Supporting Information

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SI Text

Cell Lines, Cell Culture, and Extract Preparation. To produce *Mlh1*$^{-/-}$ and *Mlh1*$^{-/-}$ *Exo1*$^{-/-}$ MEF lines, *Mlh1*$^{+/-}$ mice were first crossed with *Exo1*$^{+/-}$ mice (1). Subsequently *Mlh1*$^{+/-}$ *Exo1*$^{+/-}$ double heterozygous mice were intercrossed, and embryonic day 13.5 embryos were isolated. MEF lines were prepared from embryos by standard procedures, cultured in 10% FCS DMEM, and genotyped by PCR. Primary MEF lines of the appropriate genotypes were immortalized by continuous culture for >25 passages by using a 3T3 protocol (2).

ExoI$^{-/-}$ ES cells (3) were grown in roller bottles in DMEM (Millipore) supplemented with 15% FBS (HyClone), 2 mM glutamine (HyClone), 1,000 units/mL leukemia inhibition factor (Millipore), 0.1 mM nonessential amino acids (HyClone), 30 μM each adenosine, cytidine, guanosine, uridine, and 10 pM thymidine (Millipore), 100 units/mL penicillin, 100 μg/mL streptomycin (Millipore), and 0.1 mM β-mercaptoethanol (Millipore). *ExoI*$^{-/-}$ *Mlh1*$^{-/-}$ MEF cells were cultured in roller bottles in DMEM high glucose medium (HyClone) supplemented with 10% FBS (HyClone).

Cell extracts were prepared by a modification of the method of Roberts and Kunkel (4). Cells from 16–24 roller bottles were washed with PBS and trypsinized at 37 °C. Subsequent steps were performed at 0–4°C. Trypsinized cells were collected in PBS, the final volume was adjusted to 400 mL with PBS, and PMSF was added to 1 mM. Cells were pelleted at 370 × *g* for 6 min and resuspended in 200 mL of 20 mM Hepes·NaOH (pH 8.0), 1.5 mM $MgCl_2$, 5 mM KCl, 250 mM sucrose, 1 mM DTT, and 1 mM PMSF. Cells were collected by centrifugation as above, resuspended in 45 mL of hypotonic buffer [20 mM Hepes·NaOH (pH 8.0), 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and 1 mM PMSF], and incubated for 5–10 min. Cells were pelleted at 650 × *g*, and 5–8 mL of hypotonic buffer was added to the pellet (≈10 mL packed volume). The mixture was incubated on ice for 5 min, and cells were disrupted in a Dounce homogenizer with 10 strokes of pestle B. The extract was incubated on ice for 30 min with occasional mixing and clarified by centrifugation at 27,000 × *g* for 10 min. The supernatant was supplemented with KCl, aprotinin, leupeptin, E64, and pepstatin to final concentrations of 100 mM, 2 μg/mL, 4 μg/mL, 1 μg/mL, and 1.4 μg/mL, respectively. The extract was quick-frozen in small samples in liquid N_2 and stored at −80 °C.

Recombinant Proteins. Near-homogeneous recombinant human MutSα, MutLα, MutLαD699N, Exo1b, PCNA, and RPA were isolated according to published methods (5, 6). C-terminal His_6-tagged Fen1 was expressed in *E. coli* and isolated as described (7).

DNA sequences of the 5 human RFC genes (RFC p140, p40, p38, p37, and p36) were optimized for *E. coli* expression and synthesized by GenScript. The RFC p140 gene was inserted between the NcoI and BamHI of pCDF-1b (Novagen), yielding pCDF-1b-RFC140, placing it under control of a T7 promoter, *lac* operator, a strong ribosome-binding site, and a T7 terminator (Fig. S4). Synthetic genes corresponding p36, p37, p38, and p40 human RFC subunits were inserted as modules into pETDuet-1 (Novagen) so that expression of each was under the control of a T7 promoter, *lac* operator, and a strong ribosome binding site (Fig. S4). Sequences of these plasmids will be provided on request.

