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DNA damage, if not repaired, can become a mutation. Mutation accumulation is associated with initiation and progression of tumorigenesis. DNA mismatch repair (MMR) is required for maintaining genetic stability by repairing replication errors. Biochemical studies have shown that MMR also recognizes mismatch-causing DNA lesions, suggesting the role of MMR in preventing mutations after the exposure to DNA damaging agents. I hypothesized that MMR plays a role in preventing genotoxin – induced mutations and that the abilities of two MMR proteins, PMS2 and MLH1, to prevent mutations are not equal. The mutagenic effects of ultraviolet and oxidative stress were studied with two genetic tools: forward and reversion mutational assays using the 2.3 Kb mouse *Aprt* gene in kidney cells of PMS2 or MLH1 null mice. I report that loss of MLH1 resulted in a more significant spontaneous mutator phenotype with an increase in all types of base pair substitutions. In constrast, loss of PMS2 resulted in increased spontaneous levels of base pair substitutions and large mutational events (loss of

heterozygosity). Interestingly, half of all base pair substitutions in the Pms2 null cells were A:T → G:C transitions. Upon the exposure to DNA damaging agents, both *Pms2* and *Mlh1* deficient cells significantly increased the percentage of mutant alleles with multiple mutations. The reversion assay was used to demonstrate that the increase in A:T → G:C transitions was due to an elevated rate of formation of these mutations. Using the specificity of reversion assay to detect either C → T or CC → TT mutations, I also showed that the tandem CC → TT double substitution is the signature mutation caused by hydrogen peroxide and metal mixture (Cu/Fe). I also identified a mouse kidney cell line with G:C → C:G mutator phenotype. Cytotoxicity and microsatellite instability studies suggested that either an atypical MMR pathway or a non-MMR pathway is involved in this rare mutator phenotype. Taken together, these data suggest that MMR prevents genotoxin – induced mutations by post- replicationally repairing mismatched bases opposite DNA lesions.

Spontaneous and Enviornmental Mutagenesis in Mismatch Repair Deficient Cells

by
Chi Y. Shin-Darlak

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APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Head of the Department of Environmental and Molecular Toxicology

Redacted for Privacy

Dean of the Graduate School

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Chi Y. Shin-Darlak, Author

CONTRIBUTION OF AUTHORS

Dr. Isabel Mellon performed the analysis of transcription-coupled repair of the DHFR gene in *Pms2* –proficient cells and *Pms2*- deficient cells. Olga Ponomareva assisted in ionizing radiation–induced mutation analysis of K435 cells. Lanelle Connolly assisted in examining microsatellite instability of K435 cells.

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INTRODUCTION

Mutations, physical changes in cellular DNA, can perturb cellular functions by altering gene expression. The development of neoplasia is a multi-step process in which an accumulation of somatic mutations, that inactivates tumor suppressor genes or activates oncogenes, allows cells to gain the capacity to proliferate and to escape the restrictions that control normal cellular growth. Thus, the accumulation of mutations is the rate-limiting step for tumorigenesis. Recently it was estimated that a cancer cell may have more than 10,000 mutations (Stoler et al., 1999). Moreover, studies show that even benign or non-neoplastic lesions harbor many mutations (Gogusev et al., 2000). However, the spontaneous mutation rate of human cells is approximately 1×10^{-10} per base pair per cell generation (DeMars, 1973). Based on the number of cell divisions during an average life span, this rate cannot explain the large number of mutations found in many tumors. To account for this discrepancy, it was suggested that at some point during tumorigenesis cancer cells develop a mutator phenotype in which the mutation rate becomes significantly greater than that in normal somatic cells (Jackson and Loeb, 1998). It was also hypothesized that tumorigenesis is not driven by mutations but by clonal expansion and selective pressure (Tomlinson et al., 1999). However, it is likely

that the intrinsic genetic instability of cancer cells promotes both clonal expansion and selective advantages.

There are two phenotypes of genetic instability observed in cancer cells; chromosomal instability (CIN) and microsatellite instability (MIN). These genetic instabilities are used as diagnostic tools for tumor detection and to monitor tumors. Chromosomal instability (CIN) is manifested by aneuploidy (a change in chromosome number) and gross chromosomal aberrations such as translocations. Microsatellite instability (MIN) is manifested by frameshift mutations in microsatellites, which are short repetitive nucleotides distributed throughout the genome. The mechanism for the CIN phenotype involves inactivation of genes required for chromosomal segregation and mitotic checkpoints. The MIN phenotype involves inactivation of genes for mismatch correction. Although there is accumulating evidence that chromosome instability also promotes the mutator phenotype (Cahill et al., 1998), the mutator phenotype has been strongly associated with cancer cells exhibiting the MIN phenotype. It is possible that initiation and progression of cancer involves numerous genetic changes at the nucleotide level, which eventually give rise to the CIN or MIN phenotypes depending on the affected genes and tissue specificity. To examine this possibility, it is important to define the factors and the mechanisms that increase base pair changes in the genome, and also to define the role of cellular DNA repair systems in protecting the genetic information.

DNA damage, a major factor in increasing mutation formation, has been associated with cancer. Most DNA damaging agents are potential mutagens and carcinogens (Cleaver and Crowley, 2002; Hendricks et al., 1995; Weihrauch et al., 2002). As mentioned earlier, accumulation of somatic mutations is the rate-limiting step required for cancer development. Spontaneous base-pair mutations occur when DNA polymerases insert the wrong base across from damaged or non-damaged templates during DNA replication or even DNA repair synthesis. Biochemical studies of purified DNA polymerases have revealed a range of error rates from one out of 5000 bases for DNA pol β (Umar et al., 1996) to one out of 10^7 for pol δ and ε (Hindges et al., 1995; Syvaoja et al., 1990). The presence of proofreading machinery and the mismatch repair system reduce the rate of spontaneous mutations in normal cellular environments.

Exposure to mutagens could be a contributing factor in cancer by increasing the number of somatic mutations in critical genes such as growth-controlling genes and genes encoding DNA repair and apoptosis. There is an association between environmental (exogenous) / xenobiotic chemicals and human cancers as shown in situations where individuals have been exposed chronically to low doses or to an acute dose of a well known mutagen. The relationship between smoking and the incidence of lung cancer has been well investigated (Hurd and Ebbert, 2002). Also, some natural chemicals ingested as a part of diet can damage DNA and potentially cause mutations (Ames et al., 1990). It has also been suggested that cellular DNA is damaged by endogenous processes, which may serve as a source of multiple

mutations during the progression of tumorigenesis (Jackson and Loeb, 1998; 2001). These metabolic processes are depurination, deamination of cytosine/methylated cytosine, and generation of reactive oxygen species. Particularly in the presence of ineffective DNA repair, endogenous DNA damage could contribute to increased mutation frequencies. It was estimated that a cell undergoes 9,000 depurinations per day caused by spontaneous hydrolysis of the glycosylic bond (Nakamura et al., 1998), and if not repaired these events can cause G → T or A → T transversions. Deamination of cytosine to uridine or 5-methylcytidine to thymidine occurs at 1/500 the rate of depurination, generating C → T mutations when not repaired (Lindahl et al., 1974). The major endogenous source of DNA damage is that produced by reactive oxygen species (ROS). ROS are generated by normal cellular processes such as respiration, cell injury, hydroxylation of steroids and drugs, inflammation and phagocytosis. Oxidative stress has been implicated in cancer, aging, and degenerative diseases (Jackson et al., 1998; Beckman et al., 1998).

Although cellular DNA suffers frequent damage, there are cellular defense mechanisms that repair such damage and ensure genetic stability. The crucial roles of DNA repair systems in preventing cancer have been revealed by hereditary diseases such as Xeroderma Pigmentosum (XP) and hereditary non-polyposis colon cancer (HNPCC), which result from inactivation of nucleotide excision repair (NER) and mismatch repair (MMR), respectively (Bootsma et al., 1998; Bronner et al., 1994; Leach et al., 1993; Muller and Fishel, 2002; Papadopoulos et al., 1994). It is clear that the stability of cellular DNA is maintained at equilibrium between

DNA repair and DNA damage, such that an increase in the production of DNA damage or reduction in DNA repair results in an increased frequency of mutations.

The mismatch repair (MMR) system maintains genetic stability by repairing base-base mispairs caused by misinsertion of a nucleotide(s) or deletions/insertions due to DNA polymerase slippage (Kolodner and Marsischky, 1999). If not repaired, these mispairs would lead to base substitutions or frameshift mutations. There are the three ways for mismatched base pairs to occur. One is misincorporation of a base(s) by DNA polymerases during DNA replication; the second is physical or chemical damage to the template nucleotide, which results in change of the damaged base; and the third is heteroduplex formation during genetic recombination. The mismatch repair system prevents mutations from all three possible pathways. In addition to increased cancers in HNPCC families (Bronner et al., 1994; Peltomaki and Vasen, 1997), MMR is also lost in some sporadic human cancers (Esteller et al., 1998; Gurin et al., 1999). In these cancers, defects in a subclass of MMR genes result in either an absent or reduced ability to correct DNA replication errors (Parson et al., 1993; Umar et al., 1994). The increased rates of mutations may ultimately lead to the formation of tumors (Eshleman et al., 1998b).

MMR factors have been well studied in *E. coli* and serve as a paradigm for studying eukaryotic systems (Modrich et al., 1994; 1996). In *E. coli*, there are two major repair pathways, the methyl-directed long patch and the very short patch pathways (Friedberg et al., 1995). The methyl-directed pathway functions by

correcting base – base mispairs, small insertions, and deletions resulting from errors in DNA replication. The very short patch pathway corrects G/T mispairs in non-replicating DNA that result from deamination of 5-methylcytosine residues. In *E. coli*, the long patch MMR is comprised of activities of MutS, MutL, MutH, and MutU (UvrD). MutS is an ATPase (Haber and Walker, 1991), which functions as an homodimer in recognition of mismatched base(s) and small insertion/deletion loops up to 4 nucleotides in length (Parker et al., 1992). The C-terminus of MutS is involved in dimerization and the N-terminus is required for binding to mismatched bases. The ATP binding/hydrolysis domain is located in the C-terminus, and mutation at this domain yields a dominant negative mutator phenotype (Wu et al., 1994). It was suggested that disruption of ATP binding/hydrolysis results in irreversible binding to mismatches and further prohibits the access of repair molecules. MutL is also an ATPase, which acts as a homodimer and accelerates downstream processing steps by recruiting MutH (Ban and Yang, 1998a; 1998b; Hall and Matson, 1999). The exact function of MutL has not yet been defined. The structural study with crystallized MutL revealed that ATP binding resulted in dimerization of N-terminal domains (Ban et al., 1999). It has been suggested that conformational changes upon dimerization are required for the initial recognition and repair. It has been shown that MutL physically interacts with MutS, MutH (Hall et al., 1998), and MutU (Yamaguchi et al., 1998). The binding of MutL stimulates the function of MutH and MutU. A model for the function of MutL is that MutL coordinates the mismatch binding activity of MutS with the MutH

cleavage and MutU helicase activities, and thereby directs the strand removal process. MutH is a methylation – sensitive endonuclease which introduces nicks on newly synthesized strands of DNA. This strand –specificity of repair is provided by adenine methylation at GATC sequences. The methylation is catalyzed by the Dam methylase and occurs after DNA replication. This allows the newly synthesized DNA to transiently exist in an unmethylated form. MutH recognizes the unmethylated DNA strand of hemimethylated GATC sites resulting in incision by MutH and subsequent excision of the mismatched base on this newly synthesized strand (Au et al., 1992). MutU (UvrD) is a DNA helicase II which unwinds the duplex DNA at the nick and proceeds in a directional manner toward the mismatch (Dao and Modrich, 1998; Hall et al., 1998, Yamaguchi et al., 1998). Unwinding of DNA is necessary for the activities of the single-stranded exonucleases, the 3' to 5'exonuclease (exonuclease I and exonuclease X), and 5' to 3' exonucleases (exonuclease VII and RecJ) (Modrich and Lahue, 1996; Viswanathan and Lovett, 1998). In addition, DNA polymerases III, SSB (single strand binding proteins), and ligase are required for resynthesis and completion of repair (Modrich 1991; Modrich and Lahue, 1996).

In *E. coli*, the MMR system uses single MutS and MutL proteins that work as homodimers. However, in yeast and mammals there are multiple MutS homologs (MSH) and MutL homologs (MLH) that work as heterodimers of MutS/MutS and MutL/MutL (reviewed in Kolodner and Marsischky, 1999). These multiple homologs participate in repair of different polymerase errors. The general

steps of MMR in eukaryotes are similar to prokaryotic systems. Both consist of mismatch recognition, strand discrimination/excision, and resynthesis/ligation. The yeast *Saccharomyces cerevisiae* has six MSH (MSH1 – MSH6) and four MLH (MLH 1-3, PMS1) proteins, and mammals have five MSH (MSH2 – 6) and four MLH (MLH1, MLH3, PMS1, and PMS2) proteins. MSH1 of yeast is required for normal mitochondrial function and repairs base substitutions (Chi et al., 1994; Reenan et al., 1992) and frameshifts at poly(GT) tracts (Buermeyer et al., 1999b; Sia et al., 2000). No mammalian homolog of this protein has been identified. In both yeast and mammals, MSH2 plays a central role of recognition of mismatches by forming heterodimers with MSH6 (MutSα complex) and with MSH3 (MutSβ complex) (Acharya et al., 1996). MutSα efficiently recognizes and repairs single base pair mismatches and 1-3 nucleotide insertion/deletion mispairs (Macpherson et al., 1998; Papadopoulos et al., 1995). Larger insertion/deletion mispairs (2-8 nucleotides) are preferentially recognized by the heterodimer MutSβ (Palombo et al., 1996; Genschel et al., 1998). Eukaryotic MSH2 and MSH6 have ATP binding and hydrolysis activity in the C-terminal region. ATP binding causes the MSH2-MSH6 complex to be released from mismatches and proteolysis- sensitive conformation changes (Alani et al., 1997; Studamire et al., 1998).

To date, there are two models to account for the role of ATP binding and hydrolysis: ATP hydrolysis–independent translocation and ATP hydrolysis–dependent translocation of MSH proteins. The first model is that the ADP-bound form of the MSH complex binds to mismatches, followed by ADP-

ATP exchange. This ATP binding results in formation of a hydrolysis-independent sliding clamp that causes the translocation from the mismatch and slides along the DNA until additional components of MMR machinery are contacted (Gradia et al., 1999). The second model involves the hydrolysis of ATP to ADP, which enables the MSH complex to translocate from the mismatch–containing site (Blackwell et al., 1998). MSH4 and MSH5 form a heterodimer and specifically functions in meiotic recombination.

In yeast, loss of either MSH4 or MSH5 results in approximately a 50% reduction in meiotic crossing over, resulting in increased levels of homolog non-disjunction and decreased spore viability (Hollingsworth et al., 1995; Ross-Macdonald et al., 1994). Neither MSH4 nor MSH5 has a role in classic mismatch correction nor do they interact with MSH2 or MSH6 (Pochart et al., 1997). Moreover, as with other MSH proteins, the MSH4-MSH5 heterodimer interacts with the MLH1-MLH3 complex to promote meiotic crossing over. The mammalian homologs (Bocker et al., 1999; Her et al., 1998; 1999) of the yeast MSH4 and MSH5 have been identified. The highest expression levels of both proteins were observed in the testis and ovaries, which support a meiotic specific function (Edelmann et al., 1999; Bocker et al., 1999; Her et al., 1999).

MutL homologs also form heterodimers, MutLα and MutLβ complexes, whose functions overlap. In yeast, MutLα is comprised of MLH1/PMS1 heterodimers, which are the major molecules of MMR (Prolla et al., 1994). Along with MLH1/MLH3 heterodimers, MLH1/MLH2 heterodimers play minor roles in

MMR. In mammals, MutLα, a heterodimer of MLH1 and PMS2 (the homolog of yeast PMS1) is also the most important heterodimer (Li et al., 1995). MLH1 also dimerizes with MLH3 or with PMS1, to form MutLβ (Raschle et al., 1999). These heterodimers may also play a minor role (Prolla et al., 1998) in MMR. Like bacterial MutL, eukaryotic MLH proteins also have ATPase activity in N-terminal domains. (Guarne et al., 2001; Hall et al., 2002). Comparable to bacterial MutL, the MLH molecules are believed to couple the recognition functions of the MSH proteins to other MMR molecules.

In eukaryotic systems, the functions of MutS and MutL homologs have been studied and characterized better than the proteins that are involved in processing steps of MMR such as strand discrimination, excision, and resynthesis. For strand discrimination, PCNA (proliferating cell nuclear antigen) has been implicated since it physically interacts with MutS and MutL homologs (Johnson et al., 1996; Gu et al., 1998; Umar et al., 1996) providing a potential link between mismatch recognition and DNA polymerase-associated strand discrimination and other processing activities. This idea has been supported by studies showing that mutations in the yeast PCNA (POL30) result in as strong a mutator phenotype as seen in MMR deficient cells (Ayyagari et al., 1995). Epistasis analysis has suggested that the anti-mutator activity of PCNA works in the same pathway as the mismatch repair molecules (Johnson et al., 1996; Umar et al, 1996). Thus, PCNA may be a signaling factor for strand discrimination, directing the MMR complex to DNA polymerases at the replication fork and repair of mismatches on the nascent

strand (Umar et al., 1996). A human endonuclease, MED1, has been proposed to be a MutH functional homolog because it interacts with an MLH protein (Bellacosa et al., 1999). However, a structural study revealed that MED1 is closely related to DNA glycosylase/AP-lyase and also has significantly higher affinity to fully methylated DNA than hemi-methylated DNA (Drummond and Bellacosa, 2001). Taken together, MED1 is likely not a homolog of MutH, leaving a search for an additional MutH homolog to continue. While it is questionable if methylation is a strand discrimination signal in eukaryotes, it has been proposed that a nick on the nascent strand can serve as a signal (Fang et al., 1993; Holmes et al., 1990; Thomas et al., 1991).

For the exonuclease function, four proteins are implicated in yeast to participate in MMR activity: EXO1, RAD27 (the homolog of the yeast FEN1 flap endonuclease) for 5' to 3' exonuclease activity (Johnson et al., 1995; Tishkoff et al., 1997) and the 3' to 5' proofreading exonucleases of DNA polymerases δ and ε (Tran et al., 1999). While the involvement of RAD27 in MMR is yet to be determined (Tishkoff et al., 1997a), the role of EXO1 in MMR is clear as shown by the direct interaction of EXO1 with MSH2 (Tishkoff et al., 1997b) and MLH1 (Tran et al., 2001), and by a weak mutator phenotype upon inactivation of EXO1 (Tran et al., 2001). In mammals, a human homolog of EXO1, EXO1/HEX1 has been identified (Qui et al., 1999; Schmutte et al., 1998; Tishkoff et al., 1998; Wilson et al., 1998). The eukaryotic version of a helicase (MutU) has not been identified. For resynthesis, DNA polymerase δ and other aphidicolin – sensitive

DNA polymerases (Thomas et al., 1991), PCNA, and RPA (Lin et al., 1998) are likely to be involved. The proteins for ligation have not been identified.

This primary role of MMR in mutation avoidance is demonstrated by the mutator phenotype of MMR deficient cells, including human tumor cell lines, in which mutation rates of spontaneous frameshift and base pair substitutions at selectable loci are significantly increased over the mutation rates of normal cells (Yao et al., 1999; Baross-Francis et al., 2001). Microsatellite instability (MIN), which represents expansions or contractions of the repeat sequences, is the hallmark of mismatch-deficient cells. Microsatellites are the short repeated sequences of mono-, di-, tri-, or tetra-nucleotides. It has been shown that MMR deficient sporadic and hereditary tumors (Aaltonen et al., 1993; Boland et al., 1998; Ionov et al., 1993) exhibit microsatellite instability. As mentioned earlier, the MutSα complex preferentially recognizes base-base mismatches and small insertions/deletions (1–3 nucleotides) and large insertions/deletions (2-8 nucleotides) are recognized by MutSβ complex. Studies with Msh2 (Bhattacharyya et al., 1994) showed MMR-deficient colorectal carcinoma cell lines increased the mutation rate for microsatellite loci ranging from 0.6 to 3.8×10^{-2} while MMR-proficient colorectal carcinoma cells exhibit a mutation rate of 2.8×10^{-6}. These repeat sequences are susceptible to frameshift mutations caused by the slippage errors of DNA polymerases (Schlotterer et al., 1992) and the frameshift mutations of microsatellites are normally monitored and repaired by MMR (Strauss, 1997; 1999; Schaaper, 1993). In eukaryotes, most microsatellites are located in non-

coding regions, thus their instabilities do not influence gene function (Field et al., 1998). However, there are a few critical genes such as for *APC*, and *TGF-β*, which have the mono- repeat sequences that are sensitive to slippage mutations in MMR-deficient cells (Huang et al., 1996; Kuraguchi et al., 2000; Parsons et al., 1998). In yeast, loss of MSH2, MLH1, or PMS2 leads to a pronounced instability at poly (GT) repeats (Strand et al., 1993; 1995), while loss of either MSH3 or MSH6 has a much smaller effects (Johnson et al., 1996b; Strand et al., 1995). However, cells defective for both MSH3 and MSH6 show substantial microsatellite instability (Johnson et al., 1996b) indicating possible functional overlap between these two molecules. Several studies have suggested that MMR is the main repair system that corrects base addition or deletion in repeat sequences, while proofreading machineries of DNA polymerases are more important for preventing base pair substitutions (Strauss, 1997; Tran et al., 1997; Strand, 1993).

In addition to correction of replication errors, MMR responds to cellular DNA damage through various functions. It was suggested that MMR plays a role in DNA damage surveillance in which MMR functions as a sensor for DNA damage to the genome and promotes cell death. Several studies have shown that apoptotic cell death was decreased in *Mlh1*-deficient cells after exposure to DNA damaging agents such as alkylating agents (Hickman et al., 1999), ionizing agents (Fritzell et al., 1997), cisplatin (Lin et al., 2001), and hydrogen peroxide (Hardman et al., 2001). MMR is also required for the G2/M checkpoint after 6 – thioguanine (Buermeyer et al., 1999) and ionizing radiation exposures (Davis et al., 1998).

Lack of MLH1 allows cells to progress through G2/M resulting in increased cell survival. Increased cell survival after exposure to various DNA damaging agents has been explained by the absence of futile cycles related to the repair functions of MMR (Karran et al., 1993; Bignami et al., 2000). According to this model, MMR recognizes mismatched damaged DNA bases after replication and excises misincorporated bases on the newly synthesized strand. However, DNA polymerases repeatedly insert wrong bases opposite to the damaged bases which will be again recognized and excised by MMR. These repetitive cycles of misincorportation and excision eventually result in cell death. In contrast, in the absence of MMR, the absence of futile cycles leads to the cell survival after exposure to DNA damaging agents. Several biochemical studies have shown that MutSα complex recognizes various mismatched damaged bases indicating MMR is also responsible for preventing DNA damage – induced mutations (Ni et al., 1999; Wang et al., 1999; Mu et al., 1997; Yamada et al., 1997; Duckett et al., 1997). In agreement with these results, cells that are resistant to DNA damaging agents exhibit increased levels of mutations (Glaab et al., 1998a; 1998b; 1999; 2001; Nara et al., 2001; Xu et al., 2001). It is important to understand the exact mutation avoidance role of MMR in preventing genotoxin–induced mutations and contributing to antitumor activity. As mentioned earlier, cellular DNA is susceptible to damage. It is important to understand the precise interaction between DNA damage and repair not only because mutations caused by DNA damage

contribute to initiation and progression of tumors, but also because this interaction significantly influences cancer therapy.

In this study, the role of mammalian MMR in preventing spontaneous and genotoxin – induced mutations was investigated. The two genetic methods used for the study were forward and reversion mutational analyses employing mouse *Aprt* as the reporter locus. I report that the mutagenic consequences of loss of MLH1 versus PMS2 in mouse cells are not equal, and that full MMR activity is required for preventing DNA damage- induced mutations. With a mammalian reversion assay, I demonstrated that *Pms2*-deficient cells exhibit elevated rates of A:T → G:C mutations relative to other base-pair substitutions. I also demonstrated that hydrogen peroxide, in the presence of a metal mixture induced tandem CC → TT mutations. In MMR–deficient cells, tandem CC → TT mutations were induced at elevated levels by both oxidative stress and ultraviolet light.

Multiple Mutations are Common at Mouse *Aprt* in Genotoxin-Exposed Mismatch Repair Deficient Cells

Chi Y. Shin, Isabel Mellon, and Mitchell S. Turker

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ABSTRACT

Mismatch repair deficiency is known to contribute to elevated rates of mutations, particularly at mono- and di-nucleotide repeat sequences. However, such repeats are often missing from the coding regions of endogenous genes. To determine the types of mutations that can occur within an endogenous gene lacking susceptible repeat sequences, we examined mutagenic events at the 2.3 kb mouse *Aprt* gene in kidney cell lines derived from mice deficient for the PMS2 and MLH1 mismatch repair proteins. The *Aprt* mutation rate was increased 33 fold and 3.6 to 20 fold for *Mlh1* and *Pms2* null cell lines, respectively, when compared with a wild type kidney cell line. For the *Pms2* null cells this increase resulted from both intragenic events, which were predominantly base-pair substitutions, and loss of heterozygosity events. Almost all mutations in the *Mlh1* null cells were due to base-pair substitutions. A:T → G:C transitions (54% of small events) were predominant in the *Pms2* null cells whereas G:C → A:T transitions (36%) were the most common base-pair change in the *Mlh1* null cells. Interestingly, 4-9% of the spontaneous mutant alleles in the mismatch repair deficient cells exhibited two well-separated base-pair substitution events. The percentage of mutant alleles with two and occasionally three base-pair substitutions increased when the *Pms2* and *Mlh1* null cells were treated with ultraviolet radiation (15-21%) and when the *Mlh1* null cells were treated with hydrogen peroxide (35%). In most cases the distance separating the multiple base-pair substitutions on a given allele was in excess of 100 base pairs, suggesting that the two mutational events were not linked directly

to a single DNA lesion. The significance of these results is discussed with regards to the roles for the PMS2 and MLH1 proteins in preventing spontaneous and genotoxin-related mutations.

INTRODUCTION

A variety of mutational events ranging in size from base-pair substitution to loss of entire chromosomes can occur spontaneously in normal cells. Essentially all types of mutational events have also been observed in malignant cells (Turker, 1998), but based on the relatively low rates that these events occur in normal cells it has been proposed that an important part of malignant progression is the acquisition of mutator phenotypes (Loeb and Loeb, 2000; Loeb, 1991). Such phenotypes are characterized by increased rates of mutation accumulation over those observed in normal cells, and they are often due to loss of DNA repair and/or damage recognition pathways (Cahill et al., 1999).

Loss of DNA mismatch repair provides one type of mutator phenotype in malignant cells (Buermeyer et al., 1999; Harfe and Jinks-Robertson, 2000). DNA mismatch repair is a post-replication process that recognizes and corrects mismatched base pairs and small insertion and deletion loops (Modrich and Lahue, 1996). Many of these loops occur as a consequence of DNA polymerase slippage at mono- and di-nucleotide microsatellite regions, and a deficiency in the mismatch repair pathway increases dramatically the rate of mutation at these regions (Buermeyer et al., 1999; Ionov et al., 1993). However, a link between microsatellite instability, which is often used as a diagnostic marker for mismatch repair deficiency, and gene mutation in cancer has been shown only for a subset of cancer relevant genes including *TGF-β,* and *APC* (Huang et al., 1996; Kuraguchi et al., 2000; Parsons et al., 1995). These genes contain mononucleotide runs within their

coding regions, but such runs are absent from the coding regions of many cancer relevant loci. Because microsatellite instability is unlikely to contribute to all forms of altered gene function in mismatch repair deficient malignant cells, it is likely that elevated rates of base-pair changes are also playing causal roles in cancer progression linked to mismatch repair deficiency.

In addition to recognizing mispaired bases and small loops that result from spontaneous DNA polymerase errors, the mismatch repair proteins appear to play a role in preventing mutations that result from DNA damage (Andrew et al., 1998; Glaab et al., 1998). Evidence for this role includes reports that mismatch repair deficient cells exhibit a hypermutable response after exposure to alkylating agents (Glaab et al., 2000) and the direct binding of mismatch repair proteins to DNA damage (Li, 1999). Although the precise role for these proteins in avoiding genotoxin-induced mutations is not well defined, it may result from a combination of direct binding to damaged bases and the recognition of mismatched base-pairs that result from the insertion of an incorrect base across from a damaged base.

