Identification of hMutLβ, a Heterodimer of hMLH1 and hPMS1*

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hMLH1 and hPMS2 function in postreplicative mismatch repair in the form of a heterodimer referred to as hMutLα. Tumors or cell lines lacking this factor display mutator phenotypes and microsatellite instability, and mutations in the hMLH1 and hPMS2 genes predispose to hereditary non-polyposis colon cancer. A third MutL homologue, hPMS1, has also been reported to be mutated in one cancer-prone kindred, but the protein encoded by this locus has so far remained without function. We now show that hPMS1 is expressed in human cells and that it interacts with hMLH1 with high affinity to form the heterodimer hMutLβ. Recombinant hMutLα and hMutLβ, expressed in the baculovirus system, were tested for their activity in an in vitro mismatch repair assay. While hMutLα could fully complement extracts of mismatch repair-deficient cell lines lacking hMLH1 or hPMS2, hMutLβ failed to do so with any of the different substrates tested in this assay. The involvement of the latter factor in postreplicative mismatch repair thus remains to be demonstrated.

In lower organisms, lack of postreplicative mismatch repair (MMR) was shown to lead to frequent alterations in tracts of simple repetitive DNA sequences (1). As this so-called microsatellite instability was found also in tumors of hereditary non-polyposis colon cancer (HNPCC) kindreds, it was proposed that the latter syndrome was associated with a deficiency in MMR (2–5). This hypothesis was substantiated when germline mutations in the hMLH1 and hMLH1 genes, which encode the human homologues of the Escherichia coli MMR proteins MutS and MutL, were identified in HNPCC families (6–9). Because mutations in these two genes accounted initially for only about one half of the HNPCC kindreds (10, 11), and because biochemical (12–15) and genetic evidence (1, 16–18) demonstrated that both hMSH2 and hMLH1 proteins interact with other polypeptides in vivo, an intensive search was instigated for other genes that might function in MMR. To date, six MutS homologues (hMSH1–6) and several MutL homologues (hMLH1, hMLH1, and hPMS2, as well as a cluster of PMS-like (pseudo)genes on chromosome 7) have been identified in the human genome (19). Interestingly, this plethora of potential new cancer susceptibility genes changed the distribution of mutations only slightly: of the more than 350 kindreds documented in the HNPCC data base as of May 1999 (20), 61% were reported to carry mutations in hMLH1 and 36% in hMSH2. Only five kindreds have been described so far that appear to carry mutations in other loci: two in hMSH6 (21, 22), two in hPMS2 (23), and one individual from a family with a clear cancer predisposition could be shown to carry a mutation in the hPMS1 gene (23). The under-representation of mutations in the hMSH6 locus is explained by the biochemistry of MMR. Mismatch recognition, the initial step of the repair process, is mediated principally by a heterodimer of hMSH2 and hMSH6. This abundant complex binds both base/base mismatches and small insertion/deletion loops (IDLs) (13) and is thus involved in the correction of misinsertions, as well as of primer/template slippage errors. However, the latter lesions are addressed also by a second heterodimer, formed by hMSH2 and hMSH3. The functional redundancy of hMSH6 and hMSH3 in the repair of small insertion/deletion loops means that only mutations in the hMSH2 gene abolish MMR in its entirety; hMSH6 inactivation leads solely to the loss of repair of base/base mismatches, while a deficiency in hMSH3 appears to have no deleterious effect on MMR (24). Could the unequal distribution of mutations in the family of human mutL homologues be explained by a similar phenomenon?

Modrich and colleagues (25) were able to complement a mismatch repair-deficient extract of H6 (HCT116) cells by the addition of mismatch repair-proficient extracts of HeLa cells. By fractionating the latter extracts, they could demonstrate that the complementing activity was a heterodimer of hMLH1 and hPMS2 (15), which they named hMutLα. In subsequent studies, the mutator phenotype of tumor cell lines lacking either hMLH1 or hPMS2 appeared to be similar (26), implying that no significant functional redundancy existed between hPMS2 and other MutL homologues. However, the following pieces of evidence question this statement. The first concerns the identification of one patient with an HNPCC family background, who harbored a mutation in the hPMS1 gene (23). As the mutation could not be shown to segregate with the disease due to lack of clinical samples from the other family members, it is unclear whether it is causative. However, this finding suggested that hPMS1 might be involved in MMR. Further evidence in support of this hypothesis came from recent reports, which demonstrated that Mlh3p, the closest Saccharomyces cerevisiae homologue of hPMS1, interacts with Mlh1p in a two-hybrid assay (27) and contributes to the repair of a subset of insertion/deletion loops (28, 29).

