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Genetics

Novel human DNA alkyltransferases obtained by random substitution and genetic selection in bacteria

(O^6-methylguanine–DNA methyltransferase/alkylating agents/random mutagenesis/gene therapy/hematopoietic stem cells)

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ABSTRACT **DNA repair alkyltransferases protect organisms against the cytotoxic, mutagenic, and carcinogenic effects of alkylating agents by transferring alkyl adducts from DNA to an active cysteine on the protein, thereby restoring the native DNA structure. We used random sequence substitutions to gain structure-function information about the human O^6-methylguanine–DNA methyltransferase (EC 2.1.1.63), as well as to create active mutants. Twelve codons surrounding but not including the active cysteine were replaced by a random nucleotide sequence, and the resulting random library was selected for the ability to provide alkyltransferase-deficient *Escherichia coli* with resistance to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Few amino acid changes were tolerated in this evolutionarily conserved region of the protein. One mutation, a valine to phenylalanine change at codon 139 (V139F), was found in 70% of the selected mutants; in fact, this mutant was selected much more frequently than the wild type. V139F provided alkyltransferase-deficient bacteria with greater protection than the wild-type protein against both the cytotoxic and mutagenic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, increasing the D_{37} over 4-fold and reducing the mutagenesis rate 2.7–5.5-fold. This mutant human alkyltransferase, or others similarly created and selected, could be used to protect bone marrow cells from the cytotoxic side effects of alkylation-based chemotherapeutic regimens.**

Alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and the chemotherapeutic nitrosoureas produce cytotoxic, mutagenic, and carcinogenic adducts in DNA. These effects are mitigated in virtually all organisms by alkyltransferases, enzymes that irreversibly transfer the alkyl groups from O^6-alkylguanine and, to a lesser extent, O^4-alkylthymine in DNA to the enzyme (1). For example, *Escherichia coli* cells lacking the alkyltransferases Ada and Ogt are exquisitely sensitive to MNNG (2), and Mer$^-$ mammalian cells in culture, which lack the protein O^6-methylguanine–DNA methyltransferase (MGMT; EC 2.1.1.63), are likewise sensitive to MNNG (3) and nitrosoureas (4). Resistance to these chemicals can be restored by transforming the sensitive cells with a gene encoding an alkyltransferase. The sensitivity of normal human cells, particularly bone marrow cells, that express low levels of MGMT is a major limiting factor in chemotherapy by alkylating agents. Gene therapy holds promise in boosting the resistance of these cells to the cytotoxic effects of chemotherapeutic agents. It has recently been demonstrated that murine bone marrow cells (5, 6) and tissues (7, 8) overexpressing recombinant alkyltransferases have enhanced resistance to alkylating agents.

The mechanism of DNA repair by alkyltransferases is unique. Alkyltransferases are suicide enzymes; transfer is accomplished in an irreversible stoichiometric second-order reaction. Each molecule can execute only one transfer and is rendered inactive. Regions of all known alkyltransferases are highly conserved (9), especially the active site, which contains a hydrophobic cluster of amino acids surrounding a proline-cysteine-histidine-arginine motif (see Fig. 1 for human MGMT active site sequence). The cysteine accepts the alkyl group and is necessary for activity (10–12); the roles of the other conserved residues are not understood. It has been speculated that the proline properly orients the cysteine by inducing a bend (9), and that histidine either accepts a proton from cysteine, thereby making the cysteine an active thiolate anion (13), or is important for protein stability (11). The arginine has been implicated to interact with the DNA (12). Additional amino acids also have been implicated in alkyltransferase activity. For example, a conserved glutamic acid residue (position 172 for the human protein) is required for activity (14), as is the conserved arginine-128 (15). Although the crystal structure of the C-terminal fragment of the *E. coli* Ada alkyltransferase has been published (16), the interactions of the protein with the DNA substrate need to be delineated. Because the active cysteine is buried in a hydrophobic pocket, a conformational change in the structure of the enzyme may be required to bring the cysteine into close proximity to the alkylated guanine (16). Alternatively, the alkylated base could be flipped out from the DNA and inserted into the pocket that contains the active cysteine (17).

