Isolation of MutSβ from Human Cells and Comparison of the Mismatch Repair Specificities of MutSβ and MutSa*

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A human MSH2-human MSH3 (hMSH2-hMSH3) complex of approximately 1:1 stoichiometry (human MutSβ (hMutSβ)) has been demonstrated in several human tumor cell lines and purified to near homogeneity. In vitro, hMutSβ supports the efficient repair of insertion/deletion (I/D) heterologies of 2–8 nucleotides, which is weakly active on a single-nucleotide I/D mispair, and is not detectably active on the eight base-base mismatches. Human MutSa (hMutSa), a heterodimer of hMSH2 and hMSH6, efficiently supports the repair of single-nucleotide I/D mismatches, base-base mispairs, and all substrates tested that were repaired by hMutSβ. Thus, the repair specificities of hMutSa and hMutSβ are redundant with respect to the repair of I/D heterologies of 2–8 nucleotides. The hMutSa level in repair-proficient HeLa cells (1.5 μg/ml nuclear extract) is approximately 10 times that of hMutSβ. In HCT-15 colorectal tumor cells, which do not contain hMSH6 and consequently lack hMutSβ, the hMutSβ level is elevated severalfold relative to that in HeLa cells and is responsible for the repair of I/D mismatches that has been observed in this cell line. LoVo tumor cells, which are genetically deficient in hMSH2, lack both hMutSa and hMutSβ, and hMutSa and hMutSβ levels are less than 4% of those found in repair-proficient cells. Coupled with previous findings (J. T. Drummond, J. Genschel, E. Wolf, and P. Modrich (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10144–10149), these results suggest that hMSH2 partitions between available pools of hMSH3 and hMSH6 and indicate that hMSH2 positively modulates hMSH6 and hMSH3 levels, perhaps by stabilization of the polypeptides upon heterodimer formation.

Correction of mismatched base pairs, resulting from replication errors, contributes significantly to the genetic stability of human cells (reviewed in Refs. 1–3). Mutations in the gene that encodes the human MutS homolog MSH2 (hMSH2)1 confers genetic instability and have been implicated in hereditary nonpolyposis colon cancer and sporadic tumors (4–6). The muta-

tion rate at the HPRT locus is greatly increased in the MSH2−/− LoVo colorectal tumor cell line. The majority of the selectable HPRT mutations that occur in LoVo cells are transversions (80%), with the remainder largely single-nucleotide frame shifts in mononucleotide repeat sequences (7), although (CA)n microsatellite repeats are also highly unstable in this cell line (8). Extracts prepared from LoVo cells are deficient in repair of heteroduplexes containing either base-base or small insertion/deletion (I/D) mispairs (8, 9), and a mismatch recognition activity that restores repair of a G-T base-base mismatch and TG I/D mispair to LoVo nuclear extracts has been isolated from HeLa cells (9). This activity, designated hMutSa, is a heterodimer of hMSH2 and hMSH6 (also called p160 or GTBP (9, 10)).

Nuclear extracts of the HCT-15 colorectal tumor cell line (11) and the alkylating-tolerant MT1 lymphoblastoid cell line (12) are also deficient in mismatch repair due to MutSa deficiency (9, 13), but the MutSa defects in these cell lines are a consequence of MSH6 mutations (14). Extracts of HCT-15 and MT1 cells are deficient in repair of base-base mismatches and single-nucleotide I/D mispairs but retain partial proficiency in the correction of 2-, 3-, and 4-nucleotide I/D heterologies (9, 13). The HPRT mutation rate is elevated 60-fold in MT1 cells (12), which harbor missense mutations in both MSH6 alleles (14), and 300-fold in HCT-15 cells (15) in which both MSH6 alleles have been inactivated by frame shift mutations. HCT-15 cells also contain a sequence change in a conserved region of one copy of the gene that encodes DNA polymerase δ (11), but this mutation does not appear to contribute significantly to the HCT-15 mutator phenotype (16). Although dinucleotide repeat repeats are relatively stable in these MSH6−/− cell lines, mononucleotide repeats are prone to mutation (13, 14, 17), but not to the extent observed with HCT-116 cells (13, 15), which are defective in base-base and I/D mismatch repair (18, 19) due to mutations in both alleles of MLH1 (20).