In an attempt to find biochemical evidence of the existence of a complex of MLH1 with the second MutL homologue, we expressed the human MLH1, PMS1, and PMS2 proteins in the baculovirus system and studied their interactions in some detail. The data presented below demonstrate that, in addition to its participation in hMutLα, hMLH1 can indeed form a stable heterodimer with hPMS1, a complex that we shall refer to as hMutLβ. We further show that, although hPMS1 is expressed in human cells at low levels, it also interacts with hMLH1 in

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1 The abbreviations used are: MMR, mismatch repair; HNPCC, hereditary non-polyposis colon cancer; AD, activation domain; BD, binding domain; PMSP, phenylmethylsulfanyl fluoride; Chr., chromosome; DTT, dithiothreitol; PCR, polymerase chain reaction; wt, wild type; PVD, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; IDL, insertion/deletion loop.

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vivo. Interestingly, the affinity of hMLH1 for hPMS1, as measured in the yeast two-hybrid assay, was apparently greater than for hPMS2. However, despite the ability of hPMS1 and hMLH1 to interact, we were so far unable to demonstrate the involvement of the resulting heterodimer in the mismatch repair process.

Experimental Procedures

Cell Lines

The lines used in this study were purchased from the American Type Culture Collection (ATCC), with the exception of HEC-1A (a kind gift of Tom Kunkel), HCT116 + Chr. 3 (a kind gift of Richard Boland and Minoru Koi), and HEC-1A + Chr. 7 (a gift of Margherita Bignami). They were cultured in a humidified atmosphere containing 5% CO₂, either in Dulbecco’s modified Eagle’s medium (HyClone, HEC-1A) or McCoy’s 5A medium (HCT116), containing 10% fetal calf serum. The HCT116 cells complemented with chromosome 3 and the HEC-1A clone complemented with chromosome 7 were maintained in media containing 400 μg/ml G418.

CDNA Vectors

The pcit2e vectors containing the cDNAs of hMLH1, hPMS1, and hPMS2 were kindly provided by Andrew Buermeyer and Mike Lisbak. All recombinant DNA manipulations were performed as described (30).

Baculovirus Expression Vectors

The Bac-To-Bac baculovirus expression system (Life Technologies, Inc.) was used according to the instructions of the manufacturer.

pFastBacI-hMLH1—A BamHI/NcoI fragment of the pcit2a-hMLH1 vector containing the hMLH1 cDNA was subcloned between the BamHI/NcoI sites of pFastBacI.

pFastBacI-hPMS1—Sequencing of the hPMS1 cDNA in pcit2e vectors revealed two nucleotide differences in codons 4 (TTG → TGG) and 222 (ATG → ATC), as compared with the published hPMS1 sequence (23), which result in amino acid substitutions L28W and M222I, respectively. Both substitutions were reverted to the published sequence by site-directed mutagenesis. The sequences of the primers used for mutagenesis are available on request. The pcit2e-hPMS1wt (Leu-4, Met-222) or the pcit2e-hPMS1 (Trp-4, Ile-222) vectors were first cleaved with NcoI, the overhangs were filled-in with Klenow polymerase, and the vectors were then cleaved with XhoI. The resulting fragments were cloned between the BamHI filled-in with Klenow polymerase and XhoI sites of pFastBacI. All the experiments described in this paper were carried out with the recombinant hPMS1 protein containing the published (wt) sequence. However, all the experiments were repeated in parallel also with the heterodimer containing the hPMS1 subunit with the two mutations. No differences were found between the two factors.

pFastBacI-hPMS2—The BamHI/XhoI fragment of the pcit2e-hPMS2 vector was subcloned between the BamHI/XhoI sites of pFastBacI.

pFastBacI-His6-B—A fragment containing the polyhedrin promoter and the (His)₆ tag was amplified by PCR from the blueBacHis3 vector (Invitrogen) using primer BacUp (5′-GTTGCACTGTGGCTTGGTATA-3′) and primer BacRev (5′-AGCCGTTCAAGTCTTCCTTCT-3′), the overhangs were filled-in with Klenow polymerase, and the fragment was then cleaved with BamHI. The resulting fragment was cloned between the SnaBI site (filled-in with Klenow polymerase) and the BamHI site of pFastBacI.

pFastBacI-hMLH1(M15)—For expression of the (His)₉ hMLH1, the original translational start ATG was mutated to AGC. A fragment of the hMLH1 cDNA was amplified by PCR from pFastBacI-hMLH1 using primer mr2 (5′-GGGAGAATCTCCTTGAGGACACCTTTCCAT-3′) and mr4 (5′-CGGGATCCACGTGCTTGGGCGGAGGT-3′) and cleaved with BamHI and PvuII. The resulting fragment was cloned between the BamHI and PvuII sites of pFastBacI.

FastBacI-His6-hMLH1—A BamHI/XhoI fragment of the pFastBacI-hMLH1/XhoI vector was subcloned between the BamHI/XhoI sites of pFastBacI-His6-B.

Bacterial Expression Vectors

pET28c-hPMS2—The BamHI/SalI fragment of pcit2e-hPMS2 vector was subcloned between the BamHI/SalI sites of pET28c.

