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Significance of the conserved amino acid sequence for human MTH1 protein with antimutator activity

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ABSTRACT

8-Oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) is produced during normal cellular metabolism, and incorporation into DNA causes transversion mutation. Organisms possess an enzyme, 8-oxo-dGTPase, which catalyzes the hydrolysis of 8-oxo-dGTP to the corresponding nucleoside monophosphate, thereby preventing the occurrence of mutation. There are highly conserved amino acid sequences in prokaryotic and eukaryotic proteins containing this and related enzyme activities. To elucidate the significance of the conserved sequence, amino acid substitutions were introduced by site-directed mutagenesis of the cloned cDNA for human 8-oxo-dGTPase, and the activity and stability of mutant forms of the enzyme were examined. When lysine-38 was replaced by other amino acids, all of the mutants isolated carried the 8-oxo-dGTPase-negative phenotype. 8-Oxo-dGTPase-positive revertants, isolated from one of the negative mutants, carried the codon for lysine. Using the same procedure, the analysis was extended to other residues within the conserved sequence. At the glutamic acid-43, arginine-51 and glutamic acid-52 sites, all the positive revertants isolated carried codons for amino acids identical to those of the wild type protein. We propose that Lys-38, Glu-43, Arg-51 and Glu-52 residues in the conserved region are essential to exert 8-oxo-dGTPase activity.

INTRODUCTION

Oxygen radicals are produced during cellular metabolism and damage biologically important macromolecules. More than 20 different types of oxidatively altered purines and pyrimidines have been detected in DNA (1,2). Among them, an oxidized form of guanine base, 8-oxo-7,8-dihydroguanine (8-oxoguanine) appears to be important in mutagenesis and in carcinogenesis (3–5). This oxidized base can pair with cytosine and with adenine at almost equal efficiencies and, as a result, A:T to C:G as well as G:C to T:A transversion mutations are induced (6–9).

To counteract such mutagenic effects of 8-oxoguanine, organisms are equipped with elaborate mechanisms. In *Escherichia coli*, two glycosylases encoded by the *mutM* and the *mutY* genes function to prevent mutation caused by 8-oxoguanine in DNA. MutM protein removes 8-oxoguanine paired with cytosine while MutY protein removes adenine paired with 8-oxoguanine (10–13). Enzyme activities similar to those of MutM and MutY in mammalian cells were identified (14,15). A significant amount of 8-oxoguanine is formed in chromosomal DNA of mammalian cells, and most damaged bases are excised and excreted into the urine (16).

Oxidation of guanine proceeds also in forms of free nucleotides, and an oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis (17). Organisms possess a mechanism to prevent mutation due to misincorporation of 8-oxo-dGTP (18). MutT protein of *E.coli* hydrolyzes 8-oxo-dGTP to the monophosphate (17), and lack of the *mutT* gene increases the occurrence of A:T to C:G transversion 1000-fold over the wild type level (19,20). Human cells contain an enzyme similar to the MutT protein, and this enzyme specifically hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, with a relatively low K_m value, as compared with other deoxyribonucleoside triphosphates (21,22). The human 8-oxo-dGTPase is likely to have the same antimutagenic capability as the MutT protein, since the elevated level of spontaneous mutation frequency in *E.coli mutT*$^-$ cells was greatly reduced by expression of cDNA for the human enzyme (22,23). The human gene for 8-oxo-dGTPase, named *hMTH1* (for human *mutT* homologue), was found to be located on chromosome 7p22 (23).

A certain degree of sequence homology has been noted in *E.coli* MutT and human MTH1 protein (22,24). Genes for analogous functions were isolated from *Proteus vulgaris* and *Streptococcus pneumoniae*, bacteria distantly related to *E.coli* (25,26). The products of the latter two genes carry enzyme activity specifically degrading dGTP to dGMP and may be functionally related to the *E.coli* MutT protein. More recently, cDNAs for mouse and rat 8-oxo-dGTPase protein were isolated and their structures elucidated (27,28). Like the human MTH1 proteins, these mammalian proteins suppress the occurrence of transversion mutations in *E.coli mutT*$^-$ cells, when appropriately expressed in the mutant cells. These six proteins of prokaryotic and eukaryotic origins are of a similar size, and alignment of the sequences showed that all carry a conversed sequence in almost the same region (Fig. 5). In the conserved region (from the 36th to the 58th amino acid for hMTH1), 10 among 23 amino acid

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residues are identical. It is likely that this region constitutes an active center for the enzyme, and the secondary structure of the MutT protein, elucidated by NMR analysis (29), supports this view.

