Review

Molecular devices for high fidelity of DNA replication and gene expression

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Abstract: Certain types of DNA lesions, produced through cellular metabolic processes and also by external environmental stresses, are responsible for the induction of mutations as well as of cancer. Most of these lesions can be eliminated by DNA repair enzymes, and cells carrying the remaining DNA lesions are subjected to apoptosis. The persistence of damaged bases in RNA can cause errors in gene expression, and the cells appear to possess a mechanism which can prevent damaged RNA molecules from entering the translation process. We have investigated these processes for high fidelity of DNA replication and gene expression, by using both biochemical and genetic means. We herein describe (1) the molecular mechanisms for accurate DNA synthesis, (2) mammalian proteins for sanitizing the DNA precursor pool, (3) error avoidance mechanisms for gene expression under oxidative stress, and (4) the roles of DNA repair and apoptosis in the prevention of cancer.

Key words: DNA replication; mutagenesis; gene expression; DNA repair; apoptosis; carcinogenesis.

The maintenance of the genetic information is of the utmost importance for organisms. To this end, accurate DNA replication is executed and errors seldom occur. According to Drake,[1] the frequency of errors during a single cycle of DNA replication is less than 10^{-10} per base pair. The self-complementary nature of the double-stranded DNA molecule is certainly the basis for this accurate replication, however the fidelity of base pairing is limited to a level of 10^{-4} per base pair, due to the tautomeric nature of the purine and pyrimidine bases. Therefore, to reduce the rate of error in DNA replication, several consecutive reactions are needed, which involve the discrimination of unfavorable precursors in the DNA precursor pool, the elimination of misincorporated nucleotides at the replication fork by proofreading, and the correction of mismatched base pairs by post-replicational mismatch repair.

DNA as well as its precursor nucleotides may be damaged by both internal and environmental agents, including various chemicals and radiations. Particularly, reactive oxygen species (ROS), such as superoxide, hydroxyl radicals and singlet oxygen, which are produced through a normal cellular metabolism, are all threats to genetic materials. These radicals attack nucleic acids to generate various modified bases, among which 7, 8-dihydro-8-oxoguanine (8-oxoG) is the most abundant, and it seems to play a critical role in both mutagenesis and carcinogenesis.[2),3)] Organisms, therefore, have to be equipped with special mechanisms to repair such lesions and eliminate damaged molecules from the cell.

Sometime at an early stage of my career, I encountered the problem of the cellular response to DNA damage and had the chance to find T4 endonuclease V, the first DNA repair enzyme that functions *in vivo* without the aid of visible light.[4)] This event greatly influenced the subsequent course of my research, and I extended my studies toward elucidating the molecular mechanisms of mutagenesis as well

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Abbreviations: ROS, reactive oxygen species; 8-oxoG, 7, 8-dihydro-8-oxoguanine; PNP, polynucleotide phosphorylase.

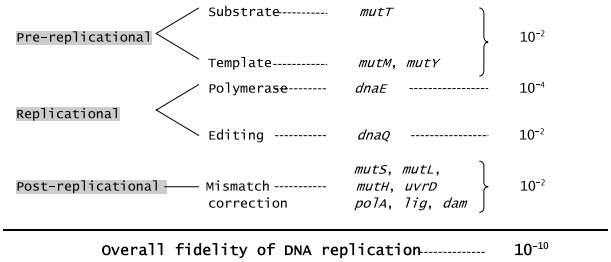


Fig. 1. Control of spontaneous mutagenesis in *E. coli*. Degrees of contribution of each step were estimated from mutation frequencies of mutants defective in particular gene functions. Note that some of mutations in a single allele sometimes affect more than one step. These values should be taken as rough estimates, in order to grasp the whole scheme.

as of carcinogenesis. This review deals with our recent studies, performed over the past fifteen years. These include (1) molecular mechanisms for high fidelity of DNA replication, as revealed in a model organism, *Escherichia coli*, (2) mammalian MutT-related proteins for preventing mutation and cancer, (3) error avoidance mechanisms for gene expression under oxidative stress, and (4) roles of DNA repair and apoptosis in the prevention of cancer.

I. Molecular mechanisms for high fidelity of DNA replication, as revealed in a model system. In *E. coli* cells, the error frequency of replication is kept within 10^{-10} per base pair,[1] and this high accuracy is achieved by the concerted actions of several replication-related proteins. If one such protein is inactivated by mutation, then the cell would exhibit an exceedingly high spontaneous mutation frequency. Therefore, these mutants, collectively termed mutators, are useful tools for elucidating cellular mechanisms related to the high fidelity of DNA replication. Once a mutant with a mutator phenotype is isolated, it can be used to clone the gene responsible and identify its protein product. This would in turn pave the way for determining the biochemical nature of the protein and further understanding its role in the entire process for accurate DNA replication.

Mutations mapped on at least 12 distinct genetic loci of the *E. coli* chromosome are known to cause mutator phenotypes (Fig. 1). Owing to recent development in molecular genetic technology, all of these genes have been cloned and their protein products identified. Based on these findings, they can be divided into several categories, from those functioning prior to or during DNA replication to those acting after passing the replication fork. A comprehensive review of the problems associated with replication fidelity was recently made by Maki,[5] and other reviews of post-replicational mismatch correction mechanisms are also available.[6),7] In this section, I would like to focus upon the elimination of oxidized guanine nucleotides from DNA and its precursor pool, which thus constitutes an important part of the accurate DNA synthesis.

(1) *Elimination of naturally occurring mutagenic substrates from the DNA precursor pool*:

Among the many mutators of *E. coli*, *mutT* has drawn particular attention. *MutT* is the first mutator found in this organism[8] and it specifically induces the transversion of adenine-thymine to cytosine-guanine (A·T→C·G).[9] As a consequence of this unidirectional mutator activity, *mutT* cells have increased the GC content levels in their chromosomal DNA.[10] Akiyama *et al.*[11] cloned the *mutT* gene and, based on a sequence analysis, identified the product as a protein with a molecular weight of 14,926. Bhatnager and Bessman[12] reported MutT protein to have a nucleoside triphosphatase activity which preferentially hydrolyzes dGTP. Since a specific form of guanine, probably the *syn* form can pair with adenine,[13] it was inferred that MutT protein may prevent misincorporation of dGTP by degrading the unfavorable substrate. Akiyama *et al.*[14] examined this possibility and found that MutT protein can indeed prevent dGTP misincorporation achieved by the DNA polymerase III core enzyme on a poly (dA) template.

Subsequently, Maki and Sekiguchi[15] found that the nucleotide which is misincorporated opposite the adenine residue of the template is not dGTP but rather its oxidized form, 8-oxo-dGTP. When 8-oxo-dGTP was added to an *in vitro* DNA replication system, 8-oxo-dGTP was incorporated opposite the adenine and cytosine residues of the template, with almost equal frequencies. The MutT protein, indeed, possesses a potent enzyme activity to degrade 8-oxo-dGTP to the monophosphate, thereby preventing the misincorporation of the oxidized form of guanine into DNA.

It is important to emphasize the importance of eliminating the oxidized form of the guanine base from DNA. 8-oxoG, which is sometimes called as the tautomeric form 8-hydroxyguanine, was first described as a minor modified base found in DNA treated with heated glucose.[2),3] It was later found that 8-oxoG is present even in untreated DNA, al-

beit at levels no higher than approximately three molecules of 8-oxoG/10^6 guanine residues in the *E. coli* chromosomal DNA.[16] It thus seems that the level of reactive oxygen species produced by cellular metabolic intermediates may be sufficient to oxidize the guanine base of the nucleotide pool, as well as that of the DNA, even in normally growing cells.

