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Nuclear Localization of the Human *mutY* Homologue *hMYH*

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Abstract The cDNA of the human *mutY* homologue (*hMYH*) was cloned from the total RNA of the tumor cell line SU-DHL-1 by reverse transcription-polymerase chain reaction (RT-PCR). Expression of *hMYH* in a plasmid can partially revert the mutator phenotype of the *Escherichia coli mutY* mutant MK609(DE3). The majority of the recombinant hMYH protein in *E. coli* was precipitated in the inclusion bodies. A minor fraction of the soluble recombinant protein was concentrated as the source of the protein in the activity assay. Recombinant hMYH displayed both glycosylase and AP lyase activity. Three independent rabbit antisera against an N-terminal peptide, HY90, a recombinant C-terminal fragment, and the full-length hMYH recombinant protein were prepared and affinity-purified, and these antisera recognized the 59 kDa endogenous hMYH protein in HeLa cells. Immunofluorescent staining experiments with these three antisera showed a consistent nuclear distribution of hMYH, excluding the nucleoli. This nuclear staining pattern was abolished if the antisera were incubated with specific peptide/protein competitors, whereas the staining pattern was unaffected if the antisera were incubated with nonspecific peptide competitors. Consistent with the immunofluorescent staining results, a flag-tagged transfected hMYH also showed a nuclear staining pattern excluding the nucleoli. These results suggest that hMYH is indeed a functional homologue of *E. coli* MutY and is localized in the nuclei of mammalian cells. J. Cell. Biochem. 77:666–677, 2000.

Key words: glycosylase; AP lyase; mismatch repair; mutator; oxidative damage

Reactive oxygen species (ROS) generated as byproducts of oxygen metabolism in cells can damage genomic DNA [Ames and Gold, 1991; Halliwell and Gutteridge, 1999]. Genomic DNA was damaged by ROS at frequencies of 10^4 and 9×10^4 incidences of oxidative damage/cell/day in human and rodent cells, respectively [Ames, 1989; Fraga et al., 1990]. Among the different oxidative-damage DNA products, 8-oxo-7,8-dihydrodeoxyguanine (8-oxoG or GO) is the most stable adduct [Ames and Gold, 1991; Cheng et al., 1992]. Both adenine and cytosine can be incorporated opposite to the 8-oxoG lesion to produce mismatched base pairs by the polymerases involved in replication [Maki and Sekiguchi, 1992; Shibutani et al., 1991]. If the 8-oxoG lesions in the DNA are not properly repaired, a high percentage of G/C to T/A transversion will occur through the 8-oxoG/A replication intermediate [Cheng et al., 1992; Moriya et al., 1991; Moriya and Grollman, 1993; Wood et al., 1990]. The GO repair system, which is composed of three enzymes, MutM, MutY, and MutT, in *Escherichia coli*, is responsible for preventing the mutagenic effect of the 8-oxoG lesions [Michaels et al., 1992; Tajiri et al., 1995; Tchou and Grollman, 1993].

The MutT protein is a nucleotide triphosphatase that eliminates 8-oxo-dGTP from the

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nucleotide pool to prevent incorporation of 8-oxoG into the genome [Maki and Sekiguchi, 1992]. The MutM protein removes 8-oxoG from the 8-oxoG/C base pair or the ring-opened purine lesions in the DNA with its glycosylase activity, to produce an abasic site that is subsequently repaired to restore the G/C base pair [Boiteux et al., 1990; Chetsanga and Lindahl, 1979; Tchou et al., 1991]. Some 8-oxoG/C base pairs escape repair by the MutM protein before replication and produce a high percentage of 8-oxoG/A base pairs because the polymerase involved in replication incorporates A opposite to 8-oxoG from five-fold to 200-fold more frequently than they incorporate C [Shibutani et al., 1991]. The MutY protein, with its dual adenine glycosylase and AP lyase activities [Gogos et al., 1996; Lu et al., 1996; Manuel and Lloyd, 1997; Tsai-Wu et al., 1992], removes the A from the 8-oxoG/A base pair. The subsequent repair synthesis mainly restores a C because the polymerases involved in repair prefer a C rather than an A across the 8-oxoG base [Shibutani et al., 1991]. The resulting 8-oxoG/C base pair provides the MutM protein a second opportunity to remove the 8-oxoG base. Consequently, mutant strains of either *mutM* or *mutY* exhibit a mutator phenotype with an increased occurrence of G/C to T/A transversion [Cabrera et al., 1988; Nghiem et al., 1988; Radicella et al., 1988].

MutY homologue activity in human HeLa cells and calf thymus has been described [McGoldrick et al., 1995; Yeh et al., 1991]. They share enzymatic characteristics with the *E. coli* MutY protein in that they recognize C/A, G/A, and 8-oxoG/A mismatches and cleave the A base, but not the G base. The cDNA of the human homologue (*hMYH*) has been cloned and sequenced [Slupska et al., 1996]. This gene is mapped to the short arm of chromosome 1, between p32.1 and p34.3, and encodes a 535-amino acid protein with 41% identity to the *E. coli* MutY protein. However, no functional analysis was presented. Here, we report on the further characterization of the hMYH protein. In vivo, expression of the hMYH protein reverted the mutator phenotype of the *E. coli mutY* mutant strain MK609. In vitro, an extract of the *mutY* mutant bacteria expressing the hMYH protein exhibited glycosylase and AP lyase activity. Immunofluorescent staining experiments with three affinity-purified anti-hMYH antisera and transfection experiments with a plasmid expressing exogenous hMYH protein both showed that hMYH is a nuclear protein. Together, these data suggest that the hMYH protein plays an important role in repairing oxidative damage to prevent formation of mutations in the nucleus.

MATERIALS AND METHODS

Bacterial Strains, Peptides, Plasmids

E. coli strain MK609 (*mutY zgd*::Tet^r) and its isogenic *mutY* wild type strain, AB1157, were provided by Dr. M. Sekiguchi [Tajiri et al., 1995]. AB1157(DE3) and MK609(DE3) lambda lysogens were prepared with a λ DE3 Lysogenization Kit (Novagen, Madison, WI) according to the manufacturer's protocol. *E. coli* strain BL21(DE3) was obtained from Novagen. *E. coli* strain RIL, whose codon usage has been optimized for the expression of eukaryotic genes, was supplied by Stratagene (La Jolla, CA).

The nonspecific peptide coilin 561 was synthesized as a free peptide with the following amino acid sequence: N terminus-RLIIESPSNTSSTEPA-C terminus. The N-terminal peptide HY90 (N terminus-YDQEKRDLPWRRRAEDEMDL-C terminus) was synthesized as a multiple antigenic peptide (MAP) based on the Fmoc MAP resins (Applied Biosystems, Foster City, CA).

