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EVIDENCE FROM THE FREQUENCY AND SPECTRUM OF MUTATIONS
THAT HUMAN FIBROBLASTS CAN REMOVE
POTENTIALLY MUTAGENIC LESIONS INDUCED BY
N-ETHYL-N-NITROSOUREA USING EITHER NUCLEOTIDE EXCISION REPAIR
OR O^6-ALKYLGUANINE-DNA ALKYLTRANSFERASE
OR BOTH KINDS OF REPAIR

presented by

LISA R. ORTQUIST

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EVIDENCE FROM THE FREQUENCY AND SPECTRUM OF MUTATIONS THAT HUMAN FIBROBLASTS CAN REMOVE POTENTIALLY MUTAGENIC LESIONS INDUCED BY N-ETHYL-N-NITROSOUREA USING NUCLEOTIDE EXCISION REPAIR OR O^6-ALKYLGUANINE-DNA ALKYLTRANSFERASE OR BOTH KINDS OF REPAIR

By

Lisa R. Ortquist

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ABSTRACT

EVIDENCE FROM THE FREQUENCY AND SPECTRUM OF MUTATIONS THAT HUMAN FIBROBLASTS CAN REMOVE POTENTIALLY MUTAGENIC LESIONS INDUCED BY *N*-ETHYL-*N*-NITROSOUREA USING EITHER NUCLEOTIDE EXCISION REPAIR OR O^6-ALKYLGUANINE-DNA ALKYLTRANSFERASE OR BOTH KINDS OF REPAIR

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N-ethyl-*N*-nitrosourea (ENU) alkylates 12 sites in DNA and is an efficient mutagen and carcinogen. Nucleotide excision repair (NER) and O^6-alkylguanine-DNA alkyltransferase (AGT) are cellular DNA repair systems that can remove ENU-induced alkyl adducts from DNA *in vitro* and in *Escherichia coli*. The function of NER and AGT in repairing alkylation damage in human cells is unclear. To investigate the role of NER and AGT in repairing such damage in human cells, I treated four populations of diploid human fibroblasts, differing in AGT and NER capacities, with ENU. I assayed the treated cells for survival and frequency of *HPRT* mutations, and sequenced mutants from each group. The results are consistent with the interpretation that ENU-induced lesions are removed in human cells by either NER or AGT, and that if both repair systems are active, NER and AGT compete for repair of these lesions.

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Finally, I would like to thank my husband, Benjamin Mosher, for his support during the course of this work.

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ABBREVIATIONS

AGT	O^6-alkylguanine-DNA alkyltransferase
DHFR	Dihydrofolate reductase
ENU	N-ethyl-N-nitrosourea
HPRT	Hypoxanthine (guanine) phosphoribosyltransferase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NER	Nucleotide excision repair
NER^-AGT^-	Deficient in NER and depleted of AGT
NER^-AGT^+	Deficient in NER but proficient in AGT
NER^+AGT^-	Proficient in NER but depleted of AGT
NER^+AGT^+	Proficient in both NER and AGT
O^6-alkG	O^6-alkylguanine
O^6-BzG	O^6-benzylguanine
O^6-EtG	O^6-ethylguanine
O^6-MeG	O^6-methylguanine
O^2-EtC	O^6-ethylcytosine
O^2-EtT	O^2-ethylthymine
O^4-alkT	O^4-alkylthymine
O^4-EtT	O^4-ethylthymine
O^4-MeT	O^4-methylthymine
TG	6-thioguanine
TG^R	6-thioguanine-resistant

INTRODUCTION

DNA damage can lead to mutations, which often result in altered gene expression or changes in protein structure and function. In this way, DNA damage can cause cell death or changes in cell growth patterns that, in a eukaryotic organism, provoke the formation of tumors. Continuous exposure to DNA-damaging agents has permitted the evolution, in the cell, of numerous DNA repair mechanisms to insure the preservation of the genetic code. One example of DNA repair is the removal of alkyl adducts from bases in DNA by the repair protein, alkylguanine-DNA alkyltransferase (AGT). Another example of the cellular response to DNA damage is the repair of UV light-induced damage, and other types of damage causing distortions in the DNA helix, via the nucleotide excision repair pathway (NER). AGT and NER have been studied extensively in *Escherichia coli*, and are now being studied in mammalian cells and organisms. By studying DNA repair, researchers are acquiring a better understanding of the origins and prevention of tumor-forming mutations in DNA.

The purpose of my research project has been to study the repair, via AGT and NER, of alkyl-DNA adducts induced by N-ethyl-N-nitrosourea (ENU) in human cells. Repair of the alkyl adducts induced in DNA by ENU has been studied in E. coli, and in transformed human cell lines. The results reported in this thesis, however, are the first to show an analysis of the repair of these lesions by AGT and NER in diploid human fibroblasts.

Chapter One of this thesis is a review of literature relevant to this project. Chapter Two is presented as a manuscript, currently in preparation for submission to the journal *Carcinogenesis*, describing the results of my research project. The O^6-benzylguanine used in depleting cells of AGT was supplied by Dr. Anthony Pegg of the Milton S. Hershey Medical Center at Pennsylvania State University. The ENU treatment of the cell strains used, the isolation of the resultant thioguanine-resistant mutants, and the purification of *HPRT* cDNA was performed by me and my colleague Lubov Lukash. DNA sequence analysis of the *HPRT* mutants was accomplished by me, L. Lukash, Dr. M. Chia-Mia Mah, Dr. Yi-Ching Wang, Dr. Janet Boldt, and Krisztina Nadas.

CHAPTER I

LITERATURE REVIEW

A. DNA Alkylating Agents

DNA alkylating agents are efficient mutagens and carcinogens (see Saffhill et al., 1985 for review) and are present in the environment. Endogenous formation of N-nitroso compounds can occur at various sites in the human body, including the gastrointestinal tract and the lungs (Bartsch et al., 1990). For example, diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) are alkylating agents found in trace amounts in human blood following ingestion of food containing nitrates (Fine et al., 1977). Nitrosoamines require metabolic activation, with enzymes in the cell converting the nitrosamine into reactive metabolites, which go on to generate alkyl adducts in DNA. Other alkylating agents, like N-ethyl-N-nitrosourea (ENU) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG), directly alkylate DNA by forming reactive intermediates spontaneously at physiological pH (Figures 1 and 2)(Montesano, 1981; Singer 1985). MNNG and ENU are direct-acting alkylating agents that are used in chemical laboratories to generate diazo compounds (Beranek, 1990). These simple alkylating agents are also useful as model compounds for the reactive forms of the metabolically-activated nitrosamines (reviewed in Saffhill et al., 1985), and have been used as such in the Carcinogenesis Laboratory to study repair of alkylation damage in the DNA of human cells.

Exposure to N-nitroso alkylating agents, including MNNG and ENU, results in the formation of a variety of alkyl-DNA adducts (Table I), but

Figure 1. Mechanisms of alkylation by N-nitroso compounds. (from Magee, 1971; Montesano, 1981; and Singer, 1985)

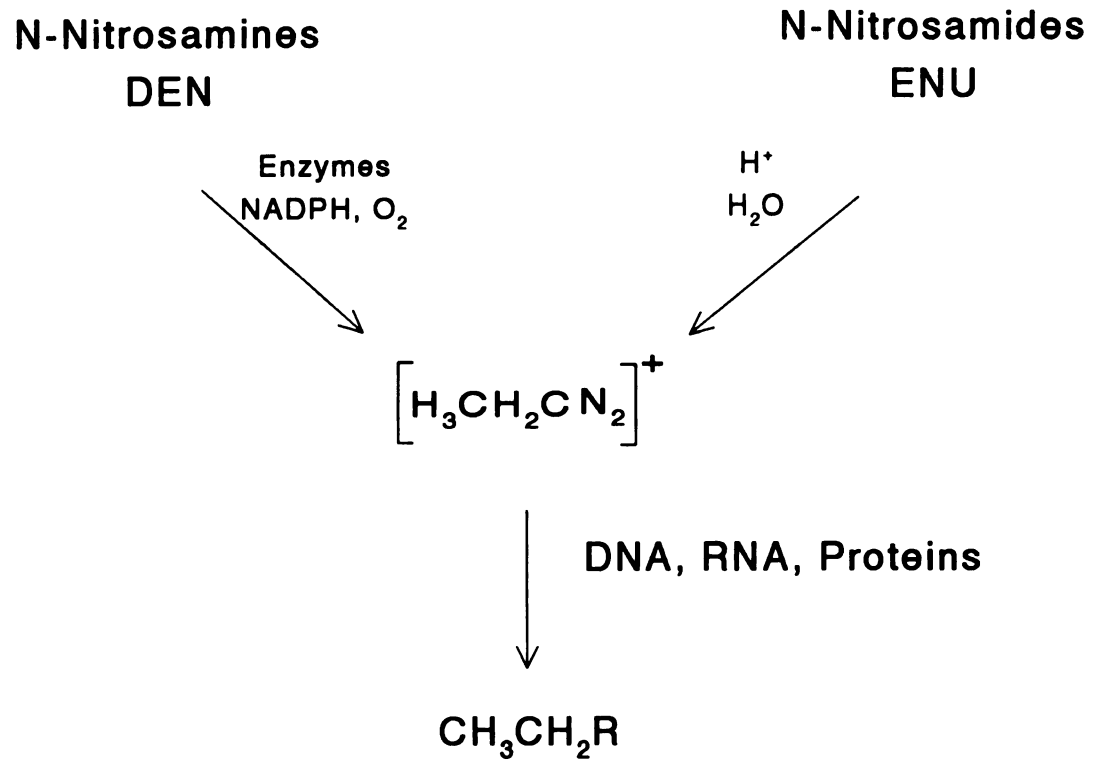
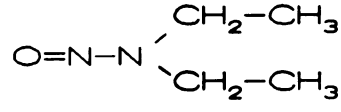
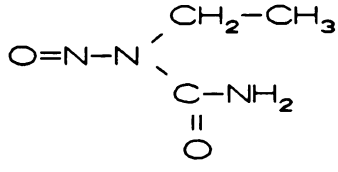


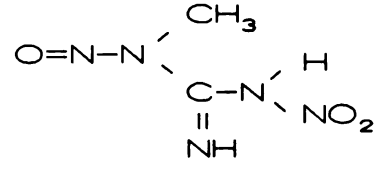
Figure 2. Chemical formulas of the N-nitroso compounds Diethylnitrosourea (DEN), N-ethyl-N-nitrosourea (ENU), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (from Singer, 1985).



DEN



ENU



MNNG

Table I. Initial *in vivo* alkylation (expressed as percent of total alkylation)

Base	Site of alkylation	MNNG[a]	ENU[b]
Adenine	N^1	--	0.1
	N^3	8.6	4
	N^7	--	0.6
Cytosine	O^2	--	2
	N^3	--	0.3
Guanine	O^6	9.2	8
	N^3	--	1.5
	N^7	82.2	12
Thymine	O^2	--	7
	O^4	--	2.5
	N^3	--	0.4
Phosphate Triester		--	58

a. From Beranek, 1990
b. From Singer and Dosanjh, 1990

whether each of these adducts is mutagenic is still being determined. The extent of DNA alkylation observed in vitro parallels the initial alkylation of DNA in cultured cells and in animals exposed to these alkylating agents (Montesano, 1981). The alkyl adducts at oxygens in DNA bases are more chemically stable in vitro, in the absence of DNA repair mechanisms, than those at nitrogens under physiological conditions (see Singer, 1979, for review). N-3 and N-7 alkyl purines are easily depurinated due to lability of their glycosyl bonds. Alkylphosphotriesters are the most stable of the alkylation adducts formed by N-nitroso compounds. These lesions are reported to be cytotoxic lesions, but there is no evidence to suggest that they are premutagenic lesions. The more potent carcinogenic alkylating agents show a greater tendency to react at oxygen sites in DNA than do the less carcinogenic agents (reviewed in Saffhill et al., 1985). Thus, it seems likely that O^6-alkG, O^4-alkT, O^2-alkT, and O^2-alkC are the most reasonable candidates for the lesions in DNA resulting in the mutations observed in MNNG- and ENU-treated cells.

Methylating agents are more reactive with DNA than ethylating agents (Singer and Grunberger, 1983), but produce adducts that are more efficiently repaired in the cell, making methylating agents less mutagenic than ethylating agents at comparable doses in vivo. (Singer, 1985). ENU and MNNG are thought to react with oxygens in DNA by an S_N1 mechanism. The reaction is dependent on the formation of an electrophilic carbocation intermediate, which is trapped by the nucleophilic oxygen in DNA, forming a covalently bound adduct (reviewed in Beranek, 1990).

