Nitrosamine-induced Cancer: Selective Repair and Conformational Differences between O⁶-Methylguanine Residues in Different Positions in and around Codon 12 of Rat H-ras

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ABSTRACT

Mammary and skin tumors induced in rodents by N-methyl-N-nitrosourea treatment have a G:C to A:T transition mutation in codon 12 of H-ras, probably resulting from alkylation of O⁶ of guanine by the carcinogen. This codon contains two guanines (5'-GGA-3'), but mutations are observed only in the central base pair of this codon. The same selectivity for mutations of -GGA-sequences has also been observed in Escherichia coli. It is known that the central G in the sequence GGA is a preferred site for alkylation, but the magnitude of chemical selectivity is insufficient to provide a complete explanation for the biological observation which is still unexplained. We have measured accurate rates of repair by the E. coli ada gene O⁶-alkylguanine-DNA-alkyltransferase of an O⁶-methylguanine in various positions in chemically synthesized 15-base pair DNA duplexes having the H-ras sequence. The rate of repair varied 25-fold, depending on the sequence flanking the methylguanine. An O⁶-methylguanine in position 2 of codon 12 was the least well repaired. The combination of this slow repair and sequence selectivity in alkylation appears to be the explanation for the selective mutation of this position. Using an antibody to probe the accessibility of the O⁶-methyldeoxyguanosine, it was shown that the rate of repair is a reflection of the conformation of the sequence containing the alkylated base, because the avidity constants between antibody and O⁶-methylguanine were also dependent on the sequence flanking the methylguanine, with the most rapidly repaired O⁶-methylguanines being those most easily bound by the antibody.

INTRODUCTION

The carcinogen N-methyl-N-nitrosourea methylates DNA. One of the products, O⁶-methylguanine, directs the misincorporation of thymine into the daughter strand during DNA replication producing G:C to A:T transition mutations. Most mammary tumors (1) and skin tumors (2) induced by MNU³ have a G:C to A:T transition mutation involving the second, but not the first, G of codon 12 (normally GGA) of the H-ras protooncogene. This mutation can be detected shortly after the administration of the carcinogen, and it is believed that it is caused by alkylation of O⁶- of guanine (3, 4). Transfection experiments have shown that a G:C to A:T transition mutation in the first G would be equally effective in transformation of NIH3T3 cells (5), and it is not known why only the second G is mutated. This selectivity is also seen in eukaryotic cell lines (6) and prokaryotes (7–10). For example, Richardson et al. (7), using the gene for xanthine-guanine phosphoribosyl-transferase (gpt gene) of Escherichia coli as target for DNA alkylation by different direct-acting nitroso-alkylating agents, found a strong bias for G:C to A:T mutations to occur when the 5'-flanking base is guanine and the 3'-base is an adenine or thymidine, and Burns et al. (9) observed that, in the E. coli lacI gene, guanines preceded 5' by a purine are 10 times more likely to be mutated than those preceded by a pyrimidine residue.

There are three possible explanations for this selectivity: (a) the second G in this sequence of DNA may be more readily alkylated than a guanine residue in other sequences; (b) the cell may be unable to repair an O⁶-alkylguanine when it is in this position; and (c) an O⁶-alkylguanine flanking by this sequence miscodes with much higher frequency than in other sequences. There has been support for all three of these possibilities. Sequence selectivity in the reaction of chemicals and DNA is well established (11–13), and a mechanism for selective alkylation of DNA by MNU has been proposed (14). Richardson et al. (15) observed 5- to 6-fold greater formation of O⁶-methylguanine when the guanine, target for alkylation, was preceded 5' by a purine than a pyrimidine. However, they observed that the sequence preference for alkylation of synthetic oligonucleotides in vitro was not directly reflected to the mutational spectra observed in vivo. Topal et al. (16) supported the second possibility, that there is specificity in the repair process, and suggested that the mutational specificity for the second G of the GGA codon 12 of H-ras occurs because an O⁶-alkylguanine in this position cannot be repaired efficiently. This was partially supported by Dolan et al. (17) who observed a 2-fold slower repair of O⁶-methylguanine in self-complementary oligonucleotides when the base 5' to the O⁶-methylguanine was a guanine rather than a cytosine. Finally, Singer et al. (18, 19) have reported that the frequency of misincorporation of thymine opposite O⁶-methylguanine during DNA replication in vitro was dependent on the flanking bases. However, the observations mentioned above are insufficient to explain the exclusive mutagenesis of the second guanine of codon 12 in H-ras. In this study we report a direct test of Topal's hypothesis that differences in DNA repair are a main contributor to this mutational specificity. Our results support this view and suggest that the selectivity for the second G in codon 12 results from a combination of selective alkylation and selective repair, with selective repair probably being the more important but neither being sufficient alone.

