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Genetics

Spectrum of cisplatin-induced mutations in *Escherichia coli*

(mutation specificity)

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ABSTRACT **Using a forward-mutation assay based on the inactivation of the tetracycline-resistance gene located on plasmid pBR322, we have determined the mutation spectrum induced in *Escherichia coli* by cisplatin [*cis*-diamminedichloroplatinum(II)], a widely used antitumor drug. Cisplatin is known to form mainly intrastrand diadducts at ApG and GpG sites. We found that cisplatin efficiently induces mutations in an SOS-dependent way (i.e., dependent upon UV irradiation of the host bacteria). More than 90% of the mutations are single-base-pair substitutions occurring at the potential sites of cisplatin adducts (ApG and GpG). Taking into account the relative proportions of ApG and GpG adducts, we found that the ApG adducts are at least 5 times more mutagenic than the GpG adducts. Moreover, a strong mutation specificity was seen at the 5′ side of the ApG adducts (A·T→T·A transversions). The observation that most mutations occur at the 5′ end of the adduct at both ApG and GpG sites is discussed in relation to recent structural data.**

Cisplatin [*cis*-diamminedichloroplatinum(II)] is widely used in the treatment of some human tumors, such as head and neck, ovarian, and testicular cancers (1). Cisplatin preferentially forms intrastrand adducts between the N-7 atoms of adjacent purines (2, 3).

Recently, several studies (4–10) focused on the characterization and quantification of the different adducts that form when cisplatin or *cis*-(1,2-cyclohexanediamine)dichloroplatinum(II) reacts with DNA or model oligonucleotides. As a result, at the lowest levels of modification that could be analyzed with cisplatin (in the range of 0.002 to 0.01 Pt adducts per nucleotide), 65% of the adducts were found to be at GpG sequences and 25% at ApG sequences, whereas GpNpG adducts could represent 6% (6).

In *Escherichia coli*, cisplatin was shown to cause filamentation (11), the induction of RecA protein synthesis (12), and the induction of prophage λ (13). It was also found to be mutagenic in *E. coli* (14, 15) and *Salmonella typhimurium* (16–20) and carcinogenic in mouse and rat (19, 20). The contribution of the different DNA adducts to the mutagenic potency is largely unknown. From results obtained with the *lacI* system developed by Miller and coworkers (21–23), Brouwer *et al.* (14) concluded that GpApG and GpCpG sequences are hot spots for cisplatin-induced base-substitution mutations.

In this paper, we present the analysis of cisplatin-induced mutations by a forward-mutation assay—namely, the inactivation of the tetracycline-resistance gene located on plasmid pBR322 (24). This assay was previously shown to respond to the different classes of mutations (25, 26).

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MATERIALS AND METHODS

Strains, Plasmids, and Culture Medium. Strains AB1186 (*uvrA*) and GW2100 (*umuC122*) and the corresponding wild-type strain AB1157 were used (27, 28). pBR322 plasmid DNA was grown in AB1157 and purified as described (24). Rich Luria–Bertani (LB) medium contained ampicillin (50 μg/ml) or tetracycline (20 μg/ml).

***In Vitro* Reaction of Cisplatin with Plasmid DNA.** Reaction of supercoiled pBR322 DNA with cisplatin was allowed to occur at 37°C overnight in buffer 1 mM sodium phosphate/2.5 mM NaCl, pH 7.4. Increasing levels of DNA modification were achieved by incubation with increasing concentrations of cisplatin. The reaction was stopped by the addition of NaCl to a final concentration of 0.15 M. The extent of modification was determined by measuring the platinum concentration by atomic absorption spectroscopy (model A560 Perkin–Elmer spectrophotometer), and the DNA-phosphate concentration, by UV spectrophotometry (nucleotide molar extinction coefficient at 260 nm = 6400). Under our experimental conditions, the extent of modification that is measured corresponds, within an error of 10%, to the molar ratio of cisplatin to nucleotide in the reaction mixture.

Restriction-Fragment-Directed Mutagenesis, UV Irradiation, Transformation of Bacteria, and Nucleotide Sequencing of the Mutants. These methods have been described in detail (24, 25).

