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Single d(ApG)/*cis*-diamminedichloroplatinum(II) adduct-induced mutagenesis in *Escherichia coli*

(single-adduct mutagenesis/mutation specificity/base substitutions)

DOMINIQUE BURNOUF*, CORINNE GAUTHIER†, JEAN CLAUDE CHOTTARD†, AND ROBERT P. P. FUCHS*‡

*Groupe de Cancérogénèse et de Mutagénèse Moléculaire et Structurale, Institut de Biologie Moleculaire et Cellulaire du Centre National de la Recherche Scientifique, 15 rue René Descartes, 67084 Strasbourg Cedex, France; and †Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, Unité Associée 400 Centre National de la Recherche Scientifique, Université René Descartes, 75005 Paris, France

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ABSTRACT **The mutation spectrum induced by the widely used antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) showed that *cis*DDP{d(ApG)} adducts, although they account for only 25% of the lesions formed, are ≈5 times more mutagenic than the major GG adduct. We report the construction of vectors bearing a single *cis*DDP{d(ApG)} lesion and their use in mutagenesis experiments in *Escherichia coli*. The mutagenic processing of the lesion is found to depend strictly on induction of the SOS system of the bacterial host cells. In SOS-induced cells, mutation frequencies of 1–2% were detected. All these mutations are targeted to the 5′ base of the adduct. Single A → T transversions are mainly observed (80%), whereas A → G transitions account for 10% of the total mutations. Tandem base-pair substitutions involving the adenine residue and the thymine residue immediately 5′ to the adduct occur at a comparable frequency (10%). No selective loss of the strand bearing the platinum adduct was seen, suggesting that, *in vivo*, *cis*DDP{d(ApG)} adducts are not blocking lesions. The high mutation specificity of *cis*DDP-{d(ApG)}-induced mutagenesis is discussed in relation to structural data.**

During the past decade, progress in molecular biology allowed the development of strategies for elucidating the molecular mechanisms involved in processing the damages introduced in DNA by physical or chemical agents. A general procedure is to determine the forward mutation spectrum induced by a particular agent on a target DNA sequence (1). This approach allows definition of the general characteristics of the mutagenic processes, such as their genetic requirements and their specificity. However, these strategies are not sensitive enough to measure the relative contribution of different adducts in repair or mutagenesis events.

This problem has been overcome by the use of synthetic oligodeoxyribonucleotides containing defined adducts located at precise sites (for review, see ref. 2). When these adducts are introduced in a replicative vector and transfected into cells, several biological endpoints can be monitored: toxicity (3, 4), mutation efficiency and specificity (3–6), effect of the repair genotype of the host (7), and effect of surrounding sequences (3, 6). Furthermore, such modified oligonucleotides became useful tools for studying the structure of the DNA helix in the vicinity of the adduct (8–13) and precise biochemistry of repair (14–16).

cis-Diamminedichloroplatinum(II) (*cis*DDP) is a widely used antitumor drug that binds to DNA, its main cellular target, by preferentially forming intrastrand crosslinks between the N7 atoms of adjacent purines (17–19). At low modification levels, the main adducts result from the chelation of two adjacent guanines [*cis*DDP{d(GpG)}], 65% of total lesions), whereas 25% of the adducts arise at 5′ d(ApG) sequences. d(GpHpG) adduct (H = A, C, or T) and interstrand crosslinks altogether represent <10% of the total lesions (20–22).

We have described the spectrum of mutations induced by *cis*DDP in *Escherichia coli* (23), using a forward mutation assay based on inactivation of the tetracycline resistance gene of pBR322 (1). We have shown that the minor adduct formed on d(ApG) sequences is at least 5-fold more mutagenic than the major *cis*DDP{d(GpG)} adduct.

To get more insight into the specificity of the mutagenic processing of these lesions, we conducted mutagenesis experiments by using a probe specifically designed to study the mutagenesis induced by a single *cis*DDP{d(ApG)} adduct.

MATERIAL AND METHODS

Strains, Vectors, and Oligonucleotides. Strain W71/18 [Δ(*lac-pro*),*rpsL*/*F′lacI*$_q$*lacZ*Δ*M15*, *proB*$^+$*A*$^+$] was used as host bacteria. Plasmid DNA was prepared and purified on cesium chloride gradients as described (24). For rapid purification of plasmids we used the minipreparation procedure of Holmes and Quigley (25). pEMBL8(−) plasmid is a gift from G. Cesarini (EMBL, Heidelberg) (26). pSM9 plasmid is a derivative of pEMBL8(−): it results from the cloning of the duplex d{(AACTAGTTC)(GAACTAGTT)} in the unique *Hin*cII site located in the polylinker region of pEMBL8(−).