The proficiency of MSH6−/− cells in the repair of I/D mismatches suggests the existence of a second mismatch recognition activity distinct from hMutSa in mammalian cells. Since hMSH2-deficient cell lines are defective in the repair of both base-base mispairs and I/D heterologies, this hMSH6-independent mismatch activity would appear to require hMSH2. There are two candidates for such an activity: a heterodimeric complex of hMSH2 and hMSH3, with the locus encoding the latter protein being the first MutS homolog gene identified in mammalian cells (21, 22), or free hMSH2 in an unknown oligomeric state. Recombinant hMSH2 has been reported to bind to I/D mismatches (23), but recent work indicates that this is an extremely low affinity interaction (24, 25), and free hMSH2 has not been detected in human cells (26). However, a complex between recombinant hMSH2 and hMSH3 polypeptides has been demonstrated (24, 25), and a similar complex has been isolated from methotrexate-resistant human cells in which the
**DHFR-MSH3 region of chromosome 5 is highly amplified (26).**

The hMSH2-hMSH6 complex has been shown to bind specifically to a G-T mispair and to 1-, 2-, and 3-nucleotide I/D mismatches and to efficiently restore repair of a base-base and a dinucleotide I/D mismatch to hMSH2-deficient nuclear extracts (9, 24). By contrast, the recombinant hMSH2-hMSH3 hMutSB complex was shown to bind weakly to a single-nucleotide insertion/deletion mismatch and with high affinity to heteroduplexes containing 2, 3, 4, or 10 unpaired nucleotides but not to the several base-base mismatches tested (24, 25). These in vitro binding specificities indicate that hMutSo and hMutSB have overlapping specificities for I/D mismatches and that the residual I/D heteroduplex repair activity observed in extracts of *MSH6−/−* cells may be due to hMutSB. We show here that this is in fact the case and also describe the specificities of hMutSo and hMutSB in strand-specific mismatch correction.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Nuclear Extracts—**HeLa S2 cells were obtained from the Lineberger Cancer Center at the University of North Carolina in Chapel Hill (E. F. Yount, 14), LeVo (8), and HL60-R (21) were cultured as described (9, 26). Nuclear extracts were prepared according to published procedures (9, 27).

**Mismatch Repair Assays—**Mismatch repair assays were performed as previously described (9, 27). Briefly, 50 μg of nuclear extract was incubated with 100 ng (24 fmol) of a f1-derived heteroduplex DNA substrate in 10–15 μl at 37 °C for 15 min. The final salt concentration in the assay was 100–110 mM KCl. Assays of immunodepleted extracts were performed similarly except that reactions contained 100 μg of extract protein, and incubation was extended to 60 min. Complementation of deficient extracts was achieved by the addition of purified hMutSB or hMutSo (9) as indicated. The hMutSB preparations used for complementation were isolated from HL60-R cells as described below. Circular phage φ1 heteroduplexes were prepared as described (28, 29) and contained a nick 181 nucleotides 3′ to the mispair (short path) in the case of the 3′ substrates or 125 nucleotides 5′ to the mispair (short path) in the case of the 5′ substrates. Heteroduplex substrates containing I/D heterologies of different sizes have the following nonpaired sequences: dA (1 nucleotide), d(CA) (2 nucleotides), d(CTCA) (3 nucleotides), d(CTGCA) (5 nucleotides), d(AACATCGA) (8 nucleotides), d(AACCACAGCTGA) (21 nucleotides). I/D heteroduplex substrates are sometimes described by the unpaired sequence that forms the loop, e.g. CA I/D. For 5′-I/D heteroduplexes the extra nucleotides were in the nicked complementary DNA strand, except for the A I/D heteroduplex, which contained an unpaired adenine in a run of six adenines within the continuous viral strand (9). For the 3′-CA I/D heteroduplex, the two extra nucleotides were 100 nucleotides 5′ to the mispair presented onto a 1-methylsulfonyl fluoride in isopropyl alcohol, 1 μg/ml leupeptin (Boehringer Mannheim), 0.5 μg/ml aprotinin (Boehringer Mannheim) containing 100 mM KCl, 1 mM dithiothreitol (DTT; Amersham Pharmacia Biotech) and incubated with nuclear extracts for 1 h at 4 °C (50 μg of extract/ml of gel). The depleted extract was recovered by pouring the slurry into a 2 ml disposable column (Bio-Rad) and collecting the eluant. Depleted extracts were used immediately.

**Purification of hMutSo and hMutSB—**hMutSo was purified to greater than 95% purity from nuclear extracts of HeLa cells as described (9). Preparations were free of detectable hMutSB as judged by Western analysis for hMSH3.

