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# DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea

(sequence specificity/$O^6$-alkylguanine/$O^4$-alkylthymidine)

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ABSTRACT **Dideoxy chain-termination DNA sequencing was used to determine the specific DNA base changes induced after *in vivo* exposure of *Escherichia coli* to *N*-methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea (ENU) using the xanthine guanine phosphoribosyltransferase (*gpt*) gene as the genetic target. The resultant mutation spectra were compared with the levels of $O^6$-alkylguanine and $O^4$-alkylthymidine in genomic DNA immediately after exposure. All (39/39) of the MNU-induced mutations were G·C→A·T transitions. In contrast, 24/33 point mutations isolated following ENU treatment were G·C→A·T transitions, 7/33 were A·T→G·C transitions, 1/33 was a G·C→C·G transversion, and 1/33 was an A·T→C·G transversion. Three large insertions, probably of spontaneous origin, were also isolated. $O^4$-alkylthymidine/$O^6$-alkylguanine ratios were 0.014 for MNU and 0.28 for ENU. These data suggest that the difference in the mutation spectrum of MNU versus ENU may be attributed, in part, to the different ratio of $O^6$-alkylguanine versus $O^4$-alkylthymidine produced in the DNA. Of the G·C→A·T transitions, 82% of the MNU- and 71% of the ENU-induced mutations occurred at the middle guanine of the sequence 5′-GG(A or T)-3′.**

Alkylating agents are thought to induce mutations through the formation of DNA adducts that result in misincorporation during replication. Numerous studies using various approaches have been done to elucidate the specific adducts that result in mutations. *In vitro* misincorporation studies (1–6), *in vivo* site-directed mutagenesis studies (7–9), and *in vivo* studies comparing specific adducts and mutation frequency (10–13) have all been reported. These studies have suggested that of the adducts examined, $O^4$-alkylthymidine ($O^4$-alkyldT) and $O^6$-alkylguanine ($O^6$-alkylG) are the principal promutagenic lesions.

An *in vivo* study comparing the concentrations of particular DNA adducts formed with the frequency of specific DNA base changes is reported in this paper. These comparisons are made utilizing a forward mutation system employing a small genetic target and dideoxy chain-termination DNA sequencing methods (14) to rapidly determine the specific DNA base changes associated with exposure to two mutagens. Specifically, *Escherichia coli* carrying the xanthine guanine phosphoribosyltransferase gene (as the genetic target) on the plasmid pSV2gpt (15–17) were exposed *in vivo* to the simple alkylating agent *N*-methyl-*N*-nitrosourea (MNU) or *N*-ethyl-*N*-nitrosourea (ENU). The type and frequency of specific DNA base changes in mutants were compared to the concentrations of $O^6$-alkylG and $O^4$-alkyldT in the genomic DNA.

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## MATERIALS AND METHODS

**Bacterial Strains.** The following K-12 *E. coli* strains were used: (*i*) GP120 [Δ(*gpt pro lac*) *PurE* suII $r^+m^+$] was a gift from J. S. Gots, University of Pennsylvania, Philadelphia). (*ii*) GP120A [Δ(*gpt pro lac*) $PurE^+$ $su^-$ $r^+m^+$]: a valine-resistant ($Val^r$) derivative of GP120 and a rifampicin-resistant ($Rif^r$) derivative of HfrH ($PurE^+$ $su^-$; ATCC e23739) were isolated and mated for 2 hr. ($Val^r$)($Rif^r$) colonies were isolated and tested for the ability to grow without added purines ($PurE^+$), the inability to grow without proline [Δ(*gpt pro lac*)], and the inability to support growth of *N*am λ phage ($su^-$). One was selected (GP120A) and used in this study. (*iii*) 7-2 [Δ(*gpt pro lac*) *PurE* $su^-$ $r^-m^-$]. ($Val^r$)($Rif^r$) colonies from the above mating were tested for their inability to grow with xanthine as a purine source [Δ(*gpt pro lac*) *PurE*] and the inability to support growth of *N*am λ phage ($su^-$). This isolate was then treated with 3 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine per ml overnight followed by transformation with unmodified pBR322 and selection in ampicillin ($amp^s$). A colony possessing the $r^-m^-$ phenotype was identified (strain 7-2) and cured of pBR322 using acridine orange (18). (*iv*) The 33694 cell line was obtained from The American Type Culture Collection (Rockville, MD).

**Plasmids.** The pSV2gpt plasmid (15–17) was the gift of R. Mulligan (Massachusetts Institute of Technology, Cambridge, MA). The tetracycline-resistant ($tet^r$) *gpt* deletion plasmids used for mapping (H20A, H30A, H40A) were constructed as follows: the *Eco*RI–*Eco*RI, *gpt*-containing fragment of deletion plasmids H20, H30, and H40 (19) was isolated and cloned into the *Eco*RI site of pBR322. These new plasmids were digested with *Pst* I, and the large *Pst* I fragment was ligated to make the new deletion plasmids $tet^r$ $amp^s$.