To understand further the significance of the conserved amino acid sequence, we carried out a systematic site-directed mutagenesis study. Use of negative and positive screening procedures led to identification of the amino acid residues essential for enzyme activity.

MATERIALS AND METHODS

Chemicals

[α-^{32}P]dCTP, [α-^{32}P]dGTP and ^{125}I-labeled protein A were obtained from Amersham Japan (Tokyo). A DNA labeling kit was purchased from Nippon Gene (Toyama, Japan). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase were obtained from Toyobo Co. (Osaka, Japan). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, phenyl-β-D-galactopyranoside and rifampicin were obtained from Sigma, and isopropyl-β-D-thiogalactopyranoside was from Wako Pure Chemical Industries (Osaka, Japan). The altered sites II *in vitro* mutagenesis system was purchased from Promega. Sources of other materials are given in the text.

Bacterial strains

Escherichia coli strain CC101 [*ara*Δ(*lac proB*)$_{XIII}$] carrying an *F lacI*$^{-}$ *Z*$^{-}$ *proB*$^{+}$ episome (30), was a gift from J. H. Miller and *mutT*$^{-}$mutation was introduced into CC101 by P1 transduction to obtain CC101T. *Escherichia coli* strain JM109 {*endA*1, *recA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_k^-, m_k^+), *relA*1, *supE*44, λ^{-}, Δ(*lac-proAB*), [*F*$^{-}$, *traD*36, *proA*$^{+}$*B*$^{+}$, *lacI*^{q} ZΔM15]} and ES1301 (*mutS*$^{-}$) {*lacZ*53, *mutS*201::Tn5, *thyA*36, *rha*-5, *metB*1, *deoC*, IN(*rrn-rrnE*)} were purchased from Promega.

Plasmids

Plasmid pALTER-1 was purchased from Promega and used for site-directed mutagenesis. Plasmid pTT100 was constructed from pTrc99A by removing the *lacI*^{q} gene sequence by digestion with *Tth*111I and *Pvu*II and resealing (23). Plasmid pALTER-MTH-ss was constructed by inserting a 327 bp *Ssp*I–*Sac*I fragment, encoding the conserved amino acid sequence of hMTH1 protein, into the *Sma*I–*Sac*I site of pALTER-1. Plasmid pTT100-hMTH1(K38X) carrying the mutant cDNA was constructed by inserting a 236 bp *Nco*I–*Sac*I mutagenized DNA fragment into *Sac*I–*Nco*I site of pTT100-hMTH1.

Oligonucleotides

Oligonucleotide primers were obtained from Greiner Labortechnik Co. Ltd (Tokyo, Japan). A mixture of 27mer oligonucleotides carrying every possible combination of nucleotides of the target 3-nucleotide region were prepared and used as primers for site-directed mutagenesis.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to the Promega technical manual of altered sites II *in vitro* mutagenesis system, with modifications (Fig. 2). The mutagenized DNA was used to transform *E.coli* ES1301 (*mutS*$^{-}$), and transformed cells were grown in an LB plate containing 50 μg/ml ampicillin. Two types of plasmids derived from template DNA and from newly synthesized DNA were recovered from the cells, and the plasmid mixture was applied to *E.coli* JM109. Transformants were selected on LB plate containing 50 μg/ml ampicillin, and Amp^{r} colonies were picked and plated on paired plates containing either 50 μg/ml ampicillin or 12.5 μg/ml tetracycline. Amp^{r}/Tet^{s} colonies were isolated and cultured for preparation of plasmid DNA. The DNA was digested with a set of restriction enzymes *Sac*I and *Nco*I, and subcloned into pTT100-hMTH1. Plasmid pTT100-hMTH1(K38X) thus obtained was applied to *E.coli* CC101T and cultured on agar medium containing minimal A salts, 0.2% glucose, 0.05% phenyl-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml)