RESULTS AND DISCUSSION

Survival of Cisplatin-Modified Plasmids. Plasmid pBR322 was randomly modified with cisplatin to various extents ranging from 0 to 40 adducts per plasmid. A dose-dependent decrease in the relative transformation efficiency was found with increasing levels of cisplatin modification. In both wild-type and *umuDC* strains, the extent of modification corresponding to one lethal hit (i.e., at a 37% survival value) was equal to ≈12 adducts per plasmid. In an excision–repair-deficient *uvrA* strain, we observed an increased toxicity of the cisplatin-modified plasmid (2–3 adducts per plasmid at the 37% survival value), showing the involvement of this pathway in the repair of these lesions. Using the same assay, Husain *et al.* (29) found that the 37% survival value was reached at ≈6 and ≈3 adducts per plasmid in wild-type and *uvrA* strains, respectively. It should be stressed that Husain *et al.* (29) used an analog of cisplatin in their study, namely *cis*-(1,2-cyclohexanediamine)dichloroplatinum.

Mutation Frequency. We used a forward-mutation assay that involves the inactivation of the tetracycline-resistance gene located on plasmid pBR322. This assay has been used previously with chemical carcinogens of the 2-acetylaminofluorene family. The assay responds both to base-pair substitution and to frameshift mutations (24–26). We used the strategy that involves the modification (in this work, with cisplatin) of the 276-base-pair *Bam*HI–*Sal* I (6S) restriction fragment that lies in the early part of the tetracycline-

resistance gene, as outlined by Fuchs *et al.* (24). The recloned platinum-modified restriction fragments were used to transform competent bacteria. The extent of cisplatin modification ranged from 0 to 7.5 platinum adducts per restriction fragment. Individual transformants were assayed for their tetracycline phenotype (plates contained tetracycline at 20 μg/ml). The mutation frequency was calculated as the fraction of tetracycline-sensitive transformants. In this assay, the background mutation frequency is $<4 \times 10^{-4}$ and equal to 6×10^{-4} for untreated plasmid DNA in non-SOS and SOS-induced bacteria, respectively (30).

When noninduced bacteria (i.e., no UV irradiation prior to the transformation step) were transformed, only a slight dose-dependent increase of the mutation frequency was seen (0.2% at the extent of modification corresponding to 7.5 adducts per restriction fragment). This could be due either to a partial induction of the SOS system or to mutations induced by direct miscoding. However, in UV-irradiated host cells (30 J/m^2 in a wild-type bacteria), a strong, dose-dependent increase in the mutation frequency was observed (Fig. 1). The mutation frequency went up to 3% at the extent of modification corresponding to 7.5 adducts per restriction fragment. The mutation frequency induced by 2-acetylaminofluorene adducts in the same mutation assay is given in Fig. 1 for comparison (25). It should be stressed that on a molecular basis, cisplatin adducts are more mutagenic than adducts involving acetylaminofluorene, a strong chemical carcinogen.

Molecular Analysis of the Mutants. Twenty-five independent pBR322 mutants were sequenced. All originated in the wild-type strain AB1157 under conditions of induced SOS functions. Twenty-one mutants are single-event mutants, three are double-event mutants, and one is a triple-event mutant. Among the multiple-event mutants, two appear to be the result of potentially linked events, since the mutations occur in close proximity (see below). On the other hand, two of the double mutants resulted from double base substitutions that occurred at remote sites; in the analysis of the data, we will therefore consider the mutations that occurred in these two mutants as having occurred independently.

Among the 25 mutations that are single-event, 24 are single-base-pair substitutions and one is a -1 frameshift mutation (Table 1). Of the substitution mutations, 18 (75%) represent transversions and $\approx$6 (25%) represent transitions.

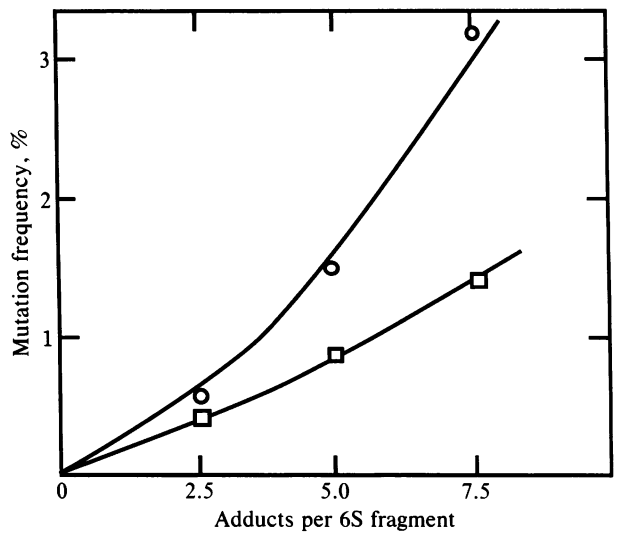

FIG. 1. Frequency of cisplatin-induced (○) and 2-acetylaminofluorene-induced (□; data from ref. 25) mutations. The frequency of tetracycline-sensitive mutants was measured as a function of the average number of adducts per 6S *Bam*HI–*Sal* I fragment in SOS-induced wild-type bacteria (AB1157 cells irradiated at 30 J/m^2).

