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Characterization of a Mammalian Homolog of the *Escherichia coli* MutY Mismatch Repair Protein

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A protein homologous to the *Escherichia coli* MutY protein, referred to as MYH, has been identified in nuclear extracts of calf thymus and human HeLa cells. Western blot (immunoblot) analysis using polyclonal antibodies to the *E. coli* MutY protein detected a protein of 65 kDa in both extracts. Partial purification of MYH from calf thymus cells revealed a 65-kDa protein as well as a functional but apparently degraded form of 36 kDa, as determined by glycerol gradient centrifugation and immunoblotting with anti-MutY antibodies. Calf MYH is a DNA glycosylase that specifically removes mispaired adenines from A/G, A/7,8-dihydro-8-oxo-deoxyguanine (8-oxoG or GO), and A/C mismatches (mismatches indicated by slashes). A nicking activity that is either associated with or copurified with MYH was also detected. Nicking was observed at the first phosphodiester bond 3′ to the apurinic or apyrimidinic (AP) site generated by the glycosylase activity. The nicking activity on A/C mismatches was 30-fold lower and the activity on A/GO mismatches was twofold lower than that on A/G mismatches. No nicking activity was detected on substrates containing other selected mismatches or homoduplexes. Nicking activity on DNA containing A/G mismatches was inhibited in the presence of anti-MutY antibodies or upon treatment with potassium ferricyanide, which oxidizes iron-sulfur clusters. Gel shift analysis showed specific binding complex formation with A/G and A/GO substrates, but not with A/A, C · GO, and C · G substrates. Binding is sevenfold greater on A/GO substrates than on A/G substrates. The eukaryotic MYH may be involved in the repair of both replication errors and oxidative damage to DNA, the same functions as those of the *E. coli* MutY protein.

Mismatches arise in DNA through DNA replication errors, through DNA recombination, and following exposure of DNA to deaminating or oxidating environments. Cells have a host of strategies that counter the threat to their genetic integrity from mismatched and chemically damaged base pairs (19). With regard specifically to mismatch repair of replication errors, *Escherichia coli* and *Salmonella typhimurium* direct the repair to the unmethylated newly synthesized DNA strand by *dam* methylation at d(GATC) sequences, using the MutHLS system (14, 45, 54). The very short patch pathway of *E. coli* is specific for the correction of T/G mismatches (a mismatch indicated by a slash) and is responsible for the correction of deaminated 5-methylcytosine (24, 30, 31, 55). The *E. coli* MutY pathway corrects A/G and A/C mismatches, as well as adenines paired with 7,8-dihydro-8-oxo-deoxyguanine (8-oxoG or GO) (3, 33, 40, 43, 53, 62). The 39-kDa MutY protein shares some homology with *E. coli* endonuclease III and contains a $[4Fe\text{-}4S]^{2+}$ cluster (35, 42, 67, 69). The MutY preparation of Tsai-Wu et al. (67) has both DNA *N*-glycosylase and apurinic or apyrimidinic (AP) endonuclease activities, whereas those purified by Au et al. (4) and Michaels et al. (40, 43) possess only the glycosylase activity. DNA glycosylase specifically excises the mispaired adenine from the mismatch and the AP endonuclease cleaves the first phosphodiester bond 3′ to the resultant AP site (4, 67). Repair by the MutY pathway involves a short repair tract and DNA polymerase I (52, 68).

The mismatch repair strategy detailed above has been evolutionarily conserved. Genetic analysis suggests that *Saccharomyces cerevisiae* has a repair system analogous to the bacterial *dam* methylation-dependent pathway (7, 56, 57, 73). A mismatch repair reaction that resembles the bacterial MutHLS system has been detected in extracts of *Drosophila*, *Xenopus*, and mammalian cells (22, 65, 70). *E. coli* MutL and MutS protein homologs have been identified in *S. cerevisiae*, mice, and humans (10, 18, 20, 23, 26, 28, 32, 48–51, 56, 61). Also, a DNA glycosylase specific for the correction of T/G mismatches has been detected in human HeLa cells (11, 71, 72). This pathway is functionally homologous to the *E. coli* very short patch pathway for the correction of deaminated 5-methylcytosine.

In this study, we further characterize an enzyme that was first identified in human HeLa cells and specifically nicks A/G mismatches (75). This protein has been partially purified from calf thymuses by ammonium sulfate precipitation and four chromatographic steps. This work reveals that the enzyme can act on A/GO- and A/C-containing DNA, in addition to A/G mismatches. This enzyme has adenine glycosylase activity and cross-reacts with anti-MutY antibodies. These findings suggest that this protein is the eukaryotic homolog of the *E. coli* MutY protein, which we have named MYH.

MATERIALS AND METHODS

Preparation of nuclear extracts from HeLa cells. Nuclear extracts from HeLa cells were prepared in a manner similar to a previously published method (17). Frozen HeLa cells (9.6 g) were resuspended in 40 ml of buffer H (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]–KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged for 15 min at 3,300 × *g*. Each pellet was resuspended in 20 ml of buffer H, and the cells were allowed to swell on ice for 10 min. The cells were lysed in a Dounce homogenizer with 30 strokes with

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a type B pestle. Nuclei were collected by centrifugation for 15 min at 3,300 × *g*, resuspended in 3.75 ml of buffer N (20 mM HEPES–KOH [pH 7.9], 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) containing 20 mM NaCl. Each sample was mixed gently with equal volume of buffer N containing 0.2 M NaCl for 30 min. Following centrifugation for 30 min at 25,000 × *g*, the supernatant was dialyzed for 2.5 h against 1 liter of buffer C (20 mM HEPES–KOH [pH 7.9], 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). The sample was centrifuged again for 20 min at 25,000 × *g*, and the supernatant was divided into small aliquots and stored at −80°C.