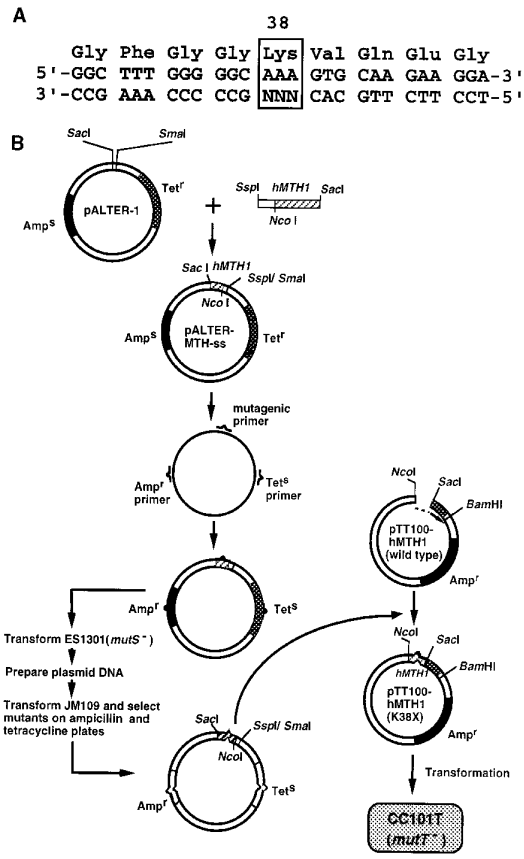

Figure 1. Construction of mutant forms of MTH1 protein. (**A**) Synthetic oligonucleotides used for site-directed mutagenesis. The first and second lines represent part of the amino acid sequence of the human MTH1 (residues 34–42) and the corresponding sequence for the sense strand of DNA, respectively. Oligonucleotide sequence for mixed primers for introducing mutations at Lys-38 is shown below. N stands for a mixture of A, T, C and G. (**B**) Schematic representation of site-directed mutagenesis. A 327 bp *Ssp*I–*Sac*I fragment carrying the conserved region of the human *MTH1* was introduced into pALTER vector, carrying Amp^{s} and Tet^{r} markers. After alkali denaturation, the mutagenic primer, an ampicillin-resistant primer and a tetracycline-sensitive primer were annealed with the single-stranded DNA, and the complementary DNA strand was synthesized with T4 DNA polymerase and sealed with ligase. The synthesized double-stranded DNA was used to transform *E.coli* ES1301 (*mutS*$^{-}$) and the cells were grown in an LB plate containing 50 μg/ml ampicillin. The plasmid DNA recovered from the lysate was applied to *E.coli* JM109 cells, and Amp^{r}/Tet^{s} colonies were isolated. The mutagenized DNA was isolated and inserted into pTT100-hMTH1 to replace the corresponding wild type fragment. The hybrid plasmid was applied to *E.coli* CC101T (*mutT*$^{-}$, *F, lacZ*^{am461}), and colonies which showed papillae were isolated and subjected to sequence analysis.

(31). After incubation for 5 days at 37°C, papillae formation was examined and appropriate samples were subjected to further analyses.

Assay of 8-oxo-dGTPase

The reaction mixture (12.5 µl) contained 20 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 40 mM NaCl, 20 µM 8-oxo-dGTP (including [α-^{32}P]8-oxo-dGTP), 80 µg/ml bovine serum albumin (BSA), 8 mM dithiothreitol (DTT), 2% glycerol and an extract of cells carrying plasmids to be examined. The reaction was run at 30°C for 20 min and terminated by adding 2.5 µl 50 mM EDTA. An aliquot (2 µl) of the reaction mixture was applied to a PEI–cellulose sheet. 8-Oxo-dGMP produced was separated from 8-oxo-dGTP on TLC with 1 M LiCl for 90 min and quantitated in Fujix Bio-image analyzer BAS2000 (Fuji Photofilm Co., Ltd, Tokyo) (22). One unit of 8-oxo-dGTPase was defined as the amount of enzyme that produced 1 pmol/min 8-oxo-dGMP at 30°C (21).

Western blot analysis

Western blotting was done as described (32,33), but with minor modification. Five or ten micrograms of protein of the bacterial cell crude extracts were subjected to 15% SDS–polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membrane (BA-83, Schleicher & Schuell, Dassel, Germany) at 25 V for 1 h in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol, pH 9.3). The nitrocellulose filter was soaked in blocking solution (5% BSA, 10 mM Tris–HCl, pH 7.4, 0.9% NaCl, 0.05% Tween 20) at 52°C for 1 h and then incubated overnight at 4°C with anti-MTH1 (1 µg/ml) (33). The filter was rinsed in buffered saline containing 0.05% Tween 20 to remove excess antibodies, then reacted with 1 µCi/ml of ^{125}I-labeled protein A for 1 h on ice. The filter was washed with radioimmune precipitation assay buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) at room temperature to remove unbound protein A. The filter was air-dried, exposed to an imaging plate for 3 h and then data were processed using a Fujix Bio-image analyzer BAS2000.