Four-subunit human DNA polymerase δ was expressed in SF9 cells by using 2 baculovirus constructs, one expressing the p125 and p12 genes and the second encoding p50 and His_6-tagged p66. The p125 gene was PCR-amplified from pVL1393/p125 (8) using primers d(GTCGGATCCGCCACCATGGATGGCAAGCGGCGGCCAG) and d(gtctctagattatcaccaggcctcaggtccag), and the p50 gene was amplified from pVL1393/p50 (8) using primers d(gtcggatccGCCACCatgttttctgagcaggctgcccag) and d(GTCTCTAGATTATCAGGGGCCCAGCCCCAGGCCTC). After digestion with BamHI and XbaI, p125 and p50 PCR products were cloned into BamHI-, XbaI-cleaved pFASTBac Dual (Invitrogen) downstream from the polyhedrin promoter to yield pFASTBacDual-p125 and pFASTBacDual-p50. The p12 gene was PCR-amplified from pVL1393/p12 (8) using primers d(GTCCTCGAGGCCACCATGGGCCGGAAGCGGCTCATC) and d(gtcgcatgcttatcatagggggatagagatgccagag), and the His_6-p66 gene was amplified from pFastBac HTA-p66 (9) using primers d(GTCCTCGAGGCCACCATGTCGTACTACCATCACCATCACC) and d(gtcgcatgcttattatttcctctggaagaagccag). After digestion with XhoI and SphI, p12 and His_6-p66 DNA fragments were inserted into XhoI- and SphI-cleaved pFASTBacDual-p125 or pFASTBacDual-p50 (downstream from the p10 promoter) to yield pFASTBacDual-p125-p12 or pFASTBacDual-p50-His_6-p66, respectively. Final constructs were verified by sequencing, and with the exception of p125, all were correct. The p125 gene contained a point mutation corresponding to a V720A amino acid substitution, which was also present in our isolates of pVL1393/p125. The mutation was reverted to wild type by using the QuikChange site-directed mutagenesis kit (Stratagene). Using the Invitrogen "Bac to bac" protocol, plasmids pFASTBacDual-p125-p12 and pFASTBacDual-p50-His_6-p66 were used to prepare 2 baculoviruses that upon coinfection of SF9 cells yielded 4-subunit human DNA polymerase δ.

Unless otherwise noted, all protein purification buffers contained 1 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride, and fractions were collected into tubes containing a set of protease inhibitors to yield final concentrations of aprotinin, leupeptin, E64, and pepstatin of 1 μg/mL, 2 μg/mL, 0.5 μg/mL, and 0.7 μg/mL, respectively. Proteins in column fractions were identified by SDS/PAGE followed by Western analysis with an appropriate antibody and/or Coomassie R-250 staining.

To express human RFC in *E. coli*, the p36-p37-p38-p40-pET-duet-1 and pCDF-1b-RFC140 plasmids were cotransformed into BLR (BL21(DE3)/recA^{-}) cells (Novagen), and transformants were selected on LB plates containing 50 μg/mL streptomycin and 50 μg/mL carbenicillin. After ≈20 h, 30 colonies were suspended in 40 mL of expression media [12 g/L of Bacto-Tryptone, 24 g/L of yeast extract, 0.4% glycerol, 50 mM KPO_4 (pH 7.2), 0.5% casamino acids] supplemented with 50 μg/mL streptomycin and 50 μg/mL carbenicillin and cultured at 37 °C to an A_{600} of ≈0.05. The culture was then diluted with 6 L of expression media lacking antibiotics and grown to an A_{600} of 0.4, at which point IPTG was added to 0.5 mM and incubation was continued for 15 h at 15 °C. Cells were collected by centrifugation at 5,900 × *g* for 10 min, resuspended in ≈200 mL of 20 mM Tris·HCl, pH 7.4, and 150 mM NaCl and collected by centrifugation at 3,300 × *g* for 10 min. The pellet was resuspended in 60 mL of 20 mM Hepes·KOH (pH 7.4), 2 mM EDTA, 5% glycerol (wt/vol), 0.01% Nonidet P-40, 50 mM NaCl, 2 μg/mL aprotinin, 4 μg/mL leupeptin, 1 μg/mL E64, 1.4 μg/mL pepstatin, 0.2 mM PMSF, 2 mM DTT, and 3 dissolved Complete Mini Protease Inhibitors Mixture tablets (Roche). After addition of 4.5 mL of 20 mg/mL lysozyme and incubation on ice for 10 min, cells disrupted by sonication. Conductivity was adjusted to that of 200 mM NaCl, and the lysate was spun at 40,000 × *g* for 25 min.