At least one of the alkyl adducts generated by N-nitroso compounds has been directly implicated in carcinogenesis. The formation of the O^6-

methylguanine (O^6-MeG) adduct in DNA following metabolic activation of the tobacco-specific nitrosoamine 4-(N-methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone (NKK) appears to be a major factor in the induction of lung tumors in rats and mice, and in the activation of the K-ras proto-oncogene in lung tumors in mice (Belinsky et al., 1986, 1990). Using monoclonal antibodies against O^6-MeG, Umbenhauer et al. (1985) found elevated O^6-MeG levels in DNA from normal esophageal tissue taken from human cancer patients living in a region in China with a documented high incidence of esophageal cancer. Goth and Rajewsky (1974) have demonstrated that the lack of repair of O^6-alkylguanine (O^6-alkG) lesions is related to tumor formation in the brains of rat neonates treated with ENU. At various times following treatment with ^{14}C-ENU, DNA was isolated from various tissues in the neonatal rats and the amount of adducts remaining in the DNA was measured. Although other adducts were rapidly lost from the DNA in brain tissue, O^6-ethylguanine was not removed rapidly from this DNA in the treated rats, and the rats eventually developed tumors of the brain. Mutations involving thymine bases are also biologically relevant. Perantoni et al. (1987) exposed pregnant rats to ENU and discovered nervous system tumors in the progeny of the treated rats. These tumors were found to contain a T·A → A·T activating mutation in the *neu* oncogene. Popp et al.(1983) also found a change in the amino acid sequence of β-globin in the progeny of an ENU-treated mouse that can be attributed to a T·A → A·T base substitution in the β-globin gene.

B. DNA Repair

Defects in DNA repair have been linked to an increased risk of cancer in humans. For example, many patients with xeroderma pigmentosum

(XP), a disease resulting from a deficiency in the nucleotide excision repair of UV-light-induced DNA damage, die at an early age of complications arising from neoplasia (Cleaver, 1990), implying that the defect in repair of UV-induced DNA damage promotes tumor formation. Extracts from cultured fibroblasts from patients with lung cancer reportedly have lower alkyltransferase activity than that observed in fibroblast cell extracts from healthy controls (Rudiger et al., 1989), suggesting that a reduced capacity to repair alkylation damage in DNA may be a risk factor for lung cancer. Furthermore, transgenic mice expressing high concentrations of the human homolog for AGT in the thymus, an organ that in mice ordinarily has low levels of alkyltransferase, are protected from the development of thymic lymphomas after exposure to the DNA alkylating agent N-methyl-N-nitrosourea (MNU) (Dumenco et al., 1993), demonstrating that DNA repair via AGT is instrumental in protecting against tumorigenic alkyl-DNA lesions.

1. Nucleotide Excision Repair

Nucleotide excision repair is one of the mechanisms by which a cell preserves the integrity of its genome. NER has been studied at the molecular level in *E. coli*, and has been shown to involve the removal of an oligonucleotide containing the damaged base(s) and repair of the resulting gap by DNA polymerase and DNA ligase (see Sancar and Sancar, 1988, for review).

In *E. coli* the NER mechanism requires at least six proteins: UvrA, UvrB, UvrC, UvrD, DNA polymerase I, and DNA ligase. The UvrA protein binds single-stranded DNA or damaged double-stranded DNA and has ATPase functional domains. The UvrB protein alone does not bind DNA or have

ATPase activity. However, when associated with the UvrA protein, UvrB increases the stability of the UvrA-DNA complex, and DNA and UvrB together stimulate the ATPase activity of UvrA. UvrC is a DNA-binding protein that has been reported to be capable of exonuclease activity. The UvrB subunit combines with two UvrA subunits in the presence of ATP, and the A_2B_1 complex binds to the DNA containing the damaged nucleotide(s). UvrC then binds to the A_2B_1-DNA complex and two incisions take place - hydrolyzing the 8th phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to the damaged base to remove a 12-13 base oligomer from the DNA. UvrD is a helicase, and works with DNA pol I and DNA ligase to release the damage-containing oligomer and fill in the resulting gap. The UvrABC complex excises UV-light-induced DNA damage from DNA as well as the base adducts formed in DNA by many chemicals, and is considered to recognize the distortion of the DNA helix caused by these types of damage.

The molecular mechanisms of nucleotide excision repair in eukaryotes have not been studied in as much detail as NER in *E. coli*. In the yeast *S. cerevisiae*, five genes essential for NER have been cloned and sequenced. One of these, *RAD3*, produces a DNA-dependent ATPase and a helicase, suggesting similarities between the mechanisms of NER in prokaryotes and eukaryotes. Cells from patients with XP have been assigned to seven complementation groups, A - G, based on their ability to perform UV-induced unscheduled DNA synthesis when fused to cells from different complementation groups (Timme and Moses, 1988). The number of different complementation groups implies that, as in *E. coli* and *S. cerevisiae*, NER in human cells involves multiple gene products. One human gene that has been cloned and shown to correct a NER defect in a rodent cell line is *ERCC1*. The *ERCC1* gene product has sequence homology with

RAD10, a yeast NER protein, and also some homology to the *E. coli* UvrC protein. Interestingly, the *ERCC1* gene does not correct the NER defect in cells from any of the XP complementation groups. Another cloned human gene, *ERCC2*, shows an amino acid sequence similar to that of the yeast RAD3, a DNA helicase. *ERCC2* has been implicated as the defective gene in XP patients from complementation group D. *ERCC3* is a cloned human gene that corrects the NER defect in cells from XP group B patients, and also appears to be a DNA helicase. The NER defect demonstrated by cells from XP patients from group A, C, or G can be corrected by the human genes *XPA*, *XPC*, and *ERCC5*, respectively (See Bootsma and Hoeijmakers, 1994 for review).

The structure of DNA in eukaryotes is more complex than that in *E. coli*, and the NER mechanisms in eukaryotes are probably more complicated than in bacteria. However, the generally accepted mechanism for NER in human cells is based on the NER pathway in *E. coli*. The lesion in DNA is recognized and a dual incision is made in the damaged DNA 27-29 nucleotides apart. Excision of the lesion-containing oligomer and subsequent repair involves DNA helicase, single-stranded binding proteins, and the activity of DNA polymerase δ or ϵ (NER in eukaryotic cells is reviewed in Sancar and Sancar, 1988; Cleaver, 1990; and Bootsma & Hoeijmakers, 1994).

Evidence suggests that there is a distinction between repair of the genome overall and repair of actively transcribed genes. It has been shown, for example, that UV-induced pyrimidine dimers are removed faster from the amplified DHFR gene in the human cell line 6A3 than from the genome overall (Mellon et al., 1986). Moreover, the transcribed strand of the amplified DHFR gene is repaired faster than the non-transcribed strand

in this same human cell line and in a rodent cell line (Mellon et al., 1987). Vrieling et al. (1989) compared the spectrum of UV-induced mutations in the *HPRT* gene of Chinese hamster cell lines proficient or deficient in repair of UV-induced lesions, and reported preferential repair of UV-induced lesions in the transcribed strand of the *HPRT* gene. Fibroblast cells from a patient of the XP complementation group C can repair UV-induced lesions in actively transcribed genes but not those in inactive regions of the genome (Venema et al., 1990). These results suggest that the defect in NER in these cells involves one or more gene products that allow the repair of damage in the inactive regions of the overall genome, implying separate mechanisms for the repair of actively transcribed genes and of transcriptionally silent regions of the genome.

The association between the NER machinery and transcription has recently been clarified (reviewed in Friedberg et al., 1994 and Bootsma and Hoeijmakers, 1994). The *ERCC3* gene product appears to be one of the components of the human basal transcription factor TFIIH, which is required for a late step in the initiation of transcription by RNA polymerase II. The human *ERCC2* gene product shares homology with the yeast *RAD3* gene product, which has been shown to be part of the yeast RNA Pol II preinitiation complex, implying a similar role for *ERCC2*. *ERCC2* and *ERCC3* gene products are reported to interact with each other. At least two , then, of the human genes directly involved in NER are believed to also associate directly with the transcription apparatus. Friedberg et al., (1994) suggest that NER proteins are coupled to the transcription apparatus in order to recognize base damage in the template strand during transcription and act as nucleation sites for the assembly of the NER machinery. They further suggest that if these NER proteins have a higher

affinity for sites of DNA damage than for the transcription initiation complex, this may serve to limit transcription initiation when cells are exposed to DNA damage. Finally, they hypothesize that there are two distinct repair complexes, one operating in transcription and repair of active genes, and the other working on DNA damage in the genome overall.

2. O^6-Alkylguanine-DNA Alkyltransferase

Another important means by which the cell rids the genome of potentially mutagenic DNA lesions is by using the repair protein O^6-alkylguanine-DNA alkyltransferase (AGT). (For a review of AGT in bacteria and mammalian cells, see Yarosh, 1985; Laval, 1990; Pegg, 1990a,b; and Sassanfar et al., 1991.) In both prokaryotes and eukaryotes, this protein repairs alkyl adducts in DNA, particularly at the O^6 position of guanine bases, by transferring the alkyl group from the DNA to a cysteine in its own amino acid sequence. A single AGT protein can act only once. AGT activity is depleted in cells treated with alkylating agents by the reaction of the protein with alkylated DNA. Once the AGT activity has been depleted from a cell in this manner, only synthesis of new AGT proteins can restore the original level of activity. In this respect AGT is not a true enzyme, as a single reaction with an alkyl substrate leaves the protein inactive. AGT binds to double-stranded DNA, and requires no other protein or cofactor to affect the removal of an alkyl group from DNA. Ethyl groups are removed from DNA by AGT more slowly than methyl groups. A mechanism has been proposed for the removal of alkyl groups from DNA by AGT: A basic residue in the protein interacts with the acceptor cysteine residue, gaining a proton from the cysteine and generating a thiolate anion. The anion then attacks the alkyl group in

the damaged base and removes it, forming S-alkylcysteine in the protein and restoring the substrate base in DNA.

AGT repair in *E. coli* has been well characterized. There are apparently two genes responsible for the AGT activity in *E. coli*. The *ada* gene product contains two domains separated by a hinge region, and has two alkyltransferase functions. At the amino terminus the protein can act on alkylphosphotriesters. The carboxy terminus acts on O^6-alkG and O^4-alkT. Binding of an alkyl adduct at the amino terminus converts the *ada* gene product into a strong activator of the *ada* gene and of the *alkA* gene, which codes for another DNA repair enzyme, N-3 methyladenine-DNA glycosylase II. Thus, *E. coli* initially exposed to an alkylating agent subsequently become resistant to a second exposure to such alkylation damage, responding with increased levels of AGT and glycosylase in what is known as the adaptive response. Another AGT protein is the product of the *ogt* gene. This protein repairs O^6-alkG and O^4-alkT lesions. It shows homology to the C-terminal domain of the *ada* gene product, but unlike *ada*, expression of *ogt* is not induced by exposure to alkylating agents. The *ogt* gene appears to be responsible for protecting *E. coli* from low levels of alkylation damage in the absence of induction of the *ada* gene. The *ada* gene product demonstrates a higher affinity for O^6-alkG lesions than the *ogt* gene product, and the *ogt* gene product has a higher affinity for O^4-alkT lesions than does the *ada* protein (Sassanfar et al., 1991), indicating that, although similar in activity, the *ada* protein and the *ogt* protein may have different repair specificities in the cell.

Although the eukaryotic alkyltransferase has not been defined in as much detail as AGT in *E. coli*, the same mechanism is apparently employed in eukaryotic cells as that demonstrated by the bacterial AGT. Transfer

of an alkyl group from the O^6 position of guanine produces an S-methyl cysteine residue in the protein, and only RNA and protein synthesis can restore AGT activity in mammalian cells depleted of AGT by exposure to alkylating agents (Pegg, 1990a, 1990b). Unlike the bacterial AGT, mammalian cell extracts with AGT activity exhibit no repair of alkylphosphotriesters and O^4-alkT (Yarosh et al., 1985). However, O^4-MeT lesions in DNA oligomers have recently been shown to inhibit, with low affinity, the activity of purified human AGT, suggesting that the human AGT may repair O^4-MeT, but with very poor efficiency (Sassanfar et al., 1991). Cells from human tissues have a higher alkyltransferase activity than cells from the corresponding tissues in rats or mice. In human tissues, liver has the highest activity, followed by the GI tract, then the lung. Brain tissues have the lowest levels of activity (Grafstrom et al., 1984). Induction of AGT activity following treatment with alkylating agents has been observed in rat liver cells, and in human tumor cell lines, but not in normal rat or human fibroblasts (Laval, 1990).

3. DNA Glycosylase

The DNA glycosylase enzymes are another cellular defense against alkylating agents. Glycosylases remove alkylated bases from DNA by catalyzing the cleavage of the sugar-base bond. In general, the *E. coli* glycosylases can remove N-3 and N-7 alkylpurines from DNA, and remove ethylated bases at these positions more slowly than methylated bases. The *tag* gene in *E. coli* codes for N-3 methyladenine-DNA glycosylase I, which excises N3-methyladenine from DNA. The *E. coli alk* gene, which is upregulated by the methylated *ada* gene product in the adaptive response, codes for the N-3 methyladenine-DNA glycosylase II enzyme. The *alk* gene

product excises N3-methylpurines and, more slowly, N-7 methylpurines and O^2-methylpyrimidines. Cell extracts from *E. coli* and from rat tissues removed O^6-MeG from DNA treated with ^{3}H-MNU, but ^{3}H-O^6-MeG was not detected as a free base, indicating that glycosylases are not involved in the repair of this lesion. Unlike the bacterial glycosylase, mammalian DNA-3-methyladenine glycosylase is not active on O^2-MeC or O^2-MeT in vitro. (Reviewed in Saffhill et al., 1985; Yarosh, 1985; Brent et al., 1988; Lindahl & Sedgwick, 1988; and Samson et al., 1988).