**hMutSB** was isolated from nuclear extracts of either HCT-15 or HL60-R cells by a procedure similar to that used for hMutSo. All steps were performed at 4 °C. Crude nuclear extract was treated with (NH₄)₂SO₄ (194 g/liter), and the precipitate was removed by centrifugation. The supernatant was again treated with (NH₄)₂SO₄ (226 g/liter). The precipitate, which contained more than 95% of the hMutSB, was collected by centrifugation; resuspended in buffer A containing 100 mM KCl, 2 mM DTT, dialyzed against the same buffer; quick-frozen in liquid nitrogen; and stored at −80 °C (fraction 1). Fraction 1 (10 ml) containing completely 100 mg of nuclear protein was thawed on ice by the addition of 10 volumes of buffer A containing 250 mM KCl, 1 mM DTT and loaded at 50 ml/h on to a Q-Sepharose column (10.5 cm × 4.9 cm², Amersham Pharmacia Biotech) equilibrated with buffer A containing 250 mM KCl, 1 mM DTT. The column was washed with 100 ml of buffer A containing 250 mM KCl, 1 mM DTT. This step is useful for separation of hMutSB from hMutSo, since hMutSo binds to Q-Sepharose under these conditions while hMutSB remains in the flow-through (approximately 400 mM KCl is required to elute hMutSo from Q-Sepharose). The effluent of the Q-Sepharose column was applied to a single-stranded DNA-agarose column (2.7 cm × 0.4 cm², Life Technologies, Inc.) equilibrated with buffer A containing 250 mM KCl, 1 mM DTT via a direct connection between the two columns. The single-stranded DNA-agarose column was disconnected from the Q-Sepharose column and washed at 50 ml/h with 50 ml of buffer A containing 300 mM KCl, 1 mM DTT and subsequently with 50 ml of buffer A containing 300 mM KCl, 1 mM DTT, 2.6 M MγCl₂. hMutSB was eluted from the single-stranded DNA-agarose with 50 ml of buffer A containing 300 mM KCl, 1 mM DTT, 2.6 M MγCl₂. hMutSB from Q-Sepharose was collected, dialyzed against buffer A containing 1 mM DTT to a conductivity equivalent to 100 mM KCl and loaded at 75 ml/h on to a Q-Sepharose column (0.35 cm × 0.28 cm²) equilibrated in buffer A containing 100 mM KCl, 1 mM DTT. The column was washed with 4 ml of the starting buffer and then eluted with 0.7 ml of buffer A containing 250 mM KCl, 1 mM DTT. The eluate (0.7 ml) was diluted with 1.1 ml of buffer A containing 1 mM DTT to a conductivity of 250 mM KCl. The protein was precipitated by the addition of 1 ml of 100 mM KCl, 1 mM DTT, 2 mM MgCl₂, 1 mM ATP (fraction II). Fraction II was diluted with buffer A containing 1 mM DTT to a conductivity equivalent to 100 mM KCl and loaded at 75 ml/h on to a Q-Sepharose column (0.35 cm × 0.28 cm²) equilibrated in buffer A containing 100 mM KCl, 1 mM DTT. The column was washed with 4 ml of the starting buffer and then eluted with 0.7 ml of buffer A containing 250 mM KCl, 1 mM DTT. The eluate (0.7 ml) was diluted with 1.1 ml of buffer A containing 1 mM DTT to a conductivity of 250 mM KCl. The protein was precipitated by the addition of 1 ml of 100 mM KCl, 1 mM DTT, 2 mM MgCl₂, 1 mM ATP (fraction III). Fraction III was dried under vacuum using a lyophilizer. The lyophilisate was dissolved in buffer A containing 100 mM KCl, 1 mM DTT, 2.6 M MγCl₂, 1 mM ATP (fraction IV). Fraction IV was digested with 0.1% (v/v) saturated phenylmethanesulfonyl fluoride in isopropyl alcohol, 1 μg/ml leupeptin, 0.5 μg/ml aprotinin, and 0.5 mg/ml bovine serum albumin (samples for electrophoretic analysis were saved prior to the serum albumin addition). Pooled fractions were dialyzed against buffer A containing 100 mM KCl, 2 mM DTT, 20% (w/v) sucrose and quick-frozen in liquid nitrogen. Small aliquots were stored at −80 °C. Eighteen g of HL60-R cells (wet weight) yielded 50–50 μg of hMutSB with a purity of 95%, and the yield from HCT-15 cells was about 5-fold lower. The hMutSB preparations obtained in this manner were free of detectable hMutSo as judged by Western blotting for MSH6.