The supernatant was loaded onto a 30-ml SP Sepharose column (GE HealthCare) equilibrated with buffer A [25 mM Hepes·KOH (pH 7.4), 0.1 mM EDTA, 5% glycerol (wt/vol), 0.01% Nonidet P-40] containing 200 mM NaCl at 3 mL/min. After a 30-mL wash

with equilibration buffer, the column was developed with a 50-ml linear gradient of NaCl (200–1,000 mM) in buffer A. RFC peak fractions, which eluted at 480 mM NaCl, were diluted to 300 mM NaCl with buffer A and loaded at 2.5 mL/min onto an 8-ml Mono S column (GE HealthCare) equilibrated with buffer A containing 300 mM NaCl. The column was washed with 10 mL of starting buffer, 10 mL of buffer A containing 350 mM NaCl, and 10 mL of buffer A containing 400 mM NaCl, and then eluted with a 20-mL gradient of NaCl (400–1,000 mM) in buffer A at 2 mL/min. RFC peak fractions, which eluted at 660 mM NaCl, were diluted with an equal volume of 100 mM KPO_4, pH 7.5, and loaded at 2.5 mL/min onto a 2-mL CHT2-I column (Bio-Rad) equilibrated with solution A [5% glycerol (wt/vol), 0.01% Nonidet P-40, 100 mM NaCl] containing 50 mM KPO_4, pH 7.5. After 5-ml wash with equilibration buffer, the column was developed with 5 ml of solution A containing 140 mM KPO_4, pH 7.5, 5 mL of solution A containing 185 mM KPO_4, pH 7.5, 5 mL of solution A containing 230 mM KPO_4, pH 7.5, 5 mL of solution A containing 275 mM KPO_4, pH 7.5, and a 5-mL linear gradient of KPO_4, pH 7.5 (275–500 mM) in solution A. RFC peak fractions, which eluted ≈255 mM KPO_4, were diluted with buffer A to a conductivity equal to that of 100 mM NaCl and loaded at 1.25 mL/min onto a 1-ml Mono Q column (GE HealthCare), equilibrated with buffer A containing 100 mM NaCl. After 5-ml wash with starting buffer the column was developed at 1 mL/min with 5 mL of buffer A containing 150 mM NaCl, 5 mL of buffer A containing 200 mM NaCl, 5 mL of buffer A containing 250 mM NaCl, and 5 mL of buffer A containing 300 mM NaCl, followed by a 5-mL linear gradient of NaCl (300–500 mM) in buffer A. RFC fractions, which eluted at ≈300 mM of NaCl, were diluted with buffer A to a conductivity equivalent to 250 mM NaCl and loaded at 1 mL/min onto a 1-mL Mono S column (GE HealthCare), equilibrated with buffer C [20 mM Hepes-KOH (pH 7.4), 0.5 mM EDTA, 5% glycerol (wt/vol), 0.01% Nonidet P-40] containing 200 mM KCl. After 5-ml wash with the starting buffer the column was eluted at 1 mL/min with 5 mL of buffer C containing 300 mM KCl, and at 0.5 mL/min with 2.5 mL of buffer C containing 350 mM KCl, 2.5 mL of buffer C containing 400 mM KCl, followed by a 5-mL linear gradient of KCl (400–1,000 mM) in buffer C. RFC peak fractions, which eluted at ≈370 mM KCl and were ≈95% pure, were pooled, quick-frozen in liquid N_2 in small aliquots, and stored at −80 °C.

