THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 9, pp. 5674–5686, February 28, 2014

The *Saccharomyces cerevisiae* Mlh1-Mlh3 Heterodimer Is an Endonuclease That Preferentially Binds to Holliday Junctions*[S]

Received for publication, November 13, 2013, and in revised form, January 16, 2014 Published, JBC Papers in Press, January 17, 2014, DOI 10.1074/jbc.M113.533810

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Background: Mlh1-Mlh3 is required for meiotic interference-dependent crossovers.
Results: We produced recombinant Mlh1-Mlh3 and show that it is an endonuclease that binds specifically Holliday junctions.
Conclusion: Mlh1-Mlh3 prefers to bind the open conformation of Holliday junctions, which infers that it acts as part of a larger complex to process Holliday junctions in meiosis.
Significance: Recombinant Mlh1-Mlh3 complexes will be invaluable for further studies.

MutLγ, a heterodimer of the MutL homologues Mlh1 and Mlh3, plays a critical role during meiotic homologous recombination. The meiotic function of Mlh3 is fully dependent on the integrity of a putative nuclease motif DQHAX_2EX_4E, inferring that the anticipated nuclease activity of Mlh1-Mlh3 is involved in the processing of joint molecules to generate crossover recombination products. Although a vast body of genetic and cell biological data regarding Mlh1-Mlh3 is available, mechanistic insights into its function have been lacking due to the unavailability of the recombinant protein complex. Here we expressed the yeast Mlh1-Mlh3 heterodimer and purified it into near homogeneity. We show that recombinant MutLγ is a nuclease that nicks double-stranded DNA. We demonstrate that MutLγ binds DNA with a high affinity and shows a marked preference for Holliday junctions. We also expressed the human MLH1-MLH3 complex and show that preferential binding to Holliday junctions is a conserved capacity of eukaryotic MutLγ complexes. Specific DNA recognition has never been observed with any other eukaryotic MutL homologue. MutLγ thus represents a new paradigm for the function of the eukaryotic MutL protein family. We provide insights into the mode of Holliday junction recognition and show that Mlh1-Mlh3 prefers to bind the open unstacked Holliday junction form. This further supports the model where MutLγ is part of a complex acting on joint molecules to generate crossovers in meiosis.

DNA repair mechanisms safeguard genome stability and ensure correct passage of genetic information during DNA replication. By preventing mutagenesis, DNA repair pathways represent a barrier to cellular transformation to prevent carcinogenesis and delay aging (1). These pathways repair accidental DNA damage caused by a variety of exogenous and endogenous agents or replication errors. Double-strand DNA (dsDNA) breaks represent one of the most cytotoxic and dangerous lesions and are repaired by either non-homologous end-joining or homologous recombination pathways. During meiosis, programmed chromosome breakage and subsequent dsDNA break repair by homologous recombination help to ensure correct chromosome segregation and promote genetic diversity of the progeny (2, 3).

The post-replicative mismatch repair (MMR)[2] corrects DNA polymerases errors that escape their proofreading activity. In *Escherichia coli*, mismatches are detected by the MutS homodimer. Upon mismatch recognition, the ADP-bound MutS is converted into an ATP-bound sliding clamp, which recruits the MutL homodimer, and both MutS and MutL proteins complexed with ATP then activate the MutH endonuclease. MutH incises the newly synthesized DNA strand at non-methylated d(GATC) sites, and this provides entry points for a DNA helicase and one of several exonucleases that degrades the error-containing strand (4). In eukaryotes the MutS and MutL homologues are represented by heterodimers (5, 6). The Msh2-Msh6 (MutSα) and Msh2-Msh3 (MutSβ) complexes recognize base-base mismatches or insertion-deletion loops, respectively. The main MutL complex involved in *Saccharomyces cerevisiae* MMR is the Mlh1-Pms1 heterodimer (MutLα, MLH1-PMS2 in humans). The other major MutL homologue complex, MutLγ (Mlh1-Mlh3), has a key function during meiotic homologous recombination (see below) but also a minor MMR role in the repair of insertion-deletion loops alongside Msh2-Msh3 (7–11). Unlike in *E. coli*, there are no MutH homologues in eukaryotes. However, MutLα has been shown to possess a cryptic endonuclease activity, which is dependent on the integrity of the DQHAX_2EX_4E motif within human PMS2 or yeast Pms1 (12–15). In contrast to MutSα and MutSβ, MutLα has very low affinity for DNA and shows no preference for mismatches (16, 17). In the reconstituted system, it was shown that the latent MutLα endonuclease is activated in a concerted reaction dependent on a preexisting nick, mismatch, MutSα, replication factor C (RFC), and proliferating cell nuclear antigen (PCNA). Likely, these factors help to trigger a conformational change in MutLα that licenses the endonuclease (18). MutLα incises the discontinuous strand and generates new entry points for the

* This work was supported by Swiss National Science Foundation Grants PP00P3 133636 (to P. C.) and PDFMP3 141759 (to J. Jiricny (University of Zurich) and P. C.).
[S] This article contains supplemental Table 1.
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[2] The abbreviations used are: MMR, mismatch repair; HJ, Holliday junction; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; MBP, maltose-binding protein; scDNA, supercoiled DNA; Exo1, Exonuclease 1.

5′-3′ dsDNA-specific Exonuclease 1 (Exo1) to degrade the strand containing the misincorporated nucleotide. Thus, the endonucleolytic activity of MutLα is critically important for MMR, which is in agreement with high mutation rates caused by point mutations (*e.g. pms1E707K*) within the *PMS1* nuclease motif (13).

Meiosis is a specialized cell division that results in the production of spores or gametes. Programmed Spo11-dependent double-strand breaks activate homologous recombination, which facilitates proper pairing of homologous chromosomes and their subsequent segregation (19). Furthermore, by crossing over, or exchanging of DNA sequences between the broken chromosome and a homologous template, homologous recombination contributes to the generation of genetic diversity during sexual reproduction (2). Meiotic crossovers are dependent on the functionally diverse group of proteins belonging to the ZMM family. These factors help to form and stabilize intermediates termed single end invasions and facilitate their conversion into double Holliday junctions (HJs) that are prerequisite for crossover formation (20–24). Both MutS and MutL family proteins have critical functions in meiotic recombination. The Msh4-Msh5 complex is a member of the ZMM group and likely has both early and late roles in meiotic recombination. Msh4-Msh5 localizes as early as leptotene to the chromosome axis, and mutant mice are defective in synapsis (25). Later in pachytene, Msh4-Msh5 might recruit Mlh1-Mlh3 (MutLγ) via its HJ binding and protein-protein interaction (26, 27). MutLγ is, together with the ZMM proteins, essential for meiotic interference-dependent crossovers (28). Joint molecule formation occurs normally in yeast *mlh1 mlh3* mutants, but crossing over is impaired, which suggests that MutLγ functions only in a late step of meiotic recombination to promote a crossover outcome (29–32). Similarly in mice, Mlh1 or Mlh3 foci on pachytene chromosomes mark future crossover sites (33–36). Mlh3 also contains the DQHA_2EX_4E metal binding motif that is critical for the MMR function of yeast Pms1 or human PMS2 (12). The pro-crossover function of MutLγ is absolutely dependent on the integrity of this motif, and *mlh3D523N* mutation that disrupts the motif confers joint molecule resolution defect that is identical to *mlh3* null mutants (29, 31). This infers that MutLγ and its nuclease activity is an integral part of a meiotic resolution pathway. The absence of other resolution activities including Mus81-Mms4 (MUS81-EME1 in humans), Yen1 (GEN1 in humans), and Slx1-Slx4 had only a modest impact on joint molecule resolution, which together with other data shows that Mlh1-Mlh3 is responsible for the majority of interference-dependent meiotic crossovers (29, 31). Furthermore, the disruption of the metal binding motif in Mlh3 resulted in a modest mutator phenotype in mitotic cells, suggesting that the anticipated endonuclease activity of Mlh3 is required for both its meiotic and MMR functions (31).

In contrast to MutLα, the analysis of the Mlh1-Mlh3 behavior was hindered by the fact that previous attempts to prepare recombinant MutLγ have been unsuccessful. Here we demonstrate the expression and purification of both yeast and human Mlh1-Mlh3/MLH1-MLH3 heterodimers from Sf9 cells. We show that yeast MutLγ is indeed a DNA endonuclease as anticipated by genetic studies. We demonstrate that MutLγ has a strong DNA binding activity with a marked preference for Holliday junctions. These recombinant complexes will be invaluable for further studies of MutLγ biochemistry.