Yeast Two-hybrid Experiments

The two-hybrid experiments were performed using the Matchmaker two-hybrid system (CLONTECH), following the instructions provided by the manufacturer. The cDNAs of hMLH1, hPMS1, hPMS2, and hPMS2 were subcloned into pAS2-1 (GAL4 DNA binding domain) or the pACT2 (GAL4 activation domain) vectors. For BD-hMLH1, the EcoRI fragment of pFastBacI-hMLH1 was subcloned into the EcoRI site of pAS2-1. For BD-hPMS1, the MunI/EcoRI fragment of pFastBacI-hPMS1wt was subcloned between the MunI/EcoRI sites of pAS2-1 (MunI cuts 7 nucleotides downstream of the translational start ATG, the fusion protein therefore lacks 3 amino acids at the amino terminus of the hPMS1 protein). For BD-hPMS2, the NdeI/SalI fragment of pET28c-hPMS2 was subcloned between the NdeI/SalI sites of pAS2-1. For AD-hMLH1, the NcoI/BamHI fragment of BD-hMLH1 was subcloned between the NcoI/BamHI sites of pACT2. For AD-hPMS2, the EcoRI fragment of pFastBacI-hPMS2 was subcloned into the EcoRI site of pACT2.

Overexpression and Purification of MLH1, hMutLo, and hMutLB

Typically, 1.6 × 10⁹ Sf9 cells were infected with either a single recombinant baculovirus or with a combination of two viruses at a multiplicity of infection of 10. 72 h after infection, the cells were harvested and total extracts were prepared as described (31). They were diluted to 15 μM salt with four volumes of buffer H (50 mM NaCl, 300 mM KCl, pH 7.6, 1 mM EDTA, filtered, and directly loaded on a 6-ml Hitrap Heparin FPLC column (Amersham Pharmacia Biotech). The proteins were eluted (hMutLo at 38.5 mS, hMutLj at 34.2 mS, hMLH1 at 19.8 mS) with a 2%/ml salt gradient. The peak fractions were pooled, diluted to 0.12 x salt, filtered, applied to a 1-ml Resource-Q column (Amersham Pharmacia Biotech), and eluted with a 2%/ml salt gradient. The fractions containing the pure proteins were pooled, dialyzed extensively against storage buffer (20 mM HEPES, pH 7.6, 0.1 mM EDTA, 150 mM NaCl, 10% sucrose, and 0.5 mM PMSF), and stored at aliquots at –80 °C.

In Vitro Mismatch Repair

These assays were carried out as described previously (32). Briefly, cytoplasmic protein extracts were prepared from HeLa, HCT116, and HEC-1A cell lines, using 5 × 10⁸ cells harvested in the exponential growth phase. After resuspension in ice-cold hypotonic buffer (20 mM HEPES, pH 7.9, 5 mM KC1, 0.5 mM MgCl₂, 0.5 mM PMSF, 1 mM DTT) at a density of 1 × 10⁶ cells/ml, the cells were allowed to swell for 10 min in a glass Dounce homogenizer on ice, and then lysed mechanically by applying four or more strokes with a tight pestle. When more than 80% of the pellets remained, the homogenate was centrifuged (12,000 × g for 10 min at 4 °C), and stored in small aliquots at –80 °C.

Wild type and mutant M13mp2 phage used to generate the mismatch-containing heteroduplexes were kindly provided by Tom Kunkel. The heteroduplex DNA contained the indicated base/base mispair or an IDL within the coding sequence of the lacZ α-complementation gene, and a nick either 5′ or 3′ from the mispair. Substrate (1 nmoI) was incubated with 50 μg of cytoplasmic extract, supplemented where necessary with 200 ng of the purified recombinant hMutLo or hMutLB. The repair reaction (25 μl) contained 30 mM HEPES, pH 7.8, 7 mM MgCl₂, 4 mM ATP, 200 μM each CTP, GTP, and UTP, 100 μM each dATP, dCTP, dGTP, and dTTP, 40 mM creatine phosphate, 100 fmol of creatine phosphokinase, and 15 mM sodium phosphate, pH 7.8. After 20 min of incubation at 37 °C, the heteroduplex DNA was purified and electrophoresed into E. coli NR9162 (mutS), plated on minimal medium in a soft agar layer containing 0.5 μM of a log culture of E. coli CSH50, 0.5 mg of isoperipl-1-thio-β-D-galactopyranoside, and 2 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. After incubation for 16 h at 37 °C, repair efficiency could be determined by analyzing the color of the plaques.

Immunoprecipitations

To produce nuclear extract for immunoprecipitations, pelleted nuclei were incubated 30 min at 4 °C in an appropriate volume of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 300 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The extracted nuclei were pelleted by centrifugation (20,000 × g for 30 min at 4 °C), and the supernatant was dialyzed against storage buffer, centrifuged (20,000 × g for 30 min...
at 4 °C and stored in small aliquots at −80 °C. In the immunoprecipitation experiments, 250 μg of nuclear extract were incubated at 4 °C with 1 μg of the indicated antibody in a total volume of 800 μl of RIPA buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM PMSF, 1× complete protease inhibitors (Roche Molecular Biochemicals), 0.1% SDS dissolved in phosphate-buffered saline). After 1 h, 20 μl of a Protein AG-agarose suspension (Santa Cruz Biotechnology) was added and incubated for 1 h at 4 °C. The immunocomplexes were precipitated, washed three times with 800 μl of RIPA buffer, resuspended in 20 μl of 1× SDS loading buffer, and analyzed by Western blotting.

