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Repair of DNA alkylation damage*°

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Alkylation damage of DNA is one of the major types of insults which cells must repair to remain viable. One way alkylation damaged ring nitrogens are repaired is *via* the Base Excision Repair (BER) pathway. Examination of mutants in both BER and Nucleotide Excision Repair show that there is actually an overlap of repair by these two pathways for the removal of cytotoxic lesions in *Escherichia coli*. The enzymes removing damaged bases in the first step in the BER pathway are DNA glycosylases. The coding sequences for a number of methylpurine-DNA glycosylases (MPG proteins) were cloned, and a comparison of the amino-acid sequences shows that there are some similarities between these proteins, but nonetheless, compared to other DNA glycosylases, MPG proteins are more divergent. MPG proteins have been purified to homogeneity and used to identify their substrates ranging from methylating agents to deamination products to oxidatively damaged bases. The ligation-mediated polymerase chain reaction has been used to study the formation of alkylation damage, and its repair in mammalian cells. We have studied DNA damage in the PGK1 gene for a series of DNA alkylating agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, Mechlorethamine, and Chlorambucil and shown that the damage observed in the PGK1 (phosphoglycerate kinase 1) gene depends on the alkylating agent used. This

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Abbreviations: ALKA protein, product of the *alkA* gene, 3-methyladenine-DNA glycosylase II; AP, abasic site, apurinic/apyrimidinic site; APDG, alkylpurine-DNA glycosylase cDNA, rat MPG protein; BER, base excision repair; BNNG, *N*-butyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; FPG protein, formamidopyrimidine-DNA glycosylase, MutM protein; LMPCR, ligation-mediated polymerase chain reaction; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MPG protein, methylpurine-DNA glycosylase; NER, nucleotide excision repair; PGK1, phosphoglycerate kinase 1; PNNG, *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine; SDS, sodium dodecyl sulfate; TAG protein, product of the *E. coli tag* gene, 3-methyladenine-DNA glycosylase.

report reviews the literature on the MPG proteins, DNA glycosylases removing 3-methyladenine, and the use of these enzymes to detect DNA damage at the nucleotide level.

Alkylation damage

The major cellular target for alkylating agents is DNA. Products formed by the reaction of these agents with DNA include modified bases, sugars, and phosphotriesters (Beranek, 1990; Singer & Grunberger, 1983). The major adducts formed by methylating agents are found at the N7 position of Gua, which is considered a relatively benign modification since methylation at this position does not directly affect DNA base pairing (Boiteux *et al.* 1984; Larson *et al.*, 1985; O'Connor *et al.* 1988). Adducts at the N3 position of Ade and the O6 position of Gua, however, interfere with Watson-Crick base pairing, and either block DNA replication (a potentially lethal event), transcription, or serve as premutagenic lesions (Lindahl *et al.* 1988; Pieper & Erickson, 1990).

Alkylation damage to DNA may arise from either endogenous or exogenous sources. Endogenous sources of DNA alkylation include the *S*-adenosylmethionine pool and lipid peroxidation products (Barrows & Magee, 1982; Marnett & Burcham, 1993; Rydberg & Lindahl, 1982). The list of exogenous alkylating agents is too extensive to include here, but some of these include nitrosamines, nitrosoureas, chemotherapeutic agents, and vinyl chloride (Singer & Grunberger, 1983). *N*-Methyl purines are repaired principally *via* the base excision repair (BER) pathway (Engelward *et al.* 1996; Krokan *et al.* 1997).

