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Analysis of Forward Mutations Induced by *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine in the Bacteriophage P22 *mnt* Repressor Gene

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We describe the isolation and genetic characterization of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced mutations in the phage P22 *mnt* repressor gene cloned in plasmid pBR322. Mutations in the *mnt* repressor gene or its operator on this plasmid, pPY98, confer a tetracycline resistance phenotype, whereas the wild-type plasmid confers tetracycline sensitivity. Cells carrying pPY98 were briefly exposed to MNNG to give 20 to 40% survival and a 50- to 100-fold increase in tetracycline-resistant cells. DNA sequence analysis showed that 29 of 30 MNNG-induced mutations were GC-to-AT transitions and one was an AT-to-GC transition. About 80% of the mutations are in three hotspots. This mutation spectrum is consistent with the proposed mechanism of mutagenic action of MNNG, which involves mispairing of an alkylated base, O^6-methylguanine. The *mnt* gene may be a useful target for determining mutagenic specificity at the nucleotide level because (i) forward mutations are easily isolated, (ii) the target size is small, and (iii) the DNA sequence changes of mutations can be determined rapidly.

To determine the mutagenic specificity of an agent, genetic alterations in a target should be easy to select and identify. The target sequence should be such that a variety of different kinds of mutations (single base-pair substitutions, frameshifts, insertions, and deletions) can be characterized by rapid DNA sequencing. We describe the mutation spectrum of mutations induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) on a plasmid target carrying the small bacteriophage P22 *mnt* repressor gene and demonstrate that this target satisfies these criteria.

MNNG belongs to a class of agents that form methylated bases in DNA both in vitro and in vivo (12, 24). In addition to producing abundant 7-methylguanine and 3-methyladenine adducts, MNNG treatment also produces a relatively high proportion (7%) of O^6-methylguanine (m^6G) (10). The m^6G residues are probably responsible for the high mutagenic activity of MNNG. One line of evidence implicating m^6G adducts in mutagenesis by alkylating agents is the DNA repair pathway termed the adaptive response (18). This response is induced by growth in the presence of sublethal concentrations of alkylating agents and confers resistance to the cytotoxic and mutagenic effects of subsequent exposures to higher concentrations of such agents. One of the proteins induced during the adaptive response is a methyltransferase that removes methyl groups from m^6G residues (17, 21). Mutant strains of *Escherichia coli* that lack this enzyme or produce it constitutively show hypermutability or reduced mutability, respectively, when challenged with MNNG.

The m^6G residues in replicating DNA can pair with either cytosine or thymine (14). Pairing with thymine should induce GC-to-AT transition mutations, and an analysis of MNNG-induced nonsense mutations in the *lacI* gene of *E. coli* supports this view (3). In the *lacI* target gene, AT-to-GC transitions were found to represent 1 to 5% of all substitutions, whereas GC-to-AT base changes composed the majority of mutations. Since these genetic studies were limited to substitutions that generate nonsense codons, we considered the possibility that the observed spectrum of mutations in the *lacI* gene was biased by sampling only those events leading to nonsense codons.

To determine the base pair changes at the nucleotide level after MNNG mutagenesis, we examined the spectrum of mutations that inactivate the 254-base-pair (bp) *mnt* repressor gene of phage P22 (20) without the constraint of sampling only nonsense mutations. The *mnt* gene, which is part of the *imm*I region of phage P22, produces a repressor that binds a single operator (o_{mnt}) to prevent transcription from the *ant* promoter (see Fig. 1). Youderian et al. (27) have described a plasmid (pMS96), containing a p_{ant}-*tetA* operon fusion, used to isolate mutations in *mnt*. Mutations on this plasmid either in the *mnt* structural gene or in its operator result in activation of transcription of the *tetA* gene from the *ant* promoter to confer a tetracycline resistance phenotype. We have used a derivative of pMS96, pPY98, that retains the p_{ant}-*tetA* fusion but is a more suitable substrate for enzymatic DNA sequencing, since it carries the phage M13 origin of replication (28). To test the utility of this plasmid as a target for determining mutational spectra, we have isolated and characterized 30 MNNG-induced mutations in *mnt*. All but one of these mutations are GC-to-AT base pair changes.