RESULTS

Isolation of negative mutants with Lys-38 substitution

Substitution of the lysine residue at amino acid position 38 was done as this residue is present in all the known MTH1-related proteins and locates within the highly conserved region of the protein (Fig. 5). Figure 1 shows the strategy for site-directed mutagenesis and subsequent mutant selection. Oligonucleotides with all possible combinations for a given codon were synthesized and used as primers for generating the complementary strand. To facilitate selective replication of the mutated plasmids, a set of oligonucleotide fragments exerting ampicillin resistance and tetracycline susceptibility were also introduced. Double-stranded DNAs were produced by *in vitro* repair reaction and then propagated in *E.coli* cells defective in mismatch repair. From the progeny plasmid a DNA fragment carrying the mutagenized region was excised and introduced into pTT100-hMTH1 to replace the corresponding region with the normal sequence. The resulting plasmids were applied to *E.coli mutT*$^-$ cells and colonies were examined.

Since the *mutT* mutator causes specifically an A:T to C:G transversion (20), we used the *lacZ* reversion assay to detect this type of mutation (30,31). When the *mutT* mutation was introduced into a tester strain CC101, the resulting strain CC101T produced many *lacZ*$^+$ revertants, as evidenced by formation of numerous papillae in a colony (Fig. 2A). This papilli formation was almost completely suppressed by expression of the human *MTH1* cDNA in CC101T cells (Fig. 2B). Application of mutagenized clones to this test yielded detection of mutants with a negative phenotype, as shown in Figure 2C. In this way we collected many independent clones of negative mutants and the sequences of 21 of these were analyzed. The substitutions detected included amino acids with acidic side-chains such as Asp, with uncharged polar side-chains such as Cys and Asn, and with nonpolar side-chains such as Ala and Pro (Table 1).

Table 1. Amino acid substitutions at Lys-38 residue of human 8-oxo-dGTPase protein

Wild type		Substitutions found in negative mutants		Substitutions found in positive revertants	
Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
Lys	AAA	Tyr	TAT (2)	Lys	AAA (8)
			TAC (2)		
		*Arg	CGC (3)		
		Val	GTG (2)		
			GTC		
		Asp	GAC (2)		
			GAT		
		Thr	ACA		
		Ala	GCA		
		Leu	CTA		
		Phe	TTC		
		Pro	CCT		
		Cys	TGC		
		Asn	AAC		
		amber	TAG		

Twenty-one negative mutants were isolated from 344 screened clones. A second cycle of site-directed mutagenesis was performed using one of the negative mutants, with a codon for Arg (marked with an asterisk), as template. Among 619 independent clones examined, eight positive revertantswere isolated. Numbers in parentheses indicate the number of independent clones obtained.