BER OF SIMPLE ALKYLATING AGENT DAMAGE IN *E. COLI*

BER in *Escherichia coli* consists of a single pathway and variations of this repair exist in all cells. Figure 1 shows a model for this DNA repair pathway. The first step of BER is the excision of the modified base by a DNA glycosylase. DNA glycosylases cleave the glycosylic bond to release the base, and in some cases also incise the phosphodiester bond with an associated AP lyase activity, which catalyzes β-elimination (Doetsch & Cunningham, 1990). There are two DNA glycosylases in *E. coli*, protecting cells from the cytotoxic effects of simple alkylating agents (methyl-, ethyl-, propyl-, and butylating agents) by the excision of damaged bases. The TAG protein is a DNA glycosylase that is constitutively expressed, and excises primarily 3-meAde and 3-meGua (Bjelland *et al.* 1993; Lindahl *et al.* 1988). The ALKA protein, the other DNA glycosylase protecting cells from simple alkylating agent damage, is induced following treatment of cells with low levels of DNA alkylating agents during the adaptive response (Lindahl *et al.* 1988). In contrast to the TAG protein, ALKA protein recognizes 3-meAde, 3-meGua, 7-meGua, and a number of other base damages. Figure 2 compares the survival of a series of *E. coli* wild type, *tagalkA-*, *uvrA*, or *tagalkAuvrA*- strains subjected to treatment with simple alkylating agents. The series from methyl to butyl alkylation by *N,N'*-nitrosoguanidines, S_N1 type alkylating agents, was examined in these mutants deficient in either the BER, nucleotide excision repair (NER), or both pathways in *E. coli*. For methylation and ethylation damage (MNNG and ENNG), the mutant defective in both the BER and NER pathways is not much more sensitive than the BER mutant alone, therefore repair of the cytotoxic lesions is effected primarily by the BER pathway. For propylation and butylation (PNNG and BNNG), however, cytotoxicity is almost equal for mutants deficient in only BER or NER. Extreme sensitivities to propylation or butylation are observed only when both the BER and NER systems are not

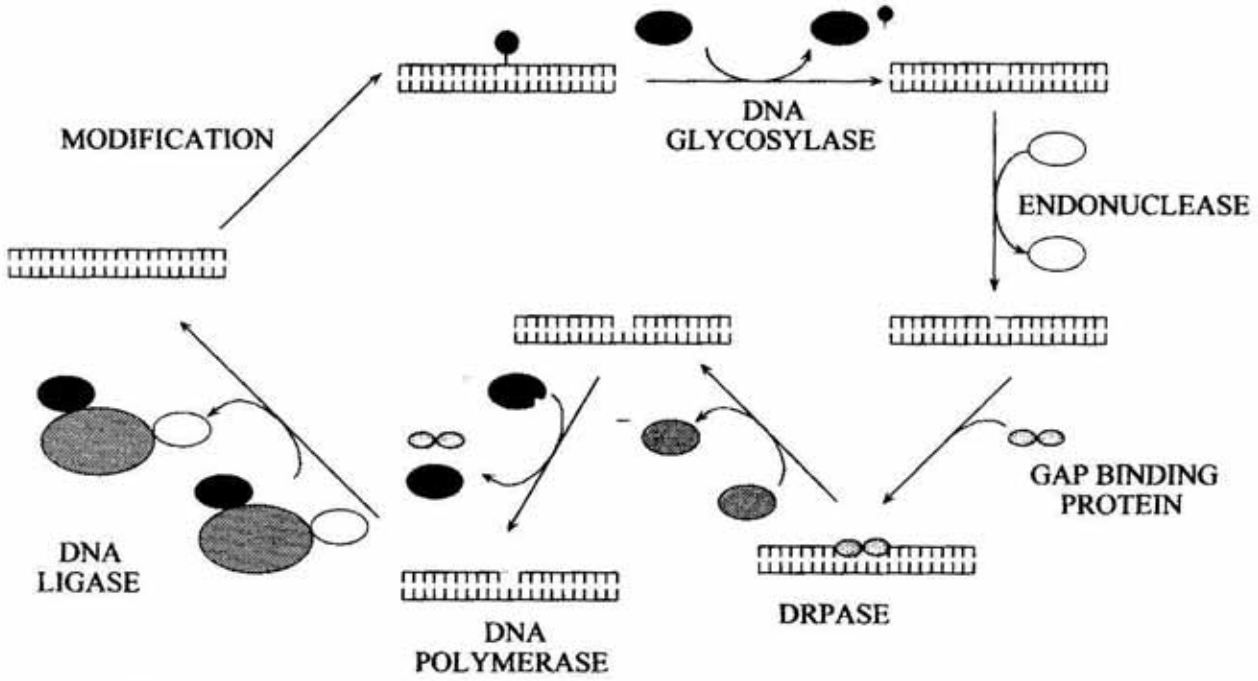

Figure 1. Base excision repair of DNA damage.