Media and Antibiotics. Bacterial suspensions are grown in Luria broth medium containing ampicillin (50 μg/ml) when needed. Transformed cells are plated on medium containing isopropyl β-D-thiogalactopyranoside (60 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (50 μg/ml), and ampicillin (100 μg/ml).

Enzymes. Restriction enzymes *Cla* I, *Sal* I, and *Spe* I were purchased from New England Biolabs and *Hin*cII was from Promega. P1 nuclease and alkaline phosphatase were from Boehringer Mannheim, DNase I was from Sigma, and snake venom phosphodiesterase and calf spleen phosphodiesterase were from Worthington. All enzymes were used as specified by the suppliers. Sequence analysis was performed using the Sequenase kit of United States Biochemical or T7 polymerase from Amersham.

Bacterial Transformation. Bacteria were made competent for transformation by the $CaCl_2$ procedure (27). Bacteria are UV-irradiated in $MgSO_4$ (0.01 M) at a fluence ranging from 50 to 70 J/m^2 to induce the SOS system (6).

Oligonucleotide Synthesis, Modification by *cis*DDP, and Purification. The 9-mer d(ApApCpTpApGpTpTpC) was synthesized by a phosphotriester method in solution (28) and purified by reverse-phase HPLC. The NH_4^+ salt of the

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Abbreviations: *cis*DDP, *cis*-diamminedichloroplatinum(II); X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

‡To whom reprint requests should be addressed.

purified nonamer was treated with an aqueous solution of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ (20 mM), freshly prepared from $AgNO_3$ treatment of the dichloro complex (29).

The reaction between the diaqua complex and the nonanucleotide (one Pt per nonanucleotide strand) was run at a concentration of $\approx 5 \times 10^{-5}$ M determined by UV absorption (estimated ε_{260}, 72,000 $cm^{-1} \cdot M^{-1}$) in doubly distilled water at pH 5.5–6. The crude reaction mixture was treated with a KCl solution to eliminate any water–platinum species and lyophilized before preparative HPLC. The reverse-phase HPLC analysis and separations were performed on a Nucleosil C_8, 5-μm, 300-Å semipreparative column (250*4). Eluent A was aqueous $CH_3CO_2NH_4$ (2×10^{-2} M), pH 6.3, and eluent B was H_2O/CH_3CN, 1:1. (Flow rate, 2 ml/min; gradient, %B = 13% for 2 min and then %B to 23% in 10 min.) HPLC monitoring of the reaction revealed the formation of a main adduct that accounted for 40% of the overall products.

The oligonucleotides were radioactively labeled using T4 polynucleotide kinase, and the phosphorylated forms were purified on 20% polyacrylamide denaturing gel. After overnight elution in 0.3 M sodium acetate buffer and precipitation with ethanol, the oligonucleotides were redissolved in water.

Construction of Vectors Used in Mutagenesis Experiments. The same general strategy, as described elsewhere (30), was followed with few modifications. pEMBL8(−) and pSM9 plasmids were linearized with *Hin*cII and *Cla* I restriction enzymes, respectively. When specified, UV-irradiated linearized pSM9 plasmid DNA was used in construction of the gapped-duplex. UV irradiation at 240 J/m^2 was performed in water in a 1-mm quartz cuvette at DNA concentration of 0.1 μg/ml, yielding an average of five to six UV lesions per plasmid strand (31). Equal quantities of linearized pEMBL8(−) and pSM9 (UV-irradiated or not) were mixed, heat-denatured, and allowed to renature as described (30). Purified 9-mer oligonucleotides were mixed with the crude mixture containing gapped-duplex molecules. After hybridization and ligation, the covalently closed circular plasmids were purified on a CsCl gradient.