**Chemicals.** Chemicals were obtained as follows: bacto-tryptone, bacto-peptone, Casamino acids, bacto-dextrose, brain heart infusion (BHI), and bacto-agar from Difco (American Scientific Products, MacGaw Park, IL); agarose, lysozyme, ammonium persulfate, thiamine, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, *N*-methyl-*N*-nitrosourea, *N*-ethyl-*N*-nitrosourea, and proteinase K from Sigma; M13 sequencing system, restriction endonucleases, cesium chloride, urea, T4 DNA ligase, NACS·52 Prepac columns, and RNase T1 from Bethesda Research Laboratories; acrylamide and bisacrylamide from Bio-Rad.

**Media and Buffers.** Solutions were constituted as follows: BHI (37 g/liter); minimal essential salts: 0.02 M $MgSO_4 \cdot 7H_2O$/0.24 M citric acid·$H_2O$/1.44 M $K_2HPO_4$/0.42 M $NaNH_4HPO_4 \cdot 4H_2O$; minimal medium: 1× minimal essential

Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; ENU, *N*-ethyl-*N*-nitrosourea; $O^6$-alkylG, $O^6$-alkylguanine; $O^4$-alkyldT, $O^4$-alkylthymidine; BHI, brain heart infusion; 7-EtG, 7-ethylguanine; 7-MeG, 7-methylguanine; TE, Tris/EDTA buffer; 7-alkylG, 7-alkylguanine; $O^4$-MedT, $O^4$-EtdT, $O^4$-methyl-, $O^4$-ethylthymidine; $O^6$-MeG, $O^6$-EtG, $O^6$-methyl-, $O^6$-ethylguanine.

salts/2% (wt/vol) bacto-dextrose/1% (wt/vol) Casamino acids/0.1 mg of thiamine per liter; λ plates, 1 g of NaCl per liter, 7.5 g of bacto-tryptone per liter, 8 g of bacto-peptone per liter, 15 g of bacto-agar per liter; minimal plates, minimal media with 15 g of agarose per liter; phosphate-buffered saline, 137 mM NaCl/2.7 mM KCl/8.1 mM $Na_2HPO_4$/1.5 mM $KH_2PO_4$; Tris/EDTA (TE), 10 mM Tris/1 mM EDTA, pH 8.0; Tris borate/EDTA buffer (TBE), 89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.0.

**Mutagenesis.** One liter of pGP120 carrying the SV2gpt plasmid was grown to a cell density of $5 \times 10^8$ cells per ml in minimal medium (pH 7.0) containing 25 μg of xanthine per ml to select against spontaneous gpt mutants in the population and 20 μg of ampicillin per ml to ensure retention of the plasmid. Immediately before treatment, hypoxanthine was added to a final concentration of 25 μg/ml to allow growth of induced $gpt^-$ mutants. The culture was then treated with 7 mM MNU or 35 mM ENU (stock solution in sodium citrate buffer—150 mM NaCl/10.8 mM $NaH_2PO_4$/9 mM citric acid at pH 4.1). Prior to ENU exposure, the cells had to be pelleted and resuspended in an appropriate volume of minimal medium at pH 7.2 to give a final volume of 1.0 liter after addition of the ENU stock.

Cells were exposed to the mutagen for 5 min at room temperature with continuous shaking in a chemical hood. The cells were pelleted by centrifugation in a Sorvall centrifuge (model RC-5B) at 5900 × *g* for 10 min, resuspended in fresh mutagen-free minimal medium containing 25 μg of hypoxanthine per ml and allowed to grow for a 3-hr expression time. The culture was then pelleted, and the plasmid DNA isolated as described below. Toxicity during the exposure and the number of cell divisions occurring during the expression period were determined by sampling aliquots of cells before and after the 5-min exposure and after the 3-hr expression time and plating on ampicillin plates. Untreated (control) cultures were carried through similar protocols to determine the spontaneous mutation frequency and to obtain spontaneous mutants for sequence analysis.

**$Gpt^-$ Mutant Isolation.** To isolate $gpt^-$ mutant plasmids using 6-thioguanine selection, treated plasmid DNA was transformed into GP120A for the following reasons: (*i*) a $PurE^+$ host is required, because exogenous purines compete with 6-thioguanine and (*ii*) treatment of GP120 with mutagens produces a high level of chromosomal mutations conferring 6-thioguanine resistance.

Plasmid DNA was isolated from control and exposed GP120 (20, 21) followed by a cesium chloride/ethidium bromide density-gradient centrifugation (22). The ethidium bromide was removed; the plasmid DNA was precipitated and stored at −20°C. One microgram of the control or treated plasmid DNA was used to transform GP120A by a modification of the method developed by Mandel and Higa (23). Following transformation, cells were grown in minimal medium for 2–4 hours, and $gpt^-$ mutants were then selected on minimal agarose plates containing 8 μg of 6-thioguanine and 20 μg of ampicillin per ml. To allow calculation of the mutation frequency, the total number of transformants was determined by plating these cultures on ampicillin plates. One mutant colony from each transformation was chosen for subsequent mapping and DNA sequencing to ensure sampling of independent events.

