Human Exonuclease I Is Required for 5' and 3' Mismatch Repair*

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We have partially purified a human activity that restores mismatch-dependent, bi-directional excision to a human nuclear extract fraction depleted for one or more mismatch repair excision activities. Human EXOI co-purifies with the excision activity, and the purified activity can be replaced by near homogeneous recombinant hEXOI. Despite the reported 5' to 3' hydrolytic polarity of this activity, hEXOI participates in mismatch-provoked excision directed by a strand break located either 5' or 3' to the mispair. When the strand break that directs repair is located 3' to the mispair, hEXOI- and mismatch-dependent gap formation in excision-depleted extracts requires both hMutSα and hMutLα. However, excision directed by a 5' strand break requires hMutSα but can occur in absence of hMutLα. In systems comprised of pure components, the 5' to 3' hydrolytic activity of hEXOI is activated by hMutSα in a mismatch-dependent manner. These observations indicate a hydrolytic function for hEXOI in 5'-heteroduplex correction. The involvement of hEXOI in 3'-heteroduplex repair suggests that it has a regulatory/structural role in assembly of the 3'-excision complex or that the protein possesses a cryptic 3' to 5' hydrolytic activity.

Human cells possess a strand-specific mismatch repair system that is similar to that of *Escherichia coli* and depends on structural and functional homologs of bacterial MutS and MutL (1–4). Inactivation of genes that encode the mammalian MutS homologs MSH2 or MSH6 or the MutL homologs MLH1 or PMS2 confers genetic instability and a predisposition to tumor development. Eleven activities have been implicated in *E. coli* methyl-directed mismatch repair, and the reaction has been reconstituted in a pure system (5–8). However, our understanding of the reaction in higher cells is limited.

Analysis of the human reaction in nuclear extracts, using model heteroduplexes in which a strand-specific single strand break directs repair to the incised DNA strand (9, 10), has indicated that the reaction occurs in several steps by a mechanism similar to that of *E. coli* mismatch correction (11, 12). Repair is initiated via mismatch recognition by hMutSα (the hMSH2-hMSH6 heterodimer) (13, 14) or hMutSβ (hMSH2-hMSH3 heterodimer) (15–17). hMutLα (hMLH1-hPMS2 heterodimer) and PCNA are also required during the earliest stages of the reaction since inactivation of either of these activities blocks repair at or prior to initiation of excision (18–20), which removes that portion of the incised strand spanning the strand break and the mispair (21, 22). Subsequent repair DNA synthesis depends on DNA polymerase δ and PCNA (23, 24). hRPA, the human single-stranded DNA binding protein, has also been implicated in mismatch repair (25), but the stages of the reaction during which this protein functions have not been defined.

The excision step of *E. coli* methyl-directed mismatch correction depends on DNA helicase II, as well as several 3' to 5' and 5' to 3' exonucleases that display specificity for single-stranded DNA (6–8, 26). Several activities have been implicated in the initiation and repair synthesis steps of eukaryotic mismatch repair, but the nature of the excision step of the reaction is less well understood. In yeast, deficiency of the 5' to 3' exonuclease EXOI (27) confers a weak mutator phenotype (28, 29) that has been assigned to the MSH2 epistasis group (29, 30). The enhancement of mutability by *exol* null defects is modest as compared with that observed upon MSH2 inactivation, but this could be due to redundant exonuclease involvement as in the bacterial reaction (29). In fact, Amin et al. (31) have recently demonstrated a synergistic potentiation of mutation rates when yeast *exol* defects are combined with weakly mutagenic, missense alleles in genes that encode MLH1, PMS1, MSH2, PCNA, or DNA polymerase δ. Since all of these activities have been implicated in mismatch repair (1–4), these findings have been interpreted in terms of direct involvement of yeast EXOI in the reaction. Yeast EXOI has also been shown to interact physically with yMSH2 and yMLH1 (29, 32), and the human EXOI homolog (33–35) interacts with hMSH2, hMSH3, and hMLH1 (36), implicating the exonuclease in one of the several genetic stabilization pathways that depend on these mismatch repair proteins.

Analysis of dinucleotide repeat instability in yeast suggested that the RAD27 5' to 3' exonuclease might also participate in mismatch repair (37); however, more recent studies indicate that the contribution, if any, of this activity to mismatch reclassification is limited (38, 39). Yeast genetic studies have also led to the suggestion that the 3' to 5' editing exonucleases of DNA polymerases δ and ε may participate in the excision step of mismatch repair (40), but this conclusion has also been questioned based on the finding that the mutator phenotype of the *pol3–01* mutant used in this study is largely due to a checkpoint defect rather than mismatch repair deficiency (41).

To further clarify the nature of the excision activities involved in the mammalian reaction, we have developed an *in vitro* assay for mismatch-provoked excision. Utilizing this

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¶ The abbreviations used are: PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol.
method we have demonstrated that hEXOI is required for mismatch repair directed by a strand break located either 3′ or 5′ to the mispair, that in vitro excision directed a 3′ or 5′ strand signals differ in their requirement for hMutLa, and that 5′ to 3′ hydrolysis by hEXOI is activated in a mismatch- and hMutSo-dependent manner in a pure system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Nuclear Extracts**—Cell lines HeLa S3, H6 (a subclone of HCT116), and MT1 were grown, and nuclear extracts were prepared as described previously (9, 15, 42). Insect cells (SF9, Invitrogen) were cultured, infected with baculovirus constructs bearing hMutLa subunits or hEXOI, and processed for protein purification as described (43). HeLa S3 nuclear extracts (9) were concentrated by precipitation with 420 glit ammonium sulfate, dialyzed against buffer A (0.05 M Hepes-POH, pH 7.5, 0.1 mM EDTA, 0.1% (v/v) phenylmethylsulfonyl fluoride (Sigma, relative to a saturated stock in isopropanol), 1 μg/ml leupeptin (Peptides International), 0.5 μg/ml E-64 (Peptides International), 0.1 μg/ml aprogin (USB)) containing 100 mM KCl and 2 mM dithiothreitol (DTT), quick-frozen in liquid nitrogen, and stored at –80 °C.

