Mouse MTH1 Protein with 8-Oxo-7,8-dihydro-2′-deoxyguanosine 5′-Triphosphatase Activity That Prevents Transversion Mutation
cDNA CLONING AND TISSUE DISTRIBUTION*

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8-Oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) is formed in the nucleotide pool of a cell during normal cellular metabolism, and when it is incorporated into DNA causes mutation. Organisms possess 8-oxo-dGTPase, an enzyme that specifically degrades 8-oxo-dGTP to 8-oxo-dGMP. We isolated cDNA for mouse 8-oxo-dGTPase, using as a probe a human MTH1 (Escherichia coli mutT homolog) cDNA. The nucleotide sequence of the cDNA revealed that the mouse MTH1 protein (molecular weight of 17,896) comprises 156 amino acid residues. When the cDNA for mouse 8-oxo-dGTPase was expressed in E. coli mutT mutant cells devoid of their own 8-oxo-dGTPase activity, an 18-kDa protein, which is cross-reactive with an anti-human MTH1 antibody, was formed. In such cells, the level of spontaneous mutation frequency that was elevated reverted to normal. High levels of 8-oxo-dGTPase activity were found in liver, thymus, and large intestine, whereas all other organs examined contained smaller amounts of the enzyme. In embryonic stem cells, an exceedingly high level of the enzyme was present.

Oxygen radicals are generated during normal cellular metabolism, and the formation of such radicals is further enhanced by ionizing radiation and by various chemicals (1). Among many classes of DNA damage caused by oxygen radicals, an oxidized form of guanine base, 8-oxo-7,8-dihydroguanine (8-oxoguanine), appears to play important roles in mutagenesis and in carcinogenesis (2–4). During DNA replication, 8-oxoguanine nucleotide can pair with cytosine and adenine nucleotides, with an almost equal efficiency, and transversion mutation ensues (5–7).

Organisms are equipped with elaborate mechanisms to counter such mutagenic effects of 8-oxoguanine. In Escherichia coli, two glycosylases encoded by the mutM and the mutY genes function to prevent mutation caused by 8-oxoguanine in DNA (8–12). MutM protein removes 8-oxoguanine paired with cytosine and MutY protein removes adenine paired with 8-oxoguanine. Enzyme activities similar to those of MutM and MutY have been identified in mammalian cells (13–16). A significant amount of 8-oxoguanine is formed in the chromosomal DNA of mammalian cells, and most damaged nucleotides are excised from the DNA and excreted into the urine (17). These enzymes may maintain spontaneous mutation frequency at certain low levels.

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 (18). Organisms possess mechanisms for preventing mutation due to misincorporation of 8-oxo-dGTP. MutT protein of E. coli hydrolyzes 8-oxo-dGTP to the monophosphate, and lack of the mutT gene increases the occurrence of A:T to C:G transversion thousands of fold over the wild type level (18–20). 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). Therefore, the human 8-oxo-dGTPase is likely to have the same antimutagenic capability as the MutT protein. Recently, the genomic sequence encoding the enzyme was isolated and the gene, named MTH1 (for mutT homolog), was seen to be located on human chromosome 7p22 (23).

To elucidate the roles of 8-oxo-dGTPase in carcinogenesis, it is necessary to construct animal models with altered levels of this enzyme activity, and it is of interest to determine whether the frequency of occurrence of tumors would increase in mice defective in the 8-oxo-dGTPase gene. As the first step toward this goal, we cloned cDNA for mouse MTH1 protein and characterized the product. We also investigated the distribution of the MTH1 protein in various organs of mice by quantitative immunoprecipitation and Western blot analysis, using an anti-MTH1 antibody.

EXPERIMENTAL PROCEDURES

Cells and Chemicals—The mouse embryonic stem cell line, CCE, was obtained from Dr. M. Katsuki, Kyushu University. C57BL/6J mice 8 ± 1 weeks old, were obtained from CLEA, Inc. (Fukuoka, J. P). Plasmid pA1 containing mouse IAP elements was provided by Dr. Y. Nakatsu, Institute for Cancer Research, Fox Chase Cancer Center (Philadelphia, PA). [α-32P]dCTP, [α-32P]dGTP, and 125I-labeled protein A were obtained from Amersham Japan (Tokyo). A DNA labeling kit was purchased from Nippon Gene (Toyama, J. P). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Klenow fragment were obtained from Toyobo Co. (Osaka, J. P). The cloning vector, pBlueScript II KS+, was purchased from Stratagene (La Jolla, CA), and the expression vector in E. coli, pTrc99A, was from Pharmacia Biotech Inc. (Uppsala, Sweden). RNA size standards and reconstituted protein molecular weight standards were obtained from Life Technologies, Inc. Rifampicin and protease inhibitors were obtained from Sigma, and IPTG was obtained from Wako Pure Chemical (Osaka, J. P). Polyethyleneimine-cellulose plates were obtained from Merck. Activated CH-Sepharose 4B and protein A-Sepharose CL-4B were purchased from Pharmacia. Dialysis membranes (Spectra/Por membrane) and B1O-DOT, SF were obtained.