O⁶-Alkylguanine is repaired by O⁶-alkylguanine-DNA-alkyltransferase [reviewed by Lindahl et al. (20) and Pegg (21)]. The exact rate constants for this repair can be determined using synthetic DNA containing alkylated bases as substrate (22, 23). We have synthesized short lengths of DNA (15-mers) having the same sequence as H-ras around codon 12, except that the first or second guanine residue of codon 12 had been replaced by an O⁶-methylguanine. We have also synthesized similar DNA sequences differing from the H-ras-sequence in that one guanine is replaced by O⁶-methylguanine and the neighboring 5' or 3' base was also changed. We measured the accurate rate constants for the repair of these DNAs by the E. coli ada gene.
O^6\text{-}alkylguanine-DNA-alkyltransferase. The rate of repair differed very greatly. In particular, an O^6\text{-}methylguanine in position 2 of the H-ras sequence was repaired 18 times more slowly than one in position 1. Previous measurements of this type have relied on HPLC to separate the alkylated from the repaired oligomers, but it was found that HPLC could not separate the 15-mers used in these experiments. Instead, the separation was done using antibodies to O^6\text{-}methyldeoxyguanosine (24, 25). This led to the unexpected finding that the avidity of the antibody to the methylguanine was influenced by the sequence in which methylyguanine was placed, and that the sequences in which the O^6\text{-}methylguanine was most accessible to the antibodies were also the sequences in which the O^6\text{-}methylguanine was most rapidly repaired. These related results show that the conformation of the alkylated base pair and the DNA around the alkylated base must be strongly dependent on the flanking sequence.

**MATERIALS AND METHODS**

Enzymes and Antibodies. T. polynucleotide kinase was obtained from Amersham International, and alkaline phosphatase and phosphodiesterase (Crotalus durissus) were from Boehringer Mannheim. Purified E. coli ada O^6\text{-}alkylguanine-DNA-alkyltransferase (M, 19,000) was provided by Dr. D. Yarosh (Applied Genetics, Inc., Freeport, NY). The method for raising polyclonal antibodies against O^6\text{-}methyldeoxyguanosine was previously described (26).

Oligodeoxynucleotides. Chemicals for oligonucleotide synthesis were obtained from Churomch, Ltd. (Glasgow, Scotland). The oligonucleotides containing O^6\text{-}methylguanine were prepared by solid-phase synthesis on a Chromach DNA synthesizer (27). The structure of the oligonucleotides used is shown in Table 1. Two of the 15-mers have the sequence of part of the rat H-ras gene with the O^6\text{-}methylguanine in either position 1 or 2 of codon 12 (i.e., -T.GmeGA.G- and -T.meGGA.G-), and two have single base changes in that codon so that the bases flanking the methylguanine are changed (i.e., -T.TmeGA.G-,-T.meGAA.G-). The dodecamers, also shown in Table 1, were used for comparative experiments. The DNA complementary to the alkylated strands and the nonalkylated analogues of the alkylated strands were synthesized by the phosphoramidite solid-phase method. The self-complementary dodecamer CCGmeGACCTGGG was synthesized in solution by the phosphotriester procedure (28). All oligomers underwent a preliminary purification on a Nensorb Prep column (Du Pont) followed by reverse-phase HPLC purification (27). The amount and purity of each oligonucleotide were measured by enzymic digestion of the oligomer to nucleosides and chromatographic comparison of the integrated areas of the UV-absorbing peaks with those of a standard.