In this study we constructed a plasmid library containing the human MGMT gene randomly mutated in the region surrounding the alkyl-accepting cysteine, and from that library we selected variants encoding active proteins. The results indicate that not only are the evolutionarily conserved residues immutable but also that a specific change in one of the hydrophobic residues near the active site confers enhanced resistance to MNNG.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from Sigma: MNNG, carbenicillin, chloramphenicol, isopropyl-β-thiogalactopyranoside (IPTG), and rifampicin. Restriction enzymes were from New England Biolabs, except for *Pin*AI, an isoschizomer of *Age*I, which was from Boehringer Mannheim. T4 DNA ligase was from GIBCO/BRL, *E. coli* DNA polymerase I (Klenow fragment) was purchased from New England Biolabs, and Ampli*taq* DNA polymerase for PCR was from Perkin– Elmer. Plasmids were isolated using the Qiagen Maxiprep kit (Chatsworth, CA) or the Promega Miniprep kit. Sequencing was done with the Promega fmol kit. Four *E. coli* strains were used: GWR111 (K-12, Δ*ada*-25 Cm^r *ogt*-1::Kan^r) (2) was a generous gift from L. Samson (Harvard University); CJ236 (*dut ung*), DH5α, and NM522 were from laboratory

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Abbreviations: IPTG, isopropyl-β-thiogalactopyranoside; MGMT, O^6-methylguanine–DNA methyltransferase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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stocks. Plasmids used were pUC118 (18) and pKT100, containing the human MGMT cDNA cloned into the *Eco*RI site of pUC9 (19) and kindly provided by S. Mitra (University of Texas, Galveston).

Oligodeoxynucleotides, supplied by Operon Technologies (Alameda, CA), unless otherwise noted, were as follows (positions in brackets are a mixture of 82% of the nucleotide indicated plus 6% of each of the other three nucleotides; positions in lowercase indicate deviations from wild-type human MGMT cDNA sequence and were designed to introduce unique translationally silent restriction sites; underlined positions indicate restriction recognition sequences). 1: 5′-AAG CCG CtC GAG CAG TGG GAG GcG CcA TGA GAG GC[A ATC CTG TCC CCA TCC TCA TCC CG]T GC[C ACA GAG TGG TC]T GCA GCA GCG GAG CCG TGG-3′. This 93-mer, corresponding to the sense strand nucleotides 374–466 of MGMT and used in conjunction with oligo 2 to make randomized duplex DNA, was supplied by Keystone Laboratories (Menlo Park, CA). It contains an *Xho*I site (coding nucleotide 380) for cloning and an *Nar*I site (nucleotide 394) for later identification purposes. 2: 5′-TCC CCA ACC GGT GGC CTT CAT GGG CCA GAA GCC ATT CCT TCA CGG CtA GcC CTC CGG AGT AGT TGC CCA CGG CTC CGC TGC TGC A-3′. This 85-mer corresponds to the antisense strand nucleotides 532–448, and contains a *Pin*AI site (nucleotide 521) for cloning and an *Nhe*I site (nucleotide 483) for identification. 3: 5′-AAG CCG CtC GAG CAG TGG GA-3′. This 20-mer corresponds to nucleotides 374–393 of the sense strand and was used to PCR amplify the randomized duplex. 4: 5′-TCC CCA ACC GGT GGC CTT CA-3′. This 20-mer corresponds to antisense strand nucleotides 532–513. It was used both to PCR amplify the randomized duplex and as a sequencing primer. 5: 5′-CGG AGA AGT GAT TTC TTA CC-3′. This 20-mer corresponds to sense strand nucleotides 324–343 and was used as a sequencing primer. 6: 5′-CAC TGC TCG aGC GGC TTT G-3′. This 19-mer corresponds to antisense strand nucleotides 390–372 and was used to introduce an *Xho*I site at nucleotide 380.