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‡ The abbreviations used are: 8-oxoguanine, 8-oxo-7,8-dihydroguanine; 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGTPase, 8-oxo-7,8-dihydro-2′-deoxyguanosine triphosphatase; IAP, intracisternal A particle; IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin; bp, base pair(s).
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from SPECTRUM (Houston, TX) and Bio-Rad, respectively. Sources of other materials are given in the text.

Isolation of Mouse 8-Oxo-dGTPase cDNA Clones—A mouse cDNA library (a gift from Dr. D. E. Rancourt, University of Utah), prepared from an embryonic stem cell line, CC1.2, was screened by plaque hybridization using a random-primed NcoI/BamHI fragment (560 base pairs) excised from p24T that contained the entire coding region for human MTH1 cDNA (22). Prehybridization and hybridization were carried out at 42°C in solution containing 4 × SSC (1 = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 × Denhardt’s solution (1 = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS, 40% formamide, and 100 μg/ml heat-denatured salmon sperm DNA (Sigma). Hybridization conditions were optimized for each of the clone DNAs. Phage DNAs were isolated from clones showing positive signals and were checked for size of the inserted DNAs. The insert of clone M-2 which contains the 5′-most region of the cDNA, and those of three others, M-1, M-8, and M-12, were subcloned into pBluescript II KS+ for further analyses. The nucleotide sequences were determined using a model 373A automated DNA sequencer (Applied Biosystems/Perkin-Elmer Japan, Chiba, Japan).

Expression of Mouse DNA in E. coli—A DNA fragment carrying the coding region for mouse MTH1 was amplified by reverse transcriptase-polymerase chain reaction with a set of primers designed from preceding sequences, 5′-GACACACCGGCAGCAGGC-3′ (Pm-1) and 5′-GGTAGATGTTGAGTGGCCGACGAC-3′ (Pm-2), using the cDNA of poly(A)+ RNA from CCE cells as a template. The synthetic oligonucleotide primer (Pm-1) was designed to introduce the NcoI site at the start codon, which was altered in the second amino acid residue, serine, to glycine. A reverse transcriptase-polymerase chain reaction product of a 521-bp DNA fragment was cloned into the EcoRI and PstI site of pBluescript II KS+ for further analyses. The nucleotide sequences of the amplified cDNA was confirmed by sequencing. After digestion with NcoI and BamHI, a 503-bp DNA fragment was excised and inserted into the NcoI/BamHI site of pTrc99a, producing pTK1.

A pair of isogenic E. coli strains, MK601 (mutT+) and MK602 (mutT−), were used. Cultures were washed with PBS and homogenized at 4°C in 5 volumes of radioimmune precipitation assay buffer, the lysates were directly applied to cDNA libraries using Oligotex-dT30 (Hoffmann-La Roche). Three μg of poly(A)+ RNA from each cell line were applied to electrophoresis on a 1% agarose-formaldehyde gel, and the RNAs that were separated were denatured in 0.5 M NaOH (denaturation solution) at room temperature for 15 min. After the denaturation, the RNAs were hybridized to the immobilized cDNA probe. The filter was washed with 2 × SSC, 0.1% SDS, and then autoradiographed on Fuji RX filter at −80°C with an intensifying screen. Phage DNAs were isolated from clones showing positive signals and were checked for size of the inserted DNAs. The insert of clone M-2 which contains the 5′-most region of the cDNA, and those of three others, M-1, M-8, and M-12, were subcloned into pBluescript II KS+ for further analyses. The nucleotide sequences were determined using a model 373A automated DNA sequencer (Applied Biosystems/Perkin-Elmer Japan, Chiba, Japan).

