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Sequence specificity of streptozotocin-induced mutations

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**ABSTRACT**

The isolation and characterization of streptozotocin (STZ)-induced mutations in the phage P22 mnt repressor gene is described. Cells carrying the plasmid-borne mnt gene were exposed to STZ to give 10-20 percent survival and at least an eleven-fold increase in mutation frequency. DNA sequence analysis showed that 50 of 51 STZ-induced mutations were GC to AT transitions, and one was an AT to GC transition. We have also compared the STZ mutational spectrum to that for N-methyl-N'-nitro-N-nitroso-guanidine (MNNG). There are sites in the mnt gene which are mutated only by STZ; only by MNNG, or by both agents. Sites at which only STZ induced GC to AT transition mutations occur were in sequences that are pyrimidine rich 5' to the mutated site and purine rich 3' to the mutated site. Induction of mutations by both STZ and MNNG should be considered to maximize the number of mutable sites.

**INTRODUCTION**

Streptozotocin (STZ) is a monofunctional nitrosourea which is a potent mutagen and carcinogen (1). It is used in the treatment of patients with islet cell tumors and can also produce insulinitis in experimental animals (2). In an effort to determine the underlying mechanisms of these drug effects, we have studied the response of Escherichia coli cells to STZ exposure.

We have previously shown that E. coli ada and alkA mutants are more sensitive to STZ, suggesting that the adaptive response is an important factor in cell survival (3). The adaptive response promotes transcription of certain genes which enhance survival and decrease mutagenesis after exposure to alkylating agents. The ada gene product acts as a positive regulator of this regulon in addition to its role as an alkyl transferase. After transfer of alkyl groups from phosphotriesters in DNA to Ada protein, the methylated Ada protein increases transcription

of ada, alkA, alkB and aidB genes. The alkA gene specifies 3-methyladenine DNA glycosylase II, while the products of the alkB and aidB genes are not yet known. As expected from the above, STZ exposure induces transcription of ada, aidB alkA, and aidD (but not aidC), suggesting that STZ causes formation of methylphosphotriesters in DNA (4).

E. coli recA but not uvrA mutants are also more sensitive to STZ than wild type (3). This indicates that the UvrABC endonuclease is not required for repair of cytotoxic STZ lesions. The requirement for RecA protein in cell survival could be its function in either post-replication repair and/or its role in the SOS response.

STZ is a potent mutagen. Our prior studies showed that pre-mutagenic lesions were repaired by O<sup>6</sup>-alkylguanine-DNA alkyl transferase since an ada mutant strain was hypermutable compared to the same strain that contained a wild type ada gene on a plasmid (3). Furthermore, an inverse correlation was observed between the amount of Ada protein in the cell and the level of mutagenesis. This mechanism of Ada-dependent mutagenesis is common among methylating agents, such as N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG), which produce O<sup>6</sup>-methylguanine (O<sup>6</sup>-MeG) alkylation (5).

We have previously determined the mutagenic specificity of MNNG using a forward mutation system (6). We characterized and sequenced mutations induced in the phage P22 mnt repressor gene present in cells on a multicopy pBR322 derived plasmid. We now have used the same DNA target to define mutational sites induced by STZ, and compared the specificity of the two mutagens. Although both agents are Ada-dependent mutagens the location of the mutations induced by each is significantly different.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

Strain GM3136 is AB1157 (7) containing F-42 and plasmid pPY97. Plasmid pPY97 is identical to pPY98 (6) except that the M13 origin of replication is inverted. Strain MM294 (8) was obtained from the Coli Genetic Stock Center, Department of Biology, Yale University, Box 6666, New Haven, CT 06520, USA.

### Isolation of tetracycline-resistant strains

Strain GM3136 was grown at 37 C in Brain Heart Infusion broth (BHA broth; Difco; 20 g per liter) containing 40 ug ampicillin per ml to approximately  $1 \times 10^8$  cells per ml. The culture was centrifuged and resuspended in minimal salts medium (9) without glucose. STZ (Sigma Chemical Corp.) was added to 100 uM final concentration and after 30 min incubation at 37 C, the cells were harvested, washed and 10 ul portions diluted into several 1 ml BHA broths and incubated overnight at 37 C. Next day, 0.1 ml from each broth culture was inoculated onto BHA agar containing 3.5 ug tetracycline per ml and incubated overnight at 37 C. A single mutant colony from each plate was purified three times on media containing 10 ug tetracycline per ml.