Random Library Construction. The human MGMT *Eco*RI cDNA fragment was moved from pKT100 (19) to pUC118. A unique translationally silent *Xho*I site was then added at coding nucleotide 379 by transforming CJ236 with the resulting construct, isolating single-strand uracil-containing plasmid, annealing oligo 6, extending the annealed oligo with DNA polymerase I (Klenow) and dNTPs, and transforming DH5α as described (20). The 141-bp fragment between the unique *Xho*I and *Pin*AI sites was replaced by a 1.7-kb stuffer fragment purified from λ DNA to assemble the "dummy" vector (see Fig. 1*A*). Two overlapping oligonucleotides, one containing the random sequence, replaced the stuffer fragment (see Fig. 1*B*) essentially as described (21). At each of the random positions in oligo 1, the wild- type nucleotide was present at an abundance of 82%—i.e., each of the other nucleotides was present at 6%. Thus, within the library the wild-type sequence constitutes ≈0.1% of the transformed clones ($0.82^{36} = 0.08\%$). The 12 amino acids randomized include the absolutely evolutionarily conserved N137, P144, H146, and R147 residues as well as V139, I141, L142, I143, V148, and V149, which are conserved in terms of hydrophobicity (9). As C145 is essential for MGMT activity (see above), it was not substituted. The 19 nt at the 3′ ends of oligos 1 and 2 were complementary. Oligos 1 and 2 were annealed, extended with Klenow and dNTPs, PCR-amplified using Ampli*taq* and oligos 3 and 4, and incubated with *Xho*I and *Pin*AI. The resulting duplex replaced the stuffer fragment in the dummy vector, resulting in the random library, which was electroporated into *E. coli* strain NM522. The number of transformed *E. coli* (4.1×10^5) was determined by plating an aliquot of the transformation mixture. The remainder of the library was amplified by growing the transformed NM522 cells overnight and recovering the plasmid. GWR111 (*ada ogt*) transformed with the amplified library was stored in aliquots at −80°C. The degree of randomization was verified by sequencing plasmid DNA from 28 unselected GWR111 library colonies.

Selection of Active Human MGMT Mutants. Active mutants were selected by treating exponentially growing cells with three sequential 10 μg/ml doses of MNNG, each separated by a 1-hr recovery period. A frozen aliquot of the library was thawed and grown at 37°C overnight in growth medium (Luria broth with 50 μg/ml carbenicillin and 25 μg/ml chloramphenicol). The overnight inoculum was diluted 1:100 in growth medium containing 50 μM IPTG and regrown at 37°C to A_{600} = 0.7 (3–4 hr) to allow expression of MGMT. Following centrifugation, 1 ml of the culture was resuspended in 1 ml of M9 salts (22) to which was added 0 or 10 μl of freshly diluted MNNG (1 μg/μl in H_2O from a 1M stock in dimethyl sulfoxide). Following incubation at 37°C for 10 min, the MNNG was removed by centrifugation. Cells were resuspended in 1 ml of growth medium + 50 μM IPTG and cultured at 37°C for 1 hr. MNNG treatment was repeated two more times, each with the addition of a fresh aliquot of MNNG. To determine survival after each dose, aliquots of the MNNG-treated and -untreated cultures were diluted in M9 salts, plated on growth medium + 50 μM IPTG agar, and incubated for 16 hr at 37°C. To select MNNG-resistant mutants, the remainder of the thrice-treated library culture was spread on growth medium + 50 μM IPTG agar and incubated for 16 hr at 37°C. Plasmids were isolated from individual colonies, and both strands of the random region were sequenced.

MNNG Survival and Mutagenesis. Cultures of GWR111 harboring either mutant or wild-type MGMT plasmid were grown as described above and treated with graded single doses of MNNG. After treatment, MNNG was removed by centrifugation and washed with M9 salts, and the cells were cultured for 1 hr in growth medium + 50 μM IPTG, diluted in M9 salts, spread on LB + 50 μg/ml carbenicillin either containing or lacking 100 μg/ml rifampicin, and incubated for 16 hr at 37°C. Mutagenesis was scored as the appearance of colonies on rifampicin plates and adjusted for the number of viable cells plated determined from survival on plates lacking rifampicin.

RESULTS

Selection of *E. coli* Expressing Active Human Alkyltransferase. The isolation of active enzymes from large plasmid libraries containing random sequence substitutions is facilitated by a stringent scheme for positive genetic complementation. We first identified conditions in which there is a substantial difference in survival between bacteria expressing and not expressing active human MGMT. *E. coli* containing deletions of the two alkyltransferase genes *ada* and *ogt* were transformed with plasmids encoding either the wild-type human MGMT gene or a dummy—i.e., the same gene containing a 1.7-kb insert designed to inactivate MGMT. Exposure of logarithmic phase cells to a single dose of 10 μg/ml MNNG reduced survival to 37% of untreated control in cells with the wild-type gene and to 1.9% in cells with the dummy, a 20-fold