Purification of MYH from calf thymus. Calf thymus tissue (380 g) was sliced into small pieces, placed in 1.5 liters of solution A (5 mM HEPES–KOH [pH 7.9], 0.2 M sucrose, 3 mM $MgCl_2$, 40 mM NaCl, 0.05 mM PMSF), and homogenized in a Waring blender with six pulses of 5 s each at 4°C. The homogenate was filtered sequentially through one, two, and four layers of cheesecloth, prewashed with chilled solution A. The filtrate was centrifuged at 3,300 × *g* for 10 min. The pellet was washed with 500 ml of solution A, resuspended in 500 ml of solution E (25 mM HEPES–KOH [pH 7.9], 10% sucrose, 0.01% Nonidet P-40, 1 mM DTT, 0.1 mM PMSF, 0.35 M KCl), and held at 4°C for 2 h with stirring. The solution was centrifuged at 10,000 × *g* for 30 min, and the supernatant was collected as nuclear extract (fraction I; 398 ml). Ammonium sulfate was slowly added to a final concentration of 45%, and the solution was stirred for 30 min at 4°C. Following centrifugation at 10,000 × *g* for 30 min, the pellet was resuspended in 40 ml of buffer A (20 mM potassium phosphate [pH 7.4], 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF) and dialyzed against 2 liters of buffer A containing 0.05 M KCl for 2.5 h (fraction II; 41 ml). Fraction II was diluted with 2.5 volumes of buffer A and loaded onto an 80-ml Bio-Rex-70 column, which had been equilibrated with buffer A containing 0.05 M KCl. After the column was washed with 160 ml of equilibration buffer, proteins were eluted with an 800-ml linear gradient of KCl (0.05 to 1.0 M) in buffer A. Fractions eluted between 0.2 and 0.46 M KCl were pooled (fraction III; 270 ml). Fraction III was loaded onto a 40-ml hydroxylapatite column equilibrated with buffer B (0.1 M potassium phosphate [pH 7.4], 10 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF). After the column was washed with 40 ml of equilibration buffer and the proteins were eluted with a 200-ml linear gradient of potassium phosphate (0.1 to 0.7 M), the flowthrough fraction and early wash fractions were pooled because they contained the MYH activity (fraction IV; 280 ml). Following dialysis against buffer A containing 0.05 M KCl for 2 h, fraction IV was loaded onto a 5-ml heparin-agarose column equilibrated with buffer A containing 0.05 M KCl. After the column was washed with 10 ml of equilibration buffer, it was developed with a 50-ml linear gradient of KCl (0.05 to 1 M) in buffer A. Fractions containing the MYH nicking activity, which eluted between 0.5 and 0.7 M KCl, were pooled and dialyzed against 1 liter of buffer S (50 mM sodium phosphate [pH 7.4], 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 10% glycerol) containing 0.1 M NaCl (fraction V; 14 ml). Fraction V was then applied to a 1-ml MonoS column which had been equilibrated in buffer S containing 0.1 M NaCl. After the column was washed with 10 ml of equilibration buffer, proteins were eluted with a linear gradient of NaCl (0.1 to 1.0 M) in buffer S. Fractions containing the MYH nicking activity, which eluted between 0.15 and 0.35 M NaCl, were pooled (fraction VI; 7.5 ml), divided into small aliquots, and stored at −80°C.

Preparation of antibodies against MutY and polypeptide. MutY was purified by the procedure of Tsai-Wu et al. (67). A synthetic peptide, Cys-Thr-Arg-Ser-Lys-Pro-Lys-Cys-Ser-Leu-Cys-Pro-Leu-Gln-Asn-Gly-Cys-Ile-Ala-Ala, corresponding to amino acid residues 192 to 211 of the MutY protein was synthesized by the Biopolymer Laboratory, University of Maryland at Baltimore, and was coupled to keyhole limpet hemocyanin as previously described (29). Antiserum to either the intact MutY protein or the KHL-coupled polypeptide was raised in rabbits by Hazleton Research Products, Inc. The anti-MutY antibodies were affinity purified by reaction with membrane-bound MutY protein; the latter was prepared by the transfer of 200 μg of purified MutY protein electrophoretically from an 8% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (27) to a nitrocellulose membrane (66). The position of the MutY protein was located by staining flanking membrane strips with Coomassie blue. The membrane strip containing MutY was excised and incubated for 24 h at 4°C with crude anti-MutY antisera. The membrane was washed three times for 10 min each in TNT (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.3% polyoxyethylenesorbitan monolaurate [Tween 20]), and the antibodies were eluted twice with 1 ml of 0.2 M glycine-HCl (pH 2.8). The eluted solutions were pooled, and 1.2 M Tris-base (pH 11.0) was added to a final pH of 8.3. Sodium azide was added to a final concentration of 0.1%, and the purified antibodies were stored at −20°C.