Plasmids pET28b+ and pET32a+ were purchased from Novagen. Plasmid pRK5F containing a FLAG epitope tag [Prickett et al., 1989] was used in the transfection experiment and was obtained from Dr. R.H. Chen [Feng et al., 1995]. Separate N- and C-terminal cDNA fragments of *hMYH* were cloned by RT-PCR from the mRNA of a lymphoma cell line, SU-DHL-l, and the full-length *hMYH* was obtained by joining of these two fragments. The N-terminal fragment was amplified with a forward primer, 5′-GGA TCC ATG ACA CCG CTC GTC TCC CGC-3′, and a reverse primer, 5′-GAG CTG CTG GGA AAC AAG GGT-3′. The C-terminal fragment of *hMYH* was amplified with a forward primer, 5′-CCA CGT ACA GCA GAG ACC CTG-3′, and a reverse primer, 5′-CAG GAT TCT CAG GGA ATG GG-3′. The *Bam*HI/*Pin*AI fragment of the N-terminal clone and the *Pin*AI/*Xho*I fragment of the C-terminal clone were joined to produce the full-length *hMYH*. The *Bam*HI/*Xho*I fragment containing the full-length *hMYH* was cloned into the

*Bam*HI/*Xho*I sites of pET32a+ to produce a recombinant protein in *E. coli*. The 700-amino-acid (molecular weight: 76,725 Da) protein produced from this construct is a fusion protein between the 6-histidine-thioredoxin epitope tag and hMYH. An independent C-terminal clone, *hMYH*-C, was amplified with PCR by use of the forward oligonucleotide 5′-CTG GCC TTG GAA GGG-3′ and the reverse oligonucleotide 5′-CAG GAT TCT CAG GGA ATG GG-3′ from the full-length *hMYH* and was cloned into a T-tailed pBluescript II KS+ (Stratagene). The *Eco*RI/*Hin*dIII fragment containing *hMYH*-C was subcloned into the *Eco*RI and *Hin*dIII sites of pET28b+ (Novagen). The recombinant *hMYH*-C contains 129 amino acid residues with an expected molecular weight of 14,007 Da.

Overexpression and Purification of the Recombinant Proteins

Protocols for overexpression and affinity purification of the insoluble recombinant proteins from *E. coli* have been reported [Wu et al., 1998]. Bacteria with the recombinant plasmids were cultured in Luxia-Bextani (LB) medium supplemented with the appropriate antibiotics. Fifty milliliters of overnight bacterial culture was added to 2 liters of LB and incubated at 37°C for 2 h. Expression of the recombinant proteins was induced by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM, and the bacterial culture was incubated for an additional 2 h. The bacteria were harvested by centrifugation in a Beckman JA-20 rotor at 7,000 rpm, 4°C, for 15 min, resuspended in 20 ml phosphate buffered saline (PBS), and lysed by sonication. The insoluble recombinant proteins in the bacterial extract were recovered by centrifugation at 15,000 rpm, 4°C, for 15 min, solubilized in 50 mM Tris-HCl pH 7.0, 8 M urea, 50 mM imidazole, and filtered through a 0.45 μm membrane. The solubilized proteins were purified with an affinity nickel column, HiTrap Chelating (Amersham Pharmacia Biotech, Uppsala, Sweden), with a linear gradient from 50 mM to 500 mM imidazole in the presence of 8 M urea at a flow rate of 1 ml/min. The protein concentration was determined with the protein assay reagent (Bio-Rad, Hercules, CA).

Preparation and Affinity Purification of Rabbit Antisera

Preparation and purification of the antiserum were based on standard protocols, with minor modifications [Harlow and Lane, 1988]. The Ni-column-purified recombinant proteins were fractionated with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie brilliant blue R-250. Gel pieces containing the recombinant proteins were sliced and used as antigen. The MAP peptide HY90 was used directly as antigen. Four-month-old male New Zealand white rabbits were immunized with a mixture of 150 μg of the antigen and Imject complete Freund's adjuvant (Pierce, Rockford, IL), followed by three boosts with a mixture of 75 μg of the antigen and Imject incomplete Freund's adjuvant (Pierce) at an interval of 3 weeks. Antisera were collected 7–10 days after each boost. The purified hMYH protein was fractionated on an SDS-PAGE gel and electroblotted to a Hybond-C Extra membrane (Amersham Pharmacia Biotech), and the strip containing the hMYH protein was excised for affinity purification of the antisera. The strip was incubated with the crude antisera at 4°C for 16 h to adsorb the antibody, washed three times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.3% Tween 20, and the bound antibodies were eluted from the strip with 0.2 M glycine solution (pH 2.0) and immediately neutralized to pH 7.0 with 1.2 M Tris-base solution. Concentrations of the affinity-purified antibodies ranged from 0.7 to 2.8 mg/ml. For immunofluorescent staining competition experiments, the antisera were incubated with 66.7 mg/ml of the synthetic peptides at 4°C for 16 h before use. These three antisera against the full-length recombinant hMYH, the hMYH C-terminal fragment, and the N terminal peptide HY90 are referred to as anti-hMYH-fl, anti-hMYH-C, and anti-HY90, respectively.

Western Blotting and Immunoprecipitation

Western blotting and immunoprecipitation procedures were standard protocols [Harlow and Lane, 1988; Sambrook et al., 1989]. A total of 1.75 μg to 7 μg affinity-purified antibodies were used for immunoprecipitation. For Western blotting, protein samples to be analyzed were fractionated on an SDS-PAGE gel, electrotransferred to a Hybond-C Extra membrane, and subjected

to detection with the rabbit antisera, followed by 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech). Dilution factors of the primary antibodies in Western blot were 10-fold to 50-fold for HeLa cell extract and 200-fold to 300-fold for the bacterial extract, respectively. Results of the Western blots were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and recorded on Hyperfilm-MP film (Amersham Pharmacia Biotech).

Preparation of the Mismatched Repair Substrate

The procedure for preparing heteroduplex DNA containing a G/A mismatch has been described [Tsai-Wu et al., 1999]. The 5′ primer (5′-ATC CGA TTC CGG CTC GTA TGT TGT GTG-3′) and the 3′ primer (5′-CCT ACC CGG GTA ACG CCA GGG TTT TCC CAG-3′) were chemically synthesized either with or without a biotin group attached at the 5′ end. Two M13mp18 phage derivatives, M13mm3 and M13mm4, which had identical DNA sequences except for a single nucleotide difference, were used as templates for preparation of the heteroduplex. To prepare the A strand, the unbiotinylated 5′ oligonucleotide and the biotinylated 3′ oligonucleotide were used as primers with Ml3mm3 as a template in the PCR reaction. In contrast, the biotinylated 5′ primer and the unbiotinylated 3′ primer were used in a second PCR reaction with M13mm4 as a template to produce the complementary G strand. The PCR reaction included 50 pg of the template DNA, 5 pmol of each of the two primers, and 1 unit of KlenTaq DNA polymerase. The PCR reaction was performed with denaturation at 95°C for 5 min, 30 cycles of 95°C for 20 s, 63°C for 20 s, 72°C for 20 s, and a final extension step at 72°C for 5 min. The unincorporated primers were removed from the 200-bp PCR product by centrifugation at 13,200g for 20 min at 4°C with Microcon 10 (Amicon, Beverly, MA). The purified PCR products were bound to streptavidin-conjugated magnetic beads M280 (Dynal, Oslo, Norway) and washed with TE buffer (10 mM Tris-HCl, pH 7.6) three times. The bound DNAs were denatured with 0.1 N NaOH for 10 min at room temperature for release of the unbiotinylated single-stranded PCR product. The unbound A strand and the unbound complementary G strand were recovered from the respective tubes, mixed in a new tube, and renatured with the addition of 0.1 N HCl at room temperature for at least 1 h. The G/A mismatch-containing heteroduplex was purified by ethanol precipitation and was then ready for use. Before labeling the heteroduplex radioactively, we performed *Hin*fI digestion to create a 3′ recessive end at the A strand. Radioactive ^{32}P-labeled nucleotide was incorporated into the 5′ end of the heteroduplex with the Klenow fragment.