Table 1. Survival of multiple MNNG treatments

Round	Wild type	Dummy	Fold difference
1	3.7×10^{-1}	1.9×10^{-2}	20
2	1.4×10^{-1}	1.8×10^{-5}	7,800
3	1.3×10^{-2}	$<7 \times 10^{-7}$	>19,000

Cultures of GWR111 cells expressing either wild-type or inactivated (dummy) human MGMT were treated with 10 μg/ml MNNG. A small amount of culture was diluted and plated after each round, the remainder allowed to recover for 1 hr, and the treatment with MNNG repeated.

difference (Table 1). Increasing the dose to 25 μg/ml increased the survival difference to 122-fold, and increasing the dose to 50 μg/ml yielded a 425-fold difference (not shown). To achieve a greater discrimination, we carried out three rounds of exposure to 10 μg/ml MNNG, each round separated by a 1-hr recovery period (Table 1). It was hypothesized that during this recovery period plasmids encoding active MGMT would repopulate their hosts with the enzyme that had been depleted during the previous MNNG exposure and would thus allow substantial resistance during the following round, whereas cells with inactive MGMTs would not be protected. Indeed, additional doses had relatively small cytotoxic effects on cells with the wild-type MGMT but a multiplicative effect on the bacteria containing the dummy MGMT. After the second round, the survival of cells encoding the wild-type MGMT dropped to 14%, whereas those with the dummy vector dropped to 1.8×10^{-5}, a 7800-fold difference. After the third round the surviving fractions were 1.3% and $<7 \times 10^{-7}$ respectively, a difference of >19,000-fold. This stringent selection scheme not only minimized false positives but also increased the probability of selecting rare mutants with enhanced MNNG resistance.

Identification of Active Mutant MGMTs. Twelve codons surrounding but not including the active cysteine of the human MGMT were randomized by replacing a cDNA segment corresponding to part of the active site with a partially random nucleotide sequence (Fig. 1). Plasmids carrying the randomized human MGMTs were transformed into alkyltransferase-deficient *E. coli*; this pool of 4.1×10^5 carbenicillin-resistant transformants was amplified by growing the bacteria and freezing aliquots. The randomized region from 28 unselected colonies was sequenced and multiple substitutions observed, averaging 4.2 amino acid changes (Fig. 2*A*) and 7.4 nucleotide changes (not shown) per plasmid. One stop codon was observed. These numbers approximate the theoretical number of substitutions expected in the absence of any selective pressure. Several different amino acid changes were observed at each position (not shown). Thus, the library contained the predicted diversity.

Two aliquots of the amplified random library were then independently subjected to three sequential rounds of 10 μg/ml MNNG selection as described above. The surviving fraction each time was $\approx 4 \times 10^{-5}$, indicating that most of the mutants are unable to confer resistance to MNNG (compared to 1% survival for wild-type MGMT; Table 1). The colonies formed were of different sizes, indicating different growth rates and probably different MGMT activities. The largest colonies, those which most closely resembled those from MNNG-untreated cultures, were picked for further analysis. Both strands of the randomized region and ≈50 contiguous bp upstream and downstream were sequenced from a total of 81 colonies. The two independent selection experiments gave similar results: (*i*) Few amino acid changes were observed in plasmids isolated from surviving colonies. From an initial library averaging 4.2 amino acid changes per mutant, the selected mutants averaged only 1.2 amino acid changes per mutant (Fig. 2*B*). This result is in accordance with the high degree of sequence conservation among alkyltransferases. (*ii*)

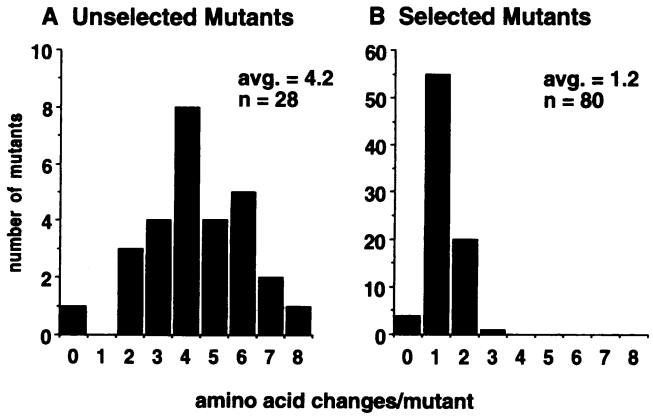

FIG. 2. Number of deduced amino acid changes. (*A*) Unselected mutants. An untreated aliquot of the random library was spread on plates, grown, plasmids recovered, and the randomized region from 28 colonies sequenced. The average number of amino acid changes per mutant is 4.2. (*B*) Selected mutants. The library was subjected to MNNG as indicated in the text and plated. The randomized region from 81 surviving colonies was sequenced. The average number of amino acid changes per surviving mutant is 1.2.