**Deletion Mapping of gpt Mutants.** Mutant plasmid DNA from 10 ml of GP120A was isolated using a modified Birnboim and Doly procedure (24). Competent strain 7-2 and strain 7-2 carrying gpt deletion plasmids H20A, H30A, or H40A [stored at −70°C (25)] were thawed on ice and then transformed with the mutant plasmid DNA. BHI was added to each culture, and the cells were incubated on a roller wheel (Bellco Glass) at 37°C for 1–2 hr. Cultures of strain 7-2 were plated on λ–ampicillin (20 μg/ml) plates and cultures of strain 7-2 carrying the deletion plasmids were plated on λ–ampicillin (20 μg/ml)–tetracycline (10 μg/ml) plates.

Two colonies were picked from each plate (one plate per transformation) and grown overnight at room temperature in 1.0 ml of BHI. The following day 5.0 ml of BHI was added to each culture, and the cultures were incubated at 37°C on a roller wheel for 4 hr. After the 4-hr incubation, 0.5 ml of the culture was transferred to a sterile microfuge tube, pelleted, washed twice with 0.5 ml of phosphate-buffered saline and resuspended in 0.5 ml of phosphate-buffered saline. A 100-μl sample of these cells was plated on minimal plates containing 25 μg of xanthine per ml, allowed to grow for 2 days at 37°C, and then scored for the presence of $gpt^+$ recombinants (0 to several hundred recombinants per plate were observed). The 7-2 cultures were used as controls to test for revertants, or the presence of "leaky" mutants. Leaky mutants were defined as mutants capable of growth in the presence of xanthine and identified by the yellow-orange lawn of cells on all plates. Mapping information was used in choosing the appropriate synthetic primer for DNA sequencing (Fig. 1).

**DNA Sequencing.** Plasmid DNA used in dideoxy chain-termination DNA sequencing was prepared from strain 33694 due to recurrent nuclease problems associated with GP120 derivatives. Briefly, plasmid was amplified overnight with chloramphenicol, isolated, and put through two rounds of cesium chloride/ethidium bromide density-gradient centrifugation. After removal of ethidium bromide, the plasmid DNA was ethanol-precipitated twice. The DNA was treated with proteinase K (0.37 unit) for 2 hr, phenol/ether extracted, and ethanol-precipitated. It was then treated with RNase T1 (10 units) for 30 min, phenol/ether extracted, and ethanol-precipitated. The DNA was applied to a Bethesda Research Laboratories NACS column equilibrated in 0.5 M NaCl in TE buffer and eluted with 2.0 M NaCl in TE buffer. The eluted plasmid DNA was then ethanol-precipitated, washed with 70% ethanol, dried, and stored at −20°C.

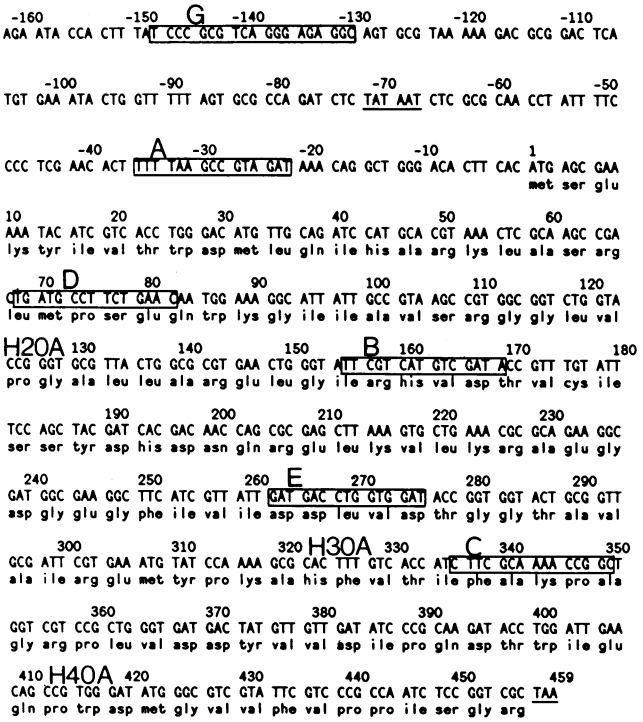


FIG. 1. Nucleotide and amino acid sequences of the *E. coli gpt* gene depicting the positions of the deletion plasmids (H20A, H30A, and H40A) and the locations and sequences of the five synthetic primers used for dideoxy DNA chain-termination sequencing (A–E and G above the boxed sequence). The Pribnow box (−72 to −67) and the TAA stop codon (457–459) are underlined.