Glycosylase/Lyase Activity Assay

Assays for glycosylase or AP lyase activity were based on published protocols, with minor modifications [Tsai-Wu et al., 1992]. The radioactively labeled DNA heteroduplex and the enzymes were incubated in 20 mM Tris-HCl, pH 7.6, 80 mM NaCl, 1 mM EDTA, 2.9% glycerol, 29 mM ammonium sulfide, and 1 mM ferrous ammonium sulfate at 37°C for 4 h. The reaction mixture was vacuum-dried and resuspended in termination solution (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol). For the glycosylase activity assay, the mixture was heated at 95°C for 5 min before loading on a 6% polyacrylamide/8 M urea sequencing gel, followed by autoradiography. For the AP lyase activity assay, the heating step was omitted.

Reversion Assay

The mutation frequencies of bacteria with or without recombinant protein expression were determined by reversion assay, with minor modifications [Tajiri et al., 1995]. Approximately 150 cfu of the bacteria to be tested were seeded in 5 ml of LB and cultured at 37°C to $OD_{600\ nm} = 0.1$. Protein expression was induced by a final concentration of 0.1 mM IPTG at 25°C for 16 h. The bacterial cultures were diluted with 1× M9 saline (42.3 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), plated on LB plates containing 100 μg/ml of rifampicin, and incubated at 37°C for determination of the mutation frequency.

Transfection

Transfection was performed with a FuGENE 6 transfection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. HeLa S3 cells were split to a final 25% confluence in a 3.5-cm culture dish and cultured for an additional 16 h

before transfection. The DNA sample (2 μg) was dissolved in 100 μl Dulbecco's modified Eagle's medium (DMEM), mixed with diluted FuGENE 6 reagent, and incubated at room temperature for 15 min before being added to 2 ml of the culture medium. The cells to be transfected were incubated in this mixture for 16 h before the immunofluorescent staining experiments.

Immunofluorescent Staining

The procedure for immunofluorescent staining was a modification of the standard protocol [Harlow and Lane, 1988]. Cells to be stained were grown on cleaned coverslips to a density of 70% confluence. Cells on the coverslip were rinsed with PBS three times, fixed with 2% paraformaldehyde at room temperature for 15 min, rinsed with PBS three times, and blocked with blocking solution (10% fetal bovine serum, 0.2% saponin, 0.05% Tween 20) at room temperature for 30 min. The blocked samples were rinsed with three consecutive 5-min PBS washes, challenged with the primary antibodies (diluted 10-fold to 50-fold) with the blocking solution for 1 h, rinsed with three consecutive 5-min PBS washes, and stained with 3.3 μg/ml Alexa 488 (or Alexa 594)-conjugated goat anti-mouse (or goat anti-rabbit) IgG antibody (Molecular Probes, Eugene, OR) at room temperature for 1 h. For staining the FLAG-transfected cells, a final concentration of 2.4 μg/ml of the anti-FLAG antibody was used. The stained specimens were rinsed with three consecutive 5-min PBS washes, mounted with anti-fading mounting medium (1 mg/ml p-phenylenediamine, 50% glycerol, 0.02% NaN_3), and stored at −20°C before examination. The specimens were examined with an Axiovert 100 TV microscope (Zeiss, Jena, Germany). The images were recorded with a cooled-CCD Quantix camera (Photometrics, Tucson, AZ).

RESULTS

Human *hMYH* cDNA, which encodes a 535-amino-acid protein with an expected molecular weight of 59,034 Da, was amplified from the cDNA of a lymphoma cell line, SU-DHL-1, with RT-PCR based on the published sequence (Accession number U63329, [Slupska et al., 1996]). The DNA sequence of our *hMYH* cDNA clone was determined on both strands to be identical to the reported sequence. The encoded hMYH protein contains an iron-sulfur cluster (C-X_3-[KRS]-P-[KRAGL]-C-X_2-C-X_5-C), which is conserved in the MutY homologues from different organisms [Lu and Fawcett, 1998]. A putative nuclear localization signal (PCS-RKKPR) is present at the C-terminus of the protein (Fig. 1). Initial attempts to overexpress the full-length hMYH protein in *E. coli* BL21(DE3) failed, presumably because of the high percentage of rare codons for *E. coli* in the cDNA. However, a C-terminal fragment, hMYH-C, with a lower percentage of rare codons can be overexpressed upon IPTG induction (Fig. 2A).

Subsequently, we were able to overexpress the full-length hMYH protein (hMYH-fl) by using an *E. coli* strain, RIL, whose codon usage had been optimized for the expression of eukaryotic genes. As shown in Fig. 2B, a 77-kDa fusion protein between hMYH and the 6-histidine-thioredoxin tag in the plasmid can be expressed upon IPTG induction, and the majority of the protein was present in the insoluble inclusion body. Both proteins were purified from the inclusion body to near homogeneity on an affinity nickel column (Fig. 2A and 2B). Rabbit antisera against hMYH-fl and hMYH-C, as well as a synthetic peptide HY90 (Fig. 1), were prepared and affinity-purified against the recombinant hMYH protein. These antisera recognized a 59-kDa protein in the HeLa extract (Fig. 3).

The *E. coli mutY* mutant exhibited a mutator phenotype; this mutator phenotype can be reversed by expression of the MutY protein or a functional homologue in the bacterium [Lu and Fawcett, 1998]. To examine whether *hMYH* is indeed a functional homologue of *mutY*, we chose to express the hMYH protein with pET32a+ in the *mutY* mutant MK609. Lambda DE3 lysogen of bacterium MK609 was prepared for the bacterium to produce hMYH from plasmid pET32a+. Figure 4 shows that a 77-kDa protein was produced upon IPTG induction, suggesting that hMYH was present in the *mutY* mutant bacterium MK609(DE3). As shown in Table I, the presence of the *hMYH* clone in the bacteria can reduce the mutation rate slightly, presumably due to leaky expression of the protein. Induction of the hMYH protein with IPTG can further reduce the mutation rate 10-fold.