(2) *Removal of oxidized bases from DNA*:

In addition to the mechanism for sanitizing the nucleotide pool, organisms possess mechanisms for repairing 8-oxoG in the DNA. The products of two *E. coli* genes, *mutM* and *mutY*, have been implicated in the repair of this lesion in DNA. MutM protein removes 8-oxoG from DNA[17, 18] while MutY protein excises adenine that may be incorporated opposite a template 8-oxoG during DNA synthesis.[19)–21]

Spontaneous mutagenesis caused by the oxidation of guanine nucleotides can be separated into two pathways: namely, one starting from oxidation of guanine in DNA and the other from oxidation of guanine nucleotide in the nucleotide pool (Fig. 2A). The oxidation of DNA forms the 8-oxoG·C pair which would induce G·C → T·A transversion if the 8-oxoG is not corrected by MutM protein. When 8-oxoG remains unrepaired until DNA polymerase arrives at the lesion, dATP would be inserted opposite the mutagenic lesion. However, in most cases, the resulting 8-oxoG·A pair is reversed back to 8-oxoG·C by the action of MutY protein which thus removes the adenine base from the 8-oxoG·A mispair. These dual defense mechanisms against mutagenesis were first proposed by Michaels *et al.*,[22] based on the findings that the combination of *mutM* and *mutY* mutations resulted in a synergistic mutator effect specific for G·C → T·A transversion (Fig. 2B) and that multicopy plasmid carrying the *mutM* gene almost completely suppressed the mutator effect of the *mutY*$^-$ mutant. The high rate of G·C → T·A mutation in the *mutM mutY* double mutator strain likely reflects the high level of 8-oxoG content.[16]

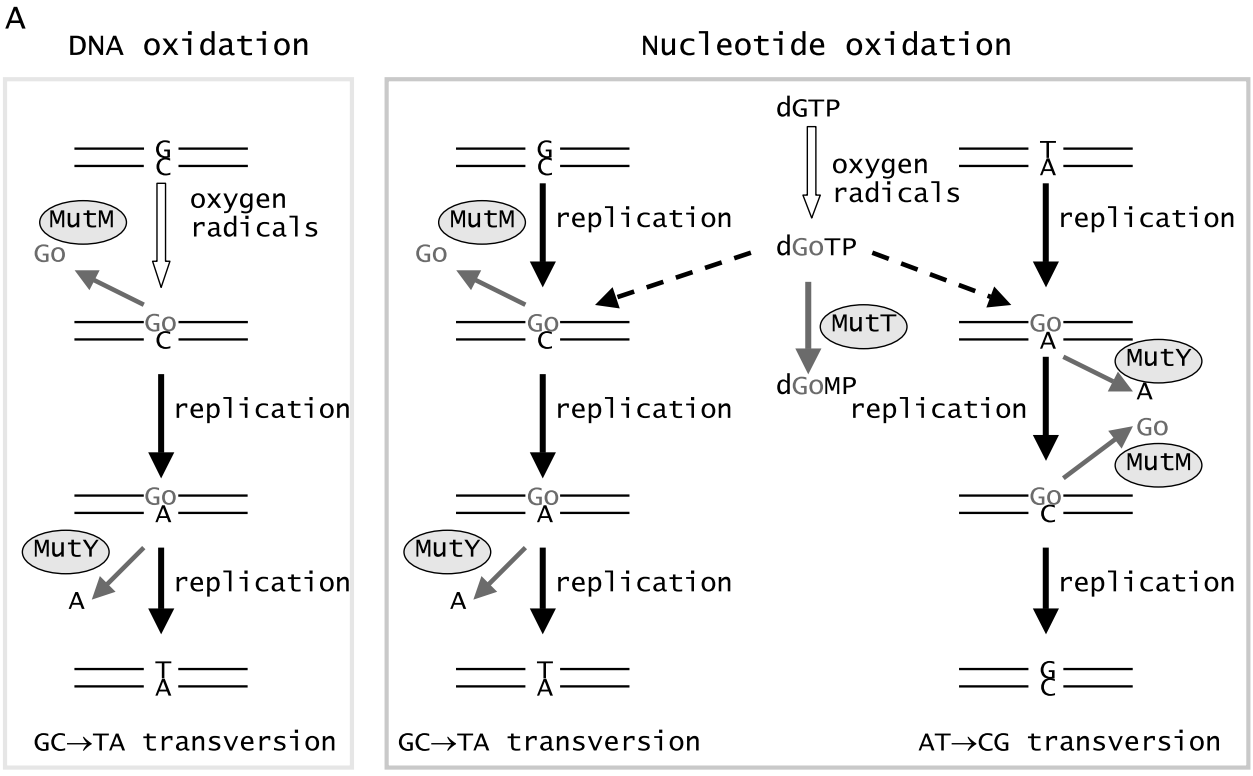
While the mutation caused by the oxidation of DNA is unidirectional, the incorporation of 8-oxo-dGTP into DNA results in base substitutions in two directions. Therefore, in *E. coli* cells, the MutT protein seems to prevent both A·T → C·G and G·C → T·A transversions by eliminating 8-oxo-dGTP from the nucleotide pool. In the spontaneous mutagenesis caused by 8-oxo-dGTP, the MutM and MutY proteins also play important roles, but their functions in the A·T → C·G pathway differ from those in the G·C → T·A pathway. The repair enzymes, especially the MutY protein, promotes the fixation of A·T → C·G transversion whereas the MutM and MutY proteins cooperate to suppress the G·C → T·A transversion, in the same manner as they do for oxidative DNA.

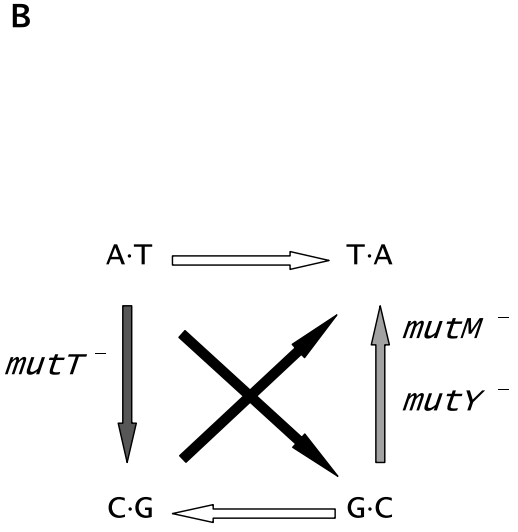
Spectrum analyses of mutations induced in cells defective in *mutT*, *mutM*, *mutY*, or those combinations,[16] showed that induction of G·C → T·A transversion by the *mutT* mutator is detectable when functions of MutM and MutY proteins are absent from the cells (Fig. 2C). The extent of the G·C → T·A mutation caused by 8-oxo-dGTP was comparable to that caused by oxidative DNA damage. It should be noted that the G·C → T·A mutation caused by 8-oxo-dGTP seems to occur at preferential sites differently distributed from those caused by the oxidation of DNA. On the other hand, the occurrence of A·T → C·G transversion in the *mutT* mutator strain was decreased to one third when the *mutM* and *mutY* mutations were introduced into the strain, and the site distribution of the mutations was also altered. Strong hotspot sites observed in the *mutT*$^-$ strain disappeared in the triple mutator strain. It thus seems likely that the hotspot mutations at these positions require a function of the MutY protein. 8-oxoG·A mispairs formed by incorporation of 8-oxo-dGMP from the nucleotide pool, and intermediate of A·T → C·G transversions induced in the *mutT* mutator cells, might thus be frequently formed at these sites, and those mispairs might be better targets for MutY protein than ones formed at other sites.

II. Mammalian proteins for sanitizing the DNA precursor pool. It is highly probable that 8-oxoG-related mutagenesis is an important part of the spontaneous mutations which occur in higher organisms and that similar mechanisms found in *E. coli* cells address the threat of oxidation of guanine residues in mammalian cells. A significant amount of 8-oxoG seems to be formed in the chromosomal DNA and amounts of 8-oxodeoxyguanosine recovered from urine have been used as a marker for oxidative stress.[23, 24]

(1) *Mammalian MTH1 with 8-oxo-dGTPase activity*:

We found an 8-oxo-dGTPase similar to the *E. coli* MutT protein present in human cells and purified it to physical homogeneity.[25, 26] The apparent *Km* of this enzyme for hydrolysis of 8-oxo-dGTP was 70 times lower than that for the degradation of dGTP, whereas the maximal reaction rates ob-





