has been implicated in an early repair step on 3′ heteroduplexes in nuclear extracts (Umar et al., 1996; Genschel and Modrich, 2003; Guo et al., 2004). However, PCNA does not suppress the anomalous 5′ to 3′ excision that occurs on a 3′ heteroduplex in the presence of MutSα, MutLα, EXOI, and RPA, nor does it activate 3′ to 5′ excision in their presence (Figure 1A, lane 3). We therefore sought additional factor(s) that confer appropriate polarity on excision directed by a 3′ strand break. Such an activity was partially purified from HeLa nuclear extract, and it copurified with the large subunit of the eukaryotic clamp loader RFC (Figure 1B), the activity responsible for loading the PCNA replication clamp onto the helix (Waga and Stillman, 1998).

Supplementation of near homogenous preparations of MutSα, MutLα, EXOI, and RPA, with RFC isolated from HeLa cells (estimated purity 55%–60%, Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/15/1/31/DC1) largely suppressed 5′ to 3′ excision on the 3′ heteroduplex (Figure 1A, lane 4), and the additional presence of PCNA activated the mismatch-dependent, 3′ to 5′ excision on this substrate, in a manner similar to that observed in HeLa nuclear extract when repair DNA synthesis is blocked by omission of exogenous dNTPs (Figure 1A, compare lanes 1, 5, and 6).

Analysis of hydrolytic products by indirect end-labeling confirmed RFC and PCNA requirements for mismatch provoked 3′ to 5′ excision. As shown in Figure 2A, activation of 3′ to 5′ hydrolysis on a 3′ heteroduplex requires both proteins. Excision produced a distribution of products corresponding to apparent termination of hydrolysis in a region 100–500 nucleotides beyond the mismatch. This product distribution was not as well defined as that observed in HeLa nuclear extracts (Fang and Modrich, 1993; Figure 2A, compare lanes 4 and 6), suggesting that the purified system may lack one or more components necessary for appropriate termination of 3′ to 5′ excision.

Indirect end-labeling was also used to assess effects of PCNA and RFC on 5′ to 3′ excision on a 3′ heteroduplex (Figure 2B). In the absence of the two proteins, extensive mismatch-dependent 5′ to 3′ hydrolysis occurred on the 3′ heteroduplex in the presence of MutSα, MutLα, EXOI, and RPA. PCNA had little effect on this reaction, but 5′ to 3′ hydrolysis was dramatically suppressed by RFC alone. This suppression was potentiated when PCNA was also present in the sense that the length of 5′ to 3′ excision tracts was significantly reduced. Nevertheless, significant 5′ to 3′ excision from the 3′ strand break does occur in the presence of both proteins, and a similar effect occurs in HeLa nuclear extract in the absence of exogenous dNTPs (Fang and Modrich, 1993; Figure 2B, lanes 7–11). The potential significance of this limited 5′ to 3′ excision in the repair of 3′ heteroduplexes will be considered below.

The mismatch and protein dependence of the 3′ to 5′ excision reaction is illustrated in Figure 3. As can be seen, the reaction is highly specific for DNA containing a mismatched base pair. As noted above, MutSα, MutLα, EXOI, and RPA support a robust 5′ to 3′ excision reaction on 5′ heteroduplexes. While MutSα is required for mismatch-dependent EXOI activation on a 5′ heteroduplex, MutLα is not, although it does enhance the mismatch specificity of the reaction (Genschel and Modrich, 2003). By contrast, MutLα is required for activation of 3′ to 5′ excision on a 3′ heteroduplex, as are MutSα, EXOI, PCNA, and RFC. RPA is not essential for 3′ to 5′ excision but does stimulate the reaction significantly, an effect that becomes more pronounced at suboptimal RFC concentrations (Figure 3). Activity levels optimal for 3′ to 5′ excision in this six component system compare favorably with those present in 50 μg of nuclear extract (Figure 3, dashed lines), which is optimal for the extract reaction.

Functions of RFC and PCNA in Mismatch-Provoked Excision

The results described above indicate that RFC and PCNA act to suppress 5′ to 3′ excision on a 3′ heteroduplex and suggest that loaded PCNA may be required