EXPERIMENTAL PROCEDURES

Preparation of Expression Plasmids and Purification of Recombinant Proteins—The sequence of all primers is listed in supplemental Table 1. The yeast *MLH3* sequence was amplified from pEAE220 (E. Alani, Cornell University) using primers 245 and 246 (31). The PCR product was digested with ApaI and XhoI restriction endonucleases and cloned into ApaI and XhoI sites of pFB-MBP-SGS1-His (37), creating pFB-MBP-MLH3-his. Similarly, the sequence of yeast *MLH1* was amplified from pEAA109 (E. Alani, Cornell University) using primers 251 and 252. The PCR product was digested by NheI and XhoI restriction endonucleases and cloned into NheI and XhoI sites of pFB-GST-TOP3 (38), creating pFB-GST-MLH1. The cloned genes were verified by sequencing. The viruses were produced using a Bac-to-Bac system (Invitrogen) according to manufacturers' recommendations. *Spodoptera frugiperda* Sf9 cells were then co-infected with optimal ratios of both viruses, and the cells were harvested 52 h after infection, washed with phosphate-buffered saline, frozen in liquid nitrogen, and kept at −80 °C until use.

Typical purification was performed with cell pellets from 3.6 liters of culture. All subsequent steps were carried out at 0–4 °C. Cells were resuspended in 3 volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1:500 (v/v) Sigma protease inhibitory mixture (P8340), 1 mM phenylmethylsulfonyl fluoride, 30 μg/ml leupeptin). Sample was stirred slowly for 15 min. Then, glycerol was added (16% final concentration). Finally, 5 M NaCl was added to 325 mM (final concentration), and the sample was stirred for 30 min. Cell suspension was centrifuged at 50,000 × *g* for 30 min to obtain soluble extract. The cleared extract was bound to pre-equilibrated amylose resin (8 ml, New England Biolabs) for 1 h batch-wise. The resin was washed extensively with wash buffer (50 mM Tris-HCl, pH 7.5, 2 mM β-mercaptoethanol, 250 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). MBP-Mlh3 and glutathione *S*-transferase (GST)-Mlh1 complex was eluted in wash buffer containing 10 mM maltose. Next, the maltose-binding protein (MBP) and GST tags were cleaved by PreScission protease (1 h) (the GST tag on Mlh1 did not improve our purification; therefore, we did not utilize it in our final protocol). The sample was applied on pre-equilibrated nickel nitriloacetic acid resin (0.7 ml, Qiagen) during 45 min of incubation in the wash buffer supplemented with 20 mM imidazole. The resin was washed with wash buffer containing 40 mM imidazole and eluted in the same buffer but with 400 mM imidazole. Pooled fractions were dialyzed against dialysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 300 mM NaCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The sample was aliquoted, frozen in liquid nitrogen, and stored at −80 °C. The sequence coding for the nuclease-deficient Mlh1-Mlh3 (D523N) mutant was amplified from plasmid pEAE282 (E. Alani, Cornell University) (31) and prepared in the same way as the wild type complex. To verify that the C-terminal His tag on Mlh3 does not affect its biochemical function reported here,

a recombinant wild type MutLγ with a His tag on the N terminus of Mlh1 rather than the C terminus of Mlh3 was also prepared. Both constructs behaved very similarly in our assays. Only the data obtained using the former construct are shown in this work. The construct for the expression of Mlh1 (N35A) was prepared using oligonucleotides 325 and 326, and the construct for the expression of Mlh3 (N35A) was prepared using oligonucleotides 327 and 328 by QuikChange site-directed mutagenesis (Agilent Technologies) following manufacturers' instructions.

The sequence of human MLH3 was amplified using primers 288 and 289 from pFB-MLH3 (11), digested with NheI and XmaI restriction endonucleases, and cloned into NheI and XmaI sites of pFB-MBP-SGS1-his, creating pFB-MBP-hMLH3-His. The pFB-MLH1 was described previously (39). The human MLH1-MLH3 complex was expressed and purified using the same procedure as the yeast homologue.

Recombinant Exo1 (D173A) was prepared as described previously (40). PCNA and RFC were expressed and purified from *E. coli* by minor modifications of previously established procedures (41, 42). We thank Robert Bambara (University of Rochester) and Manju Hingorani (Wesleyan University) for the expression plasmids.

DNA Substrates for Nuclease and Binding Assays—The oligonucleotide-based substrates were prepared as described previously (37). The sequences of all oligonucleotides used here are listed in supplemental Table 1. The oligonucleotides used for the respective substrate were: HJ (1253, 1254, 1255, 1256); dsDNA (1253, 1253C); Y-structure (1253, 1254); Nicked HJ (1253, 1254, 1255, 312, 314); Open HJ (1253, 1254, 316, 317), 3-Way junction (1253, 1254, 1255), ssDNA (1253). For endonuclease assays, negatively supercoiled pUC19 dsDNA (scDNA) was used.

Electrophoretic Mobility Shift Assays—The binding reactions (15 μl volume) were carried out in 25 mM Tris acetate, pH 7.5, 1 mM DTT, 100 μg/ml BSA (New England Biolabs), DNA substrate (1 nM, molecules), and either 3 mM EDTA or 2 mM magnesium acetate as indicated ($-Mg^{2+}$ or $+Mg^{2+}$, respectively). Where indicated, the reactions were supplemented with competitors, either dsDNA (pUC19), 3.3 ng/μl, or poly(dI-dC), 1.3 ng/μl. This corresponded to 50-fold molar excess (in nucleotides) over HJ for dsDNA competitor and a 20-fold molar excess (in nucleotides) for poly(dI-dC) competitor. Finally, the recombinant proteins were added. All reactions were assembled on ice. The reactions were then incubated for 30 min at 30 °C (yeast heterodimer) or 37 °C (human heterodimer). Upon adding 5 μl of 50% glycerol with bromphenol blue (0.25%) into each reaction, the products were separated by electrophoresis in 6% polyacrylamide gel (ratio acrylamide:bisacrylamide 19:1, Bio-Rad) at 4 °C. Gels were dried on DE81 chromatography paper (Whatman), exposed to storage phosphor screens (GE Healthcare), and analyzed by Typhoon FLA 9500 (GE Healthcare). The reactions were quantified using Image Quant software. The K_d corresponds to MutLγ concentration when 50% of the respective DNA substrate was protein-bound. The K_d is only reported when at least 90% substrate saturation was reached.

Nuclease Assays—The nuclease assays (15 μl volume) were carried out unless indicated otherwise in 25 mM Tris acetate, pH 7.5, 5 mM manganese acetate, 0.1 mM EDTA, 1 mM DTT, 100 μg/ml BSA (New England Biolabs), DNA substrate (200 ng, pUC19), and recombinant proteins as indicated. The reactions were incubated for 1 h at 30 °C and stopped with 5 μl of stop solution (150 mM EDTA, 2% SDS, 30% glycerol, 0.25% bromphenol blue) and 1 μl of Proteinase K (14–22 mg/ml, Roche Applied Science) for 15 min at 30 °C. The products were separated by 1% agarose gel electrophoresis, and DNA was visualized by staining with ethidium bromide (0.1 μg/ml) using the Alpha InnoTec imaging station.

RESULTS

Expression and Purification of S. cerevisiae and Homo sapiens MutLγ—The sequences coding for yeast Mlh3 and Mlh1 proteins were cloned into pFastBac1 vectors behind MBP or GST affinity tags, respectively (Fig. 1*A*). The heterodimer was expressed in *S. frugiperda* Sf9 cells and purified to near homogeneity (Fig. 1*B*). During purification, the MBP and GST tags were cleaved off by the PreScission protease (see "Experimental Procedures" for details). Using an identical procedure, we also prepared the Mlh1-Mlh3 (D523N) mutant with a disrupted putative endonuclease active site (Fig. 1*C*). The typical yield of the recombinant yeast Mlh1-Mlh3 heterodimers was ~ 0.5–1 mg from 3.6 liters of Sf9 culture, and the protein concentration was ~5 μM.

MutLγ Is an Endonuclease—We first set out to test whether MutLγ has an intrinsic endonuclease activity, as anticipated based on the presence of the metal binding DQHAX_2EX_4E motif within *MLH3* and on the phenotype of the putative nuclease site mutants (12, 13, 29, 31). Because MutLα exhibited a Mn^{2+}-ATP-dependent endonuclease activity on supercoiled dsDNA (13), we set out to test for a similar activity of MutLγ (Fig. 2*A*). We show here that Mlh1-Mlh3 does nick supercoiled dsDNA, whereas mutant Mlh1-Mlh3 (D523N) is devoid of this activity (Fig. 2*B*). The mutant MutLγ was prepared in exactly the same way as the wild type complex, and as we show below, both wild type and mutant complexes behave similarly with regard to DNA binding. We thus conclude that the endonuclease activity is inherent to MutLγ. As with MutLα, the endonuclease activity was dependent on manganese, as we observed almost no activity when manganese was substituted with magnesium (Fig. 2*B*). The optimal activity required at least 3–5 mM manganese (Fig. 2*C*), and magnesium added in addition to manganese had neither stimulatory nor inhibitory effect on the endonuclease activity of MutLγ (Fig. 2*D*). The endonuclease activity was inhibited by elevated levels of sodium or potassium chloride, as expected (Fig. 2*E*). We also found that MutLγ exhibits optimal endonuclease activity at pH 7.5– 8.5 (Fig. 2*F*).