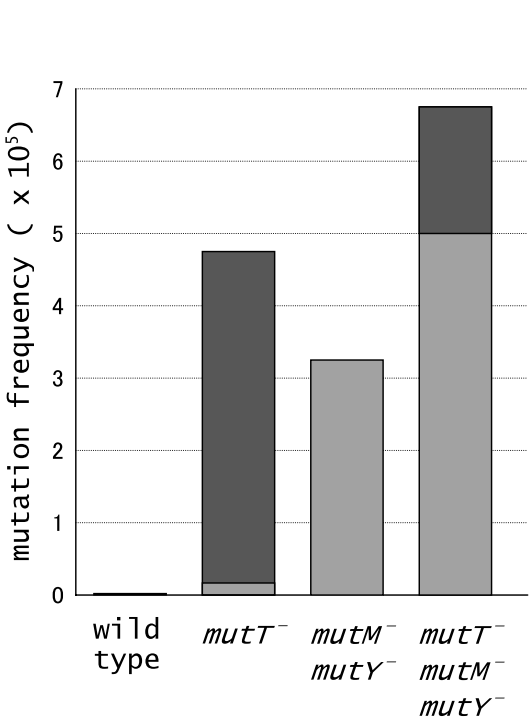


Fig. 2. Actions of *mutT*, *mutM* and *mutY* mutators. A. model for 8-oxoguanine-related mutagenesis. G_0 denotes 8-oxoguanine. B. Types of base substitutions. Base substitution mutations can be classified into transitions, as indicated by black arrows in the center region, and transversions, shown by four arrows in the rectangular form, according to orientations of the base changes. Among the four types of transversions (AT→CG, AT→TA, GC→TA, GC→CG) and two types of transitions (AT→GC, CG→TA), *mutT* mutant induces only AT→CG transversion while *mutM* and *mutY* yield specifically GC→TA transversion. C. The types of mutations arisen in mutants defective in one or more of the *mutT*, *mutM* and *mutY* genes.

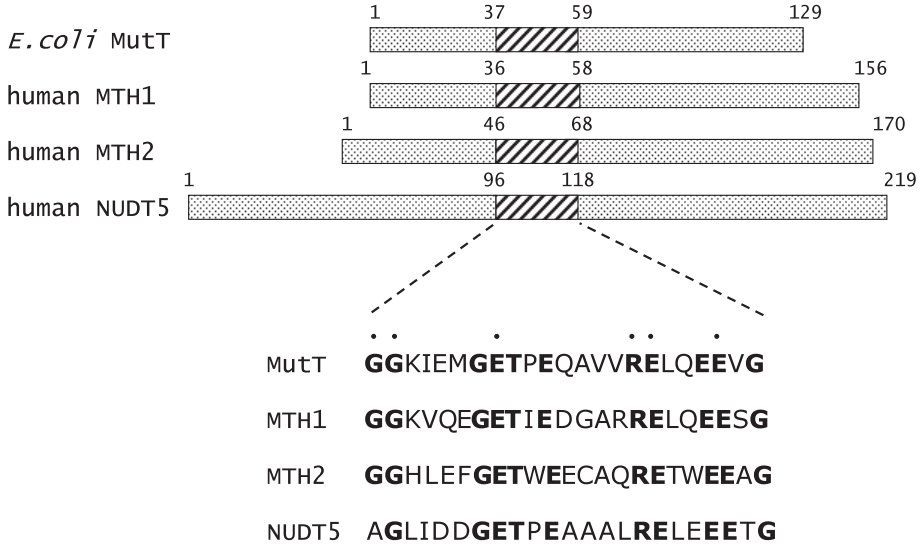


Fig. 3. Comparison of the amino acid sequences of MutT-related proteins. The 23-residues of the MutT signatures are shown below. The residues conserved are indicated in bold letters, and the essential residues for MutT catalytic activity are indicated by dots above the columns.

served with both substrates were similar. Based on the partial amino acid sequence determined with the purified protein, a cDNA for human enzyme was cloned and the nucleotide sequence was determined. The molecular mass of the protein, as calculated from the predicted amino acid sequence, was 17.9 kDa, a value close to that estimated from analysis of SDS-PAGE. When the cDNA was expressed in *E. coli* $mutT^-$ cells, the increased spontaneous mutant frequency decreased considerably.[26] Similar but more striking suppressive effects were observed when mouse or rat cDNA was expressed in the $mutT^-$ cells.[27),28] Therefore, the mammalian proteins are capable of preventing the mutations caused by the accumulation of 8-oxo-dGTP in *E. coli* cells.

The mammalian gene for 8-oxo-dGTPase has been named *MTH1* for *mutT* homolog 1.[29] The *MTH1* gene spans about 10 kb and is composed of five exons, among which the third exon contains the initiation codon. The mammalian MTH1 proteins are similar in size with the *E. coli* MutT and there is a highly conserved sequence in nearly the same region of the proteins (Fig. 3). Fourteen of 23 amino acid residues in this region are identical in both the MTH1 and MutT proteins, hence this probably constitutes an active center for the enzyme. Thus, to establish the functional significance of these residues, saturation mutagenesis of the 10 conserved residues of MutT and MTH1 proteins was done, in combination with negative and positive mutant screening, using mutagenesis primers that contain a completely degenerated codon for each residue. Shimokawa *et al.*[30] thus found that Gly^{37}, Gly^{38}, Glu^{44}, Arg^{52}, Glu^{53} and Glu^{57} in MutT protein could not be replaced by any other amino acid without losing their activity. The replacement of the corresponding residues of MTH1 protein also caused a loss of function.[31] Recently, MTH2 and NUDT5, carrying similar activities, were found, the details of which will be described in the next section.

To investigate the role of MTH1 in spontaneous mutagenesis as well as tumorigenesis, the cell and mouse lines defective in the mouse *Mth1* gene were established. The entire area of the third exon containing the initiation codon and the adjacent intron region was replaced with a *neo* cassette.[32),33] $Mth1^{-/-}$ mice thus obtained are apparently normal, but have a high susceptibility for spontaneous tumorigenesis. At the age of 18 months, more tumor were found in the lungs, livers and stomach of the $Mth1^{-/-}$ mice than in the $Mth1^{+/+}$ mice. The elevated incidence of tumor formation in the livers of the $Mth1^{-/-}$ mice correlated well with the highest content of MTH1 protein in this organ of the wild-type mice.[34] These observations indicate that the cellular level of MTH1 is an important factor in determining the susceptibility of mice to tumor induction by endogenous oxidative stress.

Mth1$^{-/-}$ ES cells were used to examine the effect of MTH1 deficiency on spontaneous mutagenesis. Mutations in the *Hprt* gene, located on the X chromosome in the mouse genome, render cells resistant to 6-thioguanine and, in this forward mutation assay, two independently isolated *Mth1*$^{-/-}$ cell lines exhibited an approximately twofold higher mutation rate, compared with the value of *Mth1*$^{+/+}$ cells.[33] As a result, MTH1 may have the potential to prevent the occurrence of mutations under normal growth conditions.

It should be noted, however, that degrees of increase in spontaneous mutation frequency, due to the loss of MTH1, are considerably lower than the value for the *E. coli mutT*$^{-}$ mutant, in which the value is 1,000 times over that of wild-type cells. Several hypotheses to explain this difference may be considered, among which the most plausible is that mammalian cells may possess enzymes capable of degrading 8-oxoG-containing nucleotides, in addition to MTH1. Recently, such enzymes were found in human cells, which will be described below.

(2) *Other proteins capable of sanitizing the nucleotide pool*:

In addition to MTH1, two mammalian proteins have been identified to degrade 8-oxoG-containing deoxyribonucleotides, the mutagenic substrates for DNA synthesis. These proteins, MTH2 and NUDT5, are similar in size to MTH1 as well as *E. coli* MutT and they thus possess the MutT module (see Fig. 3).

MTH2 with 8-oxo-dGTPase.

Based on the 23-amino acid residues conserved through MutT-related proteins, we searched the mouse EST database and isolated a cDNA sequence encoding a protein composing 170 amino acids.[35] This protein, named MTH2 (NUDT15), is capable of converting 8-oxo-dGTP to the monophosphate. The apparent *Km* values for hydrolysis of 8-oxo-dGTP and dGTP were 32 and 75 μM, respectively. Thus, 8-oxo-dGTP is a more preferred substrate for MTH2 than is dGTP, but its selectivity is less than that for the MTH1 reaction, in which *Km* for 8-oxo-dGTP is less than 2% of that for dGTP. Nevertheless, the overexpression of MTH2 in *E. coli mutT* $^{-}$ cells significantly reduced the elevated spontaneous mutation frequency in these cells, thus implying that MTH2 may act as an MTH1 redundancy factor.

NUDT5 with 8-oxo-dGDPase.