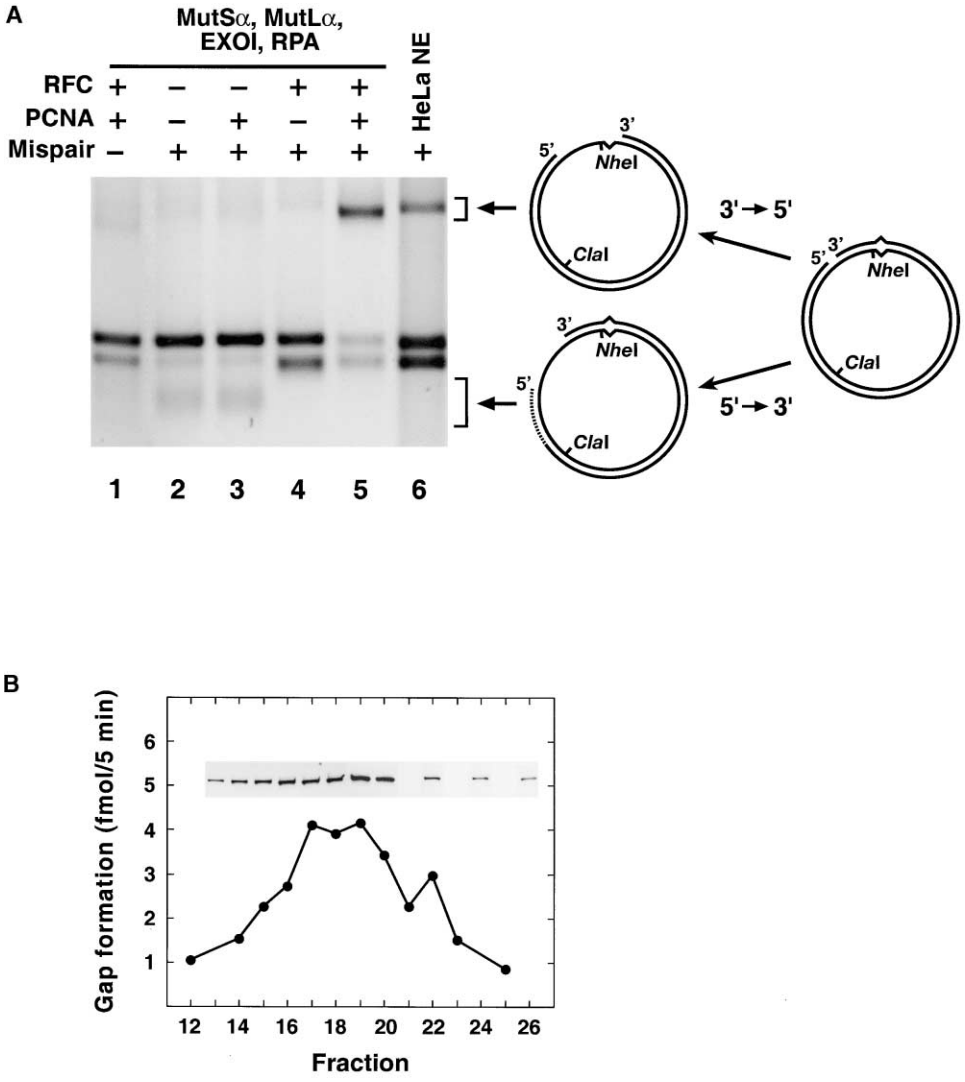

Figure 1. MutSα, MutLα, EXOI, RPA, PCNA, and RFC Support 3′ to 5′ Excision on a 3′ Heteroduplex

(A) The diagram on the right illustrates the DNA substrates used in this work. The 6440 base pair circular DNAs (Fang and Modrich, 1993; Experimental Procedures) contained a G-T mismatch or an A•T base pair in the control homoduplex and a site-specific nick in the complementary DNA strand 141 base pairs 3′ to the mispair as viewed along the shorter path joining the two DNA sites. In nuclear extracts, excision tracts span the shorter path between the two DNA sites, extending from the strand break to terminate at a number of discrete sites centered about 150 base pairs beyond the mismatch (Fang and Modrich, 1993). Cleavage of the native substrates with NheI and ClaI yields the two rapidly migrating fragments. Excision proceeding from the strand break with incorrect 5′ to 3′ polarity results in degradation of the smaller of these two restriction fragments (lower bracket in diagram). However, excision that proceeds from the nick with 3′ to 5′ polarity renders the NheI site, which is located five base pairs from the mismatch, resistant to hydrolysis (Genschel et al., 2002), and cleavage with the two endonucleases yields a pseudodiscrete species with a mobility slightly greater than that of the full-length linear duplex (upper bracket). Reactions (Experimental Procedures) contained 24 fmol 3′ G-T heteroduplex or control A•T homoduplex DNA, 100 ng (390 fmol) MutSα, 50 ng (280 fmol) MutLα, 4 ng (42 fmol) EXOI, 150 ng (1350 fmol) RPA, ~66 ng (220 fmol) human RFC (estimated assuming a purity of 55%–60%, Experimental Procedures), and 25 ng (290 fmol homotrimer) PCNA, as indicated. The reaction shown in lane 6 was performed in a similar manner except that HeLa nuclear extract (50 μg) was substituted for purified proteins. After 5 min incubation at 37°C, DNAs were deproteinized with phenol, collected by ethanol precipitation, digested with NheI and ClaI, subjected to electrophoresis through 1% agarose, and visualized with ethidium bromide. (B) HeLa nuclear extract was fractionated by ammonium sulfate precipation and chromatography on ssDNA-cellulose and phosphocellulose (Experimental Procedures). Samples of phosphocellulose fractions (2 μl) were tested by NheI-ClaI cleavage assay (A) for their ability to activate 3′ to 5′ excision on a 3′ G-T heteroduplex upon supplementation of MutSα (390 fmol), MutLα (560 fmol), EXOI (21 fmol), RPA (900 fmol), and PCNA (1160 fmol). Fractions were also scored for presence of the RFC p140 subunit by Western blot (inset).

for activation of 3′ to 5′ hydrolysis. Although yeast PCNA is nearly inactive in this system (Figure 4B, lanes 8–10), yeast RFC is effective in suppressing 5′ to 3′ hydrolysis from a 3′ strand break (Figure 4B, lanes 1–3) and suffices to activate 3′ to 5′ excision, provided that human PCNA is present (Figure 4B, lane 6). The latter observation is consistent with the finding that yeast RFC can load mammalian PCNA onto the helix (Yoder and Burgers, 1991).