The hMYH protein purified from the insoluble inclusion body was subjected to a denaturation/renaturation process in an at-

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  1 MTPLVSRLSR LWAIMRKPRA AVGSGHRKQA ASQEGRQKHA KNNSQAKPSA  50
 51 CDGLARQPEE VVLQASVSSY HLFRDVAEVT AFRGSLLSWY DQEKRDLPWR 100
101 RRAEDEMDLD RRAYAVWVSE VMLQQTQVAT VINYYTGWMQ KWPTLQDLAS 150
151 ASLEEVNQLW AGLGYYSRGR RLQEGARKVV EELGGHMPRT AETLQQLLPG 200
201 VGRYTAGAIA SIAFGQATGV VDGNVARVLC RVRAIGADPS STLVSQQLWG 250
251 LAQQLVDPAR PGDFNQAAME LGATVCTPQR PLCSQCPVES LCRARQRVEQ 300
301 EQLLASGSLS GSPDVEECAP NTGQCHLCLP PSEPWDQTLG VVNFPRKASR 350
351 KPPREESSAT CVLEQPGALG AQILLVQRPN SGLLAGLWEF PSVTWEPSEQ 400
401 LQRKALLQEL QRWAGPLPAT HLRHLGEVVH TFSHIKLTYQ VYGLALEGQT 450
451 PVTTVPPGAR WLTQEEFHTA AVSTAMKKVF RVYQGQQPGT CMGSKRSQVS 500
501 SPCSRKKPRM GQQVLDNFFR SHISTDAHSL NSAAQ              535
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Fig. 1. Amino acid sequence of *hMYH*. The amino acid residues constituting the iron-sulfur cluster typical of the endonuclease III family proteins are boxed. The amino acid sequence corresponding to the peptide HY90 used to generate the anti-peptide antiserum is underlined. The hMYH-C terminal fragment starts from Leu^{444} (arrow) and continues to the stop codon. The putative nuclear localization signal is marked with a double underline.

tempt to recover its enzymatic activity. No activity was observed in such a preparation (data not shown). Alternatively, we chose to prepare active hMYH from the soluble fraction of the *mutY* mutant extract. Figure 4 shows that hMYH can be produced in the *mutY* mutant MK609(DE3) (Fig. 4A), and the majority of the protein was in the insoluble inclusion body (Fig. 4B). A small fraction of the protein was in soluble form, which can only be detected by concentration of the protein with immunoprecipitation by use of the anti-hMYH-C antiserum, followed by Western blot (Fig. 4B). The concentrated crude extract of hMYH in the *mutY* mutant was used as the source in the activity assay. The enzyme preparation was incubated with a double-stranded DNA substrate containing a G/A mismatch that was labeled with ^{32}P at the A strand.

All reaction mixtures were treated with sodium hydroxide before fractionation in a denaturing urea gel. This sodium hydroxide treatment induces breakage of the phosphodiester backbone immediately downstream of an a-basic site and therefore can reveal the glyco-

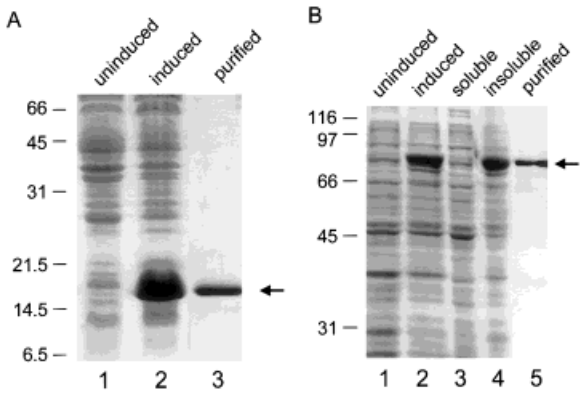

Fig. 2. Overexpression of the recombinant hMYH proteins in *E. coli*. **A:** SDS-PAGE gel stained with Coomassie blue, showing the recombinant hMYH-C fragment in BL21(DE3). **Lane 1:** Uninduced *E. coli* extract. **Lane 2:** IPTG-induced *E. coli* extract. **Lane 3:** Affinity-purified hMYH-C protein with the Ni column. **B:** SDS-PAGE gel stained with Coomassie blue, showing the recombinant full-length hMYH protein in RIL. **Lane 1:** Uninduced *E. coli* extract. **Lane 2:** IPTG-induced *E. coli* extract. **Lane 3:** Soluble fraction of the IPTG-induced *E. coli* extract. **Lane 4:** Insoluble fraction (inclusion body) of the IPTG-induced *E. coli* extract. **Lane 5:** Affinity-purified full-length hMYH from the inclusion body with the Ni column. Arrows mark the positions of the recombinant proteins.

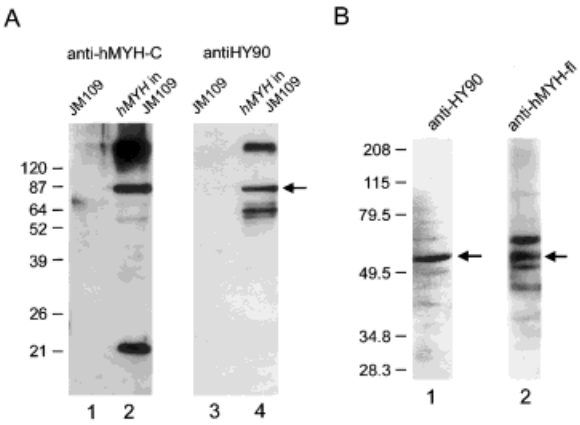

Fig. 3. Characterization of the antisera. **A:** Western blots of the unpurified anti-hMYH-C (**lanes 1 and 2**) and anti-HY90 (**lanes 3 and 4**) antisera on *E. coli* extracts. Lanes 1 and 3: Extracts of *E. coli* without the recombinant protein. Lanes 2 and 4: Extracts of *E. coli* expressing the recombinant full-length hMYH protein in plasmid pET32a+. Both antisera recognize a 77-kDa protein (arrow) that is a fusion between the full-length hMYH and the thioredoxin plasmid pET32a+. **B:** Western blots of the affinity-purified anti-HY90 antiserum (**lane 1**) and anti-hMYH-fl antiserum (**lane 2**) on HeLa cell extracts. Both affinity-purified antisera recognize the 59-kDa endogenous hMYH protein (arrows). Note that the affinity-purified anti-hMYH-fl antiserum also recognizes three other bands.