**Preparation of Excision-depleted Nuclear Extracts and Protein Isolation**—All procedures were performed at 4 °C. Protein was determined by Bradford detergent assay using bovine serum albumin as standard or by absorbance at 280 nm.

For preparation of excision-depleted extracts, MT1 (MSH6+/−) and H6 (MLH1−/−) nuclear extracts (about 200 mg of protein) were adjusted to a conductivity equivalent to 0.225 mM KCl by the addition of 3 mM KCl and loaded onto 10 ml of P-11 phosphocellulose (Whatman) equilibrated with buffer A containing 0.225 mM KCl and 1 mM DTT. The column was washed with 20 ml of starting buffer, and fractions containing protein that passed through the column were pooled. After a wash with 50 ml of buffer A containing 0.375 mM KCl and 1 mM DTT, the column was step-eluted with buffer A containing 1 mM KCl and 1 mM DTT. Protein-containing fractions passing through the column in 0.225 mM KCl were combined with those eluting in the 0.375–1 mM KCl step. This material, which is depleted of mismatch-provoked excision activity, was concentrated, dialyzed, and stored as described above for HeLa nuclear extract. In addition to removal of excision activity, this depleting procedure also results in partial removal of hMutSa and hMutLa. To ensure that these required activities were not limiting, depleted extract fractions were supplemented with a mixture of homogenous hMutLa or hMutSa prior to use. Thus, excision-depleted extract from MSH6−/− MT1 cells was supplemented with hMutLa (0.75 μg/mg protein extract), and the excision activity of MSH6−/− extract. Excision-depleted extract from MLH1−/− H6 cells was supplemented with hMutSa (3.1 μg/mg) in a similar manner.

hMutSa was purified to homogeneity from HeLa cells by a minor modification of the previously described method (13). Recombinant hMutLa was purified to homogeneity from a cleared supernatant prepared from insect cells infected with baculovirus constructs that express hMLH1 and hPM2S2 (43) using chromatography on phosphocellulose, hydroxyapatite, and MonoQ as described for isolation of HeLa hMutLa (19). To remove insect excision activities, the phosphocellulose eluate was passed over single-stranded DNA-cellulose in 0.20 mM KCl prior to loading onto hydroxyapatite.

Recombinant hEXOla and hEXOlb were isolated from SF9 cells infected with baculovirus-expressing constructs, which were prepared using the PFASTBAC system (Invitrogen) according to the manufacturer’s recommendations. hEXOla cDNA (EST433001, Ref. 33), kindly provided by R. Kolodner, was digested with EcoRV and KpnI and ligated into PFASTBAC 1 cut with Stul and KpnI. The resulting construct contains the complete open reading frame of the shorter, 803-amino acid splice variant designated hEXOla (33). PCR mutagenesis was used to generate the corresponding PFASTBAC 1 derivative of the longer, 844-residue splice variant, hEXOlb (33).

Insect cells were infected with baculovirus expressing hEXOla or hEXOlb, and cleared supernatants were prepared as described previously (43). For hEXOla preparation, cleared supernatant (150 μg of protein) was loaded onto a 2-ml Heparin HiTrap column (Amersham Biosciences) equilibrated with buffer B containing 0.1 M KCl, 1 mM DTT, and protease inhibitors as noted above. After washing with 10 ml of starting buffer, the column was eluted with a 10-step KCl gradient (0.22–0.40 M, 10-ml, and 0.02 M KCl increase per step). Fractions containing excision activity, which eluted about 0.33 M KCl, were quick-frozen in liquid nitrogen and stored at –80 °C (Fraction III). Fraction III was thawed, dialyzed against 1 liter of buffer B containing 0.20 M KCl and 1 mM DTT and supplemented with protease inhibitors as above (Fraction IV). Fraction IV was dialyzed against 2 liters of buffer B containing 0.20 M KCl and 1 mM DTT until the conductivity was equivalent to 0.3 M KCl and loaded onto a 1-ml MonoQ FPLC column (HR 5/5, Amersham Biosciences) equilibrated with buffer B containing 0.10 M KCl. After a wash with 20 ml of starting buffer, the column was eluted with a 14-step KCl gradient (0.15–0.80 M, 10-ml, and 0.02 M KCl increase per step). Fractions containing excision activity, which eluted about 0.33 M KCl, were quick-frozen in liquid nitrogen (Fraction V). Fraction V was thawed, dialyzed against 1 liter of buffer B containing with protease inhibitors and containing 0.075 M KCl and 2 mM DTT until the conductivity was equivalent to 0.1 M KCl and then loaded onto a 1-ml MonoQ FPLC column (HR 5/5, Amersham Biosciences) equilibrated with buffer B containing 0.10 M KCl, and protease inhibitors as above (Fraction VI). After a wash with 20 ml of starting buffer, the column was eluted with a 14-step gradient of KCl (0.10–0.50 M KCl). Active fractions, which eluted about 0.26 M KCl, were pooled and supplemented with protease inhibitors as above, aliquoted, quick-frozen in liquid nitrogen, and stored at –80 °C (Fraction VII). The purification is summarized in Table I.