Preparation of Extracts from Cells and Tissues—For immunodetection of MTH1 protein, extracts were prepared as follows. CCE cells and tissue samples from mice perfused with phosphate-buffered saline were homogenized at 4°C in 5 volumes of radioimmune precipitation assay buffer containing 0.5 mM phenylmethylsulfonyl fluoride and 0.5 μg/ml each of leupeptin, pepstatin, and chymostatin, followed by sonication on ice to complete disruption of the cells. The lysates were directly applied to both immunoprecipitation and slot blot analysis to determine DNA contents.

Immunodetection of Mouse MTH1 Protein—Immunoprecipitation was done as described by Harlow and Lane (30). Briefly, 1 ml of preclared extract was incubated at 4°C overnight with an excess amount of antibody anti-MTH1 (5 μg) or normal rabbit immunoglobulin G (10 μg), and protein A-Sepharose CL-4B was used to collect the immunocomplex. After washing five times with 1 ml of radioimmune precipitation assay buffer containing 0.5 mM phenylmethylsulfonyl fluoride and 0.5 μg/ml each of leupeptin, pepstatin, and chymostatin, followed by sonication on ice to complete disruption of the cells. The lysates were directly applied to both immunoprecipitation and slot blot analysis to determine DNA contents.

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RESULTS

cDNA for Mouse 8-Oxo-dGTPase—A mouse cDNA library, derived from an embryonic stem cell line, was screened for the mouse 8-oxo-dGTPase cDNA sequence, using as a probe a 560-bp Ncol/BamHI DNA fragment which contains the coding region for human MTH1 protein. Twelve out of $5 \times 10^5$ clones screened showed a strong, positive hybridization signal. After examining sizes of the inserted DNA fragments, four clones were chosen, and their inserts were subcloned into plasmid pBluescript II KS +.

Sequences of both strands were determined for more than 90% of the cDNA region (Fig. 2). Included within the approximately 950-bp cDNA is an open reading frame of 471 bp, defined by an ATG initiation codon (nucleotides 99-101) and a TAA stop codon (nucleotides 567-569), corresponding to a protein composed of 156 amino acid residues. In the 3′-untranslated region of 380 nucleotides, a putative polyadenylation signal, AATAAA, by a thick underline, was found 14 nucleotides upstream from the poly(A) stretch.

The amino acid sequence deduced from the nucleotide sequence is also shown in Fig. 2. The 3′-most region of the cDNA, while the other three, M-1, M-8, and M-12, carry the 3′-furthest region (Fig. 1).

Expression of Mouse cDNA in E. coli Cells—To confirm that the mouse cDNA we isolated indeed encodes 8-oxo-dGTPase, the cDNA was expressed in E. coli MK602 (mut + ) cells, which are devoid of their own 8-oxo-dGTPase activity. To achieve a high level of expression, the cDNA was placed downstream of the strong trc promoter in pTrc99A, and the transformed cells were incubated in the presence of IPTG. When a cell-free extract of cells carrying pTK1 with the cDNA was applied at 50 times over that of cells carrying the mouse cDNA, a band for the human enzyme was detected on SDS-polyacrylamide gel electrophoresis analysis (Fig. 3, lane 3). When the extract of cells carrying the human enzyme was digested on Western blot analysis using anti-MTH1 (Fig. 3, lane 3). Thus, approximately a 40-fold larger amount of the mouse enzyme was produced in E. coli cells, as compared with the human enzyme. For this estimation, we used partially purified human and mouse MTH1 TrpE fusion protein to assess the recognition sensitivity of anti-MTH1 antibody against human and mouse MTH1 proteins, respectively (data not shown).

Suppression of the mutT Mutator by Expression of the Mouse cDNA—We examined the effects of cDNA expression on the level of spontaneous mutation frequency toward rifampicin resistance in mutT + cells. As shown in Table I, the frequency of mutation of E. coli MK602 (mut + ) cells carrying the cDNA was as low as that of wild type cells. Such being the case, the mouse MTH1 protein probably functions in E. coli cells to prevent the occurrence of spontaneous mutations caused by accumulation of 8-oxo-dGTP in the nucleotide pool.

Northern Blot Analysis—The size of mouse MTH1 mRNA was estimated by Northern blot hybridization. The poly(A)+ RNAs from mouse fibroblast cell line, NIH3T3, and the mouse embryonic stem cell line, CCE, were hybridized with a randomly primed mouse cDNA probe. One major band of about 1.2 kilo-
bases was detected (Fig. 4A), and this size of the transcript corresponds to that estimated from the cDNA sequence. However, since the size of cDNA is slightly smaller than that of the mRNA detected by Northern blot analysis, it is likely that the cDNA lacks a certain region of the poly(A) stretch. The 1.2-kilobase band was detected in various mouse tissue samples, including kidney, liver, and small intestine (Fig. 4B).