Among the 18 transversions, 12 are A·T→T·A transversions, 5 are G·C→T·A transversions, and 1 is a G·C→C·G transversion (Table 2). The relative proportions of the transition mutations are given in Table 2.

The distribution of the mutants along the DNA sequence appears to be rather random, with the 24 substitution mutations occurring at 17 different sites (i.e., an average of 1.4 mutants per site; Fig. 2). Using the *lacI* system developed by Miller and colleagues (21–23), Brouwer *et al.* (14) concluded that cisplatin induces base-pair-mutation hot spots at GpApG and GpCpG sequences. A careful analysis of the discrepancy between these results will be discussed at the end of the present paper.

Two complex mutants were found: mutant w120-1Pt contains a triple base-pair substitution within 5 base pairs, and mutant w120-4Pt exhibits a -1 frameshift associated with a G→T transversion within a run of three guanine residues (Table 1).

Mutant w80-5Pt has a $-$C frameshift mutation at a cytosine residue that is part of a GpCpG sequence (Table 1).

Analysis of the Induced Mutations in Relation to the Potential Cisplatin Adducts. Adducts formed by the reaction of cisplatin with DNA have recently been isolated by enzymatic digestion to the level of nucleosides of platinated DNA, followed by chromatographic separation of the adducts by reversed-phase HPLC (4–6). The characterization of the adducts was achieved by comparison with model compounds. The major cisplatin adduct (65% of the adducts formed) was found to be a chelate between the N-7 atoms of two adjacent guanine residues (6). Another reaction product was characterized as being also an N-7 intrastrand crosslink, between the purines in the dinucleotide ApG (25% of the adducts formed) (6). No GpA adduct was detected (6). Diadducts involving two guanines separated by a third base (GpNpG) were also found. Very few monofunctional adducts could be detected at the low levels of modification that correspond to physiological conditions.

In general, it is of interest to compare the mutation spectrum for a given target sequence of DNA to the chemical modification spectrum. In previous work in this laboratory, the same system was used to compare the modification spectrum to the corresponding mutation spectrum of both 2-aminofluorene and 2-acetylaminofluorene adducts (25, 26, 31). Unlike acetylaminofluorene adducts, which are strong blocking lesions for the 3′→5′ exonuclease activity associated with bacteriophage T4 DNA polymerase (31), both aminofluorene (26) and cisplatin adducts (D.B. and R.P.P.F., unpublished results; ref. 49) only slow down the 3′→5′ exonuclease activity. Similarly, cisplatin adducts impede the digestion of DNA by exonuclease III at sites of adjacent guanine residues (32, 33). On the other hand, chemical treatment with hot piperidine, as used for the mapping of aminofluorene adducts (26), did not cleave cisplatin-modified DNA (D.B. and R.P.P.F., unpublished results; ref. 32).

With both aminofluorene and acetylaminofluorene adducts, the modification spectra showed that all the potential target bases (the guanine residues) were modified to similar extents (26, 31). Therefore, we will discuss the position of the cisplatin-induced mutations in the light of the potential sites where cisplatin is known to form adducts (4–8).

Among the 24 base-pair-substitution mutants that are considered in the present study, 14 occur at ApG sites, 9 occur at GpG sites, and 1 occurs at a GpCpG site. The mutations occurring at the ApG sites present a remarkable specificity, since in all cases the mutation affects the adenine residue. In about 85% of the cases (12/14) the mutation is an A·T→T·A transversion; in the remaining cases (2/14) the mutation is an A·T→G·C transition. The strong specificity for A·T→T·A transversions can be viewed in the light of *in vitro* (34) and *in vivo* (35–37) studies showing that SOS-dependent