hMutSB was immunopurified on 0.05 ml protein A-Sepharose columns charged with hMSH3-specific immunoglobulin. Columns equilibrated with buffer A containing 100 mM KCl, 1 mM DTT, 50 μg/ml aprotinin and 100 mM KCl for 2 h and the resin was then washed with 5 ml of the same buffer. Bound protein was specifically eluted by incubating the washed resin with 1 ml of a 0.2 mM solution of the hMSH3-specific peptide in buffer A containing 100 mM KCl for 1 h. The eluted protein was precipitated with trichloroacetic acid and used both for denaturing gel electrophoresis and immunoblots. For each experiment, half of the obtained protein was
used for silver-stained denaturing gel electrophoresis. One quarter each was used for separate immunoblots with antibodies against hMSH2 and hMSH3.

Protein content in nuclear extracts and purified fractions was determined using a Bradford assay with bovine serum albumin as a standard (31). The stoichiometry of hMSH2 and hMSH3 in samples of purified hMutSβ was estimated using a cooled charge-coupled device imager (Photometrics) following denaturing gel electrophoresis and staining with Coomassie Brilliant Blue.

RESULTS

Presence of hMutSβ in Extracts of Human Cells—MSH6−/−
cell lines like HCT-15 and MT1 are selectively defective in the repair of base-base mispairs and single-nucleotide I/D mismatches but are proficient in correction of I/D heterologies of 2, 3, and 4 nucleotides (9). Since hMSH6 is a required subunit of hMutSα (9, 10), we reasoned that repair of I/D heterologies larger than 1 nucleotide in these cell lines must depend on a second mismatch recognition activity. To isolate this activity, HCT-15 nuclear extract was resolved by chromatography, and fractions were tested for their ability to restore repair of a dinucleotide I/D heteroduplex to nuclear extract of LoVo colorectal tumor cells, which are devoid of hMSH2 due to partial deletion of the structural gene (8). This cell line is also free of detectable hMSH3 and contains only trace levels of hMSH6 (see below and Ref. 26).

The activity isolated in this manner (see “Experimental Procedures”) restored repair of a CA I/D heteroduplex (3.1 fmol/15 min with LoVo nuclear extract and 25 ng of purified protein) but was inactive on a substrate containing a G-T base-base mispair (<0.3 fmol/15 min in an identical assay). Activity is associated with two polypeptides of approximately 104 and 125 kDa, which were identified as hMSH2 and hMSH3 by Western blot (Fig. 1A). Apparent molecular weights of the two proteins agree well with those predicted from the cDNA sequences (10, 21). This activity was also isolated from methotrexate-resistant HL60-R promyelocytic leukemia cells (21), in which hMSH3 levels are highly elevated due to amplification of the DHRF-MSH3 region of chromosome 5 (26). Polypeptide composition and mismatch repair activity of the protein isolated from this cell line are similar to those of the HCT-15 activity, but yields are about 5-fold higher (Fig. 1A). Integration of Coomassie-stained species after denaturing electrophoresis indicated that hMSH3 and hMSH2 are present in roughly equal amounts in the HL60-R activity (0.7–0.9 mol of hMSH3/mol of hMSH2), suggesting a 1:1 complex as proposed previously for the hMutSβ complex produced from baculovirus-expressed subunits (24). The hMSH3:hMSH2 ratio was lower for the protein isolated from HCT-15 cells (0.4 mol of hMSH3/mol of hMSH2), perhaps a consequence of hMSH3 loss or proteolysis during isolation from bulk extracts of human cells was provided by immunopurification against the amino terminus (residues 81–100). A proteolytic origin from hMSH3 is consistent with electrophoretic analysis across the hMutSβ elution profile from the last Mono-Q column. Early eluting fractions from this column were free of the contaminant and characterized by a hMSH3:hMSH2 molar ratio near unity, whereas the molar ratio was reduced in later eluting fractions containing the contaminant.