C. Mutation Induction by Alkylating Agents

Of the alkyl adducts formed in DNA by ENU and MNNG, the lesions at oxygens in DNA bases are considered to be the most mutagenic lesions, resulting in miscoding bases (Singer, 1979; Montesano, 1981; Larson et al., 1985). Alkylation at the N-3 position of pyrimidines and the N-1 position of purines leads to errors in transcription and inhibition of DNA replication that may generate the cytotoxic effects of the alkylating agent. These lesions have not been implicated in mutagenesis, however, except when loss of the alkylated base, either spontaneously or due to glycosylase activity, results in an apurinic/apyrimidinic site (reviewed in Saffhill, et al., 1985).

More than 20 years ago, Loveless (1969) first implicated O^6-alkG as a potentially mutagenic lesion. Since then, in vitro site-specific studies have shown that O^6-MeG very frequently pairs with T instead of C during both DNA replication and transcription using Klenow and *E. coli* RNA polymerase I, resulting in G·C → A·T base substitution mutations (Snow et al., 1984; Toorchen and Topal, 1983; Singer and Dosanjh, 1990). DNA polymerase may mistake the alkylated G for A, since O^6-alkG resembles A in

bond angles and bond lengths (reviewed in Swann, 1990). In 1990, Singer and Dosanjh reported that prokaryotic and eukaryotic DNA polymerases, using a template containing O^6-MeG, preferentially inserted a deoxythymidine opposite O^6-MeG. Insertion of the correct nucleotide, deoxycytidine, opposite O^6-MeG results in a decrease in extension past the site of the lesion relative to the extension observed following incorporation of deoxythymidine. From two-dimensional NMR assays the O^6-MeG:C base pair appears to distort the DNA helix, while the O^6-MeG:T base pair does not (reviewed in Swann, 1990; Basu and Essigmann, 1990), and this preservation of the helix alignment may hide the erroneous base pair from any repair mechanism.

O^6-MeG in an M13 bacteriophage vector transfected into *E. coli* gives rise to G·C → A·T base substitutions in the progeny phage DNA (Loechler et al., 1984), consistent with the O^6-MeG miscoding observed in vitro. Treatment of the bacteria with MNNG prior to insertion of the O^6-MeG-containing vector depletes the cells of AGT. The mutation frequency in the progeny phage from the bacteria not depleted of AGT is reduced relative to the frequency in phage obtained from bacteria depleted of AGT by MNNG treatment. This is indicative of repair of the O^6-MeG lesion in the vector by AGT. O^6-MeG is the most frequent adduct formed at oxygens in DNA bases by treatment of DNA with MNNG in vitro (Table 1). The majority of mutations induced by MNNG in *E. coli* are G·C → A·T transitions, (Richardson et al., 1987a; Gordon et al., 1990). AGT repairs O^6-MeG lesions in vitro. The presence of AGT reduces the frequency of G·C → A·T mutations in the *gpt* gene of MNNG-treated *E. coli*, relative to the frequency observed in AGT^- bacteria (Richardson et al., 1987a), strengthening the hypothesis that in *E. coli*, MNNG-induced G·C → A·T

mutations are most likely caused by O^6-MeG lesions and demonstrating that, as seen in the in vitro studies, AGT works on O^6-MeG in bacteria.

In vitro, methylated DNA can also serve as a substrate for uvrABC excision repair (Van Houten and Sancar, 1987). The NER pathway repairs O^6-MeG in *E. coli* in the absence of the adaptive response. Samson and colleagues (1988) treated strains of *E. coli* differing in AGT and NER capacities with MNU, and compared the rate of removal of O^6-MeG from the DNA of the treated cells. They found that the rate of removal of O^6-MeG from the DNA of the treated cells was similar in strains normal in NER capability, regardless of AGT capacity, for the first hour after treatment. After the first hour, i.e. the time required for the induction of the adaptive response, cells normal in expression of the *ada* gene repaired O^6-MeG faster than *ada*$^-$ cells. Cells normal in *ada* and NER repaired these lesions faster than cells normal in *ada* but lacking NER at all time points. These authors conclude that following transient exposure to an alkylating agent that saturates the AGT proteins in the cell, NER will play a major role in the repair of O^6-MeG lesions.

Richardson et al. (1987a) have reported a strand bias for MNNG-induced O^6-MeG premutagenic lesions in treated *E. coli*. They analyzed the MNNG-induced mutations in the *gpt* gene of *E. coli* proficient or deficient in the *ada* gene. Both cell strains showed statistically significant preference for induction of premutagenic lesions on the non-transcribed strand. This could be due to less efficient repair of the O^6-MeG lesions in the nontranscribed strand by AGT, which prefers double stranded DNA. Roldna-Arjona and colleagues (1994), however, recently reported a similar bias in MNU-induced mutations in a shuttle vector gene in treated *E. coli* completely lacking AGT activity (*ada*$^-$, *ogt*$^-$). This suggests that the

strand bias observed could be due to a bias in induction of lesions on the non-transcribed strand, rather than to some property of AGT repair.

1. Mutations Caused by Ethyl Adducts *in vitro* and in *E. coli*

Unlike the pattern of methyl-DNA adduct formation caused by the methylating agent MNNG, the distribution of ethyl adducts at oxygens in ENU-treated DNA is not dominated by lesions at the O^6-position of guanine (See Table 1). Instead, O^2-EtT and O^4-EtT together account for roughly an equal percentage of the total alkylation adducts as O^6-EtG. This predicts that mutations at T's will be responsible for a percentage of the ENU-induced mutations.

Although occurring at a much lower frequency than O^6-EtG, O^2-EtC may also be mutagenic. It was observed in vitro that O^6-EtG miscodes as A, resulting in G·C → A·T base substitutions, and that AGT works on O^6-EtG, and that the frequency of G·C → A·T transitions observed in vivo decreases dramatically in cells competent in AGT relative to cells with no AGT. Therefore, O^6-EtG has been accepted as the primary cause of the G·C → A·T transition, and O^2-EtC has been ruled out by a lack of evidence indicating otherwise. However, it is conceivable that the mammalian DNA glycosylase could work on O^2-alkT or O^2-alkC in vivo, leaving apyrimidinic sites, even though this activity has not been demonstrated by mammalian glycosylase in vitro. If this were to occur, and if DNA polymerases preferentially misinserted dATP opposite the apyrimidinic sites (Kunkel, 1984), this would result in T·A → A·T or C·G → T·A mutations. A C·G → T·A base substitution mutation could be misinterpreted as a G·C → A·T mutation and attributed to O^6-EtG. In vitro analysis of the mutagenic specificity of O^2-EtC is in progress in the laboratory of B. Singer, and the results

should clarify this point.

The mutagenic specificities of O^4-EtT and O^2-EtT have been studied in vitro by inserting the modified base into an oligomer and analyzing the effects of DNA polymerases on the modified template (Singer and Dosanjh, 1990; Bhanot et al., 1992; Grevatt et al., 1992; Dosanjh et al., 1993, Menichini et al., 1994). As was shown for O^6-MeG, insertion of the correct nucleotide, deoxyadenosine, opposite O^2-EtT or O^4-EtT, obstructs the progress of the DNA polymerase past the site of the lesion. If, however, the polymerase inserts a dGTP opposite the O^4-EtT, or a dTTP opposite the O^2-EtT, replication can continue past the lesion. Two-dimensional NMR studies predict that the O^4-MeT:G base pair will not distort the helix, while O^4-MeT base-paired with A should result in a distorted helix conformation (Basu and Essigman, 1990), suggesting that, as with the O^6-alkG:T mispair, the O^4-alkT:G base pairing could effectively hide the lesion from DNA repair. O^4-EtT, then, is implicated in T·A → C·G base substitutions and O^2-EtT in T·A → A·T base substitutions in alkylated DNA in vitro.

O^4-EtT and O^2-EtT can also be connected with the T·A → G·C base substitutions observed in the DNA of ENU-treated cells. Drinkwater and colleagues (Eckert et al., 1989) suggest that T·A → G·C mutations can result from the processing of adducts that block DNA replication, i.e. via the SOS response in *E. coli*. The SOS response in *E. coli* results in induction of NER, recombination, and mutagenic repair mechanisms and inhibits cell division (Sancar and Sancar, 1988). Incorporation of dATP opposite O^4-EtT or O^2-EtT has been reported to block DNA replication (Dosanjh et al., 1993; Grevatt et al., 1992). Perhaps SOS-type processing of these replication-blocking lesions can account for the observed T·A →

G·C mutations.

Preston and colleagues (1987) have determined that O^4-MeT or O^4-EtT lesions in a phage vector introduced into an *E. coli* strain that is defective in the *ada* gene result in T·A → C·G mutations in the progeny phage, confirming the mutagenic specificity of O^4-alkT lesions observed in vitro. In *E. coli* proficient in the alkyltransferase activity encoded by the *ada* gene, the frequency of this mutation is not higher than background. O^4-MeT has been shown in vitro to be repaired by the *ada* gene product. The increase in the frequency of mutations in *ada*$^-$ bacteria relative to the frequency seen in the bacteria normal in the *ada* gene is consistent with repair of O^4-alkT by this alkyltransferase.

In *E. coli* the importance of NER increases and the importance of AGT repair decreases, as the size of the alkyl adduct increases (reviewed in Yarosh, 1985). In the *lacI* gene of normal and NER-deficient *E. coli*, treatment with ENU results primarily in G·C → A·T and T·A → C·G base substitutions mutations, probably due to persistent O^4-EtT and O^6-EtG lesions. The frequency of these mutations in the normal *E. coli* was 5-fold lower than in the NER-deficient cells, indicating that NER is involved in the repair of O^6-EtG and O^4-EtT (Burns et al., 1988). Samson et al., (1988) propose that NER is the primary pathway for the repair of O^4-EtT and O^6-EtG, and repairs O^6-MeG in the absence of the adaptive response. They treated various strains of *E. coli*, differing in NER and ada phenotypes, with ENU and MNU. Using monoclonal antibodies specific for O^4-EtT, O^6-EtG, and O^6-MeG, they then analyzed the time-dependent removal of these adducts from the DNA of the treated cells. Initially following treatment, O^6-MeG, O^6-EtG and O^4-EtT were removed from the DNA at the same rate in *ada*$^-$ and *ada*$^+$ cells. Cells expressing abnormally high

levels of the ada gene product (*ada*^{c}), however, showed a more rapid rate of removal than that of the *ada*$^{+}$ or the *ada*$^{-}$ cells. Approximately 1 hour after treatment, the rate of removal of O^6-MeG increased in the *ada*$^{+}$ cells relative to the rate in the *ada*$^{-}$ cells, indicating the induction of the adaptive response. This increase in rate of removal was not apparent for O^4-EtT or O^6-EtG adducts. In NER^{-} derivatives of the *ada*$^{-}$ and *ada*$^{+}$ cell strains, removal of O^6-MeG, O^6-EtG and O^4-EtT was slower than in the NER proficient cell strains. They conclude that in wild-type *E. coli* in the absence of the adaptive response, NER is the primary pathway for the initial repair of O^4-EtT, O^6-EtG, and O^6-MeG, and that the inducible *ada* protein can repair ethyl adducts (Samson et al., 1988).

One would expect that cells functional both in NER and AGT would be better at surviving alkylation damage and maintaining DNA free of alkylation-induced mutations than cells with only one of these repair systems operating. However, some studies in *E. coli* have shown that NER and AGT interfere with one another. For example, Rossi and colleagues (1989) found that an O^6-MeG lesion in an M13mp18 vector transfected into *E. coli* was more mutagenic in cells competent in NER and AGT than in cells competent only in AGT repair. They suggest that binding of the uvrA protein to the damaged DNA inhibits repair by alkyltransferase. Their results agree with those of Chambers et al., (1985). Chambers and colleagues placed an O^6-MeG lesion in a ϕX174 vector and transfected this vector into *uvrA*$^{-}$ cells (i.e. lacking NER but normal in AGT) and into *uvrA*$^{+}$ cells (normal in both NER and AGT). They found that the mutation frequency in the progeny phage DNA was 40-fold higher in the *uvrA*$^{+}$ cells than in the *uvrA*$^{-}$ cells, indicating that the NER apparatus interfered with the repair of O^6-MeG via AGT.

In *E. coli* treated with ENU, miscoding lesions are more likely to occur at 5'-Pu-G-3' or 5'-Pu-T-3' sites in the *gpt* gene (Richardson et al., 1987). This specificity for induction of mutations at sites with a 5' purine is characteristic of S_N1 methylating agents (Horsfall et al., 1990), and is also seen in MNNG-treated cells (Gordon et al., 1990). As reviewed by Horsfall et al. (1990), the observed site specificity may result from reduced efficiency of repair of the adduct with a 5'-flanking purine or from increased initial formation of the adduct at such a site. Burns et al., (1988) treated NER-proficient *E. coli* and NER-deficient *E. coli* with ENU. They found a bias in the formation of G·C → A·T and T·A → C·G substitution mutations in the lacI gene at guanines and thymines with G:C base pairs 5' and 3' to the mutated base in the NER-proficient cells. This bias was not present in the mutations observed in the NER-deficient cells. They conclude that, in the lacI gene of ENU-treated *E. coli*, excision repair is less efficient at removing O^6-EtG and O^4-EtT if that guanine or thymine is flanked by G:C base pairs. Wong et al. (1992) found in *in vitro* studies, using oligomers containing O^6-MeG residues, that there are localized, strand specific disruptions in the chemical structure of DNA containing O^6-MeG that make DNA containing this lesion more sensitive to the restriction enzyme *Mae*II. Differences in the 5' neighboring base and the base-pairing pyrimidine alter the disturbance in the helix caused by this lesion. These perturbations affect the activity of the cloned human AGT. They conclude that it is likely that the stacking interactions between O^6-MeG and its neighboring bases determines the kinetics of formation and/or repair of this, and other alkyl lesions.