### Western Blotting

Proteins were separated on denaturing 7.5% SDS-polyacrylamide gels, blotted onto PVDF membranes, and detected with the ECL-Kit (Amersham Pharmacia Biotech), according to the protocol provided.

Antibodies used for immunoprecipitation and Western blotting were hMLH1 (PharMingen, clone G168-728), HPMS2 (Oncogene, Ab-1), and HPMS1 (polyclonal rabbit antiserum generated at Eurogentec by immunization with a (His)6-tagged internal peptide of hPMS1 (amino acid 335–634), which was purified by affinity chromatography from BL21 bacteria transformed with plasmid pET-16b-hPMS1(aa335–643).

### Sucrose Gradients

Purified hMutLo (42 μg), hMutLoβ (42 μg), or hMLH1 (25 μg) were mixed with 50 μg of the size marker mixture (β-amylobin (200 kDa), 8.5 kDa, yeast ADH (150 kDa), 7.61 kDa, albumin (66 kDa), 4.4 kDa, Sigma), diluted with HEN buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 150 mM NaCl) to 350 μl, and loaded onto 10–30% sucrose gradients in HEN buffer and centrifuged (34,000 rpm at 4 °C for 3 h in a Sorvall TH-641 rotor). 40 fractions were collected from the bottom of the tubes. 20 μl of each fraction were analyzed on Coomassie Blue-stained, denaturing 7.5% polyacrylamide gels.

### Gel Filtration

The gel filtration experiments were performed at 4 °C on a Superose 6 FPLC column (28 cm × 1.5 cm², Amersham Pharmacia Biotech) equilibrated with HE buffer containing 150 mM NaCl, and calibrated with bovine thyroglobulin (Rt = 85.8 Å), apoferritin (Rt = 67.3 Å), yeast alcohol dehydrinase (Rt = 52.2 Å), and bovine serum albumin (Rt = 36.1 Å). 50 μg of the purified heterodimers and 37 μg of purified hMLH1 were applied onto the column at a flow rate of 0.3 ml/min. 150-μl fractions were collected and analyzed by Western blotting.

### RESULTS

**Expression of Recombinant MutL Homologues in Insect Cells—Spodoptera frugiperda** SF9 cells were infected with recombinant baculoviruses carrying cDNA inserts encoding hMLH1, hPMS1, or hPMS2. Interestingly, following infection with the individual viruses, only hMLH1 could be obtained in reasonable amounts (Fig. 1), while hPMS1 and hPMS2 were found mostly in the insoluble fraction (data not shown). We argued that this could be due to incorrect folding of the PMS proteins in the absence of their cognate partner, similar to the case of the MutS homologues (13). The SF9 cultures were therefore co-infected with all the possible combinations of the three baculovirus constructs. Co-infection of hPMS2 and hMLH1 vectors resulted in a substantial increase in the amount of soluble hPMS2 (Fig. 1), which is consistent with the hypothesis that co-expression of the two subunits of hMutLo ensures proper folding of the polypeptides. Similarly, the amount of hPMS1 was considerably increased in experiments where the latter expression vector was co-infected together with the hMLH1 baculovirus construct (Fig. 1). These results strongly implied that hMLH1 might form a heterodimer also with hPMS1.

In contrast, no noticeable increase in protein expression was apparent following co-infection of the SF9 cells with the hPMS1 and hPMS2 vectors, even though Northern blot analysis of the infected cells showed that the respective mRNAs were at least as abundant as seen in the hMLH1-hPMS1 and hMLH1-hPMS2 co-infections described above (data not shown). This implies that the low expression of the hPMS1 and hPMS2 proteins was associated most likely with post-translational processes such as protein misfolding and degradation.

The recombinant hMLH1, hPMS2, and hPMS1 proteins migrated through denaturing polyacrylamide gels (SDS-PAGE) with apparent molecular sizes of 86, 115, and 116 kDa, respectively. Similarly, migration was observed for the two former proteins in extracts of human cell lines (Refs. 15 and 33; see also Fig. 5). Correlation with the predicted molecular masses of these polypeptides (hMLH1 = 84.5 kDa, hPMS2 = 95.8 kDa, and hPMS1 = 105.8 kDa) suggests that both hPMS2 and hPMS1 might be post-translationally modified or that they migrate aberrantly through polyacrylamide gels. The molecular sizes of the three MutL homologues estimated from SDS-PAGE were confirmed by Western blotting experiments (Fig. 1, lower panels). It should be pointed out that, in these blots, the recombinant hMLH1 often appears as a doublet. Since only a single band due to hMLH1 was observed in cell extracts from several different cell lines and since recombinant hMLH1 expressed with a His₆ tag at the amino terminus could be purified as a single protein (data not shown), it would appear that the translation of hMLH1 mRNA generated from our baculovirus vector initiated at two separate, albeit closely situated sites.