MATERIALS AND METHODS

Bacterial and viral strains. GM2621 is a derivative of *E. coli* K-12 JC9239 (F⁻ *recF143 thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 hisG4 argE3 rpsL31 tsx-33 glyU44*) harboring pPY98. JC9239 was donated by A. J. Clark. Phage λ imm^{P22} hybrid 1 (hy1) contains the *immC* region of phage P22 and λ imm^{P22} *dis* contains both the *immC* and *immI* regions of phage P22 (26). These phages were gifts of N. Yamamoto. Phage λ *vir* is from laboratory stocks. Phage IR-1 was a gift of N. Zinder.

Media. BH broth contains 20 g of brain heart infusion (Difco Laboratories) per liter; BH agar contains, in addition, 16 g of agar per liter. The minimal medium used was that

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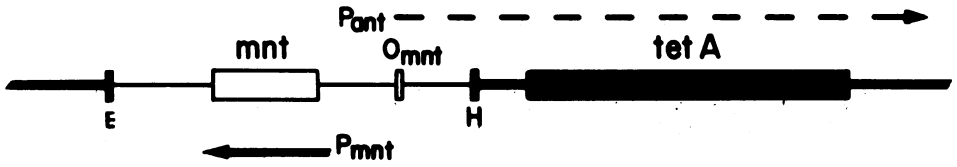

FIG. 1. The p_{ant}-*tetA* fusion operon. Mnt repressor binds to o_{mnt} (shown as O_{mnt} in Fig. 1), preventing rightward transcription of the *tetA* gene from p_{ant} (shown as P_{ant} in Fig. 1); plasmids carrying this fusion operon confer a tetracycline sensitive phenotype (27). Mutations in *mnt* or o_{mnt} prevent repressor binding and permit transcription of the *tetA* gene from p_{ant}, resulting in a tetracycline resistance phenotype. The 500-bp fragment of phage P22 DNA is indicated by a thin line. For plasmids pMQ151 and pPY98, H indicates the *Hin*dIII recognition site of pBR322, and E denotes the *Eco*RI recognition site.

described by Davis and Mingioli (4). C medium is minimal medium supplemented with 0.1% Casamino Acids (Difco). Antibiotics were used at the following concentrations: ampicillin, 40 μg/ml; chloramphenicol, 150 μg/ml; tetracycline, 10 μg/ml or 3.5 μg/ml for mutant selection.

Plasmid constructions. pMQ151 is a pBR322 derivative in which a 500-bp *Eco*RII-*Hin*dIII fragment, containing the *immI* region of phage P22 from pMS96 (27), was substituted for the 29-bp *Eco*RI-*Hin*dIII fragment of pBR322. pPY98, constructed by P. Youderian, is a derivative of pMQ151 containing the M13 *ori* region from pZ152 (28).

Preparation of plasmid DNA. Plasmid DNA was prepared essentially as described by Clewell and Helinski (2). Ethidium bromide was removed by extraction with isopropanol, and the DNA was precipitated with ethanol at −20°C overnight. After centrifugation, the DNA was dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA. The purity of plasmid DNA was routinely checked by electrophoresis in 1% agarose gels.

Isolation of tetracycline-resistant strains. GM2621 was grown in C medium at 37°C to 1×10^8 to 2×10^8 cells per ml and exposed to MNNG at 10 or 20 μg/ml for 5 min. The cells were centrifuged, washed twice, and resuspended in the original volume of minimal medium. Portions (0.01 ml) were added to 1 ml of BH broth plus ampicillin and incubated overnight at 37°C. After growth overnight, portions of 0.1 ml (about 10^8 cells) were inoculated onto BH plates containing 20 μg of ampicillin per ml and 3.5 μg of tetracycline per ml, and plates were incubated at 37°C. Single-mutant colonies were purified three times on media containing 10 μg of tetracycline per ml.