A variety of approaches have been used to define the types of spontaneous and induced gene mutations that can occur in mismatch repair deficient cells. Many studies rely on cancer-derived cell lines with mismatch repair deficiencies (Bhattacharyya et al., 1995; Harwood et al., 1991; Kato et al., 1998; Ohzeki et al., 1997; Phear et al., 1996; Tomita-Mitchell et al., 2000), but such cell lines could have additional alterations that contribute to elevated mutation rates. The construction of mice with specific mismatch repair deficiencies has allowed for

studies that avoid problems associated with cancer-derived cell lines. Many of these studies have used bacterial or Lambda phage transgenes to define the spectrum of small mutational events that are associated with mismatch repair deficiencies (Andrew et al., 1997; Andrew et al., 2000; Baross-Francis et al., 1998; Baross-Francis et al., 2001; Narayanan et al., 1997). While these systems allow large numbers of mutational events to be identified relatively rapidly, the transgenes are not expressed in mammalian cells, are present in multiple copies, and have variable integration sites. Therefore, they may offer mutagenic substrates that are different from those offered by actively expressed endogenous loci.

Most tumor suppressor genes are located on autosomes (Lasko and Cavenee, 1991; Weinberg, 1995). To examine the role of mismatch repair in mutation avoidance for an endogenous autosomal locus, I have created mouse kidney epithelial cell lines that are null for either *Pms2* or *Mlh1* and heterozygous for the selectable *Aprt* locus. Although this locus contains several mono- and dinucleotide regions, they are not of sufficient length to act as susceptible mutational targets in mismatch repair deficient cells. Therefore, mouse *Aprt* provides a useful target for the study of base-pair substitution events at an endogenous and expressed allele in a mismatch repair deficient background. These events were examined in both the *Pms2* and *Mlh1* null backgrounds after exposure of the cells to ultraviolet radiation or hydrogen peroxide, and in non-exposed cells. The PMS2 and MLH1 proteins form a functional heterodimer (MutLα) (Buermeyer et al, 1999); yet I observed that the spontaneous mutational spectra differed in the

different null backgrounds. We also observed examples of spontaneous mutant alleles (4-9%) that had two distinct base-pair substitution events. The frequencies of mutant alleles with multiple mutations rose to 15-21% when the *Pms2* and *Mlh1* null cells were exposed to ultraviolet radiation (UV) and to 35% when the *Mlh1* null cells were exposed to hydrogen peroxide. The important roles for the PMS2 and MLH1 proteins in preventing spontaneous and genotoxin-related mutations are discussed.

METHODS AND MATERIALS

Cell lines. Three clonal kidney cell lines (K1, K2 and K597S2) were derived from mice (C57BL/6) null for *Pms2* and one clonal kidney cell line (K634S2) was derived from a mouse (C57BL/6) null for *Mlh1*. These mice were also heterozygous for the selectable *Aprt* locus. The methods used to isolate cell lines are as described below in detail and also elsewhere (Horn et al., 1984; Turker et al., 1999). The cell lines were maintained in the growth medium containing the Dulbeco's Modified Eagle's Medium (DMEM, Life Technologies, Rockville, MD) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO) at 37°C, 5% CO_2.

Isolation of kidney cell lines. Kidneys from a mouse were finely chopped in chilled PBS (phosphate buffered saline, pH 7.5, Life Technologies, Rockville, MD) and digested in 5 mls of 4 mg/ml collagenase/dispase (Sigma, St. Louise, MO) in

PBS by incubating for 1 hour at 37°C. After the digestion, 9 mls of the growth medium were added to dilute the digestion enzymes. The cell suspension was centrifuged at 1000 g at room temperature for 10 minutes to remove the digestion enzymes. The cell pellets were then resuspended in 5 mls of growth medium and transferred to a T-25 flask. After a week, the primary culture was trypsinized and seeded in a 100 mm dish with standard rinsing and trypsinization methods described below. After 3 weeks, a single clone for each cell line was isolated in a cloning ring as described below and the cell line was established by passaging the cells with one to two splits for greater than 10 passages. At that time they were considered to be "established". Cell lines with 15 – 30% plating efficiencies were used for all experiments.

Passage of cell lines: rinsing and trypsinization of cells. When the established cells were confluent in a flask or a dish, they were rinsed once with 10 mls of Versene (PBS, phosphate buffered saline with 0.02% EDTA). After the aspiration of Versene, the cells were detached from the surface by incubating cells in the presence of 1ml of 0.25% trypsin (Sigma, St. Louis, MO) in PBS (a process called trypsinization) at 37°C, 5% CO_2 for 5 to 10 minutes. When the cells were detached, 5 mls of the growth medium were added to resuspend the cells. 1 ml of the cell suspension was then transferred to a new flask (T-25 for T-75) and additional growth medium was added to bring the volume up to 5 mls for T-25 flasks and 10 mls for T-75 flasks.

Isolation of cell clones. 500 cells were plated using a 100 mm dish containing 10 ml growth medium. After approximately 10 days of growth, visible clones (containing more than 100 cells) were isolated by first rinsing cells with 10 mls of PBS. After the aspiration of PBS, greased cloning rings were placed on the clones. 100 µl of 0.25% trypsin was added into an individual cloning ring and incubated at 37°C, 5% CO_2 for 5 to 10 minutes to detach the clone from the plate surface. 100 µl of the growth medium was added to a cloning ring. The cells were transferred to a well of a 24 well dish in the presence of 1 ml of the growth medium and incubated at 37°C, 5% CO_2. When the well was confluent, the cells were rinsed with 1 ml Versene and trypsinized with 0.5 ml 0.25% trypsin as described above and all the cells were transferred to a T-25 flask.

Determination of cell numbers. Cells were rinsed and detached by trypsinization as described above and resuspended in 10 to 20 mls of the growth medium in a 15 or a 50 ml tube, respectively, depending on the density of the cells. To count cells, an aliquot of suspension was added to a hemacytometer. The number of cells/ml was determined by multiplying the number of cells per square millimeter by 10,000.

Determination of *Aprt* mutation rates. For each cell line to be tested, 10 sets of 100 cells were counted as described above and plated into 10 wells of a 24-well

plate. When the cells were confluent, they were rinsed and trypsinized. All the cells in a well were transferred to a T-25 flask. When a T-25 flask was confluent, all the cells were trypsinized, transferred to a T-75 flask, and expanded in the growth medium to a final cell number of 1.5 to 2.2×10^6 cells per T-75 flask (in ten T-75 flasks). The final cell number in a T-75 flask representing each set was determined as described above. The *Aprt* mutant frequencies were then determined for each expanded cell population by plating 100,000 cells/100 mm dish (in four dishes) in 10 mls of the growth medium/dish. After 16 hours, 2,6-diaminopurine (DAP) (Sigma, St. Louis, MO) was added at a final concentration of 80 μg/ml. To determine the plating efficiency (also termed as cloning efficiency), 500 cells from each set were counted and plated in 100 mm dishes in duplicate without the addition of DAP. After 12 days, all the dishes were stained with crystal violet solution as described below. The mutation rates were determined with a formula that calculates mutation rates based on the method of Luria and Delbruck using a table developed by Capizzi and Jameson (1973). The program based on the formula was developed by Prolla et al. (1998) and used for this study.

The basics of rate calculation. The formula 29 (1) in Lea and Coulson's article provides a way to estimate the mutation rates. Based on the formula, a software program was written by Dr. Eric Bronner.

$$r = m \log (n) \qquad (1)$$

$$= (\alpha \cdot n_t) \log (C \cdot \alpha \cdot n_t) \qquad (2)$$

in which

r = the average number of mutants per sample

m = the mean number of mutations

n = the total number of mutations

α = the mutation rate/locus/generation

n_t = the number of cells per sample

C = the number of samples

log is the natural log.

If both side of the equation are multiplied by C,

$$C \cdot r = C \cdot \alpha \cdot n_t \log (C \cdot \alpha \cdot n_t) \qquad (3)$$

this will give a general form of an equation.

$$Y = X \log (X) \qquad (4)$$

in which

Y is defined as the total number of mutants.

To obtain the mutation rate (α), one needs to find X ($=C \cdot \alpha \cdot n_t$).

$$\alpha = X / (C \cdot n_t) \qquad (5)$$

One (*e. g.* the computer) solves for X by iteratively plugging in any X value until it finds an X value which satisfies the equation. Once X is identified, then mutation rate can be determined.

Counting clones in dishes. To assess either cloning efficiency or mutant numbers, the growth medium was removed from dishes and 10 mls of 0.25% crystal violet (Sigma St. Louis, MO) solution were added to dishes. Shortly after, excess crystal violet solution was rinsed off under gentle running water. The stained clones were counted by visual identification. Crystal violet solution (0.25%) was prepared by dissolving 5 g of crystal violet (Sigma, St. Louis, MO) in 500 mls of 25% ethanol and then diluted to 2 L with deionized water.

Determination of mutant frequencies after genotoxin exposure. The mutagenic responses of the cell lines were acertained by determining mutant frequencies in subclones exposed to ultraviolet (UV) radiation and hydrogen peroxide. For each cell line, 7 to 10 independent subclones were treated with each genotoxin at doses that kill 90% of the exposed cells. The 90% killing doses were determined by comparing plating efficiency of treated cells to those of non-treated cells. Independent subclones were isolated as described previously and expanded to T-75 flasks in the growth medium for the experiments. For UV radiation exposure, one million cells of each subclone were plated in a 100 mm dish and after 24 hours exposed to 9.6 J/m^2 (shortwave germicidal UV bulb, Fisher, Pittsburgh, PA), which

was measured using a UV intensity meter (VWR, Plainfield, NJ). After exposure, the cells were trypsinized and all were replated into a T-75 flask. After 6 days of recovery, 400,000 cells of each subclone were plated into four 100 mm dishes (total 4 dishes for each subclone), and after 24 hours treated with 80 μg/ml DAP to select *Aprt* mutants. For hydrogen peroxide exposure, 700,000 cells of each subclone were plated in a T-75 flask, and after 24 hours treated with 25 μM hydrogen peroxide for *Pms2*-deficient cells and 50 to 65 μM for wild type and *Mlh1*-deficient cells. The working stock solution [100 ×] of hydrogen peroxide was made in deionized water, filter-sterilized, and added to the growth medium at the appropriate final concentrations. After 24 hours, the medium was changed and the cells were allowed to recover for 6 days. The medium was changed to remove dead cells after 3 days. After recovery, 400,000 cells were plated into four 100 mm dishes (total four dishes for each subclone) and treated with 80 μg/ml DAP. For the non-exposed controls, the same number of cells was plated for DAP selection. To determine the plating efficiencies, 500 cells of treated and non-treated subclones were plated in duplicates using 100 mm dishes in the growth medium. The number of mutants was determined by counting clones stained with the crystal violet solution as described above. The mutant frequencies were determined by dividing the number of *Aprt* mutants by the number of plated cells, adjusted for plating efficiency. The equation for calculating mutation frequency (Mf) follows:

Mf = the number of *Aprt* mutants / (the number of plated cells × % plating efficiency/100)

Isolation of *Aprt* mutant cells. One or two *Aprt* mutant clones (*i.e.*, DAP resistant) were isolated per dish, with a total of 1 to 2 mutant clones isolated per subclone. The *Aprt* mutant clones were isolated by trypsinizing individual clones in cloning rings as described above. The trypsinized mutant clones were then transferred to 24 well plates in the growth medium. Cells were expanded to T-25 flasks in the growth medium and genomic DNA was isolated as described below. Mutant sib clones are defined as *Aprt* deficient clones bearing identical mutations that were isolated from the same parental subclone. When mutant sib clones were observed, the mutation for only one sib clone was included in the DNA sequence analyses shown in Tables 2, 3, and 5.

Analysis of transcription-coupled DNA repair. The biochemical assay for transcription-coupled DNA repair (TCR) was performed by Dr. Isabel Mellon, University of Kentucky, as described elsewhere (Smith et al., 2000).

Isolation of genomic DNA. *Aprt* deficient clones were expanded in T-25 flasks to confluency. After the growth medium was removed, each flask was rinsed with 5 ml PBS. 500 µl of NLB solution (1 mM Tris-HCl, pH 8.0, 2.25 mM EDTA, 0.4 M NaCl), 10 µl of 10 mM proteinase K, and 35 µL of 100 µM sodium dodecyl sulfate (SDS) were then added to each flask. The flasks were incubated overnight at 37°C. The cellular lysates were transferred to microcentrifuge tubes (1.7 ml) and 170 µl

of saturated NaCl added, followed by vigorous shaking for 15 seconds at room temperature. After microcentrifugation at the maximum speed (14,000 rpm) for 10 minutes, the supernatant fraction was transferred to a new tube (1.7 ml) and DNA precipitated by adding 2 volumes of 100% ethanol at room temperature. The DNA pellet was centrifuged down at the maximum speed (14,000 rpm) for 5 minutes and washed with 1 ml 70% ethanol. The DNA pellet was air-dried and dissolved in 35 to 50 μl of TE buffer containing 10 mM Tris-HCl (pH8.0) and 1 mM EDTA (pH 8.0). The genomic DNA was stored at 4°C.

Determination of DNA concentration. Genomic DNA was quantified by a UV spectrophotometer (SmartSpec™, Bio-Rad, Hercules, CA). Genomic DNA (2 μl) was diluted to deionized water (998 μl) 1:500 and transferred to a 1.5 ml quart cuvette (10 mm path length). The blank was 1ml deionized water. The optical density (OD) of DNA was measured at 260 nm. An OD of 1 roughly corresponds to 50 μg/ml of double stranded DNA.

The concentration of the genomic DNA (μg/ml) = OD × 50 μg/ml × dilution factor (500)

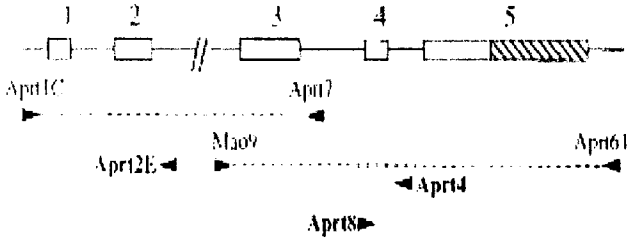
Amplification of *Aprt* allele for sequencing analysis. The basic scheme for the amplification of *Aprt* regions for sequence analysis is shown in Figure 1. This allele was amplified by using the primers Aprt1C

(CGTGAGTTTAGCGTGCTGATAC) and Aprt7 (AAGACCCTGCCCTTCCTCTAC) to amplify exons 1 to 3, and Mao9 (TCCCACAACCTTCCCTCCTTA) and Aprt 61 (GACCCATGCAACAAACTGAGGAG) to amplify exons 3 to 5. Separate reactions were performed for each primer set. The polymerase chain reaction (PCR) mixture included 1 μg of genomic DNA (determined as above), 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 μM $MgCl_2$, 200 μM of each dNTPs, 10 pmoles of sense/antisense primers (shown above), 1 unit Taq DNA polymerase (Life Technologies, Rockville, MD), and deionized H_2O to adjust the volume. The conditions for amplification were 45 seconds at 94°C and 1 minute at 72°C for extension. The annealing temperatures started at 76°C and dropped 2°C per cycle until an annealing temperature of 54°C was reached. An additional 25 cycles were then used. All annealing times were for 45 seconds.

DNA sequence analysis. The PCR products were separated by electrophoresis using a 1% agarose gel (Life Technologies, Rockville, MD), excised, and isolated with a purification kit (Qiagen, Valencia, CA) according to the manufacturer's guide. The electrophoresis was done in a 15 × 22 cm gel in TAE buffer (40 mM Tri-Acetate, pH 8.0, 1 mM EDTA) at 75 V for 4 hours. The fragments (100 ng) were then sequenced with an automated fluorescent DNA sequencer (PE/ABD377) by using 4 pmoles of primers Aprt2E (GAATGGATGGGTGTGGGGCA) for exons 1 and 2, Aprt 4 (CTTAGTCAGCACCCAGGACAG) for exons 3 and 4, and

Aprt 8 (GAAACGCTCGAGGCTAGGGA) for exons 4 and 5. The sequence analysis was performed by the Microbiology Molecular Core facility at Oregon Health and Sciences University.

Figure 1. Primers used to amplify and sequence mouse *Aprt*.



The 2.3 Kb mouse *Aprt* gene contains five exons (boxed regions) included a non-coding region (striped) in exon 5. Primers used for sequencing are shown in bold; the other primers were used to amplify the sequenced regions. See Methods and Materials section entitled 'DNA sequence analysis' for more detail.

RESULTS

Elevation of mutation rates in the *Pms2* and *Mlh1* null cells. The mutation rates for loss of *Aprt* expression were determined for kidney cell lines that were wild type for mismatch repair (KO6), null for *Pms2* (K2 and K597S2), and null for *Mlh1* (K634S2) (Table1). The mutation rates were as follows: 3.6×10^{-6} for the mismatch repair proficient KO6 cells, 1.3 and 7.4×10^{-5} for the two *Pms2* null cell lines, and 1.2×10^{-4} for the *Mlh1* null cell line. These results are consistent with those from other laboratories using different target alleles in mice that showed higher mutant frequencies for tissues of *Mlh1* null mice when compared with those of *Pms2* null mice, and that both deficiencies yielded higher mutant frequencies than those for wild type mice (Baross-Francis et al., 2001; Yao et al., 1999).

Table 1. Mutation rates at the *Aprt* locus in mismatch repair-proficient and deficient mouse kidney epithelial cells

	Mismatch repair-proficient cells	Mismatch repair-deficient cells		
		Pms2 null cells		Mlh1 null cells
	KO6	K2	K597S2	K634S2
Mutation rate (events/cell /generation)	3.6×10^{-6}	1.3×10^{-5}	7.4×10^{-5}	1.2×10^{-4}

Experimental data are supplemented in Table 15 (Appendix).

In these studies, the mutation frequencies of lacI gene increased 18 fold in *Mlh1* null mice and 13 fold in *Pms2* null mice, compared to in wild type mice (Baross-Francis et al., 2001), and also the mutation frequencies at mono-nucleotide repeat regions (microsatellites) of *Mlh1* null mice were 2-3 fold higher than those of *Pms2* null mice (Yao et al., 1999).

The spontaneous mutational spectra differ for the *Pms2* and *Mlh1* null cells.

Aprt expression can be lost via two general classes of mutational events. One class is intragenic events such as base-pair substitutions, frame shifts, small deletions, and silencing; these events leave the target *Aprt* allele essentially intact. The second class is large events such as mitotic recombination, chromosome loss, and large interstitial deletions; these events remove the target *Aprt* allele. When comparing spontaneous mutational events for the *Pms2* and *Mlh1* null cells it was observed that the vast majority of mutations in *Mlh1* null cells were intragenic events (98%) whereas most mutations within the *Pms2* null cells (70%) were large events. Large events within the *Pms2* null cells were not examined further because there was relatively little heterozygosity for microsatellite loci on chromosome 8 in these cell lines. Heterozygosity is required for a detailed analysis of large mutational events (loss of heterozygosity). The mouse *Aprt* gene is located on chromosome 8 (Kozak et al., 1975).

To identify mutations when intragenic events had occurred, sequence information was obtained for all 5 exons of *Aprt* including a 223 bp non-coding

region of exon 5, and all of introns 1, 3, and 4. Additional sequence information was obtained for approximately 100 bp of the 974 bp intron 2, for an approximate total of 1.25 kb sequenced per mutant allele (Fig. 1). The sequence analysis revealed differences when comparing spontaneous mutations from the *Pms2* and *Mlh1* null cells (Table 2). Although in both cases transition mutations represented the majority of events, they were more common in the *Pms2* null cells (72%) than the *Mlh1* null cells (52%). Moreover, the distribution of the transition mutations was markedly different, with A:T → G:C mutations (75% of transitions, 54% of total) being the most common intragenic event in the *Pms2* null cells. A:T → G:C mutations were observed less commonly in the *Mlh1* null background (31% of transitions, 16% of total) (p value, 0.0002). G:C → A:T mutations were the most common small event in the *Mlh1* null cells (69% of transitions, 36% of total). Transversion mutations were also common in the *Mlh1* null cells (48% of total), but were not common in the *Pms2* null cells (17.2 % of total).

Three spontaneous 2 bp deletions (11% of total), which were apparently due to polymerase slippage at short dinucleotide repeats in the coding region of exon 5, were observed in the *Pms2* null cells. Two of these mutations were TC deletions that occurred within the sequence TTCTCTCTCC, and the other was a TG deletion that occurred within the sequence AGTGTGTGAG. No other spontaneous deletions or insertions were observed. The relatively low percentage of these mutational events, which are common in many mutant alleles in mismatch repair deficient cells (see Introduction), demonstrates that these repeats are not of

sufficient length to act as the primary targets for mutations in mismatch repair deficient cells.

The sequence analysis revealed two examples in *Mlh1* null cells (9%) of mutant *Aprt* alleles with well-separated base-pair changes and one example in the *Pms2* null cells (4%) (Table 3A). The distances separating these mutations ranged from 374 to 1480 base-pairs, which is consistent with two independent events occurring on each mutant allele. Four of the six mutations led to altered codons and one an altered splice junction site. A mutation with no potential impact on protein function (in intron 4) was identified in one case. An additional spontaneous mutant allele was observed in a *Pms2* null cell that had two base-pair substitutions separated by a single base-pair, which makes it difficult to determine if they truly represent independent mutational events. Other authors disagree on whether these changes represent one or two mutational events (Buettner et al., 2000; Harwood et al., 1991; Strauss, 1997). Both events are included in Table 2 and shown in Table 3A.

Table 2. Mutation distribution at the *Aprt* locus of *Pms2* and *Mlh1* null cells.

Mutation	Pms2 null cells			Mlh1 null cells		
	Spontaneous (%)	UV (%)	H_2O_2 (%)	Spontaneous (%)	UV (%)	H_2O_2 (%)
Transitions						
A:T → G:C	15 (54%)	12 (36%)	5 (63%)	4 (16%)	3 (20%)	8 (30%)
G:C → A:T	5 (18%)	13 (39%)		9 (36%)	5 (33%)	10 (37%)
Transversions						
A:T → T:A	1 (3.4%)		3 (37%)			2 (7.4%)
A:T → C:G	1 (3.4%)	1 (3%)		6 (24%)	3 (20%)	3 (11%)
G:C → C:G	1 (3.4%)	1 (3%)		4 (16%)	3 (20%)	1 (3.7%)
G:C → T:A	2 (7%)	2 (6%)		2 (8%)	1 (6.7%)	2 (7.4%)
Frameshifts						
Deletion	3 (11%)	2 (6%)				1 (3.7%)
Tandem double		2 (6%)				
Multiple mutants	2	5		2	2	7
No. of total point mutations	28	33	8	25	15	27
No. of point mutants analyzed	26	26	8	23	13	20

Table 3. Examples of alleles with multiple mutations.

Subclones	Mutation events	Sequence Context (Non-transcribed)	Codon # (exon)	Dist. (bp)	Amino acid Change
(A) Spontaneous mutants					
Pms2-K1S6B-Sp	G:C → A:T A:T → G:C	CTCAGGAGCTG GACAAACAGAC	78 (3) Intron (4-5)	374	GAG (glu) → AAG (lys)
Pms2-K597S2-6E-Sp	G:C → T:A A:T → G:C	TGTGACCTGCT TGACCTGCTGC	141(5) 142(5)	1	GAC (asp) → GAA(asp) CTG (leu) → CCG (pro)
Mlh1-K634S3-14-Sp	A:T → C:G G:C → C:G	GATCGACTACA TGTCAGCCTCC	59 (2) 99 (3)	1094	GAC (asp) → GCC (ala) GCC (ala) → CCC (pro)
Mlh1-K634S4-A-Sp	G:C → A:T G:C → A:T	GCTAGGCCGTG TTGCGGCCTGT	SJ of exon2 139 (5)	1480	Loss of exon 2 GCC (ala) → ACC (thr)
(B) UV-exposed cells					
Pms2-K2S6C-UV	G:C → A:T G:C → A:T	TCCTCCCTGCC AGCTTCCATCC	SJ (1) 42 (2)	278	Loss of exon 1 TCC (ser) → TTC (phe)
Pms2-K2S7A-UV	G:C → T:A A:T → G:C G:C → C:G	GGAACCTGACT TGTTCAGGTGC GTGGAGTGACC	4 (1) 27 (1) 158 (5)	68 1795	CCT (pro) → CAT (his) AGG (arg) → GGG (gly) GAG (glu) → GAC (asp)
Pms2-K2S9C-UV	G:C → T:A G:C → A:T	GGAACCTGAGT GTCACCTGAAG	4 (1) 50 (2)	267	CCT (pro) → CAT (his) CTG (leu) → TTG (leu)
Pms2-K597S2-E-UV	A:T → G:C G:C → A:T	GGGGCTTCCTG GCTTCCTGTTT	69 (3) 70 (3)	2	TTC (phe) → CTC (leu) CTG (leu) → TTG (leu)
Pms2-K597S2-7E-UV	G:C → A:T A:T → C:G G:C → A:T	GCAGGCGCGTA GCCCTTCCCCT CCTAGCTCAGG	Intron 1-2 74 (3) 76 (3)	1189 6	Loss of exon 1 or 2 TCC (ser) → GCC (ala) GCT (ala) → GTT (val)
Mlh1-634S2-2F-UV	G:C → A:T G:C → C:G	GTTTGGCCCTT CCCGGGCAGAG	72 (3) 120 (4)	331	GGC (gly) → GAC (asp) GGG (gly) → GGC(gly)
Mlh1-634S2-6A-UV	A:T → G:C G:C → A:T	GCCCCACTGTG TGGTGGCTCAG	96 (3) Non-coding	609	ACT (thr) → GCT (ala)

Table 3 (continued)

(C) H_2O_2-exposed cells					
Mlh1-634S2-2A- H_2O_2	G:C → A:T G:C → A:T	AGTATGGGAAG AAGCGAGCTG	106 (3) SJ 3	11	GGG (gly) → AGG (arg) Loss of exon 3
Mlh1-634S2-2E- H_2O_2	A:T → G:C G:C → A:T	TTCCCAGGGAT TGGTGGCTCAG	SJ (2) Non-coding	1791	
Mlh1-634S2-2F- H_2O_2	A:T → T:A G:C → A:T	TTCCCTAGCTC CACGGGCTACC	75 (3) Intron 3-4	140	CTA (Leu) → CAA (Gln)
Mlh1-634S2-5E- H_2O_2	A:T → G:C A:T → T:A	CGACTTCCCAA GTGGATGACCT	16 (1) 127 (4)	1616	TTC (phe) → TCC (ser) GAT (asp) → GAC (asp)
Mlh1-634S2-5F- H_2O_2	A:T → G:C A:T → C:G	TGAAGTCCACG TGGCTAAGCCC	52 (2) Non-coding	1719	TCC (ser) → CCC (pro)
Mlh1-634S2-7G- H_2O_2	G:C → A:T A:T → G:C	GGGAAGGTAAG GCTCAACCCAG	107 (3) Non-coding	579	AAG (lys) → AAA (lys)
Mlh1-634S2-8B- H_2O_2	A:T → G:C A:T → C:G	GAGAGTGGTCA GCTCCAAGCAG	123 (4) Non-coding	316	GTG (val) → GCG (ala)

Ultraviolet radiation alters the mutation spectrum in *Pms2* null cells and increases the frequency of multiple mutations. A potential role for the mismatch repair pathway in the avoidance of UV induced mutations has not been resolved (Liu et al., 2000; O'Driscoll et al., 1999; Tobi et al., 1999; Wang et al., 1999). To address this issue, the *Pms2* and *Mlh1* null kidney cells were exposed to UV and *Aprt* mutant cells were isolated. As seen in Table 4 there was no increase in the frequency of mutant cells when *Pms2* subclones were exposed to UV. For this reason the mutations observed in the UV exposed cells will not be termed as "induced" mutations. Nonetheless, a sequence analysis demonstrated clearly that UV had a mutagenic effect for the *Pms2* null cells. One observation consistent with this effect was an increase in the C → T signature mutation for UV mutagenesis. For the UV-exposed *Pms2* null cells, 13 of 33 (39%) mutations were C → T substitutions (Table 2); 11 of these 13 mutations occurred at dipyrimidine sites and all occurred on the non-transcribed strand. Other studies with wild type cells also showed that dipyrimidines sites at the non-transcribed strand were predominantly mutated by UV irradiation. (Drobetsky et al., 1987; Kanjilal et al., 1993; Khattar and Turker, 1997; Takeuchi et al., 1998). In contrast, only 5 of 28 spontaneous small mutations (18%) were C → T, and in only one case did this base change occur on the non-transcribed strand. A second effect from UV exposure was an increase in the number of mutant alleles with multiple mutations (5 of 26, 19%) (Tables 2 and 3B). Two of these mutant alleles had 3 base-pair substitutions for a total excess of 7 base-pair substitutions from 26 mutant alleles (27%) as compared

with 4% for non-exposed *Pms2* null cells (p value, 0.011 or 0.032 if the questionable mutation is included, see above section). Two tandem mutations and two base-pair deletions at the short TC run in exon 5 (discussed above) were also observed in the mutants isolated from the UV-exposed *Pms2* null cells.

