Comparison of mutagenesis by \(O^6\)-methyl- and \(O^6\)-ethylguanine and \(O^4\)-methylthymine in *Escherichia coli* using double-stranded and gapped plasmids

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To compare mutagenesis by \(O^6\)-methylguanine (\(m^6G\)), \(O^4\)-methylthymine (\(m^4T\)) and \(O^6\)-ethylguanine (\(e^6G\)), and assess their genotoxicity in *Escherichia coli*, double-stranded and gapped plasmids were constructed containing a single \(m^6G\), \(e^6G\) or \(m^4T\) in the initiation codon (ATG) of a lacZ' gene. Modified base induced mutations were scored by the loss of lacZ' activity on X-gal-containing media resulting in formation of white or sectored (mutant) rather than blue (non-mutant) colonies. Genotoxicity experiments with gapped plasmids containing the modified bases indicated that \(m^4T\) produced a greater number of bacterial colonies than \(m^6G\) or \(e^6G\). \(m^4T\) was more mutagenic (45% mutant colonies) than \(m^6G\) which produced 62% mutant colonies (as was observed previously for \(e^6G\) in both wild type *E. coli* or *E. coli* deficient in both \(O^6\)-alkylguanine-DNA alkyltransferases as well as methylation-directed mismatch repair (\(ada\'-\text{o}^4\text{gt}^{-}\text{mut}S\')). \(m^6G\) in gapped plasmids produced 62% mutant colonies in wild type *E. coli*, but this percentage increased to 94% in the \(ada\'-\text{o}^4\text{gt}^{-}\text{mut}S\) strain. In double-stranded plasmids both \(m^4T\) and \(m^6G\) produced very similar distributions of mutant and non-mutant colonies in the \(ada\'-\text{o}^4\text{gt}^{-}\text{mut}S\) strain. These observations led to the conclusion that differences in the mutagenicity of \(m^6G\) and \(m^4T\) in wild type *E. coli* were a result of preferential repair of \(m^4T\) compared to \(m^6G\) by alkyltransferase and mismatch repair mechanisms, and did not reflect differences in their respective coding efficiency or their inherent obstructiveness to DNA synthesis as was observed with \(e^6G\). The combination of alkyltransferase and mismatch repair was concluded to be primarily responsible for the apparent genotoxicity of \(m^6G\) compared to \(m^4T\) in double-stranded plasmids.

Introduction

\(O^6\)-Methylguanine (\(m^6G\)), \(O^4\)-methylthymine (\(m^4T\)) and \(O^6\)ethylguanine (\(e^6G\)) are representative of adducts believed to be most responsible for the mutagenic effects of methylation and ethylating carcinogens (e.g. \(N\)-methyl- or \(N\)-ethyl-N-nitroso compounds) (1, 2). The mutagenic properties of \(m^4T\) and \(e^6G\) (3–14) and \(m^6G\) (15–17) have been examined previously in *Escherichia coli* using the site-specific mutagenesis approach (18–20). We have extended these earlier studies by comparing mutagenesis by these three adducts in double-stranded and gapped plasmids in both DNA repair competent and repair defective strains of *E. coli*. Our aims were to compare the ability of these adducts to obstruct DNA replication and thereby induce preferential use of the complementary strand in double-stranded vectors as template, to compare the ability of \(O^6\)-alkylguanine-DNA alkyltransferase (alkyltransferase) and methylation-directed mismatch repair systems to modulate mutagenesis by these adducts and to compare their respective coding properties. Additionally, we sought to establish if our *E. coli* mutagenesis system could provide qualitative and quantitative confirmation of previous observations about mutagenesis by these O-alkylated bases in very different systems (3–17).