Identification of *mnt* mutants. Tetracycline-resistant bacteria were lysogenized with λ imm^{P22} hy1. Lysogens were identified as cells immune to λ imm^{P22} hy1 but sensitive to λ *vir*. Lysogens were also challenged with λ imm^{P22} *dis*. Those lysogens harboring plasmids with operator-constitutive mutations were immune to λ imm^{P22} *dis*, whereas those with plasmids containing *mnt* mutations allowed growth of this phage.

Purification of sequencing primer. A crude preparation of primer MM-1 (5′-CTCACAATACAGGTC-3′) was obtained from K. L. Taneja and purified by electrophoresis through and elution from a 20% acrylamide–7 M urea gel (15). Further purification was achieved by chromatography over a Sep-Pak C_{18} cartridge (Waters Associates, Inc.). The *Eco*RI clockwise sequencing primer was obtained from New England BioLabs, Inc.

DNA sequencing. Purified plasmid DNA (1 μg) was digested with *Pst*I, mixed with 5 ng of oligonucleotide primer, heated at 100°C for 3 min, and chilled on ice for 30 min. Primer extension by DNA polymerase (large fragment) in the presence of deoxyribonucleoside triphosphates and dideoxyribonucleoside triphosphates and ^{32}P-labeled deoxyadenosine triphosphate was carried out as described by Sanger et al. (19) and Wallace et al. (25). The labeled oligonucleotides were separated by electrophoresis in 8% acrylamide–7 M urea gels.

RESULTS

Experimental system. The plasmid used in this study, pPY98, is a derivative of pBR322 which contains a 500-bp fragment of bacteriophage P22 DNA substituted for the smaller *Eco*RI-*Hin*dIII region of pBR322 (Fig. 1). This fragment of P22 DNA contains the *mnt* structural gene, its promoter and operator, and the *mnt*-regulated *ant* promoter (Fig. 1).

In pPY98, the 3′ end of the *mnt* gene is located 27 bp from the *Eco*RI recognition site and thus allows use of commercially available primers for sequencing from this direction. DNA sequencing from the 5′ end of the *mnt* gene was achieved with a synthetic 15-bp primer complimentary to part of the *mnt* operator. Use of both primers allows overlapping sequencing of the whole *mnt* gene. To facilitate production of template strand for DNA sequencing, pPY98 also contains the M13 *ori* region (28). Superinfection of strain GM2621 (mnt^+) with the single-stranded phage IR-1 (5) yields a mixed lysate in which about one-half the particles are transducing particles containing single-stranded plasmid DNA. For reasons which are not clear, IR-1 superinfection of *mnt* mutants of GM2621 results in at least a 1,000-fold decrease in production of single-stranded plasmid DNA. For this reason, we abandoned using IR-1 superinfection to prepare substrate DNA for sequencing and used linearized, double-stranded plasmid DNA as our substrate.

Mutation induction. Cells containing the mnt^+ plasmid, pPY98, are sensitive to tetracycline. A mutation in either *mnt* or its operator on this plasmid will allow transcription of *tetA* from p_{ant} to produce tetracycline-resistant cells. Mutations in the plasmid were induced after a 5-min exposure to 10 or 20 μg of MNNG per ml. These exposures result in about 40 and 20% survival, respectively. The mutation frequency to tetracycline resistance increased in a dose-dependent manner by factors of about 50 and 100, respectively, from a basal level of approximately 1 tetracycline-resistant mutant per 10^7 cells plated.