In wild type cells, UV-induced mutations occur mostly on the non-transcribed strand (Khattar and Turker, 1997) presumably because transcription coupled repair (TCR) selectively repairs the transcribed strand (Tornaletti and Pfeifer, 1996). The observation that mutations in the UV exposed *Pms2* null kidney cells were occurring mostly on the non-transcribed strand suggested that TCR is active in these cells. However, mismatch repair deficiency has been correlated with a reduction in TCR in *E. coli* (Mellon and Champe, 1996) and in some human tumor cells (Mellon et al., 1996). Dr. Mellon (University of Kentucky) kindly conducted an experiment to examine the TCR function in a wild type (KO6) and two *Pms2* null kidney cell lines (K2 and K9). In all cases most cyclobutane pyrimidine dimers were removed from the transcribed strand within 16 hours after UV irradiation, which demonstrates that the TCR function is not impaired in the *Pms2* null kidney cells that were tested. Figure 2 shows results for one wild type and one *Pms2* null cell line; essentially the same result was observed for the second *Pms2* deficient cell line (Dr. I. Mellon, personal communication). Figure 2 also shows that little repair occurred on the non-transcribed strand for the mouse cells, reflective of this relatively low rate of global repair.

Table 4. Mutant frequencies for unexposed and H_2O_2 and UV-light exposed cells.

Subclones	Spontaneous mutant frequency	H_2O_2- exposed mutant frequency (*)	UV- exposed mutant frequency (*)
Pms2-null cells			
K597S2-1	3.4×10^{-4}	3.2×10^{-3} (9.4 ×)	5.7×10^{-4} (1.7 ×)
K597S2-2	1.5×10^{-4}	5.2×10^{-5} (0.3 ×)	1.7×10^{-4} (1.2 ×)
K597S2-4	9.3×10^{-5}	1.2×10^{-3} (13 ×)	9.0×10^{-5} (1 ×)
K597S2-5	7.4×10^{-5}	4.1×10^{-5} (5.6 ×)	1.9×10^{-4} (2.5 ×)
K597S2-6	9.6×10^{-4}	2.3×10^{-3} (2.4 ×)	3.3×10^{-4} (0.3 ×)
K597S2-7	1.4×10^{-4}	1.2×10^{-3} (8.4 ×)	3.2×10^{-4} (2.3 ×)
K597S2-10	2.6×10^{-4}	6.4×10^{-4} (2.5 ×)	4.2×10^{-4} (1.6 ×)
Average	2.9×10^{-4}	12.3×10^{-4}	3.0×10^{-4}
{standard deviation}	{3.1}	{11.7}	{1.6}
Mlh1-null cells			
K634S2-1	1.1×10^{-3}	9.7×10^{-4} (1 ×)	9.7×10^{-4} (1 ×)
K634S2-2	6.4×10^{-4}	9.4×10^{-4} (1.5 ×)	2×10^{-3} (3 ×)
K634S2-5	2.9×10^{-3}	2.3×10^{-3} (0.8 ×)	1.6×10^{-3} (0.5 ×)
K634S2-6	1.1×10^{-5}	1.5×10^{-4} (14 ×)	ND
K634S2-7	1.2×10^{-3}	1.8×10^{-3} (1.5 ×)	ND
K634S2-8	6.5×10^{-4}	1.0×10^{-3} (1.5 ×)	1.0×10^{-3} (1.5 ×)
Average	10.8×10^{-4}	11.4×10^{-4}	14×10
{standard deviation}	{10}	{7.5}	{5}

(*) indicates the fold induction of induced mutant frequency compared to spontaneous mutant frequency.
ND indicates not determined.

Overall, my data suggested that the types of point mutations induced by UV exposure and their location on the non-transcribed strand are not affected by *Pms2* deficiency. However, the increase in multiple mutations suggests that the efficiency of correcting damage-related mismatches is impaired.

No significant increase in mutant frequencies was observed when the *Mlh1* null kidney cells were exposed to UV (Table 4), nor was a dramatic shift observed for the mutational spectrum (Table 2). These results suggest that the high background level of spontaneous mutations in the *Mlh1* cells masks any mutagenic effect from UV exposure. Two of thirteen (15%) mutant alleles examined contained multiple mutations (Tables 2 and 3B).

Multiple mutations are observed frequently in hydrogen peroxide treated *Mlh1* null kidney cells. No increase in mutant frequencies was observed when the *Mlh1* null cells were treated with hydrogen peroxide (Table 4). However, the sequence analysis revealed a quantitative difference in mutations because 7 of 20 (35%) mutant *Aprt* alleles from hydrogen peroxide-exposed *Mlh1* null cells contained double base-pair substitutions (Tables 2 and 3C). This represents a 3.5-fold increase when compared with the number of double mutations observed to occur spontaneously in the *Mlh1* null background (p value, 0.012). Of these seven mutant alleles, four contained base-pair substitutions within the non-coding region of exon 5 and one had a base-pair substitution within an intron suggesting that these were "passenger" (i.e., non-causal) mutations. Each of these five mutant

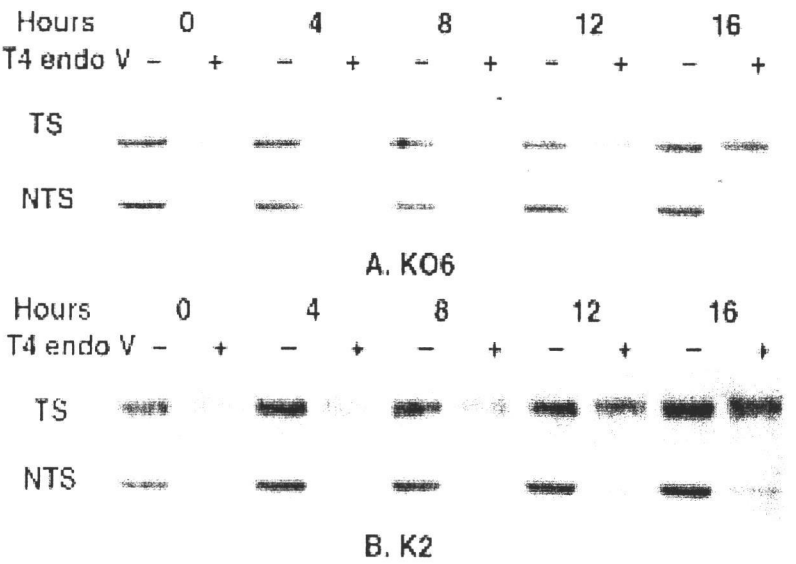
alleles also contained a base-pair substitution that altered an amino acid, with one exception. In this case, a lysine codon (AAG) was mutated to AAA, which also codes for lysine, suggesting a codon usage or splicing defect (Liu et al., 2001). Alternatively, an epigenetic event (Rose et al., 2000) or third site mutation could have occurred, though there is no evidence to support the latter possibility for mouse *Aprt*. For the two remaining mutant alleles with two mutational events, one had two codon changes and the other had a codon change and a splice junction mutation. Two additional passenger mutations were observed at the "knockout" (i.e., containing *neo* gene insert) *Aprt* allele when six hydrogen peroxide-exposed mutants where examined at this allele. There is no selective pressure for mutational events at this non-functional allele.

Hydrogen peroxide exposure increased the mutant frequencies for the *Pms2* null subclones (Table 4), but the majority of these mutations (82%) were large events that removed the selected *Aprt* allele. Only 8 independent base-pair substitutions were identified from this experiment; all occurred at A:T base-pairs (Table 2). No examples of alleles with multiple mutations were found.

Hot spots for mutations differ for *Pms2* and *Mlh1* null cells. Most of the 136 independent mutational events identified in this study occurred at unique sites. However, four sites were found to have been mutated in four to five independent mutants and will be termed "hot spots" for the purpose of this chapter. One hot spot, mentioned in previous sections, is the dinucleotide repeat sequence

TTCTCTCTCC (exon 5), for which five independent 2 base-pair deletions were observed. Of these, four were observed in *Pms2* null cells and one in a *Mlh1* derived mutant. Two additional hot spots were found to occur predominantly in the *Pms2* null cells at codons 136 (**A**TG, exon 5) and 142 (C**T**G, exon 5). The fourth hot spot is *Mlh1* specific, an adenine in codon 128 (G**A**C, exon 4) (Table 5).

Figure 2. Analysis of TCR in wild-type and *Pms2* null cells.



Transcription- coupled repair of the DHFR gene in wild-type (KO6) and PMS2 deficient (K2) cell lines. Cells were irradiated with 10 J/m^2 UV light (254 nm), incubated to allow for repair and DNA was isolated at the times indicated and DNA was isolated at the times indicated above the lanes. DNA samples were restricted with *Bam*H1, treated (+) or not treated (-) with T4 endonuclease V and electrophoresed in agarose gel and transferred to membrane and probed with ^{32}P-labeled RNA probes specific for the transcribed (T) or nontranscribed (NT) strand of the mouse DHFR gene. Following detection with film, the membrane was stripped and rehybridized using a probe for the other strand. Experimental results kindly provided by Dr. Mellon (University of Kentucky).

Table 5. Frequently mutated sites.

Codon	Location	Mutation	Genotype	Exposure
142, CTG	exon5	A:T→ G:C	*Pms2* null	none
		A:T → G:C	*Pms2* null	none
		A:T → G:C	*Pms2* null	none
		A:T → C:G	*Mlh1* null	UV
136, ATG	exon 5	A:T → G:C	*Pms2* null	none
		A:T → G:C	*Pms2* null	UV
		A:T → G:C	*Pms2* null	H_2O_2
		A:T → G:C	*Pms2* null	H_2O_2
		A:T → T:A	*Pms2* null	H_2O_2
128, GAC	exon 4	A:T → C:G	*Mlh1* null	none
		A:T → C:G	*Mlh1* null	none
		A:T → G:C	*Mlh1* null	none
		A:T → C:G	*Mlh1* null	UV
		A:T → C:G	*Mlh1* null	UV

DISCUSSION

The PMS2 and MLH1 proteins form a heterodimer (MutL α) that is required to couple mismatch recognition with the repair process (Buermeyer et al., 1999). However, our comparison of spontaneous mutagenesis in the *Pms2* vs. *Mlh1* null mice revealed that the mutation rates (Table 1) and mutant frequencies (Table 4) were higher for the *Mlh1* null cells. These results are consistent with studies showing elevated mutation frequencies for mono- and dinucleotide microsatellite sequences (Yao et al., 1999) and for base-pair changes at a *lacI* reporter transgene (Baross-Francis et al., 2001) in tissues of *Mlh1* null mice when compared with the tissues of *Pms2* null mice. These previous results have been interpreted as indicating that loss of the MLH1 protein is more mutagenic than loss of the PMS2 protein (Yao et al., 1999), presumably because MLH1 can also bind to the PMS1 and MLH3 proteins to provide limited mismatch repair function in the absence of PMS2. In contrast, there does not appear to be backup protein when MLH1 protein is lost (Buermeyer et al., 1999). The *in vivo* mouse studies also revealed differences in the spectrum of mutations. One base-pair deletions in short mononucleotide regions and C:G → T:A base-pair substitutions were more common in the *Mlh1* null background than in the *Pms2* null background (Baross-Francis et al., 2001; Yao et al., 1999). We also observed that C:G → T:A mutations are more common in the *Mlh1* kidney cells. These mutations are the most common base-pair change found in wild type mammalian cells, presumably due to deamination of cytosine

and methylcytosine (Schmutte and Jones, 1998). In contrast, the most common base-pair substitution events in the *Pms2* null cells were A:T → G:C mutations.

Additional differences in the mutational spectra were observed in our comparison of *Aprt* mutants derived from the *Pms2* and *Mlh1* null kidney cells. One was that LOH events were common in the *Pms2* null background, as they were in wild type kidney cells (Turker et al., 1999), whereas these events were rare in the *Mlh1* cells. This difference indicates that the ratio of base-pair substitution mutation rates for *Mlh1* null cells versus *Pms2* null cells is actually higher than the ratios that can be calculated from the data shown Table 1, which includes both small and large mutational events. The reduced frequency of LOH events in the *Mlh1* null cells does not mean necessarily that the rate at which these events occur is reduced, but instead that they may be more difficult to detect when the mutation rate for small events is increased dramatically. Alternatively, it is possible that the MLH1 protein is required to resolve events such as mitotic recombination. This latter possibility appears unlikely because both UV and hydrogen peroxide exposure increased the frequency of LOH events (15% and 30%, respectively) in the *Mlh1* null cells. Another difference between the two null genotypes is that the three mutational hot spots found in the *Pms2* null cells were distinct when compared with the one mutational hot spot found in the *Mlh1* null cells.

For some loci, such as *Hprt*, *Apc,* and the *supFG* transgene reporter, most intragenic events in mismatch repair deficient cells occur at mononucleotide runs of six or more bp (Andrew et al., 2000; Bhattacharyya et al., 1995; Glaab et al., 2000;

Kato et al., 1998; Kuraguchi et al., 2000; Narayanan et al., 1997). However, in our study no mutations were found at the small number of mononucleotide runs that are present in mouse *Aprt*. The largest such run consists of six cytosines, but it was located upstream of the AG splice junction site of exon 5. It is possible that slippage at this site will have no effect on splicing, and hence no effect on expression. Mononucleotide runs within the coding region of mouse *Aprt* are four or three base-pairs in length, which do not appear to be long enough to act as mutational hot spots. Five independent deletions was observed at the only run of four dinucleotides within *Aprt*, but the total percentage of mutations at this site (5 of 136, 3.7%) is relatively low when compared with hot spots found at mononucleotide runs within other genes in mismatch repair deficient cells.

Two well-separated base-pair substitutions were present in two spontaneous *Aprt* mutant alleles obtained from *Mlh1* null cells and one mutant allele obtained from a *Pms2* null cell for a total of 6% of the spontaneous mutant alleles examined. The distances separating these substitutions ranged from 374 bp to 1480 bp. Likewise, most (75%) of the multiple mutations observed in the genotoxin exposed mutants were separated by distances greater than 140 bp. The largest distance between any two mutational events in the 2.3 kb *Aprt* allele was 1795 bp. There are several overlapping ways to interpret these data. One is that well separated mutational events on a single allele represent two independent events resulting from two distinct DNA lesions. A second possibility is that the repair process for a DNA lesion can involve resynthesis of a significant portion of the *Aprt* gene. A

third possibility is that genotoxin damage interferes with the polymerase function to produce errors that are normally corrected by the mismatch repair pathway. An extension of this possibility is that mismatch repair is also important for an apoptotic response after genotoxin exposure, as has been shown for *Mlh1* null cells exposed to hydrogen peroxide (Hardman et al., 2001). It is formally possible that one mechanism will apply for UV exposure (e.g., direct damage to DNA) whereas a separate mechanism will apply to hydrogen peroxide exposure (e.g., interference with polymerase function and/or the apoptotic response). While it is not possible to distinguish between these and other possibilities at this time, it is possible to conclude statistically that genotoxin exposure increases the frequency of alleles with two or more base-pair substitution events in mismatch repair deficient cells when compared with unexposed cells (p value, 0.013). In contrast, no examples of *Aprt* alleles with multiple mutations were observed for UV or EMS-induced mutations in wild type mouse embryonic carcinoma cells (Khattar and Turker, 1997) or UV-induced wild type CHO cells (Drobetsky et al., 1987).

Analyses of multiple and silent mutations at the *TP53* gene in human cancers has led to the suggestion that many mutations found at this allele in tumor cells are not necessary for functional changes and that they may reflect error prone processes (Strauss, 1997; Strauss, 1998a; Strauss, 1998b; Strauss, 2000). Similarly, many of the excess mutations observed in this study occurred in non-coding regions. It is also interesting to note that examples of multiple mutations at *TP53* have been found both in tumor cells that exhibit microsatellite instability, a

hallmark of mismatch repair deficiency, and in tumor cells that lack microsatellite instability (Strauss, 1998a). Moreover, multiple mutations were observed on mutant human *APRT* alleles in a mismatch repair proficient colon cancer cell line (Harfe and Jinks-Robertson, 2000). The frequency of multiple mutations was also found to increase at the bacterial *lacI* locus in mouse tumors (Buettner et al., 2000). Finally, it has been shown recently that multiple mutations, which appear related to hypermutation that occurs in germinal-center B cells, are common in B-cell diffuse large-cell lymphomas (Pasqualucci et al., 2001).

Taken together, the data from this study and studies by others demonstrate that mismatch repair deficiency can increase the frequency of alleles with multiple mutations, particularly in genotoxin exposed cells, though other instability pathways can also contribute to the formation of these alleles. The data also demonstrate that the PMS2 and MLH1 proteins are not functionally equivalent in protecting genomes from mutation formation. Moreover, locus specific mutagenic effects can contribute further to the types of mutations that occur in mismatch repair deficient malignant cells. While most studies examining causal mutations in mismatch repair deficient tumors have focused on critical loci that have mononucleotide runs, the present study suggests that the spectrum of mutations at cancer relevant loci without such runs should also be examined in these tumors.

A:T → G:C Base Pair Substitutions Occur at a Higher Rate than Other Substitution Events in *Pms2* Deficient Mouse Cells

Chi Y. Shin and Mitchell S. Turker

DNA repair
In Press

ABSTRACT

The mismatch repair pathway involves multiple proteins that are required to correct DNA polymerase generated mismatches before they become mutations. It has been shown recently that the predominant base-pair substitution events leading to loss of endogenous *Aprt* activity in *Pms2* null mouse cells are A:T → G:C mutations (Shin et al., 2002; Shao et al., 2002). To determine if this observation could be explained by an increased rate of A:T → G:C mutations, as compared with other base-pair substitutions, we developed a reversion assay to examine G:C → A:T, C:G → A:T, and A:T → G:C mutations within mouse *Aprt* in a *Pms2* null mouse kidney cell line. The results demonstrated a 6-50 fold increase in the rate of the A:T → G:C mutations relative to the other base-pair substitutions. Additional work demonstrated that growth of the *Pms2* null cells in antioxidant containing medium reduced the rate of the A:T → G:C mutations. The results are discussed with regards to the role of mismatch repair proteins in preventing base-pair substitutions induced by oxidative stress.

INTRODUCTION

The mismatch repair pathway involves multiple proteins that are required to correct DNA polymerase generated errors before they become mutations. Two of these proteins, PMS2 and MLH1 form a heterodimer, MutLα, which is believed to be involved in coupling mismatch recognition to the downstream events of strand discrimination, excision, and resynthesis (Buermeyer et al., 1999). These functions may require the binding of MutLα with the mismatch binding heterodimeric protein MutSα (MSH2/MSH6) or MutSβ (MSH2/MSH3) (Buermeyer et al., 1999).

Recent work examining mutations at mouse *Aprt* in a *Pms2* null kidney cell line and in *Pms2* null splenic T cells revealed that approximately 50% of all base-pair substitutions were A:T → G:C (Shao et al., 2002; Shin et al., 2002). However, because other types of mutational events were observed including those marked by loss of heterozygosity for the expressed allele, neither study provided a direct demonstration that PMS2 deficiency led to an elevated rate of A:T → G:C mutations. To address this issue directly, we examined specific types of "reversion" base-pair substitutions at *Aprt* in *Pms2* null kidney cells. This assay involved stably transfected mutant *Aprt* constructs that would only regain functional expression if A:T → G:C, G:C → A:T or C:G → A:T mutations occurred. The results demonstrate a 6-50 fold elevation in the mutation rate for A:T → G:C mutations as compared with the other base-pair substitution events. Interestingly, the mutation rate for the A:T → G:C substitutions decreased 4-5 fold when the cells were grown in the presence of antioxidants.

MATERIALS AND METHODS

Cell lines. The kidney cell line (K588) used for the reversion assays was derived from a mouse (B6) homozygous deficient for both *Pms2* and *Aprt*. The methods used to create this cell line have been presented previously in pages 22-23. The 6-thioguanine resistant (i.e., *Hprt*-deficient) derivatives of the K588 were used for all experiments because *Hprt* expression can interfere with the selective conditions used to isolate *Aprt* revertants. The K588 cell line was cultured in the growth medium containing Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Rockville, MD) containing 10% (v/v) fetal bovine serum (FBS, Sigma, St. Louis, MO) at 37°C, 5% CO_2. Their transfectant derivatives carrying mutant *Aprt* alleles (see below) were grown in the presence of 1 μg/ml puromycin (Clontech, Palo Alto, CA) for K588 transfectants.

Creation of reversion constructs by site-directed mutagenesis. The reversion constructs to detect specific base pair substitution events were generated by creating TT → CC, T → G, and C → T base pair changes in the non-transcribed strand of *Aprt* plasmids (pSam 3.1) by site–directed mutagenesis (Figure 3). The methods were adopted from the method of Fisher and Pei (1997). The change of TTC (phenylalanine) to CCC (proline) at the wild type 67th codon (exon 3) allows the detection of both G:C → A:T (C → T) and GG:CC → AA:TT (CC → TT) base substitutions; the change of TAT (tyrosine) at the wild type 105th codon (exon 3) to

TAG (stop codon) detected C:G → A:T (G → T); the change of CAG (glutamine) at the wild type 121 st codon to TAG (stop codon) detected A:T → G:C base pair substitutions (T → C).

DNA primers used for these methods are as follows. For G:C → A:T/GG:CC → AA:TT reversion constructs, the sense primer (CCTGTTTGGCCCTTCCCTA) and the antisense primer (CCGCCCTGGAGTCTAG) were used; for C:G → A:T, the sense primer (AAGGTAAGCGAGCTGTGT) and the antisense primer (CCCCTACTCCAGAGAATA) were used; for A:T → G:C, the sense primer (GGTAGAGAGTGGTCGTTGTG) and the antisense primer (ATGAGCACACAGCCCACG) were used. 5'-Phosphorylated forms of these primers were purchased from Life Technologies, Rockville, MD.

The PCR mixtures (50 μl) included 5 to 10 ng of *Aprt* plasmid (pSam 3.1), *Pfu* reaction buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 100 μg/ml bovine serum albumin), 200 μM each dNTPs, 50 pmole of each phosphorylated sense and the antisense primer, 1.25 units *Pfu* DNA polymerase (Stratagene, La Jolla, CA), with the volume adjusted to 50 μl by adding deionized H_2O. The amplification conditions were 1 minute at 94°C and 1 minute at 60°C for annealing. The extension times were initially 7 minutes at 72°C lengthened 5 seconds per cycle, and repeated 17 cycles.

After amplification, the PCR products were electrophoresed in a 1% agarose gel (15 × 22 cm, Life Technologies, Rockville, MD) in TAE buffer at 75 V

for 4 hours, excised, and purified by a Qiagen gel purification kit according to the manufacturer's guide. To digest the methylated template plasmids, all of the purified samples (30 μl) were diluted to a final volume of 100 μl in Dpn1 buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol pH 7.9, 25°C) with 40 units Dpn1 (NEB, Beverly, MA) and incubated at 37°C for 2 hours. To purify the PCR products, an equal volume (100 μl) of phenol:chloroform:isoamyl alcohol (25:24:1) solution was added to the digestion mixture. After vigorously vortexing for a minute, the aqueous solution containing DNA was separated from phenol extracts by centrifugation at the maximum speed (14,000 rpm) for 10 minutes. The aqueous solution was transferred to a new 1.7 ml tube to which 10 μl of 7.5 M ammonium acetate (NH_4OAC) and 200 μl of 100% ethanol were added and mixed. DNA was precipitated by storing the tube at - 80 °C overnight followed by centrifugation at 4 °C at the maximum speed (14000 rpm) for 10 minutes. The DNA pellet was dried at room temperature and resuspended in 10 μl of deionized H_2O. For the ligation, the amplified products were ligated by T4 DNA ligase (Life Technologies, Rockville, MD) in ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000) in 30 μl final volume at 16 °C overnight. The ligated products were purified by increasing the volume to 100 μl by adding deionized H_2O and followed by the addition of an equal volume (100 μl) of phenol:chloroform:isoamyl alcohol (25:24:1) solution. Further purification and

precipitation procedures followed as described above. The DNA was dissolved in 30 µl of deionized H_2O and kept at –20 °C until use.

Transformation of the mutant *Aprt* plasmids. For transformation, 5 µl of the ligation mixture was added into 40 µl (≥ 5×10^8 cells) of DH5α *E. coli*. The mixture of DNA and cells was transferred to a pre-chilled 0.2 cm gap cuvette (Bio-Rad, Hercules, CA) and electroporated at 2.5 V, 25 µF, 200 Ohm. After the electroporation, 1 ml of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added, the mixture was transferred to a 15 ml tube, and incubated at 37 °C for 1 hour. 50 to 100 µl of the solution was plated on LB (Luria broth, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) plates containing 100 µg/ml ampicillin. After overnight incubation at 37 °C, ampicillin-resistant colonies were isolated, inoculated with 10 ml of LB broth containing 100 µg/ml ampicillin, and incubated at 37 °C overnight while shaking.

To identify the *E. coli* cells with mutant plasmids, the plasmids were isolated from 1.5 mls of the ampicillin resistant cells using a Qiagen Mini-Prep Kit. The isolated plasmids were quantified as described previously (page 30). To confirm that the *Aprt* gene was wild type, with the exception of the engineered mutation, 1 µg of each plasmid was sequenced with 4 pmoles of primers that were described previously (page 31-32). To subclone the mutated copy of *Aprt* into a plasmid suitable for transformation into mammalian cells, the plasmids (10 µg)

were digested with 15 units of EcoR1 in EcoR1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl) in a final volume of 100 µl (Life Technologies, Rockville, MD). The digested DNA was separated by 1% agarose gel (15 × 22 cm) electrophoresis in 1 × TAE, 75 V for 5 hours. The band of size 3.1 kilo base pairs corresponding to *Aprt* was excised and purified using a Qiagen gel purification kit. pPur, a selectable puromycin vector (Clontech, Palo Alto, CA) was also digested with EcoR1 in the same condition described above and the gel purified as described above. 2 µg of linearized vector (pPur) was dephosphorylated by alkaline phosphatase (10 units, Boehringer-Mannheim, Indianapolis, IN) in the presence of dephosphorylation buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.5 at 20°C) in a final volume of 100 µl at 37°C for 1 hour. The dephosphorylated pPur vector was further purified with phenol:chloroform:isoamyl alcohol (25:24:1) as described above, with further precipitation as described previously. 0.3 µg of *Aprt* insert was ligated and subcloned as described before into an EcoRI site of pPur vector (1 µg) by T4 ligase (10 units) in a final volume of 30 µl. 5 µl of ligation mixture was transformed into DH5α cells as described previously. The ampicillin resistant cells were isolated and the correct products were confirmed by digesting the plasmids with EcoRI, which resulted in the generation of 3.1 kb Aprt and 4.3 kb pPur vector. A large quantity of the plasmids (reversion constructs) for transfection into mammalian cells was isolated using a Qiagen Maxi-prep kit.

Transfection of mutant *Aprt* plasmids to *Pms2*-proficient and deficient cells. Transfectants were generated by electroporating the reversion constructs into *Pms2* - proficient (Dif6) and deficient cells (K588), which both lack endogenous APRT. The details of transfection follow: 15 μg of each reversion construct were linearized by digestion with ScaI (15 units, Invitrogen, Carlsbad, CA) in the presence of digestion buffer 6 (50 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 50 mM NaCl, and 50 mM KCl) in a final volume of 100 μl at 37°C for 2 hours. The linearized reversion constructs were purified and precipitated as described above. To prepare cells for the transfection, each cell line was grown in two T-75 flasks until it reached 80% confluency and harvested by rinsing the cells with Versene and incubating then with 0.25% trypsin (1.5 mls) as described previously (page 23). The detached cells were resuspended in 30 mls of the growth medium and cells were counted as described previously (page 24). The cells were centrifuged at 1000 rpm and resuspended in electroporation buffer (137 mM NaCl, 5 mM KCl, 0.8 mM Na_2HPO_4, 5.5 mM dextrose, 21 mM HEPES, pH 7.2) to yield 5×10^6 cells/ml. 10 μg of linearized reversion constructs was added to 5×10^6 (1 ml) cells and placed on ice for 10 minutes. The DNA and cell mixture were transferred to a 0.9 cm gap cuvette (Bio rad, Hercules, CA) for mammalian cells and transfected at 0.25 V, 960 μFD. Immediately after the transfection, cells were transferred to a T-25 flask containing 5 mls of the growth medium and incubated at 37°C, 5% CO_2. After 2 days of recovery, cells were rinsed with Versene and trypsinized as

described previously (page 23) and counted as described as before (page 24). 100,000 cells were plated in a 100 mm dish containing 10 ml growth medium, and puromycin-resistant subclones were selected by adding the final concentration of 1 μg/ml puromycin for K588 cells (*Pms2*-deficient).