2. How Eukaryotic Cells Respond to Alkylation Damage

O^6-alkG, O^4-alkT, and O^2-alkT are also relevant mutagenic and carcinogenic lesions in eukaryotic organisms. Lung tumors induced by ENU in mice were found to have G·C → A·T base substitutions activating the *K-ras* protooncogene, consistent with the ENU-induced formation of persistent O^6-EtG lesions (You et al., 1992). T·A → G·C and T·A → A·T mutations have also been found. In ENU-treated rats, activation of the *neu* oncogene has been shown to be due to a T·A → A·T base substitution in tumors of the peripheral nervous system (Perantoni et al., 1987). In granuloma pouch skin fibroblasts of ENU-treated rats, ENU-induced mutations in the *hprt* gene were predominantly T·A → A·T and T·A → G·C base substitutions (Jansen et al., 1994).

Since it has been shown that the repair specificities of the eukaryotic AGT and NER differ from those of the prokaryotic AGT's and NER in vitro, researchers have been studying the response of cultured eukaryotic cells to DNA damage in order to investigate NER and AGT in these cells. There are also differences in repair efficiency among eukaryotes. Human cells show greater efficiency in repairing UV-induced damage in the entire genome using NER, whereas NER in rodent cells concentrates more on the DNA in actively transcribed genes (Cleaver, 1990). Likewise, human cells have higher AGT activities than the corresponding rodent cells. Therefore, in order to understand DNA repair in the human organism, it is important to study repair in human cells.

One approach to studying DNA repair in human cells in culture is by making use of shuttle vector systems. Such systems use recombinant plasmids able to replicate in both mammalian and bacterial cells, with

genes that can be expressed in bacteria for detection and analysis of mutations. For example, Klein and colleagues (1990) used an SV40 viral-based shuttle vector carrying a single O^4-EtT lesion at a defined position. When replicated in human HeLa cells, this vector gave rise to T·A → C·G mutations, in agreement with the mutagenic specificity of this lesion observed in vitro and in *E. coli*. In a related approach, Drinkwater and colleagues (Eckert et al., 1988) used a herpes simplex virus thymidine kinase gene carried on an EBV-based shuttle vector to analyze the induction of mutations by ENU in HeLa cells. They found that, in addition to the G·C → A·T and T·A → C·G mutations that had been observed in ENU-treated *E. coli*, ENU induced T·A → A·T and T·A → G·C mutations in human cells. They did not observe any strand or sequence bias in the formation of the G·C → A·T mutations, as had been reported for the G·C → A·T mutations induced by MNNG. These investigators introduced the hypothesis that O^2-EtT is the lesion responsible for the observed T·A → A·T mutations.

Although vector systems are useful, there may be differences between repair of the shuttle vector DNA and repair of the DNA in an actively transcribed endogenous gene. Therefore, many investigators are analyzing DNA repair in endogenous genes in mammalian cells. A particularly well-studied gene is the gene that codes for the enzyme hypoxanthine (guanine) phosphoribosyltransferase (HPRT), which is involved in the purine salvage pathway, a crucial process by which a cell recycles nucleotide bases (for reviews on HPRT, see Stout and Caskey, 1985; Chinault and Caskey, 1984; Morrow, 1983). HPRT catalyzes the formation of inosine monophosphate and guanosine monophosphate via the condensation of 5-phosphoribosyl diphosphate and hypoxanthine or guanine bases. The *HPRT* gene is X-linked in mammals, making it hemizygous in males and functionally hemizygous, via

inactivation of one of the two X chromosomes, in females. The sequence of the gene is known, with an open reading frame of 654 bases coding for a protein of 217 amino acids. The enzyme has been shown to be intolerant of slight structural changes, such as those that might be caused by a single base substitution. HPRT is nonessential in cultured cells that are normal in the ability to synthesize purines de novo. $HPRT^-$ mutants can be selected for by growing cells in media containing 6-thioguanine, which adversely affects DNA replication in cells competent in HPRT, but has no measurable effect on cells with no functioning HPRT.

Maher and colleagues have spent several years studying the effects of DNA repair on the induction of $HPRT^-$ mutants by alkylating agents in a number of human fibroblast cell strains that differ in NER and AGT capacities (Simon et al., 1981; Domoradzki et al., 1984, 1985; Maher et al., 1990). With methylating agents, the presence or absence of NER was found to make little difference in the toxicity of MNNG or the frequency of induced $HPRT^-$ mutants. Diploid fibroblast cell strain XP12BE from a patient with xeroderma pigmentosum, complementation group A, which has negligible levels of NER repair (Cleaver and Bootsma, 1975), was not more sensitive than diploid human fibroblasts, designated SL68, from an apparently normal neonate to the cytotoxic and mutagenic effects of MNNG. However, the capability for AGT repair makes a large difference in the response of human fibroblasts to this carcinogen. Cells proficient in AGT activity demonstrate higher survival and lower mutation frequencies when treated with MNNG compared to the high cytotoxicity and mutagenicity of MNNG in cells that have a very low level of AGT activity.

In contrast to the results shown for MNNG, the XP12BE cells are a great deal more sensitive than the SL68 cells to the cytotoxic and

mutagenic effects of ENU. This indicates that, as in bacteria, NER plays a larger role in the repair of ethyl adducts than does AGT (Simon et al., 1981; Maher et al., 1990).

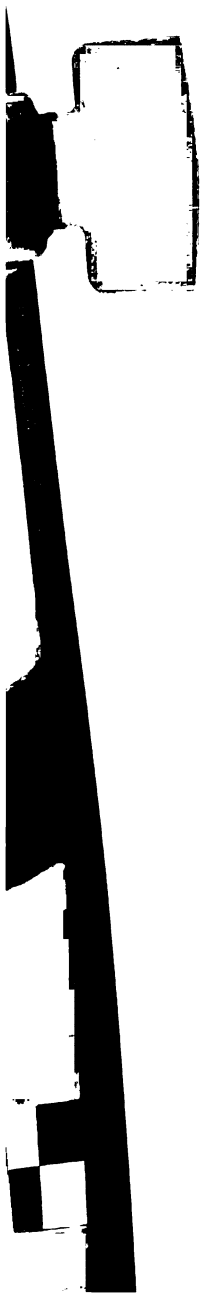
One potential problem with studying repair of alkyl-DNA lesions by AGT or NER in cell strains of different repair phenotypes that are not isogenic is the possibility that the unidentified mutation that has rendered the cell incompetent in AGT (or NER) affects other pathways that contribute tangentially to NER (or AGT). For example, GM0011 cells have an uncharacterized, spontaneous mutation resulting in the loss of AGT activity (Middlestadt et al., 1984). These cells also have a slightly decreased capacity for NER (Maher et al., 1990). It could be that these two phenomena are related. It has been shown, for example, that conversion of AGT-competent human lymphoblastoid or fibroblast cell lines to AGT-incompetent cell lines can be accompanied by simultaneous loss of expression of thymidine kinase and galactokinase (Karran et al., 1990), perhaps as a result of the loss of some common regulatory mechanism.

Dolan et al. (1990) recently reported that O^6-benzylguanine, as a free base in the media at a concentration of 5μM, could completely titrate out the AGT activity in AGT-proficient cultured human tumor cells. O^6-benzylguanine was shown to be non-toxic at this dose in these cells. Thus, O^6-benzylguanine can be used to study the repair of alkyl adducts in DNA in the presence or absence of AGT activity in cells that are isogenic. Maher and colleagues (Lukash et al., 1991) have used O^6-benzylguanine to study the affect of AGT repair on MNNG-induced cytotoxicity and mutagenicity in the diploid human cell strain SL68. O^6-benzylguanine depletes the cell of AGT to less than 1% of the normal level of activity. The AGT activity remains at this low level for up to 48 hours following

removal of the O^6-benzylguanine. Using this inhibitor, Maher and colleagues showed that the cytotoxicity and mutagenicity of MNNG is much lower in the cells with normal levels of AGT compared to the cells depleted of AGT. This confirmed that AGT is directly involved in the repair of MNNG-induced cytotoxic and mutagenic lesions in diploid human fibroblasts.

When Lukash et al. (1990) analyzed the *HPRT* mutations induced by MNNG in the AGT proficient and AGT deficient SL68 cells by sequencing the coding region of the *HPRT* gene, they found that MNNG treatment results in predominantly G·C → A·T transition mutations in either phenotype. There are also a small percentage of mutations at T's, possibly from methyl lesions at T's. If one can assume that the G·C → A·T mutations arose as the result of a methylated purine, e.g. O^6-MeG, then the majority of the G·C → A·T mutations (67%) occurred in the nontranscribed strand in the AGT-proficient cells, and 70% occurred in the nontranscribed strand in the AGT-depleted cells. This suggests that MNNG-induced premutagenic lesions in the *HPRT* gene occur preferentially in the nontranscribed strand, and that AGT repair of O^6-MeG in human fibroblasts is not strand-specific.

Skopek and colleagues (Bronstein et al., 1991) have treated EBV-transformed human lymphoblastoid cells with ENU and sequenced the coding region of the resulting HPRT-deficient mutants. The three cell lines they employed, designated X, A, and N, differed in repair capacity. The X cells, from a patient with XP, are completely deficient in NER capability, but are normal in AGT repair. The A cells are from a patient with hereditary spherocytosis and are devoid of AGT repair, but normal in NER. The N cells are normal in both kinds of repair. They found that the N cells, with NER and AGT both functioning, demonstrate a decreased



cytotoxic and mutagenic response to ENU compared to either the X or the A cells, with only AGT or NER working on the ENU-induced lesions. Interestingly, the X cells and the A cells are equally sensitive to both the cytotoxic and the mutagenic effects of ENU.

Upon sequencing the *HPRT* gene of the resulting mutants, they found that all three cell lines show transition and transversion mutations at G's and T's following ENU treatment. There was no significant neighboring-base sequence bias or strand bias in the distribution of lesions. The most significant difference they found among the three cell lines was an 8-fold increase in the frequency of G·C → A·T mutations in the A cells, and a 3-fold increase in frequency of this mutation in the X cells, compared to the frequency observed in the N cells. They attribute this increase to a lack of repair of O^6-EtG by AGT. They conclude that NER and AGT are both important in the repair of ENU-induced ethyl adducts in human cells.

Skopek and colleagues continued in their investigation of the repair of ENU-induced alkylation damage in the X, A, and N cells (Bronstein et al., 1992). They quantitated the removal of O^6-EtG, O^4-EtT and O^2-EtT adducts from the DNA of treated cells by measuring the binding of monoclonal antibodies to these lesions in DNA isolated from treated cells. They found that the O^6-EtG lesions are removed from the DNA of the treated cells only when NER and AGT are both present in the cell, i.e in the N cells. The O^4-EtT and O^2-EtT lesions are not removed from the DNA of any of the three cell lines. Skopek and colleagues conclude that only cells expressing both AGT and NER are able to efficiently remove O^6-EtG from DNA, and that neither NER nor AGT act on O^4-EtT or O^2-EtT lesions.

The results reported by Skopek and colleagues could be complicated

by the differences in the genetic background of the lymphoblastoid cell lines used. As discussed above, the different repair phenotypes observed in the lymphoblastoid cell lines could result from genetic alterations that affect multiple pathways in the cell. In our present study, we have employed O^6-benzylguanine to specifically deplete diploid human fibroblasts of AGT. This has allowed us to study DNA repair in human cells, while limiting the number of genetically different cell strains used. We have used the XP12BE cell strain, which is deficient in NER. These cells demonstrate an equal or slightly elevated capacity for AGT as that of the other cell strain used, SL68, which is normal in NER. Thus, the defect in NER of the XP12BE cells is not altering the capacity for AGT in this cell strain. By analyzing the induction and repair of alkyl adducts in normal, diploid human cells, we can obtain a better understanding of the initiation and processing of DNA lesions that lead to tumorigenic mutations in the human organism.

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CHAPTER II

Evidence from the frequency and spectrum of mutations that human fibroblasts can remove potentially mutagenic lesions induced by *N*-ethyl-*N*-nitrosourea using either nucleotide excision repair or O^6-alkylguanine-DNA alkyltransferase or both kinds of repair

SUMMARY

N-ethyl-*N*-nitrosourea (ENU), an extremely efficient mutagen, reacts with 12 sites in DNA, but O^6-ethylguanine (O^6-EtG), O^4-ethylthymine (O^4-EtT), and O^2-ethylthymine (O^2-EtT) are considered its major potentially mutagenic lesions. Early studies from this laboratory suggest that nucleotide excision repair (NER) acts on ENU-induced lesions. O^6-alkylguanine-DNA alkyltransferase (AGT) from extracts of mammalian cells is known to remove ethyl groups from O^6-EtG in DNA *in vitro*. The relative contribution of these repair systems in protecting human cells from the cytotoxic and/or mutagenic effects of ENU is unclear. Recent studies involving three EBV-immortalized human lymphoblastoid cell lines have indicated that in the absence of the other, neither AGT nor NER can remove ENU-induced lesions and that both repair systems are required.