Nudix type 5 (NUDT5) protein was originally identified as ADP-sugar pyrophosphatase.[36] Since this protein carries the 23-residue of the MutT signature, we have examined its action on 8-oxoG-containing deoxyribonucleotides.[37] The kinetic parameters of the NUDT5 enzyme, measured for the hydrolysis of several nucleotides, are given in Table I. The *Km* for the hydrolysis of 8-oxo-dGDP is ten times lower than that for dGDP, which is the second best substrate for the enzyme. 8-oxo-dGTP is only hydrolyzed by NUDT5 at very low levels under these conditions, but when a large amount of NUDT5 was used in the reaction, cleavage of 8-oxo-dGTP was detected, for which the apparent *Km* was 63 μM. It should be noted that NUDT5 has a *Km* of 0.77 μM for 8-oxo-dGDP, which is considerably lower than that for ADP sugars (32 μM for ADP-ribose, and higher values for other ADP sugars), which have previously been identified as substrates.[38] The value of NUDT5 for 8-oxo-dGDP is almost equal to that of MutT for 8-oxo-dGTP (0.48 μM). Based on these results, we concluded that 8-oxo-dGDP is a specific substrate for the NUDT5 protein.

To determine the biological significance of the cleavage of 8-oxo-dGDP, we expressed NUDT5 cDNA in *mutT*$^{-}$ *E. coli* cells, in which the spontaneous mutation frequency is elevated to the level 1,000-fold higher than that of wild-type cells. This increased mutation frequency was reduced to the wild-type level by formation of NUDT5 protein, thus implying that the human NUDT5 can function in *E. coli* cells to clean up the nucleotide pool.[37]

(3) *Exclusion of 8-oxoG-containing nucleotides from the DNA precursor pool*:

MTH1/MTH2 and NUDT5 have opposite preferences for substrates. MTH1 and MTH2 degrade 8-oxo-dGTP, but not 8-oxo-dGDP, whereas NUDT5 cleaves 8-oxo-dGDP, but not 8-oxo-dGTP. As these nucleotides are interconvertible within a cell,[39] both types of proteins are capable of reducing the elevated levels of mutation frequency caused by oxidative stress. These situations are illustrated in Fig. 4. Taking into account the parameters for these enzyme reactions, MTH2 may have a little role than do MTH1 and NUDT5. It has also been noted that 8-oxo-dGDP is a potent inhibitor of MTH1 reaction[40, 41] and, thus, NUDT5 plays another role in promoting the MTH1 reaction, namely, by removing its inhibitor, 8-oxo-dGDP.

Recent studies of *Mth1*-deficient mouse and cell lines revealed that MTH1 is involved, to some extent, in the suppression of spontaneous tumorigenesis as

Table I. Substrate specificities of MutT-related proteins

Enzyme	Substrate	*Km* (μM)	Reference
E. coli MutT	8-oxo-dGTP	0.48	Maki & Sekiguchi[15]
	dGTP	1100	
	8-oxo-dGDP	0.06	Ito *et al.*[45]
	dGDP	170	
Human MTH1	8-oxo-dGTP	12.5	Mo, Maki & Sekiguchi[25]
	dGTP	870	
Human NUDT5	8-oxo-dGDP	0.77	Ishibashi *et al.*[37]
	dGDP	7.10	

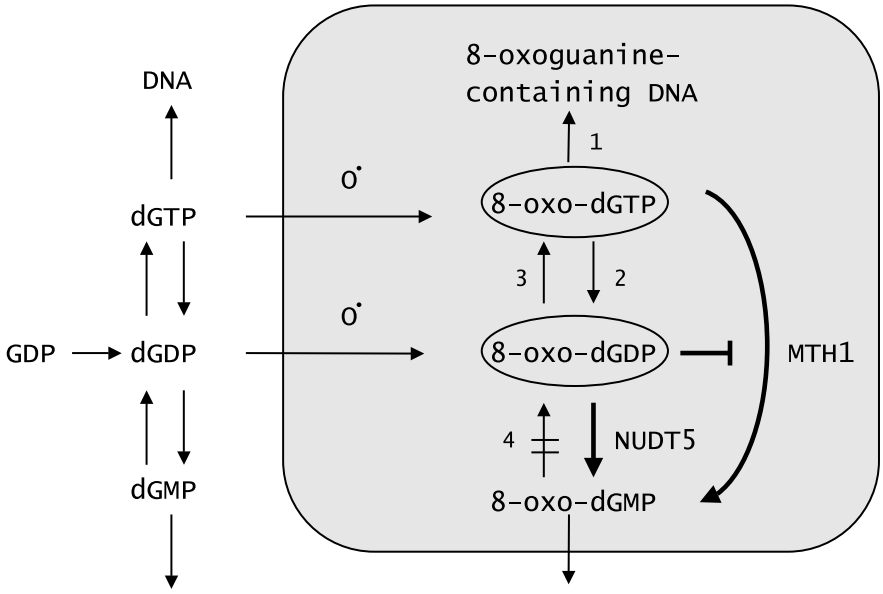


Fig. 4. The formation and elimination of 8-oxoguanine-containing deoxyribonucleotides in mammalian cells. The interconversion of oxidized guanine nucleotides, produced by oxidative stress ($O^{\cdot}$), is shown in the shaded area. (1) DNA polymerase, (2) nucleoside triphosphatase, (3) nucleoside diphosphate kinase, (4) guanylate kinase.

well as in mutagenesis.[32),42)] More definite conclusions about the biological significance of MTH1 and NUDT5 in maintaining the genetic stability might be obtained by producing mouse and cell lines deficient for NUDT5, as well as those lacking both proteins. Studies along these lines are now underway in our laboratories.

III. Error avoidance mechanisms for gene expression under oxidative stress. The basal level of spontaneous errors in RNA synthesis is estimated to be 10^{-5} per residues,[43)] which is considerably higher than that for DNA replication, and the fidelity of transcription would be worse in an aerobic state. This means that numerous erroneous proteins are synthesized even in normal cells. Furthermore, the translational fidelity would become lower when the RNA bases are modified by internal or external agents. Among such modifications, 8-oxoG is particularly important since this modified base causes base mispairing. It has been shown that the incorporation of 8-oxoG opposite the adenine residues of DNA by RNA polymerase causes a partial phenotypic suppression.[44)] In this case, although the majority of the proteins made are normal, some of the products are abnormal. Most of the erroneous proteins produced may be inactive, but some may exhibit domi-

nant characteristics that can cause disorders of some cellular functions, which could thus lead to catastrophic consequences. In mammals, many differentiated cells remain in the G_0/G_1 state and exert their cellular functions via interactions with sophisticated networks. The dysfunction of a single cell, caused by the accumulation of proteins translated from the erroneous RNA, may be amplified with increasing age. Therefore, mechanisms to control the RNA quality may thus play an important role in facilitating the normal functions of such organisms.

(1) *MutT protein for sanitization of RNA precursor pool*:

We have shown that the MutT protein is capable of degrading 8-oxoG-containing ribonucleoside triphosphate (8-oxoGTP) as well as the deoxyribonucleotide counterpart.[44] Recently, the kinetic parameters of the MutT enzyme were determined for the hydrolysis of various nucleotides, and the previous findings were confirmed and further extended.[45] The Vmax/*Km* values for 8-oxo-dGTP and 8-oxoGTP are several thousands times lower than those for dGTP and GTP.

Interestingly, the MutT protein can degrade 8-oxoG-containing deoxyribo- and ribonucleoside diphosphates as efficiently as the corresponding nucleoside triphosphates. The kinetic parameters for the hydrolysis of 8-oxo-dGDP and 8-oxoGDP by MutT enzyme are almost the same. These results clearly indicate that the MutT protein hydrolyzes four types of 8-oxoG-containing nucleotides at almost the same efficiencies. It seems, therefore, that the MutT protein functions to prevent the misincorporation of 8-oxoG into both DNA and RNA in *E. coli* cells.