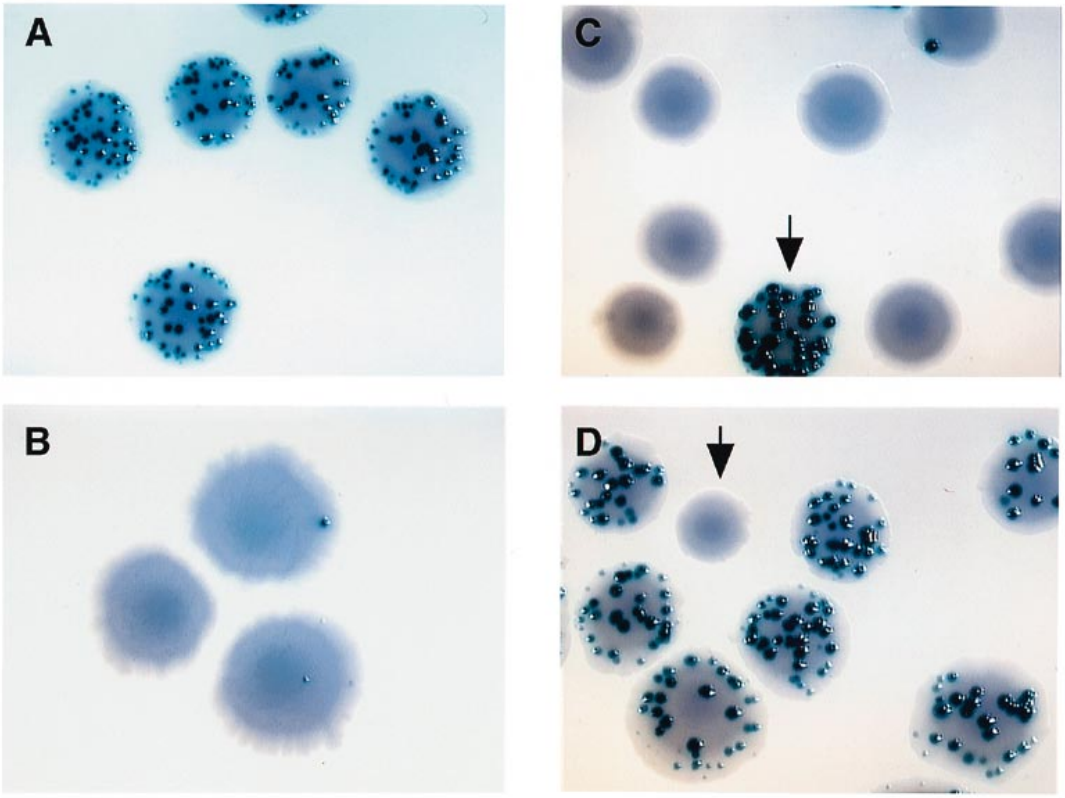

Figure 2. Isolation of mutants with a mutation at the Lys38 site. (**A**) *Escherichia coli* CC101T (*mutT*$^{-}$) cells harboring plasmid pTT100 (vector). (**B**) *Escherichia coli* CC101T (*mutT*$^{-}$) cells harboring plasmid pTT100-hMTH1 (human *MTH1* cDNA). (**C**) Screening of negative mutants. *E.coli* CC101T cells were transformed with plasmid carrying the human *MTH1* gene with mutations at the pre-determined site and placed on glucose minimal A plate containing 50μg/ml ampicillin, 0.2% glucose, 0.05% phenyl-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml) and incubated for 5 days at 37°C. An arrow indicates a colony of negative mutant. (**D**) Isolation of positive revertants. One of the negative mutants was used as the template for mutagenesis and a similar screening procedure was executed. An arrow indicates a colony of positive revertant.

Properties of mutant proteins

Western blot analysis was done with lysates of *E.coli* cells producing mutant forms of proteins (Fig. 3). Using a polyclonal antibody against hMTH1 protein, a major band was detected in all the samples, except one carrying a termination codon, at a position corresponding to the authentic 18 kDa human protein. Quantitative estimation of amounts of proteins was made by scanning the gels. Based on the standard curve obtained from the purified hMTH1 protein, we estimated the amount of mutant protein in each extract (Table 2). In all cases, immunologically reactive proteins were produced in quantities similar to or more than the wild type protein.

These mutants were assayed for 8-oxo-dGTPase activity and the result is shown also in Table 2. Little or no activity was found in extracts of cells harboring either one of the mutant clones; specific activities of mutants were <4% of the wild type.

To determine the *in vivo* biological activities of mutant proteins, mutation frequencies of cells producing various forms of hMTH1 protein were determined. As shown in Table 3, the mutation frequency of CC101T (*mutT*$^{-}$) harboring the vector plasmid was ~250-fold higher than that of CC101 (*mutT*$^{+}$) carrying the same vector. When the normal cDNA (Lys-38) was placed on the vector plasmid and properly expressed, this high level of mutation induction was completely suppressed. Mutants with tyrosine or leucine substitution at the Lys-38 site showed no capability of suppression, while those with arginine or alanine substitution exerted 10% of the suppressive effect of the wild type.

Positive revertants at the Lys-38 sites

Analyzing clones isolated by negative selection, we identified mutants with 11 different amino acid substitutions. Since it is laborious to isolate all the possible mutants using these procedures, positive selection was used to obtain revertants from individual negative mutants. The procedure was essentially the same as that described above except that one of the negative mutants was used as the template for mutagenic DNA synthesis. For this, we used the K38R mutant, in which lysine at codon 38 was converted to arginine. It was shown by Western blot analysis that the mutant produces a sufficient amount of hMTH1 protein (Table 2). After mutagenization, clones showing no papillae formation were selected (Fig. 2D) and, among the 619 examined, eight positive revertants were isolated. DNA sequence analysis revealed that all carried the codon for lysine at position 38.

Figure 4 shows data on the quantitative assay of 8-oxo-dGTPase activity exerted by wild type, one of negative mutants and its positive revertant. The enzyme activity of Arg-38 mutant (K38R) was <10% of the wild type. As expected, the enzyme level of cells harboring the positive revertant (K38R-K) was essentially the same as that of cells carrying the wild type DNA.