Table 1 Synthesized oligodeoxynucleotides containing O^6\text{-}methyldeoxyguanosine

<table>
<thead>
<tr>
<th>Pentadecamers</th>
<th>Shortened form</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGCCCTmeGGAGGGCTTG</td>
<td>-T.meGGA.G-</td>
</tr>
<tr>
<td>GGGCCCTmeGGAGGGTG</td>
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<td>-T.GGA.G-</td>
</tr>
<tr>
<td>GGGCCCTGAGGGCTTG</td>
<td>-T.TGA.G-</td>
</tr>
</tbody>
</table>

Dodecamers

| CCGmeGAGCTGGG | CCGmeGAGCAATGG |

The amount of dodecamer still containing O^6\text{-}methylguanine after the reaction was measured by HPLC (23). The amount of alkylated pentadecamer was measured by its reaction with a known quantity of E. coli ada (M, 19,000) O^6\text{-}alkylguanine-DNA-alkyltransferase.

DNA Melting Curves. These were obtained by measuring the temperature-dependent changes in absorbance at 260 nm using a Unicam SP500 spectrophotometer (Pye Unicam, Cambridge, United Kingdom), fitted with a Gilford 222 photometer and a Gilford thermoprogrammer (Gilford Instruments, Oberlin, OH). The oligonucleotide duplexes had an absorbance of 0.9 at 260 nm and 14°C in 1 M NaCl:10 mM K2HPO4 (pH 7). The temperature was increased by 1°C/min.

Measurement of the Repair of Alkylated Double-stranded DNA with E. coli O^6\text{-}Alkylguanine-DNA-alkyltransferase using an Immunoprecipitation Assay. The kinetic constants for the reaction between oligonucleotides and alkyltransferase were measured at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM DTT, 1 mM EDTA, and 200 μg/ml of bovine serum albumin (Sigma). At intervals after addition of the alkyltransferase to the methylated DNA, 20 to 50 μl of the reaction mixture were frozen by plunging in liquid N2. The amount of alkylated oligomer remaining in each sample was measured by immune precipitation using antisera to O^6\text{-}methyldeoxyguanosine raised in rabbits (24). The samples were transferred to an ice bucket and, while still frozen, 10× PBS [NaCl (40 g/liter):KCl (1 g/liter):Na2HPO4 (5.75 g/liter):KH2PO4 (1 g/liter)] and antiserum (1:10 dilution) were added, so that the final solution was 1× PBS and 1:30 final antiserum dilution. After 30-min incubation on ice, an equal volume of saturated ammonium sulfate was added. After 30 min at 0°C, the samples were centrifuged, and the supernatant was discarded. The precipitate was washed with 100 μl of 50% saturated ammonium sulfate and finally dissolved in 200 μl of 0.1 M NaOH, and the radioactivity was measured by scintillation counting.

If A0 is the initial concentration of the oligomer, then the amount of unrepaird oligomer after time t is

\[ A_t = x = (\text{cpm} \, t - \text{cpm} \, B/\text{cpm} \, 0 - \text{cpm} \, B)/A_0 \]

where cpm t is the radioactivity precipitated, cpm B is the background precipitation obtained after the oligomer had been fully repaired with excess enzyme, cpm 0 is the radioactivity precipitated before the addition of the enzyme, and x is the amount of oligomer repaired. For a second-order reaction

\[ \ln[(B_0 - x)A_0/(A_0 - x)B_0] = (B_0 - A_0)kt \]

where k is the rate constant; and B0 is the initial concentration of the enzyme. Then a graph of \( \ln[(B_0 - x)A_0/(A_0 - x)B_0] \) versus t is a straight line with a slope equal to \((B_0 - A_0)k\).