SF9 cells (1×10^6 cells/mL) were infected with p125-p12 and p50-His_6-p66 baculoviruses at multiplicity of infection of 12 each. After 48 h at 27 °C, cells were collected by centrifugation ($1,160 \times g$, 10 min) and frozen in liquid N_2. Frozen cells equivalent to 2.4 L of culture were resuspended in 50 mL of 20 mM Hepes·KOH (pH 7.4), 0.5 mM EDTA, 0.01% Nonidet P-40, 5% glycerol (wt/vol), 150 mM NaCl, 2 μg/mL aprotinin, 4 μg/mL leupeptin, 1 μg/mL E64, 1.4 μg/mL pepstatin, 0.2 mM PMSF, and 2 mM DTT, and then subjected to 5 strokes of pestle B in the dounce homogenizer. Insoluble material was removed by centrifugation at $35,000 \times g$ for 20 min. The supernatant was adjusted with 5 M NaCl to a conductivity equivalent to that of 300 mM NaCl and loaded at 3–4 mL/min onto a 10-ml Heparin column (GE HealthCare), equilibrated with the buffer C containing 300 mM NaCl. After wash with 10 mL of starting buffer, 10 mL of buffer D [25 mM KPO_4 (pH 7.0), 5% glycerol (wt/vol), 0.01% Nonidet P-40] containing 350 mM NaCl and with 10 mL of buffer D containing 400 mM NaCl, the column was eluted with 20-ml linear gradient of NaCl (400–1,000 mM) in buffer D at 1 mL/min. DNA polymerase δ peak fractions, which eluted ≈760 mM NaCl, were dialyzed against buffer E [20 mM KPO_4 (pH 7.0), 5% glycerol (wt/vol), 0.01% Nonidet P-40, 0.5 mM EDTA] containing 100 mM NaCl for 2.5 h to a conductivity equivalent to that of 210 mM NaCl. After clarification by centrifugation at $27,000 \times g$ for 10 min, the fraction was diluted with buffer E to a conductivity equal to that of 100 mM NaCl and loaded at 1.5 mL/min onto a 1-mL MonoQ column (GE HealthCare) equilibrated with buffer E containing 100 mM NaCl. After wash with 5 ml of equilibration buffer and 5 ml of buffer E containing 150 mM NaCl, the column was eluted with a 25-ml linear gradient of NaCl (150–500 mM) in buffer E at 1 mL/min. DNA polymerase δ peak fractions, which eluted at 200 mM NaCl, were mixed with 2 mL of Co^{2+}-charged agarose (Clontech) equilibrated with a buffer containing 20 mM KPO_4 (pH 7.0), 5% glycerol (wt/vol), and 100 mM NaCl (4 mM β-mercaptoethanol was substituted for DTT in buffers for Co^{2+}-charged agarose chromatography). The mixture was slowly mixed by rotation for 1 h and then transferred into an empty column. After a 5-mL wash with equilibration buffer, the column was eluted with a 20-mL linear gradient of imidazole, pH 7.5 (0–500 mM) in equilibration buffer at 0.5 mL/min. DNA polymerase δ peak fractions were pooled and loaded onto a 1-mL Heparin column (GE HealthCare) equilibrated with the buffer C containing 300 mM NaCl at flow rate of 3 mL/min. After 5-ml wash with starting buffer the column was eluted with 5 mL of buffer C containing 350 mM NaCl, 5 mL of buffer C containing 400 mM NaCl, and 5 mL of buffer C containing 450 mM NaCl, followed by a 5-ml linear gradient of NaCl in buffer C (450–1,000 mM) at 1 mL/min. DNA polymerase δ peak fractions, which eluted at 560 mM NaCl, were diluted with an equal volume of 100 mM KPO_4, pH 7.5, and loaded at 1 mL/min onto a 2-ml CHT2-I column (Bio-Rad) equilibrated with solution A containing 20 mM KPO_4, pH 7.5. The column was eluted at 1 mL/min with 5 mL of solution A containing 50 mM KPO_4, pH 7.5, 5 mL of solution A containing 100 mM KPO_4, pH 7.5, 5 mL of solution A containing 150 mM KPO_4, pH 7.5, 5 mL of solution A containing 200 mM KPO_4, pH 7.5, and a 20-ml linear gradient of the KPO_4, pH 7.5 (200–300 mM) in solution A. DNA polymerase δ peak fractions, which eluted at ≈200 mM of the KPO_4 buffer, were diluted to conductivity equal that of 100 mM KCl with buffer C and loaded onto a 1-ml Mono S column (GE HealthCare), equilibrated with buffer C containing 100 mM KCl. After a 5-ml wash with starting buffer the column was eluted at 1 mL/min with 5 mL of buffer C containing 150 mM KCl, 5 mL of buffer C containing 200 mM KCl, and 5 mL of buffer C containing 250 mM KCl, followed by a 15-ml linear gradient of KCl (250–500 mM) in buffer C. DNA polymerase δ peak fractions, which eluted at ≈270 mM KCl and were 91% pure, were pooled, quick-frozen in liquid N_2 in small aliquots, and stored at −80 °C.