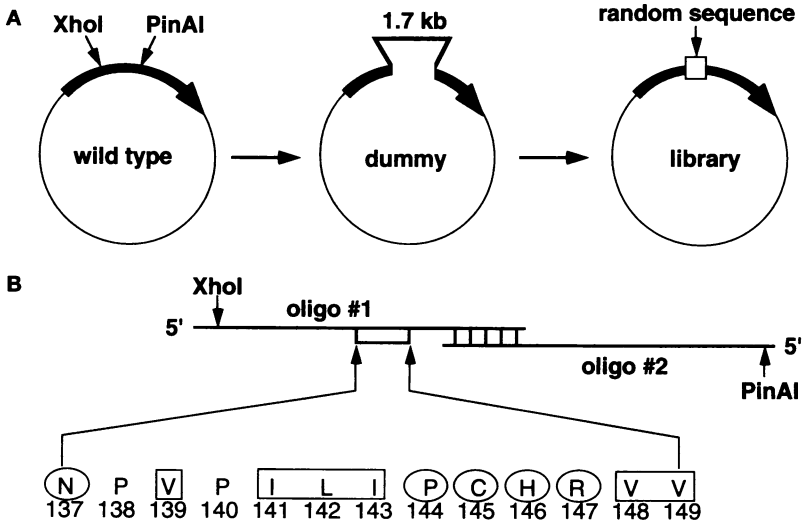

FIG. 1. Random library construction. (*A*) Plasmids. A translationally silent *Xho*I site was created at coding nucleotide 379 of the human MGMT cDNA (arrow) inserted into pUC118. The 141-bp *Xho*I–*Pin*AI fragment was replaced by a 1.7-kb stuffer fragment from λ DNA to construct the dummy vector. Replacing the stuffer fragment with the randomized cassette yielded the random library. (*B*) Randomized cassette. The relevant human wild-type codons are shown, with circled codons indicating absolute evolutionary conservation and boxed codons indicating conservation with respect to hydrophobicity and charge (9, 12). The 36 random nt in oligo 1 corresponding to amino acids 137–144 and 146–149 consisted of a mixture of 82% wild-type sequence and 6% of each of the other nucleotides; C145 was 100% wild type. Oligos 1 and 2 were annealed, extended by Klenow, and PCR-amplified. The *Xho*I–*Pin*AI fragment from the resulting pool of duplex DNA replaced the stuffer fragment of the dummy vector.

There were no amino acid substitutions at several positions (Fig. 3). All of the randomized positions that are absolutely conserved evolutionarily (N137, P144, H146, R147) were immutable, as was one other position (I143). This lack of amino acid substitutions occurred in spite of large numbers of nucleotide substitutions at each of these positions. In addition, many mutations at other positions were conservative—e.g., isoleucine for valine or leucine. (*iii*) The selected population was not significantly enriched for wild-type MGMT compared to the unselected (4/81 = 5% for selected and 1/28 = 4% for unselected; Fig. 2). (*iv*) Most unexpectedly, the same mutation, V139F, was found in 70% (57/81) of the selected colonies. Of the mutants with only 1 substitution, 43/56 had this mutation (Fig. 3*A*); likewise, V139F was one of the substitutions in 14 of the 21 selected mutants with multiple mutations (Fig. 3*B*). In summary, this selection protocol favored one mutant over all others including wild type.

The dominance of a single active mutation in this random library was not due to contamination. Of the 57 colonies harboring this mutation, only 2 shared the same nucleotide sequence throughout the randomized region. Both phenylalanine codons (TTC and TTT) were represented, the former requiring one nucleotide change from the wild-type GTC valine codon and the latter requiring two changes. Furthermore, the random library was not biased toward the V139F mutation: of the 28 unselected mutants sequenced, 11 had amino acid substitutions at this position, but only 2 of those were phenylalanine (not shown).