The domain representation and homology boxes of

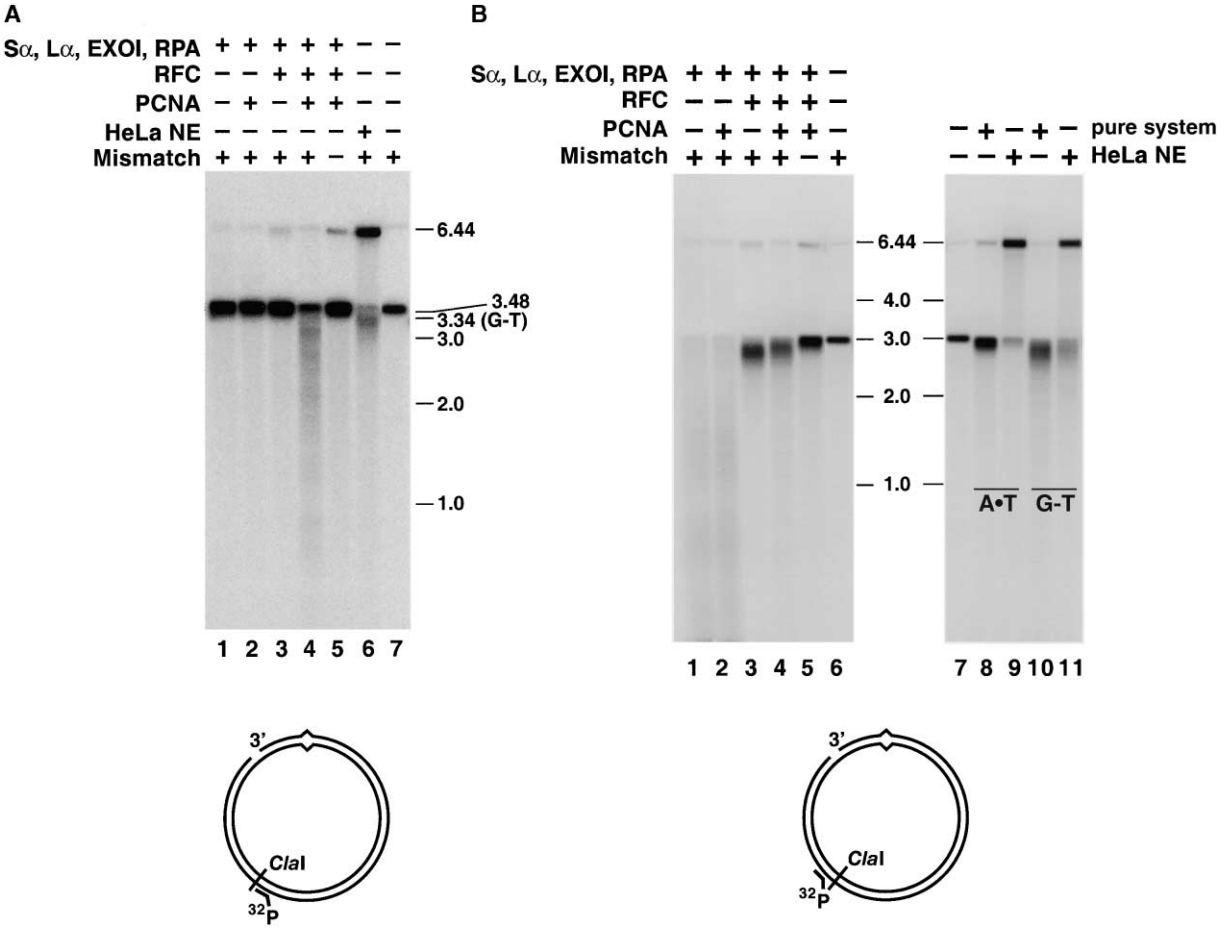

Figure 2. Excision Tract Endpoints for Hydrolysis Directed by a 3′ Strand Break

(A) Reactions containing 3′ G-T-heteroduplex (or A•T homoduplex) and proteins as indicated were performed as in Figure 1A. After digestion with ClaI, denatured products were resolved by electrophoresis through alkaline agarose and probed with 5′ ^{32}P-labeled oligonucleotide V2505 (Experimental Procedures). As illustrated schematically, this probe hybridizes to the incised complementary strand adjacent to the ClaI site in a manner that permits localization of 3′ excision tract endpoints. Numerical values on the right indicate distances from the ClaI site in kb. The 6.44 kb species observed in HeLa nuclear extract is produced by ligation of the strand break.

(B) Reactions and product analysis were as in (A) except that membranes were probed with 5′ ^{32}P-labeled oligonucleotide V2531 (Experimental Procedures), which hybridizes to the incised strand adjacent to the ClaI site in a manner that permits mapping of 5′ endpoints. Lanes 8–11 compare 5′ endpoints produced in the pure system (MutSα, MutLα, EXOI, RPA, RFC, and PCNA) with those observed in HeLa nuclear extract.

the large subunit of human and *Saccharomyces cerevisiae* RFC are shown schematically in Figure 4A (Fotedar et al., 1996; Mossi and Hubscher, 1998; Gomes et al., 2000). The N-terminal ligase homology domain is not required for RFC function as a clamp loader, nor is it necessary for DNA replication as judged by in vitro assay (Uhlmann et al., 1997; Gomes et al., 2000). As shown in Figure 4B (lane 7), yeast RFC lacking the ligase homology domain (yRFCΔN) is functional in activation of 3′ to 5′ excision on a 3′ heteroduplex provided that human PCNA is also present. However, yRFCΔN is less effective than native yRFC in suppression of 5′ to 3′ hydrolysis on 3′ substrates, an effect that is evident in the absence (Figure 4C) or presence of human PCNA (Supplemental Figure S2 on *Molecular Cell*'s website, compare lanes 5 and 6 with lanes 10 and 11). It is also noteworthy that reduced concentrations of yRFCΔN are more effective than native yRFC in activation of 3′ to 5′ excision on a 3′ heteroduplex (Supplemental Figure S2 on *Molecular Cell*'s website), consistent with the finding that deletion of the RFC ligase homology domain renders the protein more efficient as a clamp loader and activator of PCNA-dependent DNA in vitro synthesis (Uhlmann et al., 1997; Gomes et al., 2000).

A conserved domain within the large RFC subunit (residues 481–728 of human RFC p140) interacts directly with PCNA (Fotedar et al., 1996). While antibody directed against this domain had no effect on the ability of human RFC to suppress 5′ to 3′ excision on a 3′ heteroduplex, it largely abolished PCNA-dependent activation of 3′ to 5′ hydrolysis (Figure 4D). RFC thus provides multiple functions in 3′ directed excision: it is necessary for effective suppression of 5′ to 3′ excision at a 3′ strand break in a manner that depends on integrity of the ligase homology domain of the large subunit and is also required for activation of 3′ to 5′ hydrolysis in a manner that

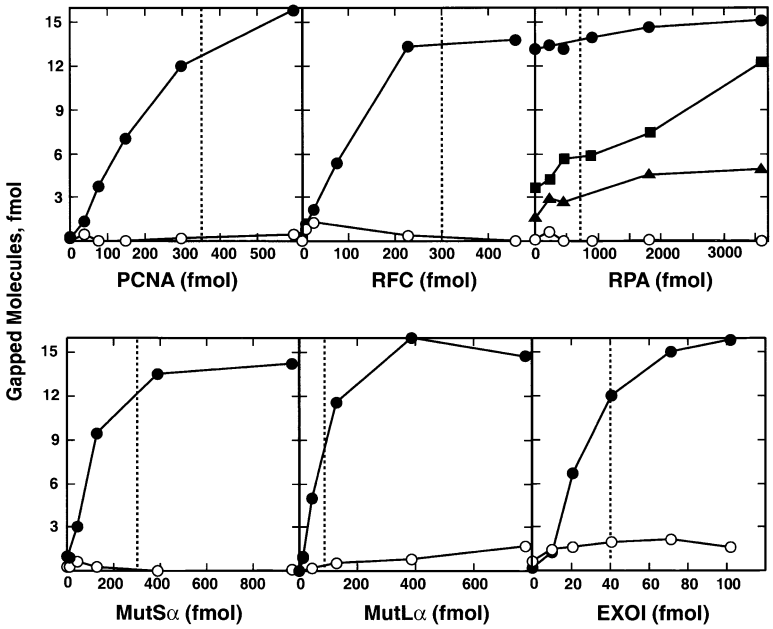

Figure 3. Dependence of the 3′ Excision Reaction on MutSα, MutLα, EXOI, RPA, PCNA, and RFC

Reactions (Experimental Procedures) contained 24 fmol of incised 3′ A•T homoduplex (○) or 3′ G-T heteroduplex (●; 141 base pair nick and mispair separation distance). Concentrations of individual activities were varied as indicated, with amounts of the other five components fixed at the concentrations specified in Figure 1A. RPA titrations (upper right) were performed in the presence of variable amounts of RFC (●; 220 fmol [standard amount]; ■, 100 fmol; and ▲, 50 fmol). 3′ to 5′ excision was scored by cleavage with NheI and ClaI (Figure 1). Dotted vertical lines indicate the amount of each protein estimated by Western blot analysis to be present in 50 μg of HeLa nuclear extract (data not shown).

depends on its interaction with PCNA. Based on the results summarized above, we infer that the latter function involves loading of PCNA onto the helix.