Table 2. 8-Oxo-dGTPase activity and immunoreactive hMTH1 protein in mutants at Lys-38 site

Type of mutants	Amino acid substitution	Codon	Immunoreactive hMTH1 protein (ng/10 μg protein)	8-Oxo-dGTPase activity (U/μg protein)	Specific activity (U/10 ng)
positive	Lys	AAA	10.2	8.6×10^3	84.3
negative	Tyr	TAT	9.0	2.5×10^2	2.8
		TAC	14.8	4.1×10^2	2.8
	Val	GTG	8.6	1.9×10^2	2.2
		GTC	8.0	1.3×10^2	1.7
	Asp	GAC	8.6	7.5×10	0.9
		GAT	30	8.1×10	0.3
	Arg	CGC	16.2	5.0×10^2	3.1
	Pro	CCT	9.4	8.4×10	0.9
	Ala	GCA	10.4	2.9×10^2	2.8
	Thr	ACA	7.8	1.2×10^2	1.5
	Leu	CTA	11.0	2.3×10^2	2.1
	Phe	TTC	9.4	1.6×10^2	1.7
	Cys	TGC	10.0	2.6×10^2	2.6
	Asn	AAC	7.6	1.2×10^2	1.6

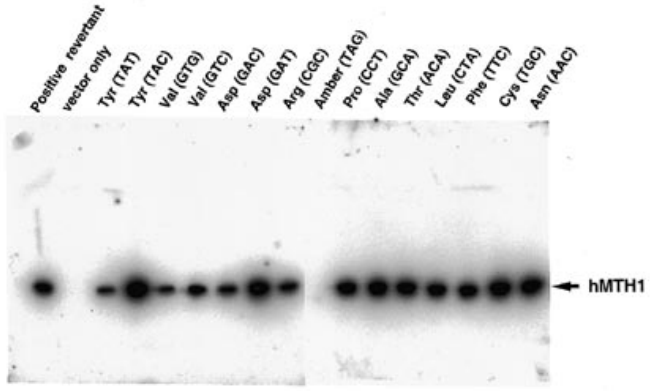

Figure 3. Western blot analysis of mutant hMTH1 proteins produced in *E.coli* cells. *Escherichia coli* CC101T cells carrying plasmids with positive revertant or mutants at position 38 were grown at 37°C to A_{600} of 0.8 and crude extracts were prepared by sonication. Ten micrograms protein of crude extracts were applied to electrophoresis on 15% SDS–polyacrylamide gels, transferred to nitrocellulose membrane and reacted with polyclonal anti-human MTH1 antibodies and ^{125}I-labeled protein A. An arrow indicates the position of human MTH1 protein. Codons for mutated clones are shown in parentheses.

Table 3. Suppression of *E.coli mutT* mutator phenotype by expression of wild type or mutant hMTH1

Escherichia coli strain	Mutation frequency
CC101 (*mutT*$^+$) with pTT100 (vector)	$2.7 \pm 0.7 \times 10^{-8}$
CC101T (*mutT*$^-$) with pTT100 (vector)	$6.7 \pm 0.3 \times 10^{-6}$
CC101T (*mutT*$^-$) with pTT100-hMTH1 (wild type)	$1.9 \pm 0.8 \times 10^{-8}$
CC101T (*mutT*$^-$) with pTT100-hMTH1 (K38Y)	$5.9 \pm 1.1 \times 10^{-6}$
CC101T (*mutT*$^-$) with pTT100-hMTH1 (K38L)	$5.4 \pm 0.6 \times 10^{-6}$
CC101T (*mutT*$^-$) with pTT100-hMTH1 (K38R)	$1.9 \pm 0.1 \times 10^{-7}$
CC101T (*mutT*$^-$) with pTT100-hMTH1 (K38A)	$1.5 \pm 0.5 \times 10^{-7}$

Analyses of other conserved residues

The negative and positive selection procedure was used for analyses of three amino acid residues located within the conserved region. Since selection of positive revertants is more versatile than selection of negative mutants, only a few negative mutants with a predefined sequence were isolated, one of which was used as a template for second round of mutagenic DNA synthesis to isolate positive revertants.

According to this protocol, Glu-43 was substituted with a few of other amino acids to form negative mutants. Using the E43R mutant as template, 18 positive revertants were isolated from 1060 clones examined. DNA sequence analysis revealed that all carried codons for glutamic acid; 13 had the original GAG and the remaining five GAA (Table 4).

Similarly, screening of positive revertants was done for the Arg-51 site. Using the R51L negative mutant as a template, 1552 colonies were screened. Twenty-two independent clones were selected as positive revertants and all carried codons for arginine, with all six types of Arg codons.

For Glu-52 substitutions, a result similar to that for Glu-43 was obtained. From 2162 clones examined, 15 positive revertants were isolated, all of which carry codons for Glu.