ATP binding and hydrolysis by MutLα are required for mismatch repair, and the endonuclease activity is strongly stimulated by ATP (13). As ATP binding and hydrolysis are equally important for the meiotic and mismatch repair functions of MutLγ in genetic assays (44), we set out to test the effect of ATP on its endonuclease activity. Initially, we observed that ATP inhibited the cleavage of scDNA by MutLγ (Fig. 2*G*). However, ATP is known to chelate divalent cations such as Mn^{2+} or Mg^{2+}. To distinguish whether ATP has a direct effect on the MutLγ endonuclease or affects it indirectly via reducing the

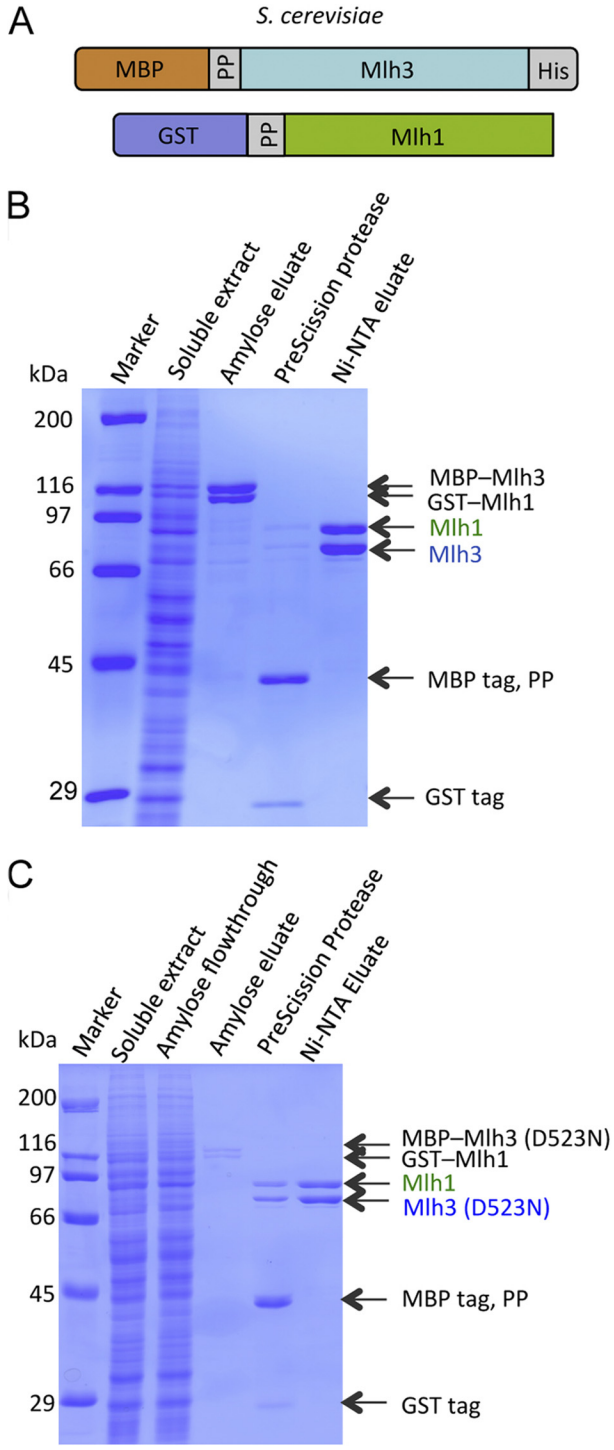

FIGURE 1. **Purification of recombinant yeast MutLγ.** *A*, a diagram of *S. cerevisiae* Mlh1 and Mlh3 constructs. *PP*, PreScission protease cleavage site. *B*, a representative Mlh1-Mlh3 purification showing fractions analyzed by SDS-PAGE. The mass of molecular weight markers is indicated on the *left*, and the positions of the respective recombinant constructs are indicated on the right. The gel was photographed upon staining with Coomassie Brilliant Blue. *C*, a representative purification as in *panel B* but with the nuclease-deficient Mlh1-Mlh3 (D523N) mutant.

free manganese concentration, we supplemented the reactions with ATP as well as an equimolar concentration of manganese acetate (Fig. 2*G*). The simultaneous addition of Mn^{2+} largely, but not completely, negated the inhibitory effect of ATP. Thus, in contrast to MutLα, ATP does not promote the endonuclease activity of MutLγ, indicating that MutLα and MutLγ nucleases are regulated differently. Furthermore, we found out that ATP binding is important for the stability of the MutLγ heterodimer. Mutations that disrupt ATP binding in Mlh1 (Mlh1 (N35A)) or are predicted to confer the same defect on Mlh3 (Mlh3 (N35A)) (44) resulted in nearly complete protein degradation in Sf9 cells (Fig. 2*H*). ATP binding was previously found to be important for the stability of human MutLα (45), and we show here that it is similar for MutLγ.

Yeast Exo1 was found in genetic assays to be required for all Mlh1-Mlh3-dependent meiotic crossovers. Surprisingly, the direct protein-protein interaction between Exo1 and Mlh1, but not the nuclease activity of Exo1, was essential for this effect (30). We set out to test whether the nuclease-deficient Exo1 (D173A) mutant stimulated the endonuclease activity of Mlh1-Mlh3 on dsDNA (46). We show in Fig. 3*A* that this was not the case; Exo1 (D173A) did not stimulate the Mlh1-Mlh3 endonuclease. Rather, we observed a decrease of the MutLγ endonuclease activity. The reason for this effect is not known; nevertheless, we point out that Mn^{2+}-dependent nicking of scDNA is unlikely the physiological condition for the MutLγ endonuclease. Therefore, we cannot exclude that Exo1 (D173A) might have a very different role on other substrates and/or under different experimental conditions.

Furthermore, the nuclease activity of MutLα was strongly promoted by RFC and PCNA in both yeast and human systems (12, 13). The effect of these proteins on the meiotic function of Mlh1-Mlh3 is unknown due to the inviability of the respective mutants. We show here that in contrast to MutLα, the endonuclease of MutLγ was not promoted by the recombinant yeast RFC and PCNA proteins (Fig. 3, *B* and *C*), not even in combination with Exo1 (D173A) (Fig. 3*D*). We also show that our preparations of RFC and PCNA were active, as demonstrated by their capacity to stimulate the endonuclease of hMutLα (Fig. 3*E*). Furthermore, we observed no magnesium-dependent endonuclease activity on either scDNA or a plasmid-based DNA substrate containing a cruciform structure resembling a Holliday junction (data not shown (43)). In summary, we demonstrate here that Mlh1-Mlh3 is indeed an endonuclease as anticipated from biochemical studies. Its activation in the context of meiotic recombination is likely to be regulated in a different manner than the nuclease of MutLα in MMR.

Mlh1-Mlh3 Preferentially Binds Holliday Junctions—Having shown that our preparation of yeast MutLγ is active as a nuclease, we next set out to analyze its DNA binding activity. To this end, we used a variety of oligonucleotide-based DNA structures and monitored DNA binding by electrophoretic mobility shift assays. In contrast to what was observed for MutLα, we show in Fig. 4*A* that MutLγ binds DNA with a very high affinity (K_d for dsDNA, Y-structure, and HJ ~1–2 nM and for ssDNA ~3 nM). Yeast MutLα was initially described to lack DNA binding activity (17). Later, DNA binding of MutLα was observed, but the apparent affinity was very low, with K_d values for oligonucleotide-based DNA in the high nanomolar or micromolar range (47–49). Initially, we did not observe significant differences between the various structures tested, and the DNA-bound Mlh1-Mlh3 complex was mostly trapped in the