5′ Directed Excision Reaction in the Presence of RFC and PCNA

Because RFC and PCNA suppress 5′ to 3′ excision when the strand break that directs hydrolysis is located 3′ to the mismatch, we have examined the effects of these two proteins on the 5′ directed reaction in the MutSα, MutLα, EXOI, and RPA system (Genschel and Modrich, 2003). In contrast to the dramatic suppression of 5′ to 3′ hydrolysis on a 3′ heteroduplex, RFC and PCNA have only a modest effect on 5′ to 3′ excision directed by a 5′ strand break (Figure 5A). The two proteins reproducibly reduced efficiency of excision by about 25%, but the 4- to 5-fold mismatch dependence characteristic of the MutSα, MutLα, EXOI, RPA system (Genschel and Modrich, 2003) was retained, and 5′ to 3′ excision tracts produced upon supplementation of these four proteins with RFC and PCNA are virtually identical to those observed in HeLa nuclear extract (Figure 5B). The six protein system described here thus supports bidirectional mismatch-provoked excision.

Requirement for EXOI Active Site in 5′ and 3′ Directed Excision

The six components shown here to be sufficient for mismatch-provoked 3′ to 5′ excision do not include a known 3′ to 5′ hydrolytic activity. In the absence of other proteins, human EXOI hydrolyzes DNA with 5′ to 3′ polarity (Lee et al., 2002). The other proteins used in this study were free of detectable 3′ to 5′ exonuclease activity (Supplemental Table S1 on *Molecular Cell*'s website). Consequently, the ability of this system to support efficient 3′ to 5′ excision was unexpected. We have entertained two explanations for the lack of dependence of the reaction on a known 3′ to 5′ exonuclease. It is possible that a required 3′ to 5′ hydrolytic function is present as a trace contaminant in one of the other five required activities. If this putative exonuclease were to depend on mismatched DNA and other repair components for its activation, it would go undetected in the exonuclease screens mentioned above. Because the MutSα, MutLα, EXOI, RPA, and PCNA preparations used in this work were all essentially homogeneous, we regard this possibility as unlikely. While the human RFC preparations employed were only 55%–60% pure, we have shown that this component can be replaced by near-homogeneous preparations of the yeast proteins (Figure 4B).

An alternate possibility is that EXOI, in addition to its 5′ to 3′ exonuclease activity, may provide a 3′ to 5′ hydrolytic function that is activated on 3′ heteroduplexes in a manner that depends on other repair components (Genschel et al., 2002). To evaluate this possibility, we have examined the ability of a mutant form of EXOI to support 5′ and 3′ directed excision in the purified system. EXOI is a member of the RAD2 family, and based on structural analysis of the RAD2 homolog FEN1 (Hosfield et al., 1998), the conserved Asp-173 is expected to play a critical role in EXOI active site function (Sokolsky and Alani, 2000). We therefore constructed an EXOI D173A mutant and evaluated activity of the mutant protein in mismatch repair. The mutant protein is defective in both 5′ and 3′ directed excision in the nuclear extract system described previously (Genschel et al., 2002; data not shown), and as illustrated in Figure 6A, in 5′ and 3′ directed excision in the six component, purified system described here. These defects do not appear to be the consequence of a gross structural rearrangement because presence of excess mutant protein inhibits excision supported by wild-type EXOI, an effect that is reversed by heat denaturation (Figure 6A). Because the