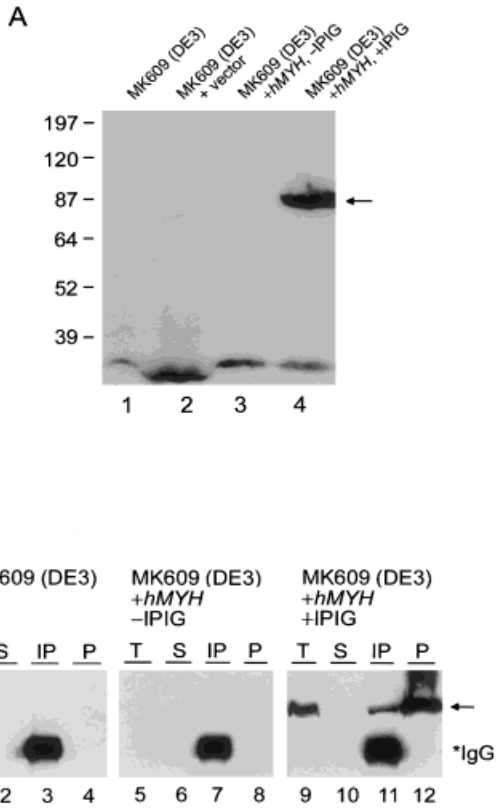

Fig. 4. **A:** Overexpression of the full-length hMYH protein in the *mutY* mutant strain MK609(DE3), as shown in a Western blot with the anti-hMYH-C antiserum. **Lane 1:** MK609(DE3). **Lane 2:** MK609(DE3) with the vector pET32a+. **Lane 3:** Uninduced *hMYH*/pET32a+ in MK609(DE3). **Lane 4:** IPTG-induced *hMYH*/pET32a+ in MK609(DE3). **B:** Presence of soluble recombinant hMYH as shown in an immunoprecipitation/Western blot. Anti-hMYH-C antiserum was used for the immunoprecipitation and the Western blot. Total (T; **lanes 1, 5, 9**), soluble (S; **lanes 2, 6, 10**), and insoluble (P; **lanes 4, 8, 12**) proteins from the same number of bacteria were loaded on the gel and probed with the anti-hMYH-C antiserum. Similar to Fig. 2, the majority of the proteins were in the inclusion body (lane 12). A small fraction of the recombinant hMYH protein can be detected in soluble form by 16-fold concentration with immunoprecipitation (**lane 11**). Arrow and asterisk mark the positions of the recombinant hMYH and the heavy chain of IgG, respectively.

sylase activity of an enzyme. Recombinant *E. coli* MutY protein was used as positive control for glycosylase activity. Addition of iron and sulfur ions to the reaction enhanced glycosylase activity, and therefore iron and sulfur ions were included in all subsequent reactions (Fig. 5A, lanes 1 and 2). The extract of MK609(DE3) *mutY* mutant did not display any glycosylase activity (Fig. 5A, lane 3), whereas the extract containing hMYH from MK609(DE3) produced a cleaved DNA fragment of the same size as that produced by the MutY protein (Fig. 5A, lane 4). This suggests that hMYH, like MutY, can recognize the G/A mismatch in the substrate and remove the adenine from the DNA double helix. However, it is noteworthy that the relative glycosylase activity on the G/A mismatch heteroduplex of the recombinant hMYH is much lower than that of MutY. To determine whether hMYH possesses lyase activity in addition to glycosylase activity, we performed the same experiment, with the exception that the sodium hydroxide treatment was omitted from the reaction. As shown in Fig. 5B, the cleavage product can still be observed without sodium hydroxide treatment, suggesting that hMYH also possesses lyase activity (lanes 3 and 4).

The computer program PSORT II (http://psort.nibb.ac.jp) suggests that hMYH has a 65% possibility of being localized in the mitochondria and a 22% possibility of being localized in the nucleus. We used affinity-purified antisera in immunofluorescent staining experiments to determine the subcellular localization of hMYH. Figure 6 shows that endogenous hMYH is concentrated in the nucleus, exclud-

TABLE I. Complementation of *hMYH* to the Mutation Frequency of the *mutY* Mutant[a]

	Mutation rate (rif^s to rif^r) ($\times 10^{-8}$)	-Fold
AB1157(DE3)	3.21	1.0
MK609(DE3)	81.8	25.5
MK609(DE3)/pET32a+	176.0	54.8
MK609(DE3)/*hMYH*	44.9	14.0
MK609(DE3)/*hMYH* + IPTG	16.8	5.23

[a]The wild-type (AB1157(DE3)), *mutY* mutant (MK609(DE3)), and *mutY* mutant with or without recombinant hMYH protein expression were spread on a rifampicin-containing plate for determining the spontaneous mutation rates of these strains. Row 1 shows the spontaneous mutation rate of *mutY* wild-type AB1157(DE3) and is defined as 1. The *mutY* mutant MK609(DE3) has a 25.5-fold increased mutation rate (rows 1 and 2). The presence of the plasmid pET32a+ increased the mutation rate twofold (rows 2 and 3). However, leaky expression of *hMYH* reduced the mutation fourfold (rows 3 and 4), and full induction of *hMYH* with IPTG reduced the mutation rate 10-fold (rows 3 and 5). These results are summarized from triplicates of six independent experiments.

ing the nucleoli, of HeLa cells, as shown by staining with anti-hMYH-fl, anti-hMYH-C, and the anti-peptide antiserum anti-HY90 (A, B, and C, respectively). The staining pattern obtained with anti-HY90 was the same in the breast cancer cell line MCF10A (Fig. 6D). The nuclear staining pattern was abolished if the anti-HY90 antiserum was preincubated with the specific peptide competitor HY90 (Fig. 6E) or if the anti-hMYH-C antiserum was preincubated with the purified hMYH (Fig. 6G). In contrast, the nuclear staining pattern was not affected if a nonspecific peptide competitor, coilin 561, was used (Fig. 6F). To consolidate the results further, we transfected a flag-tagged *hMYH* to HeLa cells and stained the cells with the anti-flag antibody. Fig. 6H is a phase contrast image of such an experiment. As shown in Fig. 6I, the transfected cell displayed a nuclear staining pattern, excluding the nucleoli, whereas no staining was observed in the untransfected cells. It is worthy to note that considerable cytoplasmic staining was observed in the transfected cells but not in the untransfected cells, suggesting a small portion of the exogenous hMYH, as detected by anti-FLAG staining, was present in the cytoplasm. This observation is consistent with the nucleus-mitochondria shuttling model of the hMYH protein as discussed in the following section.