A modified base is removed by a DNA glycosylase, followed by incision by an AP endonuclease such as the exonuclease III or the human AP endonuclease. This is followed by removal of the 5′-deoxyribose phosphate either by a lyase (e.g. FPG protein in *E. coli* or the DNA polymerase β in mammalian cells), or a deoxyribosephosphodiesterase (DRPASE) (e.g. RECJ protein in *E. coli*). The intermediates may be protected from further damage by gap binding proteins (HU protein (Castaing *et al.* 1995), or poly(ADP)ribose polymerase) DNA polymerases fill in the gap (e.g. DNA polymerase I in *E. coli*, or DNA polymerase β in mammalian cells) DNA ligase then seals the nick to complete the repair (e.g. DNA ligase III in mammalian cells). The final step involves the formation of a complex between DNA ligase, XRCC1, and poly(ADP)ribose polymerase (Caldecott *et al.* 1996). In mammalian cells DNA polymerase β is also in the complex (Klungland & Lindahl, 1997; Kubota *et al.* 1996).

functional. Therefore both systems share the repair of cytotoxic lesions induced by PNNG and BNNG.

CLONING OF EUCARYOTIC MPG PROTEINS

The same method, used to clone all of the coding sequences for the MPG proteins, has proved invaluable for studying both their genes and gene products. This procedure is based on the observation that, when plated on solid media containing methylmethane sulfonate, the BER-deficient cells are 10^5-fold more sensitive to chemical methylation compared to wild type cells (Boiteux *et al.* 1984). A recombinant plasmid expression library of the coding sequences from a specific organism is transformed into *E. coli* cells deficient in both the *tag* and *alkA* genes. Aliquots of this library are plated onto LB containing methylmethane sulfonate, and plasmids from the surviving colonies are assayed for their ability to transfer this resistance to cells not exposed to methylmethane sulfonate (Berdal *et al.* 1990; Chakravarti *et al.* 1991; Chen *et al.* 1989; Clarke *et al.* 1984; Engelward *et al.* 1993; Morohoshi *et al.* 1993; O'Connor & Laval, 1990; O'Connor & Laval, 1991; Pierre & Laval, 1986; Samson *et al.* 1991; Santerre & Britt, 1994). Collectively, the proteins isolated using such a method may be referred to as MPG proteins. Universally, the eucaryotic MPG protein coding sequences isolated to date are closer in substrate specificity to the *E. coli* ALKA protein than to the TAG protein. The coding sequences for a number of MPG pro-

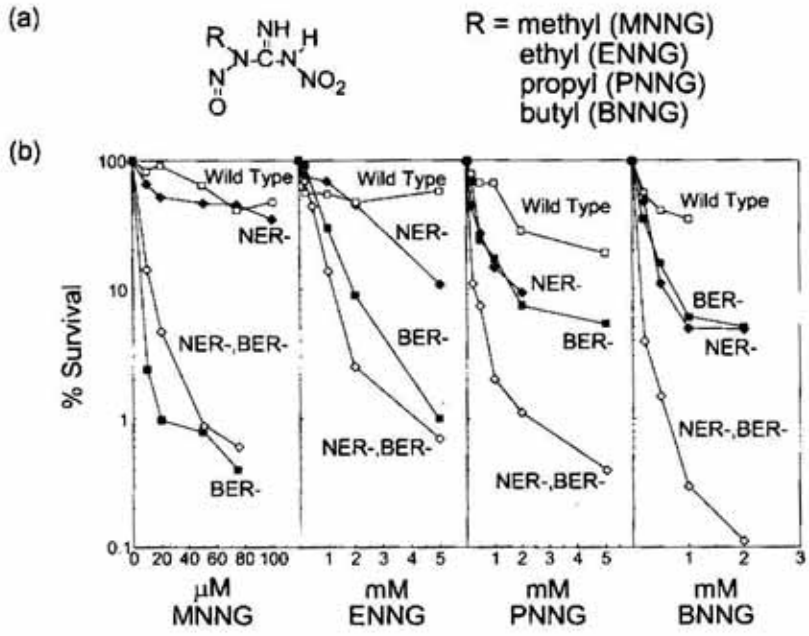

Figure 2. Role of BER and NER in the repair of cytotoxic lesions as a function of adduct size in *E. coli*.