**Purification of Recombinant hMutLo, hMutLoβ, and hMLH1**—The purification of the human MutL homologues was achieved by a two-step procedure. First, the total extract was loaded at a low salt concentration (110 mM NaCl) on a HiTrap heparin FPLC column. hMLH1 could be eluted from this column at a salt concentration of ~310 mM. In contrast, when cell extracts obtained following hMLH1-hPMS2 and hMLH1-hPMS1 co-infections were loaded, hMLH1 was retained on the column somewhat longer, eluting together with the respective PMS homologue at ~420 mM (hMLH1-hPMS2) or ~366 mM (hMLH1-hPMS1) salt. The peak fractions were pooled, diluted to 120 mM salt, and applied onto a Resource Q column. From
a typical protein partial specific volume of 0.725 cm$^3$/g (37).

For the recombinant proteins were calculated assuming sedimentation on sucrose density gradients. The molecular mass of the recombinant proteins was calculated on a Superose 6 column. While hMLH1 eluted as a monomer (calculated $M_{\text{hMLH1}} = 85$ kDa), the peaks of hMutL$\alpha$ and hMutL$\beta$ were clearly shifted toward higher molecular mass (Fig. 3). The retention of the complexes suggested that they were eluting as heterotetramers, or that they migrated faster through the matrix because of their non-globular shape. A similar atypical chromatographic behavior was observed for the H6 (HCT116) complementing activity (15), as well as for the bacterial MutL protein (34–36). The sedimentation coefficients ($s_{20,w}$) of the purified proteins were determined by band sedimentation on sucrose density gradients. The molecular masses of the recombinant proteins were calculated assuming a typical protein partial specific volume of 0.725 cm$^3$/g (37). These latter data (Table I) indicate that the hMLH1-hPMS1 and hMLH1-hPMS2 complexes are heterodimeric.

Co-immunoprecipitation of hPMS1 and hPMS2 with hMLH1—hMLH1 was immunoprecipitated from 250 μg of HeLa nuclear cell extracts with a mouse monoclonal antibody (see “Experimental Procedures”). Analysis of the precipitates by Western blotting revealed the presence of hMLH1, hPMS1, and hPMS2 (Fig. 4), which demonstrates that hMLH1 interacts stably with both hPMS1 and hPMS2. No proteins were detected in precipitates from an extract of a cell line lacking hMLH1 (HCT116) or in control experiments where the precipitating antibody was omitted (Fig. 4). Similarly, affinity-purified anti-hPMS1 antiserum could be used to precipitate hPMS1 together with hMLH1, but not with hPMS2 (data not shown). These results provide further evidence that hMLH1 interacts in vivo either with hPMS2 or with hPMS1 to form the heterodimers hMutL$\alpha$ and hMutL$\beta$, respectively.

**Interactions of hMLH1 with hPMS2 and hPMS1 in the Yeast Two-hybrid Assay**—The interactions between hMLH1 and hPMS1 or hMLH1 and hPMS2 were studied in the yeast two-hybrid system. hMLH1, hPMS1, and hPMS2 were expressed in the S. cerevisiae Y187 reporter strain as chimeric proteins (see “Experimental Procedures”). Quantitative assays, using O-nitrophenyl-β-D-galactopyranoside as substrate, revealed an approximately 15-fold stronger interaction between hMLH1 and hPMS1 than between hMLH1 and hPMS2, independent of whether hMLH1 was fused to the BD or the AD of Gal4 (Table I).
Identification of hMutLβ

II. Qualitative filter assays confirmed these results. No evidence of homodimerization of hMLH1, hPMS1, or hPMS2 was obtained in these assays.

Analysis of hMLH1, hPMS1, and hPMS2 Expression in Human Cancer Cell Lines—In HeLa cytoplasmic extracts, the anti-hPMS1 antibody specifically reacted with a protein migrating with the same size as the recombinant hPMS1 (Fig. 5). Based on the results obtained with the baculovirus-infected SF9 cells (see above), we expected to observe substantially decreased levels of hPMS1 and hPMS2 in cell lines lacking hMLH1. As shown in Fig. 5, the cell line HCT116, which carries a truncation mutation in one allele of the hMLH1 gene (9) and has been reported to express no hMLH1 and undetectable levels of hPMS2 (38), did indeed show a significantly lower amount of hPMS1, as compared with the control cells (Fig. 5, lane 2). Interestingly, restoring hMLH1 expression in HCT116 by chromosome 3 transfer (39) not only increased the levels of hPMS1 (Fig. 5, lane 3), but also that of hPMS2 (Fig. 5). Addition of 200 ng of the purified recombinant hMutL homologues was analyzed by Western blot analysis of 30 μg of cytoplasmic extracts of the different cell lines. HCT116 (deficient in hMLH1) and HEC-1A (deficient in hPMS2), which were complemented by the transfer of chromosomes 3 or 7, respectively.