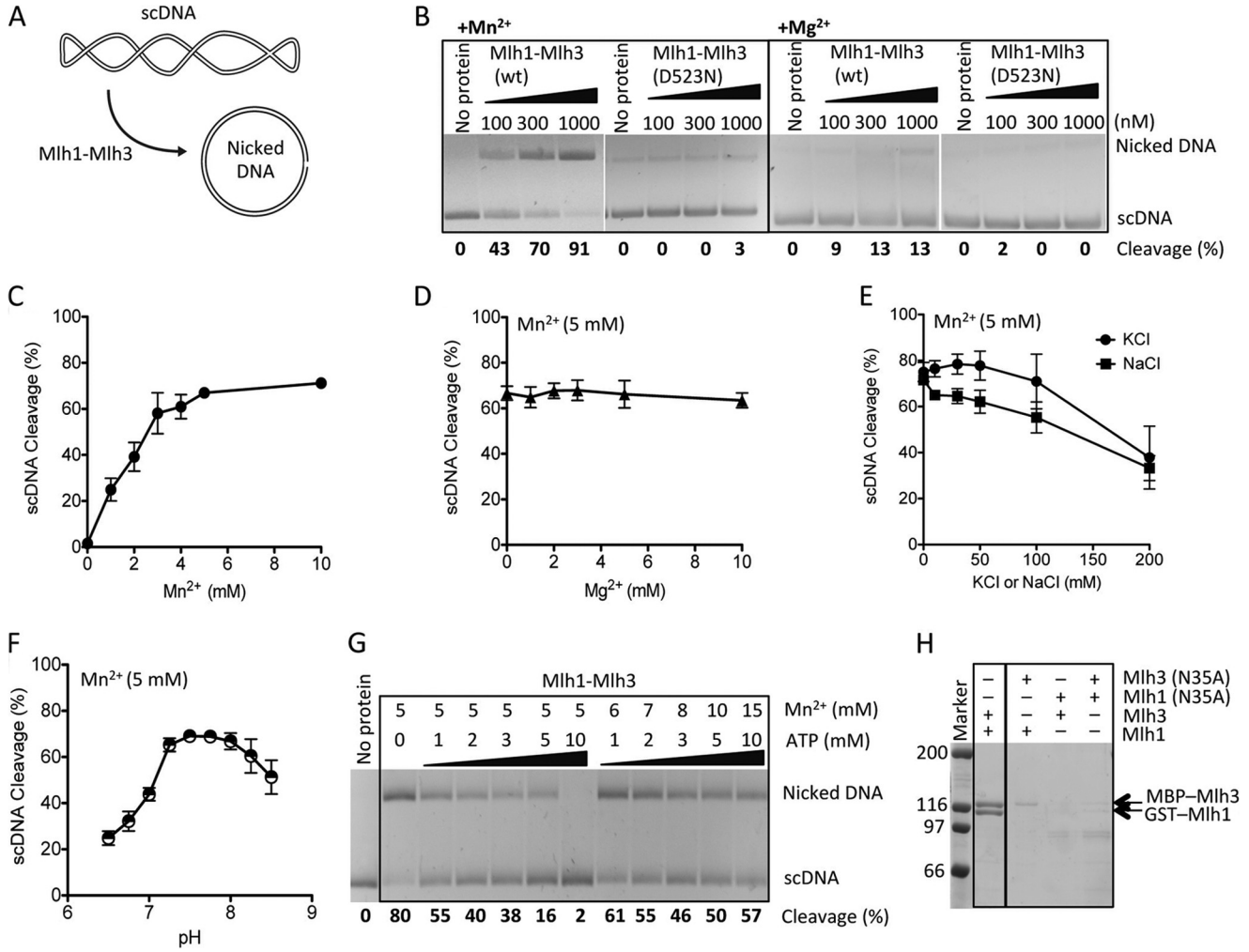

FIGURE 2. **Yeast Mlh1-Mlh3 is an endonuclease that cleaves dsDNA.** *A*, a scheme of the endonuclease assay. *B*, endonuclease assay was carried out with wild type or mutant Mlh1-Mlh3 (D523N), in a reaction buffer containing either 5 mM manganese acetate (*left side*) or 5 mM magnesium acetate (*right side*) as indicated. *Cleavage (%)*, the average value from two independent experiments. *C*, endonuclease assay with Mlh1-Mlh3 (300 nM) was carried out in the presence of various concentrations of manganese acetate as indicated. The results are based on two independent experiments; *error bars*, S.E. *D*, endonuclease assay with Mlh1-Mlh3 (300 nM) was carried out in the presence of 5 mM manganese acetate and various concentrations of magnesium acetate as indicated. The results are based on two independent experiments; *error bars*, S.E. *E*, the effect of sodium and potassium chloride on the endonuclease activity of Mlh1-Mlh3 (300 nM). The results are based on two independent experiments; *error bars*, S.E. *F*, the effect of pH on the endonuclease activity of Mlh1-Mlh3 (300 nM) in Tris acetate-based reaction buffers. The results are based on three independent experiments; *error bars*, S.E. *G*, the effect of ATP on the endonuclease activity of Mlh1-Mlh3 (300 nM). *Cleavage (%)*, the average value from two independent experiments. *H*, ATP binding is required for the stability of MutLγ in Sf9 cells. Amylose pulldown assays were carried out using extracts from Sf9 cells infected with a combination of baculoviruses coding for wild type or mutant Mlh1 or Mlh3 proteins.

wells of the polyacrylamide gels, which was suggestive of a cooperative binding or aggregation (data not shown). Thus, similarly to MutLα, the DNA binding appeared to be rather unspecific (47). The only exception was the HJ substrate, where we observed a minor protein-bound DNA species that entered the gel (data not shown).

We next supplemented the reactions with competitor DNA (pUC19 dsDNA, 3.3 ng/μl) and repeated the binding analyses. The presence of the DNA competitor lowered the apparent DNA binding affinity (Fig. 4*B*). Importantly, we could now observe a clear preference for the HJ substrate (Fig. 4, *B* and *C*). The apparent K_d for HJ was ~16 nM, which was about 5-fold lower than that for dsDNA (K_d ~ 82 nM) and 11-fold lower than for ssDNA (K_d ~ 180 nM). Furthermore, the protein-bound DNA species that entered the polyacrylamide gel was very prominent and was observed only in the case of the HJ substrate (Fig. 4*C*, indicated by a *red arrow*). We believe that this species represents the Mlh1-Mlh3 heterodimer bound specifically to the HJ structure. At higher concentrations and in the case of other DNA substrates such as dsDNA and ssDNA, the DNA was bound rather unspecifically, likely by multiple MutLγ heterodimers, and the complexes then became too large to enter the polyacrylamide gels (Fig. 4*C* and data not shown).

DNA binding by MutLγ decreased as a function of NaCl concentration, indicating that DNA binding was mediated primarily via ionic interactions (data not shown). Next we supplemented the reactions with Tween 20, which is a non-ionic detergent that reduces hydrophobic interactions that may be responsible for protein-protein aggregation. The inclusion of Tween 20 (0.5%) in the binding buffer increased the selectivity

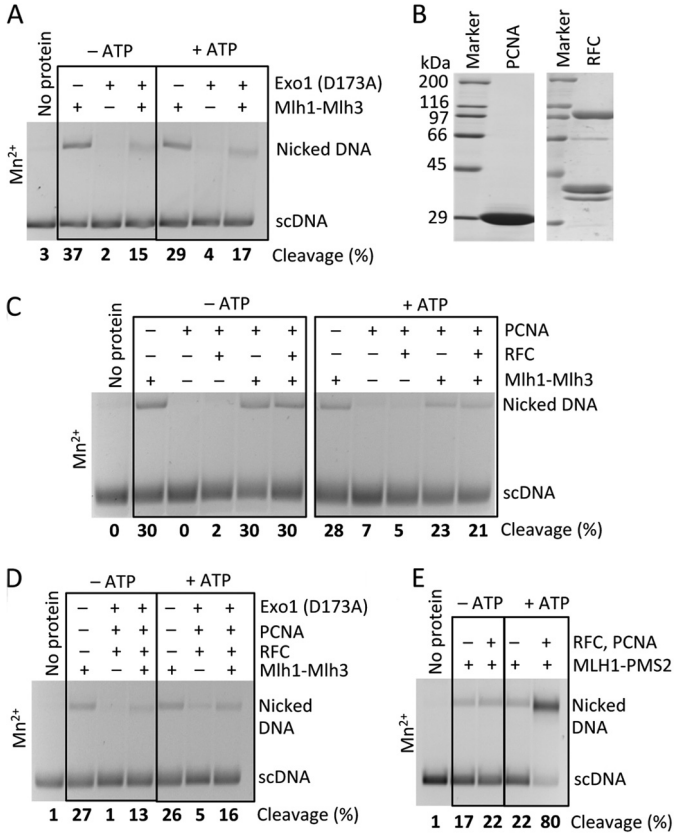

FIGURE 3. **The endonuclease activity of yeast Mlh1-Mlh3 is not promoted by either Exo1 or RFC/PCNA.** *A*, the effect of nuclease-dead yeast Exo1 (D173A, 100 nM) on the endonuclease activity of yeast Mlh1-Mlh3 (100 nM). ATP was present in the reaction buffer where indicated (1 mM). *Cleavage (%)*, the average value from two independent experiments. *B*, purified recombinant yeast PCNA and yeast RFC proteins used in this study. The gel was photographed upon staining with Coomassie Brilliant Blue. *C*, the effect of yeast PCNA (100 nM) and yeast RFC (100 nM) on the endonuclease activity of yeast Mlh1-Mlh3 (100 nM). ATP was present in the reaction buffer where indicated (1 mM). *Cleavage (%)*, the average value from two independent experiments. *D*, the effect of yeast PCNA, yeast RFC, and yeast Exo1 (D173A) on the endonuclease activity of yeast Mlh1-Mlh3 (all proteins 100 nM). ATP was present in the reaction buffer where indicated (1 mM). *Cleavage (%)*, the average value from two independent experiments. *E*, the effect of yeast PCNA (90 nM) and yeast RFC (27 nM) on the endonuclease activity of human MLH1-PMS2 (60 nM). ATP was present in the reaction buffer where indicated (1 mM). *Cleavage (%)*, the average value from two independent experiments.