Measurement of the Avidity Constants for the Binding of O^6\text{-}Methylguanine in Oligonucleotides by Antibodies to O^6\text{-}Methyldeoxyguanosine. One hundred fmol of radiolabeled double-stranded oligomer and varying amounts (over the range of 1 to 100 pmol) of the same, but not labeled, oligomer were incubated with antiserum as described above. The amount of antiserum added was sufficient to precipitate 50 to 60%
SEQUENCE SELECTIVE REPAIR AND CONFORMATION OF O\(^6\)-METHYLGUANINE

of the radioactive oligomer when the nonlabeled oligomer was not present in the solution. For some oligomers this precipitation was achieved using less antiserum than for others. In these cases, \(\gamma\)-globulin was added in order to obtain the same total protein concentration for all the experiments. The immunoprecipitation was performed as described above except that Polyethylene Glycol 6000 (24%, w/v, in water) rather than saturated ammonium sulfate was used as the precipitating agent.

The anti-O\(^6\)-methylguanine-oligomer avidity constants were determined using the method by Steward and Petty (29). Briefly the reciprocals of bound antigen \((1/b)\) and free antigen \((1/c)\) were plotted according to the Langmuir equation

\[
\frac{1}{b} = \frac{1}{Ka} + \frac{1}{Ab}
\]

where \(Ab\) is the total antigen binding sites, and \(K\) is the avidity constant. The bound and free antigens were calculated as follows

\[
b = \frac{cpm2 - cpm3/cpm1}{n}
\]
\[
c = n - b
\]

where \(cpm1\) is the total radioactivity of the reaction mixture, \(cpm2\) is the radioactivity precipitated in the absence of antiserum, \(cpm3\) is the radioactivity precipitated in the absence of antiserum, and \(n\) is the total amount of oligomer (pmol). The total antigen binding sites \((Ab)\) were determined from Equation A by extrapolation of the plot of \(1/b\) versus \(1/c\). (As \(1/c\) approaches 0, \(1/b\) approaches \(1/Ab\).) The avidity constants were then obtained from the plot of \(\log(b/Ab - b)\) versus \(\log c\) according to the Sips equation:

\[
\log(b/Ab - b) = \log K + \log c
\]

RESULTS

The purity of the synthesized oligonucleotides was determined by chromatography and nucleoside analysis. All of them gave a single peak when chromatographed by reverse-phase HPLC. Enzymic hydrolysis and chromatography of the nucleosides showed that more than 98% of the \(A_{260}\) absorbing material was associated with either dC, dG, T, dA, and dmeG. Neither 2,6-diaminopurine nor partially deprotected nucleosides were detected. A small amount of inosine was observed due to deamination of adenine by contaminating adenosine deaminase in the alkaline phosphatase. Comparison of the integrated areas of the nucleosides with the areas of a standard mixture of nucleosides showed that the base compositions of the oligomers were identical to those expected.

The melting curves of the DNA duplexes were obtained, and the \(Tm\) values were calculated as the temperature at which the hyperchromicity was half its final value. In agreement with previous observations (28, 30, 31), DNA duplexes containing \(O^6\)-methylguanine had less hyperchromicity and lower melting point than the parent duplexes. The \(Tm\) values for the \(-T.GmeGAG-\) and \(-T.meGGA.G-\) duplexes were 68°C and 66°C, respectively, compared with the 77°C of the parent \(-T.GGA.G-\) duplex (Fig. 1A). Both \(-T.mGAA.G-\) and \(-T.TmeGA.G-\) sequences had a \(Tm\) value of 63°C compared with the 75°C of the parent \(-T.TGA.G-\) sequence (Fig. 1B).