Western blot (immunoblot) analysis. Protein fractions were resolved on a SDS–10% polyacrylamide gel (27) and transferred to a polyvinylidene difluoride membrane (66). The membrane was subjected to the Enhanced Chemiluminescence analysis system from Amersham International plc according to the manufacturer's protocol, except the blocking solution contained 10% nonfat dry milk (Carnation), and the wash solution contained 1% nonfat dry milk.

DNA substrates. The 116-mer DNA substrates, with four-nucleotide overhangs at both 5′ ends, were identical to those used by Yeh et al. (75). For the synthesis of the oligonucleotide containing a single GO, the protected phosphoramidite of 7,8-dihydro-8-oxo-2′-deoxyguanosine was made from 8-bromo-2′-deoxyguanosine by the method of Roelen et al. (58) except that bis(diisopropylamino)-2-cyanoethylphosphoramidite was used to phosphitylate the nucleoside and the lactam group was not protected. The 19-mer oligonucleotides with normal bases or GO were synthesized on an Applied Biosystems 381A automated synthesizer by standard procedures. The resulting oligomer with GO was cleaved from the resin and deprotected by treatment with concentrated ammonia in the presence of 0.1 M β-mercaptoethanol at 55°C for 16 h (8). The deprotected oligonucleotides were electrophoretically purified on 20% polyacrylamide–8.3 M urea sequencing gels (39). Two complementary oligonucleotides were annealed in a solution containing 7 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, and 50 mM NaCl at 90°C for 2 min and then cooled gradually to room temperature over 30 min to form heteroduplexes as shown below (X represents GO).

(i) 5′-CCGAGGAATTAGCCTTCTG-3′
3′-GCTCCTTAAGCGGAAGACG-5′

(ii) 5′-CCGAGGAATTAGCCTTCTG-3′
3′-GCTCCTTAAXCGGAAGACG-5′

(iii) 5′-CCGAGGAATTCGCCTTCTG-3′
3′-GCTCCTTAAGCGGAAGACG-5′

The annealed duplexes, either the 116-mer (75) or the 19-mer, were radiolabeled at the 3′ end of the upper strand with Klenow fragment of DNA polymerase I for 30 min at 25°C in the presence of [α-^{32}P]dCTP (50 μCi at 3,000 Ci/mmol), 20 μM dTTP, 20 μM dATP, and 20 μM dGTP (37). The resulting blunt-ended duplex DNA was 120 or 20 bp long, respectively. The reaction mixture was passed through a Quick-Spin column (G-50 for the 120-mer and G-25 for the 20-mer) (Boehringer Mannheim). Alternatively, the upper strand of the 116-mer was labeled at its 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (37) prior to annealing with the lower strand.

Enzymatic DNA-nicking and DNA glycosylase assays. The DNA mismatch-nicking activity of *E. coli* MutY was assayed as previously described (34). Mismatch-nicking activity for calf MYH was assayed in a manner similar to that of MutY, except different buffer and incubation times were used. Protein samples were incubated with 1.8 fmol of either the 5′-end-labeled 116-mer, the 3′-end-labeled 120-mer, or the 3′-end-labeled 20-mer duplex DNA in a 20-μl reaction mixture containing 10 mM Tris-HCl (pH 7.6), 5 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 1.5% glycerol. Following a 2-h incubation at 37°C, the reaction products were lyophilized and dissolved in a solution containing 3 μl of 90% (vol/vol) formamide, 10 mM EDTA, 0.1% (wt/vol) xylene cyanol, and 0.1% (wt/vol) bromophenol blue. After being heated at 90°C for 3 min, DNA samples were analyzed on 8% polyacrylamide–8.3 M urea DNA sequencing gels (39), and the gel was then autoradiographed. Under these conditions, little cleavage (<5%) was observed on oligonucleotides containing an AP site (data not shown). The DNA glycosylase activity was monitored by adding piperidine, after the enzyme incubation, to a final concentration of 1 M. After 30 min of incubation at 90°C, the reaction products were analyzed as described above.

Enzyme binding assays. Protein-DNA complexes were analyzed on 4% polyacrylamide gels in 50 mM Tris-borate (pH 8.3) and 1 mM EDTA as previously described (34). MutY or MYH protein samples were incubated with 3′-end-labeled 20-bp oligonucleotides as in the nicking assay, except 20 ng of poly(dI-dC) was added to each reaction mixture. Bovine serine albumin (1 μg) was added as indicated to the MYH binding assay. For the binding competition assay, in addition to the 1.8 fmol of labeled 20-mer substrates, unlabeled 19-mer DNAs containing A/G, A/GO, or C · G pairings were added in excess of up to 180 fmol.

Other materials and methods. HeLa cells were provided by The National Cell Culture Center. The column matrixes were from different sources: Bio-Rex-70 and hydroxylapatite were from Bio-Rad, heparin-agarose was from Bethesda Research Laboratories, and MonoS column was from Pharmacia. The ^{32}P nucleotides were obtained from New England Nuclear, DuPont. Protein concentration was determined by the method of Bradford (9). For the antibody inhibition experiments, the protein was incubated with various concentrations of anti-MutY antibodies (0 to 2.5 μg) for 1 h on ice prior to the addition of the nicking reaction mixture. MutY was oxidized by potassium ferricyanide by incubation of the protein with various concentrations of the agent (0 to 1 mM) for 30 min on ice prior to the nicking reaction.