(a) Structure of the series of *N,N'*-nitrosoguanidines used in this experiment. (b) Survival curves for BER- and NER-deficient strains, wild type refers to wild type AB1157 cells (open squares), BER- to *tagalkA* mutants (filled squares), NER- to *uvrA* mutants (filled diamonds), and BER-, NER- to *tagalkAuvrA* mutants (open diamonds). The mutant strains were constructed from *E. coli* AB1157. Repair of methylation and ethylation damages is performed primarily by the BER pathway, whereas propylation and butylation damages are repaired almost equally by both the BER and NER pathways.

teins have now been reported, and these are compared in Fig. 3. Unlike the majority of DNA repair proteins, the MPG proteins are extremely divergent, and therefore the availability of the *Saccharomyces cerevisiae* and *E. coli* clones did not assist in cloning the mammalian MPG proteins.

EXPRESSION AND PURIFICATION OF THE MAMMALIAN MPG PROTEINS

The biochemical properties of the mammalian MPG proteins may most easily be studied by purifying these proteins to homogeneity from cells overproducing the enzyme of interest. Such overproduction has been performed for the human, mouse, and rat recombinant MPG proteins (O'Connor, 1993; Roy *et al.* 1994). Initially the AUG start codon proposed in the original report isolating the rat cDNA (O'Connor & Laval, 1990) was used to express the coding sequence; however, expression from this point in the sequence resulted in the production of a protein without any mammalian DNA glycosylase activity. Therefore, the complete rat MPG coding sequence reported in the original paper was used and an AUG inserted as the first codon. A ribosomal binding site was also added to assist in translation and the whole construct was inserted in a vector using the T7 RNA polymerase promoter (O'Connor, 1993). Figure 4 shows the fractions obtained from the MonoS FPLC column, the last step in purification of the recombinant rat MPG protein to homogeneity. The steps used to purify the rat MPG protein are identical to those used for purification of the human MPG protein (O'Connor, 1993).

SUBSTRATE SPECIFICITY OF THE MPG PROTEINS

The purification of the MPG proteins to homogeneity has permitted the identification of

numerous substrates for these enzymes. The adducts excised by the MPG proteins include not only simple alkylating agent damage, but also alkylation damage by nitrogen mustards used in chemotherapy, deamination products such as hypoxanthine, and also oxidatively damaged bases, such as 5-formyl uracil (Habraken *et al.* 1991; Margison & Pegg, 1981; Mattes *et al.* 1996; Saparbaev *et al.* 1995; Saparbaev & Laval, 1994; Singer & Brent, 1981; Singer & Hang, 1997). Table 1 documents a number of substrates for the