MTH1 Protein in Mouse Tissues—On incubation with extracts of mouse tissues in the presence of Mg2+, 8-oxo-dGTP was rapidly degraded to 8-oxo-dGMP. However, as there was concomitant formation of 8-oxo-dGDP in the reaction, the extract probably contained nonspecific nucleoside triphosphatase activity (21). Western blot analysis was the method of quantifying MTH1 protein in tissue samples, and only a faint band for the protein was detected over nonspecific interfering bands. Thus, we made use of immunoprecipitation prior to Western blotting. To optimize the experimental conditions, we analyzed an extract of CCE, a mouse embryonic stem cell line, which contains a relatively large amount of 8-oxo-dGTPase protein. As shown in Fig. 5, a distinct band for MTH1 protein was detected on the blots, and the intensities of signals, quantified by an image analyzer, were proportional to amounts of enzyme in the extract applied.

Tissue samples were obtained from mice perfused with phosphate-buffered saline, and extracts were prepared in the presence of protease inhibitors. Extracts containing 3 mg of protein were incubated with anti-human MTH1 antibody, anti-MTH1 (5 μg), and the immunocomplexes were collected and subjected to Western blot analysis using anti-MTH1. As shown in Fig. 6,
were done three times.

value for bands were quantified by a Fujix 2000 Bio-image analyzer and the determined, and values per 1
tion analysis, DNA contents of these tissue samples were also
might be cleaved by proteases.

sample derived from this organ (see Fig. 4
band in Northern blot analysis was clearly detected in the

brain, heart, lung, and stomach. Although there was no detect-

high intensity bands were detected for samples derived from
mouse thymus, liver, spleen, kidney, testis, and large intestine. Less but significant amounts of MTH1 protein are present in
brain, heart, and stomach. Although there was no detect-
able band for the small intestine on Western blot analysis, a

TABLE II

| Tissues and cells | MTH1 protein |
|------------------|--------------|
|                  | molecules/mg protein × 10¹⁰ | molecules/µg DNA × 10⁹ |
| Brain            | 0.98 ± 0.07 | 3.19 ± 0.22 |
| Thymus           | 5.80 ± 0.49 | 1.30 ± 0.11 |
| Heart            | 1.20 ± 0.17 | 5.48 ± 0.76 |
| Lung             | 1.45 ± 0.14 | 8.85 ± 0.18 |
| Liver            | 5.82 ± 0.54 | 17.83 ± 1.62 |
| Spleen           | 2.69 ± 0.20 | 1.26 ± 0.09 |
| Kidney           | 2.84 ± 0.36 | 4.46 ± 0.57 |
| Testis           | 3.24 ± 0.61 | 3.69 ± 0.69 |
| Large intestine  | 4.21 ± 0.59 | 5.11 ± 0.72 |
| Small intestine  | ND           | ND           |
| Stomach          | 1.80 ± 0.28 | 5.88 ± 0.91 |
| CCE             | 34.04 ± 2.01 | 25.02 ± 1.48 |

DISCUSSION

8-Oxo-dGTP can be generated not only by direct oxidation of
dGTP but also by phosphorylation of 8-oxo-dGDP (33). Human
cells contain a powerful nucleoside diphosphate kinase activity
that converts ribo- and deoxyribonucleoside diphosphates,
including 8-oxo-dGDP, to the corresponding nucleoside triphos-
phates (33). Thus, 8-oxo-dGDP, formed either by oxidation of
dGDP or by reduction of 8-oxo-GDP, can be converted 8-oxo-
dGTP. Once 8-oxo-dGTP is formed, it can be incorporated into
cellular DNA to yield transversion mutation. 8-Oxo-dGTPase is
present in bacteria and mammalian cells and appears to function
so as to prevent this misincorporation. The enzyme specifically
hydrolyzes 8-oxo-dGTP to the monophosphate, and the
8-oxo-dGMP thus formed cannot be rephosphorylated. Guany-
late kinase that acts on both GMP and dGMP for phosphoryl-
tion so as to prevent this misincorporation. The enzyme specifically
hydrolyzes 8-oxo-dGTP to the monophosphate, and the
8-oxo-dGMP thus formed cannot be rephosphorylated. Guany-
late kinase that acts on both GMP and dGMP for phosphoryl-

Biological significance of 8-oxo-dGTPase has been estab-
lished based on the use of E. coli mutants defective in the mutT
gene (18–20). Lack of the gene causes an increased level of A:T
to C:G transversion thousands of fold over the wild type cells.