RESULTS

An A/G mismatch-nicking endonuclease is present in the calf thymus. We have identified an A/G mismatch-nicking enzyme in nuclear extracts from calf thymus cells. Active A/G-nicking enzyme, termed MYH, was purified approximately 1,600-fold over the nuclear extract from calf thymus cells through four chromatographic steps: Bio-Rex-70, hydroxylapatite, heparin, and MonoS (Table 1). A protein gel of the most purified active fractions showed 10 to 15 bands by silver staining (data not shown). When one of the active fractions from the heparin column was fractionated through a 10 to 25% glycerol gradient, the A/G-nicking protein was found to have a molecular mass of approximately 65 kDa.

TABLE 1. Purification of MYH from calf thymus

Fraction	Step	Protein (mg)[a]	Sp act (U/mg)[b]		Ratio of activity (+Pip/−Pip)	Fold purification (−Pip)	% Recovery (−Pip)
			−Pip	+Pip			
I	Nuclear extract	708	1,102	1,333	1.21	1	
II	Ammonium sulfate	512	7,035	10,271	1.46	6	100
III	Bio-Rex-70	69.4	27,409	40,017	1.46	25	79
IV	Hydroxylapatite	4.8	110,416	160,103	1.45	100	22
V	Heparin	0.2	818,180	1,178,179	1.44	742	8
VI	MonoS	0.1	1,763,140	3,032,699	1.72	1,600	6

[a] Protein concentration was measured by the Bradford assay (9).
[b] One unit of nicking (without piperidine treatment [−Pip]) or glycosylase (with piperidine treatment [+Pip]) activity is defined as that resulting in cleavage of 1% (0.018 fmol) of the 3′-end-labeled 120-mer DNA with an A/G mismatch in 2 h at 37°C.

Calf MYH is a DNA glycosylase. On the basis of high specificity for A/G mismatches and the nicking of the mispaired adenine strand, Yeh et al. (75) suggested that the human A/G mismatch-specific endonuclease is the homolog of MutY (MYH). The MutY protein has been shown to be a DNA glycosylase (4, 67). An A/G mismatch-containing substrate, labeled on the 5′ end of the A strand, was used to assay the glycosylase activity of calf MYH protein. As seen by Tsai-Wu et al. (67) in their investigation of the MutY protein, two cleavage products (L and S) were observed (Fig. 1, lane 2) when a 5′-end-labeled DNA substrate was treated with partially purified protein from calf thymus cells. Because of its sensitivity to piperidine treatment (Fig. 1, lane 3), the L product apparently contains an AP site at its 3′ terminus. We surmise that the nicking activity observed during the MYH purification may be the combined action of glycosylase and AP endonuclease.

Copurification of A/G mismatch-specific nicking and glycosylase activities. To investigate whether the AP endonuclease activity associated with calf thymus MYH is contributed by a separate enzyme, glycosylase activities were also assayed by adding piperidine after enzyme incubation and further incubated at 90°C for 30 min during the purification steps. As shown in Table 1, both nicking (without piperidine treatment) and glycosylase (with piperidine treatment) activities copurified through multiple chromatographic steps, since the ratio between both specific activities remained constant. In a separate experiment, both DNA glycosylase and nicking activities also copurified when they were independently assayed in column fractions during the purification.

Western blot detection of MYH with anti-MutY antibodies. To test for the homology between MYH and MutY, we used polyclonal antibodies raised against the MutY protein to probe partially purified calf A/G mismatch-nicking enzyme or crude nuclear extracts from calf thymus or human HeLa cells. Western blotting with anti-MutY antibodies detected mainly a protein with an apparent molecular mass of 65 kDa and some minor bands in both nuclear extracts of calf thymus and human HeLa cells (Fig. 2, lanes 3 and 4). The 65-kDa band was also detected in partially purified A/G-nicking enzyme (Fig. 2, lane 2). The sizes of these reactive bands are consistent with those determined by glycerol gradient centrifugation. However, an additional 36-kDa protein was detected in Western blot analysis in some preparations of the MYH protein from calf thymus cells (data not shown) probably because of a protease reaction in these preparations.

The DNA nicking activity of calf MYH is inhibited by MutY antibodies. Since the MYH protein cross-reacts with antibodies directed against the MutY protein in Western blot analysis,

FIG. 1. The reaction of calf MYH involves an AP intermediate. Partially purified calf thymus MYH (fraction VI [Enz]) was incubated with 5′-end-labeled 116-bp heteroduplex DNA containing an A/G mismatch. After incubation at 37°C for 2 h, DNA was either loaded directly (lane 2) or treated with 1 M piperidine (Pip) at 90°C for 30 min before loading (lane 3) on an 8% polyacrylamide–8.3% urea sequencing gel, which was then autoradiographed. Lane 1 contains the untreated DNA. The L nicking product is believed to contain an AP site at its 3′ end, because its mobility could be increased to the position of the S product by piperidine treatment.