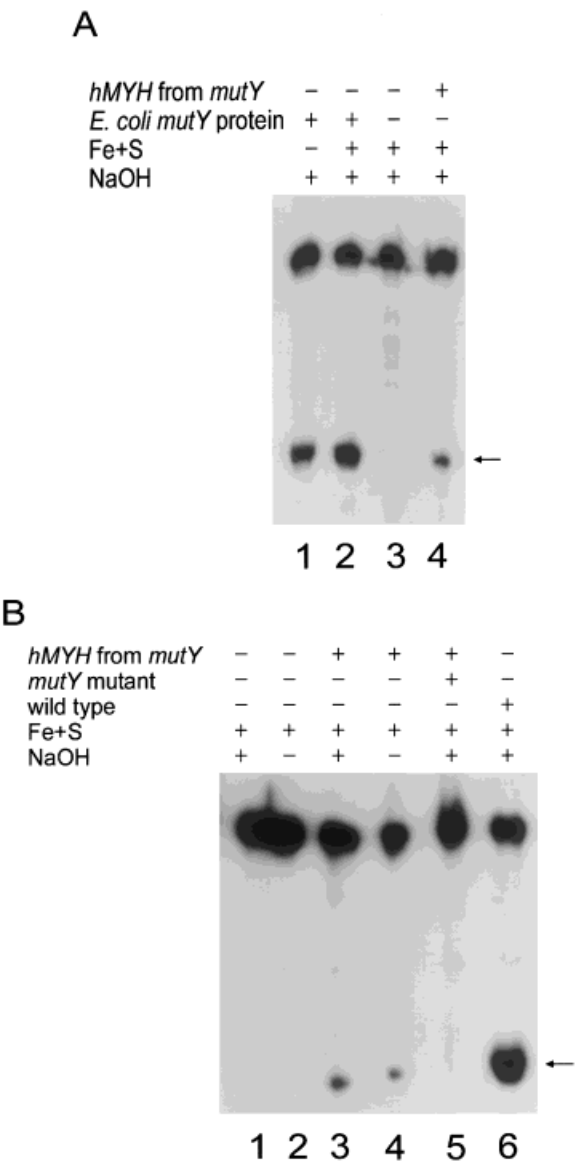

Fig. 5. **A:** Glycosylase activity of the recombinant hMYH. Soluble recombinant hMYH protein in MK609(DE3) was used as the source of enzyme (**lanes 3 and 4**). Recombinant *E. coli* MutY protein was used as positive control (**lanes 1 and 2**). Double-stranded DNA containing a G/A mismatch and ^{32}P-labeled at the A strand was used as the substrate. After incubation of the enzymes and the DNA substrate, the reaction mixture was treated with NaOH before fractionation in a 6% denaturing gel. Sodium hydroxide treatment induced breakage of the phosphodiester backbone immediately downstream of an abasic site, revealing the glycosylase activity of the enzyme. Addition of iron and sulfur enhanced the activity of the recombinant hMYH (lanes 1 and 2). Soluble recombinant hMYH possesses glycosylase activity (lanes 3 and 4). **B:** AP lyase activity of the recombinant hMYH. NaOH treatment did not induce cleavage of the DNA substrate (**lanes 1 and 2**). The extract of the *mutY* mutant MK609 did not exhibit any activity, whereas the *mutY* wild-type extract exhibited glycosylase activity (**lanes 5 and 6**). The presence of the cleavage product in the absence of NaOH/heat treatment (**lane 4**) suggests that hMYH also possesses AP lyase activity.

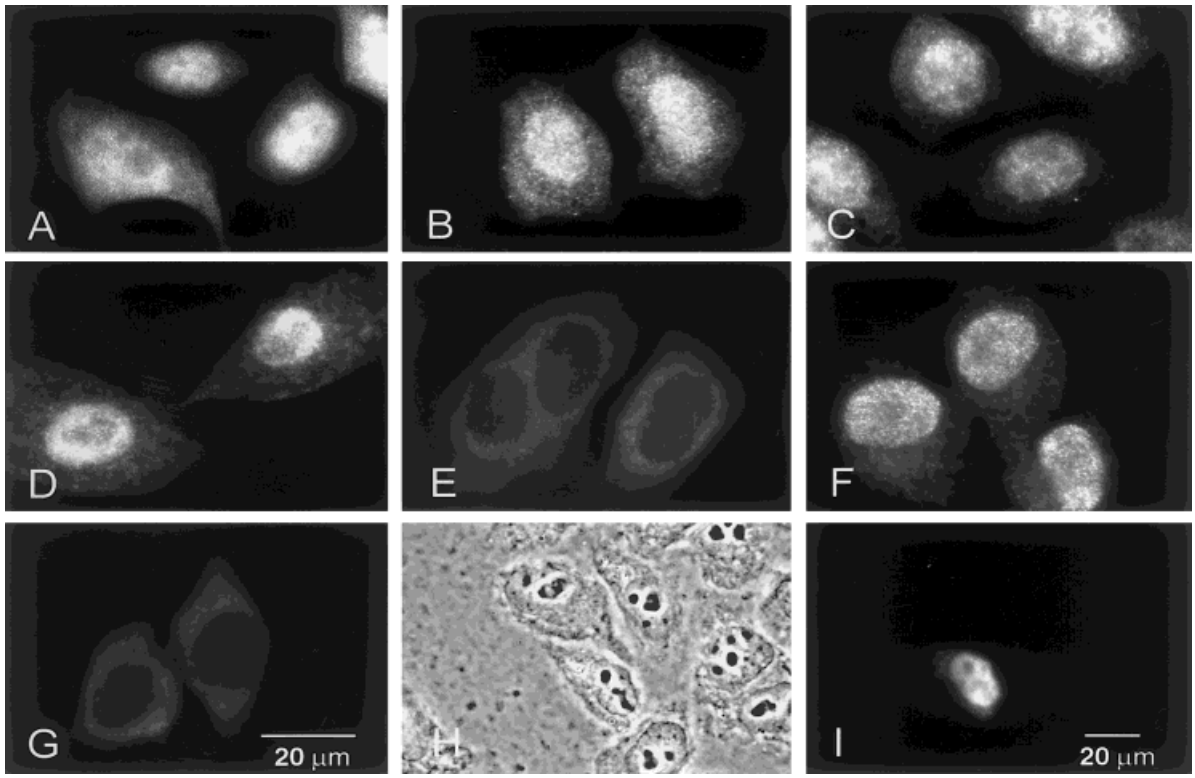

Fig. 6. Nuclear localization of hMYH in cultured cell lines. HeLa cells were used in all panels except in panel D, where MCF10A cells were used. Anti-hMYH-fl (**A**), anti-hMYH-C (**B**), and anti-HY90 (**C, D**) display a nuclear staining pattern excluding the nucleoli. This nuclear distribution pattern was unaffected if the antiserum was preincubated with a nonspecific peptide competitor (**F:** anti-HY90 + peptide coilin 561) and was abolished if the antisera were incubated with a specific competitor (**E:** anti-HY90 + peptide HY90; **G:** anti-hMYH-C + recombinant hMYH protein). Note that the background fluorescence was intentionally enhanced to show that the nucleus was devoid of staining. Panels **H** (phase contrast image) and **I** (fluorescence image) show the results for HeLa cells transfected with flag-tagged hMYH. The transfected hMYH protein also displays nuclear staining excluding the nucleoli. A–G share the same size bar, whereas H and I share another bar as shown in the figures.

DISCUSSION

MutY is a bifunctional adenine glycosylase/AP lyase responsible for repairing DNA mismatches induced by oxidative damage [Tsai-Wu et al., 1992]. It belongs to a DNA repair protein superfamily which includes the MutY family [Lu and Fawcett, 1998], the endonuclease III (Endo III) family [Thayer et al., 1995], the OGG1 family [Aburatani et al., 1997; Lu et al., 1997; Radicella et al., 1997; Roldan-Arjona et al., 1997], and the AlkA family [Labahn et al., 1996; Yamagata et al., 1996]. Two of the most conserved sequences in the Endo III and MutY families are the helix-hairpin-helix (HhH) domain and the iron-sulfur cluster. The HhH domain was identified as the thymine glycol-binding site in the crystal structure of Endo III [Kuo et al., 1992], and the iron-sulfur cluster (Cys-X_6-Cys-X_2-Cys-X_5-Cys) is important because of its glycosylase activity and binding to DNA [Porello et al., 1998].