Protein concentrations were determined by using the BioRad Protein Assay with BSA (Pierce) as standard and are expressed as moles of heterodimer for MutSα and MutLα, monomer for ExoIb, heterotrimer for RPA, homotrimer for PCNA, heteropentamer for RFC, and heterotetramer for DNA polymerase δ.

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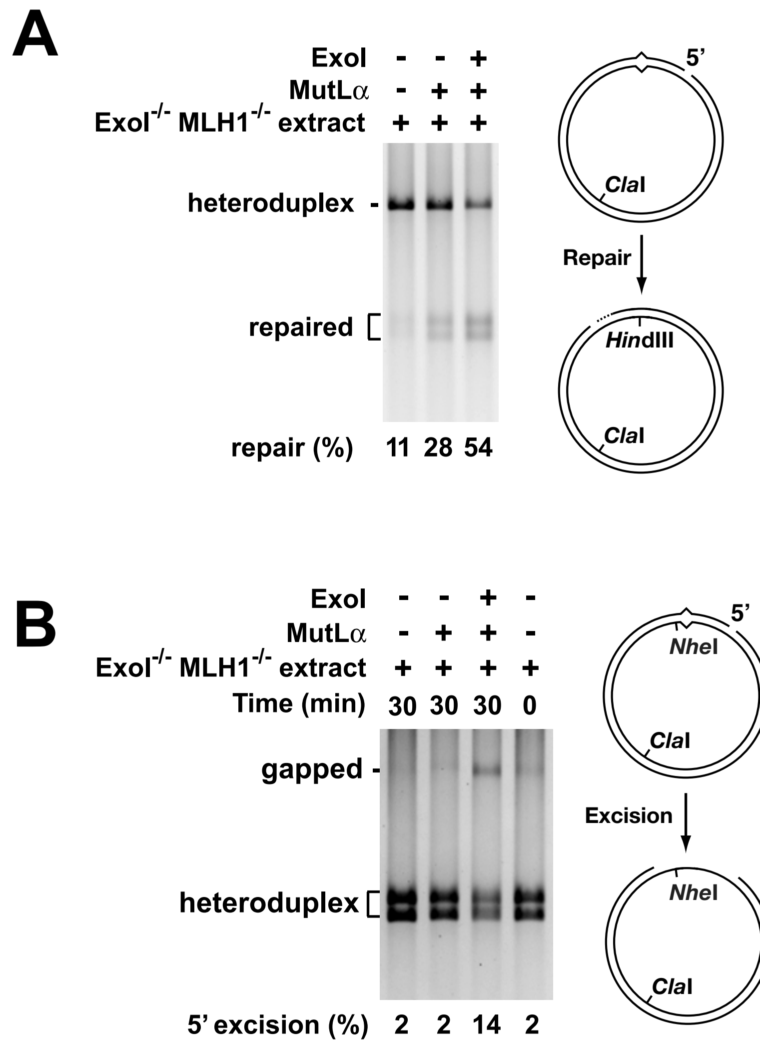

Fig. S1. ExoI-independent 5′-directed mismatch repair extracts of MEFs. Mismatch repair and mismatch-provoked excision reactions using extracts of *Mlh1*$^{-/-}$ *ExoI*$^{-/-}$ MEF cells were performed as described for Fig. 1 *A* and *B* except that the substrate was a 5′-G–T heteroduplex.