(2) *Elimination of oxidized RNA precursors in mammalian cells*:

Once 8-oxoGTP is formed in the cellular nucleotide pool, this oxidized nucleotide then becomes incorporated into RNA, thus resulting in translational errors.[46] Since 8-oxoG is placed opposite adenine in the DNA template, this misincorporation could cause a partial phenotypic suppression when an *E. coli lacZ*$^-$ strain with a $^{5'}$T·A·G$^{3'}$ stop codon is used as a tester strain (Fig. 5A). In the presence of 8-oxoGTP, the $^{3'}$A·T·C$^{5'}$ trinucleotide in the transcribed strand of the mutant could thus be copied to $^{5'}$8-oxoG·A·G$^{3'}$, which would then pair with $^{5'}$C·U·C$^{3'}$ glutamic acid anticodon. This RNA transcript would encode a wild-type β-galactosidase protein, whereas the vast majority of the mRNAs encode truncated proteins. We have used this assay system to examine the abilities of human candidate proteins to eliminate the mismatch-evoking oxidized nucleotides from the RNA precursor pool.

The β-galactosidase activity produced by a partial phenotypic suppression is relatively low, but it can be detected when the cells are cultured in the presence of X-gal. *E. coli* 101 cells carrying an amber mutation at codon 461 in the *lacZ* gene yield white colonies, since they are unable to produce an active β-galactosidase protein.[47] On the other hand, 101T cells, which carry a *mutT* mutation in addition to the *lacZ* amber mutation, produce blue colonies, probably due to the partial phenotypic suppression of the *lacZ* mutation caused by the misincorporation of 8-oxoG into mRNA. When the cDNA for either MTH1 or NUDT5 was introduced into the 101T cells, the formation of blue colonies was almost completely suppressed, thus implying that those human proteins can replace the defective MutT function in *E. coli* cells.[46] On the other hand, no suppression was induced by the expression of MTH2.

More quantitative data were obtained by measuring the actual β-galactosidase activity. As shown in Fig. 5B, approximately a 30-fold increase in the enzyme activity was observed in the *mutT*-defective 101T strain in comparison to the wild-type 101 strain. This increased level of activity was almost completely suppressed by the expression of the cDNA for either MTH1 or NUDT5 in the cells. According to the results of the colony color test, the cDNA encoding MTH2 was unable to prevent any phenotypic suppression.

The kinetic parameters of MTH1 and NUDT5 proteins were measured for the hydrolysis of the normal and oxidized forms of guanine ribonucleotides.[46] The Vmax/*Km* value of MTH1 for the hydrolysis of 8-oxoGTP is considerably higher than that for 8-oxoGDP, thus implying that the function of MTH1 is primarily to eliminate 8-oxoGTP from the RNA precursor pool. On the other hand, NUDT5 exhibited a higher Vmax/*Km* value for 8-oxoGDP than that for 8-oxoGTP. Since 8-oxoGTP and 8-oxoGDP are interconvertible within the cell,[39),48] MTH1 and NUDT5 may thus collaborate to prevent the misincorporation of 8-oxoG into RNA.

(3) *Proteins bound to 8-oxoG-containing RNA*:

The misincorporation of 8-oxoG into RNA could

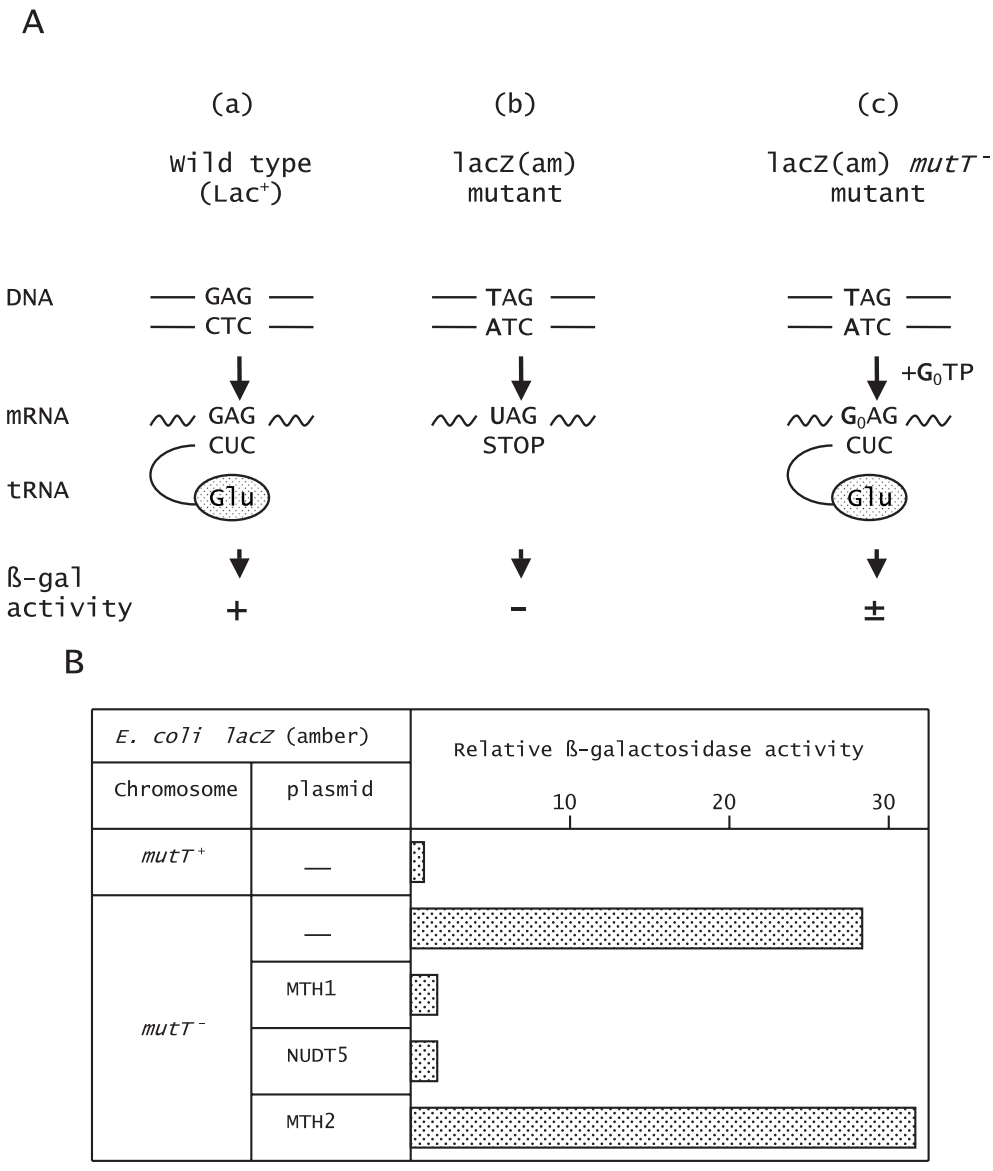


Fig. 5. Partial phenotypic suppression of an amber mutation of the *lacZ* gene of *E. coli* by expression of cDNA for human MutT-related proteins. A. model for phenotypic suppression. *E. coli* cells carrying amber mutation in the *lacZ* gene cannot produce an active β-galactosidase (b). Under the $mutT^-$ background, these cells would accumulate 8-oxoGTP (G_0TP), allowing 8-oxoguanine misincorporation into messenger RNA. This would suppress the amber mutation, thereby producing a small but significant amount of β-galactosidase protein. B. β-galactosidase activity of $mutT^+$ and $mutT^-$ cells with or without human cDNA. The data were taken from ref. 46.

be prevented by the actions of the MutT-related proteins in *E. coli* and mammalian cells. However, when guanine residues in RNA are oxidized *in situ,* this mechanism does not function. The correction of errors by excision repair depends on the double-stranded nature of DNA and is not applicable to RNA molecules, which are single-stranded in most domains. The persistence of oxidized guanine residues in RNA may lead to the formation of a large amount of error-containing proteins, which would be hazardous to the cell. One way to avoid such a catastrophe is to discriminate 8-oxoG-containing RNA from normal RNA, thus preventing the former from entering into the cellular translational machinery.

To explore such a possibility, we initiated a

search for a protein(s) that specifically binds to 8-oxoG-containing RNA.[49] To detect such a complex, ^{32}P-labeled poly (8-oxoG·A) was incubated with an *E. coli* extract, treated with RNase to remove any excess ribopolymers and then was irradiated with ultraviolet light to form cross-links between ribopolymer and the protein moieties of the complex. The materials were spread on two-dimensional gel electrophoresis and then the radioactive spot representing the binding protein was identified. From the mobility on SDS-PAGE, the molecular mass of the protein was estimated to be 77 kDa, thus corresponding to the value for polynucleotide phosphorylase (PNP) protein. This band was absent from the samples derived from *pnp* mutants which lack PNP. It was furthermore shown that a purified preparation of PNP protein binds tightly to 8-oxoG-containing RNA.