TABLE II Interactions between human MutL homologues as analyzed in a yeast two-hybrid assay

| AD         | hMLH1 | hPMS1 | hPMS2 |
|------------|-------|-------|-------|
| BD         | 0.01  | 0.02  | 0.01  |
| BD-hMLH1   | 0.01  | 0.03  | 1.47  |
| BD-hPMS1   | 0.01  | 1.24  | ND    |
| BD-hPMS2   | 0.01  | ND    | ND    |

ND, not determined.

Given that hPMS1 might compete with hPMS2 for the available hMLH1, we decided to determine the relative amounts of the three MutL homologues in HeLa nuclear cell extracts (Fig. 6). 5–20 ng of the recombinant hMutLa and hMutLβ were loaded next to 30 μg of HeLa nuclear extract onto SDS-PAGE, and the separated proteins were transferred onto a PVDF membrane. The proteins were visualized by Western blotting, and the intensities of the bands in the HeLa nuclear extract were compared with those of the purified recombinant proteins. These experiments revealed that hPMS2 was approximately 10-fold more abundant in these cells than hPMS1 (hMLH1), hPMS2: 15 ng; hPMS1: 1.5 ng). Thus, assuming that hPMS1 levels remain constant, hMLH1 appears to exist predominantly in a complex with hPMS2.

Functional Test of hMutLa and hMutLβ in Mismatch Repair Assays—We next wanted to examine the possible involvement of hMutLa in the mismatch repair process. To this end, we used extracts of the HCT116 cell line, which are defective in mismatch correction in vitro (25) due to mutations in the hMLH1 and hMSH3 genes (41) and the concomitant lack of hPMS1 and hPMS2 (Fig. 5). Addition of 200 ng of the purified recombinant hMutLa to these extracts completely restored in vitro MMR.
Identification of hMutLβ

while no complementation was evident with hMutLβ for all the substrates tested (Fig. 7A). Similar results were obtained when the reactions were additionally complemented with 200 ng of the purified recombinant hMutSβ complex (data not shown). The small increase in repair activity observed following the addition of hMutLβ to repair reactions with the G/T 3’ and Δ2 3’ substrates is most likely not significant, as increasing amounts of protein failed to further enhance the repair efficiency of the extracts, and complementation only with purified hMutSβ resulted in a similar fluctuation of the background signal of the assay. The lower efficiency of the hMutLα-complemented extracts in the repair of substrates containing larger loops is most likely due to the fact that these cells also lack functional hMutSβ.

Extracts of the HEC-1A cell line, which are deficient in hPMS2 (40), could be complemented with purified hMutLα (Fig. 7B), albeit only for loop repair. This is due to the fact that this cell line carries a mutation also in the hMSH6 gene (42). Substrate recognition in these cells is therefore mediated solely by the hMutSβ complex. Since hMutSβ binds only to IDLs, the lack of complementation with the substrates carrying base/base mismatches was anticipated (42). Correspondingly, when the extracts were supplemented with 200 ng of purified recombinant hMutSα together with 200 ng of hMutLα, efficient repair of all substrates could be observed (data not shown). No complementation was observed when hMutLα was substituted with hMutLβ in these experiments.

The lack of apparent biological activity of hMutLβ in our in vitro MMR assays was of some concern to us. Although we cannot formally eliminate the possibility that the hMutLβ heterodimer was isolated in a biologically inactive form, the fact that it was purified by a procedure identical to that used to obtain hMutLα, which was biologically fully active in the above MMR assays, makes this seem unlikely. The second point of concern was that the original cDNA clone contained two amino acid changes, L4W and M222I, as compared with the published sequence (see “Experimental Procedures”). These were corrected by site-directed mutagenesis; leucine 4 is conserved in most MutL homologues and it was thought that this amino acid is more likely to be correct. The methionine 222 is in a non-conserved region, and its substitution for another small hydrophobic residue such as isoleucine is probably phenotypically silent, but we reverted this mutation too. However, in order to eliminate the possibility that reversion of these mutations resulted in inactivation of hPMS1, we tested the L4W/M222I hPMS1-hMLH1 heterodimer in all the assays in parallel with the wt factor. As we failed to observe any differences between the two variants, we concluded that these amino acid changes are not responsible for the observed lack of biological activity of hMutLβ in the MMR assays (see also below).

DISCUSSION

Since its discovery and the concomitant identification of a mutation in this gene in one member of a family predisposed to cancer (23), hPMS1 has been little studied. This lack of attention was associated with the finding that the mutator phenotypes of cell lines lacking hMLH1 or hPMS2 were very similar, which implied that hPMS1 was unlikely to play a backup role in MMR analogous to the function of the hMSH2-hMSH3 heterodimer in IDL repair (1, 26). It even appeared possible that hPMS1 is a pseudogene. Our experiments conclusively show that this is not the case. hPMS1 is expressed in human cells and produces a polypeptide that is capable of high affinity interaction with hMLH1. However, because it is expressed in quantities approximately 10 times lower than its homologue, hPMS2, the hMutLα heterodimer predominates in human cells.