Recovery and Analysis of Mutants. The form I plasmid DNA from the different constructions was used to transform either uninduced or SOS-induced W71/18 *E. coli* cells. In the present assay, blue colonies are identified as containing a potential platinum-induced mutant plasmid. Because blue colonies can contain both a blue and a white colony-forming plasmid, an additional purification step was introduced to isolate the pure (blue) mutant plasmids. The plasmid DNAs contained in blue colonies are prepared by a boiling procedure (25) and retransformed into W71/18 bacteria. Single blue colonies resulting from the second transformation step were transferred onto nitrocellulose filters placed in X-Gal plates and grown overnight at 37°C. The plasmid DNA in the colonies was analyzed by hybridization with 5-end-labeled 15-mer oligonucleotides complementary to the region containing the 9-mer, according to the method of Wallace *et al.* (32). Sequencing, using a modified Sequenase protocol, was used to confirm results of the hybridization analysis.

RESULTS AND DISCUSSION

Strategy of Mutagenesis. The mutagenesis assay uses the *lacZ* system. The nonanucleotide d(A**ACTAGTTC**) is cloned in the unique *Hin*cII site of pEMBL8(−) plasmid. This insertion creates a unique *Spe* I restriction site (underlined) and results in incorporation of an in-frame nonsense amber codon (in boldface) in the early part of the α fragment of the *lacZ* gene (see Fig. 1). When propagated in a suppressor-free strain, this plasmid, pSM9, forms white colonies on X-Gal-containing plates. All base-substitution events that will change the nonsense codon into a sense one will give rise to blue colonies. In other words, this assay detects phenotypically (i.e., color change from white to blue) all possible base-substitution events involving the TAG triplet except the G → A transition that changes the amber codon into an ochre codon (TAG → TAA). The d(ApG) dinucleotide contained in the oligonucleotide is the target for *cis*DDP adduct formation.

Characterization of the *cis*DDP{d(ApG)} Adduct. Analysis of the main platination adduct by atomic absorption spectroscopy (Perkin–Elmer A560 with HGA-500 graphite furnace) coupled to the UV absorption (with ε assumed to be 72,000 $cm^{-1} \cdot M^{-1}$) showed that it was monomeric and contained one platinum atom.

The NMR spectrum of this adduct (0.5×10^{-3} M) was recorded in 99.95% ^2H_2O at 70°C on a Bruker WM 250 NMR spectrometer. The spectrum of the nonexchangeable protons exhibits two signals at 8.96 and 9.11 ppm (from trimethylsilyl-3-propionic acid d_4-2,2,3,3). These downfield-shifted resonances, together with the fast H–^{2}H exchange of the first one, are comparable to those observed for the adenine (H8) and guanine (H8) protons of previously described *cis*DDP-{d(ApG)} chelates (33, 34).

Enzymatic digestions were used to confirm assignment of the coordinated purines. The adduct was digested with DNase I and nuclease P1 according to Fichtinger–Schepman *et al.* (21). The UV quantification was based on HPLC analysis of the nucleosides resulting from the digestion of the nonmodified nonamer. Despite two successive 12-hr digestions with nuclease P1, analysis of the nucleosides released from the platinated 9-mer (two dA, two dC, two dT) showed that the nondigested product was *cis*-$[Pt(NH_3)_2\{d(TpApG)$-N7(2),N7(3)$\}]$. For better characterization of the main adduct, enzymatic digestions by exonucleases were done.

Digestion with snake venom phosphodiesterase, which hydrolyzes the phosphodiester linkages starting from the 3′-OH end was done according to Inagaki *et al.* (35, 36). After incubation with snake venom phosphodiesterase at room temperature followed by alkaline phosphatase treatment, measurement of the released nucleosides yielded a 1:2 ratio for dC and dT residues, respectively, showing clearly that platinum is bound to guanine (6).

Similarly, digestion with calf spleen phosphodiesterase (35, 36), which hydrolyzes the phosphodiester linkages starting from the 5′-OH end followed by alkaline phosphatase digestion yielded only cytosine and adenine nucleosides in a 1:2 ratio. Even after longer incubation times (72 hr), only a very small amount of dT could be detected, showing that calf spleen phosphodiesterase cannot hydrolyze the phosphodiester linkage at the 5′ side of the *cis*DDP{d(ApG)} chelate, whereas this phosphodiesterase does so in these conditions with an oligonucleotide containing a *cis*DDP{d(GpG)} chelate (37). The NMR and digestion data combined identify the main platination adduct as *cis*-$[Pt(NH_3)_2\{d(ApApCpTpApGpTpTpC)$-*N7(5),N7(6)*$\}]$ complex.