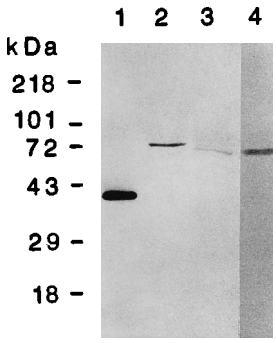

FIG. 2. The mammalian MYH protein cross-reacts to anti-MutY antibodies by Western blot analysis. Proteins were separated on an SDS–10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and allowed to react with antibodies against *E. coli* MutY. Western blotting was performed by the Enhanced Chemiluminescence analysis system from Amersham International plc. Protein fractions are purified *E. coli* MutY protein (3 ng) (lane 1), partially purified calf thymus MYH protein (2 μg; fraction VI) (lane 2), calf thymus nuclear extract (36 μg; fraction I) (lane 3), and HeLa nuclear extract (36 μg) (lane 4). Lane 4 is from a nonconcurrent experiment. The sizes of the protein bands are compared with prestained molecular mass standards from Bethesda Research Laboratories.

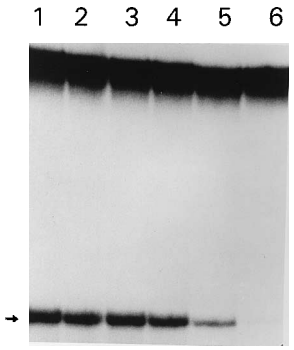

FIG. 3. Calf A/G mismatch-DNA nicking is inhibited by anti-MutY antibodies. Active calf MYH protein (fraction VI) was incubated with antibodies against the *E. coli* MutY protein prior to the reaction with a 3′-end-labeled A/G mismatch-containing 120-mer substrate. MYH protein was incubated with various amounts of anti-MutY antibodies at 0°C for 1 h as follows: lane 1, 0; lane 2, 0.025 μg; lane 3, 0.125 μg; lane 4, 0.25 μg; lane 5, 1.25 μg; and lane 6, 2.5 μg. DNA (1.8 fmol) containing an A/G mismatch was 3′ end labeled and assayed with the protein samples for 2 h at 37°C. The cleavage products, after denaturation, were analyzed on an 8% polyacrylamide–8.3 M urea sequencing gel that was then autoradiographed. The arrow marks the nicking product.

we sought to determine if the antibodies could inhibit the enzyme activity of the calf thymus MYH protein. Preincubation with increasing concentrations of the antibodies with the calf MYH resulted in reduction of nicking activity in proportion to the increasing concentration of the antibodies (Fig. 3). Similar results were obtained upon treatment of the MutY protein with anti-MutY antibodies (data not shown). No inhibition was detected when either enzyme was incubated with control serum prior to the reaction.

Mismatch-nicking specificity of the MYH protein. Yeh et al. reported that the partially purified HeLa A/G mismatch-specific endonuclease detected no nicking activity on DNA substrates containing other mismatches (75). Because *E. coli* MutY does contain a weak nicking activity on A/C mismatch-containing DNA (43, 53, 67), we checked the mismatch specificity of calf MYH. With the same 120-mer DNA substrates as used by Yeh et al. (75), the activity of the partially purified MYH from calf thymus cells was tested on DNA substrates containing A/G, A/C, T/G, or A/A mismatches or homoduplexes labeled at the 3′ end of the top strand. Sequencing gel analysis revealed that both the A/G- and A/C-containing substrates were nicked by the MYH (Fig. 4a, lanes 2 and 4, respectively) at the same position as that of MutY. Previous work showed that MutY cleaves at the first phosphodiester bond 3′ to the mispaired dA (34). Calf MYH nicked only the dA strand but not the dC strand of A/C substrate (data not shown). Densitometric analysis revealed that the activity on the A/C substrate was 30-fold lower than the activity on the A/G substrate. This observation is similar to the finding that the efficiency of *E. coli* MutY nicking is 20-fold lower (67) and the efficiency of *S. typhimurium* MutB (the *Salmonella* homolog of MutY) is ninefold lower on A/C substrates than on A/G substrates in vitro (16). Calf MYH had no nicking activity on a 20-bp substrate containing an A/I (inosine) mismatch (data not shown).

Calf MYH can cleave A/GO-containing DNA. The nicking activity of the partially purified MYH was further assessed by testing its activity on 3′-end-labeled (on the top strand) 20-bp substrates containing either A/G, A/GO, or C/GO mismatch. Calf MYH cleaves A/G or A/GO mismatch-containing DNA at the first phosphodiester bond 3′ to the mispaired adenine (Fig. 4b, lanes 3 and 6) but not the G or GO strand. Identical results were obtained for the MutY protein (Fig. 4b, lanes 2 and 5). By densitometric analysis of Fig. 4b and other experimental data, we have consistently observed a twofold-lower nicking activity on the A/GO-containing substrate than that on the A/G substrate for both calf MYH and MutY. It has been reported that the glycosylase activity of MutY protein is approximately the same on A/G- and A/GO-containing substrates (43). DNA