These results clearly indicate that Glu-43, Arg-51 and Glu-52 are essential for the hMTH1 protein to exert its function.

DISCUSSION

Studies on *E.coli* mutator genes and their products revealed that a major cause of spontaneous mutation is errors of DNA replication, and organisms are equipped with elaborate mechanisms to avoid such errors and to correct errors after DNA replication (34). Among them, the *mutT* mutator gene is involved in a novel mechanism that prevents replication errors by degrading a potent mutagenic substrate, 8-oxo-dGTP, to the unutilizable monophosphate in the nucleotide pool of the cell (17). An enzyme similar to the MutT protein was found in various prokaryotic and eukaryotic organisms, and the cDNA and the genomic sequences encoding the enzyme were isolated and their sequences determined (22,25–28). In six molecular species of the enzymes derived from bacterial and mammalian cells, there are a well conserved amino acid sequences, around the center region of the protein (Fig. 5). NMR analysis of the *E.coli* MutT protein revealed that the highly-conserved region is within loop I from residues 37 to 46 and in the C-terminus of α-helix I from positions 52 to 59 (35), and loop I, α-helix I and loop II motifs contribute significantly to the active site of MutT (29,35).

Table 4. Amino acid substitutions at Glu-43, Arg-51 and Glu-52 sites

Wild type		Negative mutants		Positive revertants	
Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
Glu-43	GAG	*Arg	AGA	Glu	GAG (13)
		Gln	CAA		GAA (5)
		Ala	GCT		
Arg-51	AGG	*Leu	CTA	Arg	AGA (7)
		Asn	AAC		AGG (1)
		His	CAC		CGT (1)
					CGC (3)
					CGA (5)
					CGG (5)
Glu-52	GAG	*Gly	GGC	Glu	GAA (8)
		Asp	GAT		GAG (7)
		Arg	AGG		

The procedure was the same as for Table 1, and each of the negative mutants with a codon marked with an asterisk was used as template for a second cycle of site-directed mutagenesis. Positive revertants for Glu-43, Arg-51 and Glu-52 were isolated from 1060, 1552 and 2162 clones examined. Numbers in parentheses indicate the number of independent clones obtained.

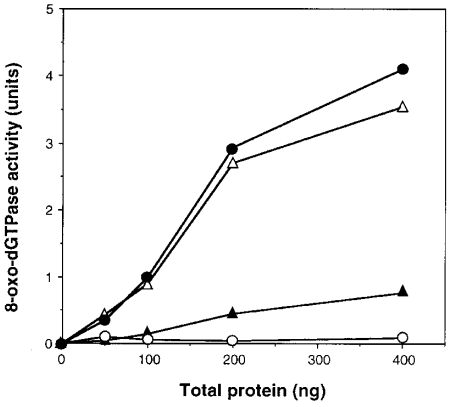

Figure 4. Expression of various forms of human *MTH1* cDNA in *E.coli* cells. *Escherichia coli* CC101T (*mutT*⁻) cells harboring various types of plasmids were grown at 37°C and extracts were prepared for assay of the enzyme activity. ●, *E.coli* CC101T (*mutT*⁻) cells harboring pTT100-hMTH1 with human 8-oxo-dGTPase (wild type); ○, CC101T (*mutT*⁻) cells harboring pTT100 vecter (control); ▲, CC101T (*mutT*⁻) cells harboring plasmids with K38R negative mutant, used as the template for mutagenesis; △, CC101T (*mutT*⁻) cells harboring plasmids with one of positive revertants (K38R-K).

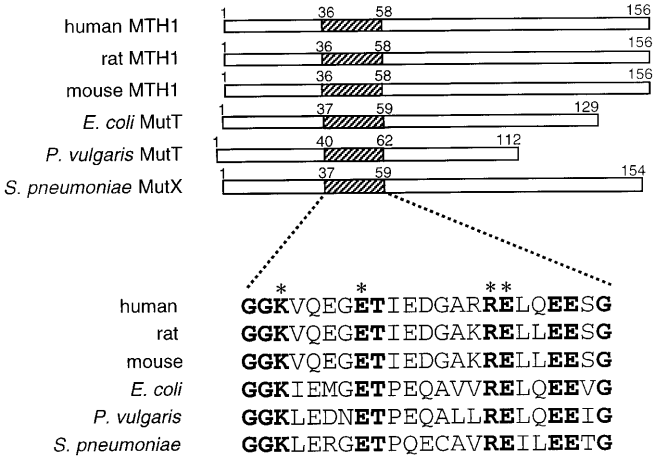

Figure 5. Conservation of the specific amino acid residues in 8-oxo-dGTPase proteins. Shaded boxes represent the regions with high degree of amino acid sequence homology, with their amino acid sequences given below. The amino acid residues conserved throughout the six species of enzyme are shown in boldface letters. Numbers correspond to the positions of amino acid residues from the N-termini. Asterisks indicate the sites of amino acid substitutions made in this study.