Table 1. Description of the mutations

Name	Extent of modification*	Mutation	Position†	Local sequence‡	Amino acid change
w120-2Pt	7.6	C·G→T·A	379	GA*GG*AT	Ile→Ile
w120-1Pt	7.6	C·G→A·T	392		Arg→Ser
w120-1Pt	7.6	A·T→G·C	395		Ile→Asp
w120-1Pt	7.6	T·A→A·T	396		Ile→Asp
w80-8Pt	5.0	C·G→G·C	402	CC*GG*CC	Ala→Gly
w80-6Pt	5.0	G·C→A·T	404	CC*GG*CA	Gly→Ser
w80-5Pt	5.0	−C	436	TG*GCG*CC	
w80-2Pt	5.0	T·A→A·T	440	AT*AG*GC	Tyr→Asn
w80-14Pt	5.0	T·A→A·T	440	AT*AG*GC	Tyr→Asn
w120-9Pt	7.6	T·A→A·T	440	AT*AG*GC	Tyr→Asn
w120-22Pt	7.6	G·C→A·T	471	TC*GG*GC	Arg→Gln
w120-10Pt	7.6	T·A→A·T	482	GA*AG*TG	Phe→Ile
w120-14Pt	7.6	T·A→A·T	482	GA*AG*TG	Phe→Ile
w120-15Pt	7.6	G·C→T·A	485	TC*GG*GC	Gly→Trp
w80-37Pt	5.0	C·G→A·T	527	AC*GGGG*CC	Pro→Thr
w80-15Pt	5.0	C·G→A·T	528	AC*GGG*GCC	Pro→His
w120-4Pt	7.6	−G	536		
w120-4Pt	7.6	G·C→T·A	537		
w120-23Pt	7.6	G·C→T·A	537	CC*GGGG*G	Gly→Val
w120-11Pt	7.6	T·A→A·T	543	AC*AG*TC	Leu→Gln
w120-18Pt	7.6	T·A→A·T	557	GG*AG*AT	Ser→Thr
w120-29Pt	7.6	T·A→C·G	606	GT*AG*GT	Leu→Pro
w120-7Pt	7.6	T·A→A·T	624	TT*AG*GA	Leu→Gln
w120-2Pt	7.6	T·A→C·G	624	TT*AG*GA	Leu→Pro
w80-8Pt	5.0	A·T→T·A	633	GG*AG*TC	Glu→Val
w80-9Pt	5.0	A·T→T·A	633	GG*AG*TC	Glu→Val
w80-36Pt	5.0	A·T→T·A	633	GG*AG*TC	Glu→Val
w80-38Pt	5.0	A·T→T·A	633	GG*AG*TC	Glu→Val
w80-7Pt	5.0	C·G→A·T	636	AT*GCG*AC	Ser→amber§
w120-31Pt	7.6	G·C→A·T	644	AA*GGG*AG	Gly→Arg

All mutants were obtained in the wild-type strain AB1157 under conditions of induced SOS functions (30 J/m^2).
*Number of platinum adducts per *Bam*HI–*Sal* I restriction fragment.
†Numbering according to ref. 50.
‡Bases involved in the potential adduct are shown in italics; the base involved in the mutation is underlined. This analysis is not made for the two "complex" mutants (w120-1Pt and w120-4Pt).
§Mutant w80-7Pt has an amber mutation giving rise to a tetracycline-sensitive phenotype despite the *supE* locus in strain AB1157. The *supE* suppressor preferentially inserts glutamine.

replication preferentially adds adenine across from certain noncoding lesions, such as apurinic sites. Aminofluorene adducts preferentially induce G·C→T·A transversions under SOS-induced conditions (26). Although it was demonstrated that no apurinic sites were introduced into the DNA during its chemical treatment with 2-hydroxyaminofluorene, we cannot rule out the possibility that apurinic sites are generated *in vivo* during the repair of aminofluorene lesions (26). On the other hand, as discussed in detail by Miller (38), the simple model of preferentially inserting an adenine across from a pyrimidine-pyrimidine photoproduct does not seem to explain the mutation specificity of UV light as observed in different systems.

The picture is less clear for the nine mutations occurring at GpG sites. Six mutations occur within runs of three or more G·C base pairs. In four such cases, the mutated guanine residue is in the middle of the repeated sequence. It is therefore not possible to know whether the mutated base is at the 3′ or the 5′ side of the potential GpG adduct. In the five unambiguous cases, however, the mutation occurred three times at the 5′ guanine and twice at the 3′ guanine. The specificity of the mutation at the GpG sites was as follows: four G·C→A·T transitions, four G·C→T·A transversions, and one G·C→C·G transversion. At the GpCpG site located at nucleotide position 636, the mutation involves the 3′ guanine residue, which is converted into a thymine residue.