Table:<ref>

| Tissues and organs | Amounts of MTH1 protein |
|-------------------|------------------------|
|                   | molecules/mg protein × 10¹⁰ | molecules/µg DNA × 10⁹ |
| Brain             | 0.98 ± 0.07 | 3.19 ± 0.22 |
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| CCE               | 34.04 ± 2.01 | 25.02 ± 1.48 |

aData non-detectable.

bCCE, embryonic stem cell.
In the present study, we obtained evidence that the level of spontaneous mutation frequency of mutT− cells, which was elevated, reverted to normal when cDNA for mouse 8-oxo-dGTPase was expressed in such cells. It seems likely that mouse 8-oxo-dGTPase has the same antimutagenic capacity as the E. coli MutT protein.

When the human cDNA was expressed in E. coli mutT− cells, only a partial suppression of the mutator phenotype was achieved, 8-fold reduction in spontaneous mutation frequency from the level of mutT− cells but 50 times higher level as compared with mutT+ cells (22). In the case of expression of the mouse cDNA, an almost complete suppression occurred, as shown in Table I. This difference may be due to the amounts of the two types of mammalian enzymes produced in E. coli cells. Fortyfold larger amounts of the mouse enzyme were produced in E. coli cells as compared with the case of the human enzyme, although essentially the same vector/expression system was used. It is likely that the mouse enzyme has structural features that are more readily formed and retained in bacterial cells.

It should be noted here that, for the complete suppression of mutT mutation, an exceedingly large amount of the mouse enzyme is needed compared with the authentic MutT protein. One possible explanation for this inefficiency may be differences in intrinsic catalytic properties of the bacterial and mammalian enzymes. The $K_v$ value for the E. coli enzyme is 0.48 $\mu M$ (18), while that for the mammalian enzyme is 12.5 $\mu M$ (21). Thus, the E. coli enzyme more efficiently degrades 8-oxo-dGTP than do the mammalian enzymes.

Another aspect to be considered is the intracellular localization of the enzyme. Enzymes involved in nucleotide metabolism are located mostly in a region where DNA synthesis occurs (34). Hence, the authentic E. coli enzyme may have specific structural features to facilitate proper subcellular localization. Foreign enzymes are likely to be devoid of such signals and may not be able to function as well as the native enzymes.

The 8-oxo-dGTPase protein, originally isolated from E. coli, is composed of 129 amino acid residues (35). Recently, analogous genes were found in two distantly related bacteria, Proteus vulgaris and Streptococcus pneumoniae (36, 37). The products of the two latter genes carry enzyme activity specifically degrading dGTP to dGMP and are structurally related to the E. coli MutT protein. Alignment of the sequences of these five mammalian and bacterial enzymes revealed that all carry a highly similar sequence in almost the same region (Fig. 7). In the conserved region (from the 36th to the 58th amino acid for the mouse enzyme), 10 among 23 amino acid residues are identical. Thus, it is reasonable to assume that this region constitutes an active center for the enzyme. The secondary structure of the E. coli MutT protein, as elucidated by NMR analysis (38), supports this view.

When examining the distribution of 8-oxo-dGTPase protein in mice, all organs except the small intestine contained substantial amounts of the protein on Western blot analysis, the highest value was seen in the liver when compared with other organs. Even a higher level of the enzyme activity was detected in embryonic stem cell line CCE, with an intense proliferative capacity. High oxygen consumption may correlate with high levels of oxidative damage (39), and the level of expression of the MTH1 gene might be regulated, in this context.

To better understand the roles of mammalian 8-oxo-dGTPase in the control of spontaneous mutagenesis as well as carcinogenesis, mice defective in their own MTH1 gene are needed. Cloning of cDNA is a first step toward this goal followed by characterization of the genomic sequence. The 8-oxo-dGTPase enzyme is part of an elaborate system of defenses of organisms against oxidative damage to genetic materials. It is of interest to determine when and in which organs of the defective mice such abnormalities could occur. Related studies are in progress in our laboratory.

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