This finding has encouraged us to search for a similar protein in mammalian cells. Although the existence of polynucleotide phosphorylase was thought to be limited to bacteria and plants, recent sequence analyses revealed that this enzyme is present in humans and other animals.[50)–52)] We cloned cDNA encoding the human PNP protein and found that the protein binds specifically to 8-oxoG-containing RNA.[53)] Although it was difficult to detect the polynucleotide phosphorylase activity in crude extracts of human cells, due to the existence of interfering enzyme activities and inhibitors, we were thus able to determine the amount of PNP protein using specific antibodies. The immunologically isolated protein indeed carried the PNP enzyme activity. Taking advantage of this assay procedure, we showed the amounts of PNP protein in the cell to change profoundly in response to oxidative stress.

Human YB-1 protein, Y box-binding protein 1 with multiple regulatory activities, has been shown to have a potential to bind to 8-oxoG-containing RNA.[54)] There is a possibility that both PNP and YB-1 function cooperatively to maintain a high fidelity of translation by sequestering the oxidatively damaged RNA molecules.

(4) *A plausible model to execute accurate gene expression under oxidative stress*:

8-oxoG can be formed in RNA by the incorporation of oxidized guanine nucleotide into RNA and also by the direct oxidation of the constituent guanine base in the RNA. In *E. coli*, the MutT protein functions to prevent the former process while the PNP protein appears to be involved in the latter.

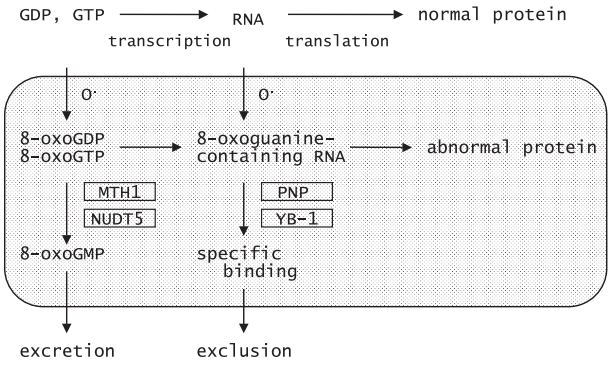


Fig. 6. A model for preventing erroneous protein synthesis by exclusion of 8-oxoguanine-containing ribonucleotides and RNA.

Similar mechanisms seem to function in mammalian cells, but the situations are much more complex in comparison to the bacterial ones. At least two proteins with different substrate preferences, MTH1 and NUDT5, are involved in the former process, and also two different types of proteins, PNP and YB-1, are implicated with the latter process. These situations are illustrated in Fig. 6.

Oxidation damage to RNA may be a prominent feature of vulnerable neurons in aged subjects. A large amount of 8-oxoG was found in the cytoplasm of neurons of Alzheimer's disease patients but not in those of normal subjects.[55)] The oxidative damage in RNA as well as in DNA has been reported to be associated with Parkinson's disease.[56)] It will be important to see if such proteins that are involved in the 8-oxoG-related RNA metabolism have some alterations in age-related diseases.

IV. Roles of DNA repair and apoptosis in the prevention of cancer. Certain types of DNA lesions are responsible for the induction of mutation as well as of cancer. Most of these lesions, however, can be removed by cellular DNA repair systems and, furthermore, the cells with the remaining DNA lesions are eliminated by apoptosis. It seems that the DNA repair acts as the first line of defense against carcinogenesis while apoptosis functions as the second line of defense. Therefore, the components of these processes may be important factors for determining the susceptibility to tumor formation. We herein show the outlines of our studies performed to reveal these mechanisms.

(1) *DNA-repair methyltransferase for preventing cell death and mutation induction*:

Alkylation of DNA at the O^6 position of gua-

nine is regarded as one of the most critical events leading to the induction of mutation and cancer in organisms. Once O^6-methylguanine is formed, it can pair with thymine during DNA replication, with the result being a conversion of the guanine·cytosine (G·C) to an adenine·thymine (A·T) pair,[57),58)] and such mutations are frequently found in the tumors of rodents induced by alkylating chemicals.[59)] To counteract such effects, the organisms possess a mechanism to repair O^6-methylguanine in DNA. An enzyme, O^6-methylguanine-DNA methyltransferase (MGMT), is present in various organisms, from bacteria to human cells, and it thus appears to be responsible for preventing the occurrence of such mutations. The enzyme transfers methyl groups from O^6-methylguanine and other methylated moieties of the DNA to its own molecule, thereby repairing the DNA lesions in a single-step reaction.[60),61)]

The amounts of methyltransferase protein contained in the cell vary with tissues, and it was pointed out that more tumors are formed in tissues with less methyltransferase in animals administered alkylnitrosourea.[62),63)] Some human tumor-derived cell lines are hypersensitive to alkylating chemicals, and these cell lines, termed Mer^- or Mex^-, have little or no methyltransferase activity.[64),65)] It was suspected that this methyltransferase deficiency might be the cause of the frequent occurrences of tumors in certain cases. To elucidate the role of methyltransferase in carcinogenesis, appropriate animal models with altered levels of the enzyme activity thus have to be developed. To achieve this goal, it was necessary to clone the cDNA for the methyltransferase protein.

The cloning of cDNA for human methyltransferase was achieved in 1990 by three groups of investigators, each using different strategies to screen the cDNA clone. Tano *et al.*[66)] isolated the cDNA clone on the basis of its rescue of a methyltransferase-deficient *E. coli* cell, and Rydberg *et al.*[67)] cloned it by screening a cDNA library with oligonucleotide probes derived from the active-site amino acid sequence of bovine methyltransferase. Hayakawa *et al.*[68)] in our group expressed a cDNA library in methyltransferase-deficient human Mer^- cells and recovered cDNA from cells which become resistant to alkylating agents. The amino acid sequence of the methyltransferase protein produced in cDNA-carrying human cells was in complete match with that deduced from the nucleotide sequence of the cDNA.[61)]

To construct mouse lines deficient in the methyltransferase, the mouse genomic sequence for the *Mgmt* gene, encoding the methyltransferase protein, was characterized. The *Mgmt* gene consists of five exons and spans over 150 kb.[69)] The sequences of exons for the mouse gene are almost the same as those for the human counterparts, though the sizes of introns differ in the genes of the two species.[70)] Using such information, we constructed mice defective in the *Mgmt* gene. The $Mgmt^{-/-}$ mice appeared normal but they exhibited hypersensitivity to the killing effect of alkylating chemicals.[71)] The LD_{50} of $Mgmt^{-/-}$ mice was 20 mg of MNU/kg of body weight, less than one-tenth of the value for the wild-type mice (300 mg/kg). We thus had to use a very low dose of alkylating chemicals to examine their tumorigenic response. At a sublethal dose (2.5 mg/kg) the $Mgmt^{-/-}$ mice produced a significant number of tumors while wild-type mice treated in the same manner had no such tumors.[72)] A similar result was obtained with $Mgmt^{-/-}$ mice exposed to dimethylnitrosoamine.[73)]

Death of $Mgmt^{-/-}$ mice after MNU administration was closely related to bone marrow damage and dysplastic mucosae of intestines together with crypt abscesses. This severe myelosuppression led to a drastic decrease in the number of peripheral leukocytes and platelets.[71)] Therefore, methyltransferase plays a vital role in protecting these organs from toxic effects of alkylating chemicals. It is notable that $Mgmt^{-/-}$ mice are considerably more sensitive to chemotherapeutic alkylating drugs presently in clinical use, such as dacarbazine and ACNU, than are wild-type mice.[74),75)]

(2) *Dissociation of killing and tumorigenic effects of alkylating chemicals due to mismatch repair deficiency*:

$Mgmt^{-/-}$ mice are hypersensitive to both the killing and to the tumorigenic actions of alkylating chemicals. These dual effects can be dissociated by the introduction of an additional defect in mismatch repair genes. Mice with mutations in both alleles of the *Mgmt* and the *Mlh1* gene, the latter encoding a protein involved in the mismatched base recognition, are as resistant to MNU as are wild-type mice, in terms of survival, but tend to develop numerous tumors after receiving sublethal doses of MNU[76)] (see Fig. 7 and 8). In contrast to $Mgmt^{-/-}$ $Mlh1^{+/+}$ mice, which showed a smaller