To investigate the role of NER and AGT in repairing ENU-induced lesions, we made use of two diploid human fibroblast cell lines: one, derived from the foreskin of a normal neonate, is proficient in NER and AGT; the other, from a skin biopsy of a xeroderma pigmentosum patient (XP12BE, complementation group A), is deficient in NER but proficient in AGT activity. To dissect the role of these two kinds of DNA repair, we pretreated the cells with 25 μM O^6-benzylguanine (O^6-BzG) to deplete cells of AGT activity prior to exposing them to ENU. This allowed us to compare the effects of ENU in (a) cells lacking NER and depleted of AGT; (b) the same cells lacking NER, but proficient in AGT repair; (c) cells proficient in NER but depleted of AGT; and (d) the same cells proficient in both NER and AGT repair. ENU-treated cells were assayed for survival of colony-forming ability and frequency of 6-thioguanine resistant mutants. The

coding region of the *HPRT* gene of ~36 unequivocally independent mutants from each group were sequenced to analyze the mutations.

The results showed that cells lacking both kinds of repair were the most sensitive. In the cells lacking NER but proficient in AGT, cell killing and mutant frequency was ~38% lower than in cells deficient in NER and AGT. The majority of the decrease in mutants reflected a decrease in G·C → A·T base substitutions, the mutation attributable to O^6-EtG. In the cells proficient in NER but depleted of AGT activity, cell killing and mutant frequency was ~62% lower than in cells deficient in both kinds of repair. The decrease in mutant frequency reflected a decrease in substitutions involving G·C and T·A base pairs. Cells proficient in both kinds of repair were not more efficient in reducing the cytotoxic and mutagenic effects of ENU than cells competent in only NER, i.e., cell killing and mutant frequency was ~67% lower than in cells devoid of both kinds of repair. Analysis of the mutations indicated that the majority of this decrease in mutation frequency reflected a decrease in T·A → C·G and T·A → G·C substitutions. There was also a decrease in G·C → A·T base substitutions, but the data suggested that NER is not efficient in repairing lesions that would give rise to such substitutions when AGT is also acting. The data are consistent with the interpretation that in diploid human fibroblasts, potentially cytotoxic and mutagenic lesions induced by ENU can be efficiently removed by either NER or AGT and that if both kinds of repair are present, AGT removes those lesions that would result in G·C → A·T transitions, while NER removes those that would result in T·A → C·G and T·A → G·C base substitutions.

CHAPTER II

INTRODUCTION

Exposure of cells to DNA damaging agents can lead to mutations, and mutations have been shown to be causally involved in tumor formation. Cells have evolved DNA repair pathways in response to continuous exposure to such agents. These pathways have been extensively studied in bacteria and yeast, but are less well understood in mammalian cells. The repair process that can deal with the broadest range of damage is nucleotide excision repair (NER), which recognizes and removes damage that causes helical deformities in DNA, such as UV-induced cyclobutane pyrimidine dimers and 6-4 photoproducts, bulky multi-ringed chemical adducts, etc. (see ref. 1 for review). O^6-alkylguanine-DNA alkyltransferase (AGT) removes alkyl groups from the O^6 position of guanine in DNA by transferring the alkyl group to a cysteine in its own amino acid sequence (see ref. 2 for review).

The alkylating agent *N*-ethyl-*N*-nitrosourea (ENU), a highly efficient mutagen, has been shown to be carcinogenic in laboratory animals (3-5) and may be carcinogenic in humans (6). ENU alkylates 12 nucleophilic sites in DNA (7,8), but O^6-ethylguanine (O^6-EtG), O^2-ethylcytosine (O^2-EtC), O^2-ethylthymine (O^2-EtT), O^4-ethylthymine (O^4-EtT), and ethylphosphotriesters, which make up 8%, 2%, 7%, 2.5%, and 58% of the products, respectively (9), are the most chemically stable in DNA *in vitro* (10). O^6-EtG and O^4-EtT have been shown *in vitro* to be mutagenic, resulting in G·C → A·T (9) and T·A → C·G (11, 12) transitions, respectively. These are the predominant mutations induced by ENU in wild-type *E. coli* (13), but T·A → A·T base

substitutions are a major contributor to the mutagenic effects ofENU in eukaryotic cells, including human cells (14-17). Site specific mutagenesis studies show that O^2-EtT produces this transversion (18).

Recent evidence indicates that in *E. coli* both NER and AGT participate in the repair of O^6-EtG and O^4-EtT lesions (19, 20). AGT from human cell extracts has been shown to remove ethyl groups from O^6-EtG in DNA in vitro (2, 21, 22) and it may also remove ethyl adducts from O^4-EtT, but very inefficiently (23). The results of a study by Simon et al. (24) suggest that in human fibroblasts NER also acts on ENU-induced lesions. However, Skopek and colleagues showed that in EBV-immortalized human lymphoblastoid cells AGT cannot remove ENU-induced lesions in the absence of NER and vice versa. Both kinds of repair are required (17, 25).

The present study was designed to examine the contribution of NER and AGT in reducing the cytotoxic and mutagenic effects of ENU in diploid human fibroblasts and to examine the nature of the potentially mutagenic lesions responsible for the mutations. To do so, we employed two fibroblast cell lines; one, derived from the foreskin of a normal neonate, is proficient in NER and AGT; the other, from a skin biopsy of a xeroderma pigmentosum patient (XP12BE, complementation group A), is devoid of NER capacity (26), but has a level of AGT activity comparable to or even higher than that seen in normal fibroblasts (21). We used O^6-benzylguanine (O^6-BzG) to deplete populations of the two cell lines of pre-existing AGT (27, 28). This approach allowed us to compare the effects of ENU in cells devoid of both NER and AGT repair; in cells devoid of NER but proficient in AGT repair; in cells devoid of AGT but proficient in NER; and in cells proficient in both kinds of repair. The cells were exposed to ENU and compared for survival and for frequency of 6-thioguanine-resistant (TG^R)

mutants. The coding region of the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene of ~36 unequivocally independent ENU-induced mutants from each of the four populations was sequenced to gain insight into the potentially mutagenic lesions induced in DNA by ENU in human cells, and to determine the role of NER and AGT repair in removing such lesions.

The results showed that the cytotoxicity and the frequency of mutants was highest in the $NER^{-}AGT^{-}$ cells, and that either AGT or NER, acting independent of the other, was able to significantly reduce these effects. NER was more effective in this process than AGT. Surprisingly, the cytotoxicity and the frequency of mutants induced in cells proficient in both repair processes was not much lower than that seen in cells proficient in NER but devoid of AGT repair. Interpretation of the spectra of *HPRT* mutations induced in these four populations, taken together with the data on mutant frequencies, suggests that AGT, in the presence or absence of NER, removes the lesion(s) responsible for G·C → A·T transitions; that NER, acting in the absence of AGT, removes lesions involving G·C and T·A base pairs; and that in the presence of AGT, NER is involved primarily in removing potentially mutagenic lesions involving T·A base pairs.

MATERIALS AND METHODS

Cells and media

Cell strain SL68 was initiated in this laboratory from the foreskin of a normal newborn. It has a normal level of NER, and the AGT activity level, determined by measuring the removal of O^6-methylguanine from a DNA template by cell extract, is 124 fmol removed per mg protein (29). Cloning efficiency ranged from 44 to 70%. Cell strain XP12BE is from a xeroderma pigmentosum patient, complementation group A, and is very deficient in nucleotide excision repair (<1% of normal) (26). The XP12BE cells show an AGT activity level of 211 fmol removed per mg protein. The cloning efficiency for these cells ranged from 20 to 30%. Cells were between passages 15 and 20 when treated with ENU, with "passage" indicating the number of population doublings from time of initiation of cell line or strain. Cells were cultured in a water-saturated incubator at 37°C in 5% CO_2. The culture medium was Eagle's minimal medium modified as described (30) or McM medium (31) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 5% supplemented calf serum; 5% fetal calf serum (Hyclone, Logan UT) (culture medium). McM medium lacking adenine or the modified Eagle's minimal medium, supplemented as above and containing 40 μM TG, was used for selection of TG^R mutants (selective medium).

Treatment of cells with ENU in the presence or absence of O^6-BzG

O^6-BzG was synthesized by Dr. Robert Moschel (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick,

MD) and stored as a solid at -20°C. Cells were plated in culture medium at a density of 1 x 10^4 cells/cm^2. After allowing time for attachment, O^6-BzG dissolved in anhydrous dimethylsulfoxide (DMSO)(Burdick and Jackson, Muskegon, MI) was added to the appropriate dishes by micropipette to give a final concentration of 25 μM. The remaining dishes received an equal volume of DMSO only. After 2 h of pretreatment with O^6-BzG, the medium in all the dishes was exchanged for serum-free Eagle's minimal essential medium, lacking bicarbonate and buffered with 20 mM HEPES (pH 7.25). O^6-BzG (25 μM) was again added as above to the appropriate set of dishes. ENU, dissolved in anhydrous DMSO immediately prior to treatment, was added to the dishes by micropipette at the indicated concentrations, and the cells were incubated for 30 min at 37°C. Following treatment, the medium was removed and the cells were rinsed with phosphate-buffered saline and refed with fresh culture medium containing serum. To insure that the level of AGT activity remained low, O^6-BzG (25 μM) was included in the appropriate dishes for an additional 24 or 48 h, after which cells were refed with fresh culture medium without rinsing. One set of the cells exposed to each dose of ENU, and the respective controls, were immediately assayed for cell survival by plating cells at appropriate cloning densities. The rest of the cells were allowed to replicate undisturbed and begin the expression period for TG resistance.

Assay for cell survival

The cytotoxic effect of ENU in the presence or absence of O^6-BzG was determined from the loss of colony-forming ability as described (30). Briefly, cells treated at the same density as that used for the mutagenesis experiments were trypsinized, plated at cloning densities, and

allowed to form colonies. Cell survival was expressed as a percent of the colony-forming ability of the appropriate set of control cells.

Assay for frequency of mutants

The procedures used were essentially as described (28), except that cells were exposed to ENU in the presence or absence of O^6-BzG, and those exposed to ENU in the presence of O^6-BzG were maintained in media containing O^6-BzG for an additional 24-48 h. The cells were maintained in exponential growth for an 8-day expression period before 0.75 to 1 x 10^6 were plated in selective medium at 500 cells/cm^2. When macroscopic TG^R colonies were visible 14 days later, these were located and isolated, and the TG^R cells in the clone were pelleted and used for preparing *HPRT*.

Synthesis of cDNA directly from mRNA in cell lysates

Cells from mutant clones (100 - 1000 per clone) were trypsinized, suspended in cold PBS (pH 7.4), and centrifuged 10 min at 4°C. The supernatant was removed, and the cell pellet was stored frozen at -80°C or used immediately. Cell pellets were resuspended in 5 μl of cDNA cocktail as described by Yang et al. (32). The reverse transcriptase reaction was performed at 37°C for 1 h to allow the first strand cDNA to be synthesized from total poly(A)-mRNA.

Amplification of HPRT cDNA and DNA sequencing

Second strand HPRT cDNA was prepared and the cDNA was amplified essentially as described (32) using PCR as follows: 30 cycles (94° C, 5 min; 94° C, 1 min; 50° C, 1 min; 72° C, 2 min) followed by 20 cycles (94° C, 5 min; 94° C, 1 min; 48° C, 1 min; 72° C, 2 min). The amplified *HPRT* DNA

product was sequenced using methods described by Yang et al. (32) or by cycle sequencing, as described below.

For cycle sequencing, we followed the procedures outlined in the cycle sequencing kit purchased from United States Biochemical (USB). Amplified double-stranded DNA was processed through a Centricon 30 tube (Amicon Corp.) to remove unincorporated dNTP's and primers. The coding region of the *HPRT* gene was sequenced using the primers shown in Figure 1. Primers were end-labeled with [γ-^{32}P] dATP (3000 Ci/mMol; NEN, DuPont) and T4 polynucleotide kinase, then mixed with approximately 100 ng of PCR product, 1 unit of *Taq* DNA polymerase, and reaction buffer supplied by USB. The template and primer mixture was divided into four tubes, each containing cold dNTP's and ddNTP's as directed by the cycle sequencing kit protocol. DNA synthesis occurred via 30 cycles as follows: (94° C, 5 min; 94° C, 1 min; 50° C, 1 min; 72° C, 2 min). The reaction was stopped by adding the stop solution supplied by USB. All samples were electrophoresed as described (32).

Figure 1: Sequence and location of primers used in cycle sequencing of *HPRT* cDNA. Sequence of primers is shown 5' → 3'. Primers 5 and 6 are complementary to the antisense strand, and primer 1* is complementary to the sense strand.