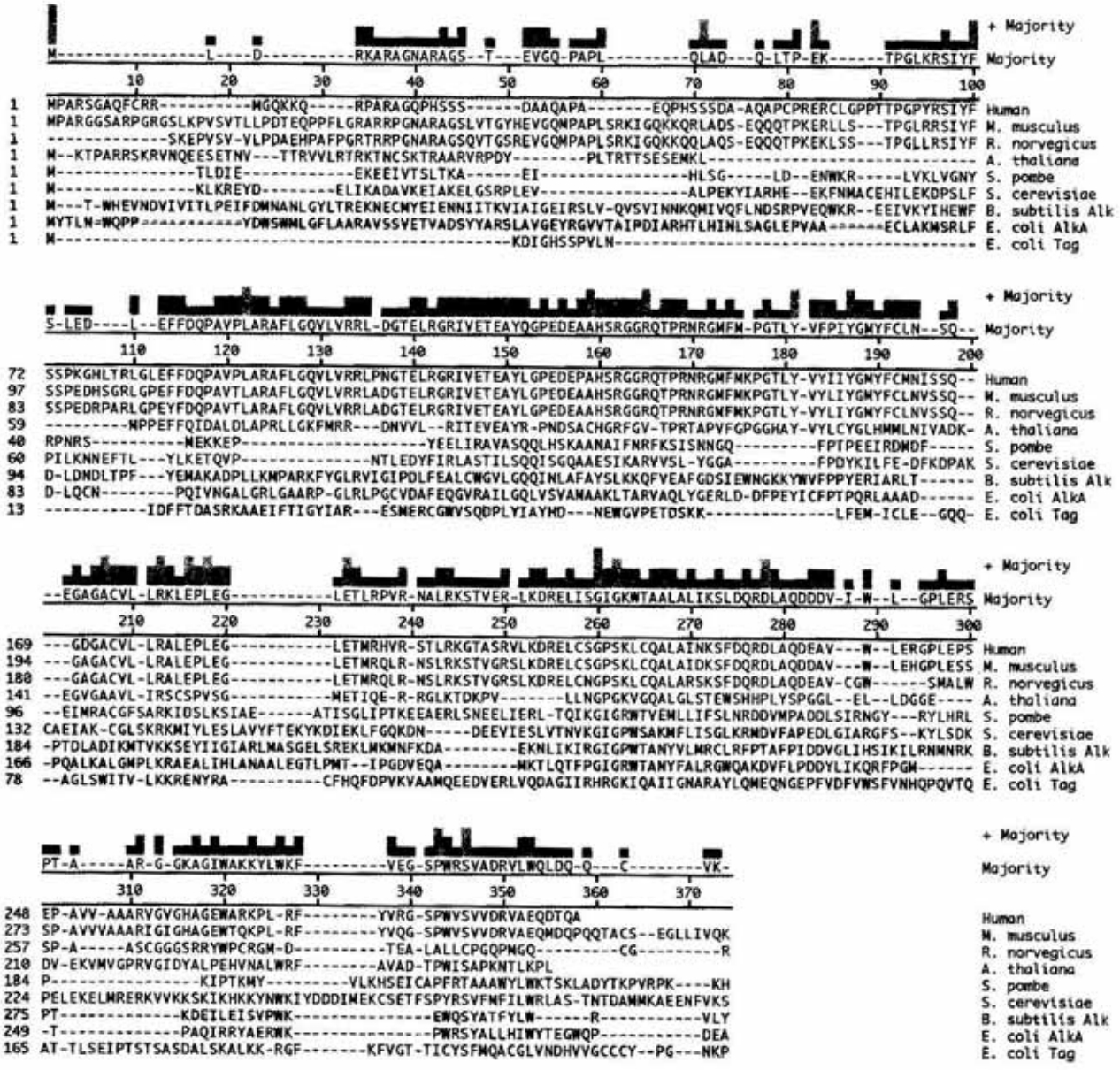

Figure 3. Comparison of MPG protein sequences from various organisms.

The comparison was constructed using the MegAlign Program in DNASTAR. The regions marked with colored histograms indicate related sequences in the different MPG proteins. The majority sequence is shown on the top line. The histograms compare the extent of homology. The higher the histogram, the greater the homology. The extent of homology is also indicated by color. The order of homology from highest to lowest is: yellow, green, light blue, and dark blue. Only one Gly in these proteins is highly conserved. It is found in the helix-turn-helix motif in the crystal structure of the ALKA protein of *E. coli*. This conserved Gly is found at position 260 in the majority sequence.

ALKA protein and human MPG protein. Since the substrate specificities for the rat and human MPG proteins are almost identical

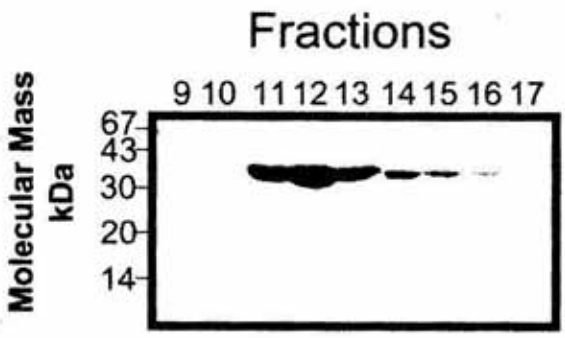

Figure 4. SDS/Polyacrylamide gel electrophoresis of the fractions of a MonoS FPLC separation as the last step in purification of the rat MPG protein.