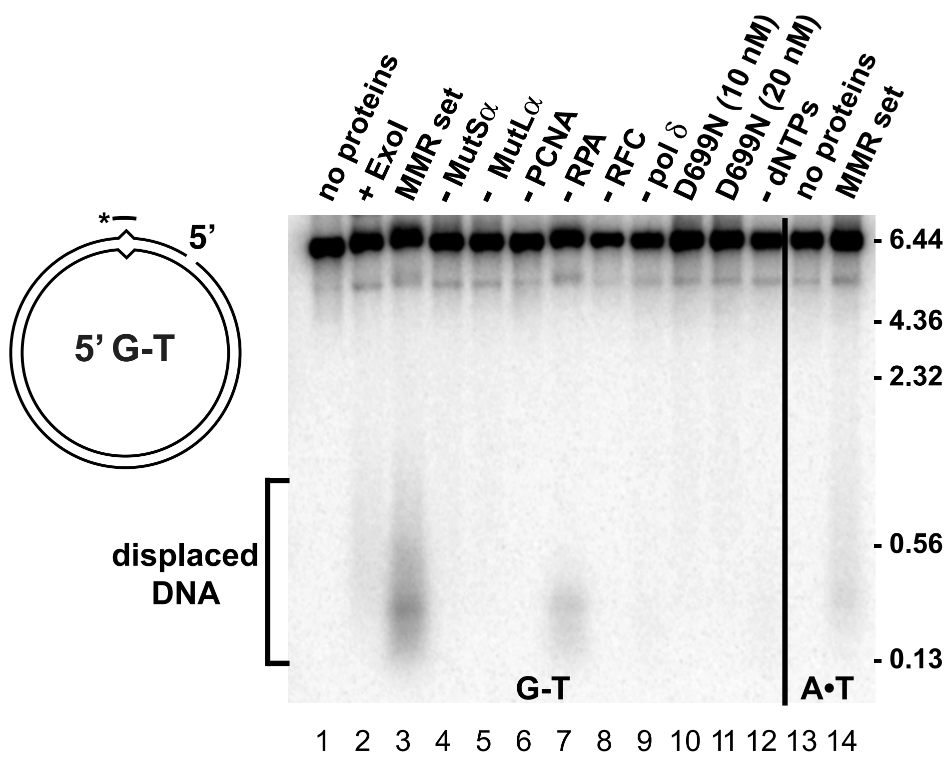

Fig. S2. Mismatch-dependent, synthesis-driven displacement of DNA segments from a 5′ G–T heteroduplex. Experimental conditions and labels are identical to those of Fig. 4 except that the substrate was a 5′ G–T heteroduplex.