Initially attempts were made to determine the rates of repair by separating the methylated oligomer from the repaired oligomer by HPLC, as had been previously done with shorter oligomers (22, 23). Those attempts were unsuccessful. Although we tried changing the buffers, the columns, the elution gradients, and the temperature to obtain a better separation, the results were unsatisfactory. However, the rate of repair can be measured by separating the DNA containing \(O^6\)-methylguanine from the repaired oligomer by immunoprecipitation with antibodies against \(O^6\)-methyldeoxyguanosine (25, 32). To validate this technique, the rate of repair of the self-complementary dodecamer, CGCmeGAGCTCGCG, by the ada \(O^6\)-alkylguanine-DNA-alkyltransferase was measured using immunoprecipitation, and the result was compared with that obtained when HPLC was used to separate the alkylated from the repaired oligomer (23).

The extent to which the dodecamer was precipitated depended on the amount of antiserum added, reaching a plateau in which 88 to 93% was precipitate when the final dilution of antiserum was 1:30 (2.5 \(\mu\)l of antiserum/75-\(\mu\)l assay volume) (Fig. 2). Complete precipitation was not achieved even when the antiserum was added undiluted to the reaction mixture.

![Fig. 1. Melting curves at 260 nm for the GGC.GCT.GmeGA.GGC.GTG (-T.GmeGAG-), GGC.GCT.meGGA.GGC.GTG (-T.meGGA.G-), GGC.GCT.GGA.GGC.GTG (-T.GGA.G-), and GGC.GCT.TmeGA.GGC.GTG (-T.TmeGA.G-) duplexes (A) and GGC.GCT.TmeGA.GGC.GTG (-T.TmeGA.G-), GGC.GCT.meGAA.GGC.GTG (-T.meGAA.G-), and GGC.GCT.TmeGA.GGC.GTG (-T.TGA.G-) duplexes (B). The oligonucleotide solutions had an initial absorbance of 0.9 \(A_{260}\) units at 14°C.](cancerres.aacrjournals.org)
methylguanine to the same extent. Seventy-seven% of double-
been fully removed by excess ada alkyltransferase. However, the antiserum did not precipitate every 15-mer containing O6-
sequences were precipitated by the antiserum (Table 2) which was similar to the precipitation of the analogous O6-
O'5-T.GmeGA.G- sequences were precipitated, but only about 36% of the duplex formed from the -T.GmeGA.G- sequence, in which the methylated base is in the position of the second G in codon 12 of rat H-ras. The single-stranded oligomers were more completely precipitated than the duplexes, but even in the single-stranded form, the -T.GmeGA.G- sequence was poorly precipitated (40% precipitated compared with 87% for the other sequences). Another oligomer, the dodecamer CGCAGmeG-
which contains the sequence around codon 12 of human N-ras with the meG again in position 2 of the codon, was also poorly precipitated in the double-stranded form (10%), but in this case the single-stranded DNA was precipitated well (87%). It seems unlikely that the poor precipitation of the -T.GmeGA.G- sequence occurred because it was impure, because when chromatographed on HPLC the oligomer was eluted as a single sharp peak, and the nucleoside analysis gave the expected base composition. However, in order to make certain that the poor precipitation of the -T.GmeGA.G- sequence was a property of that sequence rather than an artifact, the oligomer was resynthesized. The product of the second synthesis gave very similar results to those obtained from the first synthesis (Table 2).

For all DNA duplexes, including the -T.GmeGA.G- sequence, the proportion that precipitated was independent of the duplex concentration, over the range used in the experiments, meaning that the rate constant for the repair of that sequence could be as accurately determined as the rates for the sequences that were more completely precipitated (Fig. 3).