Determination of copy number. Southern blot analysis (Mummaneni et al., 1993) was used to identify cell lines with 1-2 integrated copies of the reversion constructs. Genomic DNA (15 μg) from 10 to 15 transfectants was isolated as described previously (page 29) and digested with 50 units HindIII (Life Technologies, Rockville, MD) in a final volume of 100 μl in the presence of HindIII reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) by incubating at 37°C overnight. The digested genomic DNA was purified in the equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in the presence of 7.5 M ammonium acetate and 2 volume of 100 % ethanol at –80 °C as described previously. The digested genomic DNA of each transfectant was resuspended in 15 μl Tris-EDTA buffer and electrophoresed overnight at 45 V using a 1% agarose gel (15 × 22 cm). The genomic DNA was transferred to nylon membranes (Hy;bond N+, Amersham Pharmacia, Pisscataway NJ) by a vacuum blottor (Model 785, Bio-Rad, Hercules, CA) according to the manufacturer's guide. The genomic DNA was cross-linked using a UV cross-linker (1200 J/m^2) and the membrane was air-dried and kept at 4°C until use.

The probe was generated by α ^{32}P dCTP-labeling the entire 3.1 Kb fragment (pSam 3.1) containing the *Aprt* gene with a random label kit (Life Technologies, Rockville, MD). 10 µl (100 ng) of DNA was denatured at 95°C for 10 minutes and kept on ice. In another tube, 1 µl dATP, 1 µl dTTP, 1 µl dGTP, 2 µl hexanucleotides, 1 µl Klenow were added and mixed. The total 6 µl of the mixture was added to 10 µl of denatured DNA. 5 µl (50 µCi) of α ^{32}P dCTP was added and incubated at 37°C for 2 hours. The reaction was stopped by adding 80 µl of 25 mM EDTA and this resulted in bringing the volume up to 100 µl. The probe was further purified using equilibrated Sepharose G-50 columns (Boehringer Mannheim, Indianapolis, IN). The Sepharose G-50 columns were first equilibrated by adding 100 µl of Tris-EDTA buffer and by centrifugation at 1600 g for 4 minutes three times. The labeled probe was then loaded in a column and centrifuged at 1600 g for 4 minutes. The flow through (probe) was collected in a 1.5 ml tube and kept at 4 °C until use.

For the hybridization procedure, the membrane was first prehybridized in a hybridization tube containing 15 ml Church buffer (1% bovine serum albumin, 500 mM Na_2HPO_4, 1 mM EDTA, pH 8.0, 7% SDS) while rotating the tube in a hybridization oven (Bio-Rad) at 65°C for 4 hours. All 100 µl of the labeled probe was denatured at 95°C for 10 minutes and added to the bottle containing the membrane and hybridized for 65°C overnight. After the hybridization, the membrane was washed first twice in 50 ml of Low Stringency Wash Buffer (0.5% bovine serum albumin, 1 mM EDTA, 5 % SDS, 40 mM sodium phosphate {68.4

mM Na_2HPO_4, 32 mM NaH_2PO_4}) at room temperature while shaking for 5 minutes and then twice in 500 ml of High Stringency Wash Buffer (1 mM EDTA, 40 mM Na_2HPO_4, 1% SDS) at 65 °C while shaking for 30 minutes. The membrane was air-dried, wrapped in plastic wrap, placed in a cassette with the DNA side up, overlaid with a x-ray film (Kodak, Rochester, NY) overnight at –80 °C. The film was developed by using a film developer.

For each lane, a 1.3 kb band (generated by HindIII digestion) was observed representing a common *Aprt* generated fragment. The number of additional bands was equivalent to the number of *Aprt* constructs integrated in each cell line (*i.e.*, copy number).

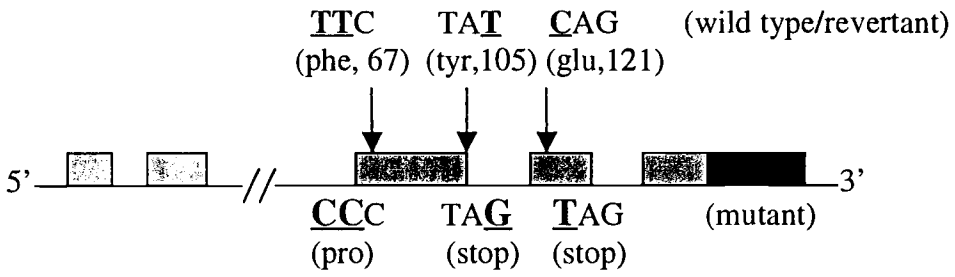
Determination of reversion rates in the presence and the absence of antioxidants. For each cell line tested, 12 to 16 "pools" of 100 to 500 cells each were expanded to approximately 5-20 × 10^6 cells and then 5-10 × 10^6 cells were plated in 3-5 dishes of 150 mm. For determination of plating efficiencies, 500 cells from each pool were plated at the same time in the growth medium. After 24 hours, the cells were exposed to growth medium contain 58 μM azaserine (Sigma, St. Louis, MO) and 76 μM adenine (Sigma, St. Louis, MO) to select for cells that had regained functional APRT enzymatic activity. The plating efficiency plates were not treated with azaserine and adenine. To study the effects of antioxidants, parallel experiments were performed by plating the cells initially in the growth medium containing 20 μM N-acetyl-cysteine (Sigma, St. Louis, MO), 0.8 mM

glutathione (Sigma, St. Louis, MO), and 5 μM Trolox (Sigma, St. Louis, MO). When the cell number increased to approximately 1×10^5, the concentrations of antioxidants were increased to 60 μM N-acetyl-cysteine, 3.2 mM glutathione, and 30 μM Trolox. The total number of cells of each pool and the revertants in each pool was counted and used to calculate the reversion (mutation) rates as described previously (page 25-27).

Isolation of revertants and DNA sequence analysis. The procedures for isolations of revertant cells and their genomic DNA were described in page 24 and 29-30, respectively. To obtain independent mutational events, only 2-5 revertant clones from each experiment were expanded for DNA sequence analysis. To detect all the base pair changes (A:T → G:C, G:C → A:T/GG:CC → AA:TT, and G:C → T:A), exon 3 and exon 4 of *Aprt* were amplified using the primers Mao9 (TCCCACAACCTTCCCTCCTTA) and Aprt4 (CTTAGTCAGCACCCAGGACAG). The PCR mixture (50 μl) included 0.5 μg of genomic DNA, 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 μM $MgCl_2$, 200 μM of each dNTP, 10 pmoles of the primers, 1 unit Taq DNA polymerase (Life Technologies, Rockville, MD), and deionized H_2O to adjust the volume. The PCR conditions were 45 seconds at 94°C and 45 seconds at 72°C for extension. The annealing temperatures started at 67°C and dropped 2°C per cycle until an annealing temperature of 51°C was reached. An additional 25 cycles were then used. All annealing times were for 45 seconds. To detect G:C → A:T/GG:CC

→ AA:TT base pair substitutions, exon 3 was amplified by the sense primer Mao9 (described above) and the anitsense primer APRT7 (AAGACCCTGCCCTTCCTCTAC). The PCR method was the same as above with the exception that the annealing temperature started at 76°C and dropped 2°C per cycle until an annealing temperature of 54°C was reached. The purified PCR fragments were sequenced with an automated fluorescent DNA sequencer (PE/ABD377) using the primer Aprt7 to detect G:C → A:T/GG:CC → AA:TT and G:C → T:A mutations (in exon 3) or Aprt 8 (GAAACGCTCGAGGCTAGGGA) to and A:T → G:C mutations (in exon 4).

Figure 3. Base substitutions introduced in mouse *Aprt* by site directed mutagenesis.



Wild type codons (non-transcribed strand) are shown above the gene schematic and specific introduced mutations are shown below. Coding regions of exons are depicted as filled boxes and 3' non-coding region in exon 5 is shown as a stippled box. Reversion mutations change mutant codons back to wild type codons with the exception of the double substitution at codon 67. In this case single substitutions (TCC, ser or CTC, leu) can also create a functional enzyme. See text for more details.

RESULTS

Elevated rate of A:T → G:C base substitutions in *Pms2* null cells. The reversion assay was used to determine the rate of formation of A:T → G:C, C:G → A:T, and G:C → A:T mutational events in a wild type cell line (DIF6) and in the *Pms2* null epithelial cell line (K588). Two independent transfectants containing one to two copies of the mutant *Aprt* genes were obtained for each construct in each cell type. For the wild type cells, the spontaneous revertant frequencies cells were less than 5 $\times 10^{-8}$. Therefore it was not possible to determine reversion rates in these cells.

In contrast to the results obtained with the wild type cells, revertant cells were detected readily for all three constructs in the *Pms2* null cells thereby confirming the importance of PMS2 in the avoidance of base pair substitution events. Moreover, the high revertant frequencies allowed mutation rates to be determined. As seen in Table 6, the mutation rates for a construct designed to measure A:T → G:C mutations were 6-50 times higher than those for constructs designed to measure the C:G → A:T and G:C → A:T mutational events.

To ensure that the mutation rates were reflecting bona fide base-pair substitution events, a sequence analysis was performed for selected revertant cells. This analysis confirmed that the C:G → A:T (N = 6) and A:T → G:C (N = 19) reversion mutations were occurring. The construct designed to measure G:C → A:T mutational events could also measure double substitutions, GG:CC → AA:TT. The sequence analysis revealed that for this construct 86 % (N=19) of the events were single substitutions and 14 % (N = 3) were double substitutions.

The A:T → G:C mutation rate is reduced in the presence of antioxidants. It has been demonstrated that the repair of oxidatively damaged thymine occurs preferentially on the transcribed strand due to transcription-coupled repair (Cooper et al., 1997). Because the majority of A:T → G:C forward mutations observed in *Pms2* null mouse cells occurred when the thymine base was on the non-transcribed strand (Shao et al., 2002; Shin et al., 2002) we speculated that oxidative damage could account for many of the A:T → G:C mutations in the *Pms2* null cells. The reversion construct used in this study had the thymine base located on the non-transcribed strand. As shown in Table 6, exposure of the K588 cells to an antioxidant mix led to a 70-80% reduction in the rate of A:T → G:C mutations. In contrast, similar reductions were not observed for G:C → A:T or C:G → A:T mutation rates under identical conditions.

Table 6. Reversion rates for *Pms2* null cells in the presence and absence of antioxidants†.

Mutation	Cell lines	Mutation rates	
		Regular medium (standard devation)	Antioxidant medium (standard devation)
A:T → G:C	*Pms2* T → C S 1	10×10^{-6} (4×10^{-6})	2.8×10^{-6} (1.5×10^{-6})
	Pms2 T → C S 15*	17×10^{-6} (6.5×10^{-6})	3.5×10^{-6} (1.7×10^{-6})
G:C → A:T	*Pms2* C → TS 1	0.43×10^{-6} (0.3×10^{-6})	0.4×10^{-6} (0.2×10^{-6})
	Pms2 C → T S 3*	0.9×10^{-6} (0.5×10^{-6})	1.4×10^{-6} (0.7×10^{-6})
C:G → A:T	*Pms2* G → T S 2	0.34×10^{-6} (0.2×10^{-6})	0.6×10^{-6} (0.3×10^{-6})
	Pms2 G → T S 5	1.7×10^{-6} (0.6×10^{-6})	1.2×10^{-6} (0.9×10^{-6})

† See Materials and Methods for antioxidant conditions.
* These cell lines have two copies of reversion constructs. The others have a single copy.
Experimental data are shown in Table 16, 17 (Appendix).

DISCUSSION

In a prior study examining mutagenesis at mouse *Aprt* in *Pms2* null kidney cells, we observed that approximately 50% of all intragenic mutations were A:T → G:C base-pair substitutions (Shin et al., 2002). A similar observation was made for *Aprt* mutant T-cells in *Pms2* null mice (Shao et al., 2002). The data were obtained in both studies with a "forward" mutation assay that detects a wide range of mutational events leading to loss of *Aprt* expression. Other investigators have not specifically noted that that the A:T → G:C transitions are prominent in *Pms2* null cells, perhaps because their mutational targets contain mono and dinucleotide runs (Andrew et al., 2000; Baross-Francis et al., 2001; Kato et al., 1998; Narayanan et al., 1997; Prolla et al., 1998; Yao et al., 1999). Such runs act as hot spots for frame shift mutations in mismatch repair deficient cells. The mono and dinucleotide runs in the coding region of mouse *Aprt* are relatively short and do not serve as true hot spots (Shin et al., 2002).

Confirmation of an elevated rate of A:T → G:C mutation in *Pms2* null cells, relative to other base-pair substitution events, might provide clues about the function of the different mismatch repair proteins in preventing mutations. Therefore, in this study I used reversion assays to determine the rate of three different base-pair substitution events in *Pms2* null cells. The results demonstrated that the rate of A:T → G:C mutations is indeed substantially higher than the rates

for two other types of base-pair substitution events, G:C → A:T and C:G → A:T in the *Pms2* null cells.

PMS2 protein binds to MLH1 to form the functional MutLα heterodimer (Buermeyer et al., 1999). However, the mutagenic consequences of losing either of these proteins are not functionally equivalent as higher mutation rates and mutant frequencies occur in *Mlh1* null cells than in *Pms2* null cells (Baross-Francis et al., 2001; Shin et al., 2002; Yao et al., 1999). These observations have led to the suggestion that a heterodimer of MLH1 and PMS1, termed MutLβ (Räschle et al., 1999), and/or of MLH1 and MLH3 can provide a small, but detectable, compensation for loss of PMS2 (Buermeyer et al., 1999). If so, a potential explanation for our results is that the compensating heterodimers lack activity to correct damagedA:C or damagedT:G mismatches, whereas they have limited activity to correct other mismatched base-pairs. Further work to measure base-pair substitution rates in cells with double knockout mutations (e.g., *Pms2* and *Pms1*) will be required to address this possibility.

The observation of a 70-80% reduction in rate of formation of the A:T → G:C mutations when the *Pms2* null cells were grown in medium containing anti-oxidants suggests a role for oxidative DNA damage. Oxidative damage to both thymine and adenine bases have been described (Cadet et al., 1999). Because the forward mutation assay suggested a strand bias in which the A:T → G:C mutations in *Pms2* null cells are mostly involved thymine on the non-transcribed strand (Shao et al., 2002; Shin et al., 2002), I propose that oxidative damage to thymine is the

promutagenic lesion. Oxidation of thymine to 5-formyluracil promotes the formation of T:G mispairs (Masaoka et al., 2001), which could account for the A:T → G:C mutations, and thymine glycols have been reported to cause A:T → G:C mutations in *E.coli* (Basu et al., 1989). However, mutations induced when 2-hydroxyadenine is the promutagenic lesion are also A:T → G:C substitutions (Kamiya and Kasai, 1997), which means that this form of oxidative damage cannot be ruled out as playing a role in mutation formation. Additional work will be required to determine the types of mutations that can be caused by the different forms of oxidatively damaged thymine and adenine bases, with particular emphasis on how damage is processed into mutational events. In this regard, the *Pms2* null cells provide a sensitive background for these studies.

Although 8-oxoguanine is known to cause C:G → A:T mutations (Moriya, 1993), the rate of formation of this mutation was not altered by growth of the *Pms2* null cells in the presence of antioxidants. G:C → A:T mutations most likely result from spontaneous deamination of cytosine (Hardeland et al., 2001), which might explain why the formation of these mutations were not altered by growth of the cells in antioxidants.

In summary, I have used a reversion assay to confirm that A:T → G:C mutations occur at a higher rate than two other types of base-pairs substitutions in *Pms2* null kidney cells, including the most common spontaneous base-pair substitution in wild type cells (Hardeland et al., 2001) and *Mlh1* null cells (Shin et al., 2002), G:C → A:T. I have also provided data suggesting that oxidative damage

is involved in the formation of A:T → G:C mutations. My interpretation is that one or more MLH1-containing heterodimers have limited ability to repair some types of mispairs when PMS2 protein is absent, but significantly less ability to repair oxidative damage dependent A:C and/or T:G mispairs. Further work with *Pms2* null cells that are deficient for additional MutL proteins should help identify these heterodimers and their role in preventing the formation of base-pair substitutions.

A Role for Mismatch Repair in the Prevention of Tandem CC → TT Substitutions Induced by Oxidative Stress and Ultraviolet Radiations

Shin CY and Turker MS

ABSTRACT

DNA mismatch repair (MMR) is important for preventing base pair substitutions induced by DNA polymerase error, oft times in response to DNA damage. I have used a reversion assay to investigate the role of MMR in preventing UV irradiation and oxidative stress- induced C → T and tandem CC → TT base pair substitutions. C → T and tandem CC → TT base pair substitutions are the signature mutations of UV irradiation and it has been suggested that they are also the signature mutations for oxidative stress. However, the induction of these mutations by oxidative stress has not been demonstrated in mammalian cells. An analysis of spontaneous mutation in *Pms2* null cells revealed that 86% were single substitutions and the remaining 14% were tandem substitutions. The percent of tandem substitutions rose to 67% and 92% for cells exposed to UV and a mixture of hydrogen peroxide and metals (Cu/Fe), respectively. Exposure to hydrogen peroxide alone did not induce tandem substitutions. Tandem substitutions were also induced by UV irradiation and the hydrogen peroxide/metal mixture in *Pms2* proficient cells, but at frequencies significantly lower than those observed in the *Pms2* null cells. The study demonstrated that MMR is required to prevent tandem CC → TT mutations in the mammalian genome induced by UV irradiation and Fenton reaction –mediated oxidative stress.

INTRODUCTION

Cellular DNA is susceptible to damage by many compounds originating from endogenous and exogenous sources. DNA damage, if not repaired, can become a mutation. Mutation formation is known to contribute to the development and progression of cancer. Considering the very low spontaneous DNA polymerase error rates in normal cells, it was suggested that DNA damage is a major contributor to the induction of base pair substitutions. The major pathway to prevent base pair substitutions is DNA mismatch repair. The primary function of mismatch repair is to maintain genetic stability by repairing replication errors such as mismatched bases and small insertions/deletions. The importance of MMR in cellular function is clinically manifested in hereditary non-polyposis colon cancer (HNPCC) resulting from a MMR defect (Altonen et al., 1993). Recent studies have shown that mutations are increased in MMR deficient cells after exposure to DNA damaging agents such as alkylating agents (Hickman et al., 1999), ionizing radiation (Xu et al., 2001), and hydrogen peroxide (Glaab et al., 2000). This suggests that MMR has a broad range of substrates to recognize and repair. Biochemical studies also showed that MMR recognized mismatched DNA lesions such as photoproducts (Wang et al, 1999) and 8-oxo-guanine (Ni et al, 1999), caused by two major mutagens, UV irradiation and oxidative stress, respectively.

UV irradiation acts as a mutagen and carcinogen by causing DNA damage such as cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)-pyrimidone photoproducts. These types of lesions are largely repaired by

nucleotide excision repair (NER) (Friedberg et al., 1995). Inactivation or reduced function of NER is associated with Xeroderma Pigmentosum (XP) (Bootsma et al., 1998). Individuals with XP are prone to develop skin cancers (van Steeg et al, 1999; 2000). The role of mammalian MMR in preventing mutations induced by UV irradiation was previously suggested with cell lines that lack both NER and MMR (Nara et al, 2001). These cells showed a significant increase in mutant frequencies when compared with a cell line that only lacked NER. However, the exact role of MMR in preventing UV-induced mutations has not been addressed.

Reactive oxygen species (ROS) causes endogenous oxidative stress, which is considered to be a major contributor to the formation of DNA damage (Ames et al, 1992). ROS is a collective term for radical and non radical oxygen intermediates such as superoxide or hydroxyl radicals and hydrogen peroxide or singlet oxygen, respectively. ROS is generated from normal cellular metabolism such as mitochondrial respiration (Chance et al, 1979) and the oxidative burst from activated immune cells (Shacter et al, 1988; Adams et al, 1992). They are also generated by other sources such as ionizing radiation (Nikjoo et al, 1994; Skov 1984) and transition metals (Halliwell et al, 1986). Of these mechanisms, ROS generation by transition metals has been of particular interest since transition metals such as iron and copper are present not only in the structure of many enzymes as prosthetic groups that catalyze redox reactions, but also are bound to chromatin in the nucleus (Marsden et al., 1979). Cellular transition metals are

involved in endogenous oxidative stress by generating hydroxyl radicals through the Fenton reaction:

$$Fe^{2+} (Cu^{+}) + H_2O_2 \rightarrow Fe^{3+} (Cu^{2+}) + OH^{-} + HO\bullet$$

A combination of metal availability and increased production of hydrogen peroxide may generate an elevated level of hydroxyl radicals that can damage cellular molecules including DNA (Imlay et al, 1988; Dizdaroggla et al, 1991; Michalik et al, 1995; Blakely et al, 1990). ROS–induced DNA damage has been implicated in pathological conditions such as cancer and degenerative diseases (Floyd 1990; Ames et al, 1992; Jenner 1994). ROS causes numerous DNA lesions with mutagenic potentials that are not clearly understood. Among ROS-induced DNA lesions, 8-oxoguanine causes G → T transversions that have been identified as a biomarker for oxidative stress (Helbock et al, 1999). Other studies have suggested that C → T transitions are the most common mutations resulting from ROS-induced DNA lesions (McBride et al., 1991), perhaps because deoxycytidine (dC) is susceptible to damage from the Fenton reaction (Lio et al., 1996). Previous studies with in vitro treated single stranded DNA have shown that a mixture of hydrogen peroxide and metals induced C → T and less frequently tandem CC → TT mutations in *E. coli* (Reid et al., 1991; 1993). These authors suggested the tandem CC → TT mutations as an additional biomarker for endogenous oxidative

stress. However, oxidative stress induced CC → TT mutations have not been reported in mammalian cells.

In mammalian systems, forward mutation analysis has been commonly used to study mutagenesis. However, scanning genes for rare mutations is a difficult task, and it is also hard to distinguish mutations of interest from a variety of other types of mutations. To investigate the potential mutagenic effects of hydrogen peroxide and transition metals on cytosines, I have developed a mammalian reversion assay specific for detecting C → T and CC → TT mutations. C → T and CC → TT mutations are well known UV- induced mutational signatures. Using this approach, I investigated the role of mismatch repair in preventing C → T and CC → TT mutations induced by oxidative stress and UV irradiation.

In this study, I demonstrated that tandem CC → TT base pair substitutions were significantly elevated in *Pms2*-deficient cells after exposure to UV irradiation and hydrogen peroxide/metals mixture.

METHODS AND MATERIALS

Cell lines. A *Pms2* -proficient cell line, Dif6, is a differentiated version of an embryonal carcinoma cell line that lacks endogenous APRT activity. Dif6 cells transfected with reversion constructs were cultured in the Dulbeco's Modified Eagle's Medium (DMEM, Life Technologies, Rockville, MD) supplemented with 5% (v/v) FBS (Sigma, St. Louis, MO), 5% (v/v) serum plus (JRH, Bioscience, Lenexa, KS), and 1.5 μg/ml puromycin (Clontech, Palo Alto, CA) at 37°C, 5%

CO_2. The *Pms2*-deficient cell line (K588) was derived from a mouse (C57BL/6 strain) homozygous deficient for both *Pms2* and *Aprt* using the procedure described on pages 22-23. Their transfectants (Pms2C → TS1 and Pms2C → TS3) were cultured in DMEM containing 10% (v/v) FBS and 1 μg/ml puromycin. Non-transfectants of both cell types were grown in the same conditions as above without puromycin.

Generation of the reversion construct and cell lines for the reversion assay.

The reversion construct (G:C → A:T/GG:CC → AA:TT) was generated by creating TT → CC base pair changes in the non- transcribed strand of *Aprt* by site – directed mutagenesis (page 57-60). To detect C → T and CC → TT base substitutions, the wild type 67^{th} codon, TTC (phenylalanine) in the exon 3 was changed to CCC (proline) (Figure 3). The mutated copy of *Aprt* was subcloned into a selectable puromycin vector (pPur, Clontech, Palo Alto, CA) (page 61). The transfectants were generated by transfecting the reversion constructs into *Pms2*-proficient and deficient cells that both lack endogenous *Aprt* via electroporation (page 62-63). A Southern blot analysis was used to identify transfectants with one or two copies of the reversion constructs as described previously (page 63-65).

Determination of revertant frequencies after UV irradiation and oxidative stress. The basic method for determining genotoxin-induced mutant frequencies was described in detail (page 27-28). For UV–induced revertants, 4×10^6 of both

Pms2-proficient and deficient cells were distributed equally into four 100 mm dishes (1×10^6/dish) per dose. After 24 hours, the cells were treated at doses ranging from 4.8 to 14.4 J/m^2. After exposure, the cells were trypsinized and replated into T-75 flasks. After 6 days of recovery, 6 million cells were plated into three 150 mm dishes (2 million cells per dish). A 150 mm dish contained 20 mls of the growth medium as a final volume. For non-treated controls, the same number of cells was grown in parallel with the exposed cells prior to plating into the 150 mm dishes. After 24 hours, the UV-exposed and untreated cells were treated with azaserine (58 μM, Sigma, St. Louis, MO) and adenine (76 μM, Sigma, St. Louis, MO) to select for *Aprt* revertants. To determine the plating efficiencies, 500 treated or non-treated cells were plated in duplicates using a 100 mm dish containing 10 ml growth medium to which azaserine and adenine were not added.

For H_2O_2 or $H_2O_2/CuSO_4/FeSO_4$-induced revertants, 7×10^5 cells were plated into T-75 flask (total 5 flasks), one for untreated control and four for treatment. After 24 hours, the cells in 4 flasks were treated with H_2O_2 alone (50 to 265 μM for wild type cells and 25 μM for *Pms2*-deficient cells) or with a $H_2O_2/CuSO_4/FeSO_4$ mixture (100/250/250 μM for wild type cells and 15/150/150 μM for *Pms2*-deficient cells). For each experiment, the stock solutions (100 × for H_2O_2, 1000 × for $CuSO_4$, 100 × for $FeSO_4$) were prepared in deionized H_2O, filter-sterilized, and added to the growth medium to obtain the final concentration. During the 6 day recovery period, the growth medium of the exposed cells was replaced once to remove dead cells and untreated control cells were split once (1:3

split). After the recovery period, 10 million cells from each flask were plated into five 150 mm dishes (2 million cells per a 150 mm dish). After 24 hours, azaserine (58 μM) and adenine (76 μM) were added to the medium to select the revertants. For the plating efficiencies, 500 cells of each treated and non-treated cells were plated in duplicate using a 100 mm dish containg 10 ml growth medium without azaserine and adenine. After 14 days of incubation, 3 to 5 *Aprt* revertants were isolated from four to five 150 mm dishes representing a T-75 flask to obtain independent revertants by following the methods described previously (page 24). The dishes were then stained with crystal violet solution as described before (page 27). The revertant frequency was determined by using the equation described previously (page 28).

Mf= the number of *Aprt* revertant / (the number of total plated cells × % plating efficiency / 100)

Isolation of genomic DNA. The genomic DNA of revertants was isolated as described before (page 29-30). The concentrations of the genomic DNA was determined as described (page 30).

Sequence analysis. The mutational events, which led to reversion were confirmed by sequencing the exon 3 as described previously in pages 66-67. To obtain independent mutational events, only 2-4 revertant clones from each experiment were expanded for a sequence analysis.