The human MutY homologue has been cloned and sequenced based on its homology to MutY [Slupska et al., 1996]. Recently, Takao et al. [1999] showed that the gene product encoded by this gene possessed glycosylase activity specific for GO/A mismatch. In this report, we also demonstrate that hMYH is indeed a functional homologue of MutY both in its ability to complement the mutator phenotype of a *mutY* mutant in vivo and in its ability to cleave a G/A-containing DNA heteroduplex in vitro. As shown in Fig. 4, most of the recombinant hMYH protein was in the insoluble inclusion body. Renaturation of the affinity-purified hMYH protein from the inclusion body failed to show a glycosylase activity (data not shown).

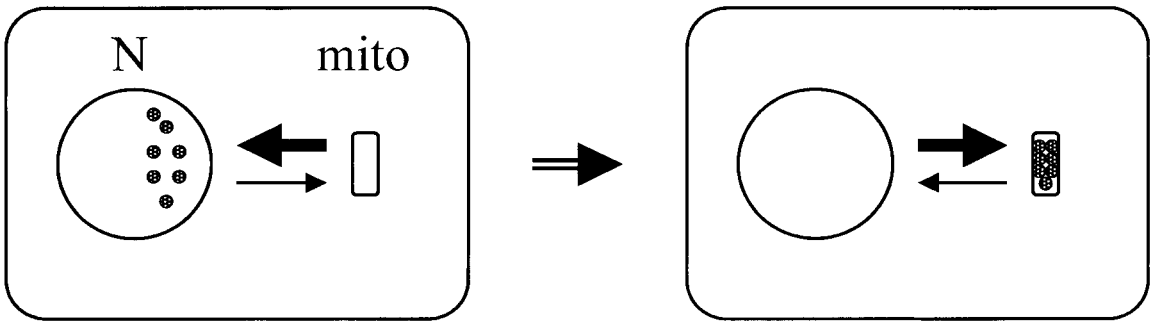

Fig. 7. Intracellular shuttling model of hMYH. The intracellular hMYH protein has a steady-state nuclear distribution (left panel). Upon induction (e.g., elevated oxidative potential in the mitochondria), the nuclear hMYH is translocated to the mitochondria to repair oxidative damage in the mitochondrial DNA. hMYH, filled circles; N, nucleus; mito, mitochondria.

Because a minor fraction of the hMYH protein was present in soluble form (Fig. 4), we chose to use the soluble hMYH protein in the *mutY* mutant bacterial extract as the source for the enzymatic assay. Recombinant hMYH protein indeed possessed an adenine glycosylase activity because, in a reaction assay followed by NaOH/heating treatment, the *mutY* mutant bacterial extract-containing hMYH protein could produce a specific cleavage product, whereas the *mutY* mutant bacterial extract alone could not produce any cleavage product. Although we showed that this cleavage product could be observed in the absence of NaOH/heat treatment, suggesting that hMYH protein might also posses AP lyase activity, it is still possible that this activity comes from another AP lyase activity in the bacterial extract. Assay with a purified enzyme will provide a definitive characterization of the activity of the hMYH protein.

MutY protein is one of the enzymes responsible for correcting DNA mismatches in the bacterial genome in preventing the occurrence of mutations [Michaels and Miller, 1992; Nghiem et al., 1988]. The functional homologue hMYH should serve the same purpose in preventing mutations in the nuclei of human cells. To serve its function properly, hMYH should be able to be imported into the nucleus. Two putative nuclear localization signals (NLSs) are present in hMYH. The first (PWRRR at 98) is found in the conserved region and thus might not be the functional NLS. Takao et al. [1998] showed that the second region (PKKPR at 505) is a functional NLS in that it can target a heterologous green fluorescent protein (GFP) into the nucleus. However, the C-terminal flag-tagged full-length hMYH, when transfected into COS cells, displayed a mitochondrial distribution. Furthermore, Takao et al. [1999] demonstrated the presence of an alternative spliced form of hMYH (type 2) that did not possess the first 14 amino-acid residues, but otherwise shared an identical amino acid sequence with the original hMYH (type 1), and this type 2 hMYH was targeted to the nucleus when transfected into COS cells. Based on this observation, they hypothesized that type 1 hMYH is a mitochondrial form, whereas type 2 hMYH is a nuclear form, and the mitochondria-targeting sequence (MTS) is functionally dominant over the NLS. This is in contrast to our observation that the full-length hMYH (type 1 in their designation) is a nuclear protein.

That the hMYH protein is a nuclear protein is supported by several lines of evidence. First, three independent anti-hMYH antisera all showed nuclear staining that could be competed out by a specific peptide. Because these antisera recognized the endogenous hMYH protein, the results of the immunofluorescent staining should reflect the authentic distribution of the hMYH protein in the cells. Second, the exogenous flag-tagged hMYH produced by transfection with use of our vector was localized to the nucleus. Exclusion of the staining from the nucleoli is particularly indicative of a specific targeting of the transfected hMYH protein. Furthermore, the only G/A-specific adenine glycosylase purified from HeLa cells were from the nuclear extract [Yeh et al., 1991].

These data support the idea that hMYH is a nuclear protein that acts to prevent the occurrence of mutations in the nucleus. The current

model, based on Takao's [Takao et al., 1999] and our observations, is that hMYH is a shuttling enzyme with a steady-state nuclear distribution (Fig. 7). This enzyme has dual functions in preventing mutations from occurring in the nucleus as well as in the mitochondria. Upon induction, presumably elevated oxidative potential in the mitochondria, MTS of hMYH can become dominant over NLS, and some unidentified nuclear factors can recognize the MTS and transport hMYH from the nucleus to the mitochondria. Whether hMYH behaves like a shuttling protein is currently under investigation.

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REFERENCES

Aburatani H, Hippo Y, Ishida T, Takashima R, Matsuba C, Kodama T, Takao M, Yasui A, Yamamoto K, Asano M. 1997. Cloning and characterization of mammalian 8-hydroxyguanine-specific DNA glycosylase/apurinic, apyrimidinic lyase, a functional *mutM* homologue. Cancer Res 57:2151–2156.

Ames BN. 1989. Endogenous oxidative DNA damage, aging, and cancer. Free Radic Res Commun 7:121–128.

Ames BN, Gold LS. 1991. Endogenous mutagens and the causes of aging and cancer. Mutat Res 250:3–16.

Boiteux S, O'Connor TR, Lederer F, Gouyette A, Laval J. 1990. Homogeneous *Escherichia coli* FPG protein. A DNA glycosylase which excises imidazole ring-opened purines and nicks DNA at apurinic/apyrimidinic sites. J Biol Chem 265:3916–3922.

Cabrera M, Nghiem Y, Miller JH. 1988. *mutM*, a second mutator locus in *Escherichia coli* that generates G.C—T.A transversions. J Bacteriol 170:5405–5407.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G—T and A—C substitutions. J Biol Chem 267:166–172.

Chetsanga CJ, Lindahl T. 1979. Release of 7-methylguanine residues whose imidazole rings have been opened from damaged DNA by a DNA glycosylase from *Escherichia coli*. Nucleic Acids Res 6:3673–3684.

