Characterization of a Mutagenic DNA Adduct Formed from 1,2-Dibromoethane by \( O^6 \)-Alkylguanine-DNA Alkytransferase

It has been proposed that the DNA repair protein \( O^6 \)-alkylguanine-DNA alkytransferase increases the mutagenicity of 1,2-dibromoethane by reacting with it at its cysteine acceptor site to form a highly reactive half-mustard, which can then react with DNA (Liu, L., Pegg, A. E., Williams, K. M., and Guengerich, F. P. (2002) J. Biol. Chem. 277, 37920–37928). Incubation of Escherichia coli-expressed human alkytransferase with 1,2-dibromoethane and single-stranded oligodeoxyribonucleotides led to the formation of covalent transferase-oligo complexes. The order of reaction determined was Gua \( \rightarrow \) Thy \( \rightarrow \) Cyt \( \rightarrow \) Ade. Mass spectrometry analysis of the tryptic digest of the reaction product indicated that some of the adducts led to depurination with the release of Ade. Mass spectrometry analysis of the Cyt, Thy, and Gua \( \rightarrow \) Gua > Gua > Gua > Gua > oligo complexes. The order of reaction determined was Gua \( \rightarrow \) Thy \( \rightarrow \) Cyt \( \rightarrow \) Ade. Mass spectrometry analysis of the tryptic digest of the reaction product indicated that some of the adducts led to depurination with the release of the G\( _{7}\) adduct. Peptide cross-linked to a Gua at the N7 position, with the site of reaction being the active site Cys145 as established by chromatographic retention time and the fragmentation pattern determined by tandem mass spectrometry of a synthetic peptide adduct. The alkytransferase-mediated mutations produced by 1,2-dibromoethane were predominantly Gua to Ade transitions but, in the spectrum of such rifampicin-resistant mutations in the \( RpoB \) gene, 20% were Gua to Thy transversions. The latter are likely to have arisen from the apurinic site generated from the Gua-N7 adduct. Support exists for an additional adduct/mutagenic pathway because evidence was obtained for DNA adducts other than at the Gua N7 atom and for mutations other than those attributable to depurination. Thus, chemical and biological evidence supports the existence of at least two alkytransferase-dependent pathways for 1,2-dibromoethane-produced mutagenicity, one involving Gua N7-alkylation by alkytransferase-5-CH\(_2\)CH\(_2\)Br and depurination, plus another as yet uncharacterized system(s).

\( O^6 \)-Alkylguanine-DNA alkytransferase (AGT) \(^1\) represents an important defense mechanism against the mutagenic, carcinogenic, and cytotoxic effects of simple alkylating agents (1–3). AGT repairs \( O^6 \)-alkylguanine adducts that are of major importance in initiating mutations and cytotoxicity. This repair process is mediated by transfer of the alkyl group from the \( O^6 \) atom of Gua to the active site Cys residue in the AGT protein. AGT is inactivated by the alkyl group transfer, and therefore additional repair requires new protein synthesis. Although an early study reported that the mutagenicity of 1,2-dibromoethane (DBE) was not affected by enzymes that repair alkylation lesions (4), there is now convincing data that AGT paradoxically promotes the toxicity of DBE (5, 6). Overexpression of human AGT (hAGT) or AGTs from other species enhances the mutagenicity and lethality of DBE in Escherichia coli (5–9) and induces growth retardation in mammalian cells (10).

Recently, we have shown that this toxicity is due to the interaction of DBE with the Cys acceptor site of AGT (Cys145 in hAGT) (11). This Cys145 residue is present in the hAGT protein as part of an extensive hydrogen-bonding network involving a water molecule and His146, Arg147, and Glu172 (12). This environment causes Cys145 to have a high reactivity with model electrophiles unrelated to alkylated DNA substrates and a very low \( k_p \) (13). It therefore reacts readily with DBE to generate a reactive intermediate, S-(2-bromoethyl)-Cys145-hAGT (11). This half-mustard intermediate is likely to cyclize forming an episulfonium ion that can react to form a covalent AGT-DNA adduct. Formation of DNA adducts is facilitated by the DNA binding properties of AGT.

DBE was used widely in gasoline as an anti-knock ingredient, as well as in pesticides and soil fumigants. Its use was drastically reduced due to reports of its toxicity. DBE is carcinogenic in rats, and toxic and mutagenic in microorganisms, plants, insects and humans (14–16). Earlier studies with DBE have indicated that toxicity can be caused by metabolites formed via microsomal metabolism (17) and by the action of glutathione S-transferase (GST) (18–20). The latter reaction generates S-(2-bromoethyl)glutathione, which undergoes a non-enzymatic dehalogenation and forms an episulfonium ion.

\(^{1}\) The abbreviations used are: AGT, \( O^6 \)-alkylguanine-DNA alkytransferase; DBE, 1,2-dibromoethane; hAGT, human AGT; GST, glutathione S-transferase; oligo, oligodeoxyribonucleotide; peptide Gly51–Arg47, GNPVILIPCHR; IPTG, isopropyl \( \beta \)-d-thiogalactopyranoside; DTT, di-thiothreitol; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; TAE-PAGE, Tris-acetate-poly-acrylamide gel electrophoresis; Me\( _2 \)SO, dimethylsulfoxide; HPLC, high performance liquid chromatography; ESI, electrospray ionization; CID, collision-induced dissociation; SRM, selected reaction monitoring; ATP\( _S \), adenosine 5'-O-thiotriphosphate; DMS, dimethylsulfate.