Further evidence for presence of the hMutSβ complex in extracts of human cells was provided by immunopurification using immobilized antibody against hMSH3. As shown in Fig. 1B, hMSH2 co-purifies with hMSH3 from nuclear cell extracts of repair-proficient HeLa cells, from the hMSH6-deficient cell line HCT-15, and from the hMSH3-overproducing cell line HL60-R. However, hMSH3 could not be isolated from nuclear extracts of the hMSH2-deficient LoVo cell line, which harbors mutations in both alleles of MSH2 (8) but is also phenotypically deficient in hMSH3 and hMSH6 (see below and Ref. 26).

hMutSβ Is More Abundant in HCT-15 than in HeLa Cells—hMSH3 and hMSH6 were quantitated via immunoblotting in nuclear extracts of HeLa, LoVo, and HCT-15 tumor cell lines (Fig. 2). Neither protein was detectable in MSH2−/− LoVo cells (<0.03 μg of MSH6 and <0.005 μg of MSH3 per mg of nuclear extract, Fig. 2), consistent with an earlier observation with this cell line (26). Since hMSH3 and hMSH6 were only detected in cell lines expressing hMSH2 and since both proteins associate...
tightly with hMSH2 during fractionation (Fig. 1 and (9, 24, 26)), immunological quantitation of hMSH3 and hMSH6 can be used to estimate levels of hMutSα and hMutSβ in nuclear extracts. In HeLa cells, the hMSH3 level is equivalent to about 0.15 μg of hMutSβ/mg of nuclear extract protein, while the hMSH6 level is equivalent to about 1.5 μg of hMutSα/mg. The hMutSα/hMutSβ ratio of 10:1 found here for HeLa cells is similar to the value of 6:1 estimated for HL-60 cells based on chromatographic resolution of the two heterodimers (26). In HCT-15 cells, the hMSH3 level was equivalent to about 0.5 μg of hMutSβ/mg of nuclear extract, but hMSH6 was undetectable. This confirms the absence of hMutSα in this cell line.

hMutSβ Is Responsible for I/D Mismatch Repair in HCT-15 Extracts—Nuclear extracts of HCT-15 cells were immunodepleted by adsorption to an anti-MSH3 support (see “Experimental Procedures”). As shown in Fig. 3, this resulted in loss of repair activity on a CA I/D heteroduplex, but activity on the dinucleotide I/D heteroduplex substrate was restored upon the addition of purified hMutSβ. By contrast, a G-T heteroduplex was not repaired by either the depleted or the mock-depleted extract, even upon the addition of hMutSβ. As expected, the addition of hMutSα to immunodepleted extracts restored repair of both the base-base and the I/D heteroduplexes. The addition of hMutSα also restored G-T heteroduplex repair activity to the mock-depleted extract, but repair of the CA I/D substrate was not significantly increased above endogenous levels. hMutSα therefore has a unique role in G-T heteroduplex repair, but hMutSα and hMutSβ are redundant with respect to correction of the dinucleotide I/D substrate. The finding that immunodepletion of HeLa nuclear extracts with the hMSH3 antiserum did not affect repair of either heteroduplex (data not shown) is also consistent with this view.

Mismatch Specificities of hMutSα and hMutSβ in Strand-specific Repair—Inasmuch as MSH2−/− LoVo cells are also phenotypically deficient in hMSH3 and hMSH6 (see Fig. 2 and Ref. 26), extracts of this cell line can be used to establish the mismatch repair specificities of hMutSα and hMutSβ, since the potential for subunit exchange is precluded. As noted above (see “Experimental Procedures”), the hMutSα and hMutSβ preparations used in this study are free of detectable cross-contamination. As shown in Fig. 4, hMutSα restored near-normal levels of repair for each of the eight base-base mismatches to LoVo extract, but comparable amounts of hMutSβ did not detectably increase repair above base line in any case. The amounts of hMutSα and hMutSβ used in these experiments was 0.8 μg/mg LoVo extract, corresponding to about 50 and 500% of the levels of hMutSα and hMutSβ in HeLa nuclear extract, respectively (see above).

Purified hMutSα and hMutSβ were also compared with respect to their ability to restore repair of I/D heteroduplexes to LoVo nuclear extract. As shown in Fig. 5, heterologies of 1, 2, or 3 nucleotides were not processed to a significant degree by LoVo nuclear extract, although repair of I/D mismatches of 5–27 nucleotides was observed, increasing with the size of the unpaired region. Since correction of I/D mismatches of 8 and 16 nucleotides has also been observed in extracts of MLH1+/− HCT-116 cells (32), we attribute this activity to a pathway distinct from the mismatch repair system that is dependent on hMutSα, hMutSβ, and hMutLα. In the experiment shown in the upper panel of Fig. 5, LoVo extract was supplemented with equivalent amounts of hMutSα or hMutSβ (0.4 μg/mg of extract, corresponding to about 25 and 250% of hMutSα and hMutSβ concentrations in HeLa extract, respectively). Since the rate of repair under these conditions is limited by the MutS homolog concentration, the values shown provide a direct comparison of the specific activities of the two heterodimers in I/D heteroduplex repair. As can be seen, hMutSα restored repair on the single-nucleotide I/D mispair, but both proteins complemented LoVo extract to increase repair on I/D mismatches containing 2–8 nucleotides. hMutSα supported repair of all I/D mismatches in this class, but hMutSβ was somewhat more active in promoting correction of the 3-, 5-, and 8-nucleotide heterologies tested. Furthermore, the repair spectrum of hMutSβ-complemented LoVo extract was virtually identical to that observed with extract prepared from MSH6+/− HCT-15 cells (compare Fig. 5 and Table I).