The protein was purified from BL21 hosting the pAPDG60 plasmid constructed using the APDG cDNA and the pTO50 vector. The purification protocol was identical to that used for the human MPG protein (O'Connor, 1993; O'Connor & Laval, 1990) Approximately 5 μl from each 1 ml fraction from the column was loaded onto the 15% SDS/polyacrylamide gel. Following migration the gel was stained with Coomassie Brilliant Blue. The rat protein in fraction 12 represents 5 μg of protein. The N-terminal sequence of the protein in the pooled fractions 11–13 was verified by Edman degradation sequencing.

we will refer to them as mammalian MPG proteins. The mammalian proteins have diverged significantly from the procaryotic enzymes, in that the mammalian MPG proteins no longer efficiently excise nitrogen mustard damage compared to the efficiency observed for the *E. coli* ALKA protein (Mattes *et al.* 1996). On the other hand, mammalian MPG proteins are more efficient than their procaryotic homologues at removal of ethenoAde and hypoxanthine (Saparbaev *et al.* 1995; Saparbaev & Laval, 1994). Recent solution of the crystal structure of the ALKA protein, and in the future, that of one of the mammalian MPG proteins, should provide explanations for the wide substrate range of this group of DNA glycosylases. The ALKA protein has a structure which favors the acceptance of a variety of substrates at the active site (Labahn *et al.* 1996; Yamagata *et al.* 1996). Whether mammalian proteins have conserved this feature is still in question.

IN VIVO FUNCTION OF THE MPG PROTEIN IN MAMMALIAN CELLS

Several studies concerning the *in vivo* function of MPG proteins in mammalian cells have been reported. Heterologous expression of coding sequences for MPG proteins has been used to attempt to show that overproduction of a protein will provide protection against DNA alkylating agents. Unfortunately, these studies using heterologous expression are ambiguous with respect to the role of MPG proteins in the protection of cells against methylating agent damage. Overproduction of the TAG protein, the ALKA protein, or the rat MPG protein in Chinese hamster ovary cells protected the cells against cytotoxic damage by DNA methylating agents. In contrast, ex-

Table 1. Specificity of human MPG protein and the bacterial ALKA protein for various substrates.

Substrates are marked (+) where there is a significant probability that the protein removes the adduct *in vivo*, or (–) when the protein would not remove an adduct *in vivo*, and (nt) indicates that excision of an adduct was not studied.

Damage	ALKA protein	Human MPG protein
N-7-Gua/N-3-Ade		
methyl	+	+
ethyl	+	+
nitrogen mustards	+	–
quinacrine mustard	–	–
EthenoAde	–	+
Hypoxanthine	–	+
5-Formyl uracil	+	–
7-(2-Chloroethyl)Gua	+	nt

pression of the human MPG cDNA in Chinese hamster ovary cells in two other studies either did not increase survival, or actually increased the amount of sister chromatid exchange, suggesting that overexpression of these sequences is either innocuous or deleterious for cells (Bramson *et al.* 1995; Coquerelle *et al.* 1995). The use of mouse embryonic stem cells deficient in the endogenous MPG protein, however, has clarified these discrepancies (Engelward *et al.* 1996). The mouse embryonic stem cells are sensitive to a variety of DNA alkylating agents and also bleomycin damage; indicating that one of the principal roles for the MPG protein in mammalian cells is to protect cells against alkylation damage at the ring nitrogens of purines. Use of these cells to produce transgenic mice should elucidate the *in vivo* role of the MPG protein.