The progress of the repair differed significantly between the sequences examined (Fig. 4). In order to quantitate the observed differences, the removal of the methyl group by the alkyltransferase was considered a second-order reaction (23, 24), and graphs like these in Fig. 5 were obtained. Subsequently the rate constants were calculated (Table 3). The rate of the repair of O6-methylguanine was 18 times faster when present at position 1 (-T.meGGA.G- sequence) than at position 2 (-T.GmeGA.G- sequence) of codon 12 of the ras-related oligomers. Substitution of the guanine preceding the O6-methylguanine in the -T.GmeGA.G- sequence by a thymine (-T.TmeGA.G- sequence) increased the rate of the reaction by 25 times. Substitution of the second guanine of the -T.meGGA.G- sequence with an

This treatment with antiserum successfully separated the alkylated from the repaired oligomer. Using 1:30 final antiserum dilution, less than 2% of the oligomer was precipitated after the O6-methyl group had been removed by the ada gene alkyltransferase. The proportion of the oligomer precipitated was independent of changes in its concentration over a range of 50 to 200 fmol/75-µl assay volume, and addition of nonmethylated parent oligomer over the same concentration range did not affect the precipitation of the methylated oligonucleotide (Fig. 3).

Using the immunoprecipitation method to separate the methylated and the nonmethylated parent oligomer, produced during the repair process, we obtained a rate constant of $2.2 \times 10^7$ M$^{-1}$sec$^{-1}$ for the repair of the self-complementary 12-mer, which is virtually identical to that obtained ($2.5 \times 10^7$ M$^{-1}$sec$^{-1}$) for the same oligomer when HPLC was used to separate the methylated from the repaired oligomer (23). These results indicated that immunoprecipitation might be used for kinetic studies of the repair of the H-ras-related sequences.

Only 0.6% and 0.5% of the nonmethylated -T.GGA.G- and -T.TGA.G- sequences were precipitated by the antiserum (Table 2) which was similar to the precipitation of the analogous O6-methylguanine-containing 15-mer after the methyl group had been fully removed by excess ada alkyltransferase. However, the antiserum did not precipitate every 15-mer containing O6-methylguanine to the same extent. Seventy-seven% of double-

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Double strand (%)</th>
<th>Single strand (%)</th>
<th>After total repair (%)</th>
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<td>-T.GmeGA.G-</td>
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<td>1.0</td>
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<td>-T.TGA.G-</td>
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</tr>
<tr>
<td>CGCmeGAGCTCGCG*</td>
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<td>86</td>
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<tr>
<td>CGCmeGAGCAATGC</td>
<td>61</td>
<td>86</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Two different synthesis of the GmeGA sequence.
* Self-complementary oligomer.

Fig. 3. Immunoprecipitation of different quantities of CGCmeGAGCTCGCG, the self-complementary 12-mer, and the GGC.GCT.GmeGA.GGC.GTG (-T.GmeGA.G-) duplex by a 1:30 final antiserum dilution.

Fig. 4. Differential repair for the ras-related sequences by the E. coli ada alkyltransferase. Four hundred fmol of ada protein were incubated with 60 fmol of each oligomer in a volume of 40 µl as described in "Materials and Methods." O, -T.TmeGA.G- duplex; @, -T.meGGA.G-; □, -T.meGAA.G-; and ■, -T.GmeGA.G- duplexes.
To discover whether the rapid repair of the self-complementary dodecamer were 2 orders of magnitude less than that observed for the oligomer and enzyme, respectively; $v$ is the amount reacted after time $t$. The slope of the line is equal to $k(\Delta E - x)$, where $k$ is the rate constant.

There was a 2-fold difference in the amount of the -T.GmeGA.G- and -T.TmeGA.G- duplexes (33, 34). Fig. 6 shows the precipitation of the -T.GmeGA.G- and -T.TmeGA.G- duplexes. PEG has the advantage of precipitating low avidity as well as high avidity complexes, whereas ammonium sulfate is mainly studied using either saturated ammonium sulfate or PEG. In this paper, we report that the repair of the O6-methylguanine by E. coli ada alkyltransferase is highly sequence dependent. Using synthetic 15-mer as substrate for the E. coli ada alkyltransferase, the rate of the repair of O6-methylguanine was found to differ as much as 25-fold, depending on the flanking base sequence (Table 3). Surprisingly, the DNA duplexes -T.TmeGA.G- and -T.meGGA.G-, which have the same bases flanking the alkylated base, were repaired with rates differing by 3.6 times. A possible explanation for this observation could be a next-to-neighbor effect (16, 36). The -T.GmeGA.G- sequence was the most poorly recognized by the alkyltransferase and was repaired 18 times slower than the -T.meGGA.G- sequence.