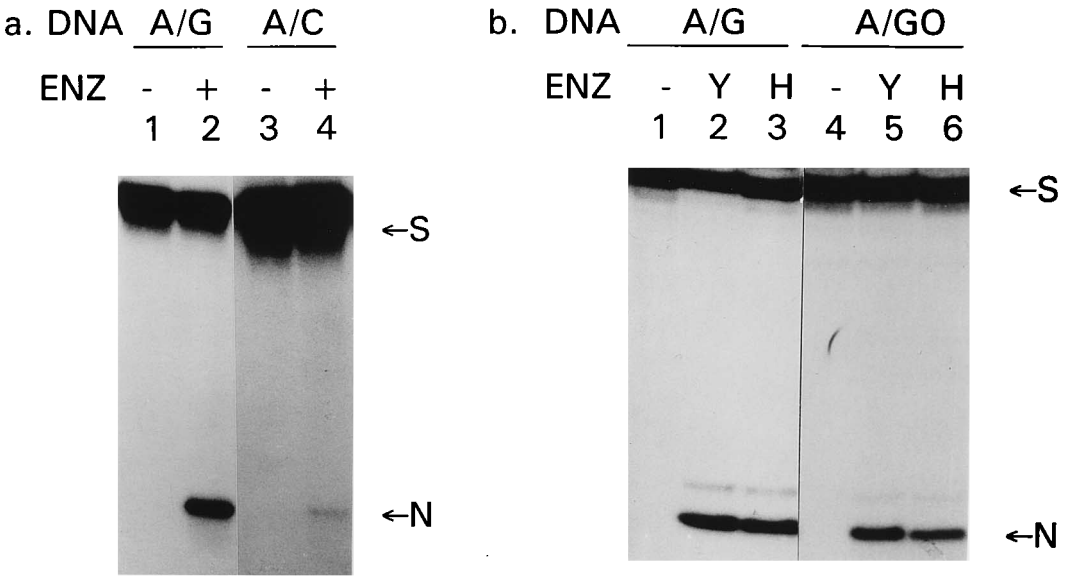

FIG. 4. Mismatch-nicking specificity of calf MYH. (a) 3′-end-labeled 120-bp DNA (1.8 fmol) containing mismatch A/G (lanes 1 and 2) or A/C (lanes 3 and 4) were incubated with the partially purified calf thymus MYH protein (fraction VI [ENZ]) at 37°C for 2 h (lanes 2 and 4). Untreated samples are shown in lanes 1 and 3. Results of lanes 3 and 4 were from an X-ray film exposed six times longer than that of lanes 1 and 2 in the same gel. (b) Enzymes (ENZ) used are purified *E. coli* MutY (Y) and partially purified MYH from calf thymus cells (H) (fraction VI). MutY and MYH were incubated with the 3′-end-labeled 20-mer DNA with A/G (lanes 1 to 3) or A/GO (lanes 4 to 6) at 37°C for 0.5 or 2 h, respectively. Untreated samples were shown in lanes 1 and 4. The uncut DNA substrate (S) and nicking product (N) are indicated. A minor band above the N product is an impurity in the DNA substrate.

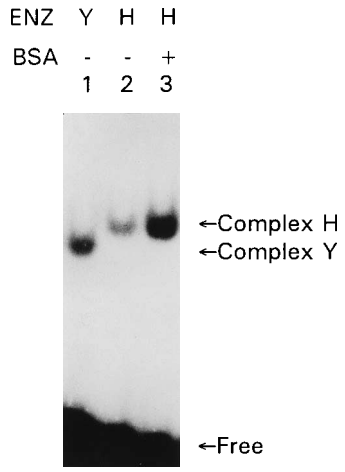

FIG. 5. Binding complex of the calf MYH protein and A/G mismatch-containing 20-mer DNA. Following incubation with a 3′-end-labeled 20-bp substrate containing an A/G mismatch, the products were resolved on a 4% native polyacrylamide gel. For the enzyme (ENZ) used, the MYH protein (H) (fraction V) forms an apparently larger complex with A/G mismatch-containing DNA (lanes 2 and 3) than the MutY protein (Y, lane 1). Reaction in lane 3 contains 50 μg of bovine serine albumin (BSA) per ml. Free DNA and protein-DNA complexes are marked to the right of the gel.

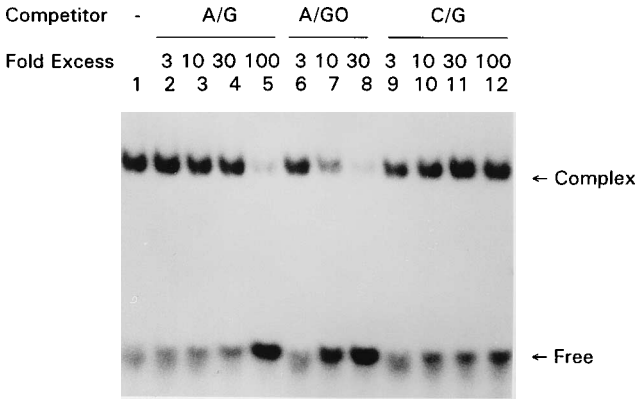

FIG. 6. MYH binding competition. Gel shift analysis, as described in the legend to Fig. 5, was used to monitor the specificity of calf MYH binding to a 3′-end-labeled A/GO-containing 20-bp substrate (1.8 fmol). All reaction mixtures contain 50 μg of bovine serum albumin per ml. No unlabeled competitor DNA (−) was added to the reaction in lane 1. Unlabeled A/G-containing 19-mer DNAs were added to reaction mixtures in lanes 2 to 5 to give 3-, 10-, 30-, and 100-fold excesses over the labeled DNA as shown. Lanes 6 to 8 contain 3-, 10-, and 30-fold excesses of A/GO-containing 19-mer DNA in addition to the labeled substrate. Unlabeled homoduplex (C/G 19-mer) DNAs were added to the reaction mixtures in lanes 9 to 12. Free DNA and protein-DNA complex are marked to the right of the gel. These experiments were performed three times. Competition was observed in 100-fold excess of A/G, 10-fold excess of A/GO, or 30-fold excess of A/GO with 30 to 60%, 50 to 80%, and 20 to 40% of remaining binding activity.