Feng XH, Filvaroff EH, Derynck R. 1995. Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. J Biol Chem 270: 24237–24245.

Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci USA 87:4533–4537.

Gogos A, Cillo J, Clarke ND, Lu AL. 1996. Specific recognition of A/G and A/7,8-dihydro-8-oxoguanine (8-oxoG) mismatches by *Escherichia coli* MutY: removal of the C-terminal domain preferentially affects A/8-oxoG recognition. Biochemistry 35:16665–16671.

Halliwell B, Gutteridge JMC. 1999. Free radicals in biology and medicine. New York: Oxford University Press.

Harlow E, Lane D. 1988. Antibodies: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Kuo CF, McRee DE, Fisher CL, O'Handley SF, Cunningham RP, Tainer JA. 1992. Atomic structure of the DNA repair [4Fe-4S] enzyme endonuclease III. Science 258: 434–440.

Labahn J, Scharer OD, Long A, Ezaz-Nikpay K, Verdine GL, Ellenberger TE. 1996. Structural basis for the excision repair of alkylation-damaged DNA. Cell 86:321–329.

Lu AL, Fawcett WP. 1998. Characterization of the recombinant MutY homolog, an adenine DNA glycosylase, from yeast *Schizosaccharomyces pombe*. J Biol Chem 273:25098–25105.

Lu AL, Yuen DS, Cillo J. 1996. Catalytic mechanism and DNA substrate recognition of *Escherichia coli* MutY protein. J Biol Chem 271:24138–24143.

Lu R, Nash HM, Verdine GL. 1997. A mammalian DNA repair enzyme that excises oxidatively damaged guanines maps to a locus frequently lost in lung cancer. Curr Biol 7:397–407.

Maki H, Sekiguchi M. 1992. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355:273–275.

Manuel RC, Lloyd RS. 1997. Cloning, overexpression, and biochemical characterization of the catalytic domain of MutY. Biochemistry 36:11140–11152.

McGoldrick JP, Yeh YC, Solomon M, Essigmann JM, Lu AL. 1995. Characterization of a mammalian homolog of the *Escherichia coli* MutY mismatch repair protein. Mol Cell Biol 15:989–996.

Michaels ML, Miller JH. 1992. The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J Bacteriol 174:6321–6325.

Michaels ML, Tchou J, Grollman AP, Miller JH. 1992. A repair system for 8-oxo-7,8-dihydrodeoxyguanine. Biochemistry 31:10964–10968.

Moriya M, Grollman AP. 1993. Mutations in the *mutY* gene of *Escherichia coli* enhance the frequency of targeted G:C→T:A transversions induced by a single 8-oxoguanine residue in single-stranded DNA. Mol Gen Genet 239:72–76.

Moriya M, Ou C, Bodepudi V, Johnson F, Takeshita M, Grollman AP. 1991. Site-specific mutagenesis using a gapped duplex vector: a study of translesion synthesis past 8-oxodeoxyguanosine in *E. coli*. Mutat Res 254:281–288.

Nghiem Y, Cabrera M, Cupples CG, Miller JH. 1988. The *mutY* gene: a mutator locus in *Escherichia coli* that generates G.C—T.A transversions. Proc Natl Acad Sci USA 85:2709–2713.

Porello SL, Leyes AE, David SS. 1998. Single-turnover and pre-steady-state kinetics of the reaction of the adenine glycosylase MutY with mismatch-containing DNA substrates. Biochemistry 37:14756–14764.

Prickett KS, Amberg DC, Hopp TP. 1989. A calcium-dependent antibody for identification and purification of recombinant proteins. Biotechniques 7:580–589.

Radicella JP, Clark EA, Fox MS. 1988. Some mismatch repair activities in *Escherichia coli*. Proc Natl Acad Sci USA 85:9674–9678.

Radicella JP, Dherin C, Desmaze C, Fox MS, Boiteux S. 1997. Cloning and characterization of *hOGG1*, a human homolog of the OGG1 gene of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 94:8010–8015.

Roldan-Arjona T, Wei YF, Carter KC, Klungland A, Anselmino C, Wang RP, Augustus M, Lindahl T. 1997. Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase. Proc Natl Acad Sci USA 94:8016–8020.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Shibutani S, Takeshita M, Grollman AP. 1991. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 349:431–434.

Slupska MM, Baikalov C, Luther WM, Chiang JH, Wei YF, Miller JH. 1996. Cloning and sequencing a human homolog (*hMYH*) of the *Escherichia coli mutY* gene whose function is required for the repair of oxidative DNA damage. J Bacteriol 178:3885–3892.

Tajiri T, Maki H, Sekiguchi M. 1995. Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. Mutat Res 336:257–267.

Takao M, Aburatani H, Kobayashi K, Yasui A. 1998. Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage. Nucleic Acids Res 26: 2917–2922.

Takao M, Zhang QM, Yonei S, Yasui A. 1999. Differential subcellular localization of human MutY homolog (hMYH) and the functional activity of adenine:8-oxoguanine DNA glycosylase. Nucleic Acids Res 27:3638–3644.

Tchou J, Grollman AP. 1993. Repair of DNA containing the oxidatively-damaged base, 8-oxoguanine. Mutat Res 299:277–287.

Tchou J, Kasai H, Shibutani S, Chung MH, Laval J, Grollman AP, Nishimura S. 1991. 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. Proc Natl Acad Sci USA 88:4690–4694.

Thayer MM, Ahern H, Xing D, Cunningham RP, Tainer JA. 1995. Novel DNA binding motifs in the DNA repair enzyme endonuclease III crystal structure. EMBO J 14: 4108–4120.

Tsai-Wu JJ, Liu HF, Lu AL. 1992. *Escherichia coli* MutY protein has both N-glycosylase and apurinic/apyrimidinic endonuclease activities on A.C and A.G mispairs. Proc Natl Acad Sci USA 89:8779–8783.

Tsai-Wu JJ, Su MJ, Fang W, Wu CH. 1999. Preparation of heteroduplex DNA containing a mismatch base pair with magnetic beads. Anal Biochem 275:127–129.

Wood ML, Dizdaroglu M, Gajewski E, Essigmann JM. 1990. Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. Biochemistry 29:7024–7032.

Wu CH, Tsai-Wu JJ, Huang YT, Lin CY, Lioua GG, Lee FJ. 1998. Identification and subcellular localization of a novel Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis*. FEBS Letters 439:192–196.

Yamagata Y, Kato M, Odawara K, Tokuno Y, Nakashima Y, Matsushima N, Yasumura K, Tomita K, Ihara K, Fujii Y, Nakabeppu Y, Sekiguchi M, Fujii S. 1996. Three-dimensional structure of a DNA repair enzyme, 3-methyladenine DNA glycosylase II, from *Escherichia coli*. Cell 86:311–319.

Yeh YC, Chang DY, Masin J, Lu AL. 1991. Two nicking enzyme systems specific for mismatch-containing DNA in nuclear extracts from human cells. J Biol Chem 266: 6480–6484.