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This ion then reacts rapidly with cellular nucleophiles such as DNA (21). The structures and mutagenic effects of major DBE-DNA adducts formed in this way have been studied extensively (19, 22, 23). Of these, S-2-[N'-guanyl]ethylglutathione accounts for up to 95% of total DNA adducts (24) and its formation produces G:C to A:T transition mutations (25). GST was shown to enhance DBE genotoxicity in Salmonella typhimurium strains TA100 and TA1535, where mutations at specific guanines are needed to produce reversions (22, 26, 27). The extent to which the various pathways for activation of DBE (activation by microsomes, by GST or by AGT) contribute to its genotoxicity are not yet clear.

While previous studies provide a plausible model for the hAGT-mediated mechanism of DBE toxicity, there has been no direct evidence for the formation of hAGT-DNA adducts in vivo, structural characterization of hAGT-DNA adducts, or investigations of the types of mutations caused by these adducts. In the work described in this study, we describe evidence that AGT-DNA covalent cross-links are formed in cells, compare the reactivity of hAGT-DBE intermediates with different DNA bases, identify the mutations produced by DBE in cells lacking and expressing AGT, and characterize one of the hAGT-DNA adducts. Our results show that the predominant reaction is with Gua and that, in the presence of AGT, DBE treatment leads to a large increase in the frequency of G:C to A:T transitions and G:C to T:A transversions in E. coli. This evidence supports the involvement of both depurination and alternate mechanisms (e.g. error-prone DNA synthesis across sites) with hAGT adducts.

**EXPERIMENTAL PROCEDURES**

*Materi als—DBE, dimethylsulfate (DMS), piperidine, porcine spleen DNase II, white potato acid phosphatase II, isoproxy 1,3-bis(2-thiacyclallophosphate (IPTG), dithiothreitol (DTT), and bovine intestinal mucosal alkaline phosphatase were purchased from Sigma Chemical Co. Adenosine 5′-[γ-32P]thiotriphosphate (triethyl amnomium salt) (γ-32P[ATP]γS) was purchased from Amersham Biosciences. DNase I was purchased from Roche Applied Science, Indianapolis, IN. The Photo- topeme- HRP Detection Kit for immunoblot analysis was purchased from Cell Signaling Technology (Beverly, MA). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). The peptide GN-Arg147 was obtained (HPLC-purified) from SynPeptide (Dublin, CA).

Recombinant C-terminal histidine-tagged wild-type hAGT was purified by C-term affinity chromatography using a BioCad Sprint Perfusion system (PerSeptive Biosystems) as described previously (28). E. coli TRG8 cells transformed with pNi-hAGT or an empty pNi vector (29) were grown at 37 °C under the selection of 50 μg/ml ampicillin and kanamycin (11) unless otherwise specified.

Most oligodeoxyribonucleotides (oligos) used in this study were synthesized by Invitrogen. The complementary 16-mer oligonucleotides 5′-d(TGCGTGAAGTGAGTGA)-3′ and 5′-d(GTTGCGCAGAAGCTTCCACTCAGTTTCG)3′ used for MS analysis were purchased from Midland Certified Reagent Co. (Midland, TX) (GFP identity, and purity confirmed by MALDI-TOF MS). These oligonucleotides were dissolved in H2O, equilibrated to 0 °C, mixed, heated to 90 °C, and then allowed to cool to room temperature (4 h time) to form a double-stranded oligomer for cross-linking studies.

**Analysis of DBE-dependent AGT Binding to DNA in Vitro—Oligodeoxyribonucleotide 16-mers 5′-d(C10C3′)-, 5′-d(TGTG)-3′, or 5′-d(AAG)-3′ were labeled with [γ-32P]ATPγS at the 5′-end as described previously (11). Radiolabeled oligonucleotides (0.5 pmol) were incubated with various unlabeled oligonucleotides (0–1000 pmol) for 60 min at 37 °C. The 15 μl reaction mixtures also contained 20 mM DBE and wild-type hAGT (2 μg) in AGT buffer (50 mM Tris (pH 7.6) and 0.1 mM EDTA). Reactions were terminated by adding the addition of 40 mM Tris (pH 6.8), 100 mM DTT, 2% SDS (w/v), 0.1% bromophenol blue (w/v) and 10% glycerol (v/v). The mixture was then incubated for 1 h at 37 °C and analyzed on a 7% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) using 10 μl Tris acetate, 1 mM EDTA, pH 8.0 (TAE-PAGE) using 20% gels. Gels were vacuum dried and analyzed using a PhosphorImager SI system.

**Identification of DBE-mediated Formation of Covalent DNA-AGT Complexes—E. coli TRG8 cells transformed with an empty pNi vector or wild-type pNi-hAGT were grown in 40 ml of LB at 37 °C to an OD 600 nm of 0.5. The expression of the hAGT protein was induced using 0.2 mM IPTG. After a 1-h induction period, cells were exposed to concentrations of 0.035 or 0.2 mM DBE or vehicle (dimethylsulfoxide (Me2SO)) for 90 min under constant shaking. Cells were collected by centrifugation and washed with 20 ml of 1× M9 salt solution (90 mM Na2HPO4, 25 mM KH2PO4, 10 mM NaCl, and 20 mM NH4Cl). The cell pellets were stored at −80 °C until analysis.