Analysis of V139F in *E. coli*. A V139F single mutant was analyzed for its ability to protect GWR111 from MNNG. The entire MGMT gene was sequenced from this mutant and shown to contain no other coding differences from wild type. The expression levels of V139F and wild-type MGMT, as detected by [^{35}S]methionine pulse-chase, were indistinguishable (data not shown). In one set of experiments, cells were treated with varying doses of MNNG and the surviving fraction determined. Survival of *E. coli* harboring V139F was greater than those harboring the wild-type plasmid (Fig. 4*A*). The D_{37}s, defined as the dose at which survival on the exponential portion of the curve is reduced to 37% and taking into account any threshold dose (23), were: V139F, 17 μg/ml; wild type, 4 μg/ml; and dummy, 2 μg/ml. This improved survival provides an independent verification of the results presented above in which the V139F mutant was selected from the random library much more frequently than the wild type. In a second set of experiments, cells were treated with varying doses of MNNG and the fraction of viable cells able to form colonies on rifampicin-containing medium determined. Resistance to rifampicin results from mutations of RNA polymerase (24) and is often used as an indicator of cellular mutation frequencies. The mutation frequency was lower (between 2.7-fold and 5.5-fold for different MNNG concentrations) in V139F than in wild type (Fig. 4*B*), implying enhanced repair of MNNG-induced promutagenic lesions. Thus, V139F makes *E. coli* more resistant to both the cytotoxic and mutagenic effects of the methylating agent MNNG.

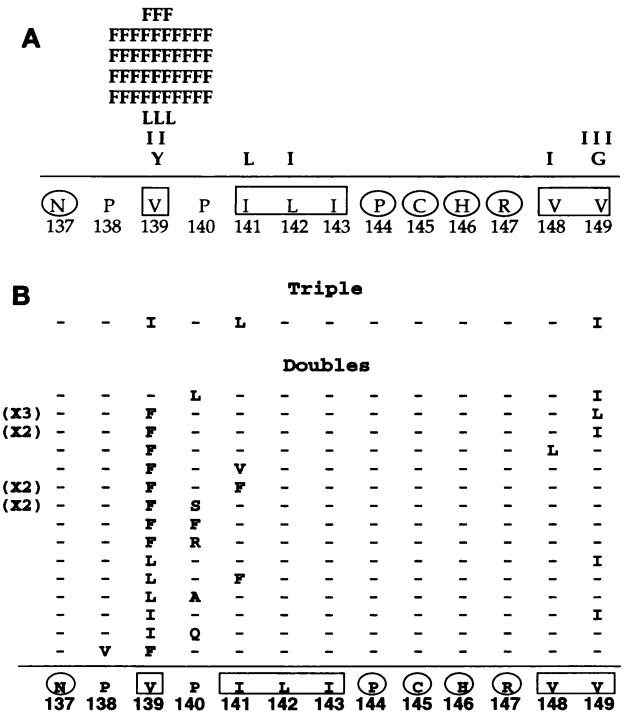

FIG. 3. Deduced amino acid changes in active MGMT mutants selected by MNNG resistance. The wild-type sequence is indicated below the solid lines and the evolutionarily conserved positions labeled as in Fig. 1. (*A*) Mutants with only one coding change from wild type. Each of the 56 single mutants is represented by a single entry. (*B*) Mutants with two or three amino acid changes. The double mutants recovered more than once are indicated (×2 or ×3).

DISCUSSION

We have used random sequence substitutions to alter the active site of the human gene that encodes O^6-methylguanine-DNA methyltransferase. This protein repairs DNA damaged by alkylating agents commonly used in cancer chemotherapy, and presumably by endogenous cellular metabolic processes that generate active alkyl groups that damage DNA. Inactivation of alkyltransferases in virtually all cells examined has been shown to render them susceptible to the cytotoxic and mutagenic effects of alkylating compounds. Conversely, overexpression of alkyltransferases renders cells resistant to alkylating agents. Although alkylating agents have been very effective in the treatment of human malignancies, their use has been limited by toxicity to bone marrow and other tissues. The introduction and enhanced expression of human MGMT in mouse bone marrow cells has been reported to protect cells both *in vitro* and *in vivo* from nitrosourea (5, 6). These results suggest the feasibility of using MGMT in gene therapy to reduce cytotoxic side effects and/or to allow the use of increased amounts of alkylating agents in chemotherapy. The use of the human MGMT is advantageous because it minimizes an immunological response. Gene therapy directed at the protection of bone marrow may be more easily achieved than that directed at obliteration of tumor cells. Theoretically, transfection of only a fraction of the stem cell population may