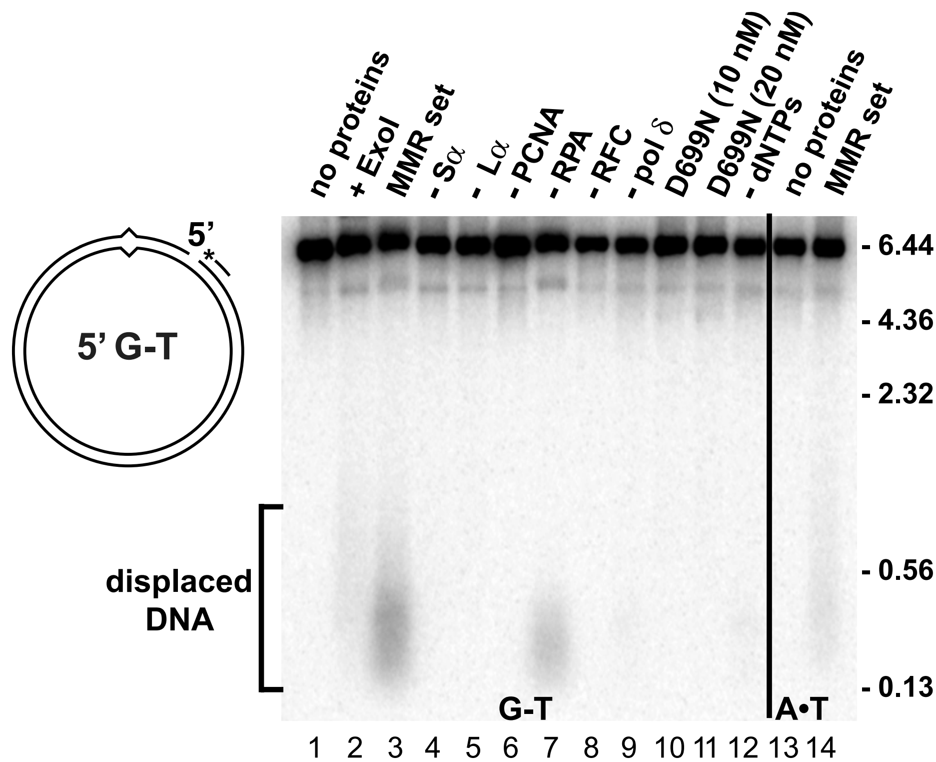

Fig. S3. Mismatch-dependent, synthesis-driven displacement of DNA segments from a 5′-G–T heteroduplex. Reactions containing 5′-G–T heteroduplex DNA (or A·T homoduplex control DNA, lanes 13 and 14) were as in Fig. 4. The ^{32}P-labeled oligonucleotide probe [d(tcg ccctgatagacggtttttcgc)] was complementary to the incised DNA strand adjacent to the strand break as illustrated in the diagram on the left.