Table 1. Spontaneous, MNU-induced, and ENU-induced mutation frequencies

| Treatment | Divisions, no. | Survival, % | Mutation frequency, no. × $10^{-5}$ |
|---|---|---|---|
| None | ≈3.5 | 100 | 0.55 (spontaneous) |
| MNU 7 mM | ≈2.5 | 56 | 8.7 ± 4.7 (average of 4) |
| ENU 35 mM | ≈2.5 | 50 | 4.7 ± 3.6 (average of 4) |

Survival of *E. coli* GP120 following the 5-min exposure to MNU or ENU and the subsequent number of cell divisions occurring during the 3-hr expression time. The table also lists the mutant frequency of plasmid isolated from GP120 and transformed into GP120A.

Synthetic DNA primers spanning the *gpt* gene were made by Applied Biosystems (Foster City, CA) and used to sequence control and gpt$^-$ mutants according to the method of Sanger *et al.* (14) as described in the M13 sequencing system manual (Bethesda Research Laboratories), with modifications to allow sequencing of double-stranded plasmid.

One-half microliter of plasmid DNA (0.2–1.0 μg of DNA per μl in TE buffer), 0.5 μl of 10× polymerase reaction buffer (Bethesda Research Laboratories), and 2.5 μl of the synthetic primer (18 ng) were mixed and then drawn into a 5-μl capillary pipette. The ends of the pipette were heat-sealed. The entire pipette was then immersed in a 95°C water bath for 3 min and subsequently buried in ice for 15 min. The hybridized template and primer were added to 6.5 μl of 1× polymerase reaction buffer, 1 μl of 0.1 M dithiothreitol, and 1 μl of Klenow fragment (1 unit/μl) in a 1.5-ml Microfuge tube. The contents were centrifuged briefly, and 1.0 μl of [$^{32}$P]dATP (400 Ci/mmol, 10 mCi/ml; 1 Ci = 37 GBq; Amersham) was added. Three microliters of this solution were then pipetted on one side of each of the four reaction tubes (G, A, T, C), and 2 μl of the combined diluted deoxy- and dideoxynucleotide mixes were pipetted on the other side of the tubes (the recommended concentration of dideoxyATP in the mixes was changed from 0.5 mM to 0.128 mM). The contents were briefly centrifuged to start all reactions simultaneously. The initial sequencing and the chain elongation reaction conditions were as described (M13 sequencing system manual). However, the A reaction was stopped by adding 5 (instead of 10) μl of the formamide/dye solution.

One microliter from each sequencing reaction was loaded onto an 8% acrylamide/urea/TBE sequencing gel. The gels were run at 40 mA and autoradiographed for 1–3 days at −70°C using Kodak XAR-5 x-ray film and Kodak Lightning Plus intensifying screens (Eastman Kodak).

**Alkylation.** One liter of GP120 at a density of 5 × $10^8$ cells per ml was exposed to 7 mM MNU or 35 mM ENU for 5 min (procedure as described in the mutagenesis section). Following exposure, cells were pelleted and frozen in liquid nitrogen. The frozen pellet was kept at −70°C until the DNA was isolated.

DNA was isolated by chloroform/isoamyl alcohol/phenol extraction and purified by hydroxylapatite chromatography (26). The amounts of 7-alkylguanine (7-alkylG) and $O^6$-alkylG

Table 2. DNA alterations in the *gpt* gene observed for spontaneous, 7 mM MNU-induced, and 35 mM ENU-induced mutants