While experiments with equal amounts of hMutSα and hMutSβ are useful for determination of relative activities of the proteins on particular substrates, these conditions do not accurately reflect the situation in the repair proficient cell lines, which, as noted above, contain considerably more hMutSα than hMutSβ. Supplementation of LoVo nuclear extract with disparate amounts of hMutSα and hMutSβ (2 and 0.4 μg/mg of extract, corresponding to about 125 and 250% of hMutSα and hMutSβ levels in HeLa extract, respectively) resulted in significant increases in the level of hMutSα-promoted I/D mismatch relative to that observed with hMutSβ. As shown in the lower panel of Fig. 5, such increases were evident in all cases except for the 5-nucleotide heterology.

Although hMutSβ did not support significant repair of the single-nucleotide I/D mismatch under standard assay conditions, low level hMutSβ-directed repair of the single-nucleotide heterology was detected in HCT-15 nuclear extract and in hMutSβ-supplemented LoVo extract at reduced ionic strength (reduction of KCl concentration from 110 to 70 mM). However, under reduced salt conditions, the rate of hMutSβ-supported correction of the dinucleotide I/D heteroduplex was 4–10 times that observed with the single-nucleotide I/D mismatch, as judged by competition experiments in which both substrates were present in the same reaction. hMutSβ therefore displays low but detectable activity on single-nucleotide I/D mispairs.

Like hMutSα, hMutSβ Supports Bidirectional Mismatch Repair—Human strand-specific mismatch repair can be directed by a single-strand break located either 5′ or 3′ to the mispair on the incised strand (29). The experiments summarized in Figs. 4 and 5 used 5′-heteroduplexes. In similar experiments, supplementation of LoVo extract with either hMutSα or hMutSβ restored repair of a 3′-CA I/D heteroduplex (3.9 fmol/15 min for hMutSα and 4.2 fmol/15 min for hMutSβ). However, repair of a 3′ G-T heteroduplex was restored only upon the addition of hMutSα (4.9 fmol/15 min for hMutSα and <0.3 fmol/15 min for hMutSβ). Like hMutSα (9), hMutSβ therefore supports bidirec-

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2 S. J. Littman and J. Genschel, unpublished results.
repair specificities of hMutSα.

Extracts from HCT-15 were treated using either serum containing hMSH3-specific antibodies (mock) or preimmune serum (mock) to the lesion (G-T) or a CA I/D mismatch and a nick 5' to the lesion (CA). We have shown that hMutSα supports repair of each I/D mispair tested but not hMutSβ-dependent repair. This indicates extensive overlap in I/D mismatch specificity for the two activities, although we cannot exclude the possibility that hMutSα and hMutSβ may differentially respond to other I/D mispairs depending on sequence context or the nature of the unpaired nucleotides.

hMutSα and hMutSβ repair spectra are also in general accord with chromosome transfer experiments in which chromosome 5 bearing MSH3+ or chromosome 2 bearing MSH2+ and MSH6+ genes was introduced into the MSH3−/− MSH6−/− HUAA double mutant cell line. Mono-, di-, tri-, and tetranucleotide repeats are unstable in HUAA cells (34). Introduction of MSH3 on chromosome 5 stabilized a tetranucleotide repeat and dinucleotide repeats, but failed to stabilize a d(A)n repeat (34). HUAA extracts are defective in repair of base-base and I/D mismatches, but extracts prepared from HUAA cells containing wild type MSH2 and MSH6 genes repair base-base mismatches as well as 1-, 2-, and 4-nucleotide I/D mismatches (35). Extracts derived from HUAA cells harboring a wild type copy of MSH3 were inactive in base-base mismatch repair and is weakly active on the single-nucleotide I/D mismatch tested but supports efficient repair of I/D mismatches of 2 to ~8 unpaired nucleotides. It is noteworthy that hMutSα supported the repair of each I/D mispair that was also corrected in a hMutSβ-dependent reaction. This indicates extensive overlap in I/D mismatch specificity for the two activities, although we cannot include the possibility that hMutSα and hMutSβ may differentially respond to other I/D mispairs depending on sequence context or the nature of the unpaired nucleotides.