Genomic DNA from TRG8 cells was prepared using a Qiagen blood and cell culture DNA Maxi kit (Valencia, CA). The manufacturer’s protocol for genomic DNA preparation from Gram-negative bacteria was adapted with minor modifications. Heat-inactivated RNase and lysozyme were added to buffer B1 to lyse the cells. Protease K was employed to rid the cell pellets of hAGT protein, and NaCl (250 mM) was added to buffer B1 and B2 to reduce the noncovalent binding of proteins to DNA. E. coli genomic DNA was precipitated from the Qiagen, Valencia, CA elution solution using a mixture of (w/v) 70% isoproxy alcohol and 30% 0.4 M NaCl, followed by centrifugation at 14,000 × g for 15 min. The DNA was dissolved in 100–300 μl of deionized water by rotation-mixing at room temperature overnight.

Aliquots of E. coli genomic DNA (4 μg) were partially digested with DNase I (0.4 unit/μg DNA) at 37 °C for 30 min. The mixtures were resolved by electrophoresis using 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The presence of hAGT protein was determined using a specific monoclonal antibody (Clone MT 3.1; Lab Vision, Fremont, CA) and visualized using the Phototope-HRP Detection Kit.

**Analysis of Mutants Induced by DBE in the hisG Gene of S. typhimurium and the lacI Gene of E. coli—For studies of S. typhimurium, the strain YT108 (derivative of TA1535, lacking both endogenous AGT genes (30)) was transformed with pNi and pNi-AGT vectors. For studies in E. coli, strain TRG8 (8) was transformed with pPiBA2-AGT (which produces active AGT) or pPiBA2-C145A, which produces an inactive mutant. These plasmids were constructed by PCR amplification from pNi-AGT and pNi-C145A plasmids (31) using the upper primer 5′-GC-AGCACTCTTGATATTTAGGAGAAGAGG-3′, which introduces an XbaI restriction site just upstream of the ribosome binding site and downstream of the AGT ORF, and the lower primer 5′-GGTGGCCGAAAGCGTGCCATG-3′, which changes a BamHI site to a HindIII site downstream of the stop codon. The amplification reaction generated 680 bp DNA fragments that were cut with XbaI and HindIII. The restricted fragments were ligated to a pASK-Hind vector (IRA GMBH, 60 μl for 16-h ligation) were infected with various mutated colonies in the presence of sodium dodecyl sulfate (SDS-PAGE) using 5 μg/ml and analyzed using a PhosphorImager SI system. The oligonucleotide bands were quantified using ImageQuant software (11). The percentage of oligonucleotide in the lower mobility hAGT-oligonucleotide complex forms was determined from these measurements and plotted against the concentrations of unlabeled oligonucleotides used in the reaction. Oligonucleotide 16-mers 5′-d(TGCGTGAAGTGAGTGA)-3′ containing a Gua or an O6-methylguanine at the G+ position were 5′-end labeled using [γ-32P]ATPγS. Mixtures of 6.5 pmol of [32P]-labeled and 20 pmol of unlabeled oligonucleotides were added to 0–70 min to a mixture of 2 μg of hAGT protein and 20 mM DBE. After the addition of oligonucleotides, the incubation was allowed to proceed for another 60 min. Reaction mixtures were then separated by SDS-PAGE and analyzed using a PhosphorImager SI system.
cin, 40 µg/ml histidine) (32) and incubated for 2–3 days at 37 °C. The percentage survival was quantified by the number of colonies of bacteria exposed to DEB over that of untreated bacteria. For mutant isolation, after DEB treatment, undiluted cells were plated on M9 minimal medium plates (32) containing 0.7 mg/ml phenyl-β-D-galactoside as the sole carbon source for selection of mutants that synthesize β-galactosidase constitutively (lacI or lacO) or Vogel-Bonner minimal medium (33) with excess biotin and trace amounts of t-histidine for isolation of S. typhimurium hisG revertants (34). The plates were incubated at 37 °C for 3 days, and the number of colonies on the plates was determined. The mutation frequency was calculated by the number of colonies grown on the selective media over 105 survivors grown on the glucose-containing plates.

The following day, selective plates were purified by streaking on Petri dishes containing the same selective agent. These bacterial colonies were used in a PCR amplification reaction using the cells directly instead of purified DNA as a template. After incubation for 10 min at 95 °C to break the cells and release template DNA, Ffu ultra DNA polymerase was added, and amplification carried out with primers 5′-TGAAGTTGAACAGCTAAC-3′ and 5′-GGCTACTGGCCGGTTTCCTACG-3′ for the analysis of E. coli lacI gene and 5′-GGTTAGACACACTCCGCTTACGC-3′ and 5′-GGAGGTTGGGATGATGAGGTAGC-3′ for the analysis of S. typhimurium hisG gene. The amplification products were purified using the QIAquick PCR purification kit (Qiagen) and both strands sequenced using the same primers employed for amplification in separate reactions at the Vanderbilt Core DNA Sequencing Facility, Vanderbilt University Medical Center.