| Site | Leaky | Sequence 5′-3′ | Change | | Number | | |
|---|---|---|---|---|---|---|---|
| | | | Base pair | Amino acid | MNU | ENU | Spontaneous |
| | | | Transitions | | | | |
| −93 | − | ATACTG G TTTTTA | GC→AT | Promotor | 1 | 0 | 0 |
| −67 | − | CTATAA T CTCGCG | AT→GC | Pribnow | 0 | 2 | 0 |
| 3 | − | TCACAT G AGCGAA | GC→AT | Met→Ile | 0 | 1 | 1 |
| 27 | − | CACCTG G GACATG | GC→AT | Trp→**TGA** | 3 | 2 | 0 |
| 37 | − | ATGTTG C AGATCC | GC→AT | Glu→**TAG** | 2 | 0 | 0 |
| 82 | − | TCCATT G TTCAGA | GC→AT | Gln→**TAA** | 0 | 1 | 0 |
| 87 | − | ACAATG G AAAGGC | GC→AT | Trp→**TGA** | 1 | 0 | 0 |
| 109 | − | GTAAGC C GTGGCG | GC→AT | Arg→His | 1 | 0 | 0 |
| 110 | − | TAAGCC G TGGCGG | GC→AT | Arg→His | 0 | 1 | 0 |
| 116 | − | GTGGCG G TCTGGT | GC→AT | Gly→Asp | 6 | 2 | 0 |
| 128 | − | TACCGG G TGCGTT | GC→AT | Gly→Asp | 4 | 0 | 0 |
| 176 | − | CCGTTT G TATTTC | GC→AT | Cys→Tyr | 0 | 2 | 0 |
| 181 | − | TGTATT T CCAGCT | AT→GC | Ser→Pro | 0 | 1 | 0 |
| 262 | − | GTTATT G ATGACC | GC→AT | Asp→Asn | 1 | 0 | 0 |
| 274 | + | CTGGTG G ATACCG | GC→AT | Asp→Asn | 1 | 1 | 0 |
| 281 | − | ATACCG G TGGTAC | GC→AT | Gly→Asp | 1 | 0 | 0 |
| 402 | +/− | TACCTG G ATTGAA | GC→AT | Trp→**TGA** | 8 | 5 | 0 |
| 418 | + | CCGTGG G ATATGG | GC→AT | Asp→Asn | 10 | 9 | 0 |
| 419 | + | CGTGGG A TATGGG | AT→GC | Asp→Gly | 0 | 4 | 0 |
| | | | Transversions | | | | |
| −67 | + | CTATAA T CTCGCG | AT→CG | Pribnow | 0 | 1 | 0 |
| 110 | − | TAAGCC G TGGCGG | GC→CG | Arg→Pro | 0 | 1 | 0 |
| 406 | − | TGGATT G AACAGC | GC→TA | Glu→**TAA** | 0 | 0 | 1 |
| | | | Insertions | | | | |
| | +/− | | | | 0 | 3 | 7 |
| | | | Deletions | | | | |
| | − | | | | 0 | 0 | 8 |

The table shows the nucleotide positions of mutants (Fig. 1), the mapping data as to whether or not the mutation was leaky, the altered base with the surrounding 5′ and 3′ nucleotides, the base change, the amino acid change or nonsense codon (boldface), and the number of times the mutation occurs in spontaneous, ENU-induced or MNU-induced mutants. −, Nonleaky mutant; +, leaky mutant; +/−, some mutants were leaky, whereas others were not. The mutation spectrum was determined for 17 spontaneous, 36 ENU-induced, and 39 MNU-induced mutants.

in the DNA were quantitated using HPLC separation coupled to fluorescence detection as previously described (27). $O^4$-alkyldT concentrations were determined using sensitive $O^4$-methylthymidine ($O^4$-MedT) and $O^4$-ethylthymidine ($O^4$-EtdT) polyclonal antibodies in a competitive radioimmunoassay (28). Since the thymine/guanine ratio in *E. coli* DNA is 1:1 (29), results were expressed as pmol of $O^6$ per μmol of guanine, pmol of $N^7$ per μmol of guanine, and pmol of $O^4$ per μmol of thymidine.

## RESULTS

Treatments with 35 mM ENU or 7 mM MNU produced 5- and 10-fold increases, respectively, in mutation frequency and resulted in about a 50% survival (Table 1). Assuming 10 copies of plasmid per cell, these treatments produced $4.75 \times 10^7$ ENU-induced mutants and $9.75 \times 10^7$ MNU-induced mutants in the original treated cultures. These large numbers ensured that the mutants selected for sequence analysis arose independently. MNU- and ENU-exposed cells divided about 2.5 times (versus 3.5 times for the control) during the 3-hr expression time (Table 1).

Sequencing results of the spontaneous, 7 mM MNU-induced, and 35 mM ENU-induced mutations are presented in Table 2. The mutation spectrum resulting from MNU exposure showed four or more mutations occurring at positions 116, 128, 402, and 418. The mutation at position 402 resulted in a nonsense mutation, whereas mutations at the other positions resulted in missense mutations. A leaky mutation was observed at position 418. All 39 MNU-induced mutations were G·C→A·T transitions.

ENU induced a different mutation spectrum with 4 or more mutations occurring at positions 402, 418, and 419. The mutation at 402 resulted in a nonsense codon, whereas those at positions 418 and 419 were leaky missense mutations. One other leaky site at position 274 was observed, but only one mutant occurred at this site. Following ENU exposure, 24/36 mutations observed were G·C→A·T transitions and 7/36 were A·T→G·C transitions (Table 2). One of the A·T→G·C transitions occurred at position 181, 2 occurred in the Pribnow box at position −67, and the other 4 occurred at position 419. Positions 181 and 419 gave rise to missense mutations. The rest of the ENU mutation spectrum consisted of 1 G·C→C·G transversion at position 110, 1 A·T→C·G transversion at position −67 (Pribnow box), and 3 mutants containing large insertions (Table 2). Given the 5-fold increase over the spontaneous mutation frequency (Table 1) and the nature of spontaneous mutants (see below), there is a high probability that these insertion mutations were spontaneous in origin.