Identification of mnt^- plasmids. To distinguish between mutations in *mnt* or its operator, tetracycline-resistant strains were lysogenized with a hybrid λ phage (λ imm^{P22} hy1) which contains the *immC* region of phage P22 (26). These lysogens were superinfected with λ imm^{P22} *dis*, which contains both *immI* and *immC* regions of P22 (26). Lysogens harboring plasmids with operator-constitutive mutations are immune to λ imm^{P22} *dis* because they produce active *mnt* product, but mnt^- lysogens permit growth of λ imm^{P22} *dis*. Mnt protein indirectly regulates early gene expression by regulating the level of antirepressor formed by the

superinfecting phage (23). If antirepressor is produced (i.e., in the absence of *mnt* protein), P22 c2 repressor is inactivated and the phage grows lytically. Alternatively, in the presence of *mnt* protein, c2 product prevents transcription from the early P22 promoters, and the superinfecting phage fails to develop.

Approximately 50% of the MNNG-induced isolates were found to carry *mnt* mutations as judged by this test; however, about 10 to 20% of these isolates were subsequently shown to carry operator-constitutive mutations by DNA sequence analysis.

We also isolated 40 independent tetracycline-resistant derivatives of our starting strain that arose spontaneously. All of these carry operator-constitutive mutations. This result indicates that MNNG expands the mutation spectrum to include *mnt* mutations and that such mutations have a high probability of arising from the mutagenic treatment. Operator-constitutive mutations were not considered further because they arise by changes in only a very limited number of base pairs and are representative of the background of spontaneous mutation.

DNA sequence analysis. The results from sequencing 30 independent MNNG-induced *mnt* mutations are shown in Table 1. All are transition mutations; 29 are GC-to-AT base pair changes, and 1 is an AT-to-GC base pair change. Although the *mnt* gene comprises 252 bp, mutations were found only in the first 109 bp, which code for the amino-terminal end of the repressor. No mutations were found in the *mnt* promoter. Three prominent hotspots (at positions 24, 40, and 52 and 53) are evident. One of these (position 24) changes the initiation codon for fMet to Ile, presumably prevents initiation of *mnt* translation, and represents 17% of all mutations analyzed. The second hotspot, at position 40, changes His6 to Tyr and represents 23% of all mutational changes. Since other data (27) strongly suggest that this amino acid is in direct contact with DNA, this mutation probably results in a loss of affinity of Mnt repressor for its operator. The third hotspot (40% of all mutations analyzed) includes positions 52 and 53, which are in the codon for Arg10, and results in substitution of Cys and His for Arg. At present, it is not known why this residue is important for Mnt function. It may be in direct contact with DNA or it might be critical for determining the native conformation of the repressor. The three hotspots account for 80% of all mutations. The change at position 109 produces an ochre mutation. A GC-to-AT transition at bp 118 should also result in an ochre mutation, but this change was not detected. No amber mutations were expected because the host strain carries the *glnU44* (*supE44*) amber suppressor, and no amber mutations can be generated in the first 120 bp of *mnt* by AT-to-GC or GC-to-AT transitions. The single AT-to-GC transition occurs at bp 28 (Arg2 to Gly).

TABLE 1. Analysis of 30 mutations induced by MNNG in the *mnt* gene[a]

Base pair	No. of mutations	Codon change	Amino acid change
24	5	ATG → ATA	fMet → Ile
28	1	AGA → GGA	Arg2 → Gly
40	7	CAC → TAC	His6 → Tyr
52	9	CGT → TGT	Arg10 → Cys
53	3	CGT → CAT	Arg10 → His
91	1	GCG → ACG	Ala24 → Thr
110	2	TCA → TTA	Ser29 → Leu
121	1	GAG → AAG	Glu33 → Lys
130	1	CAA → TAA	Gln36 → Stop

[a] The transcription start site is designated base number 1. The gene comprises 252 bp and codes for a protein with 82 amino acids.

DISCUSSION

This work demonstrates that the *mnt* gene is a suitable target for the analysis of mutation spectra. The selection of tetracycline-resistant derivatives of cells carrying pPY98 is not difficult, and the development of a rapid spot test to discern *mnt* mutations from operator mutations reveals a spectrum of mutations easily distinguished from the background spontaneous spectrum.