### Identification of mnt mutants

Each tetracycline-resistant colony was tested genetically as described (6), to identify those containing mutations in the mnt region.

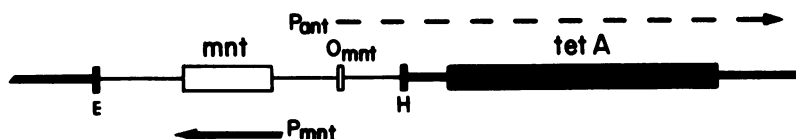
### DNA sequencing analysis

Plasmid DNA from tetracycline-resistant mnt derivatives of strain GM3136 was isolated (10) and used to transform strain MM294 to tetracycline-resistance. This step ensured that plasmid DNA for sequencing was derived from a single molecule, and in addition, DNA isolated from an enda mutant strain (MM294) gave more consistent DNA sequencing results. Isolation of plasmid DNA was as described (11,12) and sequenced according to the enzymatic method of Sanger et al (13) as previously described (8).

## RESULTS

### Experimental system

The experimental system to identify mutations has been described in detail (6,8) and therefore only a summary is given here. We use the 252 base-pair mnt gene as a target to monitor mutations. This gene is present on a pBR322 derivative (pPY97) as an operon fusion (Figure 1). Mnt repressor binds to its operator preventing transcription from the ant promoter and thus cells which are Mnt<sup>+</sup> are tetracycline sensitive. Mutations in mnt or its operator prevent repressor binding and permit transcription of the tetA gene from the ant promoter, resulting in a



**Figure 1.** Genetic map of the mnt-tetA fusion region. The heavy lines indicate pBR322 DNA and E and H denote the EcoRI and HindIII recognition sites respectively. The thin line indicates phage P22 DNA and shows the mnt gene and its operator (O<sub>mnt</sub>), and the promoters for the ant and mnt genes. Mutation in mnt promotes transcription from the ant promoter through the tetA gene to yield a tetracycline-resistant phenotype.

tetracycline-resistant phenotype. A simple genetic test can distinguish mutations in mnt from those in the operator region (6). Plasmid DNA from mnt mutant strains is isolated by a rapid mini-prep method (11,12) and subjected to enzymatic DNA sequencing (13).

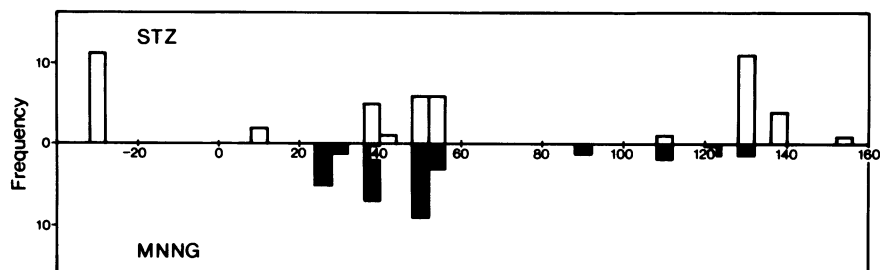
#### Mutation induction

Cells were exposed to 100  $\mu$ M STZ for 30 min which resulted in 10-20 percent survival. The mutation frequency increased by

**Table 1.** Location of mutations in the mnt gene arising after induction with STZ.

Number	Position	Codon change	Amino Acid Change
13	-33	G to A	Promoter
2	+12	G to A	Ribosome binding site
5	+40	CAC to TAC	His6 to Tyr
1	+41	CAC to CGC	His6 to Arg
6	+52	CGT to TGT	Arg10 to Cys
6	+53	CGT to CAT	Arg10 to His
2	+110	TCA to TTA	Ser29 to Leu
11	+130	CAA to TAA	Gln36 to Stop
4	+139	CAA to TAA	Gln39 to Stop
1	+152	TCA to TTA	Ser43 to Leu

Bases are numbered with respect to the starting point of transcription (+1) of the mnt gene. The translation initiation codon is at position 21-23.