Materials and methods

All of the enzymes and reagents used in this study were as described previously (10–12) unless specified below. The syntheses of the \(O^6\)-methylguanine- and \(O^6\)ethylguanine-containing oligonucleotides as well as the thymine- and uracil-containing complementary oligonucleotides were described (10–12). These oligonucleotides were repurified by reverse phase HPLC using the conditions described for detritylated oligonucleotides (21) prior to use. An oligonucleotide containing a single \(O^6\)-methylthymine derivative of \(O^6\)-methylthymidine was obtained from Glen Research, Sterling, VA. Following synthesis using automated synthesis procedures the controlled pore glass support carrying the \(O^4\)-dimethoxymethyl (DMT) protected oligonucleotide was transferred to a glass vial and was treated with 10 ml of methanol/1.88-diethylaminoethanol (5:4:0.95-7-ene (DBU)) at room temperature for 5 days to remove base protecting groups and to cleave the oligonucleotide from the support. The oligonucleotide was precipitated from the DBU solution with ether. The DMT-protected oligonucleotide was purified by reverse phase HPLC, was detritylated and the detritylated oligonucleotide was subsequently purified by reverse phase HPLC. Digestion of a sample of the oligonucleotide to nucleosides, followed by HPLC analysis (21) gave the following nucleoside composition (observed/expected): \(dC = 5.68/6, dG = 3.16/3, T = 1.02/1, dA = 5.21/5\) and \(dm^4G = 0.93/1\). m^4T eluted at 23.2 min. The oligonucleotide extinction coefficient was 145 000 cm/M at 260 nm. A sample of the oligonucleotide was further purified by preparative polycrylamide gel electrophoresis (21) prior to phosphorylation with T4 polynucleotide kinase as described (10). Oligonucleotides were incorporated into the plasmid pGP10 (Figure 1) containing either thymine (T) or uracil (U) residues to enable studies with double-stranded or gapped plasmid vectors (see below).

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Results and discussion

Our plasmid system (Figure 1), allows us to position a single modified base in the initiation codon of a lacZ’ gene. Adduct induced mutations inactivate the gene leading to the growth of white or sectored bacterial colonies on X-gal-containing media. In the absence of mutation, blue colonies result. With double-stranded plasmids containing a single modified base, DNA synthesis over the modified base-containing strand may be inhibited or blocked by the modified base while synthesis over the unmodified complementary strand occurs preferentially (23). This bias in favor of the unmodified strand serving as template leads to the growth of mostly blue colonies in our system. By positioning four uracil residues in the complementary strand of our plasmids (Figure 1B), cellular uracil-DNA glycosylase and AP endonucleases create a gap in the complementary strand (Figure 1D) forcing translational DNA synthesis past the modified base (12). However, if uracil-DNA glycosylase deficient bacteria (ung-) are transformed with U-containing plasmids, the plasmids remain double-stranded unless they are pretreated with uracil-DNA glycosylase in vitro to produce abasic plasmids (Figure 1C). These abasic plasmids are converted to gapped plasmids in ung- bacteria through the action of AP endonucleases.

Sected colonies originate in our system from an E.coli cell that harbors both mutant and normal plasmids. This situation primarily arises from replication of a double-stranded plasmid if DNA synthesis past a modified base in one strand directs incorporation of a base that differs from that encoded by its unmodified congener and synthesis over the unmodified complementary strand produces a non-mutant plasmid. Sected colonies can also be produced, albeit infrequently, from gapped plasmids if the product of gap filling i.e., a plasmid carrying a modified base in one strand and a mutation in the complement, were somehow repaired (e.g. by alkyltransferase) after gap filling or after subsequent rounds of DNA synthesis. In addition, if the modified base in subsequent rounds of DNA synthesis directed incorporation of the base from the same strand as its unmodified congener, then a non-mutant complement would result and a sectored colony would arise. However, this scenario is also expected to be infrequent.

White colonies are derived from a host cell that harbors only mutant progeny plasmids. These colonies are primarily derived from gapped plasmids when a modified base in the template strand directs incorporation of a base that is not encoded by its unmodified congener during gap filling. This results in a plasmid carrying a modified base in one strand and a mutation in the complement which would be expected to lead to mutant progeny during subsequent rounds of DNA synthesis. White colonies arise less frequently from a modified base-containing double-stranded plasmid if the plasmid resulting from synthesis over the unmodified complementary strand was somehow lost and only the mutant plasmid replicated successfully.

To determine the relative genotoxicity of these modified bases, U-containing plasmids that were either untreated (i.e. double-stranded) or uracil-DNA glycosylase treated in vitro (abasic plasmids) were used to transform ung- strain GP190 (12). Bases that were more genotoxic would be expected to produce fewer bacterial colonies when ung- cells were transformed with abasic plasmids compared to double-stranded U-containing plasmids (12). Data for these experiments are summarized in Table I. As shown, similar numbers of colonies were produced when double-stranded vectors containing either a guanine, thymine or an m6G, eG or m6G residue were incorporated in the ATG initiation codon of our plasmid. The same is true for abasic plasmids containing a guanine and thymine residue in the ATG initiation codon. However, compared to the control, 71, 50 and 44% colonies were produced from abasic plasmids containing m6T, m6G and eG residues, respectively, which suggests that m6T was less genotoxic than either m6G or eG.