To determine the specificity of various mutagenic agents, several genes have been used as targets. These include *lacI* (3), the bacteriophage lambda repressor gene, *c*I (22), the alpha-complementing subunit of the *lacZ* gene in bacteriophage M13 (11), and the *tetA* gene of pBR322 (9). The *c*I and *lacZ* systems are those which have been used most extensively for DNA sequence analysis of mutational alterations. Mutations in *mnt* are as easy to detect and isolate as in the other two systems and can be analyzed as readily at the DNA sequence level. The *mnt* system can be manipulated by conventional genetic techniques (23), and revertants with a Mnt^+ phenotype can be isolated from mnt^- strains (P. Lucchesi and M. G. Marinus, unpublished data).

Another major conclusion of this work is that MNNG produces predominantly GC-to-AT base pair changes. This conclusion is in accord with previous genetic and biochemical data (12, 24) that support the hypothesis that m^6G is responsible for the high mutagenicity of MNNG. We have shown that 29 of 30 mutational changes induced by MNNG are GC-to-AT transitions. Loechler et al. (13), using a different approach, have shown that all 60 changes at a *Pst*I recognition site induced by m^6G are GC-to-AT transitions.

One of the mutations in *mnt* was an AT-to-GC transition. We cannot exclude the possibility that this represents a spontaneous mutation, but we think this is unlikely. Spontaneous *mnt* mutations are rare. None were detected among 40 spontaneous tetracycline-resistant derivatives isolated from GM2621. It seems more probable that it resulted from MNNG action, perhaps due to formation of O^4-methylthymine. Presumably, the latter methylated base is capable of pairing with guanine to produce an AT-to-GC transition. In this respect, Coulondre and Miller (3) detected MNNG-induced AT-to-GC transitions (1 to 5%) at about the same frequency as in this work (3%).

In this study, a *recF* host was used to prevent multimer formation of plasmid DNA. Such multimers can introduce sequencing errors because in other experiments with *dam* mutants, we have detected several instances of multimers composed of wild-type and mutant monomers. The formation of such multimers obscures the DNA sequence analysis of mutational sites. We have no reason to believe that $recF^-$ affects MNNG mutagenesis, since we have found that the kinetics of mutation induction is the same in $recF^+$ and $recF^-$ strains (unpublished data). The only difference we have found between $recF^-$ and $recF^+$ strains is that the spontaneous level of mutation to tetracycline resistance is 5- to 10-fold higher in the $recF^-$ strain.

The rate-limiting step in this study was purifying plasmid DNA for DNA sequencing. We had hoped to use the single-strand phage superinfection method described by Dente et al. (5) and Zagursky and Berman (28). For reasons that are not clear, plasmid DNA was not packaged at high frequency in cells with mnt^- plasmids. The insertion of

tandem transcription terminators at the end of the *tetA* gene did not help, nor did changing the multiplicity of infection.

The present data also show that all the mutations analyzed mapped to the region corresponding to the amino-terminal half of the repressor. This may indicate that, like lambda *c*I product (8), the amino-terminal portion of *mnt* may be more important in binding to DNA. Perhaps the carboxy-terminal end of the protein is involved in multimer formation. The *mnt* mutations described here may aid in determining the mechanism of repressor binding to its operator. Surprisingly, no mutations were detected in the *mnt* promoter. GC-to-AT or AT-to-GC transitions in the −10 or −35 regions should decrease promoter strength enough to affect Mnt phenotype (7, 23). We have no explanation as to why promoter mutations were not recovered other than a difference in target size.

We found in this study that MNNG produced only a single base change in the *mnt* gene of each mutant. This contrasts with earlier data showing that MNNG induces multiple closely linked mutations (6). We believe that the high dose (100 μg/ml) and amount of time (60 min) in standard treatment protocols (1, 16) are responsible for this effect. In the studies reported in this paper, 10 μg of MNNG for 5 min in *recF*$^-$ (or wild-type, unpublished data) cells results in substantial mutagenesis.

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