**Figure 2.** Sites in the *mnt* gene mutated by STZ and MNNG. The number and position of identified mutations induced by STZ and MNNG are shown above and below the base line respectively.

at least 11-fold from a basal level of approximately 1 tetracycline-resistant mutant per  $10^8$  cells plated.

#### STZ-induced mutations

Table 1 shows the results from sequencing 51 independent STZ-induced mutations. All are transition mutations: 50 are GC to AT base pair changes, and one is an AT to GC base pair change. Of these mutations, 13 are in the promoter, 2 in the region corresponding to the 5'-untranslated leader mRNA, and the rest in the coding sequence of the *mnt* gene. Mutations in the coding region are restricted to the first 152 bases of the 252 base pair gene. This region of the *mnt* gene codes for the amino terminal end of the repressor which is required for DNA binding.

Table 1 also shows that nine GC base pairs at various sites in the DNA were mutated by STZ. Of these, six G residues are in the non-transcribed strand, and three on the transcribed strand, suggesting a strand preference for mutability. On the other hand, the total number of mutations occurring on the transcribed and non-transcribed strands, was 21 and 29, respectively.

Two sites (-33 and +130) are major "hotspots" for mutation. One of these is in the -35 region of the *mnt* promoter, and decreases promoter strength. The change at site 130 results in the generation of a translational stop codon. Together the mutations at the hotspots comprise 24/51, or about half of all the mutations (Fig. 2).

#### Sequence specificity

Fig. 2 and Table 2 show the sites in the *mnt* gene mutated by

Table 2. Effect of surrounding nucleotide sequence on mutational specificity of MNNG and STZ.

Sites mutable by MNNG only	Sites mutable by MNNG and STZ	Sites mutable by STZ only
+24 CATG G GCTA	+40 AAGT G CGGA	-33 TATT G AGAA
+91 CAGG G CGGA	+52 ATAC G GAAG	+12 CTTG G AGTG
+121 CTCC G AGTT	+53 TTCC G TATG	+139 TCTT G GACG
	+110 CATT G ATCT	+152 TTTT G ATAG
	+130 ATTT G TACC	

The numbers refer to sites where G to A mutations have occurred and these are indicated by the middle G in each sequence. For each sequence the four upstream and the four downstream nucleotides surrounding the mutated G are included.

STZ and MNNG. These sites can be divided into three types: those mutable by STZ only; or by MNNG only; or by both agents (Table 2). To examine the effect of flanking DNA sequence on mutation specificity, the number of purines and pyrimidines four bases before and after the mutated guanine residue has been determined. For the MNNG only class, at sites 24, 91 and 121, there are a total of 5 purines and 7 pyrimidines 5' to the mutated G and 7 purines and 5 pyrimidines on the 3' side. For sites mutated by both agents, there are 7 purines and 13 pyrimidines 5' to the mutated G and 12 purines and 8 pyrimidines 3'. That is, the frequency of purines and pyrimidines is about the same upstream or downstream of the mutational site. For the STZ only class, however, there is a striking difference in purine and pyrimidine frequency compared to the other two classes. There are two purines and 14 pyrimidines upstream of the mutated G, and 13 purines and 3 pyrimidines downstream. Therefore, sequences containing a central GC base pair with a 5' pyrimidine-rich region and a 3' purine-rich region are particularly prone to mutation by STZ.

## DISCUSSION

STZ produces predominantly GC to AT base pair changes. This observation is in accord with the hypothesis that O<sup>6</sup>-MeG is respo-

nsible for the high mutagenicity of STZ. O<sup>6</sup>-MeG pairing with thymine (14) produces GC to AT transition mutations (6,15,16). Our previous data (4), showing that ada mutants are hypermutable by STZ and that overproduction of Ada protein reduces mutagenesis, are in agreement with this hypothesis. O<sup>6</sup>-MeG has been detected in DNA after exposure of cells to STZ (17).

One of the mutations induced in the mnt gene was an AT to GC transition. Although we cannot exclude the possibility that this mutation arose spontaneously, it is more probable that it resulted from the formation of O<sup>4</sup>-methylthymine. This modified base can pair with guanine to produce AT to GC transitions. Alternatively, the presence of N<sup>3</sup>-methyladenine could provoke an SOS-dependent mutational event.