Primer	Sequence
5	$_{169}$ATG GGA GGC CAT CAC ATT G
6	$_{414}$C ACT GGC AAA ACA ATG CAG
1*	$_{337}$C CCC TGT TGA CTG GTC ATT

RESULTS

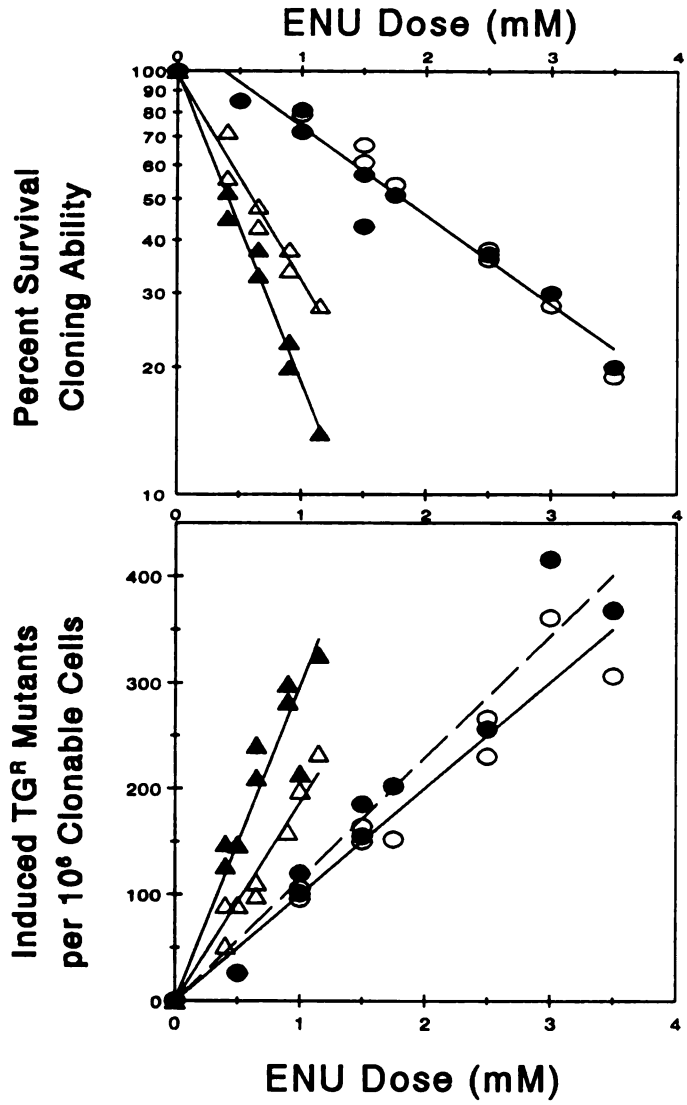
The present investigation was designed to determine the effect of NER and AGT repair on the cytotoxicity of ENU in diploid human fibroblasts and on the frequency of and spectrum of base substitution mutations induced. Previous studies in this laboratory showed that XP12BE fibroblasts, which have abundant AGT activity (21) but are extremely deficient in NER (26), are not more sensitive than normal fibroblasts to the cytotoxic or mutagenic effects of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (21). The XP12BE cells are much more sensitive than normal fibroblasts to the effects of ENU (24). These results suggest that NER is not involved in protecting human fibroblasts from methylating agents, but plays an important role in removing ethyl adducts. More recently, Lukash et al. (28) showed that depleting normal diploid fibroblasts of AGT activity significantly increases their sensitivity to the cytotoxic and mutagenic effects of MNNG. In that study the fibroblasts exposed to 25 μM O^6-BzG for a total of 27 hours showed levels of AGT activity that were <1% of the activity in the control cells for at least 48 hours after removal of the O^6-BzG inhibition. In the present study we allowed an additional 24 hours of incubation in the presence of O^6-BzG to deplete cells of AGT for at least 96 hours following treatment with ENU. This exposure to O^6-BzG did not alter the colony-forming ability of either SL68 cells or XP12BE cells that were not exposed to ENU.

Effect of repair on the cytotoxicity and frequency of mutations induced by ENU in diploid human fibroblasts

As shown in the upper panel of Figure 2, the cytotoxic effect of ENU was highest in the cells lacking both NER and AGT repair. ENU was less toxic in cells devoid of NER but proficient in AGT activity, strongly suggesting that AGT repair, in the absence of NER, removes potentially cytotoxic lesions. The cytotoxic effect of ENU was still lower in cells devoid of AGT activity but proficient in NER, strongly suggesting that excision repair acting in the absence of AGT is capable of removing potentially cytotoxic lesions induced by ENU. However, when both types of repair were present in the cells, this did not further decrease the cytotoxic effect.

A similar outcome was observed for the mutagenic effect of ENU (Figure 2, bottom panel). The cells lacking both kinds of repair showed the highest frequency, with 300 mutants induced per 10^6 cells per mM. In cells deficient in NER but with a high level of AGT activity, the frequency was significantly lower (187 mutants per 10^6 cells per mM), strongly suggesting that AGT in the absence of NER removes a significant fraction of the potentially mutagenic ENU-induced lesions before they can be converted into mutations. The frequency of mutants was significantly lower in cells proficient in NER but depleted of AGT activity, with 115 mutants induced per 10^6 per mM. This strongly suggests that NER in the absence of AGT can excise potentially mutagenic lesions induced by ENU and can do so even more efficiently than AGT acting in the absence of NER. Cells having both types of repair failed to exhibit a still lower frequency of mutants. This implies that, instead of being additive, one repair system interfered with the other, e.g. AGT interfered with the

Figure 2. Survival and induced mutant frequency in ENU-treated diploid human fibroblasts differing in NER and AGT capacities.
○ $NER^{+}AGT^{+}$; ● $NER^{+}AGT^{-}$; △ $NER^{-}AGT^{+}$; ▲ $NER^{-}AGT^{-}$.



excision of lesions by NER.

Spectra of mutations induced by ENU in diploid human fibroblasts

ENU produces 12 different alkyl adducts in DNA, but O^6-EtG, O^4-EtT, and O^2-EtT are the most likely premutagenic lesions (33,34). *In vivo* site-specific mutagenesis studies and *in vitro* replication assays showed that these ethylated bases readily yield G·C → A·T , T·A → C·G, and T·A → A·T base pair substitutions, respectively (11,18,33-36). Although O^2-EtC is also a relatively stable alkylation product in DNA (9), the ability of this lesion to miscode has not yet been examined by site-specific mutagenesis assay. It is possible that O^2-EtC lesions result in G·C → A·T transitions, but there is as yet no evidence to support this hypothesis.

In wild-type E. coli, ENU produces mainly G·C → A·T and T·A → C·G base substitutions (73% and 21% of the base substitutions observed, respectively) (13), suggesting that O^6-EtG and O^4-EtT are the principal premutagenic lesions induced by ENU. However, in a variety of eukaryotic cells assay systems all six possible base pair substitutions have been found (14-17). To examine the types of mutations induced by ENU in diploid human fibroblasts we sequenced the coding region of the *HPRT* gene of 143 mutants representing each of the four populations shown in Figure 2. The results are shown in Tables I through IV. The 123 base pair substitutions observed in the 143 mutants were distributed over 89 sites. Loss of an exon, presumably due to a base substitution at a splice site, was seen in 33 mutants. No G·C → C·G transversions were seen. Substitution mutations were found twice at positions 49, 64, 125, 134, 149, 194, 202, 214, 284, 299, 464, 530, 541, 547, 566, 575, 580, and 614; three times at positions 110, 473, 539, and 542; and five times at

Table I. Kinds and locations of mutations induced by ENU in the coding region of the *HPRT* gene in the absence of nucleotide excision repair and alkyltransferase activity

Mutant	ENU (mM)	Site	Exon	Mutation	Strand with Affected G or T[a]	Neighboring bases[b]	Amino acid Change
XBE49	0.4	149	3	GC → TA	T	CTT GCT CGA	ALA to ASP
XBE53[c]	1.15	154	3	GC → TA	NT	CGA GAT GTG	ASP to TRY
XBE14[c]	1.0	574	8	GC → TA	NT	TAT GCC CTT	ALA to SER
XBE11	0.5	91	2	GC → AT	NT	GAG GAT TTG	ASP to ASN
XBE5	1.5	134	2	GC → AT	NT	GAC AGG ACT	ARG to LYS
XBE48	0.4	134	2	GC → AT	NT	GAC AGG ACT	ARG to LYS
XBE24[c]	1.5	142	3	GC → AT	T	GAA CGT CTT	ARG to CYS
XBE52	1.15	208	3	GC → AT	NT	AAG GGG GGC	GLY to ARG
XBE114	1.15	514	7	GC → AT	NT	AGT GTT GGA	VAL to ILE
XBE3	0.5	580	8	GC → AT	NT	CTT GAC TAT	ASP to ASN
XBE14[c]	1.0	580	8	GC → AT	NT	CTT GAC TAT	ASP to ASN
XBE53[c]	1.15	77	2	TA → CG	T	CCT AAT CAT	ASN to SER
XBE46	0.4	194	3	TA → CG	NT	GCC CTC TGT	LEU to PRO
XBE101	1.15	214	3	TA → CG	NT	GGC TAT AAA	TYR to HIS
XBE13	0.5	220	3	TA → CG	NT	AAA TTC TTT	PHE to LEU
XBE116	1.15	241	3	TA → CG	NT	CAT TAC ATC	TYR to HIS
XBE107	1.15	258	3	TA → CG	NT	CTG AAT AGA	No change[d]
XBE2	0.5	308	3	TA → CG	T	CTG AAG AGC	LYS to ARG
XBE128	1.0	475	6	TA → CG	T	GTC AAG GTC	LYS to GLU
XBE100	1.15	542	8	TA → CG	NT	GGA TTT GAA	PHE to SER
XBE127	1.0	602	8	TA → CG	T	AGG GAT TTG	ASP to GLY
XBE34	1.0	466	6	TA → AT	T	CCA AAG ATG	LYS to Stop
XBE125	1.0	548	8	TA → AT	NT	GAA ATT CCA	ILE to ASN
XBE18	0.5	590	8	TA → AT	T	AAT GAA TAC	GLU to VAL
XBE129	1.0	49	2	TA → GC	NT	GGT TAT GAC	TYR to ASP
XBE126	1.0	49	2	TA → GC	NT	GGT TAT GAC	TYR to ASP
XBE24[c]	1.5	410	6	TA → GC	NT	ATA ATT GAC	ILE to SER
XBE8[c]	0.5	494	7	TA → GC	NT	CTG GTG AAA	VAL to GLY
XBE8[c]	0.5	595	8	TA → GC	NT	TAC TTC AGG	PHE to VAL
XBE14[c]	1.0	625	9	TA → GC	T	ATT AGT GAA	SER to ARG

[a]T, transcribed; NT, nontranscribed.

[b]The sequence written 5′ to 3′ is that of the nontranscribed coding strand.

[c]This mutant contained more than one mutation.

[d]No other mutation was detected.

Table I. (Continued)

Mutant	ENU (mM)	Missing Exon
XBE103	1.0	4
XBE31	1.0	4
XBE104	0.5	4
XBE19	0.5	5
XBE130	1.0	7
XBE131	1.0	7
XBE132	1.0	7
XBE133	1.0	7
XBE111	1.15	8
XBE17	0.5	8
XBE23	1.0	17 bp missing from the first part of exon 9

Table II. Kinds and locations of mutations induced by ENU in the coding region of the *HPRT* gene in the absence of nucleotide excision repair, but in the presence of alkyltransferase activity

Mutant	ENU (mM)	Site	Exon	Mutation	Strand with Affected G or T[a]	Neighboring bases[b]	Amino acid Change
XE124[c]	1.0	172	3	GC → TA	NT	ATG GGA GGC	GLY to Stop
XE25	1.0	197	3	GC → TA	NT	CTC TGT GTG	CYS to PHE
XE4	0.4	202	3	GC → TA	T	GTG CTC AAG	LEU to ILE
XE43[c]	0.9	400	5	GC → TA	NT	GTG GAA GAT	VAL to PHE
XE128	1.0	419	6	GC → TA	NT	ACT GGC AAA	GLY to VAL
XE121	1.0	197	3	GC → AT	NT	CTC TGT GTG	CYS to TYR
XE126	1.0	197	3	GC → AT	NT	CTC TGT GTG	CYS to TYR
XE44	0.9	464	6	GC → AT	T	ATT CCA AAG	PRO to LEU
XE2	0.5	110	2	TA → CG	NT	TTT ATT CCT	ILE to THR
XE103	1.15	219	3	TA → CG	T	TAT AAA TTC	PHE to LEU
XE27	1.0	290	3	TA → CG	NT	ACT GTA GAT	VAL to ALA
XE23[c]	0.5	290	3	TA → CG	NT	ACT GTA GAT	VAL to ALA
XE124[c]	1.0	383	4	TA → CG	T	GGA AAG AAT	LEU to TRP
XE125	1.0	499	7	TA → CG	T	AAA AGG ACC	ARG to GLY
XE48	1.15	530	7	TA → CG	T	CCA GAC TTT	ASP to GLY
XE29	1.5	533	8	TA → CG	NT	GAC TTT GTT	PHE to SER
XE6	1.0	592	8	TA → CG	NT	GAA TAC TTC	TYR to HIS
XE23[c]	0.5	605	8	TA → CG	NT	GAT TTG AAT	LEU to SER
XE122	0.75	95	2	TA → AT	NT	GAT TTG GAA	LEU to Stop
XE30	1.5	104	2	TA → AT	NT	AGG GTG TTT	VAL to GLU
XE47	1.15	215	3	TA → AT	T	GGC TAT AAA	TYR to PHE
XE40	0.65	247	3	TA → AT	T	ATC AAA GCA	LYS to Stop
XE31	1.5	290	3	TA → GC	NT	ACT GTA GAT	VAL to GLY
XE123	0.75	290	3	TA → GC	NT	ACT GTA GAT	VAL to GLY
XE42	0.9	473	6	TA → GC	NT	ATG GTC AAG	VAL to GLY
XE127	0.75	473	6	TA → GC	NT	ATG GTC AAG	VAL to GLY
XE43[c]	0.9	541	8	TA → GC	NT	GGA TTT GAA	PHE to VAL
XE5	0.5	547	8	TA → GC	T	GAA ATT CCA	ILE to LEU
XE26	1.0	547	8	TA → GC	T	GAA ATT CCA	ILE to LEU

Mutant	ENU (mM)	Missing Exon
XE101	1.15	4
XE11	0.5	6
XE24	1.0	7
XE36	0.65	8

[a]T, transcribed; NT, nontranscribed.