### Analysis of Rifampicin-resistant Mutants Induced by DEB—TRG8 cells were grown in 50 ml of LB media containing 0.2 mM IPTG to an OD600 of 0.5. Cells were pelleted by centrifugation and resuspended in 2 ml of M9 salts. Aliquots of cells (0.5 ml) were then exposed to various concentrations of DEB (0.035–0.1 mM) or to the solvent Me2SO at 37 °C for 90 min. The cells were washed with M9 salts and resuspended in 0.5 ml of the same solution. In order to derive rpoB gene mutants, cells were plated on LB media plates supplemented with 100 µg/ml rifampicin. Undiluted cell suspensions (100 µl) were plated either immediately after the treatment or following an additional overnight culture in LB media. The cells were grown for 3 h until discrete colonies appeared. In addition, the overnight cultures were diluted 1:1.2 × 105-fold and plated onto LB plates lacking rifampicin to determine the number of viable cells. For cells plated immediately after treatment, a 1:104-106 fold dilution was utilized. The mutagenesis frequency of the number of viable cells. For cells plated immediately after treatment or following an additional overnight culture in LB media.

### Modification of Peptide Gly 136

The synthetic peptide was reacted with DBE and dGuo to generate modifications of the peptide with -CH2CH2-dGuo and -CH2CH2-(N7)Gua at Cys145 of the peptide, for reacted with DBE and dGuo to generate modifications of the peptide —

### RESULTS

#### Relative Reactivity of Bases with hAGT and DBE—The formation of a covalent complex between hAGT, DBE, and 5'-[32]S-labeled oligonucleotides (11) was used to investigate the relative reactivity of the four deoxyribonucleosides in forming hAGT-DNA adducts. The ability of unlabeled 16-mers modified with DEB and dG were used. A section of the rpoB gene mutants, cells were plated on M9 minimal glucose-containing plates. In order to derive hAGT-DNA adducts. The ability of unlabeled 16-mers containing a cytosine (cystine) and by ethylene links. Collision-induced dissociation (CID) of the suspected ions yielded the apparent b and y fragmentation patterns (see Fig. 5 and Supplementary Data).

Cross-linking of hAGT and Double-stranded Oligomer for MS Analysis of Depurinated Products—hAGT (10 nmol, in 0.40 ml total volume) was incubated with 23 nmol of double-stranded 16-mer oligonucleotide (from Midland, see above) and 20 nm DEB at 37 °C for 60 min. The solution was dialyzed at 4 °C over a 3-day period versus 100 volumes of 80 mM sodium acetate (pH 5.0) to remove residual DEB (3 changes). The HAGT precipitated and was recovered by centrifugation (10 × g, 5 min). The pellet was suspended in 200 µl of 10 mM sodium phosphate (pH 7.6) containing 0.9% SDS (w/v) and heated at 90–95 °C for 60 min, to depurate DNA adducts. A 100-µl aliquot (1/2) was diluted to a volume of 2.0 ml in 10 mM NaHCO3 (pH 8.0), bringing the SDS concentration to 0.045% (w/v). To this sample was added 100 µl of 10 mM NaHCO3 (pH 8.0) (to precipitate hAGT) by weight and incubated at 37 °C for 14 h, followed by another addition of the same amount of trypsin and incubation for 7 h more at 37 °C. SDS-PAGE showed extensive proteolysis, and the digested sample was used for HPLC/MS-ESI analysis under conditions similar to those used for the analysis of the standard peptide (Gly136–Arg140) modified with DEB and dG (see above).

Cross-linking of hAGT and Double-stranded Oligomer for Nucleosome Digestion and MS Analysis—An incubation was set up as in the case of the depurination experiment, except that the amounts of each component were doubled (0.80 ml volume). After 60 min, 200 µl of 1 mM NaHCO3, (pH 8.7) and 100 µl of 0.4 mM dithiothreitol were added, and the reaction was allowed to stand at 92 °C for 2 h, to conjugate residual DEB. The sample was dialyzed overnight (at 4 °C) versus 200 ml of 50 mM Tris-HCl buffer (pH 8.7) containing 15 mM MgCl2. The sample was then incubated for 4 h (37 °C) with 80 µg of phosphodiesterase I; then 80 µg of alkaline phosphatase was added, and the incubation proceeded for another 4 h at 37 °C. The sample was dialyzed overnight versus 100 volumes of 50 mM potassium phosphate buffer (pH 8.0) and HAGT was recovered by applying the sample to a 0.4 ml Ni2+-nitrilotriacetic column pre-equilibrated with 20 mM potassium phosphate (pH 8.0), washing with 15 ml of the equilibration buffer, and eluting with the same buffer containing 200 mM imidazole (at room temperature). The eluted fraction containing hAGT was analyzed by A280 measurements, recording UV spectra on a Cary 14/OLIS spectrophotometer (OLIS, Bogoat, GA) were pooled (total volume ~3 ml) and dialyzed versus 250 ml of 10 mM NaHCO3 buffer (pH 8.5) overnight at 4 °C. The sample was concentrated to dryness by lyophilization and reconstituted in 1/10 the original volume. Trypsin was added (2.5 µg, MATRIX) to HAGT by weight and the reaction proceeded overnight at 37 °C; another 2.5 µg of trypsin was added and the reaction continued for 8 h at 37 °C. The sample was frozen at −20 °C until HPLC/MS analysis (10 days later).