DNA DAMAGE AND REPAIR AT THE NUCLEOTIDE LEVEL IN HUMAN CELLS

To date studies on repair of DNA damage by alkylating agents have been limited to global DNA repair, or repair at the level of the gene in viable mammalian cells (Lawley *et al.* 1986; Scicchitano & Hanawalt, 1989; Wang *et al.* 1995). At the nucleotide level, only cloned DNA fragments, procaryotes, or yeast have been used to study DNA alkylation damage. Mammalian genomes, however, are too large to study DNA damage at the nucleotide level using methods applied to procaryotes and lower eucaryotes. LMPCR (Fig. 5, left) permits the detection of damage at the nucleotide level, by a number of DNA damaging agents (Denissenko *et al.* 1996; Gao *et al.* 1994; Pfeifer *et al.* 1989, 1991; Tornaletti & Pfeifer, 1994; Wei *et al.* 1995, 1996). This genomic sequencing method depends on the generation of a 5′ ligatable phosphate group at the site of the DNA damage. The use of DNA glycosylases to create 5′ ligatable phosphates has facilitated studies on DNA repair, since the enzymatic removal of modified DNA bases generates lower background in LMPCR (Pfeifer *et al.* 1993). This is due to the low level of depurination observed when removing DNA bases enzymatically, in contrast to the harsh conditions needed to detect bases by chemical methods. Another method involving single-stranded ligation polymerase chain reaction has also been developed, but this method is much less sensitive than LMPCR, and is difficult to use for the study of DNA repair in viable mammalian cells (Grimaldi *et al.* 1996).

We have recently studied the repair of dimethyl sulfate induced damage in normal human fibroblast cells using LMPCR (Ye, N., Holmquist, G.P. & O'Connor, T.R., manuscript in preparation), and a similar method should be applicable to the study of other DNA damages such as those generated by MNNG, mechlorethamine, and chlorambucil as shown in Fig. 5b for the human PGK1 gene in HelaS3 cells. MNNG, a simple S_N1 agent methylates DNA, and chlorambucil and mechlorethamine are clinically used nitrogen mustards. Individual cultures of the HelaS3 cells were treated with either MNNG, mechlorethamine, or chlorambucil. Following treatment the cells were harvested and subjected to terminal digestion using the ALKA protein of *E. coli* and cleavage at abasic sites using a mild piperidine treatment. The data show that the degree of alkylation observed at a given site depends on the alkylating agent used. Comparison of these damages shows that damage at Ade residues is generally greater for chlorambucil damaged DNA than for mechlorethamine damaged DNA, even though the overall global DNA damage by each agent is similar. Damage by MNNG shows more modification at N-7 of Gua in the major groove of DNA than modification at the N-3 position of Ade in the minor groove. This suggests that the *in vivo* chlorambucil, a nitrogen mustard containing an aromatic moiety, shows a similar specificity to that observed in *in vitro* studies on DNA fragments (Wang *et al.* 1991). Further studies on the damage and repair by these and other al-