**DNA Substrates and Mismatch-directed Activity Assays**—For mismatch-directed activity assays described previously (9) except that reaction volume was increased to 20 μl, salt concentration was adjusted to 0.1 M KCl, and incubation was for 15 min at 37 °C. Assay of mismatch-dependent gap formation was performed in the same manner except that dNTPs were omitted and incubation was reduced to 5 min. Reactions contained 100 ng (23 fmol) of a bacteriophage β-3 derived G-T or C-A, insertion/deletion heteroduplex (Fig. 1 and Ref. 21) or an otherwise identical A-T homoduplex, and as indicated, 64 μg of excision-depleted MSH6−/− or MLH1−/− extract, 200 ng of hMutSa, 50 ng of hMutLa, and purified excision activity or recombinant hEXOI. DNA recovered from gap formation reactions was digested with 2 units each of Nhel and Bsp106I, and restriction products were resolved by electrophoresis through 1% agarose gels, which were stained with ethidium bromide. DNA was quantitated by ethidium fluorescence using a cooled, photometric grade CCD imager (Photometrics). As summarized in Fig. 1, gap formation activity is defined as the amount of substrate linearized by Bsp106I but resistant to cleavage by Nhel. Mapping of excision tract end points was performed by indirect end-labeling as described (21) except that results were visualized and quantitated using a PhosphorImager (Molecular Dynamics, Inc.).

Cells were transiently transfected as described previously (9) except that transient transfections were performed in the absence of the expression vector encoding hMutLa. Cells were transfected with multiple antigen peptide conjugated peptides, and sera were obtained using methods described before (17). Antibodies were raised against hEXOI-derived peptide 1 (TLPSSKEKVERSRRQQANL, amino acids 81–100) and peptide 2 (RASGLSKPKASQRRKHNA, amino acids 762–781). The resulting antibodies recognize both hEXOla and hEXOlb and are referred to as AB-1 and AB-2, respectively. Antibodies raised against onto a 1-ml MonoS column (HR 5/5, Amersham Biosciences) equilibrated with buffer B containing 0.19 M KCl. This column was washed and developed using the same protocol as for the Heparin column. hEXOla fractions, which eluted about 0.4 M KCl, were pooled, supplemented with 2 mM DTT and protease inhibitors as described above, quick-frozen in liquid nitrogen, and stored at –80 °C.

hEXOlb was purified by the same method except that the insect cell-cleared lysate (about 300 mg of protein) was first loaded onto a 15-ml Q-Sepharose column equilibrated with buffer B containing 0.15 M KCl, 1 mM DTT, and protease inhibitors as noted above. After washing with 75 ml of starting buffer, the column was eluted with a gradient from 0.0 to 0.4 M KCl in buffer B containing 1 mM DTT and supplemented with protease inhibitors as above, hEXOlb-containing fractions, which eluted at 0.27 M KCl, were pooled, diluted 2-fold with buffer A containing 10% (v/v) glycerol, and subjected to fractionation on Heparion Hitrap and MonoS columns as described above. hEXOlb eluted at ~0.3 M KCl on both of the latter columns. Final purities of hEXOla and hEXOlb obtained in this manner were ~95% (Fig. 4).
peptide 3, which is only present in hEXOIb (KKPLSPVRDNIQLTPEAEED, residues 811–830), are referred to as AB-3. Preimmune and specific IgG fractions were obtained by purification on protein A-Sepharose (Amersham Biosciences). Specific IgGs were further purified on affinity supports prepared by cross-linking individual peptide antigens to Reacti-Gel 6X (Pierce). For the cross-linking, 25 mg of peptide was incubated with 1 ml of activated resin in 0.1 M sodium carbonate, pH 10, for 24 h at 4 °C. The reaction was stopped by incubation with 1 M ethanolamine, pH 10, for 24 h. After extensive wash, peptide columns were loaded with the protein A-purified IgG and incubated overnight at 4 °C. The columns were washed extensively and then eluted with 0.05 M glycine, pH 2.5, and 0.15 M NaCl. Fractions were immediately neutralized with 0.1 volume of 1 M Tris-HCl, pH 8.0, pooled, dialyzed against 0.025 M Hepes-KOH, pH 7.5, 0.15 M KCl, aliquoted, quick-frozen in liquid nitrogen, and stored at −80 °C. AB-1, AB-2, and AB-3 used in the work described below were all affinity-purified in this manner.

Gel electrophoresis and Western blotting were performed as described previously (17). For immunodepletion, 20 μg of AB-2 was bound to 2 μl of protein A-Sepharose suspension and incubated with HeLa nuclear extract (0.45 mg) in a total volume of 15 μl on ice for 4 h. The resin was then removed by centrifugation, and the supernatant was used directly in gap formation or mismatch repair assay. In mock depletion experiments, AB-2 was replaced with preimmune IgG obtained from the same rabbit.

RESULTS

Partial Purification of a Mismatch Repair Excision Activity—Previous assays for mismatch-provoked excision are time-consum ing (20, 21) and unsuitable as a routine assay for isolation of the required excision components. To circumvent this problem we developed a rapid method that relies on the observation that excision tracts produced on an incised heteroduplex extend from the strand break to 90–170 nucleotides beyond the mismatch (21). When repair DNA synthesis is blocked, excision renders an NheI site just beyond the mismatch single-stranded and hence resistant to endonuclease cleavage. As shown in Fig. 1, gap formation scored by this NheI-resistant assay depends on the presence of a mismatch within the nicked, circular substrate.

Fractionation of HeLa nuclear extract indicated that one or more activities involved in the excision step of mismatch repair elute from phosphocellulose between 0.23 and 0.38 M KCl. This observation was exploited to prepare a crude fraction that was depleted of excision activity for use as receptor extract for isolation of excision activity by in vitro complementation (see “Experimental Procedures”). To avoid artifacts during fractionation, receptor extracts were prepared from mismatch-repair deficient MSH6−/−/MLH1−/− H6 cells (hMutSα-deficient) or MLH1−/−/H6 cells (hMutLo-deficient), and gap formation was scored as bona fide only if it required depleted extract, hMutSα (or hMutLo), and the partially purified fraction in question. Since the fractionation method for depletion of excision activity also results in partial removal of hMutSα and hMutLo, extracts prepared in this manner were supplemented with the appropriate activity to ensure that it was not rate-limiting for repair, i.e. depleted extract derived from MSH6−/−/MT1 cells was supplemented with hMutLo and is referred to here as hMutSa-deficient depleted extract, while that derived from MLH1−/−/H6 cells was supplemented with hMutLo and is referred to as hMutLo-deficient depleted extract (see “Experimental Procedures”).