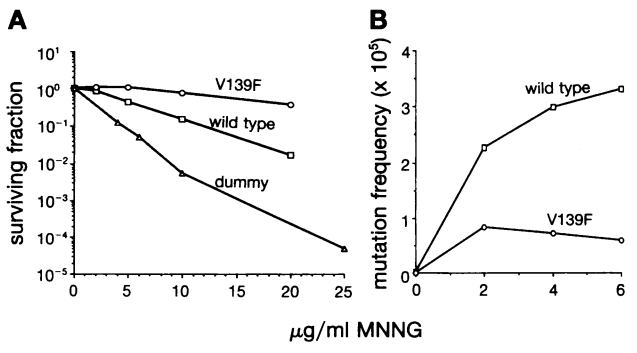

FIG. 4. MNNG-induced cytotoxicity and mutagenesis. GWR111 cultures containing the dummy vector (see *Materials and Methods*) or plasmids expressing either the wild-type or mutant V139F human MGMT were treated with graded amounts of MNNG and spread on (*A*) growth media plates to determine survival or (*B*) plates containing 100 μg/ml rifampicin to determine mutation frequency (not done for dummy strain). Average values of at least two independent experiments are shown. □, wild type; ○, V139F; (Δ) dummy.

give them a selective advantage in repopulating the bone marrow after exposure to toxic agents.

As a first step toward creating an MGMT protein with increased activity and with directed substrate specificity, we employed random sequence substitutions and selected for new active MGMTs. The concept is to create a large library of mutant genes that contain random nucleotide substitutions at designated regions and to use positive genetic selection to obtain new active molecules (25). In previous studies with β-lactamase we (26) and others (27) obtained a large number of different active mutants. In the case of herpes thymidine kinase, we obtained over 3000 new active mutants from a total of four libraries in which we screened more than 4×10^6 transformants (28, 29). In contrast, only a few substitutions yield an active human MGMT. This finding is in accordance with the high degree of conservation around the active site and is also consistent with the results of two previous studies on the human MGMT (11) and *E. coli* Ogt (12) in which single codons were randomly substituted. The lack of flexibility implies rigid functional requirements for protein-substrate interactions. Interestingly, the reported crystal structure for the *E. coli* Ada C-terminal fragment shows the active cysteine to be buried (16). The authors suggest that a conformational change is necessary for activity, but it is also possible that the alkylated base could be flipped out 180° from the DNA and into the active site (17) in much the same way that substrates for methylases that add methyl groups to DNA behave (30, 31). Base flipping may be a common mechanism by which proteins precisely modify nucleotides in DNA (17), and has been directly observed or strongly suggested by the crystal structures of the repair proteins uracil–DNA glycosylase (32, 33), exonuclease III (34), and photolyase (35). If alkyltransferases conduct their transfer reactions in such a way, the stringent amino acid requirements would not be surprising.

The consistent selection of a single MGMT mutant that provides enhanced alkylation resistance is surprising. Why hasn't this mutant, which requires only a single nucleotide change, been selected during natural evolution? Conceivably this mutant could be selected against during the course of evolution due to uncharacterized deleterious effects unrelated to O^6-alkylguanine repair.

The V139F mutant MGMT described here, or others similarly created and selected, might be advantageous for gene therapy directed at the protection of bone marrow against the toxic effects of alkylating agents. The normally limiting low transfection efficiencies of gene therapy may suffice when followed by the positive selection provided by the alkylation treatment, in contrast to the negative selection for tumoricidal gene therapy or the lack of genetic selection in the case of gene replacement. Positive selection could allow a relatively small number of resistant cells to repopulate the bone marrow. In addition, MGMT can be introduced with a strong promoter allowing overexpression of the human MGMT as has been demonstrated for the wild-type human MGMT in mouse bone marrow cells (6). Furthermore, resistance could be further enhanced by cotransfecting other proteins that scavenge alkylating agents in the cytoplasm (36). Optimized gene therapy strategies will likely involve mutant proteins created by methods such as the one described here and have the potential to allow dose escalation while reducing side effects.

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