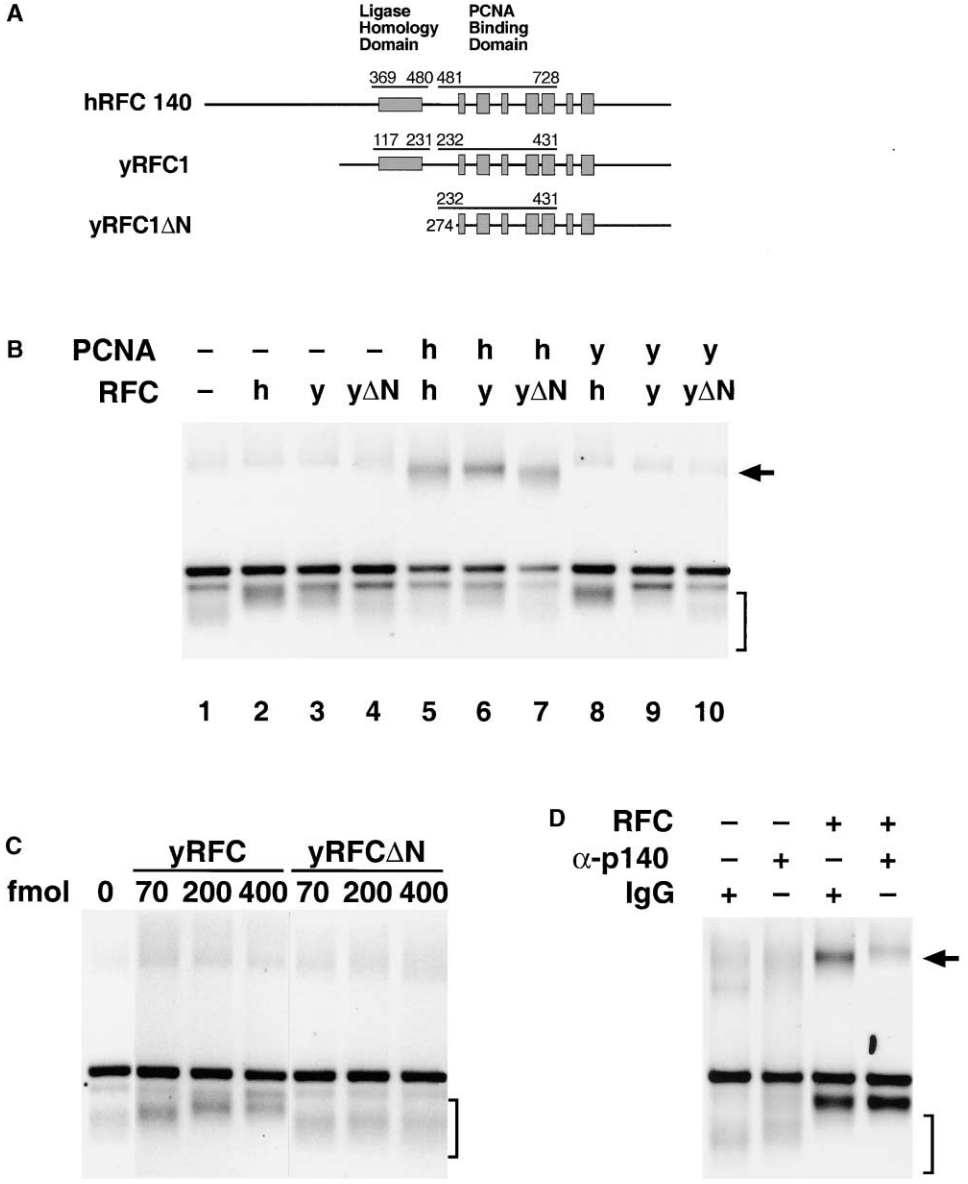

Figure 4. Yeast RFC Supports the Human 3′ to 5′ Excision Reaction but Yeast PCNA Does Not

(A) Schematic domain representation and homology boxes of the large subunit of human and *Saccharomyces cerevisiae* RFC, as well as the yeast protein yRFCΔN, from which the N-terminal ligase homology domain has been deleted (Fotedar et al., 1996; Mossi and Hubscher, 1998; Gomes et al., 2000).

(B) Excision on a 3′ G-T heteroduplex was performed as described in Figure 1A in the presence of MutSα, MutLα, EXOI, and RPA. Reactions also contained as indicated 260 fmol human (h) or yeast (y) RFC or yRFCΔN and 1160 fmol human (h) or yeast (y) PCNA. Products of 3′ to 5′ excision are indicated by an arrow and those of 5′ to 3′ hydrolysis by a bracket (see Figure 1A).

(C) Reactions were as in (B) except that PCNA was omitted and yRFC or yRFCΔN were present as indicated.

(D) Reactions as in (B) contained human PCNA and 21 fmol EXOI. Human RFC, anti-p140 antiserum directed against RFC p140 residues 481–728 (800 ng), or control serum (800 ng) was present as indicated.

mutant protein is well behaved and purifies like wild-type protein (data not shown), these findings strongly suggest that the catalytic center of EXOI is required for excision directed by either a 5′ or 3′ strand break.

As noted above, our results indicate that loaded PCNA is required for activation of 3′ directed, EXOI-dependent excision, suggesting that interaction of the two proteins may be involved in this effect. This possibility was addressed by far Western analysis. As shown in Figure 6B, PCNA interacts strongly with RFC and MutSα but more weakly with MutLα, confirming previous observations to this effect (Bellacosa, 2001; Kleczkowska et al., 2001).

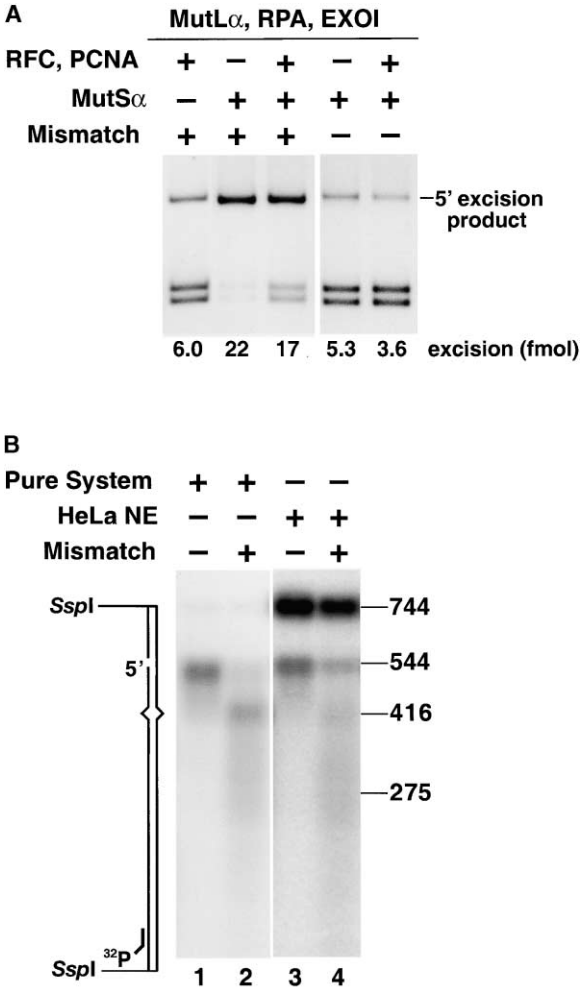

Figure 5. 5′ Directed Excision in the Presence of RFC and PCNA

(A) Reactions (Experimental Procedures) contained 24 fmol of 5′ G•C homoduplex or 5′ G-T heteroduplex (128 base pair separation between nick and mispair; Genschel and Modrich, 2003), 280 fmol MutLα, 42 fmol EXOI, 1800 fmol RPA, and 390 fmol MutSα. RFC (220 fmol) and PCNA (290 fmol) were present as indicated. Incubation was for 5 min at 37°C and 5′ to 3′ excision was scored by NheI assay as described (Genschel and Modrich, 2003). Extents of excision are indicated below each lane.

(B) 5′ excision reactions contained MutSα, MutLα, EXOI, RPA, RFC, and PCNA in amounts indicated in (A) (Pure System) or 50 μg of HeLa nuclear extract. After 5 min incubation at 37°C, DNA was deproteinized, digested with SspI, denatured, subjected to electrophoresis through 1.8% alkaline agarose, and transferred to a nylon membrane, which was probed with 5′ ^{32}P-labeled oligonucleotide V5216 (Experimental Procedures). As indicated in the diagram, this probe hybridizes to the incised complementary strand adjacent to the SspI site and can be used to locate 5′ excision tract endpoints. Coordinates shown on the right correspond to length in nucleotides. The 544 nucleotide species corresponds to the location of the nick and 416 to the location of the mismatch. The 744 nucleotide species observed in HeLa nuclear extract is the product of ligation.