of MutLγ binding to HJ (Fig. 4*E*). Tween 20 reduced the binding affinity to dsDNA about 2-fold ($K_d \sim 160$ nM), whereas it had a minimal effect on the apparent K_d for HJ ($K_d \sim 20$ nM). Thus, in the presence of Tween 20, MutLγ preferred HJ over dsDNA >8-fold. Based on these results, we conclude that DNA binding by MutLγ is mainly ionic in nature and that unspecific DNA binding is promoted by protein aggregation mediated largely by hydrophobic interactions. Next we analyzed the DNA binding in the presence of the synthetic polymer poly(dI-dC) (1.3 ng/μl). When using both poly(dI-dC) competitor and 0.5% Tween 20, MutLγ preferred binding to HJ >10-fold over dsDNA (Fig. 4, *D* and *F*). We also show that the fraction of the specifically bound HJs was very prominent (up to about 70% of the DNA substrate) and was apparent over a wide range of Mlh1-Mlh3 concentrations (Fig. 4*F*). In contrast, no specific binding to dsDNA was observed. Such binding selectivity is in agreement with the anticipated role of MutLγ in the processing of meiotic double Holliday junctions. It, however, stands in contrast with the behavior of MutLα, which shows no specific binding to mismatched DNA (17). Such behavior is rather reminiscent of MutSα or MutSβ factors, which show a similar binding preference for mismatched over homoduplex DNA (50, 51). We also analyzed DNA binding of the Mlh1-Mlh3 (D523N) mutant. As shown in Fig. 4, *G* and *H*, the mutant preferred to bind HJs similarly to the wild type protein. Although the binding affinity was lower than that of the wild type protein, the experiment shows that the integrity of the putative endonuclease active site does not affect the DNA binding selectivity. In summary, MutLγ has a strong affinity for DNA and exhibits a striking preference for binding to Holliday junctions.

Mlh1-Mlh3 Prefers to Bind the Unstacked Form of a Holliday Junction—We next analyzed the DNA binding by Mlh1-Mlh3 in a reaction buffer supplemented with magnesium. The inclusion of magnesium had a relatively modest effect on the binding affinity to dsDNA ($K_d \sim 155$ nM *versus* ~ 82 nM, decrease of binding affinity less than 2-fold). In contrast, magnesium lowered the binding affinity to HJ ~8-fold ($K_d \sim 130$ *versus* ~16 nM). Thus, in the presence of magnesium, the binding preference of MutLγ to HJ-like structures was strongly reduced (data not shown). The loss of binding preference to HJ in the presence of magnesium was, however, not complete, as revealed by a competition experiment. We prebound MutLγ to a ^{32}P-labeled HJ and then challenged the complex with an excess of either unlabeled HJ or dsDNA. As shown in Fig. 5*A*, the HJ competitor was more effective in disrupting the MutLγ-HJ complex than the dsDNA competitor. Preference for binding HJs in reactions with magnesium was further revealed in the presence of poly(dI-dC) competitor and Tween 20. Under these conditions, MutLγ preferred binding to HJs over dsDNA ~3-fold (Fig. 5, *B* and *C*). We could also clearly detect the specific MutLγ-HJ complex (Fig. 5, *B* and *C*). Nevertheless, the ~3-fold preference for HJs over dsDNA was still significantly smaller than that observed in the absence of magnesium (Fig. 4, *D* and *F*, ~10-fold). Supplementing the reaction with ATP affected neither the affinity for DNA nor the preference for binding HJs by Mlh1-Mlh3 (Fig. 5, *E* and *F*).

We believe that the lower preference for binding HJs in the presence of magnesium reflects an altered HJ structure. Holliday junctions are known to exist in two major conformations. In the absence of metal ions such as Mg^{2+}, HJ adopts an open planar structure with a 4-fold symmetry. In the presence of Mg^{2+}, HJ stacks into a closed antiparallel structure with a 2-fold symmetry (52, 53). Under our experimental conditions, HJ adopts the open or closed conformation depending on the presence of magnesium (data not shown) as expected. Our observation that Mlh1-Mlh3 shows a stronger preference for HJs in the absence of magnesium suggests that MutLγ prefers to bind the open unfolded HJ or a similar structure.

To characterize the binding selectivity of MutLγ in greater detail, we constructed additional oligonucleotide-based DNA substrates, including a three-way junction, a nicked HJ, and a four-way junction with a non-complementary core (open HJ). We next performed electrophoretic mobility shift assays in the presence or absence of magnesium. The most notable results

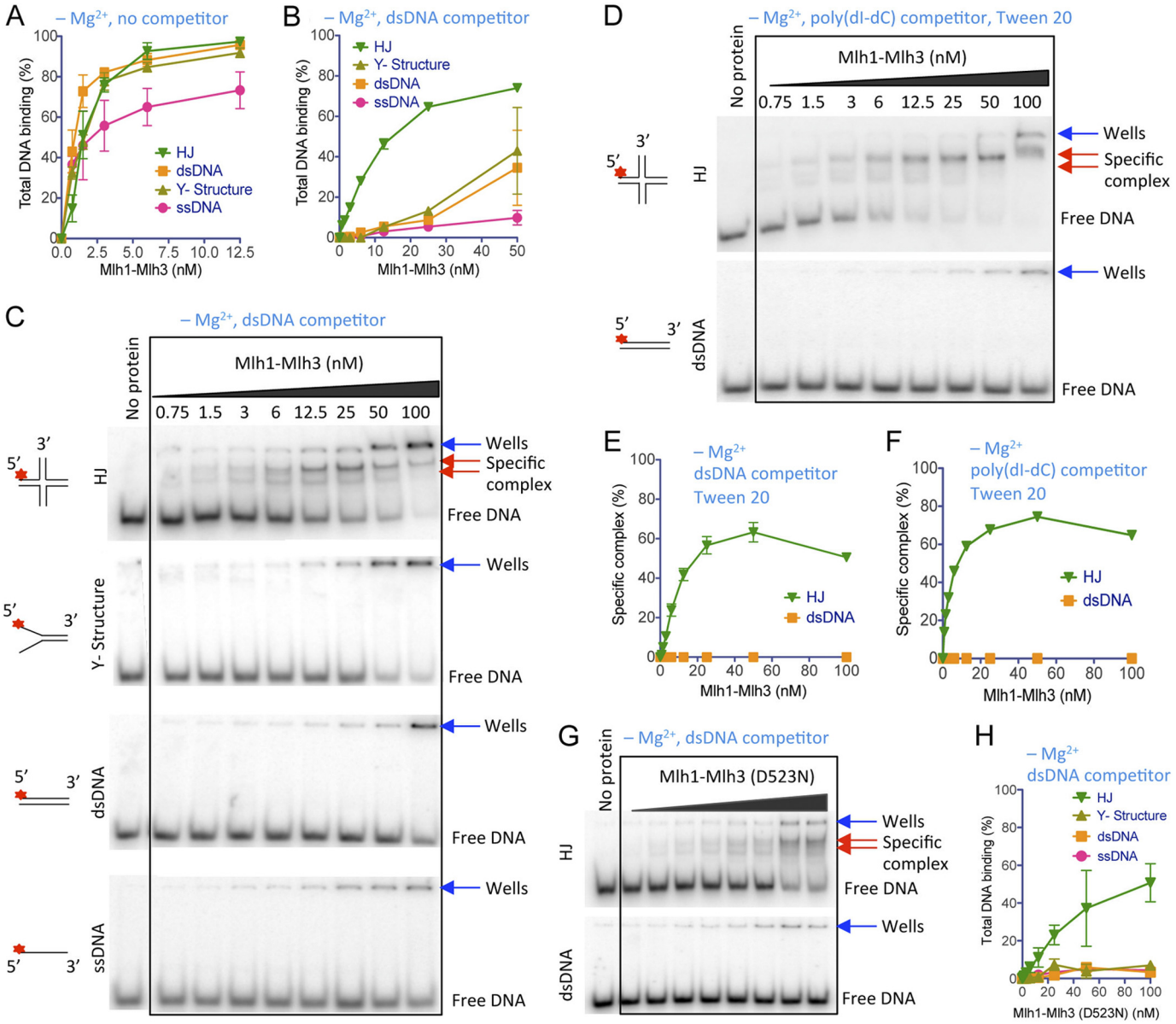

FIGURE 4. **Yeast Mlh1-Mlh3 has a high affinity for DNA and prefers to bind Holliday junctions.** Electrophoretic mobility shift assays were carried out with oligonucleotide-based DNA substrates, as indicated. All oligonucleotides were 50-nucleotides long. *A*, quantitation of assays carried out in a buffer containing 3 mM EDTA and no DNA competitor. The curves show the disappearance of the substrate band and are based on three independent experiments; *error bars*, S.E. *B*, electrophoretic mobility shift assays were carried out in a buffer containing 3 mM EDTA and a dsDNA competitor. The curves show the disappearance of the substrate band and are based on two independent experiments; *error bars*, S.E. *C*, representative experiments from the condition described in *panel B*. The species representing Mlh1-Mlh3 bound specifically to the Holliday junction is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *D*, non-ionic detergent increases the fraction of specifically bound HJ by Mlh1-Mlh3. An electrophoretic mobility shift assay was carried out as in *panel C* but in a buffer supplemented with 0.5% Tween 20 and poly(dI-dC) competitor instead of dsDNA. Shown are representative experiments. The species representing Mlh1-Mlh3 bound specifically to the Holliday junction is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *E*, quantitation of the fraction of specifically bound DNA from experiments carried out in a buffer containing 3 mM EDTA, dsDNA competitor, and 0.5% Tween 20. Results are based on two independent experiments, and *error bars* show S.E. *F*, quantitation of the specific complex from *panel D*. Results are based on two independent experiments, and *error bars* show S.E. *G*, representative experiments such as in *panel C* but with the nuclease-deficient Mlh1-Mlh3 (D523N) mutant (0.8–100 nM). The species representing Mlh1-Mlh3 (D523N) bound specifically to the Holliday junction is indicated by an *arrow* and is denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *H*, quantitation of total DNA binding from assays such as in *panel G*. Results are based on two independent experiments, and *error bars* show S.E.