MISMATCH REPAIR (FMOL/60 MIN)

FIG. 4. hMutSα but not hMutSβ restores repair of base-base mismatches to LoVo nuclear extracts. MSH2+/− LoVo nuclear extract was assayed for repair of base-base mismatches as described under “Experimental Procedures” in the absence (solid bars) or in the presence of 40 ng of either hMutSα (gray bars) or hMutSβ (cross-hatched bars) or hMutSβ (gray bars). The heteroduplex substrates used in these experiments contained a single-strand break 5’ to the mispair.

DISCUSSION

Two phenotypes have been described for mismatch repair-deficient human cell lines with mutations in genes that encode MutS homologs. MSH2 mutations confer hypermutability at selectable loci and destabilize simple repeats such as (A)n and (CA)n (5, 14). MSH6−/− mutants, on the other hand, show an increased HPR mutant rate and instability of single-nucleotide repeat sequences, but mutations in dinucleotide repeats are rare (13–15, 17). hMSH3 deficiency has been reported in hematological malignancies (33) and sporadic cancers (34, 44), but the effect of hMSH3 deficiency on genetic stability has not been carefully evaluated.

The differential nature of the mutation spectra of MSH2−/− and MSH6−/− human cells can be understood in terms of the repair specificities of hMutSα and hMutSβ that we describe here. We have shown that hMutSα supports repair of all eight base-base mismatches, as well as each I/D mispair tested ranging from 1 to 8 unpaired nucleotides. By contrast, hMutSβ is inactive in base-base mismatch repair and is weakly active on the single-nucleotide I/D mismatch tested but supports efficient repair of I/D mismatches of 2 to ~8 unpaired nucleotides. The effect of sequence context, since we have observed weak but significant hMutSβ-directed repair of an A I/D mismatch under conditions of reduced ionic strength. Furthermore, while mononucleotide repeats are highly unstable in hMSH6-deficient human cells (13, 14, 17), the degree of destabilization of such sequences is not as great as that observed with MLH1−/− cells (13, 15), suggesting that some processing of mononucleotide

FIG. 3. Immunodepletion of nuclear extracts from HCT-15 with a hMSH3-specific antibody. Nuclear extracts from HCT-15 were treated using either serum containing hMSH3-specific antibodies (mock) or preimmune serum (mock) and assayed for mismatch repair as described under “Experimental Procedures.” Assays were supplemented with 100 ng of hMutSα or hMutSβ as indicated. Heteroduplex substrates contained a G-T mispair and a nick 5’ to the lesion (G-T) or a CA I/D mismatch and a nick 3’ to the lesion (CA).
otide repeats appear to be relatively stable in Proce-
sures" without addition (35).

shown that hMutS heterodimers produced by 100 ng of hMutSα or hMutSβ were used. The lower panel shows results obtained with 100 ng of hMutSα and 20 ng of hMutSβ. Heteroduplex substrates contained a single-strand break 5’ to the I/D mispair. Sequences of the unpaired I/D heterologies are given under “Experimental Procedures.”

mismatches by hMutSβ does occur. Interestingly, mononucleotide repeats appear to be relatively stable in MSH6−/− murine cells (35).

While the mismatch repair specificities of hMutSα and hMutSβ described here are consistent with mutation spectra and in vitro assay of hMSH6-deficient and hMSH3-deficient cells, they differ significantly from hMutSα specificity deduced from gel shift assay using a heterodimer produced by in vitro transcription/translation (25). These experiments failed to detect interaction of hMutSα with I/D heteroduplexes of 2 or 10 unpaired nucleotides, whereas hMutSβ produced in a similar manner was found to bind to both. Since other experiments have shown that hMutSα efficiently binds I/D heteroduplexes with one, two, and three unpaired nucleotides (9, 24), it is possible that hMutSβ produced by in vitro transcription/translation is not fully active. In addition, since other activities are involved in mismatch rectification, mismatch binding may not provide a completely accurate indicator of the specificities of hMutSα and hMutSβ in the overall repair reaction. Repair, on the other hand, is expected to reflect the influence of these other activities.