The effect of sequence on the rate of repair seems to be a reflection of the conformation and accessibility of the alkylated base, because the association of antibodies to O6-methyldeoxymethylnosine with the DNA duplexes was also affected by the base sequence flanking the alkylated base. Those duplexes which were repaired most slowly were also those where the methylnosine was least accessible to the antibody (Table 3). A more subtle correlation exists between stability of DNA duplexes, as reflected in the Tm, and protein-DNA interactions, as reflected in the avidity between duplex and antibody and rate of repair (Table 3). Recently, Voigt and Topal (37) observed that sequences, similar to those used in the this study, have different electrophoretic mobilities on a nondenaturing polyacrylamide gel. In this case, however, it appeared to be a direct correlation between the Tm and the effect on the DNA structure. These observations indicate that O6-methylguanine could have a global effect in the structure of DNA which is greatly dependent upon the flanking base sequence.

The most poorly repaired methylguanine and the one most weakly interacted with the antibody to O6-methyldeoxymethylnosine was in the sequence with the alkylated base surrounded by the same sequence as the second G of codon 12 of H-ras gene. Therefore, our results argue strongly for a contribution of repair specificity in the observed nonrandom distribution of mutations observed...
in both eukaryotes and prokaryotes. However, two previous observations have seemed inconsistent with the existence of repair specificity in vivo. When Mitra et al. (38) put the rat H-ras oncogene containing an O6-methylguanine placed in either position 1 or 2 of codon 12 into a shuttle vector and studied the mutations caused upon transfection of Rat 4 (TK-) cells, they found that the mutation frequency, although very low (1%), was independent of the position of the O6-methylguanine. In this system, however, a small number of plasmids enter each cell, and the alkyltransferase molecules would always be in a vast excess over the number of alkylated bases. This is not comparable to the situation where mammary tumors are induced by administration of MNU (39), where more O6-methylguanine residues are produced than the available alkyltransferase molecules. Under such conditions, the difference in rates of the repair observed could have a disproportionate and greater effect in the mutation frequency because the enzyme molecules would be exhausted on the repair of the most favored sites, while the least repairable sites would remain untouched, and thus, a higher frequency of mutations at these positions is to be expected.

In another study, a similar mutational spectrum was obtained in the gpt gene of the pSV2gpt plasmid grown in unadapted, i.e., low ada alkyltransferase content, adapted, i.e., high alkyltransferase content, or alkyltransferase-deficient E. coli cells and then exposed to N-methyl-N-nitrosoguanidine (40). An unusual aspect of these results was that the mutations observed were almost exclusively at the antisense strand, and such strand specificity was not observed when the chromosomal E. coli lacI gene was used as target for alkylation (8–10). In experiments with mammalian mer* and mer− cells (44), there are some indications that preferential repair had taken place. In the latter, Sikpi et al. (44) treated the E. coli supF gene with MNU before its insertion into a shuttle vector and the subsequent transfection of mer* and mer− cells. Positions 2 of the gpt gene (40) and 2 of the supF gene (44) appeared to be stronger mutagenic hotspots in the repair-proficient cells, while positions 402 and 123, respectively, appeared to be stronger mutagenic hotspots in the repair-deficient cells. Such differences would be expected only if a nonrandom repair process follows a nonrandom alkylation process.