#### Relative Reactivity of Bases with hAGT and DBE—The formation of a covalent complex between hAGT, DBE, and 5-\[32\]S-labeled oligonucleotides (11) was used to investigate the relative reactivity of the four deoxyribonucleosides in forming hAGT-DNA adducts. The ability of unlabeled 16-mers consisting of a covalent complex between hAGT, DBE, and 5-\[32\]S-labeled oligonucleotides (11) was used to investigate the relative reactivity of the four deoxyribonucleosides in forming hAGT-DNA adducts. The ability of unlabeled 16-mers containing a cytosine (cystine) and by ethylene links. Collision-induced dissociation (CID) of the suspected ions yielded the apparent b and y fragmentation patterns (see Fig. 5 and Supplementary Data).
ing of repeats of a single nucleoside or two nucleosides to prevent the formation of this radioactive complex was used to determine their reactivity (Fig. 1). As shown in Fig. 1A, hAGT was linked to [35S]d(C16) forming complexes with either one (complex 1) or two (complex 2) hAGT proteins (Fig. 1A). Approximately 28% of the d(C16) was present in complexed forms (in the absence of any unlabeled oligonucleotide), and the amount of radiolabeled DNA-hAGT complexes declined as the levels of unlabeled d(C16), d(T16), or d(A16) increased from 0 to 350 pmol (Fig. 1A). d(T16) competed most effectively and the d(A16) was least effective (Fig. 1B), indicating an apparent order of reactivity of T>C>H>A for hAGT-DNA conjugation.

Studies with d(G16) could not be carried out due to problems in the synthesis of such homopolymers; therefore, reactivity of Gua was compared with Thy using [35S]d(TG)8 in the presence of unlabeled oligonucleotides (Fig. 1, C and D). The latter was more effective indicating that Gua is more reactive than Thy. Taken together, these data indicate that the overall order of reactivity for the four bases is Gua>Thy>Cyt>Ade. Similar results were obtained using other combinations of labeled and unlabeled oligonucleotides; e.g. we confirmed that Thy is more reactive than Ade by comparing the reactivity of d(TG)8 and d(AG)8 (Fig. 1, E and F).

AGT repairs O6-methylguanine and the possibility was considered that the DBE-mediated conjugation of the protein might be greatly influenced by the presence of an O6-methyl-Gua. This experiment is complicated by the possibility that hAGT would repair the O6-methylGua lesion rather than react with DBE. Therefore, hAGT was incubated with DBE for 0–60 min prior to addition of oligonucleotides, 5'-[35S]-d(TGCGTm6GAAGGAGTGA)-3' or 5'-[35S]-d(TGCGTGGAAGGAGTGA)-3' (where m6G is O6-methyldeoxyguanosine). The two oligonucleotides reacted in a similar manner with the reactive intermediate (Fig. 2). The levels of complex formation did not differ at any of the time points tested. However, the amounts of complex declined as the length of the preincubations were extended, suggesting that the intermediate loses activity with time. This result is consistent with the proposed instability of
activated hAGT-Cys\textsuperscript{145}-SCH\textsubscript{2}CH\textsubscript{2}Br, which is converted to hAGT-Cys\textsuperscript{145}-SCH\textsubscript{2}CH\textsubscript{2}OH in the absence of DNA or other reaction substrates (11).

On the basis of many previous studies of reactions of alkylating agents with Gua in DNA and particularly the episulfonium ion formed from S-(2-bromoethyl)glutathione (21, 24), reaction with the N\textsuperscript{7} position of Gua is a potentially major site of attack. Lesions where Gua is modified at N\textsuperscript{7} can be identified by using the ability of piperidine to cleave at apurinic sites derived from thermal release of the adduct (22, 35). Therefore 5'-\textsuperscript{[\textsuperscript{35}S]d(C)\textsubscript{7}G(C)\textsubscript{8}}-3\textsuperscript{'} or 5'-\textsuperscript{[\textsuperscript{35}S]d(T)\textsubscript{16}} was reacted with DBE and hAGT and also with DMS, which causes alkylation prevalently on the N\textsuperscript{7}-position of guanine (35) to act as a positive control. The products were then treated with piperidine and separated by TAE-PAGE (Fig. 3). No alteration in mobility was seen when d(T)\textsubscript{16} was used but a small part of the 5'-d(C)\textsubscript{7}G(C)\textsubscript{8}-3' was cleaved to form the expected 7-mer when reacted with both DBE and hAGT. More extensive cleavage was observed with the 5'-d(C)\textsubscript{7}G(C)\textsubscript{8}-3' after it had been reacted with DMS (Fig. 3). These results are consistent with at least part of the covalent adducts formed by DBE-activated hAGT with Gua being at the N\textsuperscript{7} position.