We initiated systematic mutational studies by replacing the amino acid at Lys-38 site by site-directed mutagenesis of the cloned human *MTH1* cDNA. Various amino acid substitutions were introduced at the site and mutants showing the negative phenotype (formed papilli colonies) were selected. From one of the negative mutants, which was used as the template for the next round of mutagenic DNA synthesis, revertants showing the positive (wild type) phenotype were isolated, and sequence analysis revealed that all the revertants carry the original codon AAA for lysine at the site. To ascertain that the synonymous AAG codon for lysine has the same potential as the original AAA codon in exerting the activity, we constructed a mutant sequence with AAG codon by oligonucleotide-mediated site-directed mutagenesis. Cells carrying the mutant sequence with AAG codon showed the positive phenotype, and there was no difference in the enzyme activity (data not shown). Bias in codon distribution in this particular experiment may be incidental and, indeed, similar mutagenesis experiments at other sites yielded various types of codons in revertant populations.

Western blots analysis revealed that all of the negative mutants produced large amounts of protein indistinguishable from the wild type protein, though they did exhibit no or only little enzyme activity. The biological activities of some mutant proteins were also tested by expressing the cDNA in *E.coli mutT*⁻ cells and determining mutation frequencies. As was shown in Table 3, expression of a positive revertant suppresses completely the occurrence of specific A:T to C:G transversion mutation, while mutants with amino acid substitutions exhibit no suppression capacity. Our results are consistent with the results of Frick *et al.* (35,36) that *E.coli* MutT protein with a lysine to glutamine change at codon 39 (the codon corresponding to Lys-38 of human MTH1) shows an 8-fold decrease in K_{cat} and a 5.3-fold increase

in K_m (dGTP) in the Mg^{2+}-activated dGTPase, resulting in a 42-fold overall decrease in K_{cat}/K_m. Taken together, a lysine residue at this site is strictly required for 8-oxo-dGTPase activity.

Amino acid substitutions were performed at Glu-43, which correspondingly locates in loop I of *E.coli* MutT protein (35). In this case as well, only codons for the original amino acid residue were found in a collection of the revertant clones. Thus, glutamic acid at this site must be conserved to retain the enzyme activity.

Studies were extended to analysis of Arg-51 and Glu-52, which correspond to Arg-52 and Glu-53 of *E.coli* MutT and are located within α-helix I of the protein (35). For these two sites, positive revertants obtained were found to carry codons for the original amino acid with different codons. Among 22 revertants for the mutated arginine residue, all six different types of codons were found, while 15 revertants for the mutated glutamic acid possessed one of the two codons. As were the cases for Lys-38 and Glu-43, it was concluded that these two sites are essential to exert the enzyme activity. Frick *et al.* (36) demonstrated that a mutation of the Arg-52 residue of *E.coli* MutT, R52Q, results in almost complete loss of dGTPase activity.

According to the NMR studies of *E.coli* MutT protein, a cluster of five glutamic acid residues, containing Glu-52 (corresponding to Glu-53 of hMTH1 protein), forms a patch of strongly negative electrostatic potential, likely constituting the metal binding site (29,37). In this study, mutant hMTH1 proteins having other amino acid residues at this site showed no capacity to suppress the *mutT* mutator phenotype. It is likely that these mutant proteins cannot hydrolyze 8-oxo-dGTP due to their inabilities to bind divalent cations, which are essential for exerting the catalytic activity. In the present study, we obtained evidence that Lys-38 and Glu-43, located in loop I, and Arg-51 and Glu-52, which are within α-helix I of the protein, cannot be replaced by other amino acids. This region probably constitute an active center for the enzyme. This view is consistent with the results obtained with the NMR studies (29,35,37).

Recently, a G to A transition at codon 83, which causes replacement of valine with methionine, was found in certain human-derived cell lines and tumor samples (38). The variant form of protein was less heat-stable as compared with normal protein, in terms of both biochemical and physicochemical properties (Yakushiji *et al.*, in preparation). There may be a certain interaction between the Val-83 and amino acid residues within the active center of the protein. Elucidation of the detailed structure of human MTH1 protein is necessary to determine the unique catalytic activity of the protein.

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