containing the C/GO base pair was not nicked on the upper strand but was nicked on the GO strand by fraction V. This activity is similar to the *E. coli* MutM (or FPG protein) activity (13, 64) and MutM-like activities in human or rodent nuclear extracts (6, 38). The MutM-like activity could be separated from the MYH activity during MonoS chromatography (data not shown).

Specific binding activity of the MYH enzyme. Gel retardation assays were performed to determine the binding affinity of the calf thymus MYH enzyme. The calf MYH enzyme forms a specific binding complex with the A/G-containing 20-mer DNA (Fig. 5, lanes 2 and 3). The formation of the MYH-DNA binding complex was enhanced by the addition of bovine serum albumin to the reaction for an unknown reason (Fig. 5, lane 3). There was a greater mobility shift for the MYH binding to the DNA substrate than for MutY binding. This observation probably reflects the difference in their sizes: the molecular masses of MutY and MYH are 39 and 65 kDa, respectively. The 36-kDa fragment from some preparations of calf MYH, detected in the Western blot with anti-MutY antibodies, formed a complex migrating faster than the MutY-DNA complex (data not shown). The calf MYH also formed a specific binding complex with a 20-bp DNA substrate containing a A/GO mismatch (Fig. 6, lane 1), but not C · GO and C · G (data not shown). Densitometric analysis revealed that the complex formation of MYH with A/GO substrate was approximately sevenfold stronger than that with the A/G substrate.

To clarify further the binding preference of the calf MYH protein, binding competitions were performed in which the 3′-end-labeled A/GO-containing 20-bp substrate was incubated with excess unlabeled A/G, A/GO, and C · G substrates (Fig. 6). Analysis revealed that 100- and 10-fold excess concentrations of the unlabeled A/G (Fig. 6, lanes 2 to 5) and A/GO substrates (Fig. 6, lanes 6 to 8), respectively, were necessary to inhibit the binding to the labeled A/GO substrate. The unlabeled C · G substrate had no effect on the binding of MYH to the labeled A/GO substrate (Fig. 6, lanes 9 to 12). In complementary experiments, the binding of MYH to the labeled A/G 20-mer could be inhibited with 15- and 1.5-fold excesses of unlabeled A/G and A/GO substrates, respectively (data not shown). Taken together, these results indicate approximately seven-fold stronger binding to A/GO-containing DNA than A/G-containing DNA.

Inhibition of MYH enzyme nicking activity. The MutY protein has homology to *E. coli* endonuclease III, a $[4Fe\text{-}4S]^{2+}$-containing DNA repair enzyme (15, 42). Further investigations of the MutY protein have determined that like endonuclease III, it contains an $[4Fe\text{-}4S]^{2+}$ cluster (35, 67). Oxidation of protein-bound $[4Fe\text{-}4S]^{2+}$ clusters by potassium ferricyanide causes the loss of one iron from the cluster, producing $[3Fe\text{-}4S]^{1+}$ (5). Treatment of the partially purified calf thymus MYH protein with various concentrations of potassium ferricyanide prior to the reaction with a 3′-end-labeled 120-mer A/G-containing substrate led to inhibition of enzyme activity in proportion to increasing concentrations of the oxidizing agent (data not shown). Similar results were obtained upon treatment of MutY protein with potassium ferricyanide (data not shown). In a Western blot analysis, antibodies raised against a polypeptide (residues 192 to 211) of the MutY protein can cross-react with a 65-kDa protein from nuclear extracts of calf thymus and human HeLa cells (data not shown), similar to the results with anti-MutY antibodies (Fig. 1). The amino acids within the region of residues 192 to 211 of MutY include four cysteines, which are conserved between MutY and endonuclease III and are apparently involved in the ligation to $[4Fe\text{-}4S]^{2+}$ cluster (42, 69). These results suggest that the calf MYH protein may also contain a similar iron-sulfur cluster.