Preliminary sequencing of several spontaneous mutants indicated that there were large insertions or deletions (several hundred nucleotides) in the gene. Further work involving restriction enzyme analysis (data not shown) showed that of the 17 spontaneous mutants isolated, 8 were deletions, 7 were insertions and 2 were point mutations (Table 2). One of the point mutations was a G·C→A·T transition at position 3, and the other was a G·C→T·A transversion at position 406. The transition at position 3 altered the start codon, and the transversion at position 406 resulted in a nonsense mutation. A partial sequence of one of the insertions was submitted to IntelliGenetics (Mountain View, CA). No homology to any sequences on file with IntelliGenetics was seen.

32/39 MNU and 17/24 ENU G·C→A·T transition mutations occurred at the middle guanine of the sequence 5′-GG(A or T)-3′ (Table 2). A computer search of the *gpt* sequence was performed to determine the total number of 5′-NGN-3′ sequences in the *gpt* gene (sense and antisense strands) that could give rise to amino acid changes or stop codons upon mutation of that guanine. Of the 171 sites found, only 16% (27 sites) are the sequence 5′-GG(A or T)-3′ (Table 3). In this experiment, MNU induced mutations at 10 of the 171 sites. Seven of these 10 sites had the sequence 5′-GG(A or T)-3′ and comprised 82% of all the G·C→A·T transitions. Similarly, ENU induced mutations at 9 of the 171 sites. Four of these 9 sites had the sequence 5′-GG(A or T)-3′ and comprised 71% of all the G·C→A·T transitions (Table 3). A strand preference for altered guanines was also observed (Table 3).

Table 3. Mutations occurring at 5′-NGN-3′ sequences

| Seq | Sites in *gpt* Anti (A) ; sense (S) | 7 mM MNU, mut/sites A ; S | 35 mM ENU, mut/sites A ; S | Spontaneous, mut/sites A ; S |
|---|---|---|---|---|
| GGC | 8 ; 4 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| GGG | 5 ; 2 | 3/1 ; 0/0 | 2/1 ; 0/0 | 0/0 ; 0/0 |
| AGT | 1 ; 5 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| AGA | 2 ; 2 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| TGA | 2 ; 10 | 1/1 ; 0/0 | 0/0 ; 1/1 | 0/0 ; 2/2 |
| TGT | 7 ; 2 | 0/0 ; 0/0 | 2/1 ; 1/1 | 0/0 ; 0/0 |
| CGA | 8 ; 4 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| CGT | 14 ; 2 | 0/0 ; 0/0 | 2/1 ; 0/0 | 0/0 ; 0/0 |
| GGA | 5 ; 4 | 20/4 ; 0/0 | 15/3 ; 0/0 | 0/0 ; 0/0 |
| GGT | 11 ; 7 | 11/3 ; 0/0 | 2/1 ; 0/0 | 0/0 ; 0/0 |
| CGC | 6 ; 7 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| AGG | 3 ; 2 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| AGC | 6 ; 4 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| TGG | 8 ; 5 | 0/0 ; 0/0 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| TGC | 6 ; 7 | 0/0 ; 2/1 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| CGG | 4 ; 8 | 0/0 ; 1/1 | 0/0 ; 0/0 | 0/0 ; 0/0 |
| Total | 171 | 38/11 | 24/9 | 1/1 |

This table gives the number of 5′-NGN-3′ sites (antisense and sense strands) in the *gpt* gene that could result in amino acid changes or nonsense mutations due to mutation of the guanine. The number of times these triplets occur in the *gpt* gene is given in column 2. Columns 3–5 indicate the number of times a mutation occurs at a particular triplet and how many of the sites from column 2 are represented by these mutations. One spontaneous mutation had a G·C→T·A transversion at the sequence 5′-TCA-3′, and 1 ENU-induced mutation had a G·C→C·G transversion at the sequence 5′-CGT-3′. Seq, sequence 5′–3′.

The concentrations of $O^6$-alkylG, 7-alkylG, and $O^4$-alkyldT after the 5-min exposure to MNU and ENU are presented in Table 4. The amount of $O^6$-methylguanine ($O^6$-MeG) was 147 pmol/μmol of guanine and the ratio of $O^6$-MeG/7-methylguanine (7-MeG) was 0.12. The amount of $O^6$-ethylguanine ($O^6$-EtG) was 41.5 pmol/μmol of guanine [amounts of 7-ethylguanine (7-EtG) were too low to be quantitated]. The ratio of $O^4$-MedT/$O^6$-MeG was 0.014, whereas the ratio of $O^4$-EtdT/$O^6$-EtG was 0.28 (Table 4). These results are consistent with those reported following *in vivo* and *in vitro* exposures (1).