were obtained with the non-complementary core junction (open HJ). As shown in Fig. 6, *A* and *B*, in the absence of magnesium, the open junction was as good a substrate for MutLγ as the HJ substrate ($K_d \sim 10$ nM). Upon the inclusion of magnesium (Fig. 6, *C* and *D*), the open junction, which cannot stack due to a lack of complementarity, became the preferred substrate for MutLγ binding ($K_d \sim 35$ nM). In summary, we demonstrate here that the binding preference of MutLγ to HJs is reduced in the presence of magnesium that stacks HJs into a closed conformation. Our results indicate that MutLγ prefers to bind the unstacked form of HJs. By inference, we believe that MutLγ *in vivo* acts in a complex with other factors that facilitate its access to the junction under physiological conditions when magnesium is present (see "Discussion").

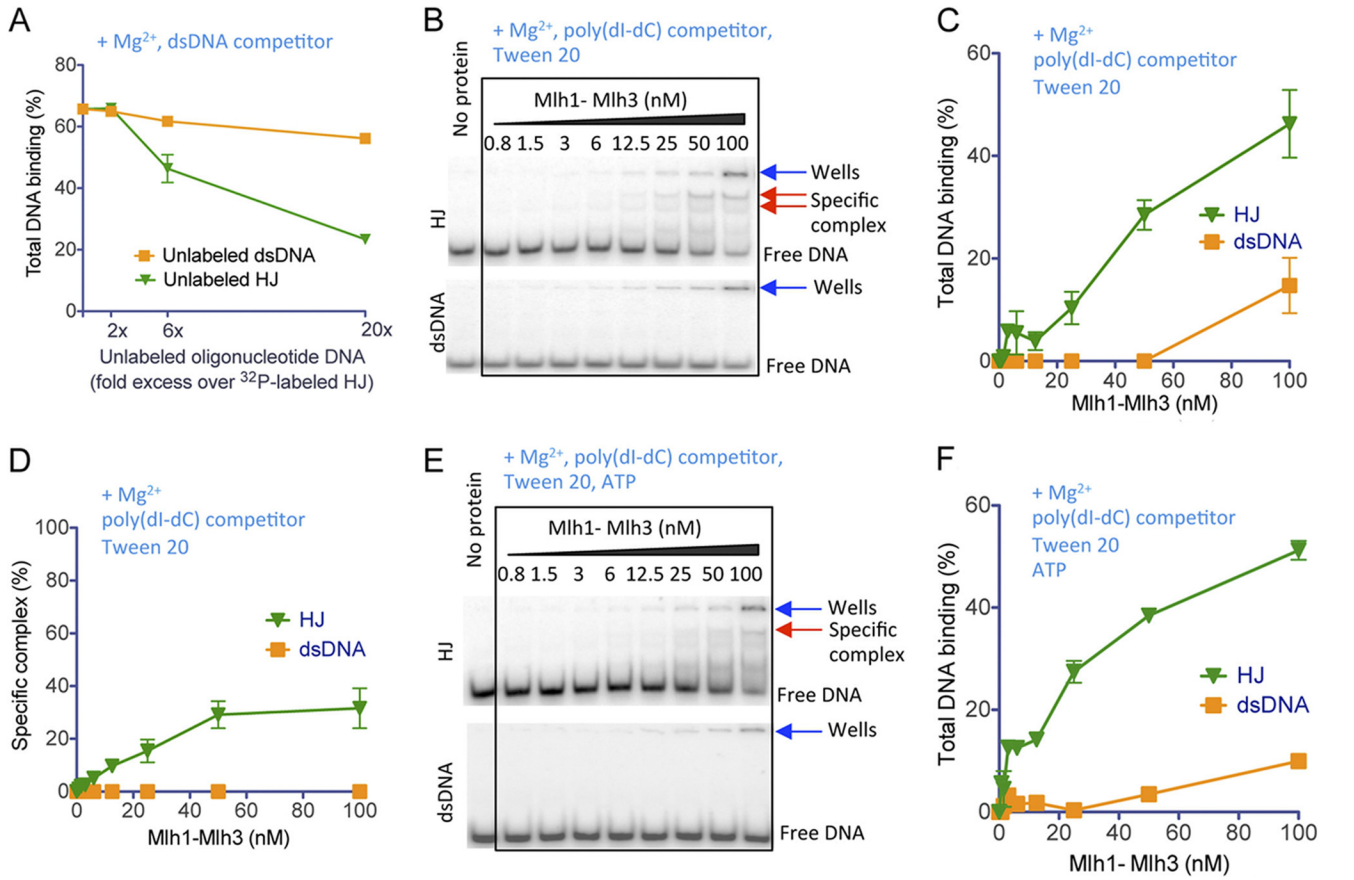

FIGURE 5. **Magnesium lowers the specificity of yeast Mlh1-Mlh3 binding to Holliday junctions.** *A*, HJ is effective as a DNA competitor in the presence of magnesium. The Mlh1-Mlh3 heterodimer (100 nM) was prebound for 15 min to ^{32}P-labeled HJ in a buffer containing 2 mM Mg^{2+}. The complex was then challenged with an excess of unlabeled dsDNA or HJ as indicated and incubated for an additional 15 min. The reaction products were then analyzed by electrophoresis. Results are based on two independent experiments, and *error bars* show S.E. *B*, electrophoretic mobility shift assays were carried out in a buffer with 2 mM Mg^{2+}, 0.5% Tween 20, and poly(dI-dC) competitor. Shown are representative experiments. The species representing Mlh1-Mlh3 bound specifically to Holliday junction is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *C*, quantitation of the experiments such as shown in *panel B*, based on the disappearance of the substrate band. Three independent experiments were done, and *error bars* show S.E. *D*, quantitation of the fraction of specifically bound DNA from experiments such as shown in *panel B*. Results are based on three independent experiments, and *error bars* show S.E. *E*, assays were as in B but additionally supplemented with ATP (1 mM). Representative experiments are shown. The species representing Mlh1-Mlh3 bound specifically to the Holliday junction is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *F*, quantitation of the experiments such as shown in *panel E*, based on the disappearance of the substrate band. Three independent experiments were done, and *error bars* show S.E.

Specific Holliday Junction Binding Is a Conserved Property of Eukaryotic MutLγ Proteins—To test whether the preference for HJ binding is conserved in evolution, we expressed the human MutLγ heterodimer. The sequence coding for hMLH3 was cloned behind a MBP affinity tag (Fig. 7*A*) and co-expressed with untagged hMLH1 in Sf9 cells. The typical yield of the human recombinant heterodimer was ~0.1 mg from 3.6 L Sf9 cells, and the protein concentration was ~645 nM (Fig. 7*B*). Next, we analyzed its DNA binding activity. In the absence of magnesium, the human complex also clearly preferred binding to HJs and related structures (Fig. 7, *C* and *D*). Upon supplementing the reaction buffer with magnesium, the apparent affinity to DNA was decreased, and the complex clearly preferred binding to the open junction structure with the non-complementary core, similarly to the yeast homologue (Fig. 7, *C* and *E*). In contrast to the yeast protein, however, the human MutLγ-bound DNA species remained trapped in the wells of the acrylamide gel, which likely reflects a greater propensity of hMutLγ to multimerize upon DNA binding (Fig. 7*C*). The lower protein concentration of our human MLH1-MLH3 preparation did not allow us to reliably establish the apparent K_d values for all substrates tested; however, the data presented here strongly suggest that the human and yeast MutLγ complexes behave similarly with regard to preferred HJ binding.

DISCUSSION

Here we present the first biochemical characterization of Mlh1-Mlh3. We show that the heterodimer can be expressed in Sf9 cells and purified to near homogeneity. Our analysis reveals that MutLγ has an unexpectedly strong affinity for DNA with a marked preference for Holliday junctions. This behavior stands in sharp contrast to the MMR-specific MutLα (Mlh1-Pms1 in yeast or MLH1-PMS2 in humans) and defines a novel paradigm for a function of a MutL homologue in eukaryotes. We also demonstrate that yeast Mlh1-Mlh3 endonucleolytically cleaves dsDNA and that the regulation of this endonuclease activity is distinct from that of MutLα.