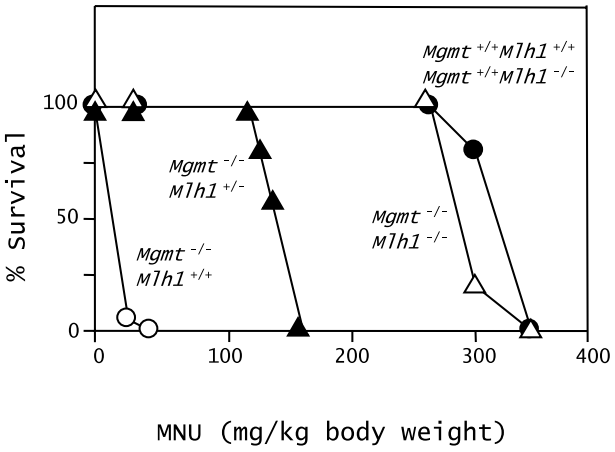


Fig. 7. The survivals of the mice given various doses of MNU. Six-week-old mice were given various doses of MNU i. p. For each dose 7 to 12 mice were used and data on survivors at 30 days after the administration were plotted.

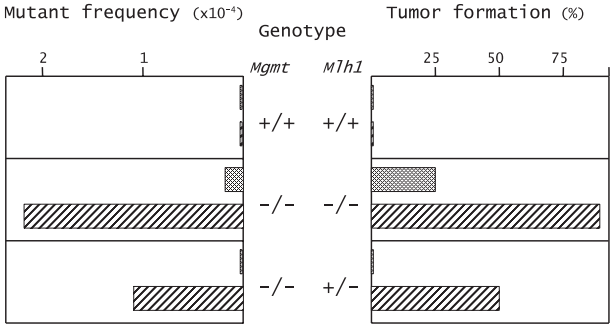


Fig. 8. Effects of MNU on the induction of mutation and tumor under various genetic backgrounds. Dotted bars represent non-treated control values, while hatched bars indicate values after MNU treatment (1 mM MNU for mutant frequency assay and 30 mg of MNU/kg of body weight for tumor formation). Data were from ref. 76, 83, 85.

thymus and hypocellular bone marrow after MNU administration, no conspicuous change was found in the *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ mice treated in the same manner. Thus, introduction of a mismatch repair defect renders *Mgmt*$^{-/-}$ mice resistant to the lethal action of alkylating chemicals, still maintaining the high susceptibility to tumor formation. Such mice may be useful for evaluating the carcinogenic effects of various substances, including those for therapeutic application.

In this context, there is a problem that these doubly deficient mice had a small but significant number of tumors even without exposure to MNU.[76] This phenomenon is common to mice defective in mismatch repair,[77),78)] and such a high incidence of tumors is characteristic of HNPCC patients, which are defective in one of the mismatch repair genes.[79)–82)] In our studies, this complexity was resolved by introducing the *Mlh1*$^{+/-}$ mutation, instead of *Mlh1*$^{-/-}$, in the methyltransferase-deficient mice.[83)] *Mgmt*$^{-/-}$ *Mlh1*$^{+/-}$ mice, with about half the amount of MLH1 protein as *Mgmt*$^{-/-}$ *MLlh1*$^{+/+}$ mice, were resistant to the killing action of MNU, up to the level of 100 mg/kg body weight. From the survival curves shown in Fig. 7, the LD_{50} values were estimated to be 20, 120, and 280 mg/kg body weight for *Mgmt*$^{-/-}$ *Mlh1*$^{+/+}$, *Mgmt*$^{-/-}$ *MLlh1*$^{+/-}$ and *Mgmt*$^{-/-}$ *MLlh1*$^{-/-}$ mice, respectively. Eight weeks after the administration of 30 mg/kg of MNU, 40% of MNU-treated *Mgmt*$^{-/-}$ *Mlh1*$^{+/-}$ mice had tumors, and there were no tumors in those mice not given the treatment. It seems that the cellular content of MLH1 protein is therefore a critical factor for determining whether the damaged cells enter into the pathway leading to mutation induction or apoptotic cell death.

The products of four genes, *MSH2*, *MSH6*, *MLH1* and *PMS2*, form a complex and play an important role in recognizing the mismatched bases to induce apoptosis,[84),85)] and it is generally assumed that a defect in any one of these genes may lead to defects in mismatched base-induced apoptosis. As a result, there is a possibility that mutations in genes other than *Mlh1* might cause a similar phenotype observed with *Mlh1*$^{+/-}$ mice. However, after treatment with O^6-benzylguanine, an inhibitor of O^6-methylguanine methyltransferase, *Msh2*$^{+/-}$ cells were reported to be as sensitive as *Msh2*$^{+/+}$ cells to alkylating agents.[84)] This apparent difference may be due to different levels of expression of the genes or to different modes of action for these proteins.

(3) *Apoptosis of cells carrying base mismatches*:

To better comprehend the roles of the methyltransferase and the mismatch repair proteins in alkylation mutagenesis and carcinogenesis, it is relevant to perform experiments with the use of cell lines genetically defective in the defined genes. Therefore, the fibroblasts obtained from lung tissue specimens of the gene-targeted mice were transformed by the introduction of SV40-derived vector, and cell lines with various genotypes were established.[85)] In concordance with responses of *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ mice to alkylating carcinogens, cells derived from these doubly defective mice exhibit an increased resistance to the lethal action of MNU.

The mutant frequency of *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ cells increased significantly after a brief exposure to MNU. On the other hand, the value for wild-type cells did not increase with the same treatment. This finding coincides well with the result of MNU-induced tumorigenicity in wild-type and *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ mice. Whereas the two types of mice exhibit the same high level of resistance to the killing action of MNU, *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ mice showed an increased susceptibility to the tumorigenic effects of the agent, as compared with wild-type mice.[76] These situations are illustrated in Fig. 8, in which the tumor induction of the mice and the mutation induction in cells are compared. In this figure, the heteroinsufficient nature of the *Mlh1* gene was also noted.

Similar results were obtained with the cell lines derived from human tumors. HeLa MR, which was derived from HeLa S3 (wild-type) and is devoid of any methyltransferase activity, is hypersensitive to MNU as well as to dacarbazine, the latter being a chemotherapeutic agent with alkylation capacity.[86] On the other hand, SW48 cells, obtained from human colorectal adenocarcinoma, is as resistant to the agents as is HeLa S3, in spite of the fact that SW48 cells lack any methyltransferase activity. This acquired resistance to alkylating chemicals of SW48 is ascribed to its apparent lack of mismatch repair capacity. It has been shown that SW48 cells are devoid of MLH1 as well as MSH6 proteins, due to the transcriptional silencing of the genes.[87),88] These results for the mouse and human cell lines imply that the mismatch repair-related function is required for the execution of apoptosis triggered by O^6-methylguanine.

In this regard, we must emphasize that, in mammalian cells, the mismatch repair proteins may be involved in two different processes, one to repair the replication-associated errors in a strand-specific manner and the other to signal the induction of apoptosis in cells with mismatched DNA bases. This was clearly shown by recent findings that certain MSH2 or MSH6 missense mutations can cause a deficiency in mismatch repair, whereas retaining the signaling functions that confer sensitivity to chemotherapeutic agents.[89),90] The molecular mechanism of the former process, mismatch repair, has been elucidated by analyzing the interactions of components and reconstructing the protein complex *in vitro*.[91] However, studies on the mechanism of the latter process, modified base-induced apoptosis, have been hampered mainly due to the lack of appropriate systems for investigating the processes occurring in cells. In this regard, human cell lines defective in the MGMT function are thus considered to be noteworthy, since the exposure of such cells to simple alkylating agents would induce a large number of O^6-methylguanine lesions in the chromosomal DNA, which would trigger apoptosis. The O^6-methylguanine thus formed would persist in the DNA through the progression of the cell cycle and, hence, the conditions and timing required for the function of an apoptosis-related protein complex could be followed in the cell. Moreover, by comparing the results obtained with O^6-methylguanine-DNA methyltransferase-proficient and -deficient cells, it is possible to ascertain whether the molecular events observed are related to a specific DNA lesion, O^6-methylguanine.