## DISCUSSION

It is evident from this work that MNU and ENU give rise to different mutational spectra. Specifically, MNU induced all (100%) G·C→A·T transitions, whereas ENU induced 73% G·C→A·T transitions, 21% A·T→G·C transitions, and 6% transversions. In contrast, spontaneous mutants were 41% insertions, 47% deletions, 6% G·C→A·T transitions, and 6% G·C→T·A transversions. The predominance of point mutations observed after MNU and ENU treatments is consistent

Table 4. Concentrations of MNU- and ENU-induced adducts in *E. coli* genomic DNA

| Mutagen | 7-AlkylG, pmol/μmol | $O^6$-alkylG, pmol/μmol | $O^4$-alkyldT, pmol/μmol | $O^4/O^6$ |
|---|---|---|---|---|
| MNU (7 mM) | 1203 | 147 | 2.1 | 0.014 |
| ENU (35 mM) | ND | 41.5 | 11.7 | 0.28 |

Exposure to MNU and ENU was 5 min. The $O^4/O^6$ ratios were determined from the above concentrations. ND, not determined.

with simple base pair mismatches induced by specific DNA adducts known to be generated by these alkylating agents (1). Two of these adducts, $O^6$-alkylG and $O^4$-alkyldT are considered to be major promutagenic lesions and have been shown to cause mispairing during DNA replication *in vitro* (2–5). In support of this theory, the different ratios of 0.28 for $O^6$-EtG/$O^4$-EtdT and 0.014 for $O^6$-MeG/$O^4$-MedT (Table 4) are consistent with the detection of A·T→G·C transitions after ENU exposure (7/33 mutations), but not after MNU exposure (0/39 mutations). These results support the idea that the relative ratios of promutagenic lesions in the DNA are important in determining the actual base pair changes induced by compounds (30). Other mechanisms may also affect this mutation spectrum. It has been shown that free alkylated deoxynucleotides can be incorporated into DNA during replication (5, 31). It has also been shown that some of the mutations induced by ENU, but not by MNU, mutagenesis result from SOS processes (32–34). It is interesting to note that SOS-induced mutations are primarily transversions (35) and that transversions were observed in the ENU-induced, but not the MNU-induced, spectrum.

Although less MNU (by a factor of 5) was used, total $O^6$-alkylG plus $O^4$-alkyldT alkylation due to MNU was 2.8-fold higher than that due to ENU (Table 4). This suggests a 14-fold higher reactivity of MNU with DNA at these sites, which is in agreement with previous results (1). However, these concentrations of $O^6$-alkylG plus $O^4$-alkyldT resulted in comparable mutation frequencies (Table 1). This suggests that the ethylated adducts are more efficient promutagenic lesions. The increase in efficiency may be due to enhanced mispairing by ethylated adducts and/or less efficient repair (36).

Mutagenesis by both MNU and ENU exhibits sequence specificity with 82% of the MNU- and 71% of the ENU-induced G·C→A·T mutations occurring at the middle guanine of the sequence 5′-GG(A or T)-3′ (Table 3). Many examples of mutation sequence specificity with other compounds have been reported (37–40).

The sequence specificity observed with MNU and ENU could be due to preferential alkylation of bases, local repair deficiencies, and/or alterations in mispairing capabilities due to the local DNA environment. Briscoe and Cotter (41, 42) using synthetic polynucleotides have shown that the base sequence is important in determining the percent of alkylation at specific base sites. Boiteux and Laval (43) have shown that repair capabilities of $O^6$-methyltransferase are reduced when DNA is in the Z-conformation. In addition, Skopek and Hutchinson (37) have shown that 5-bromouracil results in different frequencies of mutations depending upon the site of incorporation.

The system described in this paper allows rapid scoring and sequencing of forward mutations in the *E. coli gpt* gene. The positive selection for mapping allows the detection of any type of mutation that results in partial or total inactivation of *gpt*. Thus, this system was able to detect insertions, deletions, missense mutations (including leaky mutations), and nonsense mutations [including the TGA (opal) codon (Table 2)]. Leaky mutations and the TGA (opal) codon are not currently detected in other systems (37, 44, 45). In this study two hotspots (sites 418 and 419) were leaky mutations. Five of the ten sites that could result in nonsense mutations due to transitions were observed with three of these five resulting in the TGA (opal) codon.

A further advantage of this system is the ability of the *gpt* gene on plasmid pSV2gpt to function in mammalian cells (15–17). Thus, prokaryotic and eukaryotic mutation spectra can be compared. Recently, Ashman *et al.* (46) have isolated and sequenced three gpt mutants recovered from mammalian chromosomal DNA.

It is interesting to note that 100% of activated Ha-*ras*-1 oncogenes from MNU-induced rat mammary tumors had a G·C→A·T transition at the middle guanine of the sequence 5′-GGA-3′ (47). This is the same sequence specificity observed after MNU mutagenesis of the plasmid-carried *gpt* gene reported in this paper. This suggests that knowledge of the sequence specificity of chemical mutagens may be important in understanding their ability or inability to activate specific oncogenes.