An activity that restored mismatch-dependent gap formation to these excision-depleted extracts was purified 250-fold from HeLa nuclear extract. As summarized in Table I, gap formation activity on 3′- and 5′-heteroduplex substrates co-purified, with activity profiles on the two types of substrate co-eluting during the three-column steps (Fig. 3 and data not shown). The purified material (Fraction IV) was analyzed for its activity on homoduplex and several heteroduplex DNAs using excision-depleted extract derived from MSH6−/− or MLH1−/− cells. Only a background level of gap formation occurred with homoduplex DNA when incubated with hMutSa- or hMutLo-deficient depleted extract, and homoduplex background activity was enhanced only to a limited degree by supplementation with hMutSα, hMutLo, and purified excision activity (Fig. 2, open bars). By contrast, gap formation on 3′-G-T and 3′-CA heteroduplexes was dramatically enhanced in excision-depleted extract
**TABLE I**

Isolation of mismatch-provoked gap formation activity

Gap formation assays (see “Experimental Procedures”) contained 64 μg of excision-depleted extract derived from MLH1−/− H6 cells, 50 ng of hMutLo, and samples of fractions shown. Activities were determined from that portion of the assay curve where product formation was linear with added protein. Note that gap-forming activity on 3'- and 5'-heteroduplexes co-purify. P-Cell, phosphocellulose.

| Fraction       | Total protein | Specific activity | Yield |
|----------------|---------------|------------------|-------|
|                | mg            | fmol/mg          | %     |
| I. Extract     | 2320          | 30/85            | (100/100) |
| II. P-Cell     | 135           | 390/790          | 76/71 |
| III. DNA-Cell  | 7.7           | 3,400/7,200      | 37/36 |
| IV. MonoQ      | 1.6           | 8,300/16,000     | 19/17 |

**FIG. 2.** Gap formation activity supported by partially purified excision activity requires a mismatch. Reactions (see “Experimental Procedures”) contained as indicated 64 μg of hMutSo-deficient excision-depleted extract derived from MS6−/− MT1 cells or hMutLo-deficient depleted extract derived from MLH1−/− H6 cells, 200 ng of hMutSo, 50 ng of hMutLo, 0.6 μg of excision activity (MonoQ fraction), and 100 ng of homoduplex DNA (open bars), G-T heteroduplex (black bars), or CA, dinucleotide insertion/deletion heteroduplex (gray bars). Gap formation was scored as NheI-resistant DNA after digestion with NheI and Bsp106I (Fig. 1). Results shown in the upper panel were obtained with DNAs containing a nick in the viral DNA strand at the gpII cleavage site, while those shown in the lower panel used DNAs containing a single strand break in the complementary DNA strand at the Sau96I cleavage site. Activity on the CA, dinucleotide insertion/deletion heteroduplex observed in MS6−/− extracts upon supplementation with only purified excision activity may be due to MutSβ (MSH2-MSH3 heterodimer), which is absent in H6 cells due to MSH3 mutation (51)

**MSH6−/-** extracts upon supplementation with hMutSo and the purified excision activity, and a similar enhancement of gap formation on 3'-substrates was observed with MLH1−/- extract in the presence of excision activity and hMutLo. A comparable enhancement of gap formation on 5'-heteroduplexes that required both hMutSo and purified excision activity was observed with depleted extract derived from MS6−/− MT1 cells (Fig. 2, lower panel). However, 5'-heteroduplex results obtained with depleted extract derived from MLH1−/- H6 cells were surprising. In this case and in contrast to results obtained with 3'-substrates, mismatch-dependent gap formation required only depleted extract and the purified excision activity; hMutLo was not necessary. These observations suggest that although in vitro gap formation on a 5'-heteroduplex depends on a mismatch and requires hMutSo, this reaction can occur in the absence of hMutLo. Additional evidence supporting this view will be presented below.

**hEXOIb Co-purifies with Mismatch Repair Excision Activity**—The excision activity isolated by the procedure shown in Table 1 is not pure and attempts to further fractionate the material failed due to activity loss. However, we have identified a required excision activity present in this partially purified material. Previous work in Saccharomyces pombe and S. cerevisiae has demonstrated that inactivation of the EXOI gene, which encodes a 5’ to 3’ double-stranded exonuclease (27, 28), results in a modest increase in mutability (28, 29) that is epistatic to the larger increase in mutability associated with msh2 mutations (29, 30). cDNAs corresponding to two splice variants of a human EXOI homologue have been identified, both contain the putative exonuclease hydrolytic center (33–35), and the human EXOI polypeptide has been shown to interact with MTSH2 and MTH1 (36). The two hEXOI splice variants, hEXOIa and hEXOIb, encode polypeptides of 803 and 846 amino acids, respectively, and both contain the putative exonuclease hydrolytic center (33). The 802 amino-terminal residues of the two variants are identical, with the two polypeptides differing in their C-terminal 1 and 44 amino acids (33). Potential presence of hEXOI polypeptides in partially purified excision activity was evaluated by use of anti-peptide hEXOI polyclonal antibodies (see “Experimental Procedures”). Antibodies AB-1 and AB-2 recognize both splice variants, whereas AB-3 recognizes the unique C-terminal extension hEXOIb (see “Experimental Procedures”).