RESULTS

Validation of the reversion assay using UV exposure in *Pms2*-proficient cells. The validity of C → T/CC → TT reversion assay was confirmed by UV irradiation of *Pms2*-proficient cells. UV causes C → T and CC → TT base pair substitutions at dipyrimidine sites. The TT → CC base changes introduced at the 67th codon resulted in 5 consecutive cytosines (5'-GC *TTC* CT-3' → 5'-GC *CCC* CT-3'), which provided a substrate for detecting UV-induced C → T and CC → TT base pair substitutions. The revertant frequencies for *Pms2*-proficient cells (Dif6S6) with one copy of the reversion construct increased in a dose dependent manner, 11, 20, and 35×10^{-6} at doses of 9.6, 12, and 14.4 J/m^2, respectively (Table 7). The dose dependent response demonstrated that the exposure to UV was responsible for regain of APRT function. The cells (Dif6S2) with 2 copies of the reversion constructs increased the revertant frequency 5 to 6 times higher than the cells with one copy. The reason for this elevated response, which should have been only 2 fold, is not known. The cell requires reversion of only one mutant copy to restore APRT activity and the presence of mutated inactive *Aprt* copies will not interfere with the selection for a reverted functional *Aprt* copy. The sequence analysis showed that induced revertants were due to both single C → T changes (85%) resulting in CTC (leucine) and tandem CC → TT (15%) resulting in TTC (phenylalanine) and CTT (leucine) (Table 8). These relative percentages are

Table 7. The reversion frequency of spontaneous and induced C → T/CC → TT in *Pms2*-proficient cells (Dif6S6)

A. Ultraviolet (UV) light		
	Dif6S6 (one copy)	Dif6S2 (two copy)
Control (untreated)	$< 2 \times 10^{-7}$	$< 3 \times 10^{-7}$
9.6 J/m^2	11×10^{-6}	53×10^{-6}
12 J/m^2	20×10^{-6}	132×10^{-6}
14.4 J/m^2	35×10^{-6}	ND
B. H_2O_2		Dif6S6 (one copy)
Control (untreated) (Expt 1-7)		$< 4 \times 10^{-8}$
Expt 1 265 μM		$< 1 \times 10^{-7}$
Expt 2 265 μM		$< 1 \times 10^{-7}$
Expt 3 265 μM		$< 1 \times 10^{-7}$
Expt 4 265 μM		0.6×10^{-6}
Expt 5 265 μM		0.4×10^{-6}
Expt 6 180 μM		$< 1 \times 10^{-7}$
Expt 7 50 μM		$< 1 \times 10^{-7}$
C. H_2O_2/ $FeSO_4$/ $CuSO_4$ (100/ 250/ 250 μM)		Dif6S6 (one copy)
Control (untreated) (Expt 1-9)		$< 4 \times 10^{-8}$
Experiment 1		1×10^{-6}
Experiment 2		$< 1 \times 10^{-7}$
Experiment 3		$< 1 \times 10^{-7}$
Experiment 4		0.7×10^{-6}
Experiment 5		$< 1 \times 10^{-7}$
Experiment 6		0.2×10^{-6}
Experiment 7		$< 1 \times 10^{-7}$
Experiment 8		$< 1 \times 10^{-7}$
Experiment 9		1×10^{-6}

All the frequencies were adjusted by cloning forming efficiency.
ND indicates not determined.
Experimental data are provided in Table 14 (Appendix).

Table 8. The reversion spectrum of mutated codon 67 (CCC;pro) in *Pms2*-proficient cells.

	Pms2-proficient cells			
	Sponta-neous	UV	H_2O_2	H_2O_2/ Metals
A Single C → T				
GC CCC CT→ GC TCC CT (Pro) (Ser)				
GC CCC CT→ GC CTC CT (Pro) (Leu)		17 (85%)	5 (100%)	
		85%	*100%*	*0%*
B. Tandem CC → TT				
GC CCC CT→ GC TTC CT (Pro) (Phe)		2 (10%)		21 (100%)
GC CCC CT→ GC CTT CT (Pro) (Leu)		1 (5%)		
GC CCC CT→ GT TCC CT (Pro) (Ser)				
		15%	*0%*	*100%*
C. The number of revertants analyzed		20 (100%)	5 (100%)	21 (100%)
D. The number of independent pots.(revertants obtained from)		4	2	3

consistent with those observed with a forward mutational assay at *Aprt* in wild type cells exposed to UV (Khattar et al, 1997). Spontaneous reversion was not detected in *Pms2*-proficient cells (Table 7).

The UV results not only validated the reversion assay, but also demonstrated the usefulness of codon 67 to detect both C → T and CC → TT mutations. This allowed me to compare the relative frequency of both types of mutational events in cells exposed to oxidative stress.

Induction of C → T and CC → TT mutations by oxidative stress is rare in *Pms2* – proficient cells. After confirming the reversion assay with UV exposure, I investigated whether C → T and CC → TT mutations could be induced by oxidative stress including hydrogen peroxide alone and hydrogen peroxide with transition metals. In *Pms2*–proficient cells, the induction of revertants was low, barely measurable above background (Table 7). With hydrogen peroxide alone, only two of 7 trials yielded revertants with frequencies, ranging 0.4 to 0.6×10^{-6}. By adding all the numbers from the seven trials, I determined a hydrogen peroxide induced frequency of 0.14×10^{-6}. After exposure to the hydrogen peroxide/metal mixture, the induction of revertants was still low with only 4 of 9 trials yielding revertants with frequencies, ranging 0.2 to 1×10^{-6}. The accumulated frequency induced by hydrogen peroxide/metals was 0.32×10^{-6}. No spontaneous revertants were observed from a total 5×10^{8} cells that were tested. However, a sequence analysis of the revertants showed the mutagenic effects of hydrogen peroxide/metal

mixture was potentially different from those of hydrogen peroxide alone (Table 8). Hydrogen peroxide induced C → T mutations, whereas the hydrogen peroxide/metal mixture induced CC → TT mutations.

UV and H_2O_2/transition metals-induced tandem CC → TT base substitutions are increased in *Pms2* null cells. Unlike in the *Pms2*-proficient cells, the spontaneous revertant frequencies were significantly elevated in the *Pms2*–deficient cells (Table 9). The sequence analysis showed that the spontaneous revertants resulted from predominantly single C → T changes (86 %). The change of proline (CCC) to leucine (CTC, 54%) was observed more frequently than to serine (TCC, 32%). Spontaneous tandem CC → TT mutations were also detected in *Pms2* – deficient cells at a lower percentage (14%).

To investigate the role of MMR in preventing UV-induced C → T and CC → TT mutations, I irradiated the *Pms2* null cells with various doses of UV. As shown for the Pms2-proficient cells the revertant frequencies were increased in the *Pms2*-deficient cells (Pms2C → TS1). The sequence analysis (Table 8,10) of UV-induced revertants revealed that the percentage of tandem CC → TT mutations were higher in the *Pms2* deficient cells (67%) for the non-exposed *Pms2* null cells (14%). The tandem mutations resulted in mainly TTC (phenylalanine, 58%) and TCC (serine, 8.3%) in which the adjacent cytosine was also changed to thymine.

Table 9. The revertant frequencies of Pms2- deficient cell in spontaneous and induced conditions

		Pms2C → TS1 (one copy)	Pms2C → TS3 (two copy)
A. Ultraviolet (UV) light			
Control (untreated)		0.9×10^{-6}	21×10^{-6}
4.8 J/m^2		ND	369×10^{-6}
9.6 J/m^2		39×10^{-6}	ND
B. H_2O_2 (25 μM)			
Expt 1	Control	4.5×10^{-6}	8×10^{-6}
	Treated	14×10^{-6}	22×10^{-6}
Expt 2	Control	7×10^{-6}	66×10^{-6}
	Treated	2×10^{-6}	91×10^{-6}
Expt 3	Control	7×10^{-6}	ND
	Treated	1×10^{-6}	ND
C. $H_2O_2/FeSO_4/CuSO_4$ (15/150/150 μM)			
Expt 1	Control	0.7×10^{-6}	15×10^{-6}
	Treated	2.1×10^{-6}	52×10^{-6}
Expt 2	Control	18×10^{-6}	ND
	Treated	20×10^{-6}	ND
Expt 3	Control	138×10^{-6}	ND
	Treated	32×10^{-6}	ND
Expt 4	Control	1×10^{-6}	ND
	Treated	2.5×10^{-6}	ND

ND indicates not determined.

Table 10. The reversion spectrum of mutated codon 67 (CCC;pro) in *Pms2*-deficient cells.

	Pms2- deficient cells			
	Sponta-neous	UV	H_2O_2	H_2O_2/ Metals
A Single C → T				
GC CCC CT→ GC TCC CT (Pro) (Ser)	7 (32%)	2 (8.3%)	5 (36%)	2 (8%)
GC CCC CT→ GC CTC CT (Pro) (Leu)	12 (54%)	6 (25%)	6 (43%)	
	86%	*33%*	*79%*	*8%*
B. Tandem CC → TT				
GC CCC CT→ GC TTC CT (Pro) (Phe)	3 (14%)	14 (58.4%)	3 (21%)	24 (92%)
GC CCC CT→ GC CTT CT (Pro) (Leu)				
GC CCC CT→ GT TCC CT (Pro) (Ser)		2 (8.3%)		
	14%	*67%*	*21%*	*92%*
C. The number of revertants analyzed	22 (100%)	24 (100%)	14 (100%)	26 (100%)
D. The number of independent pots.(revertants obtained from)	14	6	5	9

The mutant frequencies were not elevated consistently in the *Pms2*-deficient cells exposed to hydrogen peroxide alone and the hydrogen peroxide/metal mixture (Table 9). However, a mutagenic effect was still observed for cells exposed to hydrogen peroxide and metals when a sequence analysis of revertants was performed. In this case 92% of the revertant mutations were tandem substitutions as compared with only 14% of spontaneous reversion events and 23% for cells exposed only to hydrogen peroxide. This represents as increase of approximately 63 times (20×10^{-6}, 92% of 22×10^{-6}), when compared the induction of CC $\rightarrow$ TT in *Pms2*-proficient cells.

DISCUSSION

Damage to DNA bases can cause base pair substitutions if they escape DNA polymerases and/or DNA repair make errors (Kunz et al., 2000). Mismatch repair represents an important mechanism for avoiding such mutations (Buermeyer et al., 1999). The primary function of MMR is to recognize and repair mismatched bases and small insertion/deletion loops formed during replication (Modrich, 1991; 1994). Although MMR has been implicated in mutation avoidance after cellular exposure to DNA damaging agents, direct evidence is still required to demonstrate a role of MMR in preventing specific types of DNA damage–induced base pair substitutions. I used a reversion assay to evaluate the role of MMR in preventing UV and oxidative stress-induced C → T and tandem CC → TT base pair substitutions. In my study, the role of MMR in preventing these base pair changes after exposure to UV irradiation and oxidative stress was shown by increased reversion frequencies and altered mutational spectra.

In prokaryotic cells, mismatch repair deficient bacteria showed two fold induction of UV-induced C → T and T → C base pair changes relative to wild type and a 25 fold increase in UmuC background, indicating an antimutagenic role for MMR in UV irradiation induced mutagenesis (Liu et. al., 2000). In my study, *Pms2*-deficient cells showed hypermutability to UV irradiation as demonstrated by an elevated revertant frequency compared to *Pms2*-proficient cells. Additional evidence for an anti-mutagenic role of MMR for UV mutagenesis was revealed by the sequence analysis of the revertants. Upon UV irradiation, *Pms2* deficient cells

increased tandem mutations to 67% from 15% for spontaneous revertants in *Pms2*-deficient cells and 15% for UV- induced revertants in *Pms2*-proficient cells (Table 8, 10).

UV irradiation produces CPDs and (6-4) photoproducts on cytosines resulting in C → T and CC → TT changes either by insertion of an A or AA opposite the damaged cytosine(s) during DNA replication (Armstrong et al., 1990; Brash et al., 1997; Canella et al., 2000) or by deamination of the CPDs (Peng and Shaw, 1996). The induction of tandem CC → TT mutations by UV irradiation in wild type cells occurs in approximately only 5-15% of mutant cells (Drobetsky et al., 1994; Khattar and Turker, 1997; Kusewiett et al., 1998). However, tandem CC → TT mutations increased in DNA nucleotide excision repair deficient tumors and non-tumor tissues (Spatz et al, 2001). Mutation analyses on p53, INK4a-ARF, and PTCH genes in NMSC from XP individuals showed 50 to 67% tandem mutations, whereas non-XP tumors exhibited 10-15% tandem mutations (Dumaz et al., 1993; Bodak et al., 1999; Giglia et al, 1999; Soufir et al, 2000). A study with XPA knockout mice also showed that CC → TT mutations were detected at higher frequency in the rpsL transgene in the UVB-irradiated XPA- knockout mice than in irradiated wild type mice (Tanaka et al., 2001). XP, a hereditary disease is caused by inactivation of nucleotide excision repair (NER) that is required for repairing UV- induced base damages. As a consequence, increased mutation frequencies are observed upon UV-induced DNA damage (Daya-Grosjean et al., 1993), and individuals with XP suffer from highly elevated levels of sunlight-induced skin

cancers (Kraemer, 1997). These studies showed that NER is required for preventing UV-induced tandem mutations.

Several pathways prevent the formation of UV-induced mutations. NER repairs photoproducts before DNA replication initiates and prevents mutations. If damage persists during replication, error- free DNA polymerase η inserts correct bases opposite unrepaired photoproducts reducing the number of UV-induced mutations (Washington et al., 2000; Johnson et al., 1999; 2000). XPV (XP variant) is caused by inactivation of this error free polymerase η (Masutani et al., 1999). XPV patients have functional NER but show high incidence of skin cancers (Spatz et al., 2001). My work showed tandem CC → TT mutations were elevated in *Pms2* deficient cells at a level similar to NER-deficient tumor cells indicating an important role for MMR to provide additional protection against UV-induced mutations along with NER and polymerase η.

Previous studies reported that a mixture of hydrogen peroxide and metals (Fe or Cu) induced CC → TT mutations in *E. coli* and suggested that these tandem mutations can serve as oxidative stress induced signature mutations (Reid et al., 1991; 1993). Despite inconsistent induction by oxidative stress in the *Pms2*-proficient cells (Table 7), the sequence analysis showed that the hydrogen peroxide/metal mixture induced CC → TT mutations while hydrogen peroxide alone did not (Table 8). In *Pms2*- deficient cells, the induction of revertants showing tandem CC → TT mutations was elevated 54 fold compared to *Pms2*-proficient cells (Table 10). My data confirms hydrogen peroxide/metal mixture

induces tandem CC → TT mutations and MMR may be required to prevent these mutations.

Oxidative stress is mediated by reactive oxygen species. It was suggested that base damage depended on the types of reactive oxygen species (McBride et al., 1992). For instance, cytosine residues are susceptible to hydroxyl radicals (Luo et al., 1996). Hydroxyl radicals are produced by the Fenton-reaction in the presence of both hydrogen peroxide and metals (Cu and Fe). Damage to cytosines produces various chemically modified products (Luo et al., 1996) with single C → T as the most common mutation (Tkeshelachvili et al, 1991, McBride et al., 1991). At this point, I do not know if the induction of CC → TT by oxidative stress and UV irradiation resulted from the same types of damage to cytosine or it was simply due to the "A" rule of DNA polymerases. It is noted that CC → TT mutations also occur spontaneously in *Pms2*- deficient cells along with the predominant C → T mutations. I believe that deamination of cytosines or methyl-cytosines are responsible for spontaneous C → T and CC → TT mutations. Deamination induced tandem CC → TT mutations were induced by sodium bisulfite treatment (Chen et al., 1994).

My data strongly suggest that MMR is required for preventing both UV and oxidative stress induced tandem CC → TT base pair changes. Further work will be required to determine the types of damage that are involved and whether error prone DNA synthesis contributes to these mutations.

A Mouse Kidney Cell Line with a G:C → C:G Transversion Mutator Phenotype.

Chi Y. Shin, Olga Ponomareva, Lanelle Connolly, and Mitchell S. Turker

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ABSTRACT

I report the identification of a mouse kidney epithelial cell line (K435) in which G:C → C:G transversion mutations occur at an elevated rate and are the predominant spontaneous events observed at the selectable *Aprt* locus. Of three genotoxins tested, ultraviolet radiation (UV), ionizing radiation, and hydrogen peroxide, only UV exposure was able to alter the spectrum of small mutational events. To determine if the G:C → C:G mutator phenotype was due to a deficiency in the mismatch repair pathway, the K435 cells were tested for resistance to 6-thioguanine, cisplatin, and MNNG. Although the K435 cells were as resistant to 6-thioguanine and cisplatin as *Pms2* and *Mlh1* null kidney cells, they were hypersensitive to MNNG. Moreover, the K435 cells do not exhibit microsatellite instability, a hallmark of mismatch repair deficiency. These results suggest that a novel mechanism, which does not include a classical deficiency in mismatch repair, accounts for the G:C → C:G mutator phenotype.

INTRODUCTION

Base-pair substitution mutations originate with the insertion of an incorrect base by a DNA polymerase, oft times augmented by the presence of a damaged base on the template strand. Mutation avoidance can be achieved by proofreading removal of the misinserted base (Kunkel and Bebenek, 2000), or if this polymerase-related function fails, by action of the mismatch repair pathway (Buermeyer et al., 1999). The rates at which different base-pair substitutions occur are a function of several variables including the fidelity of the polymerases for correct insertion of the four bases, the proofreading abilities of the polymerases, the different susceptibilities of the DNA bases to undergo spontaneous damage, and the repair of this damage (Kunkel and Bebenek, 2000), For example, the most common base-pair substitution in mammalians cells, the C:G → T:A transition, is believed to be due in part to deamination of cytosine, particularly when it is methylated (Schmutte and Jones, 1998). The mutation rate for this transition event is increased further in cells deficient for the *Mlh1* mismatch repair gene (Baross-Francis et al., 2001; Shin et al., 2002). G:C → T:A transversions are increased in yeast cells deficient for the OGG1 protein (Scott et al., 1999), which removes oxidatively damaged guanine bases from DNA. Therefore, the observation of a mutator phenotype for a specific base-pair event can sometimes suggest the presence of a specific DNA repair defect.

In this study, I report the identification of mouse kidney cell line (K435) that exhibits an elevated rate of spontaneous G:C → C:G transversions. Of three

mutagens tested (ultraviolet radiation, ionizing radiation, and hydrogen peroxide), only UV irradiation was able to induce additional base-pair substitutions events (G:C → A:T) demonstrating radiation mutagenesis in K435 cells. Although the K435 cells are as resistant to 6-thioguanine and cisplatin as mismatch repair deficient cells, it is hypersensitive to MNNG suggesting that a novel mechanism underlies the transversion mutator phenotype.

METHODS AND MATERIALS

Cell lines. The K435 cells were clonally derived from a kidney cell preparation obtained from a B6D2 hybrid mouse using methods described on pages 22-23. This mouse was heterozygous for *Aprt*, but wild type for other loci. Additional wild type (KO6), *Pms2*-deficient, and *Mlh1*-deficient kidney cell lines that are used in this study were reported previously (Shin et al., 2002; Turker et al., 1999). These cell lines are also heterozygous for *Aprt*. The KO6 cell line is defined as wild type based on a comparison of the mutational spectrum for this cell line with the mutational spectrum for normal kidney cells *in vivo* (Ponomareva et al., 2002). All cell lines were maintained in the growth medium containing Dulbeco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO) in 5% CO_2, at 37°C.

Determination of mutation rate. The *Aprt* mutation rate for the K435 cell line was determined by following the methods that were described previously (page 24-

27). 10 "pots" of 100 cells were expanded to a final cell number of approximately 1.5×10^6 cells. The *Aprt* mutant frequencies were obtained for each set by selecting for *Aprt*-deficient cells in medium containing 80 μg/ml 2,6-diaminopurine (DAP).

Genotoxin exposure and determination of mutant frequencies. The mutagenic response of the K435 cells was examined by determining mutant frequencies in 7 – 10 subclones exposed to ultraviolet radiation (Turker et al., 1995), hydrogen peroxide (Turker et al., 1999), and ionizing radiation (Turker et al., 1995). UV and hydrogen peroxide-induced mutant frequencies were determined by following the methods described previously (page 27-28). For the ionizing radiation treatment, a cell suspension (in 5 ml of growth medium in a 15 ml tube) of about one million cells of each subclone was exposed to 550 rad in a Shepard cesium irradiator (^{137}Cs). After exposure, the cells were plated in a T-75 flask and allowed to recover for 6 days. After 3 days of recovery, the growth medium was changed to remove dead cells. After recovery, 400,000 cells were plated using 100 mm dishes (total 4 dishes for each subclone) and treated with 80 μg/ml DAP. The remaining procedure and determination of *Aprt* mutant frequency followed the methods described previously (page 27-28). The methods for isolating *Aprt* mutants and genomic DNA were given on page 29-30.

Sequence analysis of *Aprt* mutants. The approach used to identify specific base-pair changes in the *Aprt* gene has been described in detail (page 30-32).

Determination of cytotoxic effects of 6-thioguanine, 1-methyl-3-nitro-1-nitroso guanine (MNNG), and cisplatin. The relative sensitivities of wild type, mismatch repair-deficient, and K435 cells to the DNA damaging agents 6-thioguanine (Sigma, St. Louis, MO), 1-methyl-3-nitro-1-nitroso guanine (MNNG, Sigma, St. Louis, MO), and cisplatin (Sigma, St. Louis, MO) were determined using cloning (plating) efficiency methods described below. The working stock solutions of each cytotoxic agent were prepared by dissolving 6-thioguanine in 0.05 M NaOH, cisplatin in deionized H_2O, and MNNG in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO), and filter-sterilized before use. The cells were plated in the growth medium at cloning densities (600 to 800 cells) in 100 mm dishes containing 10 ml growth medium. After 24 hours, the stock solution of each cytotoxic agent was added to the dishes at concentrations ranging from 0.1 to 3 μM for 6-thioguanine, 0.1 to 0.7 μM for cisplatin, and 1 to 13 μM for MMNG. Each experiment included non-treated controls to determine the cytotoxic effects on the treated cells. The final concentrations of NaOH or DMSO in the cell culture medium did not affect cell survival. Six days later, the media containing the cytotoxic agents were replaced by the growth media that did not contain any cytotoxic agents and the cells were incubated for the additional 7 days at 37°C, 5% CO_2. The number of surviving clones in dishes was obtained by staining dishes with crystal violet solution as described previously (page 27). The cytotoxic effects were determined

by comparing the number of clones in the treated dishes with one in non-treated control dishes.

Cytotoxic effects (measured in clone forming efficiency %) = (the number of clones in a dish at a given dose / the number of clones in non-treated dishes) × 100%

RESULTS

The K435 cells exhibit an elevated mutation rate. The K435 kidney cell line was derived from a mouse heterozygous for the selectable *Aprt* locus. No other genetic alterations were known to be present in this mouse. Surprisingly, the K435 exhibited an elevated rate of *Aprt* mutation (2.6×10^{-5}) (Table 11) when compared with another *Aprt* heterozygous kidney cell line (KO6) derived from a different wild type mouse (3.6×10^{-6}). The elevated rate for the K435 cells was instead closer to that observed for kidney cell lines derived from mice null for the *Pms2* (1.3×10^{-5} for K2 cells and 7.4×10^{-5} for K597S2 cells). and *Mlh1* (1.2×10^{-4}) mismatch repair genes.

Table 11. Comparison of mutation rates at *Aprt* for K435, wild type, and mismatch repair-deficient cells.

	Mismatch repair proficient cells[a]	K435 cells	Mismatch repair-deficient cells*	
	KO6		Pms2 null (K2 &K597S2)	Mlh2 null (K634S2)
Mutation rate (events/cell /generation)	3.6×10^{-6}	2.6×10^{-5}	1.3×10^{-5} (K2) 7.4×10^{-5} (K597S2)	1.2×10^{-4}

* These data are also included in Table 1.
Experimental data are shown in Table 15 (Appendix).

Table 12. Mutational spectra for intragenic events at *Aprt* locus in unexposed and exposed K435 cells*.

Mutational events	Spontaneous	UV	H_2O_2	IR exposure
Transitions				
A:T → G:C	2 (11 %)	1 (6 %)		
G:C → A:T	1 (5 %)	5 (29 %)	1 (11%)	
Transversions				
A:T → T:A				
A:T → C:G	1 (5 %)	1 (6 %)	1 (11%)	
G:C → C:G	14 (74 %)	6 (35 %)	7 (78%)	6 (100%)
G:C → T:A		1 (6 %)		
Frameshifts				
Deletion	1 (5 %)	1 (6 %)		
Tandem mutation		1 (6 %)		
Multiple mutants		1		
Number of mutations identified	19 (100%)	17 (100%)	9 (100%)	6 (100%)

* Cells were exposed to 9.4 joules/m^2 of UV, 65 μM hydrogen peroxide, and 6 Gy of ^{137}Cs-gamma radiation.

The K435 mutator phenotype is due to G:C → C:G transversion mutations.

Aprt mutations fall into two very broad classes: small events that leave the target allele intact such as base-pair substitutions, frameshifts, and gene silencing, and large events that remove the target allele such as chromosome loss, large deletions, and mitotic recombination. Large events (loss of heterozygosity) predominate in wild type cells (Turker et al., 1999) and in *Pms2* null cells, whereas small events

predominate in *Mlh1* null cells (Shin et al., 2002). Surprisingly, 85% of spontaneous mutants obtained from subclones of the K435 cells were due to small mutational events, which is inconsistent with this cell line being wild type cell with regards to mutagenesis. A sequence analysis of 19 independent small mutational events revealed that 14 (74%) were G:C → C:G transversions (Table 12). These 14 mutations were distributed to all 5 exons of *Aprt* (not shown). However, the G:C → C:G mutations were not distributed evenly between the non-transcribed and transcribed strands. G → C changes occurred on the non-transcribed strand 11 times (79%) and C → G changes occurred on the non-transcribed strand 3 times (21%). Four of the five remaining mutations were 1 bp substitutions and the other mutation was a 1 bp deletion.

Exposure to UV alters the spectrum of mutations in the K435 cells. The K435 cells were exposed to UV, which induces base-pair changes (Khattar and Turker, 1997), and to hydrogen peroxide and ionizing radiation, which mostly induce large mutations (Turker et al., 1999; Turker et al., 1997) when *Aprt* is the selectable target in mouse cells. All three exposures increased the average *Aprt* mutant frequencies over those observed in untreated cells (Table 13), although as noted previously in work with other mouse cells lines (Turker et al., 1995; Turker et al., 1999) significant interclonal variation was observed for both the spontaneous and induced *Aprt* mutant frequencies. For UV exposed cells, the percentage of G:C → C:G mutations was 35% for the UV exposed cells, with a characteristic UV-

induced transition (C→ T) accounting for 29% of the mutations (5% for non-treated cells, p value 0.035). These C → T changes, which were observed in UV-induced mutants isolated from five independent K435 subclones, occurred at dipyrimidine sites on the non-transcribed strand as expected for UV-induced mutations in transcription-coupled-repair (TCR) competent cells (Vreeswijk et al., 1998; Vrieling et al., 1998). One mutant allele was found to have two mutations; a C → T and a G → C mutation on the non-transcribed strand. In contrast to the UV data, small events observed in mutant cells isolated from the hydrogen peroxide and ionizing radiation exposed cells were still predominantly G:C → C:G transversions. A strand bias was again observed with G → C changes being detected on the non-transcribed strand for 12 of 13 mutant alleles isolated from the hydrogen peroxide and ionizing radiation exposed cells. These results suggest that the G:C → C:G mutations represented the spontaneous background, or that the exposures induced events that were resulted from the same pathway that caused spontaneous G:C → C:G mutations.

Table 13. Mutant frequencies for unexposed and genotoxin-exposed K435 cells*

A) Subclones	Spontaneous mutant frequency	UV exposure mutant frequency (fold induction ×)		H_2O_2 exposure mutant frequency (fold induction ×)	
K435S1	9×10^{-5}	49×10^{-5}	(5 ×)	104×10^{-5}	(11 ×)
K435S2	1.7×10^{-5}	15×10^{-5}	(9 ×)	5.3×10^{-5}	(3 ×)
K435S3	4.4×10^{-5}	6.5×10^{-5}	(1.5 ×)	14×10^{-5}	(3 ×)
K435S4	15×10^{-5}	24×10^{-5}	(1.6 ×)	133×10^{-5}	(9 ×)
K435S5	41×10^{-5}	3.4×10^{-5}	(0.1 ×)	61×10^{-5}	(1.5 ×)
K435S6	16×10^{-5}	98×10^{-5}	(6 ×)	39×10^{-5}	(2.5 ×)
Average {standard deviations}	15×10^{-5} {14}	33×10^{-5} {36}		59×10^{-5} {50}	

B) Subclones	Spontaneous mutant frequency (fold induction ×)	IR exposure mutant frequency (fold induction ×)	
K435S11	27×10^{-5}	100×10^{-5}	(3.7 ×)
K435S12	44×10^{-5}	30×10^{-5}	(0.7 ×)
K435S16	10×10^{-5}	23×10^{-5}	(2.3 ×)
K435S17	22×10^{-5}	58×10^{-5}	(2.6 ×)
K435S10	8×10^{-5}	15×10^{-5}	(1.9 ×)
Average {standard deviations}	22×10^{-5} {15}	45×10^{-5} {35}	

* K435 subclones were grown until growth within the T75 flasks was confluent. The cultures were then split into two (for IR exposure) or three flasks (for UV and H_2O_2 exposures), with one or two flasks treated as indicated, respectively. In each case one flask was not treated for determination of spontaneous mutant frequency. Standard deviations are shown in { }.