Table 2. Occurrence of base-pair substitutions

	Number of mutants	Relative proportion, %
Transitions	6/24	25
G·C→A·T	4/24	17
A·T→G·C	2/24	8
Transversions	18/24	75
A·T→T·A	12/24	50
G·C→T·A	5/24	20
G·C→C·G	1/24	4
A·T→C·G	0/24	0

As expected for a random DNA sequence, the target DNA sequence in our mutation assay contains about twice as many GpG sites as ApG sites (56 and 28, respectively). About 60% (14/24) of the mutations occur at potential ApG sites (with a strong preference for A·T→T·A transversions), whereas most (9/24) of the other mutations occur at potential GpG sites. Since ApG sites are at least 3 times less reactive with cisplatin than the GpG sites (6), the mutation cross-section (defined as the frequency at which an adduct is converted into a mutation; see ref. 25) of the ApG adduct is ≈5 times higher than that of the GpG adduct.

It is also surprising that most mutations occur at the 5′ side of the potential cisplatin adduct (Table 3). This is the case for all the mutations observed at ApG sites and for some mutations occurring at GpG sites. The mutations described by Brouwer *et al.* (14) at GpApG and GpCpG sites also occurred predominantly at the 5′ guanine residue. This

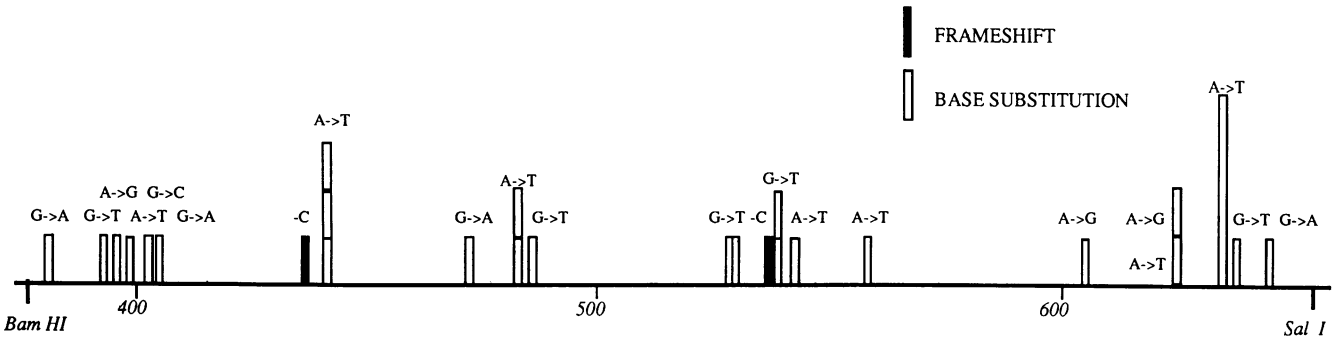

FIG. 2. Spectrum of cisplatin-induced mutations. The position and the nature of the cisplatin-induced mutations is given along the 276-base-pair *Bam*HI–*Sal* I restriction fragment (numbering shown is according to ref. 50). Open and shaded bars represent base-substitution and frameshift mutations, respectively.

implies that during replication, the incorporation of a nucleotide opposite to the first base involved in the adduct (i.e., the base on the 3′ side) is correct and that the mutation is made at the second base involved in the adduct. With regard to the structure of cisplatin adducts, NMR studies with oligonucleotides containing a single GpG adduct favor a kinked base-paired structure for the helix (39–42). Molecular-mechanics calculations provide models of comparable energies with either a kinked helix axis (43) or an unperturbed axis with a large tilt of the 5′ coordinated guanine residue (44). No data are available so far for the corresponding ApG adduct. However, at the dinucleotide level, NMR studies support a large similarity between the GpG and ApG adducts for both the configuration of the bases and the conformation of their ribose (45). Our mutation data suggest that little perturbation might be seen at the 3′ end of the adduct, since the coding properties of the guanine remain intact. Moreover, the preferential incorporation of an adenine residue opposite to the 5′ coordinated adenine (or opposite to the 5′ coordinated guanine residue in some GpG adducts) is reminiscent of the mutation specificity that is induced by noncoding lesions such as apurinic sites. On the other hand, from the data obtained with UV light in different systems (38, 46, 47), it appears that there is a severalfold preference for mutation at the 3′ residue of a pyrimidine-pyrimidine photoproduct.