As shown in Fig. 3, hEXOI polypeptide(s) recognized by both AB-1 and AB-2 co-purify with excision activity, co-eluting with excision activity as a 114-kDa species during each chromatographic step (Fig. 3 and data not shown). Since antibody AB-3 is specific for hEXOIb, it is clear that this splice variant co-purifies with excision activity. We have been unable to determine whether hEXOIa also co-purifies with excision activity due to the fact that only a single positive band was observed in the appropriate size range on Western blots using antibody AB-1 that recognizes both splice variants (Fig. 3, lower panel); however, this may be due to the small difference in the electrophoretic mobilities of the two forms of the enzyme (see below). The AB-1-positive minor species (100 kDa) evident in phosphocellulose and DNA cellulose fractions is not hEXOIa and may be derived from hEXOIb by proteolysis since it was not detected in extracts and reacts with antibody specific for this variant.

**Recombinant hEXOI Supports Mismatch-directed Gap Formation**—To assess the role of hEXOI in the excision step of mismatch repair, cDNAs for hEXOIa and hEXOIb were expressed in a baculovirus system, and the two proteins were isolated in near homogeneous form (Fig. 4, “Experimental Procedures”). The electrophoretic mobilities of these two polypep-
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Fig. 3. hEXOI co-purifies with mismatch repair excision activity. Upper panel, gap formation activity was assayed across the MonoQ FPLC elution profile (see ‘Experimental Procedures’ and Table I). Reactions contained 64 μg of excision-depleted extract from MLH1−/− H6 cells, 50 ng of hMutLa, 1 μl of column fraction, and 100 ng of 3′- (closed circles) or 5′-G-T (open circles) heteroduplex. The inset depicts a Western blot across the gradient (5 μl, each fraction) probed with anti-hEXOI antibody AB-1 (see “Experimental Procedures”) that recognizes both a and b splice variants. The mobility of the AB-1-positive species corresponds to a molecular mass of 114 kDa. Lower panel, samples of extract (340 μg), phosphocellulose (24 μg), DNA cellulose (7 μg), and MonoQ (3 μg) fractions (Table I) were resolved on a 7.5% SDS acrylamide gel and transferred to a polyvinylidene difluoride membrane that was probed with antibody AB-1 that recognizes a and b splice variants (α-hEXOI, see ‘Experimental Procedures’). In a similar experiment DNA cellulose (7 μg), and MonoQ (3 μg) fractions were analyzed by Western blot using antibody AB-3, which recognizes only hEXOIb (α-hEXOIb). The smaller 100-kDa immune-positive polypeptide evident in phosphocellulose and DNA-cellulose eluates, and to a lesser extent in the MonoQ fraction, was presumably derived from hEXOIib by proteolysis during fractionation since it was not present in the extract.

tides in the presence of sodium dodecyl sulfate correspond to molecular mass of 115 kDa and 112 kDa, somewhat greater than those predicted from primary sequence (94 kDa for hEXOIb and 89 kDa for hEXOIa); however, the mobility of recombinant hEXOIb is essentially identical to that of antibody AB-1 and AB-3-positive, 114-kDa material in purified excision activity. The specificities of these two antibodies for the several forms of the protein were confirmed using near homogeneous preparations of the recombinant activities (Fig. 4, lower panel). Quantitative comparison of Western signals from HeLa extract with those obtained using known quantities of purified hEXOIb indicates that nuclear extract contains about 50–100 ng of hEXOIb/mg total protein (data not shown).

The possibility that recombinant hEXOI might substitute for purified excision activity was tested. As shown in Table II, 10 ng of near homogeneous hEXOIb was nearly as efficient in restoring mismatch-dependent gap formation to excision-depleted extracts as was 0.6 μg of the purified excision activity (Fig. 2), which contains 7–15 ng of hEXOI as judged by quantitative Western blot. As observed with the partially purified excision activity, hEXOIb-supported gap formation on a 3′-heteroduplex required both hMutSα and hMutLa, while efficient excision on a 5′-substrate was hMutSα-dependent but largely independent of the presence of hMutLa. Gap formation activity of depleted MLH1−/− extract was increased 12-fold by addition of hEXOIb, but the additional presence of hMutLa further enhanced activity by only 20%. Experiments like those summarized in Table II have shown that the recombinant form of the hEXOIa also supports mismatch-provoked excision (data not shown), implying that hEXOIa and hEXOIb splice variants are both active in this reaction.

Immunological Depletion of hEXOI Reduces the Efficiency of Gap Formation and Repair of Both 5′- and 3′-Heteroduplexes—The use of excision-depleted extracts in the experiments described above suggests that hEXOI is required for excision directed by a strand break located either 5′ or 3′ to the mismatch. However, this interpretation is compromised by the fact that the crude fractionation procedure used to produce depleted extract removes a large number of proteins. To further clarify the requirement for hEXOI in the human strand-specific repair reaction, affinity-purified, polyclonal anti-hEXOI peptide antibody AB-2 (see “Experimental Procedures”) was used to deplete HeLa nuclear extract of the activity. As compared with mock depletion with preimmune IgG, hEXOI immunodepletion reduced mismatch-provoked gap formation by 70% on 5′- and 3′-heteroduplexes and reduced the efficiency of mismatch repair on the two types of substrate by 50–60% (Table III). The somewhat lower reduction in the efficiency of mismatch repair may be due to the fact that incubation time for repair assays was three times that of excision assays. Supplementation with near homogeneous hEXOIb efficiently restored mismatch repair and mismatch-dependent gap formation to normal levels.

hEXOI Participates in Excision-directed by Strand Signals Located 3′ or 5′ to the Mismatch—hEXOI and hEXOIa have been shown to catalyze 5′ to 3′ hydrolysis of duplex DNA (27, 29, 35), but the results shown in Table II suggest that the
human enzyme is involved in mismatch-provoked excision directed by a strand break that can be located either 5′ or 3′ to the mismatch. To clarify the nature of the hEXOI-dependent excision events occurring on 3′- and 5′-heteroduplexes, excision tract end points were mapped using an indirect end-labeling procedure (21).