Recently, hPMS1 has been brought into the limelight by two reports. The first compared the phenotypes of mice deficient in the Mlh1, Pms1, or Pms2 genes (43). While most animals deficient in Mlh1 or Pms2 developed tumors by 1 year of age, mice deficient in Pms1 stayed tumor-free during this time. Because the tumor spectrum was different in Mlh1- and Pms2-deficient mice (the former developed mostly lymphomas, intestinal adenomas, and adenocarcinomas, whereas the latter displayed only lymphomas), we cannot rule out the possibility that Pms1 modulates the mutation spectra in these animals in a way similar to MLH3 in yeast (28). Such an altered mutation spectrum might lead to the inactivation of different tumor suppressor genes and thus, in turn, to a different tumor spectrum. Initially, the likelihood of this happening appeared to be

Fig. 6. Relative abundance of hMLH1, hPMS1, and hPMS2 in HeLa nuclear cell extracts. The relative amounts of the human MutL homologues in HeLa cells were estimated by Western blotting. 30 μg of the nuclear extract were loaded next to increasing amounts (5, 10, 15, and 20 ng) of purified hMutLα (left) or hMutLβ (right). After electrophoresis in a 7.5% SDS-polyacrylamide gel, the proteins were blotted onto a PVDF membrane and visualized with an anti-hMLH1 antibody (lower panel). The membrane was then stripped, cut, and re-probed with an anti-PMS2 (upper, left) or an anti-hPMS1 antibody (upper, right).

Fig. 7. In vitro mismatch repair. Cytoplasmic extracts of the hMLH1-deficient cell line HCT116 (panel A) or of the hPMS2-deficient cell line HEC-1A (panel B) were complemented with 0.2 μg of purified hMutLα or hMutLβ and analyzed for mismatch repair proficiency. The repair efficiencies on heteroduplex substrates containing a G/T mismatch, a 1-, 2-, or a 4-base loop with a nick located either 5’ of 3’ of the mismatch were determined. (•), loop located in the circular, covalently closed viral DNA strand; (•), loop located in the nicked, complementary strand.
Bacterial MutL

MLH Family

MLH1 (ac)  
MLH3 (ac)  

PMS Family

MutL (ap)  
Hhr1  
Pms1p (ac)  
Mlh1p (ac)  
Mlh3p (ac)  

Identification of hMutLβ

**FIG. 8.** Schematic representation of the regions conserved among the MLH and PMS family members. The figure shows the evolutionarily conserved regions among homologues of the bacterial MutL protein as described in Ref. 27. All homologues share the ATP binding domain (MLH, black) at the amino terminus. Among members of the PMS family, three subdomains (SD1, SD2, and SD3, gray) were identified. The MLH family members possess a stretch of 13 amino acids at the carboxyl terminus, which is completely conserved (CTH, dark gray).

low, as both MLH1- and PMS2-deficient mice showed a high degree of microsatellite instability (43) and all the tested tissues displayed an approximately 100-fold increase in mutation rates (44). However, recent detailed analysis of the above knock-out mouse models have shown that the mutator phenotype in microsatellites of Mlh1-/- mice is 2-3-fold higher than that of Pms2-/- animals (45), suggesting that the loss of the latter gene does not completely inactivate the repair of IDLs. This leaves open the possibility that Mlh1 might act, either alone or following interaction with another polypeptide (e.g. Pms1), in the correction of a small subset of IDLs.