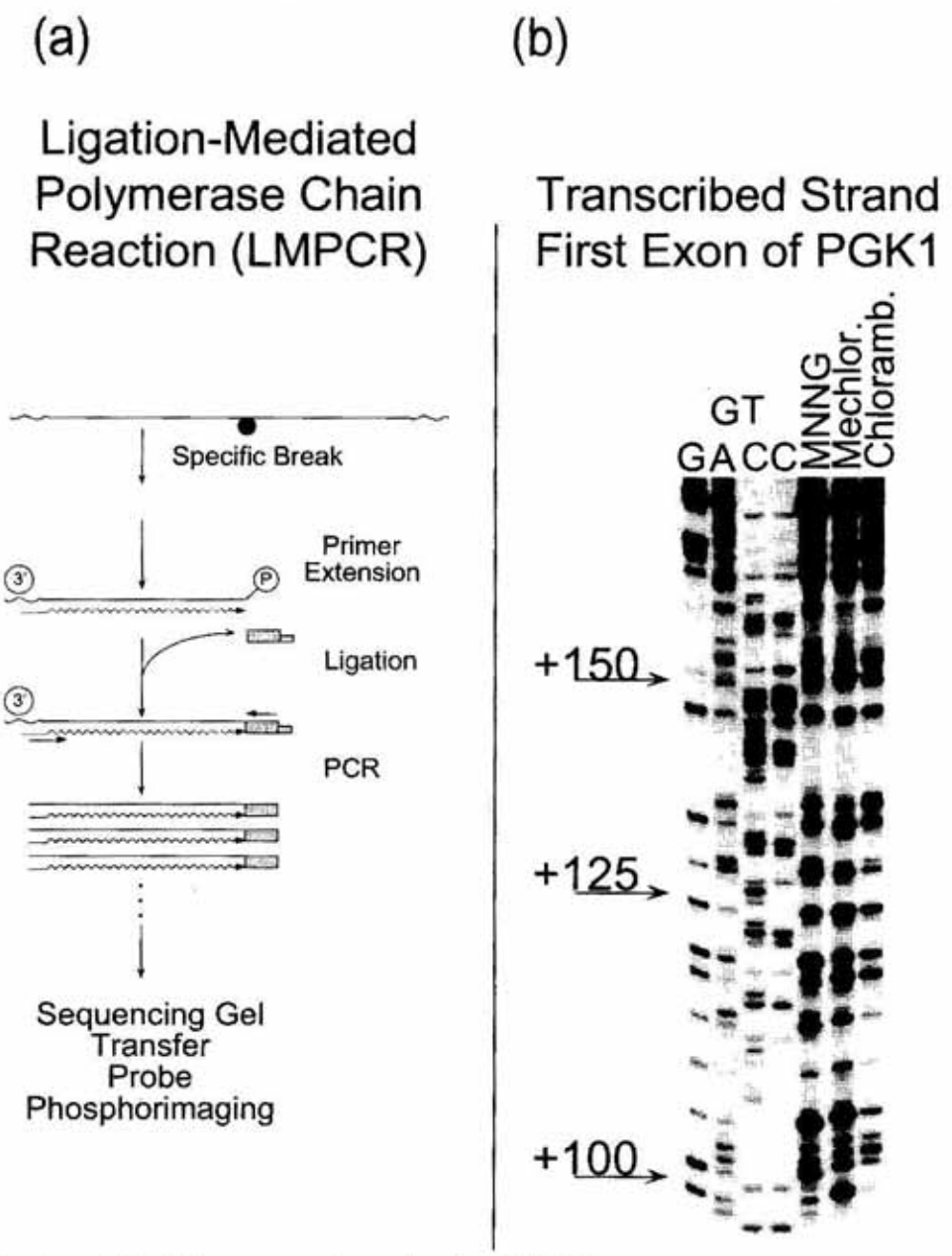

Figure 5. Detection of DNA damage and repair using LMPCR.

(Left) Diagram of the LMPCR technique. A specific break is induced in DNA which leaves a 5′ ligateable phosphate. An oligonucleotide primer is then annealed to the sequence of interest and extended to the break using a DNA polymerase, normally Sequenase. A linker is then ligated to the DNA so that a PCR reaction with a second primer inside the first is used to increase the specificity of the technique. The PCR products are size fractionated on a DNA sequencing gel and for greater sensitivity transferred to a charged nylon membrane. This membrane is probed with a single-stranded DNA probe and the data is recorded either by autoradiography or phosphorimaging. (Right) Modification of Hela S3 DNA *in vivo* by either MNNG (30 min, 500 μM treatment), Mechlor. (Mechlorethamine, 60 min, 40 μM treatment), or Chloramb. (Chlorambucil, 60 min, 40 μM treatment) at room temperature, following which the cells were rinsed with phosphate-buffered saline and the DNA was isolated. DNA was treated with ALKA protein (10 mg ALKA protein per mg DNA) for 20 min, followed by 1 M piperidine treatment for 15 min at 37°C) LMPCR was performed on the samples on the transcribed strand in the open reading frame of exon1 of the PGK1 gene (Rodriguez *et al.* 1995). The numbers indicated by the arrows on the left-hand side of the autoradiogram refer to the nucleotide positions with respect to the transcription start site of the gene. The lines on the right-hand side refer to Ade positions in the sequence.

kylating agents in various genes are currently in progress.

LMPCR has been used to study molecular epidemiology of the *p53* gene, but is also a useful tool for the study of DNA damage and repair at the nucleotide level (Denissenko *et al.* 1996; Gao *et al.* 1994; Pfeifer *et al.* 1989; Tornaletti & Pfeifer, 1994). This technique provides the means to link damage and repair reactions both *in vivo* and *in vitro*, which may eventually allow the prediction of sites which are prone to mutations in either oncogenes, or tumor suppressor genes. This is a necessary step to understand somatic cell mutations leading to cancer.

The study of DNA alkylation damage benefits from the multidisciplinary approach of studying the lesions induced in DNA, the genes and proteins involved in repairing the adducts, as well as the cellular processes for removal of DNA damage. Now that the tools to study these phenomena are available, much more should be revealed about the process of repair of alkylation damage in human cells.

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