With a 5′-G-T heteroduplex (Fig. 5, left panel) excision tracts produced in HeLa nuclear extract extended from the substrate strand break to about 150 nucleotides beyond the mismatch (lane 5), as observed previously (21). As can be seen, the control AT homoduplex did not support production of this material (lane 6). Similar excision products were observed with excision-depleted extracts upon hEXOIb supplementation provided that extract derived from MSH6+/− cells was also supplemented with hMutSa (lane 8). By contrast, hEXOIb was sufficient to restore mismatch-dependent 5′-excision to MLH1+/− deficient extract; hMutLa was not required (compare lanes 2 and 3), confirming results to this effect obtained by the restriction protection assay described above.

A similar type of experiment with 3′-heteroduplex and homoduplex substrates is shown in the right panel of Fig. 5, but in this case HeLa nuclear extract was immunologically depleted for hEXOI using antibody AB-2 or mock-depleted using preimmune IgG. No significant excursion was evident on homoduplex DNA, but excursion products were produced on the G-T heteroduplex in mock-depleted nuclear extract (G-T panel of upper gel, lane 16). The yield of these products (with a mobility corresponding to an average length of about 2900 nucleotides) was reduced substantially when the extract was depleted of hEXOI using antibody AB-2 (lane 17) but was restored to normal levels upon supplementation with purified hEXOIb (lane 18). Thus, hEXOI participates in mismatch-provoked excision on a 3′-heteroduplex that occurs with a directionality that is formally 3′ to 5′.

hMutSa Activates the 5′- to 3′-Hydrolitic Activity of hEXOI in a Mismatch-dependent Fashion—As described above, mismatch-provoked gap formation on 3′-heteroduplexes in depleted extract requires hMutSa, hMutLa, and either purified excision activity or recombinant hEXOI. The gap produced under these conditions is localized to the shorter path spanning the mismatch and the strand break that directs repair. Upon cleavage with Bsp106I and NheI, this gapped product migrates just slightly faster than full-length linear DNA due to its NheI resistance and the presence of a small gap (Fig. 6, upper panel). Because hEXOI has been reported to interact with hMSH2 and hMLH1 (36), we have examined the effects of hMutSa and hMutLa on the hydrolytic activity of hEXOIb in a purified system. Surprisingly, incubation of heteroduplex with just hMutSa, hEXOI, and ATP (in the presence or absence of hMutLa) led to the mismatch-dependent production of a DNA product of unusual mobility (Fig. 6, lower panel). Production of this species (arrow), occurred at the expense of the larger of the two restriction fragments (asterisk) that results from cleavage of the heteroduplex with Bsp106I and NheI. This observation suggested that this DNA species was produced by mismatch-dependent, 5′- to 3′-hydrolisis from the strand break. Analysis of sensitivity to other restriction endonucleases was consistent with this view, and more direct evidence for this conclusion is presented below.

Although not readily evident in photographic reproduction...
shown in the lower panel of Fig. 6, background hEXOI hydrolysis resulted in a low level of similar products with both A-T homoduplex and with G-T heteroduplex, in the latter case in the absence of hMutS\(a\). Quantitation of this data indicates that presence of a mispair and hMutS\(a\) enhances the activity of hEXOIb 5–6-fold on these 3′-substrates. This analysis also suggests that hMutLa enhances hEXOIb activity on these DNAs, but this effect, which is independent of hMutS\(a\) and a mispair, is a modest 2-fold. As noted above, the hMLH1 subunit of hMutL\(a\) has been shown to interact physically with hEXOI (36).

The nature of the hydrolytic products produced in a pure system with nicked heteroduplex DNA in the presence of hEXOIb, hMutS\(a\), and hMutLa was examined using indirect end-labeling (21) to map the location of 5′ termini produced in the reaction (Fig. 7). A basal population of 3′-end-labeled hydrolytic products was observed in all cases where hEXOIb was present. However, the additional presence of hMutS\(a\), or both hMutS\(a\) and hMutLa, led to enhanced production of a higher mobility, relatively discrete population of fragments with 5′ termini that mapped to the vicinity of the Bsp106I cleavage site (arrows in Fig. 7). This restriction site is located 3200–3300 bp from the strand break in the two types of heteroduplex, indic...
that degradation of the incised strand was exclusively demonstrated that recovery of of incised heteroduplex strands in Fig. 7 were present in large former protein was also present (Fig. 7, compare hEXOIb, DNA product size was somewhat larger when the This suggests that hMutL heteroduplex-derived restriction fragment that was recovered in reduced yield when hMutS and hEXOI were both present (2nd and 4th in gel on the right). The presumed nature of the DNA products indicated by arrows is illustrated in the diagram on the right. For the experiment in the lower panel, values below each lane indicate the yield (in fmol) of the product indicated by the arrow.

cating that the extent of excision under these conditions is substantial. Although hMutLa did not visibly alter the yield of degradation products observed in the presence of hMutSα and hEXOIb, DNA product size was somewhat larger when the former protein was also present (Fig. 7, compare lanes 4 and 5). This suggests that hMutLa may to some degree modulate hMutSα-mediated hEXOI activation.