[b]The sequence written 5′ to 3′ is that of the nontranscribed coding strand.

[c]This mutant contained more than one mutation.

Table III. Kinds and locations of mutations induced by ENU in the coding region of the *HPRT* gene in the presence of nucleotide excision repair, but in the absence of alkyltransferase activity

Mutant	ENU (mM)	Site	Exon	Mutation	Strand with Affected G or T[a]	Neighboring bases[b]	Amino acid Change
BEN122	2.5	6	1	GC → TA	NT	ATG GCG ACC	No change[c]
BEN77	1.5	634	9	GC → TA	NT	ACT GGA AAA	GLY to Stop
BEN117	2.5	197	3	GC → AT	NT	CTC TGT GTG	CYS to TYR
BEN11	1.0	209	3	GC → AT	NT	AAG GGG GGC	GLY to GLU
BEN29[d]	2.5	336	4	GC → AT	NT	ACA GGG GAC	No change[d]
BEN103	1.8	464	6	GC → AT	T	ATT CCA AAG	PRO to LEU
BEN137	3.5	539	8	GC → AT	NT	GTT GGA TTT	GLY to GLU
BEN141	3.5	539	8	GC → AT	NT	GTT GGA TTT	GLY to GLU
BEN46	2.5	635	9	GC → AT	NT	ACT GGA AAA	GLY to GLU
BEN108	1.8	65	2	TA → CG	NT	TTA TTT TGC	PHE to CYS
BEN112	2.5	122	2	TA → CG	NT	GGA CTA ATT	LEU to PRO
BEN114	2.6	125	2	TA → CG	NT	CTA ATT ATG	ILE to THR
BEN120	2.5	203	3	TA → CG	NT	GTG CTC AAG	LEU to PRO
BEN138	3.5	236	3	TA → CG	NT	CTG CTG GAT	LEU to PRO
BEN110	2.5	530	7	TA → CG	T	CCA GAC TTT	ASP to GLY
BEN29[d]	2.5	66	2	TA → GC	NT	TTA TTT TGC	PHE to LEU
BEN48	2.5	64	2	TA → AT	NT	TTA TTT TGC	PHE to ILE
BEN76	1.5	110	2	TA → AT	NT	TTT ATT CCT	ILE to ASN
BEN35	1.5	216	3	TA → AT	NT	GGC TAT AAA	TYR to Stop
BEN66	1.5	245	3	TA → AT	NT	TAC ATC AAA	ILE to ASN
BEN139	3.5	284	3	TA → AT	NT	CCT ATG ACT	MET to LYS
BEN107	1.8	284	3	TA → AT	NT	CCT ATG ACT	MET to LYS
BEN15	1.0	573	8	TA → AT	NT	GGA TAT GCC	TYR to LEU
BEN55	1.0	614	9	TA → AT	NT	CAT GTT TGT	VAL to ASP
BEN39	1.5	299	3	TA → GC	NT	TTT ATC AGA	ILE to SER
BEN113	2.6	330	4	TA → GC	T	CAG TCA ACA	No change
BEN140	3.5	395	5	TA → GC	NT	TTG ATT GTG	ILE to SER
BEN101	2.6	473	6	TA → GC	NT	ATG GTC AAG	VAL to GLY
BEN4	0.5	522	7	TA → GC	NT	GGA TAT AAG	TYR to Stop
BEN37	1.5	542	8	TA → GC	NT	GGA TTT GAA	PHE to CYS
BEN102	2.6	543	8	TA → GC	NT	GGA TTT GAA	PHE to LEU
BEN96	2.5	614	9	TA → GC	NT	CAT GTT TGT	VAL to GLY

[a]T, transcribed; NT, nontranscribed.

[b]The sequence written 5′ to 3′ is that of the nontranscribed coding strand.

[c]No other mutation was detected.

[d]This mutant contained more than one mutation.

Table III. (Continued)

Mutant	ENU (mM)	Missing Exon
BEN51	2.5	4
BEN68	2.5	6
BEN44	2.5	8
BEN100	1.8	8
BEN8	0.5	8
BEN104	1.8	8
BEN40	2.5	14 bp missing from the first part of exon 9 (610-623)
BEN64	2.5	17 bp missing from the first part of exon 9 (610-626)

Table IV. Kinds and locations of mutations induced by ENU in the coding region of the *HPRT* gene in the presence of nucleotide excision repair and alkyltransferase activity

Mutant	ENU (mM)	Site	Exon	Mutation	Strand with Affected G or T[a]	Neighboring bases[b]	Amino acid Change
EN105	1.8	96	2	GC → TA	NT	GAT TTG GAA	LEU to PHE
EN58[c]	1.5	112[d]	2	GC → TA	T	ATT CCT CAT	PRO toTHR
EN42[c]	1.5	130	2	GC → TA	NT	ATG GAC AGG	ASP to TYR
EN48	1.0	197	3	GC → TA	NT	CTC TGT GTG	CYS to PHE
EN41	1.5	539	8	GC → TA	NT	GTT GGA TTT	GLY to VAL
EN44	1.5	575	8	GC → TA	T	TAT GCC CTT	ALA to ASP
EN11	1.5	575	8	GC → TA	T	TAT GCC CTT	ALA to ASP
EN80	3.5	119	2	GC → AT	NT	CAT GGA CTA	GLY to GLU
EN55	1.5	149	3	GC → AT	T	CTT GCT CGA	ALA to VAL
EN83	3.0	151	3	GC → AT	T	GCT CGA GAT	ARG to Stop
EN50	1.0	202	3	GC → AT	T	GTG CTC AAG	LEU to PHE
EN102	2.6	334	4	GC → AT	NT	ACA GGG GAC	GLY to ARG
EN63[c]	2.5	429	6	GC → AT	NT	ACA ATG CAG	MET to ILE
EN63[c]	2.5	601	8	GC → AT	NT	AGG GAT TTG	ASP to ASN
EN110	1.8	125	2	TA → CG	NT	CTA ATT ATG	ILE to THR
EN51	1.0	170	3	TA → CG	NT	GAG ATG GGA	MET to THR
EN100	1.8	214	3	TA → CG	NT	GGC TAT AAA	TYR to HIS
EN37	1.5	110	2	TA → AT	NT	TTT ATT CCT	ILE to ASN
EN82	3.5	194	3	TA → AT	NT	GCC CTC TGT	LEU to HIS
EN2	1.0	198	3	TA → AT	NT	CTC TGT GTG	CYS to Stop
EN85	3.5	290	3	TA → AT	NT	ACT GTA GAT	VAL to GLU
EN22[c]	2.5	537	8	TA → AT	NT	TTT GTT GGA	No change[c]
EN112	2.6	541	8	TA → AT	NT	GGA TTT GAA	PHE to ILE
EN22[c]	2.5	542	8	TA → AT	NT	GGA TTT GAA	PHE to TYR
EN3	1.0	566	8	TA → AT	NT	GTT GTA GGA	VAL to GLU
EN62	2.5	566	8	TA → AT	NT	GTT GTA GGA	VAL to GLU
EN84	3.5	64	2	TA → GC	NT	TTA TTT TGC	PHE to VAL
EN58[c]	1.5	111[d]	2	TA → GC	NT	TTT ATT CCT	ILE to MET
EN43	1.5	136	3	TA → GC	T	AGG ACT GAA	THR to PRO
EN66	2.5	185	3	TA → GC	NT	CAC ATT GTA	ILE to SER
EN81	3.5	299	3	TA → GC	NT	TTT ATC AGA	ILE to SER
EN53	1.5	449	6	TA → GC	NT	TTG GTC AGG	VAL to GLY

[a]T, transcribed; NT, nontranscribed.

[b]The sequence written 5′ to 3′ is that of the nontranscribed coding strand.

[c]This mutant contained more than one mutation.

[d]This mutation is next to a second mutation in the same mutant. If the lesions involved a G and a T, these were on opposite strands.

Table IV. (Continued)

Mutant	ENU (mM)	Missing Exon
EN60	2.5	4
EN111	1.8	4
EN17	I.5	7
EN13	1.5	8
EN108	2.6	8
EN42[c]	1.5	8
EN32	1.0	8
EN39	1.5	8
EN21	2.5	21 bp missing from the first part of exon 8 (533-553)
EN106	1.8	8 bp from the last part of intron 2 have been inserted between exon 1 & 2, probably due to a change in a splice site

positions 197 and 290. In ENU-treated E. coli, ~80% of the base substitutions involve guanines or thymines in the sequence 5'-Pu(G/T)-3' (13, 20). We found no significant neighboring base effect in human fibroblasts. Only 60% of the guanines and 63% of the thymines involved in base substitutions were 3' to a purine base.

Effect of DNA repair on the types of base pair substitutions induced by ENU in human fibroblasts.

In analyzing the effect of repair on the spectra of mutations induced in the *HPRT* gene by ENU, we made three assumptions. First, we assumed that XP12BE cells differ from SL68 cells principally in their lack of NER capability. Since the two cell lines do not differ significantly in their response to MNNG (21), it is likely that they do not differ in levels of endonucleases involved in repair of apurinic or apyrimidinic sites. Second, we assumed that the premutagenic lesions responsible for the base pair substitutions we observed consisted of an ethylated guanine or thymine. Finally, we assumed that the distribution of the five classes of base pair substitutions observed in each of the four groups of independent mutants analyzed is representative of the distribution present in the population of mutants induced by ENU at a given dose. For example, as shown in Table V, we determined the nature of 30 base substitutions of the NER^-/AGT^- cells. This population demonstrated a TG^R mutant frequency of 300×10^{-6} per mM. We assigned the proportion of each class of base substitution in the total population as a ratio of that seen in the 30 substitutions identified.

The distribution of the classes of base substitutions observed in the NER^-/AGT^- cells can serve as a standard against which we can compare

Table V. Summary of base substitution mutations observed in the coding region of the *HPRT* gene in ENU-treated diploid human fibroblasts differing in NER and AGT repair capacities.

Base change	NER$^-$ / AGT$^-$			NER$^-$ / AGT$^+$			NER$^+$ / AGT$^-$			NER$^+$ / AGT$^+$		
	Mutations seen	Mutations per mM (per 10^6)		Mutations seen	Mutations per mM (per 10^6)		Mutations seen	Mutations per mM (per 10^6)		Mutations seen	Mutations per mM (per 10^6)	
G → T	3	30		5	32		2	7		7	22	
(37%) 11			110	(28%) 8		51	(28%) 9		32	(44%) 14		44
G → A	8	80		3	19		7	25		7	22	
T → C	10	100		10	65		6	22		3	9	
T → A	3	30		4	26		8	29		9	28	
(63%) 19			190	(72%) 21		136	(72%) 23		83	(56%) 18		55
T → G	6	60		7	45		9	32		6	18	
TOTAL	30	300		29	187		32	115		32	100	

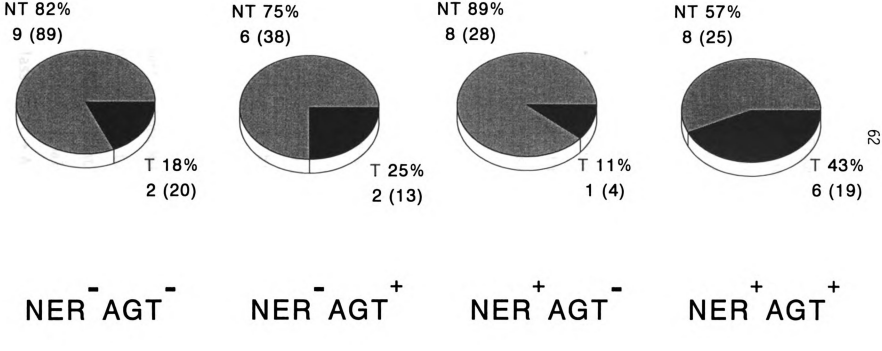
the distribution of substitutions seen in the other three ENU-treated populations. As shown in Table V, 63% involve T·A base pairs and 37% involve G·C base pairs.

In the NER^-/AGT^+ cells, the frequency of mutants decreased by ~35% relative to that observed in the NER^-/AGT^- cells (Figure 2). If one assigns the fractions of mutations in this population per mM dose as described above, one can analyze the distribution for decreases in particular classes of mutations to gain insight into the effect of AGT repair (see Table V). The majority of the decrease in mutations involved lesions producing G·C → A·T base substitutions. This could be interpreted to indicate that the lesion produced in this class of base substitution, i.e. O^6-EtG, was removed by AGT. There was also a decrease in the frequency of T·A → C·G transitions compared to the frequency in the NER^-/AGT^- cells. This suggests that AGT can also remove ethyl groups from O^4-EtT to some extent. There was little or no decrease in the contribution of classes other than G·C → A·T and T·A → C·G. In particular, there was no decrease in the contribution to the overall mutant frequency of lesions resulting in T·A → A·T transversions compared to that seen in the NER^-/AGT^- cells.