DISCUSSION

In this study, we have shown that the calf thymus contains an A/G-nicking enzyme similar to the one found in human HeLa cells by Yeh et al. (75). We have partially purified the protein from calf thymus cells by ammonium sulfate precipitation and four chromatographic steps. Several lines of evidence indicate that the mammalian A/G-nicking enzyme (MYH) is the ho-

molog of the *E. coli* MutY protein. First, the mammalian A/G-nicking enzyme, like MutY, is a DNA glycosylase that removes the mispaired adenine from the mismatch (Fig. 1). An AP endonuclease activity is copurified with the glycosylase and cleaves the first phosphodiester bond 3′ to the generated AP site. Second, immunoblotting with polyclonal antibodies raised against either intact MutY protein or a polypeptide containing residues 192 to 211 of the MutY protein detects a protein of 65 kDa and a functional but degraded form of 36 kDa (Fig. 2). The size of the A/G-nicking enzyme was established further by glycerol gradient centrifugation. These findings suggest that the native calf MYH protein is a monomer. The immunological cross-reactivity between *E. coli* MutY and mammalian MYH is noteworthy. These proteins must contain some conserved domains, especially in the region of residues 192 to 211 of MutY, that may be involved in binding to an $[4Fe\text{-}4S]^{2+}$ cluster (42, 69). Third, nicking analysis indicates that the calf enzyme is active on A/G, A/GO, and A/C mismatches (Fig. 4), but the activity is twofold lower on A/GO mismatches and 30-fold lower on A/C mismatches than on A/G mismatches. This substrate specificity is similar to the MutY protein (43, 67). Interestingly, the A/C-nicking activity is undetectable in HeLa extracts (75). Given the sensitivity limits of the present analysis, the HeLa activity may be too weak to be detected. Finally, enzyme activity is inhibited by treatment with anti-MutY antisera or upon treatment with the oxidizing agent, potassium ferricyanide. The former observation supports the cross-reactivity of the MutY antibodies with the calf thymus protein; the latter suggests that the protein may contain an iron-sulfur cluster. Combining all of these observations, we conclude that the enzyme activity we have discovered is homologous to that of the *E. coli* MutY protein.

A role for the MutY pathway in *E. coli* is the removal of adenines misincorporated opposite GO lesions following DNA replication (41, 63). Oxidative stress and metabolic processes produce active oxygen species, which react with DNA to produce GO, and have been implicated in cancer and aging (2, 25). Adenines are frequently incorporated opposite GO bases during DNA replication in vitro (60) and in vivo (74). A second round of replication through this mismatch will subsequently lead to a G · C-to-T · A transversion (12, 46, 47, 74). In *E. coli*, MutY, MutM, and MutT are involved in defending against the mutagenic effects of GO lesions (41, 63). The MutT protein has nucleotide triphosphatase activity, which is 3 orders of magnitude more active on 8-oxo-dGTP than on dGTP (36). The MutT protein thus eliminates 8-oxo-dGTP from the nucleotide pool (36). The MutM protein (FPG protein) provides a second level of defense by removal of both ring-opened purine lesions and the mutagenic GO adducts (13, 64). MutM removes the GO lesions efficiently from C/GO but poorly from A/GO (64). MutY corrects the replicative errors that result from misincorporation of dA opposite GO in DNA. DNA polymerase I has tendency to restore C/GO pairs (60), which are substrates for MutM. However, misintroduction of 8-oxo-dGTP opposite a template adenine would transform replicative errors into permanent T · A-to-G · C mutations by MutY action. Active MutT protein prevents A/GO or A/G mismatches with dA in the parental strand so that MutY would rarely encounter them.

Similar mechanisms to prevent accumulation of GO in DNA are present in mammalian cells. As shown here, both humans and calves possess a MutY homolog that cross-reacts with anti-MutY antibodies. We show that mammalian MYH is also active in removing adenines from A/GO-containing DNA. The nicking on A/GO-containing DNA is twofold weaker than that on A/G-containing substrates, but the binding is sevenfold greater to the A/GO-containing substrate. This greater binding to A/GO substrate may limit the actions of MutM homolog on the GO-containing strand. An activity similar to MutM has been detected in human and rodent cells (6, 38), and indeed, we have detected MutM-like endonuclease activity in some of our MYH preparations from a calf thymus (data not shown). Human cells have an enzyme similar to the *E. coli* MutT, which hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP (44). The human 8-oxo-dGTPase has some sequence homology to the *E. coli* MutT protein (59). Therefore, the DNA repair pathway that protects the cell from the mutagenic effects of 8-oxoG are apparently highly conserved among diverse organisms.

The relative nicking activity of MYH on A/G- and A/GO-containing substrates suggests that the MYH-dependent pathway has dual biological functions. In contrast to the 2-orders-greater activity on 8-oxo-dGTP than on dGTP for the human MutT homolog (44), calf MYH is twofold less active on A/GO-containing DNA than on A/G-containing substrates. As with *E. coli* MutY, it is likely that one role of MYH protein is the repair of A/GO mispairs. Another likely role of MYH is to repair A/G or A/C mismatches, which arise from replicative errors or genetic recombination on undamaged DNA. Mammalian MYH may be responsible for the reduction of G · C-to-T · A transversions or G · C-to-A · T transitions by repairing the misinserted adenines opposite the template guanines or cytosines. Repair of A/G or A/C may be involved in gene conversion in regions of heteroduplex DNA formed during genetic recombination.

Recent studies have determined that germ line mutations in human *hMSH2* or *hMLH1*, homologs of bacterial *mutS* or *mutL*, respectively, lead to genetic instability in microsatellite repeat sequences in hereditary nonpolyposis colon cancer (1, 10, 18, 28, 49, 50). It is interesting to note that G · C-to-T · A transversions occur frequently as mutations of the *p53* tumor suppressor gene in human lung, breast, and liver cancers (21). Thus, the MYH pathway may be linked to protection against certain cancers. Further cDNA cloning and mutation analyses of hMYH in these tumors will elucidate this notion.

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