The rate constants for the repair of O6-methylguanine, obtained in this study, are one to two orders of magnitude slower than the rates determined previously (18, 22, 23) and show a much greater effect of the sequence than was found by Dolan et al. (17). The reason for the difference in results seems to be that previous authors have all used shorter and self-complementary oligomers. Comparison of the rate of the repair of a non-self-complementary and a self-complementary 12-mer with the same bases flanking the O6-methylguanine showed that the self-complementary oligonucleotide was repaired with unusual speed. The reason is probably that, in repair assays a low concentration of oligomer, a low-salt concentration, and a 37°C temperature are used. Under these conditions, self-complemen-

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### Table 3 Relationship between the rate of repair and recognition by the anti-O6-methyldeoxyguanosine antibodies

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Rate of repair</th>
<th>Avidity constant</th>
<th>ΔTm °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGCAGGCTGAGGCGG</td>
<td>1.1 x 10^8 M⁻¹ s⁻¹</td>
<td>0.24 x 10^9 M⁻¹</td>
<td>9°C</td>
</tr>
<tr>
<td>CGCAGGCTGAGGCGGT</td>
<td>7.7 x 10^8 M⁻¹ s⁻¹</td>
<td>No data available</td>
<td>12°C</td>
</tr>
<tr>
<td>CGCAGGCTGAGGCGG</td>
<td>2.0 x 10^8 M⁻¹ s⁻¹</td>
<td>0.86 x 10^9 M⁻¹</td>
<td>11°C</td>
</tr>
<tr>
<td>CGCAGGCTGAGGCGG</td>
<td>2.8 x 10^8 M⁻¹ s⁻¹</td>
<td>1.85 x 10^9 M⁻¹</td>
<td>12°C</td>
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<tr>
<td>CGCAGGCTGAGGCGG (single strand)</td>
<td>0.9 x 10^10 M⁻¹ s⁻¹</td>
<td>No data available</td>
<td>&gt;20°C</td>
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<tr>
<td>CGCAGGCTGAGGCGG (free nucleoside)</td>
<td>2.0 x 10^8 M⁻¹ s⁻¹</td>
<td>5.0 x 10^9 M⁻¹</td>
<td></td>
</tr>
<tr>
<td>CGCAGGCTGAGGCGG (free nucleoside)</td>
<td>2.2 x 10^8 M⁻¹ s⁻¹</td>
<td>1.5 x 10^9 M⁻¹</td>
<td></td>
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* The ΔTm was calculated by subtracting the Tm value of the parent duplexes from each observed value.

* Value calculated by subtracting the Tm value of the -TGA- duplex which has the same flanking bases.

* Value calculated from Li and Swann (28).

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Fig. 6. Comparison of the amount of the -T.TmeGA.G- and -T.GmeGA.G- duplexes precipitated using either saturated (NH₄)₂SO₄ (amm. sulphate) or PEG as precipitating agents. All the samples had the same total protein concentration of 30 mg/ml.

Fig. 7. Sips plots for the DNA-anti-DNA interactions of the -T.TmeGA.G-, -T.TmeGA.G-, and -T.TmeGA.G- duplexes. The avidity constants of Table 3 were obtained from the γ-intercept of these graphs as described in “Materials and Methods.”
tary oligonucleotides exist mainly in a hairpin-loop conformation (45–48). The avidity constant between the antibodies against the O6-methyldeoxyguanosine and the self-complementary oligomer we used is very high and close to that between the antibody and the free nucleoside (Table 3), suggesting that, as one would have suspected, the O6-methylguanine in a hairpin-loop oligomer is probably mostly frayed out of the helix rather than stacked into it and, hence, could be more accessible and removed faster by the alkyltransferase.

In summary, it seems likely that the high specificity for activating mutations in the H-ras gene results as a combination of selectivity of alkylation and inefficient repair, with the latter amplifying to a great extent the effect.

ACKNOWLEDGMENTS

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REFERENCES

Nitrosamine-induced Cancer: Selective Repair and Conformational Differences between $O^6$-Methylguanine Residues in Different Positions in and around Codon 12 of Rat H-ras

Panagiotis Georgiadis, Clive A. Smith and Peter F. Swann


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