Mutagenesis experiments also supported the conclusion that m6T was less genotoxic than m6G or eG. In these experiments (Figure 2) wild type bacteria were transformed with double-
Table I. Percentage of colonies resulting from transformation of ung- E.coli with equal amounts of the indicated plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Initiation codon sequence</th>
<th>Double-stranded plasmids</th>
<th>Abasic plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG</td>
<td>100$^d$</td>
<td>93.7 ± 22.7</td>
<td></td>
</tr>
<tr>
<td>AT$m^6$G</td>
<td>115 ± 13</td>
<td>50.2 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>AT$e^6$G</td>
<td>91.3 ± 7.5</td>
<td>44.3 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>AM$m^4$G</td>
<td>113 ± 12</td>
<td>71.3 ± 19.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Values are the result of three determinations ± the standard deviation.
$^b$The complementary strand contained U residues.
$^c$Abasic plasmids were prepared from U-containing plasmids as previously described (12).
$^d$This value is defined as 100 for each experiment. The actual number of colonies per experiment ranged from between 392 and 1669.

Fig. 2. Percentage of blue, white and sectored colonies produced in the DNA repair competent strain GP102 following transformation with double-stranded plasmids (T residues were present in the complementary strand) containing the indicated modified base. The data are the average of three or more experiments. Error bars represent the standard deviation. Blue colonies are indicated by solid bars, white colonies are indicated by open bars and sectored colonies are indicated by hatched bars. Numbers associated with each bar are the percentage values.

Fig. 3. Percentage of blue, white and sectored colonies produced in the DNA repair competent strain GP102 or the ada-ogt-mutS- strain GP220 following transformation with gapped plasmids (U residues were originally present in the complementary strand) containing m$^6$G or m$^4$T. The data are the average of three or more experiments. Error bars represent the standard deviation. Blue colonies are indicated by solid bars, white colonies are indicated by open bars and sectored colonies are indicated by hatched bars. The data for m$^6$G containing plasmids are reproduced from (12) for comparison. Numbers associated with each bar are the percentage values.

Modified Base in the Plasmid

stranded T-containing plasmids harboring either an m$^6$G, $e^6$G or m$^4$T. The m$^6$G- or $e^6$G-containing plasmids produced primarily blue colonies while the m$^4$T-containing plasmids produced 45.1% mutant and 54.9% blue colonies.

We showed previously that the large percentage of blue colonies produced by double-stranded $e^6$G-containing plasmids results from $e^6$G inducing the preferential use of the unmodified complementary strand as template (12). The high percentage of blue colonies seen with the m$^6$G containing double-stranded plasmid (Figure 2) could be the result of a similar induced bias or it could reflect a combination of such bias together with repair. To evaluate the contribution of strand bias and/or repair to mutagenesis by m$^6$G and m$^4$T, wild type E.coli and a strain deficient in both alkyltransferase repair, as well as methylation-directed mismatch repair (ada-ogt-mutS-) (12) were transformed with U-containing plasmids harboring m$^6$G or m$^4$T. With these plasmids, a gap is created in the complementary strand forcing synthesis past the modified base. Figure 3 shows the distribution of colony phenotypes observed with these plasmids. Compared to the findings with double-stranded plasmids (Figure 2) the mutagenicity of m$^6$G was greatly enhanced in the wild type strain using gapped plasmids (Figure 3). Additionally, its mutagenicity was further enhanced by the elimination of alkyltransferase and methylation-directed

DNA Repair Genotype
mismatch repair. The individual contributions of alkyltransferases and mismatch repair to m^4 T mutagenesis were determined previously (12). m^4 T was also more mutagenic in repair competent bacteria and mismatch repair to m^6 G mutagenesis were determined that the phenotypic distribution differences produced by m^4 T were not a result of a greater obstruction to DNA synthesis over the unmodified complementary strand as a template during DNA synthesis.

In summary, the results of our experiments together with previous results (12), lead to the following conclusions about mutagenesis by m^6 G, e^6 G and m^4 T in E.coli. With regard to coding potential, our studies indicate that all three adducts are highly instructional lesions although their coding properties are different from those of the unmodified congener. m^4 T codes for G incorporation during translesional DNA synthesis while m^6 G and e^6 G (10–12) code for T incorporation.

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References
m^6G, e^6G and m^4T mutagenesis in *E. coli*


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