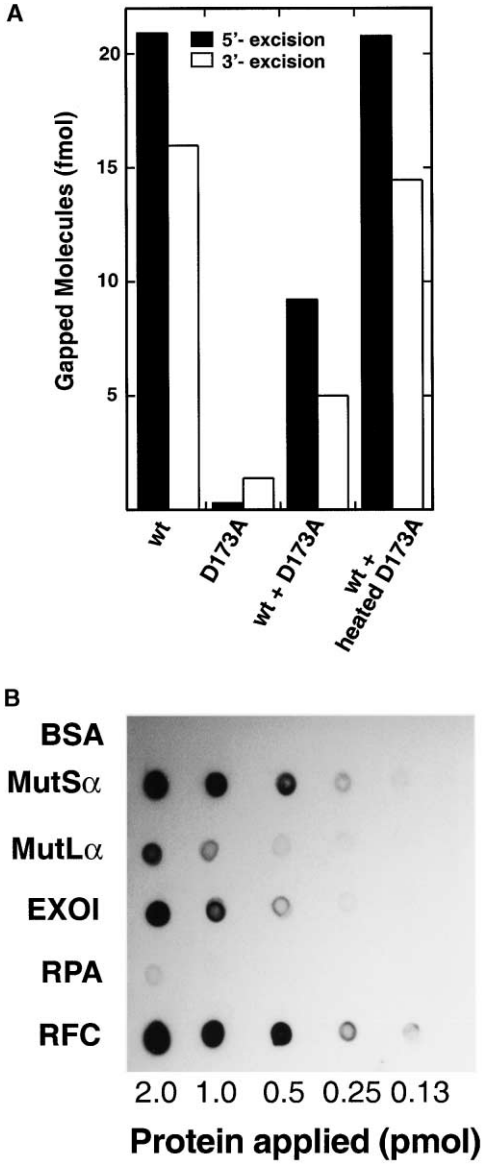

Figure 6. EXOI Involvement in 5′ and 3′ Directed Excision

(A) Reactions containing 24 fmol 3′ G-T heteroduplex (white bars) or 5′ G-T heteroduplex (black bars), MutSα, MutLα, RPA, RFC, and PCNA were performed as described in Figures 1A and 5A, respectively. Reactions also contained as indicated 42 fmol of wild-type EXOI, 106 fmol EXOI D173A, or 42 fmol wild-type EXOI plus 1060 fmol of EXOI D173A. The reactions shown on the right contained 42 fmol wild-type EXOI plus 1060 fmol of heat-denatured (100°C 15 min) EXOI D173A.

(B) Far Western analysis of PCNA interactions with mismatch repair proteins was performed as described in the Experimental Procedures. Indicated quantities of BSA, MutSα, MutLα, EXOI, RPA, and RFC were applied to a nitrocellulose membrane, which was then incubated with PCNA. Membrane-bound PCNA was visualized immunochemically.

However, PCNA also displays a strong interaction with EXOI as judged by this method. A similar interaction with PCNA has been demonstrated for the RAD2 family member FEN-1, and this interaction has been implicated in activation of nuclease activity (Gomes et al., 2000).

Discussion

In contrast to the previously described four component system that supports only mismatch-provoked 5′ to 3′ hydrolysis (Genschel and Modrich, 2003), the six com-

ponent system described here is capable of bidirectional excision, supporting hydrolysis directed by a strand break located either 5′ or 3′ to the mispair. RFC and PCNA provide multiple functions in this bidirectional system. RFC is necessary for the PCNA-dependent activation of 3′ to 5′ hydrolysis on a 3′ heteroduplex, an effect that we attribute to its function as a clamp loader (Waga and Stillman, 1998). RFC is also required for orientation-dependent suppression of 5′ to 3′ hydrolysis by EXOI that occurs on a 3′ heteroduplex, a function that is potentiated by PCNA and depends on integrity of the ligase homology domain of the large RFC subunit. At physiological salt concentration, assembly of the RFC•PCNA complex occurs preferentially on gapped or nicked circular DNA as compared to covalently closed molecules (Podust et al., 1995). Inasmuch as isolated ligase homology domains of *Drosophila* and human RFC preferentially bind to recessed 5′ phosphoryl termini (Allen et al., 1998; Mossi et al., 2000), an interaction of this sort may be involved in the suppression of 5′ to 3′ hydrolysis when the nick that directs excision is located 3′ to the mispair. The ligase homology domain of yeast RFC1 is not essential for viability (Gomes et al., 2000), and a transposon insertion within this region of the *RFC1* gene confers dinucleotide repeat instability (Xie et al., 1999). However, it is not clear whether this effect is related to function of the ligase homology domain in mismatch repair.

Despite its ability to support 3′ to 5′ excision on a 3′ heteroduplex, the system described here contains only the single DNA hydrolytic activity EXOI, which degrades DNA with 5′ to 3′ polarity in the absence of other activities (Lee et al., 2002). Attempts to identify a 3′ to 5′ hydrolytic activity associated with other components of the system yielded negative results (Supplemental Table S1 on *Molecular Cell*'s website), and we have found that EXOI D173A is defective in excision on both 5′ and 3′ heteroduplexes. This observation, coupled with the finding that EXOI D173A inhibits excision supported by the wild-type enzyme, strongly suggests that EXOI is responsible for the hydrolysis that occurs on both 5′ and 3′ substrates. We postulate that a cryptic EXOI 3′ to 5′ hydrolytic activity, which could conceivably share active site residues with the 5′ to 3′ hydrolytic function of the enzyme, is activated by other components of the repair system, such as PCNA and/or MutLα, but only when the strand break that directs excision is located 3′ to the mispair.

A similar differential activation of 5′ to 3′ or 3′ to 5′ excision systems according to 5′ or 3′ placement of the strand signal has been described for *E. coli* mismatch repair. Four exonucleases have been implicated in the bacterial reaction, and these function to some extent in a redundant manner (Burdett et al., 2001; Viswanathan et al., 2001). Interestingly, one of these activities (EXOVII) is capable of supporting hydrolysis directed by either a 5′ or 3′ strand break. While mammalian EXOI is apparently sufficient to provide the hydrolytic function for 5′ or 3′ directed mismatch-provoked excision, nuclear extract depletion studies (Genschel et al., 2002) and analysis of *Exo1*$^{-/-}$ mouse cells (Wei et al., 2003) suggest that redundant activities may also exist in mammalian cells. We therefore regard the reconstituted excision system described here as a minimal system and are currently seeking other activities that may participate in the reaction.