A vast body of *in vivo* data from a number of organisms including yeast, mice, and humans identified MutLγ as a central player in meiotic homologous recombination (26, 29,

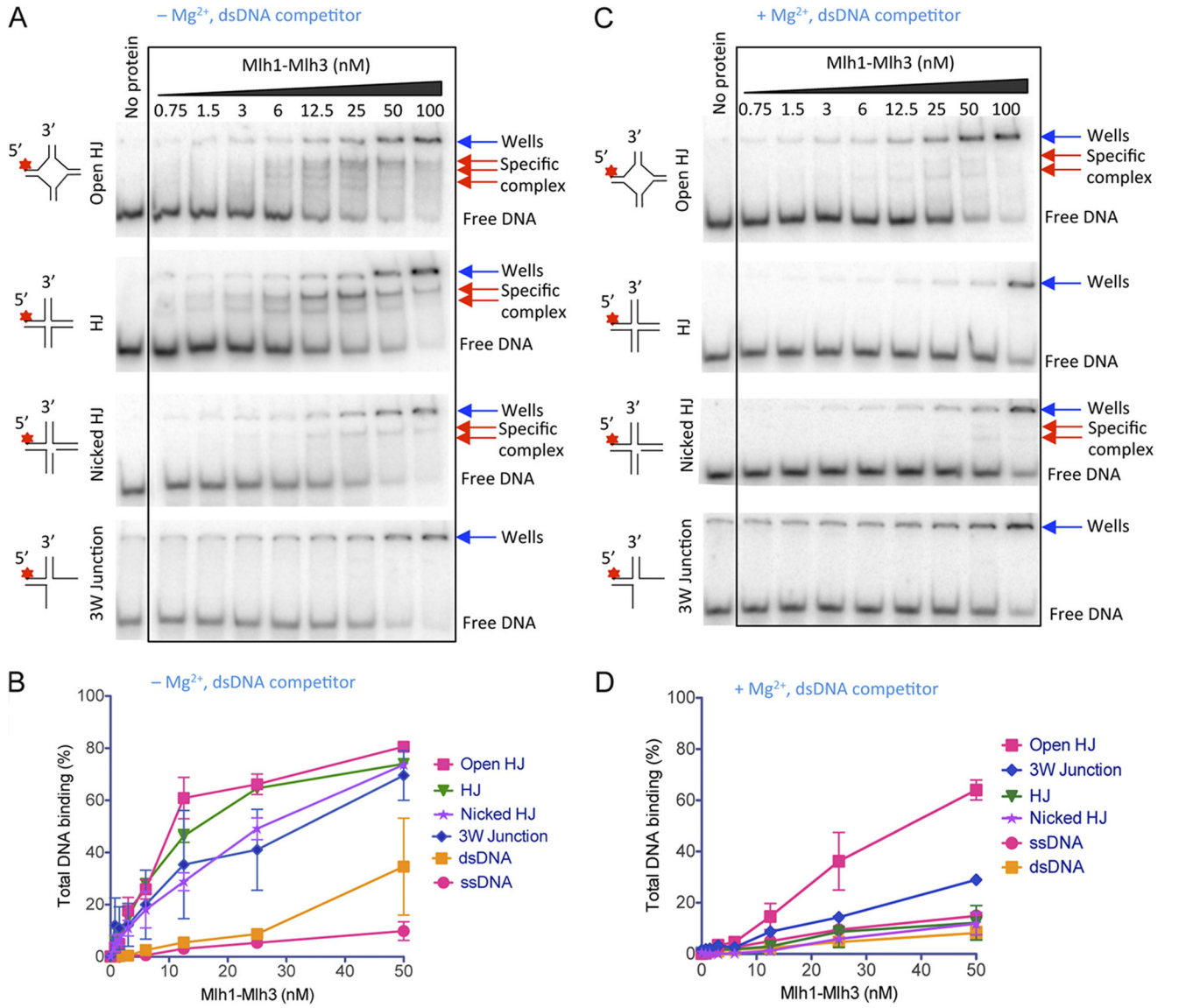

FIGURE 6. **Yeast Mlh1-Mlh3 prefers to bind the open conformation of a Holliday junction.** *A*, an electrophoretic mobility shift assay was carried out in a buffer containing 3 mM EDTA ($-Mg^{2+}$), dsDNA competitor, the respective DNA substrate as indicated on the left, and a range of Mlh1-Mlh3 concentrations. Shown are representative experiments. The image showing Mlh1-Mlh3 binding to HJ is the same as in Fig. 4*C* and is shown here again for reference. The species representing Mlh1-Mlh3 bound specifically to DNA is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *B*, quantitation of the experiments such as shown in *panel A*. The curves show the disappearance of the substrate band and are based on two independent experiments; *error bars*, S.E. *C*, an electrophoretic mobility shift assay was carried out as in *panel A* but with 2 mM magnesium acetate ($+Mg^{2+}$) instead of 3 mM EDTA. The species representing Mlh1-Mlh3 bound specifically to DNA is indicated by an *arrow* and denoted as *Specific complex*. A *blue arrow* indicates the position of wells. *D*, quantitation of the experiments such as shown in *panel C*. The curves show the disappearance of the substrate band and are based on two independent experiments; *error bars*, S.E.

31–34, 54, 55). Available evidence infers a late function, likely in the processing of joint molecules such as double Holliday junctions into crossover recombination products. It was shown that MutLγ is responsible for the majority of meiotic crossovers (29). In addition, MutLγ likely has a minor role in post-replicative MMR (7–11). Understanding the molecular mechanism of MutLγ function is a major challenge, as this complex has been very difficult to obtain. The analysis of the full-length recombinant MutLγ heterodimer presented here thus represents a major step toward that goal.

As first shown by Kunkel and co-workers (16, 47), the MMR-specific yeast MutLα is a DNA-binding protein. However, the affinity of Mlh1-Pms1 for DNA is very low. In the absence of a DNA competitor, the apparent K_d is in the high nanomolar or micromolar range, which represents 2–3 orders of magnitude lower affinity than what we demonstrate here for MutLγ. Although the MutLα heterodimer shows a modest preference for binding to ssDNA, the binding is lost upon supplementing the reaction with a competitor (47). The complex shows no preference for binding either mismatched DNA or a Holliday junction (17, 47). Thus, the DNA binding by MutLα is believed to be unspecific, and it has no direct role in mismatch recognition. This function is carried out by either the MutSα or the MutSβ heterodimers. Although MutLα may increase the affinity of MutSα or MutSβ for mismatched DNA (17, 56), MutLα is not believed to come into contact with the heteroduplex. Yet