The decrease in lesions responsible for G·C → A·T transitions, presumably O^6-EtG, does not show a bias for repair of the transcribed strand. Rather it shows greater loss from the nontranscribed strand, the strand initially containing the majority of these lesions (compare Tables I and II). The ratio of this lesion in the transcribed strand relative to the nontranscribed strand is 12:88 in the NER^-/AGT^- cells and 33:67 in the NER^-/AGT^+ cells. This decrease in strand bias is reflected in Figure 3.

When normal cells proficient in NER but depleted of AGT by O^6-BzG are

Figure 3. Strand distribution of guanines involved in ENU-induced base substitutions. NT = Non-transcribed strand; T = Transcribed strand; number outside of parenthesis = number of observed base substitutions at G·C base pairs; number in parenthesis = number of base substitutions at G·C base pairs within the larger population (see Table V).



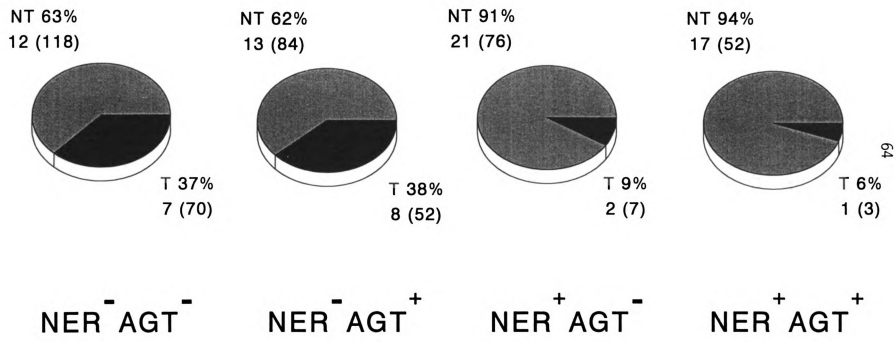
compared with the cells lacking both kinds of repair, the overall frequency of mutants decreases ~62% (Figure 2). A similar analysis of the decreases in classes of base substitutions (Table V) strongly suggests that in these cells, NER is able to repair lesions resulting in G·C → A·T, G·C → T·A, T·A → C·G, and T·A → G·C base substitutions. There was no decrease in T·A → A·T base substitutions, suggesting little or no excision repair of the lesions resulting in this class of base substitution, presumably O^2-EtT. NER appears to be as efficient as AGT alone in removing lesions leading to G·C → A·T transitions, i.e. O^6-EtG, but far more efficient than AGT in removing lesions that lead to T·A → C·G transitions (probably O^4-EtT) and to T·A → G·C transversions (lesion unknown).

There was no evidence in NER^+/AGT^- cells of a preferential removal of O^6-EtG from the nontranscribed strand, as there was no change in strand bias from that seen in the NER^-/AGT^- cells. Analysis of the change in strand distribution of the lesions resulting in T·A → C·G substitutions, presumably O^4-EtT, supports the hypothesis that strand-specific excision repair occurred. (See Tables I and III.) The ratio of these lesions in the transcribed vs. nontranscribed strand is 40:60 in the NER^-/AGT^- cells and 14:86 in the NER^+/AGT^- cells. The bias is reflected in Figure 4.

Evidence that NER and AGT acting in the same cell do not have an additive effect.

In the cells proficient in both NER and AGT, the overall frequency of mutants decreased by ~67% relative to the NER^-/AGT^- cells (Figure 2). This overall decrease is not significantly greater than that observed in NER^+/AGT^- cells (Figure 2). However, analysis as above (Table V) indicates that the decrease in the class of G·C → A·T transitions is very similar to

Figure 4. Strand distribution of thymines involved in ENU-induced base substitutions. NT = Non-transcribed strand; T = Transcribed strand; number outside of parenthesis = number of observed base substitutions at T·A base pairs; number in parenthesis = number of base substitutions at T·A base pairs within the larger population (see Table V).



what was seen in NER^-/AGT^+ cells, as if AGT repaired these lesions. Consistent with this interpretation is the change in strand distribution of these lesions (compare Tables I, II, and IV). The data show a bias in the loss of such lesions from the nontranscribed strand, as was seen when AGT was acting in the absence of NER. This bias is reflected in Figure 3.

In contrast, the NER^+/AGT^+ cells reduced the contribution of lesions leading to T·A → C·G transitions (i.e. O^4-EtT) significantly, to a frequency lower than was observed in the NER^+/AGT^- cells. This loss of O^4-EtT lesions showed a strong preference for the transcribed strand (ratio of transcribed strand:nontranscribed strand, 40:60 in NER^-/AGT^-, 0:100 in NER^+/AGT^+). This supports the hypothesis that excision repair is responsible for the decrease in this class of substitutions. This change in bias is reflected in Figure 4. Once again, there was no evidence that either AGT or NER decreased the contribution of lesions giving rise to T·A → A·T (i.e. O^2-EtT).

DISCUSSION

We found that both NER and AGT are important in repairing ENU-induced lesions in the *HPRT* gene in human fibroblasts. Alkyltransferase acting independent of NER was shown to be operative in the repair of ENU-induced lesions, as we saw that the cytotoxicity and mutagenicity of ENU decreased in the NER^-/AGT^+ cells relative to that in the NER^-/AGT^- cells. NER acting independent of AGT was shown to be instrumental in repair of ENU-induced premutagenic lesions, because in the NER proficient SL68 cells the cytotoxicity and mutagenicity of ENU were greatly reduced compared to that observed in the NER deficient XP cells. Our results in diploid human fibroblasts are not similar to those reported by Bronstein, et al. (17, 25). They reported that the constitutive absence of AGT in lymphoblasts normal in NER significantly increases the cytotoxic and mutagenic effects of ENU relative to cells normal in NER and AGT. Yang et al. also report a similar finding in fibroblasts (15). In our NER proficient cells, depletion of AGT did not significantly increase the ENU-induced cytotoxicity or mutation frequency.

One explanation for the differences between our observations and theirs could be that O^6-benzylguanine did not adequately deplete the SL68 cells of AGT. Dolan et al. (27) have shown, however, that treatment of cells with as low as 2.5 μM O^6-BzG results in a rapid decrease in AGT activity. It was shown previously in our laboratory that a 2 hour pre-treatment of SL68 cells with 25 μM O^6-BzG titrates out the AGT activity in these cells to less than 2% of the original activity, and an additional 24 hours of O^6-BzG treatment leaves the levels of AGT at <1% of the control for up to 28 hours after removal of inhibition (28). We incubated ENU-

treated cells in the presence of O^6-BzG for 48 hours, allowing a total of 96 hours with no appreciable AGT activity following ENU treatment. Therefore, even though cells can rapidly regenerate AGT (37), it is unlikely that regeneration or inadequate depletion of AGT can explain the similar toxic and mutagenic effect of ENU observed in the NER^+/AGT^+ and NER^+/AGT^- cells. Another possible explanation for the difference between our observations and those of Bronstein et al. and Yang et al. of the effect of AGT on ENU toxicity and mutagenicity is that the genetic change resulting in a constitutive lack of AGT activity also affects other pathways of DNA repair. Karran et al. (38), for example, demonstrated that the loss of AGT activity in human lymphoblastoid or fibroblast cell lines could be accompanied by simultaneous loss of expression of thymidine kinase and galactokinase, possibly due to loss of some common regulatory mechanism. GM0011, the human fibroblast cell strain used by Yang et al. (15), is constitutively devoid of AGT, and has also been shown in our laboratory to be somewhat lacking in NER capacity compared to the NER proficient SL68 cells used in our present study (29). It may be that the small difference in NER capability between SL68 and GM0011, as measured by survival of cloning ability following UV treatment, becomes a larger disadvantage in the presence of alkylation damage.

Our results show that NER^-/AGT^+ cells were able to repair the cytotoxic and mutagenic lesions caused by ENU, indicating that cooperation between NER and AGT was not absolutely required in the repair of these lesions. It could be argued that the SL68 cells and XP12BE cells differ in more than just NER capacity. The SL68 and XP12BE cells, however, showed nearly the same cytotoxic and mutagenic response to MNNG (28), indicating that these cells respond in a similar manner to alkylation when

NER is nonessential for repair of this damage. Although one could also argue that there is some residual NER activity in our NER^-/AGT^+ cells, this capacity for repair would be shared by the NER^-/AGT^- cells. Therefore, the most likely explanation for the decrease in mutant frequency and increase in survival in the NER^-/AGT^+ cells compared to the NER^-/AGT^- cells is that AGT is repairing ENU-induced lesions independent of NER activity.

The human AGT, like the bacterial AGT, repairs O^6-EtG by transferring the alkyl group irreversibly to a cysteine in its own amino acid sequence (reviewed in 23). The bacterial AGT is able to repair O^4-alkylT lesions (19). Recent studies have indicated that human AGT can also repair O^4-alkylT *in vitro*, but much less efficiently than O^6-alkylG (39,40). The sequencing data in our present study (Table V) indicate that there is a decrease in the frequency of T·A → C·G mutations in the NER^-/AGT^+ cells relative to the frequency in the NER^-/AGT^- cells, suggesting repair of the O^4-EtT lesions by AGT. Again, these results in fibroblasts conflict with those reported by Skopek and colleagues, who observed that lymphoblasts deficient in NER but proficient in AGT are not able to repair O^6-EtG or O^4-EtT lesions.

In bacteria, UvrABC can repair O^6-EtG and O^4-EtT (19). Skopek and colleagues reported recently that in human lymphoblasts, NER is not able to efficiently repair O^6-EtG lesions in genomic DNA in the absence of AGT (17,25). Our results indicate, however, that in human fibroblasts NER can effectively repair O^6-EtG lesions in the *HPRT* gene in the absence of AGT. Skopek and colleagues reported that lymphoblasts were unable to repair O^4-EtT lesions regardless of NER or AGT (25), and that the difference in spectra between NER^+/AGT^+ lymphoblasts and lymphoblasts deficient in NER or AGT was primarily due to G·C → A·T transitions (17). However, we show

that NER, in the absence of AGT activity, significantly reduced the frequency of the T·A → C·G transitions likely resulting from O^4-EtT lesions, and also that NER reduced the T·A → G·C transversions caused by an unknown lesion compared to the frequency of these mutations in the NER$^-$/AGT$^-$ cells.

In vitro studies have indicated that DNA polymerases can incorporate deoxythymidine opposite O^2-EtT, resulting in T·A → A·T transversions (18). This transversion mutation is believed to account for mutational activation of the *neu* proto-oncogene in ENU-treated rats (3), and to be the causative mutation in the mouse α- and β-globin genes in the progeny of the ENU-treated mouse (4,41). In ENU-treated rats, the majority of the mutations observed in pouch skin fibroblasts were T·A → A·T transversions, with G·C → A·T, T·A → C·G, and T·A → G·C substitution mutations occurring at low frequency (42). This is the expected result if repair of O^2-EtT is slower than that of the other potentially mutagenic lesions induced by ENU. There appears to be some removal of these lesions, as measured by decay of O^2-EtT adducts over time, in human lymphoblasts (25). Human fibroblasts in our study did not demonstrate reduction in frequency of T·A → A·T transversions regardless of NER or AGT repair capacity compared to the frequency in the NER$^-$/AGT$^-$ cells, indicating that NER and AGT were not involved in the repair of the O^2-EtT lesions. If this is a very persistent lesion, it may be that fibroblasts in the rat skin "pouch" (42) had time to repair the other ENU-induced lesions before replication, leaving the T·A → A·T transversion as the most frequently observed mutation.

It has been demonstrated that in eukaryotes, components of the NER machinery are coupled to the transcription machinery (reviewed in 43), and NER has been shown to work more efficiently on UV-induced damage in the

transcribed strand vs. the non-transcribed strand (44-46). In both prokaryotic and eukaryotic systems there have also been reports indicating preferential repair of the transcribed strand by AGT (42,47,48). Our results suggest that NER preferentially removed ENU-induced damage in the transcribed strand of the *HPRT* gene of diploid human fibroblasts. AGT appeared to concentrate on removal of the O^6-EtG lesions in the nontranscribed strand, most likely because that was the strand initially containing the most O^6-EtG. This is in agreement with the results seen for MNNG-induced O^6-MeG premutagenic lesions by Lukash et al. (28) showing that AGT exhibits no bias for repair of lesions at G's in the transcribed strand.

Interference, rather than cooperation, between NER and AGT has been observed in bacteria (49,50). If, in human cells, NER and AGT were working cooperatively on ethyl lesions at G's as suggested by Skopek and colleagues (17,25), then we would expect to see the frequency of mutations at G's in the NER^+/AGT^+ cells decrease relative to the frequencies observed in NER^-/AGT^+ or NER^+/AGT^- cells. Instead, we saw that the frequency remained nearly the same. We would also expect that in the cells competent in NER and AGT, the lesions remaining at G's would be found predominately on the nontranscribed strand, since NER would be working diligently on the transcribed strand and AGT would be working randomly on either strand. The strand distribution we saw, however, did not fit this pattern, but showed an unexpected inversion in strand distribution of the lesions remaining at G's in the NER^+/AGT^+ cells compared to the NER^+/AGT^- cells (see Figure 3). We speculate that NER and AGT, when both present in the cells, compete for the repair of premutagenic lesions at G's.

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