Our findings and the chromosome transfer experiments discussed above also suggest that significant differences exist between the specificities of hMutSα and hMutSβ and their Saccharomyces cerevisiae counterparts. As in the case of hMutSα, genetic evidence and gel shift data support a role for yeast MSH2-MSH6 in the repair of base-base mispairs and single-nucleotide and dinucleotide I/D mismatches (36–38). Yeast MSH2-MSH3 is also able to support the repair of single-nucleotide and dinucleotide I/D mismatches, but only this complex appears to be involved in the repair of larger I/D heterologies (39–41).

Heterodimer formation between hMSH2 and hMSH6 or between hMSH2 and hMSH3 results in two activities with distinct, partially overlapping specificities that are highly active in mismatch binding and repair (9, 10, 24, 25). What determines the mispair binding of these two complexes? An intriguing possibility is that both subunits of the heterodimer contribute elements to the mismatch binding site. In this model, the mismatch binding site created in the hMSH2-hMSH6 complex would be able to accommodate both I/D and base-base heterologies, whereas only I/D heterologies would fit well into the binding site created by hMSH2-hMSH3 heterodimerization. Alternatively, the mismatch binding site may reside within one subunit, with the other subunit serving to activate this binding center.

This and other studies suggest that hMutSα and hMutSβ participate in a common mismatch repair pathway. We previously demonstrated that hMutSα is present in a 6-fold molar excess over hMutSβ in exponentially growing HL-60 cells (26) and have shown here that in mitotically active HeLa cells, 90% of the nuclear hMSH2 is present in the hMutSα heterodimer. The partitioning of hMSH2 between these two complexes appears to be important for genetic stabilization, since overproduction of hMSH3, which increases the hMutSβ pool at the expense of hMutSα, is associated with a large increase in mutation rate (26). As shown here, the hMutSβ pool is also elevated in HCT-15 cells, which do not produce hMutSα due to genetic inactivation of MSH6. Several other lines of evidence indicate regulation of hMutSα and hMutSβ pools in human cells. MSH2−/− LoVo cells, which lack detectable levels of the hMSH2 polypeptide, are phenotypically deficient in hMSH3 and hMSH6 proteins (Fig. 2 and Ref. 26). The distribution of the common hMSH2 subunit between hMutSα and hMutSβ may be a consequence of mass action, with hMSH2 partitioning between hMSH3 and hMSH6 according to pool size and heterodimer formation stabilizing the hMSH3 and hMSH6 subunits. However, other forms of regulation have not been ruled out.

The relative abundance of hMutSα and its broad mismatch specificity suggest that the hMSH2-hMSH6 heterodimer is the primary mismatch recognition activity for correction of DNA biosynthetic errors. While hMutSβ may complement the specificity of hMutSα for I/D heterologies, depending on sequence context of the mismatch and the nature of the unpaired nucleotides, it is also possible that the former activity may have distinct functions in DNA metabolism. For example, Haber and

![Graph](https://via.placeholder.com/150)

**Fig. 5.** Restoration of I/D mismatch repair to hMSH2-deficient LoVo extract by hMutSα or hMutSβ. LoVo nuclear extract was assayed for repair of I/D mismatches as described under “Experimental Procedures” without addition (solid bars) or in the presence of exogenous hMutSα (cross-hatched bars) or hMutSβ (gray bars). In the experiments shown in the upper panel, 20 ng of either hMutSα or hMutSβ were used. The lower panel shows results obtained with 100 ng of hMutSα and 20 ng of hMutSβ. Heteroduplex substrates contained a single-strand break 5’ to the I/D mispair. Sequences of the unpaired I/D heterologies are given under “Experimental Procedures.”

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**TABLE I**

| Size (in nucleotides) of insertion/deletion | Repair<sup>a</sup> | fmol/15 min |
|------------------------------------------|------------------|-------------|
| <0.3                                      | 8.1              | 6.8         |
| 2                                         | 8.4              | 6.4         |
| 5                                         | 3.3              |             |

<sup>a</sup> Mismatch repair assays were performed as described under “Experimental Procedures.”

<sup>b</sup> Heteroduplex substrates were used in these experiments containing a single-strand break 5’ to the mismatch. The sequence of the unpaired region in each substrate is given under “Experimental Procedures.”

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*Primary mismatch recognition activity for correction of DNA biosynthetic errors.* While hMutSβ may complement the specificity of hMutSα for I/D heterologies, depending on sequence context of the mismatch and the nature of the unpaired nucleotides, it is also possible that the former activity may have distinct functions in DNA metabolism. For example, Haber and
cell lines, and M. McAdams for the synthesis of peptides.

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