The basal activity of hEXOIb in the absence of hMutSα on 5′-substrates is about 3-fold greater than that observed with 3′-DNAs (Fig. 7 and data not shown), and a similar phenomenon is evident with 5′- and 3′-homoduplex DNAs in extracts (Tables II and III). The basis of this effect is not known, but it could be the consequence of sequence context differences at the strand breaks in the two types of substrate. The modest enhancement of hEXOI activity by hMutLα and hMutSα (50 ng for 3′-substrates and 5 ng for 5′-substrates) were present as indicated. Reactions were terminated and DNA products were denatured (restriction digestion with NheI and Bsp106I was omitted) and subjected to electrophoresis through 1% agarose in the presence of 50 mM NaOH. DNA was transferred to nylon membranes and indirectly end-labeled (21) using 32P-end-labeled oligonucleotides corresponding to complementary strand residues 5778–5780 (3′-substrates) or viral strand residues 5761–5777 (5′-substrates). The former probe (gray bar, upper map) hybridizes to the 3′-terminus of incised viral strand in 3′-substrates, while the latter (gray bar, lower map) hybridizes to the 3′-end of the incised complementary strand of 5′-DNAs. Recovery of 3′-terminal, hybridization probe-positive material was estimated by quantitation of total radiolabel in each lane. When normalized to total label present in lane 7 (DNA only), recovery of 3′-termini for lanes 2–6 was 103 ± 9% (upper gel, ± one standard deviation) and 100 ± 6% (lower gel), implying little if any hydrolytic removal of the 3′ terminus at the strand break in either heteroduplex. Arrows indicate the position of probe-labeled marker products (not shown) derived from unreacted 3′- and 5′-DNAs upon digestion with Bsp106I. These markers correspond to 3285 and 3210 nucleotides for 3′- (upper panel) or 5′- (lower panel) heteroduplexes, respectively. The asterisk in the lower panel indicates a species that was produced at elevated levels in the presence of hEXOI and hMutLa (lane 3).

Since the oligonucleotide probes used to label the 3′ termini of incised heteroduplex strands in Fig. 7 were present in large excess, the labeling intensities observed correspond to the recovery of these 3′-ends. Integration of hybridized label in the heteroduplex lanes shown in upper and lower panels of Fig. 7 demonstrated that recovery of 3′ termini in all reactions that contained hEXOI was essentially 100% as compared with control DNA incubated in the absence of protein. Coupled with the results above, this retention of incised strand 3′ termini implies that degradation of the incised strand was exclusively 5′ to 3′ irrespective of 3′ or 5′ orientation of the heteroduplex. This finding is consistent with the reported 5′ to 3′ polarities of yeast and human EXOI (27, 29, 35). Thus, while mismatch-, hMutSα-, hMutLa-, and hEXOI-dependent excision on a 3′-heteroduplex in crude extract occurs with a polarity that is formally 3′ to 5′ from the strand break, mismatch-, hMutSα-, and hEXOI-dependent excision on this substrate in a pure system occurs from the nick with 5′ to 3′ polarity, effects that are depicted schematically in Fig. 6. Furthermore, while hEXOI-dependent
excision in extracts is restricted to several hundred base pairs spanning the shorter path between the strand break and the mismatch (Fig. 5), excision by the mismatch- and hMutSα-activated form of hEXOI rapidly removes several thousand nucleotides (Fig. 7). These observations imply that the polarity and the extent of hEXOI-dependent excision during mismatch repair are regulated by as yet unidentified extract factors.

DISCUSSION

The experiments described here indicate that hEXOI participates in the excision step of the human strand-specific mismatch repair reaction, observations consistent with genetic studies in yeast that have implicated yEXOI in a mutation avoidance pathway that involves yMSH2, yMLH1, and yPMS2 (29–32) and the finding that hEXOI interacts with both hMSH2 and hMLH1 (36). Although yeast and human EXOI have been shown to be 5’ to 3’ exonuclease (27, 29, 35), the work described here indicates that the activity has important roles in both 5’- and 3’-heteroduplex repair. If excision on these two types of heteroduplex is assumed to be exonucleolytic initiating at the strand break as it does in the bacterial system (6–8, 44), excision in the human system will require both 5’ to 3’ and 3’ to 5’ hydrolytic activities. While hEXOI clearly possesses a 5’ to 3’ activity, its involvement in 3’-heteroduplex repair suggests that the enzyme has a cryptic 3’ to 5’ activity or that it is necessary for activation of a distinct activity that is responsible for excision on 3’-heteroduplexes.

Using near homogeneous components, we have found that hMutSα activates the 5’ to 3’ hydrolytic activity of hEXOI in a mismatch-dependent manner, an effect that is most simply understood in terms of a physical interaction of the two proteins on a heteroduplex. We have also observed a limited enhancement of hEXOI activity by hMutLα, but this effect does not require a mismatch. While these simple systems are of interest in terms of potential modes of mismatch-dependent activation of downstream repair activities, it is also clear that hEXOI participation in the repair reaction must be regulated by other factors that remain to be identified. Despite its reported 5’ to 3’ polarity, hEXOI is involved in the 3’ to 5’ excision reaction that occurs on 3’-heteroduplexes in nuclear extract. While hMutSα is sufficient to activate hEXOI hydrolysis on a 3’-heteroduplex in a pure system, excision under these conditions occurs with 5’ to 3’ polarity. Repair components other than hMutSα or hMutLα must therefore regulate the activity of hEXOI when the strand break that directs correction lies 3’ to the mispair. The hEXOI-dependent mismatch-provoked excision tracts produced in crude fractions on the substrates used here are several hundred nucleotides in length, spanning the shorter path between the strand break and the mismatch and terminating as a fairly discrete species about 150 nucleotides beyond the mispair (Fig. 5 and Ref. 21). By contrast, mismatch-dependent hydrolysis observed in the purified hMutSα-hEXOI system (in the presence or absence of hMutLα) extend several thousand nucleotides from the strand break. Thus, in addition to orientation-dependent regulatory effects, unidentified repair components also control the extent of hEXOI-dependent excision on 5’- and 3’-heteroduplexes. The identity of these factors is under study.