The results with STZ contrast sharply to the spontaneous mutation spectrum in the same bacterial strain. Spontaneous mutations are mostly IS1 insertions and a minor class are deletions in the operator region (8,18). Single base changes have never been detected. This indicates that the base changes in mnt isolated from STZ-exposed cells are the direct result of drug exposure and not due to spontaneous mutation.

MNNG-induced mutations in the mnt gene did not show any strand specificity (6). STZ mutable sites, however, are twice as frequent on the nontranscribed strand (Table 2), although it should be noted that the sample size is small. In studies with MNNG by others, a strand preference could be demonstrated for the cI gene of phage lambda (19) and the gpt gene of *E. coli* (20) but not for lacI (21). Burns et al (21) reported that the most mutable sites induced with MNNG in lacI were those with a 5' flanking purine. In contrast, the most mutable sites in mnt after MNNG or STZ exposure are those with a 5' flanking pyrimidine (Table 2).

STZ-induced mutational sites could be classified into two classes. In one class are those mutations specific for STZ, while the other class consists of sites mutable by both STZ and MNNG. The latter are in DNA sequences that do not appear to differ from MNNG mutable sites whereas the former show a distinct pattern of a pyrimidine-rich stretch of bases followed by a set of purines. The four sites mutated by STZ only are not the only

ones in mnt showing this feature. The sequences surrounding the guanines at positions +63, +89, and +120 are CTAT G GAAG, TTCA G GGCG, and ACTC G GAGT, respectively. Mutation of the G at +120 to an A would conserve the serine codon and would thus be silent. Changes at +63 and +89, however, should result in replacement of methionine to isoleucine, and arginine to lysine, respectively. Whether these positions are not mutated by STZ or if they are mutated but that the amino acid replacements are permissible is not yet known. The latter alternative is distinctly possible since G to A mutations at positions +63 and +89 have never been isolated in our studies with this gene.

It should be stressed that the sample size in the above analysis is small, a problem common to all such studies of this type. What was striking, however, is that within such a small sample both STZ and MNNG each showed site preferences for mutation despite their action through a common mechanism. We suggest that the use of STZ as an adjunct to MNNG is advantageous in order to broaden the mutation spectrum.

The causes underlying differences in mutational specificity of STZ and MNNG are unclear. Among the possibilities are that differences exist in the sites of methylation by each drug and/or in subsequent processing of lesions. For example, MNNG produces base mismatches subject to Dam-dependent mismatch repair (22) but STZ does not (3). This may indicate that MNNG produces certain types of modification that are not formed by STZ. Another difference is that MNNG induces transcription of the aidC gene while STZ does not (4). While the function of aidC is not known, it is apparent that expression of other genes involved in DNA repair might also be induced by one drug and not the other, thus causing differences in the mutation spectrum.

There are two differences between the experimental systems used for the MNNG and STZ studies. First, the plasmid used in the STZ study is identical to that for the MNNG spectrum (6) except that its M13 origin of replication is inverted. Since it has been shown that the M13 origin has no effect on mutation spectrum in mnt (8,18), this possibility seems unlikely to explain differences in spectrum. Second, a recF143 strain was used for the MNNG study while a wild type strain was used for the STZ



spectrum. Although the recF gene product has been implicated in ultra-violet light mutagenesis (5), we know of no published evidence for its involvement in Ada-dependent mutagenesis. As previously reported (6), we could not detect a difference in MNNG-induced mutation frequency in mnt between recF143 and wild type strains. Therefore, although we cannot exclude the possibility that recF affects the spectrum of alkylation-induced mutagenesis, we believe it is unlikely.

Lastly, although structurally distinct from MNNG, STZ is also an N-nitroso compound that methylates DNA. While the sites of alkylation do not differ among a wide spectrum of methylating agents, the extent of methylation at specific sites may vary (23). Thus, differences in the extent to which specific lesions are formed, whether as a result of varying intracellular drug concentrations or chemical reactivity, may also explain our results. These issues are currently being addressed.

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