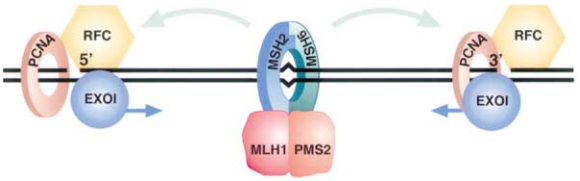

Figure 7. Model for Differential Activation of EXOI-Dependent 5′ or 3′ Directed Excision

Movement of the MutSα•MutLα complex away from the mismatch and along the helix has been postulated to signal excision system activation at the strand break that directs repair (Allen et al., 1997; Galio et al., 1999; Blackwell et al., 2001; Acharya et al., 2003). PCNA is loaded with a defined orientation at 3′ termini at a double strand-single strand junction (Tsurimoto, 1999), i.e., the labeled PCNA face in the diagram is uniquely oriented relative to the 3′ end. Thus, a mobile MutSα•MutLα complex will encounter different orientations of the PCNA trimer depending on whether the strand break is located 5′ or 3′ to the mispair. In view of the ability of PCNA to interact with MutSα, MutLα, and EXOI, this could result in orientation-dependent protein-protein interactions leading to differential activation of 5′ to 3′ or 3′ to 5′ hydrolytic activities of EXOI.

Although PCNA has been suggested to function as a strand signal during mismatch repair (Umar et al., 1996; Pavlov et al., 2003), our results are more consistent with a role for the protein in the differential activation of the appropriate hydrolytic system according to strand signal placement 3′ or 5′ to the mispair. A number of interactions between mismatch repair activities have been described (Bellacosa, 2001). MutSα interacts with MutLα, PCNA, and EXOI; MutLα interacts with EXOI and PCNA; and as shown here, EXOI also interacts with PCNA. It seems likely that these interactions and the temporal order of their occurrence control the sequence of events during mismatch repair, including the ability of the system to differentially respond to strand signals located to either side of the mispair. Although many of the mechanistic implications of these interactions remain to be defined, it is interesting to note that PCNA is loaded at primer termini in an orientation-dependent manner with its C side facing the direction of elongation (Tsurimoto, 1999). Given that movement of MutS•MutL and MutSα•MutLα complexes along the helix has been invoked in signaling between the mismatch and the strand signal that directs repair (Allen et al., 1997; Galio et al., 1999; Blackwell et al., 2001; Acharya et al., 2003), the nature of PCNA loading suggests a simple mechanism whereby orientation-dependent encounter of PCNA at a strand discontinuity by the mobile MutSα•MutLα complex results in differential hydrolytic responses according to 3′ or 5′ placement of the discontinuity (Figure 7).

The significance of one aspect of 3′ to 5′ reaction that occurs on a 3′ heteroduplex remains uncertain. In the purified system and in HeLa nuclear extract under conditions of DNA synthesis block, limited 5′ to 3′ excision occurs at the strand discontinuity on 3′ substrates (Fang and Modrich, 1993; Figure 2B). This 5′ to 3′ hydrolysis may represent a necessary step in the activation of the 3′ to 5′ hydrolytic system that subsequently processes the 3′ heteroduplex. However, the limited 5′ to 3′ excision observed on such heteroduplexes could be a con-

sequence of adventitious exonuclease attack due to prolonged exposure of the gapped 3′ excision product, an intermediate that would otherwise have a relatively short lifetime due to repair DNA synthesis.

Using an extract depletion approach, Yuan et al. (2004) have recently presented evidence for involvement of the high-mobility group box 1 protein (HMGB1) in human mismatch repair, with the protein implicated at an early step in the reaction. While we cannot rule out potential presence of HMGB1 (or a functional homolog) as a trace contaminant in one of the protein preparations used in our work, we regard this possibility as unlikely in view of purities of the proteins used. A plausible explanation for the differential HMGB1 requirement in these two systems is based on the presence of other DNA binding activities in the nuclear extract system. Such proteins may restrict access of repair activities to the mismatch and/or strand discontinuity in heteroduplex DNA, and HMGB1 may function to reverse this type of effect.

Experimental Procedures

Proteins and Nuclear Extracts

HeLa S3 cells were grown and nuclear extracts were prepared as described (Holmes et al., 1990). All protein fractionation steps were at 0°C to 4°C and were performed in the presence of a set of protease inhibitors (final concentrations: 0.5 μg/ml aprotinin, 1 μg/ml E64, 1 μg/ml leupeptin, 5 μg/ml pepstatin, 100 μg/ml pefabloc, and 0.1% phenylmethylsulfonyl fluoride). SDS gel electrophoretic profiles of the proteins used in this study are presented in Supplemental Figure S1, available on *Molecular Cell*'s website.

Recombinant human MutSα, MutLα, and EXOIb (purity 95% or better) were isolated from baculovirus-infected Sf9 insect cells as described (Blackwell et al., 2001; Genschel et al., 2002), except that MutSα purification was modified by addition of a MonoS chromatography step. The MutSα MonoQ eluate was diluted with 25 mM HEPES-KOH (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol (DTT) to a conductivity equivalent to that of 0.1 M KCl and loaded onto a 1 ml MonoS column (HR5/5, Amersham Biosciences). The column was developed with a 0.1–0.37 M KCl gradient in this buffer. MutSα eluted at about 0.22 M KCl.

The EXOIb D173A mutant was generated by PCR-based mutagenesis of the pFastbacI EXOIb plasmid (Genschel et al., 2002). The codon for Asp-173 was changed from GAT to GCT, resulting in a substitution to alanine. Mutant EXOI was expressed in Sf9 insect cells and isolated (95% purity) as described for the wild-type protein (Genschel et al., 2002).