In these studies we have used nuclear extract depleted of hEXOI by fractionation or immunological removal. Depleted extract obtained by fractionation retains 10–20% of the mismatch-provoked excision activity observed in hEXOI-supplemented reactions (Fig. 2 and Table I) or in undepleted extract fractions (data not shown), while immunologically depleted extract retains 30% residual activity (Table III). It is not clear whether this residual is due to low levels of remaining hEXOI in the depleted fractions or whether alternate activities can also support excision, as is the case in the bacterial system where redundant exonuclease involvement has been demonstrated (6–8). The fact that EXOI-deficient S. cerevisiae strains are considerably less mutable than msh2 mutants (29, 30, 32) may indicate that alternate activities can support the excision step in this lower eukaryote as well. However, E. coli strains deficient in all four of the exonucleases required for the bacterial reaction are similarly less mutable than MutS- or MutL-deficient strains (8), an effect that has been attributed to under-recovery of mutants due to chromosome loss in the exonuclease-deficient background (7). The possibility that the low mutability of yeast exoI mutants might be due to a similar phenomenon has not been addressed.

Recent genetic studies in S. cerevisiae have suggested several roles for yEXOI in mismatch repair. Synergistic potentiation of mutation rates has been observed in double mutants harboring exoI mutations and weakly mutagenic alleles of genes encoding yMLH1, yPMS1, yMSH2, yPCNA, and yeast DNA polymerase δ (31), all of which have been implicated in eukaryotic mismatch repair (1–4). In addition to its hydrolytic function, these effects have been interpreted in terms of a structural role for the yEXOI polypeptide in stabilization of a multiprotein mismatch repair assembly (31). Overexpression of yEXOI has been shown to suppress the conditional phenotypes associated with msh2-L560S mutation, whereas overexpression of mutant forms of the protein containing amino acid substitutions within the conserved nuclease domain do not. Based on these observations, Sokolsky and Alani (30) have concluded that yEXOI has a catalytic role in yMSH2-dependent mismatch repair. Synergistic mutability effects have also been observed between S. cerevisiae exoI mutants and yMutLα ATPase mutants with amino acid substitution in mlh1 and pms1 ATPase motifs, findings that have also been interpreted in terms of a hydrolytic role for yEXOI (32). Our results are consistent with hydrolytic and structural functions for hEXOI in mismatch repair. The finding that hEXOI is activated by hMutSα in a mismatch-dependent manner strongly suggests that the protein functions as an exonuclease in the repair reaction. Although we cannot rule out the possibility that the protein possesses a cryptic 3’ to 5’ hydrolytic activity, the hEXOI requirement for 3’-heteroduplex repair could be indicative of a structural or regulatory function of the hEXOI polypeptide in correction of this type of substrate.

The use of excision-depleted extracts from MSH6−/− and MLH1−/− cells has shown that while mismatch-provoked excision on 3’-heteroduplexes requires both hMutSα and hMutLα, excision on 5’-heteroduplexes depends on hMutSα but can occur in the absence of hMutLα (Fig. 2). Since the biochemical depletion procedure used to prepare these extracts results in removal of a large set of proteins, it is possible that this observation is an artifact of depleted extract preparation. However, normal excision tracts are produced in such extracts upon supplementation with hEXOI in the absence of hMutLα (Fig. 5, left panel).

There is also a precedent for hMutLα-independent 5’-heteroduplex repair in some hMLH1-deficient cells. In vitro analysis of hMutLα-deficient human cell lines has demonstrated that they define two classes with respect to the biochemical nature of the repair defect. One class of hMutLα-deficient cell lines includes the mismatch repair-deficient, drug-resistant derivatives of A2780 ovarian tumor cells and the sporadic colorectal and endometrial tumor cell lines RKO, Vaco6, and AN2CA. Each of these cell lines is devoid of hMLH1 polypeptide and hMutLα due to epigenetic silencing of MLH1 loci by promoter methylation (45–47). Extracts prepared from these cell lines are defective in correction of 3’-heteroduplexes. However, they
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display near normal levels of 5'-heteroduplex repair, and gap formation on 5'-DNAs in such extracts is mismatch-dependent (48).\textsuperscript{2} HmutLo supplementation restores 3'-heteroduplex repair to wild type levels in extracts, but the high level of residual 5' repair activity is enhanced only to a modest degree.

The second class of HmutLo-deficient cell line is typified by the colorectal tumor cell lines HCT116 (and its subclone H6) and Vaco481. H6 cells harbor nonsense mutations in the two MLH1 alleles (49), while the PMS2 alleles in Vaco481 are inactivated by frameshift and nonsense mutation (50). In vitro assay of extracts from these two cell lines has shown them to be defective in repair of both 3'- and 5'-heteroduplexes, although low residual levels of 5'-heteroduplex repair has been observed in H6 extracts with certain mispairs (18, 19, 50). 3'- and 5'-heteroduplex correction is restored to normal levels with both cell lines upon addition of purified HmutLo.

While the basis of the distinct repair phenotypes of the two classes of HmutLo-deficient cell lines has not been established, the selective repair defect in epigenetically silenced cells demonstrates that HmutLo-independent 5'-heteroduplex repair can occur in unfractionated nuclear extracts. It is pertinent to note in this context that the failure of MLH1\textsuperscript{−/−}/H6 extracts to support 5'-heteroduplex repair is not due to the presence of a simple diffusible inhibitor (18).\textsuperscript{3} Nevertheless, we have observed apparent normal, mismatch-dependent gap formation on 5'-heteroduplexes in excision-depleted extracts derived from H6 extracts upon supplementation with hEXO1. This may indicate that the failure to observe 5'-heteroduplex repair in unfractionated H6 extracts is due to a more complex form of inhibition, for example, the stoichiometric and essentially irreversible sequestration of a required repair activity that is disrupted upon fractionation.

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\textsuperscript{2} G.-M. Li, J. Drummond, L. Bazemore, S. Littman, and P. Modrich, unpublished observations.
\textsuperscript{3} S. Littman and P. Modrich, unpublished observations.