Table 3. Relation between potential adducts and mutation specificity

	No. of occurrences
ApG adducts	
Total	14
At 5′ base	14
At 3′ base	0
Mutation specificity	
A·T→T·A	12
A·T→G·C	2
GpG adducts	
Total	9
At 5′ base	3
At 3′ base	2
At 5′ or 3′ base	4
Mutation specificity	
G·C→A·T	4
G·C→T·A	4
G·C→C·G	1
GpCpG adducts	
Total	1
At 5′ base	1
At 3′ base	0
Mutation specificity	
G·C→T·A	1

The conclusions on mutation specificities should be taken with some caution, since our assay relies on the phenotypic detection of the mutants (tetracycline sensitivity). A total of 28 ApG sites are present on the target DNA sequence, of which 7 were detected as sites of cisplatin-induced mutations. It is likely that many more ApG sites could be detected if a larger collection of mutants was analyzed. The observed specificity of mutation at adenine vs. guanine in the ApG sites could simply reflect a bias in the number of detectable mutations at adenine vs. guanine. This is probably not the case, since among all the ApG sites, guanine residues appear 16 times in the first position in a codon, vs. 9 times for the adenine residues. Moreover, among the seven ApG sites that were detected in this assay, three are such that adenine is second and guanine first in the corresponding codon. At least in these cases, any mutation at the guanine residue is detectable.

As far as the specificity of ApG vs. GpG sites is concerned, one can speculate that if the adduct chelates the first two nucleotides of a codon, there is a given chance that a mutation will be detectable. The conclusion that ApG sites are more mutable than GpG sites is likely to be real, since there is a comparable proportion of ApG and GpG sites located so that a potential adduct chelates the first two nucleotides of a codon (32% and 39%, respectively).

Comparison of Our Mutation Data with the Other Available Data. The molecular nature of cisplatin-induced mutations was previously investigated by using either the histidine reversion assays in *Salmonella typhimurium* (16–20, 48) or the *lacI* forward-mutation assay in *E. coli* (14). From the reversion assays in *S. typhimurium*, it was concluded that cisplatin was able to revert base-pair-substitution strains, provided plasmid pKM101 was present, but not frameshift-mutation strains (48).

In the present work, we analyzed all the mutants leading to the inactivation of the tetracycline-resistance gene. Previous studies with chemical carcinogens belonging to the family of aromatic amines have shown that the assay responds both to base-pair substitutions (26) and to frameshift mutations (25), deletions, and insertions. Our results show that cisplatin induces a majority of A·T→T·A transversions, mainly at ApG sites. Brouwer *et al.* (14) found that cisplatin induces base-pair-substitution hot spots in the *lacI* gene at GpApG and GpCpG sequences (70% of all the nonsense mutants), the mutations being either G·C→A·T transitions or G·C→T·A transversions. At most, but not all, sites the mutated guanine residue was the one at the 5′ side. The *lacI* mutation assay used by Brouwer *et al.* (14) is such that among all the induced mutants, only the molecular nature of the base substitutions leading to nonsense codons is analyzed. As found by these authors, this subclass of mutants represents only 13% of the collection of induced *lacI* mutants.

The lack of detection of A·T→T·A transversions at the ApG sites in the *lacI* system is due to the fact that only in-frame ApGpA triplets can lead to a TpGpA stop codon. In fact the only in-frame AGA codon in the entire *lacI* gene is at position 1004, a position that is too close to the COOH-terminal end of the protein to inactivate the gene product. We checked our target DNA sequence for potential GpApG and GpCpG sites that we know to respond phenotypically. Four such sites are available in the *Bam*HI–*Sal* I restriction fragment. There are three GpCpG sites at which the G·C→T·A transversion of the 5′ guanine residue was phenotypically detected. There are also a GpCpG and a GpApG sequence for which the G·C→T·A transversion of the 3′ guanine residue was phenotypically detected. These data stem from our collection of sequenced mutants, which contains about 35 sites that respond to base substitutions. It is probable that we have not yet saturated the mutation map. Among the possible sites for mutagenesis at GpCpG sequences, only one cisplatin-induced mutation was found (Table 2).

In conclusion, using an assay that is not limited to the detection of nonsense codons, we have shown that the previously detected hot spots at GpCpG and GpApG sites are not really hot spots. Moreover, the most mutagenic lesion is at the ApG sites, giving predominantly A·T→T·A transversions.

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