**MS of hAGT Modifications**—A number of technical difficulties are associated with analysis of nucleic acid-protein cross-links. MS of peptide-nucleic acid complexes has been very limited because optimal conditions developed for either peptides or nucleic acids (either MALDI-TOF or ESI) are negated by the presence of the other component. Chemical methods of digestion following modification of Cys145 under extensive conditions, including a residue in peptide 148–165 (presumably due to alkylation of Cys\textsuperscript{150}, modified ~10% of the extent of G136-R147).

hAGT was reacted with calf thymus DNA in the presence of 20 mM DBE for 1 h and then subjected to trypsin digestion. In the MALDI-TOF spectrum (Fig. 4), a peak with an m/z of 1493 corresponded to the G136-R147 fragment cross-linked to a guanine base. A peak at m/z 1359 corresponded to the 2-hydroxyethyl adduct at Cys\textsuperscript{145} described above.

Preliminary ESI analysis was also consistent with the presence of the +2 and +3 charged ions of this adduct. This data would correspond to depurination, presumably of an N\textsuperscript{7}-guanyl adduct. Although a number of other procedures were tried (see Supplementary Data), we found that we could best characterize this adduct by optimizing the protocols to those presented in the Experimental Procedures section. Briefly, a 60-min incubation of hAGT with DBE and a double-stranded oligonucleotide was followed by a 60 min heat step (95°C, neutral pH), solubilization of the denatured protein in SDS with heating, dilution to lower the SDS concentration, trypsin digestion, and HPLC/MS-ESI with comparison to a sample prepared from the reaction of the synthetic peptide (Gly\textsubscript{136}-Arg\textsubscript{147}) with DBE and dGuo at alkaline pH. The same product was identified from an experiment in which hAGT, DBE, and the double-stranded 16-mer were incubated following reaction with DTT at alkaline pH to conjugate residual DBE, dialysis at alkaline pH, digestion with phosphodiesterase I and then alkaline phosphatase (at alkaline pH), isolation of the hAGT using Ni\textsuperscript{2+}-nitrilotriacetate chromatography (elution with imidazole), dialysis, lyophilization, and digestion with trypsin. The resulting data are presented in Fig. 5 and confirm the depurination product.

The sample prepared from the incubation of synthetic peptide (Gly\textsubscript{136}-Arg\textsubscript{147}) DBE, and dGuo was also used as a reference material to search for hAGT-derived peptide cross-linked to dGuo and other nucleosides in the above sample that had been digested enzymatically. Only in one experiment could we tentatively identify dGuo nucleoside adducts (and were unable to further verify this by fragmentation analysis), and we did not detect any of the other three nucleosides in adducts. The absence of these adducts may be due to limits of sensitivity, which may vary depending upon the nucleoside. Further, the standard peptide Gly\textsubscript{136}-Arg\textsubscript{147}-CH\textsubscript{2}CH\textsubscript{2}dGuo adduct (see Supplementary Data) apparently degraded to the corresponding Gua adduct during handling and storage.

**Formation of hAGT-DNA Adducts in Vivo**—E. coli TRG8 cells, which lack endogenous AGT protein, were transformed with an empty vector control plasmid or a plasmid leading to expression of hAGT. The cells were treated with DBE or vehicle (Me\textsubscript{3}SO) for 90 min under constant shaking. Genomic DNA was isolated using a procedure in which any non-covalent hAGT-DNA complexes were disrupted by the presence of the detergent and 250 mM NaCl. The DNA was digested with DNase I, separated by SDS-PAGE, and then subjected to immunoblot analysis using a monoclonal antibody specific for hAGT.
signal was detected only in the cells expressing hAGT and treated with DBE and was much stronger in the cells treated with the higher concentration of DBE (Fig. 6). The smear is probably due to incomplete digestion of all of the DNA since a low concentration of DNase was used. This also may account for the presence of complexes in Fig. 6 that clearly contain two hAGT molecules, which were mostly noticeable in cells treated with 0.35 mM DBE for 90 min. However, this may also indicate that two hAGT molecules are linked to each other. This is consistent with findings that binding of AGT to DNA is highly cooperative placing two molecules in close proximity to each other (36) and such dimers were seen in reaction of hAGT with DBE and oligos in vitro (11) (Fig. 1). The covalent AGT-DNA adduct was not seen when DNA was prepared from cells exposed to Me$_2$SO vehicle or 1,2-dichloroethane, a chemical that does not react with hAGT in vitro, nor form hAGT-DNA adducts (data not shown).

**Determinations of Mutation Spectra Caused by DBE and hAGT**—The ability of AGT to mediate mutations by DBE at G:C pairs was demonstrated using S. typhimurium YG7108, a strain derived from TA1535 with additional alterations that inactivate endogenous AGT genes (30). The strain contains mutation hisG46 imparting a requirement for histidine that results from an inactivating mutation forming a CCC codon. This can be reverted by any base change at the first base and by a G:C to A:T transition and G:C to T:A transversion at the second base of the codon. The expression of hAGT caused a large increase in the formation of revertants in response to DBE (Fig. 7A). The hisG gene of some mutants obtained was PCR amplified and sequenced. In all cases, the mutations mediated by hAGT were G:C to A:T transitions in the first or the second position (29% were TCC and 71% were CTC). This can be reverted by any base change at the first base and by a G:C to A:T transition and G:C to T:A transversion at the second base of the codon. The expression of hAGT caused a large increase in the formation of revertants in response to DBE (Fig. 7A). The hisG gene of some mutants obtained was PCR amplified and sequenced. In all cases, the mutations mediated by hAGT were G:C to A:T transitions in the first or the second position (29% were TCC and 71% were CTC).