K435 cells do not exhibit microsatellite instability. One of the mutational hallmarks for mismatch repair deficiency is instability at microsatellite repeat sequences (Buermeyer et al., 1999). To determine if the K435 cells exhibited microsatellite instability I examined 4 markers on chromosome 8 that exhibited microsatellite instability in *Mlh1* deficient cells. As seen in Table 14, none of these markers exhibited microsatellite instability in a total of 48 K435 subclones.

Table 14. K435 cells do not exhibit microsatellite instability for chromosome 8 loci†.

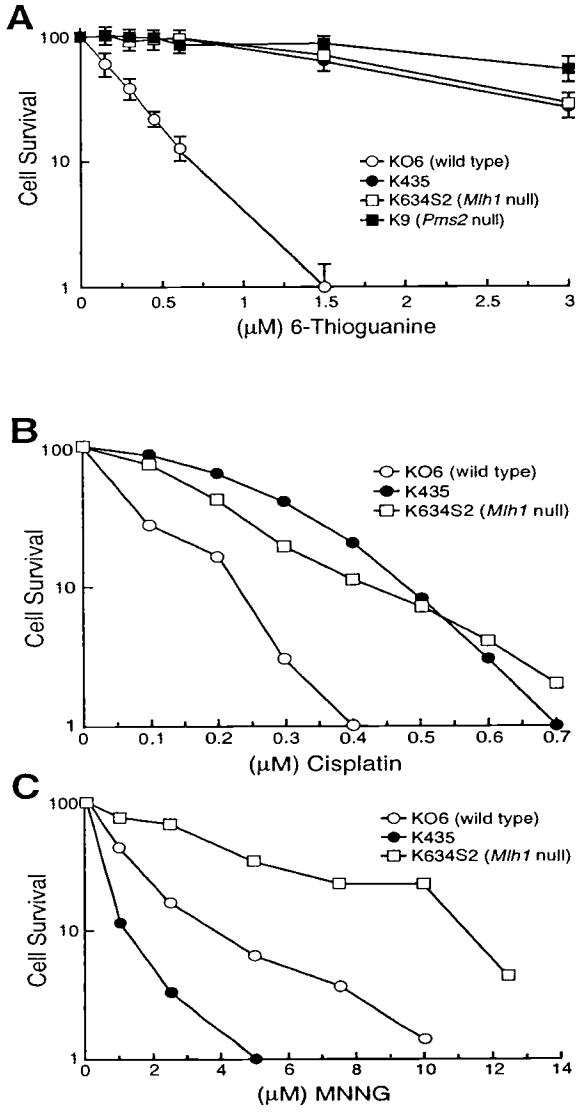
Locus	K435 subclones	# instability*	%*	*Mlh1* subclones	# instability*	%*
124	48	0	0	21	10	48
125	48	0	0	21	3	14
56	48	0	0	21	1	5
Aprt	48	0	0	15	4	27

† Microsatellite instability is defined as an altered band size for a given amplified CA repeat region.
* Number and percentage, respectively, of clones exhibiting instability for marker tested.

K435 cells exhibit resistance to 6-thioguanine and cisplatin, but are hypersensitive to MNNG. Mismatch repair deficient cells exhibit resistance to alkylating agents such as MNNG and to other genotoxins such as 6-thioguanine (6-

TG) and cisplatin (Buermeyer et al., 1999). To determine if the mutator phenotype in the K435 cells could be due to a deficiency for a mismatch repair protein, cell survival assays were used to determine relative levels of resistance to MNNG, 6-TG, and cisplatin. Wild type cells and mismatch repair deficient cells (*Pms2* and *Mlh1*) were used as sensitive and resistant controls, respectively. The K435 cells demonstrated resistance to 6-TG and cisplatin at levels (Fig. 4A and 4B) that were similar to those for the mismatch repair deficient cells. However, the 435 cells were hypersensitive to MNNG (Fig. 4C), even when compared with the wild type control cell line.

Figure 4. Cell survival assays



The K435, KO6 (wild type), K9 (Pms2 null) and K634S2 (Mlh1 null) cell lines were plated at clonal densities and exposed to the indicated doses of 6-thioguanine (A), cisplatin (B), and MNNG (C) for 24 h. Two weeks later, the plates were stained with crystal violet and surviving colonies were counted. The 6-thioguanine experiment was performed twice in duplicate. The cisplatin and MNNG experiments were performed once in duplicate after initial experiments were performed to determine effective range and suggest resistance and sensitivity, respectively. Y-axis indicates cell survival (%).

DISCUSSION

I have described a mouse kidney epithelial cell line (K435) with a G:C → C:G transversion mutator phenotype. Spontaneous G:C → C:G transversions are rare mutational events in prokaryotic and most eukaryotic systems (Zhang et al., 1998). An exception is that relatively high percentages of these mutations (18-38%) have been reported to occur at some loci in wild type *S. cerevisiae* (Kunz et al., 1998). Nonetheless, transversion mutations of any type are rare in mammalian cells and there has been only a single report of a mammalian cell line (human colon cancer) with an increased rate of transversion mutations (Eshleman et al., 1998). However, this mutator phenotype was for a variety of transversion events in contrast to the narrow G:C → C:G mutator phenotype in the K435 cell line.

In mammalian cells, only a few genetic deficiencies have been associated with increased rates of spontaneous base-pair substitutions; most of these deficiencies are for mismatch repair proteins or proteins that interact with them (Buermeyer et al., 1999). Therefore, my analysis of the K435 cells included a comparison with known traits of mismatch repair deficient cells. In wild type mouse cells most spontaneous mutations causing second step loss of *Aprt* expression (in obligate heterozygotes) are large events such as mitotic recombination and chromosome loss (Shao et al., 1999; Turker et al., 1999). Moreover, a significant number of spontaneous small events in wild type cells result from epigenetic silencing (Cooper et al., 1991; Rose et al., 2000). In contrast most mutational events in the K435 cells were base-pair substitutions (Shin et al.,

2002). Similarly, most mutational events for *Aprt* in *Mlh1* deficient cells are base-pair substitutions. However, the limited spectrum of base pair change for the K435 cell line (i.e., G:C → C:G) differs from the very broad spectrum of base-pair mutations observed for *Mlh1* deficient kidney cells. Mutations affecting *Aprt* in *Pms2* null cells differ from those observed in both the K435 cells and *Mlh1* null cells because large events are almost as common in the *Pms2* null cells as those observed in wild type cells. However, unlike wild type cells, most small events in the *Pms2* cells are base-pair substitutions, with approximately half of these events being A:T → G:C transitions (Shin et al., 2002). Results from a variety of other mismatch repair deficient mammalian systems have failed to link any specific protein deficiency with a G:C → C:G mutator phenotype (Andrew et al., 2000; Baross-Francis et al., 2001; Kato et al., 1998; Leach et al., 1996; Phear et al., 1996; Tomita-Mitchell et al., 2000).

Some spontaneous *Aprt* mutant alleles were found to have two mutations when the cells they were derived from were deficient for the PMS2 and MLH1 proteins. Both UV and hydrogen peroxide exposures increased the frequency of these mutant *Aprt* alleles with multiple mutations (Shin et al., 2002). Neither genotoxin produces *Aprt* alleles with multiple mutations in a wild type background (Drobetsky et al., 1987; Khattar and Turker, 1997; Turker et al., 1999). Although no spontaneous mutant alleles with two mutations were obtained from the K435 cells, one UV-induced mutant allele with two mutations was observed suggesting a potential repair defect.

Frame shift mutations at mono- and dinucleotide runs within genes are common in mismatch repair deficient cells (Buermeyer et al., 1999), but there are only a small number of these runs in mouse *Aprt* and they are too short to act as hot spots for mutations (Shin et al., 2002). Therefore, it was not possible to evaluate microsatellite instability mutations at the *Aprt* locus in the K435 cells. Instead I examined instability at several microsatellite loci on mouse chromosome 8 that were unstable in *Mllh1* cells. These loci were stable in the K435 cells.

Mismatch repair deficient cells exhibit resistance to a variety of genotoxic agents when compared with wild type cells (Lage and Dietel, 1999). A comparison of drug resistance between the mismatch repair deficient cells and the K435 cells yielded mixed results. The K435 cells were as resistant to 6-thioguanine and cisplatin as the mismatch repair deficient cells, but hypersensitive to MNNG even when compared with wild type KO6 kidney cell line. MNNG hypersensitivity has been reported in cells lacking O^6- methylguanine transferase (MGMT) (Suzuki et al., 1997). However, MGMT deficient cells are not resistant to cisplatin (Preuss et al., 1996) and preferentially accumulate G:C → A:T mutations (Esteller et al., 2001; Esteller et al., 2000; Psaroudi and Kyrtopoulos, 2000). Finally, it is important to note that the K435 cells do not exhibit altered sensitivity to all genotoxins as they were found previously to be normally sensitive to UV, hydrogen peroxide, and ionizing radiation (Gage et al., 2001).

Considered as a whole, the comparison of the K435 cells and mismatch repair deficient cells suggests that the G:C → C:G mutator phenotype is not due to

a classical deficiency for mismatch repair. Nonetheless, the initial formation of C:C or G:G mispairs provides the most plausible explanation for the presence of the G:C → C:G mutations. Based on my data and additional data from the literature, I suggest that G:G mispairs are responsible. Significantly, DNA polymerase errors cause C:C mispairs far less frequently that G:G mispairs (Mendelman et al., 1989). Moreover, I have shown that a strand bias exists for the G:C → C:G mutational events because in most cases the affected G base was located on the non-transcribed strand. Mutational strand biases are most often due to transcription-coupled repair (TCR) of bulky DNA adducts (Vrieling et al., 1998). If TCR is intact in the K435 cells, as suggested by the UV mutational data, it is possible that a bulky adduct attached to guanine is involved in the formation of mispairs. In bacteria, it has been suggested that the MutY and MutM proteins prevent G:G mispairs resulting from oxidative damage (Zhang et al., 1998) suggesting a potential role for such damage in the K435 mutator phenotype. Oxidative damage is preferentially repair on the transcribed strand due to the effects of TCR (Cooper et al., 1997). A defect in repair of guanine damage could also explain sensitivity to MNNG, which causes extensive damage to guanine bases. Additional work will be required to determine both the types DNA damage and/or the repair defect that may combine to account for the novel G:C → C:G mutator phenotype and drug resistance/sensitivity profile in the K435 cells.

CONCLUSIONS

Overlapping but Different Roles of MLH1 and PMS2 in Preventing Mutations.

Accurate replication of genetic information is critical for normal cellular function and survival of the organism. However, during replication, DNA polymerase errors can arise that cause misinsertion of bases or deletion/insertion loops (Kunkel, 1986; Kunkel and Alexander, 1986). Unrepaired, these errors result in base pair substitutions and frameshift mutations, respectively. Mismatch repair is a post-replicative repair process that is primarily involved in repairing the errors caused by DNA polymerases. Defects in mismatch repair significantly increase point mutations, such as base pair substitutions and frameshifts.

The complexity of mismatch repair is shown by many constituent proteins forming different heterodimers to carry out partially overlapping repair functions. PMS2 and MLH1 form a heterodimer, called MutLα that participates in preventing base pair substitutions and small deletion/insertion loops. My work showed that the mutagenic consequence of loss of the PMS2 and MLH1 proteins is not functionally equivalent. In this study, *Mlh1* null cells showed a stronger mutator phenotype as evidenced by increased mutation rates for a variety of base-pair substitutions when compared with *Pms2* null cells (Table 1). A stronger mutator phenotype for *Mlh1* deficient cells was also suggested by studies from other laboratories that showed increased levels of microsatellite instability and base pair

substitutions in *Mlh1*-deficient cells relative to *Pms2*-deficient cells (Yao et al., 1999; Baross-Francis et al., 2001).

Interestingly, the molecular bases of mutator phenotypes for *Mlh1* null cells and *Pms2* null cells were also different. All types of base pair substitutions were increased in the *Mlh1* null cells whereas *Pms2* null cells increased both large events (*e.g.*, mitotic recombination) and base pair substitutions, particularly A:T → G:C. By using a reversion assay, an elevated spontaneous rate of A:T → G:C mutations in the *Pms2* null cells was demonstrated relative to G:C → A:T and G:C → T:A base pair substitutions. Moreover, significantly decreased mutation rates for the A:T → G:C base pair substitutions, but not for other base pair substitution events, in the presence of antioxidants indicated that oxidative damage to either adenine or thymine was responsible for A:T → G:C base pair substitutions in the *Pms2* null cells (Table 6).

Based on current models on the role of mismatch repair proteins in preventing base pair substitutions, the loss of either PMS2 or MLH1 should have been equivalent (Jiricny, 2000). However, my study suggests that the absence of PMS2 does not lead to complete loss of function in preventing base pair substitutions. Therefore, MLH1 may still partner with other MutL proteins in the absence of PMS2 to partially prevent the formation of base pair substitutions. In contrast, in the absence of MLH1, the PMS2 protein may be unable to partner with other MutL proteins, leading to complete loss of MMR activity and resulting in increase of base pair substitutions.

In addition, my study demonstrated that loss of PMS2, even though it does not lead to complete loss of MMR activity in preventing base pair substitutions, results in a specific increase of oxidative stress-induced A:T → G:C mutations in *Pms2*-deficient cells. This suggests that heterodimers of MLH1 with other MutL proteins may be unable to repair damaged adenine:C or damaged thymine:G mismatches. PMS2 may have a specific role in either recognizing or repairing damaged adenine:C or damaged thymine:G mismatches. The role of PMS2 in recognition of such mismatches could be tested by a gel shift assay employing labeled oligonucleotides containing damaged adenine:C or damaged thymine:G mismatches and cell free extracts from *Pms2*-proficient and deficient cells. If PMS2 is required for recognizing the mismatches, one would expect to observe retarded migration of a band corresponding to oligonucleotides in *Pms2*-proficient cell free extracts, but not in *Pms2*-deficient cell free extracts. To determine the role of PMS2 in repairing the mismatches, one can employ *in vitro* mismatch repair assays (Duckett et al., 1999; Wang and Hays, 2002). In their recent study, Wang and Hays (2002) have reported a MMR excision assay applicable to DNA mismatches or lesions in any sequence context. Based on these techniques, if PMS2 plays a role in repair, the lesions such as damaged adenine:C or damaged thymine:G mismatches should be repaired in the presence of cell free extracts from *Pms2*-proficient cells, but not in *Pms2*-deficient cells. To further understand the mechanism of mutation avoidance function of MMR, the determination of the precise role of PMS2 in avoiding A:T → G:C base pair substitutions is important.

Mutagenesis in Mismatch Repair Deficient Cells.

Base pair substitution mutations can occur by replication errors involving non-damaged templates and, more frequently, damaged DNA bases (Loeb and Cheng, 1990). DNA undergoes continuous damage from exogenous and endogenous processes, which may result in the same mutagenic effects. Because a significant portion of spontaneous mutations is caused by endogenous DNA damage (Jackson et al., 1998; 2001), it is sometimes difficult to distinguish spontaneous mutations from those induced by exogenous DNA damage. Damage-modified DNA bases can either block DNA replication resulting in cell death or alter the pairing property of base resulting in misincorporation of a nucleotide by DNA polymerases. Mutations can result from these mispairings.

There are two general mechanisms that a cell uses to cope with DNA damage: removal of the damage or tolerance of the damage. Cells avoid mutations by repairing damaged DNA bases via direct reversal of damage (*e.g.*, alkyltransferase, photolyase) or excision of a damaged nucleotide (base excision repair and nucleotide excision repair) before replication initiates or during replication. If the damage is persistent, cells then avoid mutations by recombination and DNA polymerase template switching during replication or by DNA damage tolerant pathways that involve replication of non-damaged DNA strand instead of the DNA strand with damage (Bayton and Fuchs, 2000). However, cells may accumulate mutations by using another DNA damage tolerant

pathway, translesion DNA synthesis, which involves error-prone replication through the damaged site (Kunz, 2000).

Recent studies have suggested that translesion synthesis is the main source of base pair substitution mutations after DNA is damaged by endogenous and exogenous genotoxins (Quah et al., 1980, Gibbs et al., 1998; 2000). The highly mutagenic nature of translesion synthesis is explained by the properties of translesion DNA polymerases (reviewed in Woodgate, 1999). Unlike replicative DNA polymerases that possess high fidelity accompanied by proofreading exonuclease activities and high processivity, translesion DNA polymerases have low fidelity, lack 3'to 5' proofreading activity (Matsuda et al., 2000; Ohashi et al., 2000), and exhibit low processivity (Ohashi et al., 2000), all of which may be necessary for damage tolerance. A current model for translesion synthesis (TLS) is that when a highly processive DNA polymerase is arrested at a lesion in DNA, the replicative machinery is replaced by translesion bypassing polymerases. When the lesions are bypassed, the replicative machinery is reinstalled and DNA synthesis continues (Sander and Samson, 2000).

Replication errors are significantly reduced both by the proofreading exonuclease activity of replicative DNA polymerases (Simon et al., 1991) and by DNA mismatch repair (reviewed in Umar and Kunkel, 1996). My study suggests the importance of mismatch repair (MMR) for preventing the accumulation of spontaneous base pair substitutions. In the absence of proofreading activity during translesion synthesis, DNA mismatch repair may be the only mechanism that can

prevent DNA damage-induced base substitution mutations. Several studies have suggested the mutation avoidance role of mismatch repair by showing that mismatch repair proteins recognized mismatched DNA damaged bases (Fishel et al., 1994, Mazurek et al., 2002, Ni et al., 1999, Wang et al., 1999) and that mismatch repair deficient cells exhibit increased mutation frequencies upon exposure to DNA damaging agents (Andrew et al., 1998, Aquillina et al., 1993, Glaab et al., 1998; 1999a; 1999b, Goldmacher et al., 1986, Liu, et al., 1999, Nara et al., 2001, Toft et al., 1999, Xu et al., 2001).

In my study, forward and reversion mutation analyses of the *Aprt* locus in mismatch repair-proficient and deficient mouse cells were used to investigate the role of mismatch repair in preventing DNA damage-induced base pair substitutions. As described in Chapter 2, exposure to DNA damaging agents such as UV and oxidative stress was shown to significantly increase the percentage of mutant alleles with multiple mutations in mismatch repair-deficient cells (Table 2, 3). These mutations were separated from each other by distances ranging from a few bases to well over 1000 bases. A recent study reported spontaneous multiple mutations in *Msh2*-deficient cells (Zhang et al., 2002). Multiple mutations at a single allele are rare in wild type cells even after exposure to DNA damaging agents. However, a cancer cell has multiple mutations throughout its genome including cancer relevant loci, reflecting genetic instability in cancerous cells. Endogenous oxidative stress-induced DNA damage (Jackson and Loeb, 2001) and

increased replication errors (Strauss, 1997) have been suggested to be contributing factors for the accumulation of multiple mutations in a cancer cell.

In normal cells, there may exist an equilibrium state among DNA damage, DNA repair, and translesion synthesis. However, when this equilibrium is compromised I suggest that the multiple mutations will accumulate. Excessive DNA damage alone does not induce multiple mutations, as wild type cells do not show multiple mutations upon the exposure to DNA damaging agents (Drobetsky et al., 1987,1994, Khattar and Turker et al., 1997) This indicates the essential role of DNA repair proteins for removing premutagenic lesions before they exert mutagenic effects. My study showed that in the absence of DNA mismatch repair, a post-replicational repair pathway will lead to the accumulation of multiple base pair substitutions at *Aprt* upon exposure to genotoxins.

A causal role for translesion synthesis in inducing multiple mutations in *S. cerevisiae* was shown by a study that reported the induction of multiple mutations by error prone TLS DNA polymerase ζ when bypassing spontaneous DNA damage (Harfe et al. 2000b). In this study, nucleotide excision repair (NER)-deficient cells showed multiple mutations whereas wild type cells did not. Disruption of REV3 (DNA polymerase ζ) led to a complete loss of multiple mutations in NER-deficient cells, indicating that TLS DNA polymerase ζ was responsible for generating the multiple mutations. The notable difference between multiple mutations observed in *S. cerevisiae* from the ones I observed in mismatch repair deficient cells was the distance between mutations. Harfe et al. (2000b) showed that the multiple

mutations occurred within a range of 3 to 10 nucleotides. However, in my study, the multiple mutations were often well separated by as many as 100 to 1500 bp. This may be due to the different reporter systems used in my study and the study by Harfe et al. Harfe et al. (2000b) used a small reversion reporter that spanned only 150 bases and had a run of three Ts near the base (G) which detected G → T base pair substitutions. The multiple mutations included G → T base pair substitutions and frameshift mutations at the runs of Ts. When the guanine (G) base was removed the frameshift mutations were also absent, suggesting that DNA base damage to guanine caused misinsertion of A followed by slippage errors caused by DNA polymerase ζ. My study employed a forward mutational analysis of *Aprt*, which can detect a variety of mutational events. The well-separated multiple mutations in *Aprt* suggest either a single processive translesion synthesis event past the damage and beyond creating additional mutations at a distance, or multiple translesion syntheses for independent DNA damage events. Biochemical studies have shown that the in vitro processivity of translesion DNA polymerases in the absence of any other protein factors is extremely low ranging from 1 to 25 nucleotides (Ohashi et al., 2000) and accompanied by high error rates of misincoporation of a base ranging from 3.5×10^{-2} (pol η) to 6×10^{-3} (pol κ). It will be important to determine in vivo processivity of translesion DNA polymerases and their contribution to accumulation of mutations. Considered together, my study and the study by Harfe et al. (2000b) suggest that a decrease in pre-replicational DNA repair results in increased numbers of unrepaired, premutagenic lesions for

error-prone translesion synthesis, and that a decrease in post-replicational DNA mismatch repair results in fixation of DNA polymerase error-induced mutations.

In my studies, the role of mismatch repair in preventing DNA damage-induced base pair substitutions was shown not only by increased multiple mutations at the *Aprt* locus (Table 2, 3), but also by an increase in specific base pair substitutions upon exposure to DNA damaging agents (Table 10). To demonstrate this further, a mammalian reversion assay was developed to detect specific base pair substitutions that were induced by genotoxin exposure in the mammalian genome. By using this reversion assay, I demonstrated the mutation avoidance function of mismatch repair for preventing both endogenous and exogenous DNA-damage-induced specific base substitution mutations, particularly tandem mutations.

I mentioned earlier that endogenous oxidative stress-induced A:T → G:C mutations were prevented by mismatch repair in a PMS2-dependent pathway. My work also showed that mismatch repair-deficient cells were hypermutable to UV and hydrogen peroxide/metal mixture-induced tandem CC → TT mutations. It is not known whether mismatch repair recognizes mismatched tandem bases better than a mismatched base. However, the results indicate that mismatch repair prevents tandem mutations after exposure to DNA damaging agents. The nature of oxidative stress-induced DNA damage that caused CC → TT mutations has not been identified. However, ultraviolet light induces cyclobutane pyrimidine dimers (CPDs) that are responsible for CC → TT mutations (Reid et al., 1991, 1993).

Misincorporation of AA opposite CPDs during replication and accelerated deamination of CPDs (Peng and Shaw, 1996) both are known to cause ultraviolet-induced CC → TT mutations. I suggest that the hypermutability of mismatch repair-deficient cells to CC → TT mutations is likely due to a lack of MMR-dependent correction of the misincorporation during translesion synthesis rather than deamination, since there is no evidence suggesting that deamination of CPDs is accelerated in mismatch repair-deficient cells.

In addition, this was the first study to demonstrate that tandem CC → TT mutations can be induced by oxidative stress in mammalian cells. Oxidative stress-induced mutations have been implicated in the initiation and/or the progression of proliferative diseases such as cancer (Ames et al., 1992, and Jackson and Loeb 1999). It has been estimated that oxidative stress might chemically induce a number of DNA adducts (Marnett, 2000). However, only a few oxidative stress-induced DNA adducts and their mutagenic consequences have been identified, such as 8-oxo-guanine resulting in G → T mutations (Moriya, 1993) and thymine glycols resulting in T → C mutations in *E. coli* (Basu et al., 1989). In vivo, these may represent just a small portion of potentially mutagenic lesions. Further study will require the biochemical identification of the types of oxidative stress-induced DNA adducts prevalent in vivo, their repair efficiencies, and genetic determination of the mutagenic effects. My work suggests that the role of mismatch repair in preventing these types of mutations should be addressed.

The failure to remove premutagenic DNA lesions before the initiation of replication contributes to the enhancement of damage-induced base substitution mutations. My work suggests that the failure to remove mismatched premutagenic DNA lesions during replication also significantly contributes to damage-induced base substitution mutations. Mismatch repair proteins do not have any activity to remove damaged bases from the template strand as excision repair proteins do (Mu et al., 1997), indicating that removal of a misinserted base opposite a damaged base by mismatch repair contributes to the mutation avoidance. Thus, I hypothesize that the formation of DNA lesions can be initially repaired before replication. However, unrepaired damage will be bypassed by translesion synthesis DNA polymerases during replication. Because the translesion DNA polymerases are error-prone, misincorporations will occur generating mismatched DNA lesions, which can be recognized by mismatch repair and be repaired. Thus, the unrepaired DNA lesions will have another chance to be removed by DNA repair proteins, resulting in prevention of further mutagenic effects. According to my hypothesis, this backup function will be lost in MMR deficient cells. It has also been suggested that a damaged base, when paired with an incorrect base after error-prone translesion synthesis, is more readily removed by excision repair system, resulting in fixation of mutation (Mu et al., 1997). If so, mismatch repair prevents mutations by removing the misincorporated base in the nascent strand before excision repair removes the damaged base.

In total, my studies underscore the importance of mismatch repair for preventing DNA damage-induced base substitutions, and suggest that the mutation avoidance function of mismatch repair is clearly dependent on other pathways involved in DNA damage removal, DNA damage tolerance, and the fidelity of these processes. It is important to unravel how these pathways are cooperating to ensure genetic stability. Moreover, the participation of mismatch repair in correcting DNA repair derived errors and the contribution of error-prone repair pathways in the accumulation of mutations need to be determined. There is increasing evidence of novel translesion DNA polymerases that contribute to mutagenesis. To understand both spontaneous and damage-induced mutagenesis better, it will be important to determine the *in vivo* fidelity and processivity of translesion DNA polymerases, the factors that determine error-free vs. error-prone translesion synthesis, and the exact interaction of mismatch repair machinery with translesion synthesis machinery.

Based on Harfe's study (2000b) and the proposed roles of translesion DNA polymerases in DNA-damage-induced mutagenesis, defective error-prone translesion synthesis should result in a decrease of multiple mutations and also certain DNA damage-induced base pair substitutions in *Aprt* locus of MMR-deficient cells. A genetic assay such as the *Aprt* reversion assay in cells of MMR-deficient/ translesion DNA synthesis pathway-proficient, MMR-deficient/ translesion DNA synthesis pathway deficient backgrounds could be used to determine the role of mismatch repair in preventing mutagenesis caused by error-

prone translesion DNA polymerases. In this system, the absence of a translesion DNA synthesis pathway should lead to reduced or loss of multiple mutations, or decrease of a specific base pair substitutions (*e.g.*, CC → TT) in MMR-deficient cells. Continued use of such mutational assays along with biochemical assays will contribute to these goals.

BIBLIOGRAPHY

Aaltonen LA, Peltomak P, Leach F, Sistonen P, Pylkkanene SM, Mecklin JP, Javinene H, Powell SM, Jen J, Hamilton SR et al. 1993. Clues to the pathogenesis of familial colorectal cancer. Science 260, 812-816.

Acharya S, Wilson T, Gradia S, Kane MF, Guerretre S, Marsischky GT, Kolodner R, and Fishel R. 1996. HMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci USA 93, 13629-13634.

Alani E, Sokolsky T, Studamire B, Miret JJ, and Lahue RS. 1997. Genetic and biochemical analysis of Msh2p-Msh6p: role of ATP hydrolysis and Msh2p-Msh6p subunit interactions in mismatch base pair recognition. Mol Cell Biol 17, 2436–2447.

Ames BN, Profet M, and Gold LS. 1990. Nature's chemicals and synthetic chemicals: comparative toxicology. Proc Natl Acad Sci USA 87, 7782 – 7786.

Ames BN and Shigenaga MK. 1992. Oxidants are a major contributor to aging. Ann N Y Acad Sci 663, 85-90.

Andrew SE, McKinnon M, Cheng BS, Francis A, Penney J, Reitmair AH, Mak TW, and Jirik FR. 1998. Tissues of MSH2-deficient mice demonstrate hypermutability on exposure to a DNA methylating agent. Proc Natl Acad Sci U S A 95, 1126-1130.