Human RPA was isolated from an *E. coli* overproducing strain (Henricksen et al., 1994) by modification (Genschel and Modrich, 2003) of the published procedure. *S. cerevisiae* PCNA was isolated as described (Ayyagari et al., 1995). Human PCNA was prepared from *E. coli* BL21(DE3)/PT7hPCNA (a gift from Bruce Stillman, Cold Spring Harbor) by a modification of a published procedure (Fien and Stillman, 1992). In brief, PCNA-containing fractions from the Phenyl-Sepharose step were pooled; dialyzed against 25 mM HEPES-KOH (pH 7.5), 0.1 mM EDTA, 1 mM DTT; and loaded onto a MonoQ column. The column was washed and eluted with a 0.2–0.6 M NaCl gradient, and PCNA eluted at about 350 mM NaCl. Isolated RPA and PCNA had estimated purities of better than 98%.

Human RFC was isolated from a 35%–65% ammonium sulfate fraction derived from HeLa nuclear extract as described (Tsurimoto and Stillman, 1989) except that the column sequence was ssDNA-cellulose, phosphocellulose, hydroxylapatite, and MonoQ. RFC was monitored during fractionation by Western blot using antibody against the p140 subunit (a gift from Ulrich Hübscher, University of Zürich), and in some cases by assay of mismatch-provoked excision activity. Material eluting from the MonoQ column was estimated to be 55%–60% pure by Coomassie-stained SDS gels.

S. cerevisiae RFC (yRFC, >95% pure) was purified as described previously (Gerik et al., 1997). Yeast RFC lacking the N-terminal ligase homology domain of the large subunit (amino acids 2–273, yRFCΔN) was isolated from an *E. coli* overproducer as described (Gomes et al., 2000), except that the PCNA affinity column step was replaced by chromatography on ssDNA-cellulose. The yRFCΔN eluate from SP-Sepharose was diluted with HEG buffer containing 0.05% ampholytes 3.5–10 and loaded onto a 1 ml ssDNA-cellulose equilibrated with HEG_{200} containing 0.05% ampholytes. The column was eluted with HEG_{800}-0.05% ampholytes, and the yRFCΔN eluate subjected to Mono S chromatography as in the original procedure. Final preparations were estimated to be >95% pure and contained stochiometric equivalents of the five subunits.

Mismatch-Provoked Excision Assays

5′ G-T heteroduplex and a control homoduplex containing a G•C base pair at position 5632 were prepared using phages f1MR1 and f1MR3 as described (Fang and Modrich, 1993). These DNAs contained single-strand break in the complementary strand 128 base pairs 5′ to the mismatch in the heteroduplex, as viewed along the shorter path between the two sites in the circular molecule.

3′ G-T heteroduplex and A•T homoduplex DNAs were prepared in a similar manner using phages f1MR59 and f1MR60. These phage were derived from f1MR1 and f1MR3 (Su et al., 1988), respectively, by oligonucleotide mutagenesis to introduce a C to G base substitution at position 5491 to produce an ApaI cleavage site. The nature of these ApaI-containing derivatives of f1MR1 and f1MR3 was confirmed by sequence analysis and fragment switch. 3′ A•T control homoduplex was prepared by hybridization of the complementary strand of ApaI-cleaved f1MR59 replicative form DNA with circular f1MR59 viral strand. 3′ G-T heteroduplex was prepared in a similar manner by hybridization of the complementary strand of ApaI-cleaved f1MR60 replicative form DNA with f1MR59 circular viral strand. Resulting hybrids contained single-strand break in the complementary strand at the ApaI cleavage site 141 base pairs 3′ to the mismatch.

Gap formation assays were performed by a minor modification of previously described methods (Fang and Modrich, 1993; Genschel et al., 2002). Proteins were diluted into 7.5 mM HEPES-KOH (pH 7.5), 0.2 M KCl, 1 mM DTT, 0.5 mg/ml bovine serum albumin (BSA), 10% (vol/vol) glycerol. Reactions were assembled by mixing indicated proteins in a total volume of 8 μl diluent with 12 μl of 33 mM Tris-HCl (pH 7.6), 75 mM KCl, 2.5 mM ATP, 1.66 mM glutathione, 8.3 mM $MgCl_2$, 80 μg/ml BSA, and 2 nM DNA. Incubation was at 37°C for 5 min. Reactions were stopped by adding 40 μl of 30 mM EDTA, 180 μg/ml proteinase K, and 0.4 mg/ml glycogen, followed by incubation at 55°C for 60 min. DNA recovered after phenol extraction and ethanol precipitation was digested with NheI and ClaI and products separated on 1% agarose gel. After ethidium bromide staining, the NheI-resistant fraction was quantitated using a photometric grade CCD camera and NIH image software. Positive images obtained in this manner were inverted in Adobe Photoshop to enhance visibility of excision products.

Excision tract endpoints were mapped by indirect end labeling (Fang and Modrich, 1993; Genschel et al., 2002) after cleavage with ClaI. Reaction products were resolved by electrophoresis through 1% alkaline agarose, and DNA was subjected to depurination and strand breakage in the gel by 10 min incubation in 0.2 N HCl and then 0.4 N NaOH. After transfer to nylon membranes, products were probed with ^{32}P-labeled oligonucleotides that hybridize to complementary strand sequences on either side of the ClaI site. Probe V2505 [d(CGCTACTGATTACGGTGCTGCT), viral strand nucleotides 2505–2527], which hybridizes to the 5′ side of the ClaI site, was used to localize 3′ termini. Probe V2531 [d(ATGGTTTCATTGGT GACGTTTC), viral strand nucleotides 2531–2553] hybridizes to the 3′ side of the ClaI site and was used to map 5′ excision termini. Mapping of 5′ excision tracts on 5′ heteroduplex/homoduplex DNAs was performed as described above after SspI cleavage and resolution of denatured DNA fragments by electrophoresis through 1.8% alkaline agarose. The hybridization probe was V5216 [d(ATTGTTCT GGATATTACCAGC)], corresponding to viral strand nucleotides 5216–5236.

Far Western Analysis

BSA, MutSα, MutLα, EXOI, RPA, and RFC were spotted as indicated onto Hybond ECL nitrocellulose membranes (Amersham). Dried

membranes were blocked for 1 hr at room temperature with 0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100 containing 5% milk solids (all subsequent incubations and washes were performed in this solution). After incubation with 0.36 μM PCNA for 3.5 hr at 4°C, the membrane was washed three times and then incubated with anti-PCNA mouse IgG (PC10, Pharmingen, 1 μg/ml) for 1 hr at room temperature followed by 1 hr incubation with secondary peroxidase-conjugated sheep anti-mouse antibody (Amersham). The ECL system (Amersham) was used for signal visualization.

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