These results were further supported by studies of the mutation spectra of mutants produced by 1 mM DBE in the lacI gene. Expression of active AGT, but not the inactive C145A mutant, increased the frequency of such mutations by more than 100-fold (Fig. 6B). All 44 mutants analyzed were single base pair substitutions, 43 were transitions and predominantly (41/43) G:C to A:T base pairs. There were also two A:T to G:C transitions, and the other base substitution corresponded to a G:C to T:A transversion. These mutants were distinct from those spontaneous mutants detected in cells not containing hAGT and were not randomly distributed along the lacI open reading frame. Instead, six nucleotide residues clustered two thirds of the substitutions where two or more mutations were found. Mutation of the mid base of codon 222 was the most frequent (nine independent mutants), followed by codons 118 (six mutants), 68 and 58 (four mutants each), 27 (three mutants) and 187 (two mutants).

In order to obtain a more complete picture of the mutation spectrum, the occurrence of rifampicin resistant mutants, which result from alterations in the rpoB gene, was examined. The rpoB gene, which encodes the $\beta$-subunit of RNA polymerase II, has been widely used as a marker to examine the spectra of mutants arising endogenously or through induction by exogenous reagents (38, 39). The frequency of rifampicin-resistant (Rif$^+$) mutants produced by DBE was greatly increased by the expression of hAGT (Fig. 7C). Sequence analysis of the mutants (Table I) indicated that in DBE-treated cells expressing hAGT nearly all of the mutations were G:C to A:T transitions (76%) or G:C to T:A transversions (20%). The spectrum of mutations was quite different from that observed in cells lacking hAGT (but treated with DBE) where 39% of mutations correspond to A:T to C:G transversions, 33% of G:C to A:T transitions, 17% A:T to T:A transversions, 5% A:T to G:C transitions and 5.5% G:C to T:A transversions (Table I). The sites of mutation in cells expressing hAGT were also different from those in cells lacking hAGT (Table I). Exposure of cells to solvent Me$_2$SO did not cause any significant increase in mutations in the rpoB gene irrespective of hAGT status. Also, the spectra of background mutants in TRG8 cells did not reveal any specific induction of G:C to A:T or G:C to T:A mutations (results not shown) and was similar to that seen in a recent large study (39).

**DISCUSSION**

Previous work by other groups established the paradoxical enhancement of the mutagenicity of DBE by expression of AGT (5–7). These results were confirmed in one of our laboratories (8), and subsequently we reported the cross-linking of AGT to DNA, with the reaction initiated by the conjugation of AGT with DBE (11). The reaction of AGT with DBE is due to the high reactivity of the Cys$^{145}$ thiolate anion with alkylating agents (13) and the formation of a half-mustard and probably episulfonium ion (21) that can cross-link to DNA (11). However, further information about how this process could lead to mutations was not available. We now provide evidence for an N$^7$-Gua/depurination mechanism to explain part of the mutations.

As shown in Fig. 1, the intermediate formed by the reaction of DBE with hAGT in vitro can react with all four nucleotides. Reaction was strongest with Gua and the second most reactive base in vitro was Thy. These bases are the sites of AGT reaction (2, 40), but the difference in reaction may simply result from the availability of appropriate nucleophilic sites at locations that can react with the episulfonium ion intermediate. Although reaction with Gua was the most favored, the in vitro studies showing reaction with all four bases contrast somewhat with the mutations seen in vivo which were virtually exclusively at G:C pairs. The Cys$^{145}$ acceptor site of hAGT is located in an internal pocket and nucleoside “flipping” induced by the AGT protein is needed to allow the potential substrate to be placed in proximity to this residue (12, 41). Even allowing for the additional size of the activated DBE adduct and the possible distortion in the protein that may arise from its formation, it is unlikely that covalent attachment of the activated AGT could occur readily without this change on the DNA structure. Many aspects of the mechanism by which AGT finds lesions in DNA are unclear, for example whether it samples the DNA by flipping every base or is directed toward some sites by minor distortions in the DNA. The preference for Gua seen in our in vitro experiments may therefore be magnified in cellular DNA, which is predominantly double stranded. The presence of an O$^6$-methylguanine residue had no effect on the covalent attachment of DBE-activated AGT to DNA in vitro (Fig. 2). This result is not very surprising because hAGT does not bind much more strongly to DNA containing O$^6$-methylguanine than to DNA.
without methyl adducts (36, 42, 43). However, the result makes the important point that the formation of hAGT-DBE-DNA cross-links is not dependent on other processes such as endogenous or exogenous methylation generating sites for AGT action.

Definitive proof that AGT can be cross-linked to DNA in the presence of DBE is provided by the MS results. First, the data shown in Figs. 4 and 5 support the assignment as hAGT-derived peptide 136–147 bound to -CH2CH2-Gua through modification at Cys145. Such release of the Gua adduct under conditions of neutral heating is very consistent with the evidence shown for the hot piperidine treatment results in Fig. 3. The MS results are only consistent with the linkage of the Gua at the N7 atom, leading to depurination.