Andrew SE, Reitmair AH, Fox J, Hsiao L, Francis A, McKinnon M, Mak TW, and Jirik FR. 1997. Base transitions dominate the mutational spectrum of a transgenic reporter gene in MSH2 deficient mice. Oncogene 15, 123-129.

Andrew SE, Xu XS, Baross-Francis A, Narayanan L, Milhausen K, Liskay RM, Jirik FR, and Glazer PM. 2000. Mutagenesis in PMS2- and MSH2-deficient mice indicates differential protection from transversions and frameshifts. Carcinogenesis 21, 1291-1295.

Aquilina G, Biondo R, Dogliotti E, and Bignami M. 1993. Genetic consequences of tolerance to methylation DNA damage in mammalian cells. Carcinogenesis 14, 2097-2103.

Armstrong JD and Kunz BA. 1990. Site and strand specificity of UVB mutagenesis in the *SUP4-o* gene of yeast. Proc Natl Acad Sci USA 87, 9005-9009.

Au KG, Welsh K, and Modrich P. 1992. Initiation of methyl-directed mismatch repair. J Biol Chem 267, 12142-12148.

Ayyagari R, Impellizzeri KJ, Yoder BL, Gary SL, and Bergers PM. 1995. A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. Mol Cell Biol 15, 4420 –4429.

Ban C and Yang W. 1998a. Crystal structure and ATPase activity of MutL: implication for DNA repair and mutagenesis. Cell 95, 541-552.

Ban C and Yang W. 1998b. Structural basis for MutH activation in E. coli mismatch repair and relationship of MutH to restriction endonuleases. EMBO J 17, 1526-1534.

Baross-Francis A, Andrew SE, Penney JE, and Jirik FR. 1998. Tumors of DNA mismatch repair-deficient hosts exhibit dramatic increases in genomic instability. Proc Natl Acad Sci U S A 95, 8739-8743.

Baross-Francis A, Makhani N, Liskay RM, and Jirik FR. 2001. Elevated mutant frequencies and increased C : G-->T : A transitions in *Mlh1-/-* versus *Pms2-/-* murine small intestinal epithelial cells. Oncogene 20, 619-625.

Basu AK, Loechler EL, Leadon SA, and Essigmann JM. 1989. Genetic effects of thymine glycol: site-specific mutagenesis and molecular modeling studies. Proc Natl Acad Sci U S A 86, 7677-7681.

Bayton K and Fuchs RPP. 2000. Lesions in DNA: Hurdles for polymerases. Trends in Biol Sci 25, 74-79.

Beckman KB and Ames BN. 1998. The free radical theory of aging matures. Physiol Rev 78, 547-581.

Bellacosa A, Cicchillitti L, Schepic F, Riccio A, Yeung AT, Matsumoto Y, Golemis EA, Genuardi M, and Neri G. 1999. MED1, a novel human methyl-CpG binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci USA 96, 3969–3974.

Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, and Meuth M. 1994. Mutator phenotype in human colorectal carcinoma cell lines. Proc Natl Acad Sci USA 91, 6319-6323.

Bhattacharyya NP, Ganesh A, Phear G, Richards B, Skandalis A, and Meuth M. 1995. Molecular analysis of mutations in mutator colorectal carcinoma cell lines. Human Mol Genet 4, 2057-2064.

Bignami M, O'Driscoll M, Aquilina G, and Karran P. 2000. Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. Mutat Res 462, 71-82.

Blackwell LJ, Martik D, Bjornson KP, Bjornson ES, and Modrich P. 1998. Nucleotide-promoted release of hMutSα from heteroduplex DNA is consistent with an ATP-dependent translocation mechanism. J Biol Chem 48, 32055-32062.

Bodak N, Queille S, Avril MF, Boudadjar B, Drougard C, Sarasin A, and Daya-Grosjean L. 1999. High levels of patched gene mutations in basal-cell carcinomas from patients with xeroderma pigmentosum. Proc Natl Acad Sci USA 96, 5117-5122.

Boland CR, Thibodeau SN, Hamilton SR, Siransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodrigues-Bigas MA, Fodde R, Ranzani GN, and Srivastava SA. 1998. National cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 58, 5248-5257.

Bocker T, Barusevicius A, Snowden T, Rasio D, Guerrette S, Robbins D, Schmidt C, Burzak J, Croce CM, Copeland T, Kovatich AJ, and Fishel R. 1999. HMSH5 a human MutS homologue that forms a novel heterodimer with hMSH4 and is expressed during spermatogenesis. Cancer Res. 59, 816 – 822.

Bootsma D, Kraemer KH, Cleaver JE, and Hoeijimakers JHJ. 1998. Nucleotide excision repair syndromes: xeroderma pigmentosum, Cokayne syndrome and trichothiodystrophy. In Vogelstein B and Kinzler KW (eds) The genetic basis of human cancer. McGraw-Hill, New York, pp. 245 – 274.

Brash DE. 1997. Sunlight and onset of skin cancer. Trends Genet 13, 410 – 414.

Brash DE, Rudolph JA, Simon JA, Lin A, Mckenna GJ, Gaden HP, Halerin AJ, and Ponten J. 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci USA 88, 10124 – 10128.

Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Iescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, Tannergard P, Bollag RJ, Godwin AR, Ward DC, Nordenskjold M, Fishel R, Kolodner R, and Liskay RM. 1994. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 368, 258-261.

Buermeyer AB, Wilson-Van PC, Baker SM, and Liskay RM. 1999. The human MLH1 cDNA complements DNA mismatch repair defects in *Mlh1*-deficient mouse embryonic fibroblasts. Cancer Res 59, 538-541.

Buermeyer AB, Deschenes SM, Baker SM, and Liskay RM. 1999. Mammalian DNA mismatch repair. Annu Rev Genet 33, 533-564.

Buettner VL, Hill KA, Scaringe WA, and Sommer SS. 2000. Evidence that proximal multiple mutations in Big Blue transgenic mice are dependent events. Mutat Res 452, 219-229.

Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, and Sauvaigo S. 1999. Hydroxyl radicals and DNA base damage. Mutat Res 424, 9-21.

Cahill DP, Kinzler KW, Vogelstein B, and Lengauer C. 1999. Genetic instability and Darwinian selection in tumors. Trends Cell Biol 9, M57-60.

Canella DA and Seidman MM. 2000. Mutation spectra of *supF*: approaches to elucidating sequence context effects. Mutat Res 450, 61-73.

Capizzi RL and Jameson JW. 1973. A table for the estimation of the spontaneous mutation rate of cells in culture. Mutat Res 17, 147-148.

Chen C, Merrill BJ, Lau PJ, Holm C, and Kolodner RD. 1999. *Saccharomyces cerevisiae* pol30 (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair. Mol Cell Biol 19, 7801-7815.

Chen H and Shaw BR. 1994. Bisulfite induces tandem double CC → TT mutations in double- stranded DNA. 2. Kinetics of cytosine deamination. Biochemistry 33, 4121- 4129.

Chi NW and Kolodner RD. 1994. Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. J Biol Chem 269, 29984 – 29992.

Cleaver JE and Crowley E. 2002. UV damage, DNA repair and skin carcinogenesis. Front Biosci 7, 1024-1043.

Cooper GE, DiMartino DL, and Turker MS. 1991. Molecular analysis of APRT deficiency in the mouse P19 teratocarcinoma stem cell line. Somat Cell Mol Genet 17, 105-116.

Cooper PK, Nouspikel T, Clarkson SG, and Leadon SA. 1997. Defective transcription-coupled repair of oxidative base damage in Cockayne syndrome patients from XP group G. Science 275, 990-993.

Dao V and Modrich P. 1998. Mismatch-, MutS-, MutL-, and helicase II-dependent unwinding from the single-strand break of an incised heteroduplex. J Biol Chem 273, 9202-9207.

Davis TW, Wilson-Van Patten C, Meyers M, Kunugi KA, Cuthill S, Reznikoff C, Garces C, Boland CR, Kinsella TJ, Fishel R, Boothman DA. 1998. Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. Cancer Res 58, 767-778.

Daya-Grosjean L, Robert C, Drougard C, Suarez HG, and Sarasin A. 1993. High mutation frequency in ras genes of skin tumors isolated from DNA repair deficient xeroderma pigmentosum patients. Cancer Res 53, 1625 – 1629.

DeMars R. 1973. Mutation studies with human fibroblasts. Environ Health Perspect 6,127 – 136.

Drobetsky EA, Moustacchi E, Glickman BW, and Sage E. 1994. The mutational specificity of simulated sunlight at the *aprt* locus in rodent cells. Carcinogenesis 15, 1577 – 1583.

Drobetsky EA, Grosovsky AJ, and Glickman BW. 1987. The specificity of UV-induced mutations at an endogenous locus in mammalian cells. Proc Natl Acad Sci U S A 84, 9103-9107.

Drummond JJ and Bellacosa A. 2001. Human DNA mismatch repair in vitro operates independently of methylation status at CpG sites. Nucleic Acids Res 29, 2234-2243.

Duckett DR, Bronstein SM, Taya Y, and Modrich P. 1999. hMutSα- and hMutLα-dependent phosphorylation of p53 in response to DNA methylator damage. Proc Natl Acad Sci USA 96, 12384-12388.

Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, and Modrich P. 1996. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci USA 93, 6443-6447.

Dumaz N, Drougard C, Sarasin A, and Grosjean LD. 1993. Specific UV- induced mutation spectrum in the *p53* gene of skin tumors from DNA repair deficient xeroderma pigmentosum patients. Proc Natl Acad Sci USA 90, 10529 – 10533.

Edelmann W, Cohen PE, Kneitz B, Winand N, Lia M, et al. 1999. Mammalian MutS homologue 5 is required for chromosome pairing in meiosis. Nat Genet 21,123 –127.

Eshleman JR, Casey G, Kochera ME, Sedwick WD, Swinler SE, Veigl ML, Wilson JK, Schwartz S, and Markowitz SD. 1998a. Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of *p53*. Oncogene 10, 33-37.

Eshleman JR, Donover PS, Littman SJ, Swinler SE, Li GM, Lutterbaugh JD, Willson JK, Modrich P, Sedwick WD, Markowitz SD, and Veigl ML. 1998b. Increased transversions in a novel mutator colon cancer cell line. Oncogene 16, 1125-1130.

Esteller M, Levin R, Baylin SB, Ellenson LH, and Herman JG. 1998. MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic eudiometrical carcinomas. Oncogene 17, 2413-2417.

Esteller M, Risques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, and Herman JG. 2001. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in *p53* in human colorectal tumorigenesis. Cancer Res 61 4689-4692.

Esteller M, Toyota M, Sanchez-Cespcdes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, and Herman JG, 2000. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethlyation is associated with G to A mutations in *K-ras* in colorectal tumorigenesis. Cancer Res 60, 2368 – 2371.

Fang WH and Modrich P. 1993. Human strand- specific mismatch repair occurs by a bi-directional mechanism similar to that of the bacterial reaction. J Biol Chem 268, 11838–11844.

Field D and Wells C. 1998 Abundant microsatellite polymorphism in *Saccharomyces cerevisiae* and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae*, result from strong mutation pressures and a variety of selective forces. Proc Natl Acad Sci USA 95, 1647-1652.

Fishel R, Ewel A, and Lescoe MK. 1994. Purified human MSH2 protein binds to DNA containing mismatched nucleotides. Cancer Res 54, 5539-5542.

Fisher CL and Pei GK. 1997. Modification of a PCR-based site-directed mutagenesis method. Biotechniques 23, 570-571.

Friedberg EC, Walker GC, and Siede W. 1995. DNA repair and mutagenesis. American Society for microbiology, Wahcington, DC.

Fritzell JA, Narayanan L, Baker SM, Bronner CE, Andrew SE, Prolla TA, Bradley A, Jirik FR, Liskay RM, and Glazer PM. 1997. Role of DNA mismatch repair in the cytotoxicity of ionizing radiation. Cancer Res 57, 5143-5147.

Gage BM, Alroy D, Shin CY, Ponomareva ON, Dhar S, Sharma GG, Pandita TK, Thayer MJ, and Turker MS. 2001. Spontaneously immortalized cell lines obtained from adult *Atm* null mice retain sensitivity to ionizing radiation and exhibit a mutational pattern suggestive of oxidative stress. Oncogene 20, 4291-4297.

Genshcel J, Littman SJ, Drummond JT, and Modrich P. 1998. Isolation of MutSβ from human cells and comparison of the mismatch repair specificities of MutSβ and MutSα. J Biol Chem 273, 19895-19901.

Gibbs PEM, McGregor WG, Maher VM, Nisson P, and Lawrence CW. 1998. A human homolog of the *Sacharomyces cerevisiae* REV3 gene, which encodes the catalytic subunit of DNA polymerase ζ. Proc Natl Acad Sci USA 95, 6876-6880.

Gibbs PEM, Wang XD, Li Z, McManus TP, McGregor WG, Lawrence CW, and Maher VM. 2000. The function of the human homolog of *Sacharomyces cerevisiae* REV1 is required for mutagenesis induced by UV light. Proc Natl Acad Sci USA 97, 4186 - 4191.

Giglia G, Bouffet D, Jouvet A, Ohgaki H, Kleihues P, and Sarasin A. 1999. Molecular analysis of glioma and skin tumor alterations in a xeroderma pigmentosum child. Int J Cancer 81, 345-350.

Glaab WE, Kort KL, and Skopek TR. 2000. Specificity of mutations induced by the food-associated heterocyclic amine 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine in colon cancer cell lines defective in mismatch repair. Cancer Res 60, 4921-4925.

Glaab WE, Risinger JI, Umar A, Barrett JC, Kunkel TA, Tindall KR. 1998a. Resistance to 6-thioguanine in mismatch repair-deficient human cancer cell lines correlates with an increase in induced mutations at the *HPRT* locus. Carcinogenesis 19, 1931-1937.

Glaab WE and Skopek TR. 1999a. Cytotoxic and mutagenic response of mismatch repair-defective human cancer cells exposed to a food-associated heterocyclic amine. Carcinogenesis 20, 391-394.

Glaab WE, Tindall KR, and Skopek TR. 1999b. Specificity of mutations induced by methyl methanesulfonate in mismatch repair-deficient human cancer cell lines. Mutat Res 427, 67-78.

Glaab WE, Risinger J, Umar A, Barrett JC, Kunkel TA, and Tindall KR. 1998b. Cellular resistance and hypermutability in mismatch repair-deficient human cancer cell lines following treatment with methylmethane sulfonate. Mutat Res 398, 197-207.

Goldmacher VS, Cuzick RA, and Thilly WG. 1986. Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and N-methyl-N'-nitro-N-nitrosoguanine. J Biol Chem 261, 12462-12469.

Gogusev J, Bouquet de Joliniere J, Telvi L, Doussau M, de Manori S, Stojkoski A, and Levardon M. 2000. Genetic abnormalities detected by comparative genomic hybridization in a human endometriosis- derived cell line. Mol Hum Reprod 6, 821-827.

Gradia S, Subramanian D, Wilson T, Acharya S, Makhov A, Griffith J, and Fishel R. 1999. HMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA. Mol Cell 3, 255 – 261.

Grilley M, Welsh KM, Su S-S, and Modrich P. 1989. Isolation and characterization of the *Escherichia coli* mutL gene product. J Biol Chem 264, 1000-1004.

Gu L, Hong Y, McCulloch S, Watanabe H, and Li GM. 1998. ATP-dependent interaction of human mismatch repair proteins and dual role of PCNA in mismatch repair. Nucleic Acids Res 26, 1173-1178.

Guarne A, Junop MS, and Yang W.2001. Structure and function of the N-terminal 40 KDa fragment of a human PMS2: a monomeric GHL ATPase. EMBO J 20, 5521-5531.

Gurin CC, Federici MG, Kang J, and Boyd J. 1999. Causes and consequences of microsatellite instability in endometrial carcinoma. Cancer Res 59, 462-466.

Hall MC, Jordan JR, and Matson SW. 1998. Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. EMBO J 17, 1535 – 1541.

Hall MC and Matson SW. 1999. The *Escherichia coli* MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. J Biol Chem 274, 1306-1312.

Hall MC, Shcherbakova PV, and Kunkel TA. 2002. Differential ATP binding and intrinsic ATP hydrolysis by amino-terminal domains of the yeast *Mlh1* and *Pms1* proteins. J Biol Chem 277, 3673-3679.

Harber LT and Walker GC. 1991. Altering the conserved nucleotide binding motif in the Salmonella typhimurium MutS mismatch repair protein affects both its ATPase and mismatch binding activities. EMBO J. 10, 2707-2715.

Hardeland U, Bentele M, Lettieri T, Steinacher R, Jiricny J, and Schar P. 2001. Thymine DNA glycosylase. Prog Nucleic Acid Res Mol Biol 68, 235-253.

Hardman RA, Afshari CA, and Barrett JC. 2001. Involvement of mammalian MLH1 in the apoptotic response to peroxide-induced oxidative stress. Cancer Res 61, 1392-1397.

Harfe BD and Jinks-Robertson S. 2000a. DNA mismatch repair and genetic instability. Annu Rev Genet 34, 359-399.

Harfe BD and Jinks-Robertson S. 2000b. DNA polymerase ζ introduces multiple mutations when bypassing spontaneous DNA damage in *Saccharomyces cerevisiae*. Mol Cell 6, 1491-1499.

Harwood J, Tachibana A, and Meuth M. 1991. Multiple dispersed spontaneous mutations: a novel pathway of mutation in a malignant human cell line. Mol Cell Biol 11, 3163-3170.

Havraken Y, Sung P, Prakach L, and Prakach S. 1998. ATP-dependent assembly of a ternary complex consisting of a DNA mismatch and the yeast MSH2-MSH6 and MLH1-PMS1 protein complexes. J Biol Chem 273, 9837 – 9841.

Helbock HJ, Beckman KB, and Ames BN. 1999. 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. Methods Enzymol 300,156-166.

Hendricks JD, Shelton DW, Loveland PM, Pereira CB, Bailey GS. 1995. Carcinogenicity of dietary dimethylnitrosomorpholine, N-methyl-N'-nitro-N-nitrosoguanidine, and dibromoethane in rainbow trout.Toxicol Pathol 23, 447-457.

Her C and Doggett NA. 1998. Cloning, structural characterization, and chromosomal localization of the human orthologue of *Saccharomyces cerevisiae* MSH5 gene. Genomics 15, 50 – 61.

Her C, Wu X, Wan W, and Doggett NA. 1999. Identification and characterization of the mouse MutS homolog 5:Msh5. Mamm Genome 10, 1054 – 1061.

Hickman MJ and Samson LD. 1999. Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. Proc Natl Acad Sci USA 96, 10764 – 10769.

Hinges R and Hubscher U. 1995. Production of active mouse DNA polymerase delta in bacteria. Gene 158, 241 – 246.

Hollingsworth NM, Ponte L, and Halsey C. 1995. MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Saccharomyces cerevisiae* but not mismatch repair. Genes Dev 9, 1728 – 1739.

Holmes J, Calrk S, and Modrich P. 1990. Strand-specific mismatch correction in nuclear extracts of human and Drosophila melanogaster cell lines. Proc Natl Acad Sci USA 87, 5837 – 5841.

Hurd RD and Ebbert JO. 2002. Preventing lung cancer by stopping smoking. Clin Chest Med 23, 27-36.

Horn PL, Turker MS, Ogburn CE, Disteche CM, and Martin GM. 1984. A cloning assay for 6-thioguanine resistance provides evidence against certain somatic mutational theories of aging. J Cell Physiol 121, 309-315.

Huang J, Papadopoulos N, McKinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JK, Markowitz SD, Morin P, Kinzler KW, Vogelstein B, and Dunlop MG. 1996. APC mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci USA 93, 9049-9054.

Ionov Y, Peinado MA, Malknhosyan S, Shibata D, and Perucho M. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363, 558- 561.

Jackson AL and Loeb LA. 1998. On the origin of multiple mutations in human cancers. Sem Cancer Biol 8, 421-429.

Jackson AL and Loeb LA. 2001. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res 477, 7-21.

Jackson AL, Chen R, and Loeb LA. 1998. Induction of microsatellite instability by oxidative DNA damage. Proc Nat Acad Sci USA 95, 12468-12473.

Jiricny J. 2000. Mediating mismatch repair. Nat Genet 24, 6-8.

Johnson RE, Kovvali GK, prakash L, and Prakash S. 1995. Requirement of the yeast RTH1 5'to 3'' exonuclease of the stability of simple repetitive DNA. Science 269, 238 –240.

Johnson RE, Prakashi S, and Prakash L. 1999. Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Polη. Science 283, 1001-1004.

Johnson RE, Washington MT, Prakash S, and Prakash L. 2000. Fidelity of human DNA polymerase η. J Biol Chem 275, 7447 – 7450.

Johnson RE, Kovvali GK, Guzder SN Amin NS, Holm C, Habraken Y, Sung P, Prakash L, and Prakash S. 1996a. Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair. J Biol Chem 271, 27987 –27990.

Johnson RE, Kovvali GK, Prakash L, and Prakash S. 1996b. Requirement of the yeast MSH3 and MSH6 genes for MSH2-dependent genomic stability. J Biol Chem 271,7285-7288.

Kamiya H and Kasai H. 1997. Mutations induced by 2-hydroxyadenine on a shuttle vector during leading and lagging strand syntheses in mammalian cells. Biochemistry 36, 11125-11130.

Kanjilal S, Pierceall WE, Cummings KK, Kripke ML, and Ananthaswamy HN. 1993. High frequency of *p53* mutations in ultraviolet radiation-induced murine skin tumors: evidence for strand bias and tumor heterogeneity. Cancer Res 53, 2961-2964.

Karran P, Macphersen P, Ceccoti S, Dogliotci E, Griffin S, and Bignami M. 1993. O6-methylguanine residues elicit DNA repair synthesis by human cell extracts. J Biol Chem 268, 15878-15886.

Kato T, Yatagai F, Glickman BW, Tachibana A, and Ikenaga M. 1998. Specificity of mutations in the *PMS2*-deficient human tumor cell line HEC-1-A. Mutat Res 422, 279-283.

Khattar NH and Turker MS. 1997. A role for certain mouse *Aprt* sequences in resistance to toxic adenine analogs. Somat Cell Mol Genet 23, 51-61.

Kolodner RD adn Marsischky GT. 1999. Eukaryotic DNA mismatch repair. Curr Opin Genet Devolp 9, 89-96.

Kozak C, Nichols E, and Ruddle FH. 1975. Gene linkage analysis in the mouse by somatic cell hybridization: assignment of adenine phosphoribosyltransferase to chromosome 8 and alpha-galactosidase to the X chromosome. Somatic Cell Genet 1, 371-382.

Kraemer KH. 1997. Sunlight and skin cancer: another link revealed. Proc Natl Acad Sci USA 94, 11-14.

Kroutil LC, Register K, Bebenek K, Kunkel TA. 1996. Exonucleolytic proofreading during replication of repetitive DNA. Biochemistry 35, 1046-1053.

Kunkel TA. 1986. Frameshift mutagenesis by eucaryotic DNA polymerases in vitro. J Biol Chem 261, 13581-13587.

Kunkel TA and Alexander PS. 1986. The base substitution fidelity of eucaryotic DNA polymerases. Mispairing frequencies, site preferences, insertion preferences, and base substitution by dislocation. J Biol Chem 261, 160-166.

Kunkel TA and Bebenek K. 2000. DNA replication fidelity. Annu Rev Biochem 69, 497-529.

Kunz BA. 2000. DNA damage-induced mutation tolerance via translesion synthesis. Mutat Res 451, 169-185.

Kunz BA, Straffon AFL, and Vonarx EJ. 2000. DNA damage-induced mutation: tolerance via translesion synthesis. Mutat Res 451, 169-185.

Kunz BA, Ramachandran K, and Vonarx EJ. 1998. DNA sequence analysis of spontaneous mutagenesis in *Saccharomyces cerevisiae*. Genetics 148, 1491-1505.

Kuraguchi M, Edelmann W, Yang K, Lipkin M, Kucherlapati R, and Brown AM. 2000. Tumor-associated *Apc* mutations in *Mlh1-/- Apc*1638N mice reveal a mutational signature of *Mlh1* deficiency. Oncogene 19, 5755-5763.

Kusewiett DF, Dyble J, Sherburn TE, Ryan SL, and Ji YL. 1998. Altered UV resistance and UV mutational spectrum in repair –proficient murine fibroblasts expressing endonuclease V. Mutat Res 407, 157 – 168.

Lage H and Dietel M. 1999. Involvement of the DNA mismatch repair system in antineoplastic drug resistance. J Cancer Res Clin Oncol 125, 156-165.

Lasko D and Cavenee W. 1991. Loss of constitutional heterozygosity in human cancer. Annu Rev Genet 25, 281-314.

Lea DE and Coulson CA. 1949. The distribution of the number of mutants in a bacterial populations. J Genetics 49, 264-285.

Leach EG, Narayanan L, Havre PA, Gunther EJ, Yeasky TM, and Glazer PM. 1996. Tissue specificity of spontaneous point mutations in lambda *supF* transgenic mice. Environ Mol Mutagen 28, 459-464.

Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomaki P, Sistonen P, Aaltonen LA, Nystrom-Lahti M, et al. 1993. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 75, 1215-1225.

Li GM. 1999. The role of mismatch repair in DNA damage-induced apoptosis. Oncol Res 11, 393-400.

Li. GM and Modrich P. 1995. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc Natl Acad Sci USA 92, 1950 –1954.

Lie H, Hewitt SR, and Hays JB. 2000. Antagonism of ultraviolet – light mutagenesis by the methyl-directed mismatch repair system of *Escherichia coli.* Genetics 154, 503-512.

Lin X, Ramamurthi K, Mishima M, Kondo A, Christen RD, and Howell SB. 2001. P53 modulates the effect of loss of DNA mismatch repair on the sensitivity of human colon cancer cells to the cytotoxic and mutagenic effects of cisplatin. Cancer Res 61, 1508-1516.

Lin YL, Shivji MK, Chen C, Kolodner R, Wood RD, and Dutta A. 1998. The evolutionarily conserved zinc finger motif in the largest subunit of human replication protein A is required for DNA replication and mismatch repair but not for nucleotide excision repair. J Biol Chem 273, 1453-1461.

Lindahl T, and Nyberg B. 1974. Heat – induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry 13, 3405 – 3410.

Liu H, Hewitt SR, and Hays JB. 2000. Antagonism of ultraviolet-light mutagenesis by the methyl-directed mismatch-repair system of *Escherichia coli.* Genetics 154, 503-512.

Liu HX, Cartegni L, Zhang MQ, and Krainer AR. 2001. A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. Nat Genet 27, 55-58.

Loeb KR and Loeb LA 2000. Significance of multiple mutations in cancer. Carcinogenesis 21, 379-385.

Loeb LA. 1991. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res 51, 3075-3079.

Loeb LA and Cheng KC. 1990. Errors in DNA synthesis: a source of spontaneous mutations. Mutat Res 38, 297-304.

Luo Y, Henle ES, and Linn S. 1996. Oxidative damage to DNA constituents by iron-mediated Fenton reactions. J Biol Chem 271, 21167-21176.

Luria SE and Delbruck M. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28, 491-511.

Macpherson P, Humbert O, and Karran P. 1998. Frameshift mismatch recognition by the human MutSα complex. Mutat Res 408, 55-66.

Marnett LJ. 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361-370.

Marsden MPF and Laemmli UK 1979. Metaphase chromosome structure: evidence for a radical loop model. Cell 17, 849 – 858.

Masaoka A, Terato H, Kobayashi M, Ohyama Y, and Ide H. 2001. Oxidation of thymine to 5-formyluracil in DNA promotes misincorporation of dGMP and subsequent elongation of a mismatched primer terminus by DNA polymerase. J Biol Chem 276, 16501-16510.

Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, and Hanaoka R. 1999. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η. Nature 399, 700 – 704.

Matsuda T, Bebenek K, Masutani C, Hanaoka F, and Kunkel TA. 2000. Low fidelity DNA synthesis by human DNA polymerase-η. Nature 404, 1011-1013.

Mazurek A, Berardini M, and Fishel R. 2002. Activation of human MutS homologs by 8-oxo-guanine DNA damage. J Biol Chem 277, 8260-8266.

McBride TJ, Preston BD, and Loeb LA. 1991. Mutagenic spectrum resulting from DNA damage by oxygen radicals. Biochemistry 30, 207 – 213.

Mellon I and Champe GN. 1996. Products of DNA mismatch repair genes must and mutL are required for transcription-coupled nucleotide-excision repair of the lactose operon in *Escherichia coli*. Proc Natl Acad Sci USA 93, 1292-1297.

Mellon I, Rajpal DK, Koi M, Boland CR, and Champe GN. 1996. Transcription-coupled repair deficiency and mutations in human mismatch repair genes. Science 272, 557-560.