Taking advantage of this system, we have investigated an early step of apoptosis triggered by O^6-methylguanine.[92] In O^6-methylguanine-DNA methyltransferase-deficient HeLa MR cells treated with MNU, we detected a protein complex composed of MutSα, MutLα and PCNA on damaged DNA by immunoprecipitation method using chromatin extracts, in which the protein-protein interactions were stabilized by chemical crosslinking. Time-course experiments revealed that MutSα, consisting of MSH2 and MSH6 proteins, and PCNA bind to damaged DNA to form an initial complex, and the MutLα, composed of MLH1 and PMS2, binds to the former to make the mismatch complex for triggering apoptosis. This sequential mode of binding was further confirmed by the findings that the association of PCNA-MutSα complex on chromatin was observed even in cells that lack MLH1, whereas in the absence of MSH2 no association of MutLα with the chromatin was achieved. Moreover, reduction in the PCNA content by siRNA or inhibition of DNA replication by aphidicolin, an inhibitor of DNA polymerase α, significantly reduced the levels of the PCNA-MutSα-MutLα complex and also suppressed an increase in the casepase-3 activity, a hallmark for the induction of apoptosis.

The O^6-methylguanine-cytosine pair, produced by the action of alkylating chemicals, can be converted to an O^6-methylguanine-thymine pair after one round of DNA replication. Our finding that at least one cycle of DNA replication is required for the complex formation implies that the O^6-

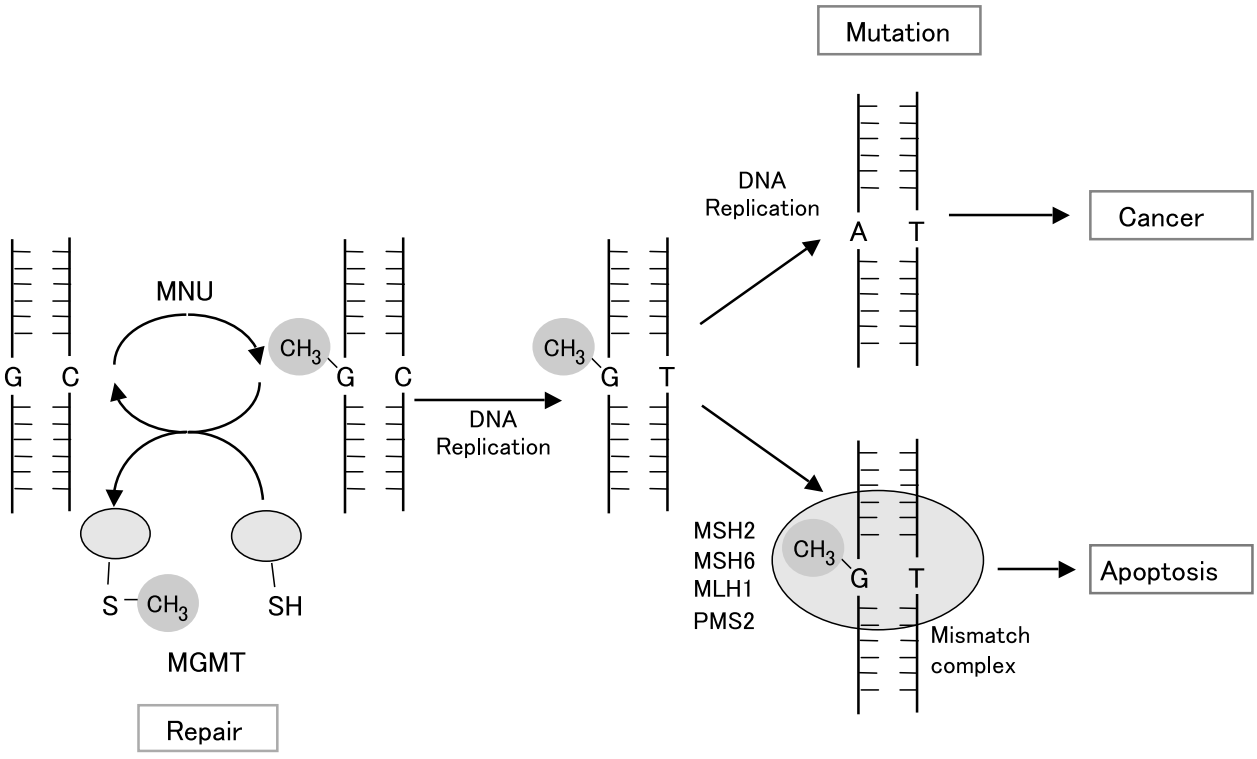


Fig. 9. Roles of DNA repair and apoptosis in preventing mutations and cancer.

methylguanine-thymine pair may be the target for the MutSα-PCNA complex. This notion was supported by recent *in vitro* DNA binding studies, which revealed that MutSα specifically recognizes the O^6-methylguanine-thymine mispair but not the O^6-methylguanine-cytosine pair.[93]

Fig. 9 illustrates the relationship of the three distinct processes triggered by alkylation of the O^6 position of guanine base in DNA. Primarily, O^6-methylguanine can be repaired by the MGMT protein carrying the intrinsic methyltransferase activity. If it is not repaired, the O^6-methylguanine-cytosine pair can be converted to an O^6-methylguanine-thymine mispair, which is recognized by the mismatch repair-related proteins to induce apoptosis. If the O^6-methylguanine-thymine pair would escape from the mismatch recognition process, it is further converted to an adenine-thymine pair. The GC to AT transition has been shown to be the prominent feature of alkylation mutagenesis. When multiple mutations arise in such ways in genes controlling the cell cycle, the cells would escape from tightly regulated cell cycle control, thus uncoupling DNA synthesis and cell division and inducing chromosomal instability. This would accelerate the transformation of the cell to malignancy.

Considering these findings, it appears to be important to elucidate how the O^6-methylguanine-triggered signal is delivered to the downstream events for apoptosis. To resolve this question, the components functioning immediately downstream of mismatch repair proteins must thus be identified. It is also important to clarify whether the mechanism concerned with O^6-methylguanine-related mispair can be applied to processes caused by other types of lesions. It is known that mismatch-deficient cells are moderaltely more resistant to oxidative agents than are wild-type cells[94),95)] and, thus, 8-oxoguanine-induced mismatch might be processed through a pathway similar to that revealed with the O^6-methylguanine-related processes.

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Profile

Mutsuo Sekiguchi obtained his doctor degree from Osaka University, Japan, in 1960, and conducted his postdoctal works in biochemistry and molecular genetics at Kanazawa University Medical School, Japan, University of Pennsylvania School of Medicine, and Purdue University Indiana, USA, under the guidance of Prof. Y. Takagi, S. S. Cohen and S. Benzer, respectively. He became Associate Professor of Biochemistry at Kyushu University School of Medicine, Fukuoka, Japan, in 1965 and started his work on molecular mechanisms of DNA repair. Sekiguchi and his colleagues searched for enzymes responsible for repair of DNA, and found T4 endonuclease V, the first DNA repair enzyme that functions *in vivo* to repair ultraviolet-damaged DNA without the aid of visible light. He further extended his studies on the enzymic repair of DNA damaged by alkylating chemicals. These earlier parts of his studies have been described in the Historical Reflection in DNA Repair (A quest to understand molecular mechanisms for genetic stability, DNA Repair, **5**, 750–758, 2006).

Sekiguchi was appointed Professor of Molecular Genetics at Kyushu University Faculty of Science in 1969 and later Professor of Biochemistry, Kyushu University School of Medicine. He was subsequently appointed Director of the Medical Institute of Bioregulation, Kyushu University and worked there until 1996, when he retired from the national university. During these years, he and his associates conducted several lines of studies regarding the molecular mechanisms for the control of spontaneous and induced mutagenesis and carcinogenesis.

Sekiguchi served as Director of the Biomolecular Engineering Research Institute, which is located in Osaka and operated by the funds from the Japanese government and major pharmaceutical companies. He is presently Professor of the Frontier Research Center, Fukuoka Dental College, Fukuoka, Japan. He is extending his studies on the mechanisms of high fidelity of DNA replication as well as gene expression, some of which are dealt with this article.

Dr. Sekiguchi served as the President of the Molecular Biology Society of Japan (1989–1991) and also as the President of Genetic Society of Japan (1995–1999). He was awarded the Japan Academy Prize in 1997, and received various other prizes for these studies.