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1. Singer, B. & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens* (Plenum, New York).
2. Singer, B., Spengler, S. J., Fraenkel-Conrat, H. & Kusmeirek, J. T. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 28–32.
3. Saffhill, R., Margison, G. P. & O'Conner, J. P. (1985) *Biochim. Biophys. Acta* **823**, 111–145.
4. Saffhill, R. (1985) *Chem.-Biol. Interact.* **53**, 121–130.
5. Saffhill, R. & Hall, J. A. (1985) *Chem.-Biol. Interact.* **56**, 363–370.
6. Singer, B., Sagi, J. & Kusmierek, J. T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4884–4888.
7. Flavell, R. A., Sabo, D. L., Bandle, E. F. & Weissmann, C. (1974) *J. Mol. Biol.* **89**, 255–272.
8. Shortle, D. & Nathans, D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2170–2174.
9. Loechler, E. L., Green, C. L. & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6271–6275.
10. Beranek, D. T., Heflich, R. H., Kodell, R. L., Morris, S. M. & Cusciano, D. A. (1983) *Mutat. Res.* **110**, 171–180.
11. Heflich, R. H., Beranek, D. T., Kodell, R. L. & Morris, S. M. (1982) *Mutat. Res.* **106**, 147–161.
12. Newbold, R. F., Warren, W., Medcalf, A. S. C. & Amos, J. (1980) *Nature (London)* **283**, 596–598.
13. Hu, Y. C. & Guttenplan, J. B. (1985) *Carcinogenesis* **6**, 1513–1516.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
15. Mulligan, R. C. & Berg, P. (1980) *Science* **209**, 1422–1427.
16. Mulligan, R. C. & Berg, P. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2072–2076.
17. Mulligan, R. C. & Berg, P. (1981) *Mol. Cell. Biol.* **1**, 449–459.
18. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 104–106.
19. Richardson, K. K., Fostel, J. & Skopek, T. R. (1983) *Nucleic Acids Res.* **11**, 8809–8816.
20. Clewell, D. B. & Helinski, D. R. (1970) *Biochemistry* **9**, 4428–4440.
21. Clewell, D. B. (1972) *J. Bacteriol.* **110**, 667–676.
22. Meselson, M., Stahl, F. W. & Vinograd, J. (1957) *Proc. Natl. Acad. Sci. USA* **43**, 581–588.
23. Mandel, M. & Higa, A. (1970) *J. Mol. Biol.* **153**, 159–162.
24. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
25. Morrison, D. A. (1979) *Methods Enzymol.* **68**, 327–328.
26. Beland, F. A., Dooley, K. L. & Casciano, D. A. (1979) *J. Chromatogr.* **174**, 177–186.
27. Bedell, M. A., Lewis, J. G., Billings, K. C. & Swenberg, J. A. (1982) *Cancer Res.* **42**, 3079–3083.
28. Richardson, F. C., Dyroff, M. C., Boucheron, J. A. & Swenberg, J. A. (1985) *Carcinogenesis* **6**, 625–629.
29. Lehninger, A. L. (1975) *Biochemistry* (Worth, New York).
30. Jensen, D. E. (1978) *Biochemistry* **17**, 5108–5133.
31. Singer, B., Chavez, F. & Spengler, S. J. (1986) *Biochemistry* **25**, 1201–1205.
32. Yoshikawa, K., Nakadate, M., Watabe, T., Ishidate, M. & Kondo, S. (1980) *Mutat. Res.* **79**, 319–325.
33. Garner, R. C., Pickering, C. & Martin, C. N. (1979) *Chem.-Biol. Interact.* **26**, 197–205.
34. Walker, G. C. (1984) *Microbiol. Rev.* **48**, 60–93.
35. Miller, J. H. & Brooks-Low, K. (1984) *Cell* **37**, 675–682.
36. Pegg, A. E., Scicchitano, D. & Dolan, M. E. (1984) *Cancer Res.* **44**, 3806–3811.
37. Skopek, T. R. & Hutchinson, F. (1982) *J. Mol. Biol.* **159**, 19–33.
38. Skopek, T. R., Wood, R. D. & Hutchinson, F. (1985) *Environ. Health Perspect.* **62**, 157–161.
39. Coulondre, C., Miller, J. H., Farabaugh, P. J. & Gilbert, W. (1978) *Nature (London)* **274**, 775–780.
40. Miller, J. H. (1983) *Annu. Rev. Genet.* **17**, 215–238.
41. Briscoe, W. T. & Cotter, L. (1984) *Chem.-Biol. Interact.* **52**, 103–110.
42. Briscoe, W. T. & Cotter, L. (1985) *Chem.-Biol. Interact.* **56**, 321–331.
43. Boiteux, S. & Laval, F. (1985) *Carcinogenesis* **6**, 805–807.
44. Coulondre, C. & Miller, J. (1977) *J. Mol. Biol.* **117**, 577–606.
45. Lewin, B. (1975) *Gene Expression: Plasmids and Phages* (Wiley, New York).
46. Ashman, C. R., Jagadeeswaran, J. & Davidson, R. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3356–3360.
47. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D. & Barbacid, M. (1985) *Nature (London)* **315**, 382–385.