The above findings agree with those of the third report, which described the interaction of *S. cerevisiae* Mlh1p with Mlh3p (ORF YPL164c) in a two-hybrid assay. The latter polypeptide was suggested by phylogenetic analysis to be the functional homologue of hPMS1 (27, 28). It contains three motifs (SD1, SD2, and SD3) near its COOH terminus, which share a high degree of similarity with sequences in all the PMS homologues (27, 46) (Fig. 8). These motifs are not found in homologues of hMLH1, and their presence can thus be used to subdivide MutL homologues into PMS and MLH families. As a deletion of these sequences in Pms1p (functional homologue of hPMS2) destroyed the interaction with Mlh1p, it was suggested that all three motifs are necessary for the interaction (27). Interestingly, we could identify only SD2 and SD3 in hPMS1, which implies that SD1 is not necessary for heterodimerization with hMLH1. The contribution of MLH3 to the genomic stability of *S. cerevisiae* was studied by epistatic analysis (28). The mih3 mutant showed no substantial increase (1.2-fold) in the rate of accumulation of Can^r mutations and only a modest increase in the reversion rate of hom3-10 and lys2-Bgl alleles (3.3- and 2.2-fold, respectively). The effect on the hom3-10 reversion was most evident in a msh6 mutant background (68-fold), indicating an involvement of Mlh3p in MSH3-dependent correction of extrahelical bases. However, the contribution of the Mlh3p to IDL repair is small, as the mutation rate of the mlh3 msh6 double mutant was at most only 15% of that reported for an msh2 or an msh3 msh6 double mutant. This implies that the greater part of IDL repair in *S. cerevisiae* is mediated by the Mlh1p-Pms1p (yeast MutLo) heterodimer. Based on the above findings, we wanted to test whether hMutLβ participates in IDL repair, as suggested for the Mlh1p-Mlh3p heterodimer and as implied by the microsatellite instability studies in the transgenic mouse models. As shown in Fig. 7 above, the hMutLβ heterodimer failed to complement MMR-deficient extracts lacking hMutLo with all the substrates tested, which included base/base mismatches and IDLs of +1, -1, and -2. The results obtained with the recombinant factor were substantiated by the data shown in Fig. 7B, where extracts of the cancer cell line HEC-1A were found to be completely deficient in mismatch and IDL repair *in vitro*. This cell line carries mutations in hPMS2 and hMSH6 (42), and its extracts thus contain only hMutSβ (hMSH2-hMSH3 heterodimer) and hMutLβ. The fact that IDL repair in these cell extracts could be restored by the addition of recombinant hMutLo indicated that they contained a functional hMutSβ heterodimer and that they were competent for MMR. hPMS1 expression in these cells was comparable to other human cell lines (Fig. 5), and hPMS1 and hMLH1 were confirmed to be wild type by direct sequencing (47). These findings, coupled with the fact that the HEC-1A cells display a severe mutator phenotype with microsatellite instability (40, 47), provide compelling evidence that, like the recombinant factor, the wild type hMutLβ present in the HEC-1A cells does not play a major role in postreplicative mismatch correction. However, we cannot exclude the possibility that hMutLβ participates in the correction of a limited subset of IDLs such as the hom3-10 frameshift reversion, as suggested by the mutational spectra of the mlh3 yeast strains (28). We failed to see any contribution of hMutLβ toward the repair of single nucleotide insertion and deletion mutations in a run of Ts, i.e. substrates that closely resemble the hom3-10 mutation hotspot. However, there remains the possibility that the involvement of hMutLβ in the repair of IDLs in this region of the *S. cerevisiae* genome is affected by their sequence context; this was not tested here. In addition, the sensitivity of the *in vitro* MMR assay is rather low and we cannot therefore exclude the possibility that the contribution of hMutLβ to the repair process was so small as to remain undetected. These issues thus require further study before a definite conclusion can be made.

Could hPMS1 be involved in human malignancy? Our findings, coupled with the microsatellite stability and lack of tumors in the PMS1-deficient mice (43) suggest that the protein is not a major player in postreplicative mismatch repair. However, due to the fact that hPMS1 apparently interacts with hMLH1 with higher affinity than hPMS2, we could foresee it affecting mismatch repair efficiency. We show above that hPMS2 is expressed in amounts approximately 10-fold higher than hPMS1. This suggests that hMLH1 exists predominantly in a complex with hPMS2. Should hPMS1 expression be increased through, for example, transcriptional deregulation, gene translocation, or amplification, the overexpressed hPMS1 protein might out-compete hPMS2 for the available hMLH1 and hMutLβ would promote. As this complex is quite certain to be inactive in the repair of most types of mismatches, the cells overexpressing hPMS1 would acquire a mismatch repair-deficient phenotype similar to cells lacking hMutLo. This situation is not without precedent. The hMSH3 gene could recently be shown to co-amplify with the DHFR locus, which resulted in the sequestration of hMSH2 in a complex with hMSH3 rather than hMSH6. This led, in turn, to deficiency in the repair of base/base mismatches and caused a strong mutator phenotype in the hMSH3-overexpressing cells (24, 48). We are currently examining the expression levels of hPMS1 in

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**MLH Family**

- MLH1 (ac)
- MLH3 (ac)

**PMS Family**

- MutL (ap)
- Hhr1
- Pms1p (ac)
- Mlh1p (ac)
- Mlh3p (ac)
- Mlh1p (ac)
- Mlh3p (ac)
Identification of hMutLβ

Identifications of tumor suppressor genes have been vital tools for understanding the molecular basis of cancer. The finding that many tumor suppressor genes function to maintain genomic integrity by preventing mutations that can cause uncontrolled cell proliferation and development of tumors has led to the identification of a large number of genes with roles in DNA repair and DNA replication. hMutLβ has been shown to interact with hMutSα and hPMS2 in both bacteria and mammalian cells, suggesting a conserved role in DNA repair. Here, we report the identification of the human homolog of the mouse mutLβ gene, which we have called hMutLβ. We show that hMutLβ is an integral component of the heterotrimeric complex with hMutSα and hPMS2, and we provide evidence that hMutLβ is required for the completion of homologous recombination in mammalian cells. These findings suggest that hMutLβ plays a critical role in the maintenance of genomic stability in human cells.