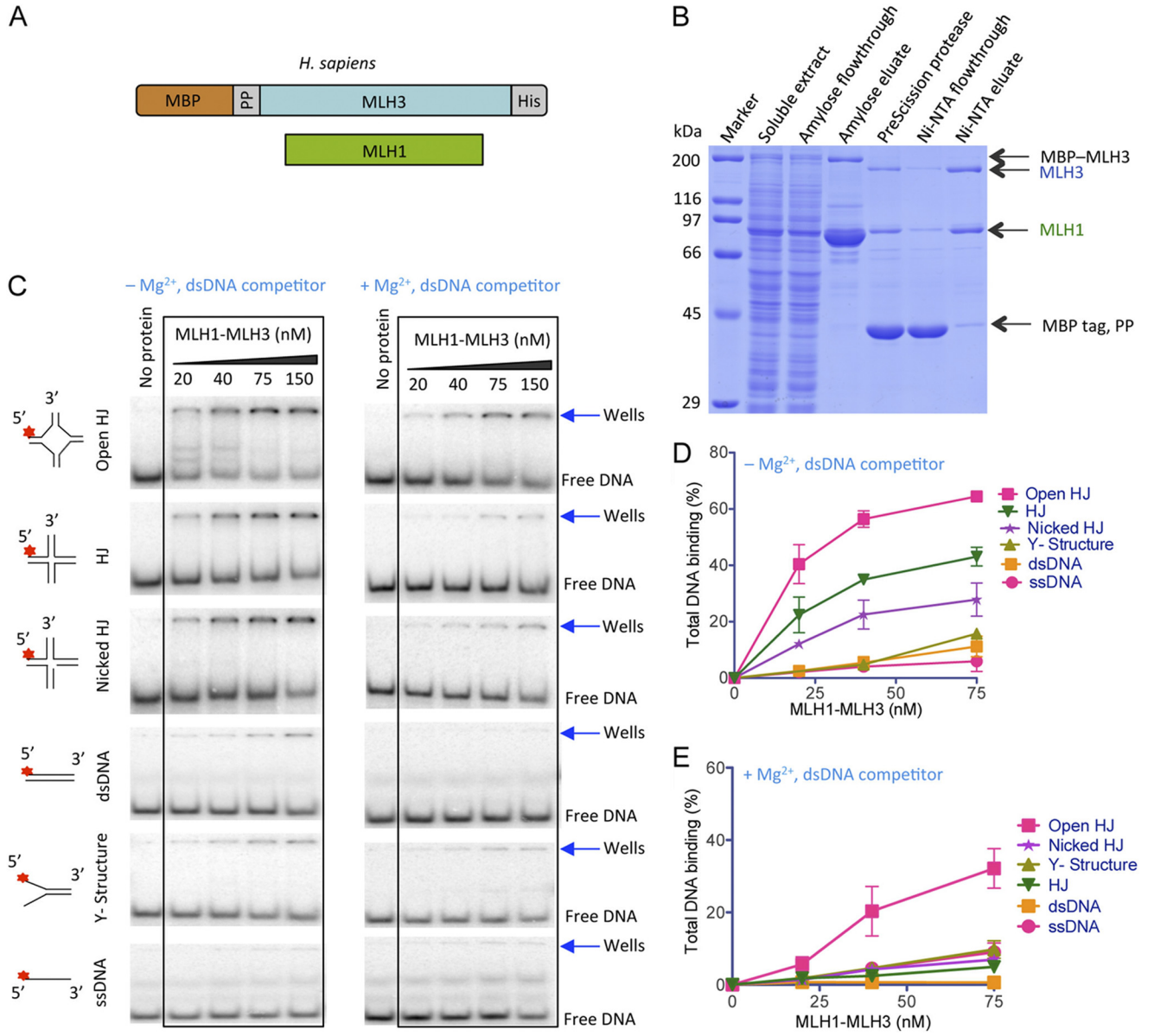

FIGURE 7. **Human MLH1-MLH3 prefers to bind Holliday junctions.** *A*, a diagram of *H. sapiens* MLH1 and MLH3 constructs. *PP*, PreScission protease cleavage site. *B*, a representative MLH1-MLH3 purification showing fractions analyzed by SDS-PAGE. The mass of molecular weight markers is indicated on the *left*, and the positions of the respective recombinant constructs on the *right*. The gel was photographed upon staining with Coomassie Brilliant Blue. *PP*, PreScission protease. *C*, an electrophoretic mobility shift assay was carried out in a buffer containing 3 mM EDTA ($-Mg^{2+}$) or 2 mM Mg^{2+} ($+Mg^{2+}$) as indicated, pUC19 dsDNA competitor, the respective DNA substrate as depicted on the *left*, and a range of MLH1-MLH3 concentrations. Shown are representative experiments. *D*, quantitation of the experiments with 3 mM EDTA such as shown in *panel C*. The curves show the disappearance of the substrate band and are based on three independent experiments; *error bars*, S.E. *E*, quantitation of the experiments with 2 mM Mg^{2+} such as shown in *panel C*. The curves show disappearance of the substrate band and are based on three independent experiments; *error bars*, S.E.

the modest DNA binding activity of MutLα is important *in vivo* as revealed by mutator phenotypes of *mlh1* and *pms1* mutants lacking the DNA binding capacity (16, 49). It may be important downstream of mismatch recognition for the movement of MutLα along the DNA contour before engagement of its endonuclease activity (57, 58).

The strong and specific binding of HJ substrates by MutLγ reported here contrasts with the behavior of MutLα. We demonstrate that MutLγ shows up to a 10-fold preference for binding HJs over dsDNA. This value is very similar to the reported preference of either MutSα (Msh2-Msh6) or MutSβ (Msh2-Msh3) toward binding heteroduplex over homoduplex dsDNA in the presence of the same competitor (50, 51). Analysis of the human MLH1-MLH3 complex further reveals that specific HJ binding by MutLγ is conserved in evolution. This infers that, during meiosis, MutLγ may directly contact HJs. Together with previously published compelling genetic data, our results further support the hypothesis that MutLγ is part of a meiosis-specific HJ resolvase (29, 31, 32).

The conformation of HJs is strongly dependent on the presence of divalent metal ions such as magnesium. In the absence of magnesium, HJs assume an open, 4-fold symmetrical structure. In the presence of magnesium, the core of the HJ folds into a stacked, X-like structure (52). We observed that the prefer-

ence of Mlh1-Mlh3 binding to HJ over dsDNA was greater in the absence (up to ~10-fold; Fig. 4) than in the presence of magnesium (up to ~3-fold; Fig. 5). This revealed that Mlh1-Mlh3 prefers to bind the unstacked, open form of a Holliday junction. This was further supported by the analysis of Mlh1-Mlh3 binding to a HJ structure with a non-complementary core. We show that Mlh1-Mlh3 bound this structure with a high affinity even in the presence of magnesium, showing that the conformation of the HJ, and not the absence of magnesium, results in the high binding affinity. The affinity of MutLγ to ssDNA is very low (Fig. 4); thus the preferred binding to the unstacked form of a HJ cannot be explained by binding to exposed ssDNA. Furthermore, no specific binding was observed to a 3-way junction, showing that a junction with all four arms is the favored substrate of MutLγ (Fig. 6).

Preferred binding to the open conformation is rather unusual for HJ resolvases. Typically, as it has been observed with *e.g.* the canonical *E. coli* resolvase RuvC or the mitochondrial *S. cerevisiae* resolvase Cce1, these enzymes bind equally well the stacked and the unstacked forms of HJs (59, 60). Upon binding, however, these proteins open the core of the HJ so that the resolvase-bound HJ in the presence of magnesium resembles more the conformation of the protein-free structure observed without magnesium rather than the stacked structure (59, 60). We believe that the simplest explanation of our results is that Mlh1-Mlh3 does not bind HJs alone but rather in complex with other factors, such as Exo1, Msh4-Msh5, or Sgs1, which may facilitate its access to HJs. Msh4-Msh5 is an obvious candidate for this role. The human heterodimer was shown to form a complex with HJs that was stable in the presence of magnesium. Upon HJ binding and ADP → ATP exchange, the MSH4-MSH5 complex turns into a sliding clamp that slides away from the HJ (27, 61). It remains to be established whether MSH4-MSH5 makes the HJ more accessible for MLH1-MLH3 binding and whether yeast Msh4-Msh5 behaves similarly. Furthermore, Sgs1 and its helicase activity is part of the crossover-specific pathway together with Mlh1-Mlh3[3] (29, 32). As Sgs1 shows a preference for unwinding HJs and it interacts with Mlh3 during meiosis (37, 62), it is possible also that the Sgs1 helicase may act in complex with Mlh1-Mlh3 to melt the HJ structure. Finally, Exo1 has a non-catalytic role in promoting joint molecule resolution by Mlh1-Mlh3. However, the molecular mechanism of this function remains unknown (30). We anticipate that some of these proteins, possibly in combination with yet-unidentified factors, may help to recruit MutLγ to the joint molecules.

During MMR, human and yeast MutLα exhibit a Mg^{2+}-dependent endonuclease activity that nicks dsDNA and that is activated in a concerted reaction requiring a pre-existing strand discontinuity (*i.e.* a nick), a mismatch, and the MutSα, RFC, and PCNA proteins. In addition, MutLα exhibits a rather unspecific Mn^{2+}-dependent endonuclease activity that nicks supercoiled dsDNA. The presence of manganese bypasses the requirement for the presence of the above reaction components (12, 13). Thus, the analysis of the Mn^{2+}-dependent nuclease reveals elements of the specific reaction. To this point, it was demonstrated that the MutLα endonuclease is strongly stimulated by ATP as well as by RFC and PCNA. We show here that MutLγ exhibits a similar, Mn^{2+}-dependent endonuclease activity. We show that, similarly to MutLα, ATP binding by either Mlh1 or Mlh3 is required for the stability of the MutLγ heterodimer. In contrast to MutLα, however, ATP does not stimulate the endonuclease of MutLγ. We also show that RFC and PCNA also do not promote the MutLγ endonuclease. Thus, the endonucleases of MutLα and MutLγ differ dramatically with regard to how their activity is regulated in a physiological context.

It is anticipated that the physiological substrate for the MutLγ endonuclease are double HJs. As MutLγ and its partners process these structures into specifically crossovers, the key question is what determines the crossover-specific resolution. Double HJs may not be fully matured (*i.e.* ligated), and the position of nicks may indicate the directionality of cleavage. Furthermore, asymmetric protein binding (such as Msh4-Msh5 or other ZMM family members, Exo1, Sgs1) may direct MutLγ cleavage. Finally, it is possible that the structure of the double HJ itself, in particular when both HJs are in close proximity, may activate MutLγ in a structure-specific manner. The availability of recombinant MutLγ will prove instrumental toward further understanding of this important and evolutionarily conserved pathway.

Acknowledgments—We thank J. Jiricny (University of Zurich), U. Rass (FMI Basel), E. Alani (Cornell University), R. Bambara (University of Rochester), W. Heyer (UC Davis), and M. Hingorani (Wesleyan University) for reagents. We thank E. Alani (Cornell University) and N. Hunter (UC Davis) for discussing unpublished data. We thank to J. Jiricny, M. Levikova, L. Mlejnkova, E. Cannavo (all University of Zurich), and C. Pinto (ETH Zurich) for helpful comments on the manuscript and M. Awad (University of Virginia) for technical assistance.

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