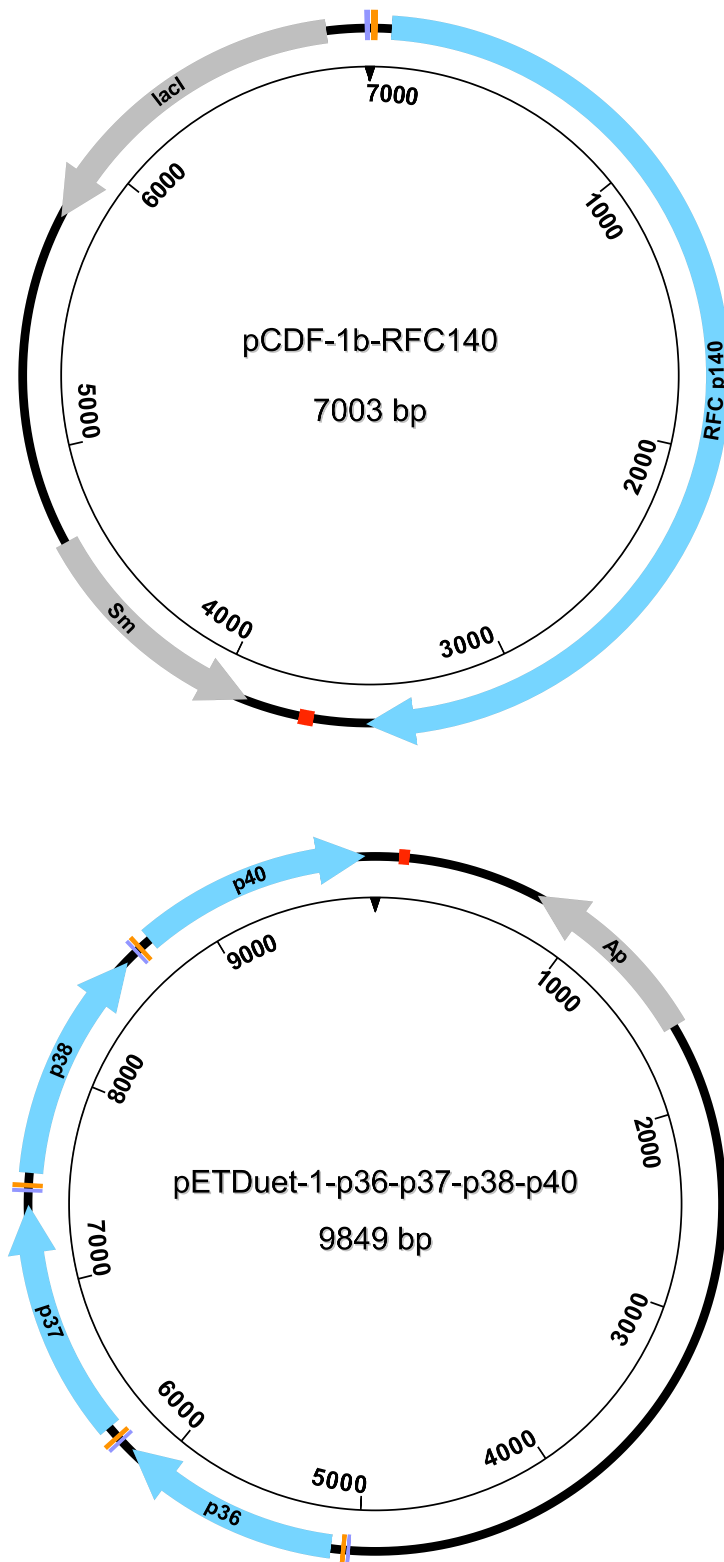

Fig. S4. Plasmids for expression of human RFC in *E. coli*. A synthetic gene for human RFC140 optimized for *E. coli* expression was inserted into NcoI and BamHI-cleaved pCDF-1b (Novagen) downstream from T7 promoter (purple box), *lac* operator (orange box), and a strong ribosome binding site. The red box downstream of the p140 gene is a T7 transcription terminator. Synthetic genes corresponding p36, p37, p38, and p40 human RFC subunits were inserted as modules into pETDuet-1 (Novagen) so that expression of each is under the control of a T7 promoter (purple boxes), *lac* operator (orange boxes), and a strong ribosome binding site.

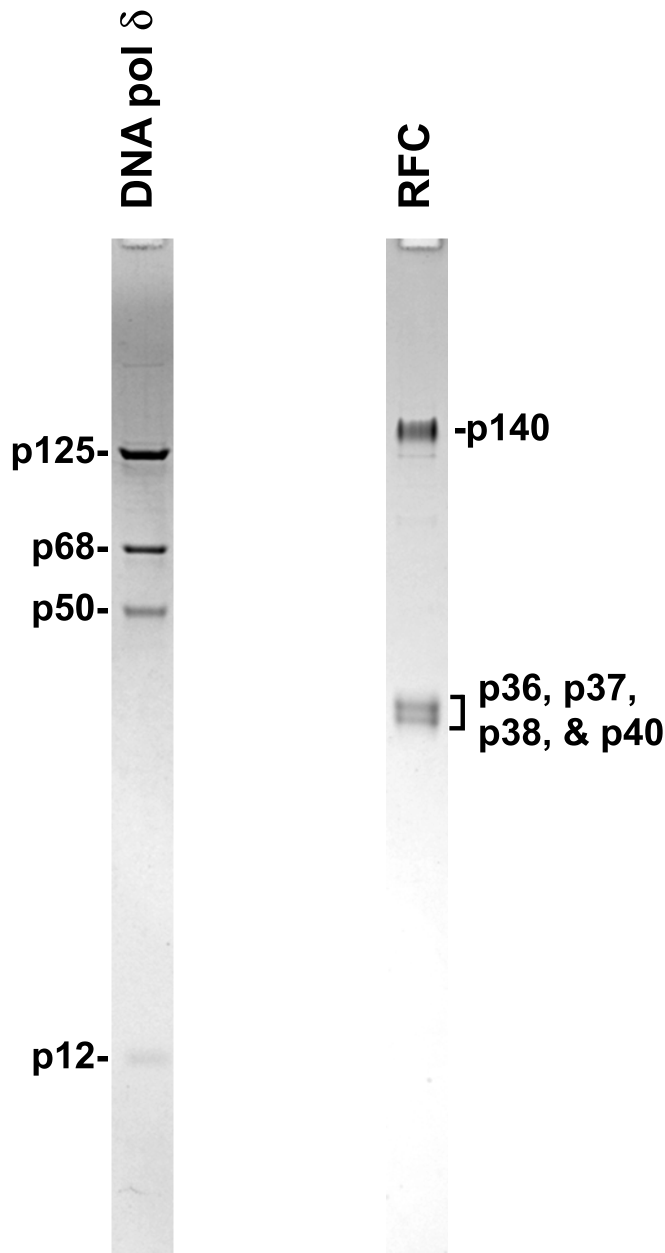

Fig. S5. DNA polymerase δ and RFC isolates used in this study. Purified DNA polymerase δ (0.2 μg) and RFC (0.5 μg) were analyzed by electrophoresis on 10% SDS gels.