N3-Ade adducts also depurinate, but we did not find MS evidence for any Ade-containing adducts when we searched the expected m/z ions.

However, not all of the binding at Gua can be explained by the guanyl N7-modification. In other experiments (not shown), hAGT and calf thymus DNA were reacted with [14C]DBE. The stoichiometry is somewhat problematic because of the difficulty in correcting for hydroxyethyl-hAGT (not bound to DNA), but in several experiments a mean of ~1/3 of the radioactivity bound to DNA (precipitated by centrifugation after 16 h at 10^5 g) remained bound after neutral thermal depurination. No N7-Gua adducts should have remained following the 60 min heat step (95 °C) at neutral pH (24, 44). Additional evidence for the existence of other DNA adducts was obtained in the piperidine cleavage experiments where only a fraction of the material was cleaved (Fig. 3).
All of the studies on the mutation spectra are consistent with these mutations being derived from a Gua alteration caused by DBE in the presence of AGT. The presence of AGT caused a massive increase in His revertants in E. coli GWR109 (derived from F26) (11) similar to the effect of exposure to N-methyl-N'-nitro-N-nitrosoguanidine, which is well-known to produce predominantly G to A transitions (45). All of the reversions of hisG46 in S. typhimurium YG7108 were G to A transitions. Similarly, G:C sites represented the overwhelming majority of mutants in the lacI gene (41/43) and in the rpoB gene (24/25).

The rpoB gene is now firmly established as a suitable target for the facile detection of all classes of mutations (39). Both G:C to A:T transitions and G:C to T:A transversions leading to rifampicin resistance were observed in AGT-mediated response to DBE (Table I). The G to T transversions, which represent 20% of the mutations observed in the rpoB gene are consistent with depurination of the Gua adduct and subsequent processing of the abasic site (46, 47). However, in both the rpoB and the lacI genes, the most common mutations were G to A transitions. At present, the lesion leading to this type of mutation is unknown but as discussed above, there is clearly one or more additional adducts that we were not able to identify in the MS experiments. Although currently we have no evidence for the formation of O6-Gua adducts, it is worth pointing out that DBE treatment not only converts AGT into a DNA damaging agent but it also depletes the AGT activity and would therefore retard the repair of any O6-Gua adducts that are substrates for AGT.

The results described here provide strong support for the hypothesis that AGT increases the mutagenicity of DBE by reacting with it to form a reactive complex that leads to the attachment of AGT to DNA. Such a complex was detected in vivo (Fig. 6) after treatment with DBE and unequivocal evidence for a guanine adduct formed by the reaction of DBE and hAGT with DNA in vitro was obtained by MS analysis (Figs. 4 and 5). Also, 20% of the DBE-induced rifampicin resistant mutations that were dependent on hAGT expression were G:C to T:A transversion mutations that are likely to have arisen from the depurination of N7 adducts to generate abasic sites. Such sites are cytotoxic and highly mutagenic giving such versions of hisG46 in S. typhimurium YG7108 were G to A transitions. Similarly, G:C sites represented the overwhelming majority of mutants in the lacI gene (41/43) and in the rpoB gene (24/25).

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transversions if not properly repaired. At present, we cannot definitively identify the adduct or adducts that give rise to the majority of the mutants which were G:C to A:T transitions and this is the subject of ongoing investigation. These could arise from an AGT protein-DNA cross-link at either the O6 or N2 positions. The extent to which by-pass DNA polymerases (37, 48) are able to extend past such protein-DNA cross-links is unclear and there may be an initial “repair” event that removes part of this bulky adduct and leaves a lesion that is more readily by-passed. In addition to the possible effect from abasic sites described above, the large increase in cell killing by DBE mediated via AGT (5–9) may be due to the difficulties of DNA replication on a template that contains protein-DNA cross-links.

A scheme for the role of AGT in the genotoxicity of DBE is shown in Fig. 8. These mechanisms may provide a very efficient way of causing DNA damage, because the reactive species generated at the active site of AGT can be directed to DNA by the DNA binding properties of the protein. It will be important to determine the extent to which AGT-mediated effects contribute to the toxicity of DBE as compared with the other known activation mechanism (GST) by using cells/tissues containing physiological levels of these activation mechanisms (Fig. 8). Our observations of the AGT-dependent mutation spectra combined with those previously published of the GST- and microsomal mediated mutations will provide tools to carry out this analysis. Future studies are also needed to test the role that other DNA repair pathways play in protecting from DBE (4, 6).

In summary, we have characterized the existence of a Gua-N7 adduct involving hAGT and its Cys145 and provide evidence that depurination can lead to mutations in bacterial cells. The work also provides evidence that this is not the exclusive pathway for AGT-enhanced mutations induced by DBE (Fig. 8). Further studies on the non-apurinic site mechanism are ongoing, as well as work with other bis-electrophiles that might involve similar mechanisms. These phenomena are likely to occur in mammalian cells as well as bacteria, although the relative contribution of the pathways shown in Fig. 8 remains to be elucidated.

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Characterization of a Mutagenic DNA Adduct Formed from 1,2-Dibromoethane by $O^6$-Alkylguanine-DNA Alkyltransferase
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