Mendel man LV, Boosalis MS, Petruska J, and Goodman MF. 1989. Nearest neighbor influences on DNA polymerase insertion fidelity. J Biol Chem 264, 14415-14423.

Modrich P. 1991. Mechanisms and biological effects of mismatch repair. Annu Rev Genet 25, 229 –253.

Modrich P. 1994. Mismatch repair, genetic stability, and cancer. Science 266, 1959 – 1960.

Modrich P and Lahue R. 1996. Mismatch repair in replication fidelity, genetic recombination and cancer biology. Annu Rev Biochem 65, 101 – 133.

Moriya M. 1993. Single-stranded shuttle phage plsmid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G:C-->T:A transversions in simian kidney cells. Proc Natl Acad Sci USA 90, 1122-1126.

Mu D, Tursun M, Duckett DR, Drummond JT, Modrich P, and Sancar A. 1997. Recognition and repair of compound DNA lesions (base damage and mismatch) by human mismatch repair and excision repair systems. Mol Cell Biol 17, 760-769.

Muller A and Fishel R. 2002. Mismatch repair and the hereditary non-polyposis colorectal cancer syndrome (HNPCC). Cancer Invest 20, 102-109.

Mummaneni P, Bishop PL, and Turker MS. 1993. A cis-acting element accounts for a conserved methylation pattern upstream of the mouse adenine phosphoribosyltransferase gene. J Biol Chem 268, 552-558.

Nakamura J, Walker VE, Upson PB, Chiang SY, Kow YW, and Swenberg JA. 1998. Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. Cancer Res 58, 222-225.

Nara K, Nagashima F, and Yasui A. 2001. Highly elevated ultraviolet-induced mutation frequency in isolated Chinese hamster cell lines defective in nucleotide excision repair and mismatch repair proteins. Cancer Res 61, 50-52.

Narayanan L, Fritzell JA, Baker SM, Liskay RM, and Glazer PM. 1997. Elevated levels of mutation in multiple tissues of mice deficient in the DNA mismatch repair gene *Pms2*. Proc Natl Acad Sci USA 94, 3122-3127.

Ni TT, Marsischky GT, and Kolodner RD. 1999. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in *S. cerevisiae*. Mol Cell 4, 439 – 444.

O'Driscoll M, Martinelli S, Ciotta C, and Karran P. 1999. Combined mismatch and nucleotide excision repair defects in a human cell line: mismatch repair processes methylation but not UV- or ionizing radiation-induced DNA damage. Carcinogenesis 20, 799-804.

Ohashi E, Bebenek K. Matusda T, Feaver WJ, Geralch VL, Friedberg EC, Ohmori H, and Kunkel TA. 2000. Fidelity and processivity of DNA synthesis by DNA polymerase κ, the product of the human DINB1 gene. J Biol Chem 275, 39678-39684.

Ohzeki S, Tachibana A, Tatsumi K, and Kato T. 1997. Spectra of spontaneous mutations at the *hprt* locus in colorectal carcinoma cell lines defective in mismatch repair. Carcinogenesis 18, 1127-1133.

Palombo F, Gallinari P, Iaccarino I, Lettieri T, Hughes M, D'Arrigo A, Truong O, Hsuan JJ, and Jiricny J. 1995. GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science 268, 1912-1914.

Palombo F, Iaccarino I, Nakajima E, Ikejima M, Shimada T, and Jiricny J. 1996. hMutSβ, a heterodimer of hMsh2 and hMsh3, binds to insertion/deletion loops in DNA. Curr Biol 6, 1181- 1184.

Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, et al. 1994. Mutation of a mutL homolog in hereditary colon cancer. Science 263, 1625-1629.

Parker BO and Marinus MG. 1992. Repair of DNA heteroduplexes containing small heterologous sequences in *Escherichia coli*. Proc Natl Acad Sci USA 89, 1730-1734.

Parsons R, Li GM, Longley MJ, Fang W-H, Papadopoulos N, Jen J, de la Chappelle A, Kinzler KW, Vogelstein B, and Modrich P. 1993. Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell 75, 1227-1236.

Parsons R, Myeroff LL, Liu B, Willson JK, Markowitz SD, Kinzler KW, and Vogelstein B. 1995. Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. Cancer Res 55, 5548-5550.

Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, and Dalla-Favera R. 2001. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 412, 341-346.

Peng W and Shaw BR. 1996. Accelerated deamination of cytosine residues in UV-induced cyclobutane pyrimidine dimers leads to CC → TT transitions. Biochemistry 35, 10172-10181.

Peltomaki p and Vasen HF. 1997. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The international collaborative group on hereditary nonpolyposis colorectal cancer. Gastroenterology 113, 1146-1158.

Phear G, Battacharyya NP, and Meuth M. 1996. Loss of heterozygosity and base substitution at the *APRT* locus in mismatch-repair-proficient and -deficient colorectal carcinoma cell lines. Mol Cell Biol 16, 6516-6523.

Pochart P, Woltering D, and Hollingsworth NM. 1997. Conserved properties between functionally distinct MutS homologs in yeast. J Biol Chem 272, 30345-30349.

Ponomareva ON, Rose JA, Lasarev M, Rasey J, and Turker MS. 2002. Tissue-specific deletion and discontinuous loss of heterozygosity are signatures for the mutagenic effects of ionizing radiation in solid tissues. Cancer Res 62, 1518-1523.

Preuss I, Thust R, and Kaina B. 1996. Protective effect of O6-methylguanine-DNA methyltransferase (MGMT) on the cytotoxic and recombinogenic activity of different antineoplastic drugs. Int J Cancer 65, 506-512.

Prolla TA, Christie DM, and Liskay RM. 1994. Dual requirement in yeast DNA mismatch repair for MLH1 and PMS1, two homologs of the bacterial mutL gene. Mol Cell Biol 14, 407-415.

Prolla TA, Baker SM, Harris AC, Tsao JL, Yao X, Bronner CE, Zheng B, Gordon M, Reneker J, Arnheim N, Shibata D, Bradley A, and Liskay RM. 1998. Tumour susceptibility and spontaneous mutation in mice deficient in *Mlh1*, *Pms1* and *Pms2* DNA mismatch repair. Nature Genet 18, 276-279.

Psaroudi MC and Kyrtopoulos SA. 2000. Toxicity, mutation frequency and mutation spectrum induced by dacarbazine in CHO cells expressing different levels of O^6-methylguanine-DNA methyltransferase. Mutat Res 447, 257-265.

Quah SR, von Borstel RC, and Hastings PJ. 1980. The origin of spontaneous mutation in *Saccharomyces cerevisiae*. Genetics 96, 819-839.

Qui J, Qian Y, Chen V, Guan MX, and Shen B. 1999. Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal, and mutation avoidance. J Biol Chem 274, 17893- 17900.

Raschle M, Marra G, Nystrom-Lahti M, ScharP, and Jiricny J. 1999. Identification of h MutLβ, a heterodimer of hMLH1 and hPMS1. J Biol Chem 274, 32368 – 32375.

Reenan RA and Kolodner RD. 1992. Characterization of insertion mutations in the *Saccharomyces cerevisiae* MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. Genetics 132, 975 – 985.

Reid TM and Loeb LA. 1992. Mutagenic specificity of oxygen radicals produced by human leukemia cells. Cancer Res 52, 1082 – 1086.

Reid TM and Loeb LA. 1993. Tandem double CC → TT mutations are produced by reactive oxygen species. Proc Natl Acad Sci USA 90, 3904 – 3907.

Rose JA, Yates PA, Simpson J, Tischfield JA, Stambrook PJ, and Turker MS. 2000. Biallelic methylation and silencing of mouse *Aprt* in normal kidney cells. Cancer Res 60, 3404-3408.

Ross-Macdonald P and Roeder GS. 1994. Mutation of a meiosis- specific MutS homolog decreases crossing over but not mismatch correction. Cell 79, 1069 – 1080.

Sander M and Samson LD. 2000. Great leaps forward: translesion synthesis gets unstalled. Trends Cell Biol 10, 159-162.

Schlotterer C and Tautz D. 1992. Slippage synthesis of simple sequence DNA. Nucleic Acids Res 20, 211-215.

Schmutte C, Marinescu RC, Sadoff MM, Guerrette S, Overhause J, and Fishel R. 1998. Human exonuclease I interacts with the mismatch repair protein hMSH2. Cancer Res 58, 4537 – 4542.

Schmutte C and Jones PA. 1998. Involvement of DNA methylation in human carcinogenesis. Biol Chem 379, 377-388.

Scott AD, Neishabury M, Jones DH, Reed SH, Boiteux S, and Waters R. 1999. Spontaneous mutation, oxidative DNA damage, and the roles of base and nucleotide excision repair in the yeast *Saccharomyces cerevisiae*. Yeast 15, 205-218.

Shaaper RM. 1993. Base selection, proofreading, and mismatch repair during DNA replication in *Excherichia coli*. J Biol Chem 268, 23746 –23765.

Shao C, Deng L, Henegariu O, Liang L, Raikwar N, Sahota A, Stambrook PJ, and Tischfield JA. 1999. Mitotic recombination produces the majority of recessive fibroblast variants in heterozygous mice. Proc Natl Acad Sci USA 96, 9230-9235.

Shao C, Yin M, Deng L, Stambrook PJ, Doetschman T, and Tischfield JA. 2002. Loss of heterozygosity and point mutation at *Aprt* locus in T cells and fibroblasts of *Pms2* (-/-) mice. Oncogene 21, 2840-2845.

Shin CY, Mellon I, and Turker MS. 2002. Multiple mutations are common at mouse *Aprt* in genotoxin-exposed mismatch repair deficient cells. Oncogene 21, 1768-1776.

Sia EA, Butler CA, Kominska M, Greenwell P, Fox ED, and Petes T. 2000. Analysis of microsatellite mutations in the mitochondrial DNA of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 97, 250 – 255.

Simon M, Giot L, and Faye G. 1991. The 3' to 5' exonuclease activity located in the DNA polymerase delta subunit of *Saccharomyces cerevisiae* is required for accurate replication. EMBO J 10, 2165-2170.

Smith ML, Ford JM, Hollander MC, Bortnick RA, Amundson SA, Seo YR, Deng CX, Hanawalt PC, and Fornace AJ Jr. 2000. *p53*-mediated DNA repair responses to UV radiation: studies of mouse cells lacking *p53*, *p21*, and/or *gadd45* genes. Mol Cell Biol 20, 3705-3714.

Soufir N, Daya-Grosjean L, De La Salmoniere P, Moles JP, Dubertret L, Sarasin A, and Basset-Seguin N. 2000. Correlation between mutation in *p16INK4a*, *p14 ART*, and *p53* genes in skin carcinomas from xeroderma pigmentosum patients. J Natl Cancer Inst 92, 1841-1847.

Spatz A, Giglia-Mari G, Benhamou S, and Sarasin A. 2001. Association between DNA repair-deficiency and high level of *p53* mutations in melanoma of Xeroderma Pigmentosum. Cancer Res 61, 2480-2486.

Stoler DB, Chen N, Bsik M, Kahlenberg MS, Rodriguez-bigas MA, Petrelli NJ, and Andersion GR. 1999. The onset and extent of genomic instability in sporadic colorectal tumor progression. Proc Natl Acad Sci USA 96, 15121 – 15126.

Strand M, Earley MC, Crouse GF, and Petes T. 1995. Mutations in the MSH3 gene preferentially lead to deletions within tracts of simple repetitive DNA in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 92, 10418-10421.

Strand M, Prolla T, Liskay RM, and Petes T. 1993. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. Nature 365, 274-276.

Strauss BS, Sagher D, and Acharya S. 1997. Role of proofreading and mismatch repair in maintaining the stability of nucleotide repeats in DNA. Nucleic Acids Res 25, 806-813.

Strauss BS. 1999. Frameshift mutation, microsatellites and mismatch repair. Mutat Res 437, 195 – 203.

Strauss BS. 1997. Silent and multiple mutations in *p53* and the question of the hypermutability of tumors. Carcinogenesis 18, 1445-1452.

Strauss BS. 1998a. Hypermutability and silent mutations in human carcinogenesis. Semin Cancer Biol 8, 431-438.

Strauss BS. 1998b. Hypermutability in carcinogenesis. Genetics 148, 1619-1626.

Strauss BS. 2000. Role in tumorigenesis of silent mutations in the TP53 gene. Mutat Res 457, 93-104.

Studamire B, Quach T, and Alani E. 1998. *Saccharomyces cerevisiae* Msh2p and Msh6p ATPase activities are both required during mismatch repair. Mol Cell Biol 18, 7590 – 7601.

Suzuki M, Sugimoto Y, Tsukahara S, Okochi E, Gottesman MM, and Tsuruo T. 1997. Retroviral coexpression of two different types of drug resistance genes to protect normal cells from combination chemotherapy. Clin Cancer Res 3, 947-954.

Syvaoja J, Suomensaari S, Nishida C, Goldsmith JS, Chui GSJ, Jain S, and Linn S. 1990. DNA polymerase α, δ, and ε: three distinct enzymes from HeLa cells. Proc Natl Acad Sci USA 87, 6664-6668.

Takeuchi S, Nakatsu Y, Nakane H, Murai H, Hirota S, Kitamura Y, Okuyama A, and Tanaka K. 1998. Strand specificity and absence of hot spots for *p53* mutations in ultraviolet B-induced skin tumors of XPA-deficient mice. Cancer Res 58, 641-646.

Tanaka J, Kamiuchi S, Ren Y, Yonemasu R, Ichikawa M, Murai H, Yoshino M, Taeuchi S, Saijo M, Nakatsu Y, Miyauch-Hashimoto H, Horio T. 2001. UV-induced skin carcinogenesis in xeroderma pigmentosum group A (XPA) gene-knockout mice with nucleotide excision repair-deficiency. Mutat Res 477, 31–40.

Thomas DC, Roberts JD, and Kunkel TA. 1991. Heteroduplex repair in extracts of human HeLa cells. J Biol Chem 266, 3744-3751.

Tishikoff DX, Filosi N, Gaida GM, and Kolonder RD. 1997a. A novel mutation avoidance mechanism dependent on *S. cerevisiae* RAD27 is distinct from DNA mismatch repair. Cell 88, 253 –263.

Tishkoff DX, Boerger AL, Bertarand P, Filosi N, Gaida GM. 1997b. Identification and characterization of *Saccharomyces cerevisiae* EXO1, a gene encoding an exonuclease that interacts with MSH2. Proc Natl Acad Sci USA 94, 7487-7492.

Tisssieer A, Frank EG, McDonald JP, Iwai S, Hanaoka F, and Woodgate R. 2000. Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota. EMBO J 19, 5259-5266.

Tkeshelashvili LK, McBride T, Spence K, and Loeb LA. 1991. Mutation spectrum of copper-induced DNA damage. J Biol Chem 266, 6401 – 6406.

Tobi SE, Levy DD, Seidman1 MM, and Kraemer KH. 1999. Sequence-dependent mutations in a shuttle vector plasmid replicated in a mismatch repair deficient human cell line. Carcinogenesis 20, 1293-1301.

Toft NJ, Winton DJ, Kelly J, Howard LA, Dekker M, te Riele H, Arends MJ, Wyllie AH, Margison GP, and Clarke AR. 1999. *Msh2* status modulates both apoptosis and mutation frequency in the murine small intestine. Proc Natl Acad Sci USA 96, 3911-3915.

Tomita-Mitchell A, Kat AG, Marcelino LA, Li-Sucholeiki XC, Goodluck-Griffith J, and Thilly WG. 2000. Mismatch repair deficient human cells: spontaneous and MNNG-induced mutational spectra in the *HPRT* gene. Mutat Res 450, 125-138.

Tomlinson I and Bodmer W. 1999. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat Med 5, 11-12.

Tornaletti S and Pfeifer GP. 1996. UV damage and repair mechanisms in mammalian cells. Bioassays 18, 221-228.

Tran HT, Godenin DA, and Resnick MA. 1999. The 3'-5' exonucleases of DNA polymerases δ and ε and the 5'-3' exonuclease Exo1 have major roles in postreplication mutation avoidance in *Saccharomyces cerevisiae*. Mol Cell Biol 19, 2000-2007.

Tran HT, Keen JD, Kricker M, Resnick MA, and Gordenin DA. 1997. Hypermutability of homonucleotide runs in mismatch repair and DNA polymerase proofreading yeast mutants. Mol Cell Biol 17, 2859-2865.

Tran PT and Liskay RM. 2000. Functional studies on the candidate ATPase domain of *Saccharomyces cerevisiae* Mutlalpha. Mol Cell Biol 20, 6390-6398.

Tran PT, Simon JA, and Liskay RM. 2001. Interactions of Exo1p with components of MutLalpha in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 98, 9760-9765.

Turker M, Walker KA, Jennings CD, Mellon I, Yusufji A, and Urano M. 1995. Spontaneous and ionizing radiation induced mutations involve large events when selecting for loss of an autosomal locus. Mutat Res 329, 97-105.

Turker MS. 1998. Estimation of mutation frequencies in normal mammalian tissues and the development of cancer. Seminars in Cancer Biology 8, 407-419.

Turker MS, Gage BM, Rose JA, Elroy D, Ponomareva ON, Stambrook PJ, and Tischfield JA. 1999. A novel signature mutation for oxidative damage resembles a mutational pattern found commonly in human cancers. Cancer Res 59, 1837-1839.

Turker MS, Mummaneni P, and Bishop PL. 1991. Region and cell type-specific de novo DNA methylation in cultured mammalian cells. Somat Cell Mol Genet 17, 151-157.

Turker MS, Pieretti M, and Kumar S. 1997. Molecular evidence for the induction of large interstitial deletions on mouse chromosome 8 by ionizing radiation. Mutat Res 374, 201-208.

Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Liskay RM, and Kunkel TA. 1996. Requirement for PCNA in DNA mismatch repair at a step proceeding DNA resynthesis. Cell 87, 65-73.

Umar A and Kunkel TA. 1996. DNA- replication fidelity, mismatch repair and genomic instability in cancer cells. Eur J Biochem 238, 297 – 307.

Van Steeg H, and Kraemer KH. 1999. Xeroderma pigmentosum and the role of UV-induced DNA damage in skin cancer. Mol Med Today 5, 86 – 94.

Van Steeg H, Mullenders LH, and Vijg J. 2000. Mutagenesis and carcinogenesis in nucleotide excision repair- deficient XPA knock out mice. Mutat Res 450, 167-180.

Viswanathan M and Lovett ST. 1998. Single-stranded DNA-specific exonucleases in Escherichia coli. Roles in repair and mutation avoidance. Genetics 149, 7-16.

Vreeswijk MP, Overkamp MW, Westland BE, van Hees-Stuivenberg S, Vrieling H, Zdzienicka MZ, van Zeeland AA, and Mullenders LH. 1998. Enhanced UV-induced mutagenesis in the UV61 cell line, the Chinese hamster homologue of Cockayne's syndrome B, is associated with defective transcription-coupled repair of cyclobutane pyrimidine dimers. Mutat Res 409, 49-56.

Vrieling H, van Zeeland AA, and Mullenders LH. 1998. Transcription-coupled repair and its impact on mutagenesis. Mutat Res 400, 135-142.

Walker KA, Jennings CD, Pulliam J, Ogburn CE, Martin GM, Urano M, and Turker MS. 1997. A cell line selected for resistance to ionizing radiation exhibits cross-resistance to other genotoxic agents and a mutator phenotype for loss of heterozygosity events. Somat Cell Mol Genet 23, 111-121.

Wang H and Hays JB. 2002. Mismatch repair in human nuclear extracts. J Biol Chem 277, 26136-26142.

Wang H, Lawrence CW, Li GM, and Hays JB. 1999. Specific binding of human MSH2:MSH6 mismatch-repair protein heterodimers to DNA incorporating thymine- or uracil-containing UV light photoproducts opposite mismatched bases. J Biol Chem 274, 16894-16900.

Weihrauch M, Bader M, Lehnert G, Wittekind C, Tannapfel A, Wrbitzky R. 2002. Carcinogen-specific mutation pattern in the p53 tumour suppressor gene in UV radiation-induced basal cell carcinoma. Int Arch Occup Environ Health 75, 272-276.

Weinberg RA. 1995. The molecular basis of oncogenes and tumor suppressor genes. Ann NY Acad Sci 758, 331-338.

Wilson DM, Carney JP, Coleman MA, Adamson AW, Christensen M, Lamerdin JE. 1998. Hex1: a new human Rad2 nuclease family member with homology to yeast exonuclease 1. Nucleic Acids Res 26, 3762 –3768.

Wu TH and Marinus MG. 1994. Dominant negative mutator mutations in the *mutS* gene of *Escherichia coli.* J Bacteriol 176, 5393 – 5400.

Xu XS, Narayanan L, Dunklee B, Liskay RM, and Glazer PM. 2001. Hypermutability to ionizing radiation in mismatch repair –deficient, *Pms2* knockout mice. Cancer Res 61, 3775-3780.

Yamada M, O'Regan E, Brown R, and Karran P. Selective recognition of a cisplatin-DNA adduct by human mismatch repair proteins. 1997. Nucleic Acids Res 25, 491-496.

Yamaguchik M, Dao V, and Modrich P. 1998. MutS and MutL activate DNA helicase II is a mismatch – dependent manner. J Biol Chem 273, 9197 – 9201.

Yao X, Buermeyer AB, Narayanan L, Tran D, Baker SM, Prolla TA, Glazer PM, Liskay RM, and Arnheim N. 1999. Different mutator phenotypes in *Mlh1*- versus *Pms2*-deficient mice. Proc Natl Acad Sci USA 96, 6850-6855.

Zeng M, Narayanan L, Xu XS, Prolla TA, Liskay RM, and Glazer PM. 2000. Ionizing radiation- induced apoptosis via separate *Pms2*-and *p53* – dependent pathways. Cancer Res 60, 4889 – 4893.

Zhang QM, Ishikawa N, Nakahara T, and Yonei S. 1998. *Escherichia coli* MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C-->C:G transversions. Nucleic Acids Res 26, 4669-4675.

Zhang S, Lioyd R, Bowden G, Glickman BW, and de Boer JG. 2002. Thymic lymphomas arising in *Msh2*-deficient mice display a large increase in mutation frequency and an altered mutational spectrum. Mutat Res 500, 67-74.

APPENDIX

Table 15. Supplemented data for forward mutation rate studies

Cell lines	Pots	Number of Cells (10^6)*	Number of Mutants*
KO6	1	2.31	0
(wild type)	2	2.30	173
	3	2.53	0
	4	1.63	0
	5	3.00	0
	6	2.70	0
	7	2.00	109
	8	2.42	0
K2	1	1.54	150
(Pms2 null)	2	1.76	11
	3	2.09	264
	4	1.65	10
	5	1.76	46
	6	1.89	23
	7	3.30	228
	8	1.76	528
	9	2.09	57
	10	1.32	118
K597S2	1	1.04	102
(Pms2 null)	2	1.68	519
	3	1.98	803
	4	1.43	193
	5	1.65	296
	6	1.32	205
	7	1.72	5489
	8	1.98	592
	9	1.98	193
	10	1.54	172

Table 15 continues.

Cell lines	Pots	Number of Cells (10^6)	Number of Mutants
K634S2 (Mlh1 null)	1	3.30	3378
	2	2.50	1274
	3	1.90	1276
	4	3.50	1707
	5	2.70	2475
	6	9.00	1200
	7	2.40	1288
	8	1.60	4297
	9	2.00	710
	10	2.90	4669
K435	1	1.70	184
	2	2.31	303
	3	2.86	129
	4	2.86	64
	5	2.97	300
	6	2.53	282
	7	3.52	1627
	8	1.65	387

* The number of cells and the number of mutants were adjusted by plating efficiencies.

Table 16. Supplemented data for reversion mutation rate studies in regular medium.

Cell lines	Pots	Number of Cells (10^6)	Number of Revertants
Pms2T→CS1	1	13.0	149
	2	15.0	6492
	3	10.0	352
	4	11.0	160
	5	34.0	725
	6	24.0	512
	7	18.4	310
	8	12.0	1363
	9	12.0	870
	10	8.4	176
Pms2T→CS15	1	6.0	4078
	2	4.3	180
	3	8.0	555
	4	6.0	209
	5	6.0	414
	6	8.2	346
	7	8.0	158
	8	8.0	112
	9	6.2	136
Pms2C→TS1	1	41.0	36
	2	47.0	52
	3	45.0	47
	4	38.0	15
	5	66.0	65
	6	56.4	72
	7	24.4	48
	8	20.0	60
	9	35.2	66
	10	26.4	25
	11	47.0	16
	12	24.4	74
	13	26.6	28
	14	26.0	606

Table 16 continues.

Cell lines	Pots	Number of Cells (10^6)	Number of Revertants
Pms2C→TS3	1	8.1	79
	2	45.3	32
	3	23.1	9
	4	8.0	0
	5	18.3	334
	6	19.5	0
	7	43.0	271
	8	35.0	311
	9	50.0	179
	10	43.0	175
	11	52.0	382
	12	39.0	285
	13	56.0	292
	14	39.0	208
Pms2G→TS2	1	24.0	74
	2	38.0	70
	3	39.0	75
	4	42.0	32
	5	29.0	25
	6	33.0	61
	7	27.0	17
	8	29.0	48
	9	25.0	30
	10	25.0	67
	11	31.0	63
Pms2G→TS5	1	24.0	41
	2	26.0	140
	3	33.0	26
	4	23.0	59
	5	21.0	0
	6	17.0	39
	7	16.0	35
	8	22.0	852

* The number of cells and the number of mutants were adjusted by plating efficiencies.

Table 17. Supplemented data for reversion mutation rate studies in antioxidant medium.

Cell lines	Pots	Number of Cells (10^6)	Number of Revertants
Pms2T→CS1	1	17.2	80
(Antioxidant)	2	13.0	161
	3	12.2	222
	4	13.0	129
	5	18.3	522
	6	11.0	615
	7	9.0	39
	8	10.0	45
	9	10.0	132
	10	9.3	54
Pms2T→CS15	1	12.0	72
(Antioxidant)	2	10.0	22
	3	12.4	151
	4	12.0	87
	5	11.0	40
	6	12.0	154
	7	10.2	202
	8	12.4	1124
Pms2C→TS1	1	34.4	122
(Antioxidant)	2	48.0	88
	3	41.2	49
	4	42.0	44
	5	43.0	136
	6	45.2	180
	7	62.4	106
	8	53.2	113
	9	45.6	42
	10	42.0	14
Pms2C→TS3	1	46.0	507
(Antioxidant)	2	48.0	327
	3	58.0	366
	4	52.0	733
	5	56.0	515
	6	32.0	248
	7	23.0	774
	8	65.0	327
	9	49.0	170

Table 17 continues.

Cell lines	Pots	Number of Cells (10^6)	Number of Revertants
Pms2G→TS2	1	114.0	145
(Antioxidant)	2	86.4	120
	3	80.0	711
	4	55.5	506
	5	69.0	107
	6	81.0	100
	7	85.0	278
	8	81.0	335
	9	101.0	280
	10	76.0	269
Pms2G→TS5	1	26.0	93
(Antioxidant)	2	24.0	99
	3	25.0	299
	4	27.0	566
	5	19.0	1084
	6	30.0	350
	7	14.0	20
	8	30.0	135
	9	32.0	62
	10	26.0	39

* The number of cells and the number of mutants were adjusted by plating efficiencies.

Table 18. Supplemented data for reversion frequency of *Pms2*-proficient cells.

		Revertants	Total # of cells	% Clone Efficiency
A. UV				
Dif6S6	9.6 J/m^2	98	22×10^6	40%
	12 J/m^2	64	8×10^6	40%
	14.4 J/m^2	231	22×10^6	30%
Dif6S2	9.6 J/m^2	52	7×10^6	15%
	12 J/m^2	208	10×10^6	15%
B. H_2O_2				
	Expt 1	0	25×10^6	56%
	Expt 2	0	20×10^6	50%
	Expt 3	0	20×10^6	37%
	Expt 4	3	16×10^6	30%
	Expt 5	2	15×10^6	30%
	Expt 6	0	25×10^6	35%
	Expt 7	0	25×10^6	50%
C. $H_2O_2/CuSO_4/FeSO_4$				
	Expt 1	46	110×10^6	40%
	Expt 2	0	30×10^6	30%
	Expt 3	0	20×10^6	47%
	Expt 4	4	18×10^6	30%
	Expt 5	0	25×10^6	50%
	Expt 6	1	20×10^6	26%
	Expt 7	0	30×10^6	35%
	Expt 8	0	20×10^6	45%
	Expt 9	47	140×10^6	28%
Non treated controls including all the experiments (A – C)		0	$>5 \times 10^8$	40-50%