Construction of Vector. The strategy shown in Fig. 1 is similar to that described earlier (6, 30). Four different plasmids were constructed as described in *Material and Methods* and designated p9, p9Pt, $p_{uv}9$, and $p_{uv}9Pt$. Plasmids p9 and p9Pt result from the incorporation of the unmodified or *cis*-DDP-monomodified nonanucleotide in the gapped-duplex molecule, respectively. In plasmids $p_{uv}9$ and $p_{uv}9Pt$, the same oligonucleotides were incorporated in gapped-duplex molecules, bearing an average of five to six UV lesions in the strand opposite to the *cis*-platinum-containing strand. Previous papers have shown that platinum adducts inhibit the activity of different restriction endonucleases (38, 39). The presence and position of the platinum adduct in the reconstructed plasmids were confirmed by specific inhibition of the *Spe* I restriction enzyme activity on the *cis*-DDP-containing plasmids (Fig. 2).

Mutagenesis Experiments. The reconstructed closed circular plasmids, purified in CsCl gradients, were used to trans-

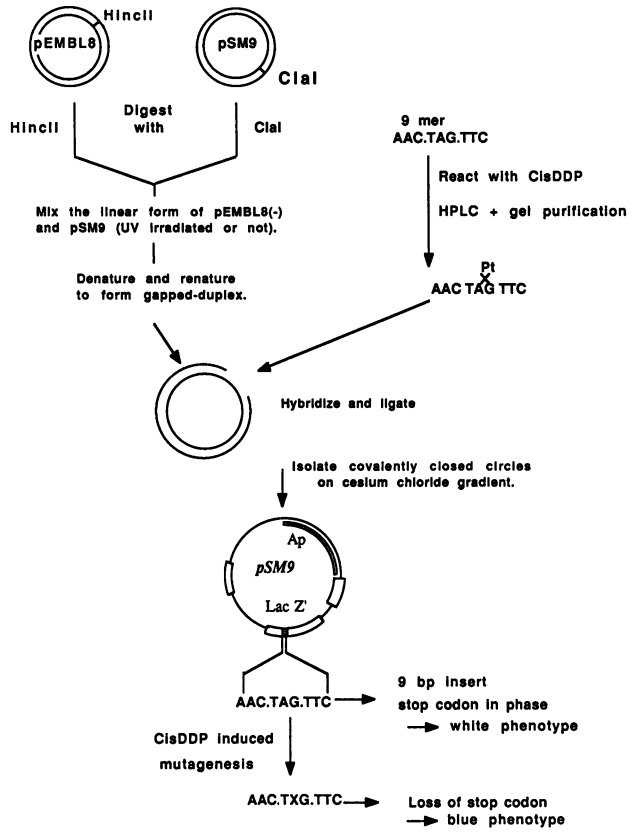
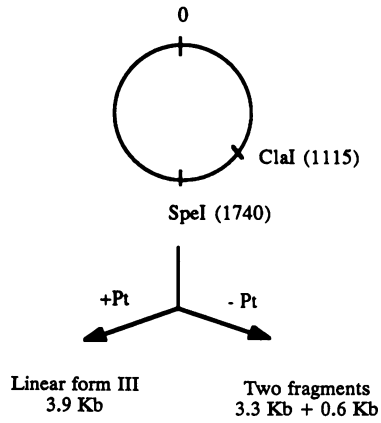


FIG. 1. Strategy for single-adduct mutagenesis experiment and detection of mutational events. bp, Base pair.

form uninduced or SOS-induced *E. coli* cells. Potential mutant colonies were selected on X-Gal-containing medium as blue colonies. Some blue colonies resulted from a small fraction of contaminating pEMBL8(−) plasmid DNA that had escaped the restriction steps and cosedimented with fully ligated reconstructed pSM9 plasmid on the CsCl gradient. These nonmutant blue colonies were easily detected and discarded from further sequence analysis by a simple hybridization assay. Indeed, an oligonucleotide probe [d(GTCAAC-TAGTTCGAC)] containing the 9-mer insert (underlined) of plasmid pSM9 will hybridize to a true mutant but not to a pEMBL8(−) contaminant. Among the clones that were found positive in this hybridization assay we could further analyze the mutation by using three different oligonucleotides d(GTCAACTBGTTCGAC) (B = G, T, or C), which differ by one base at the eighth nucleotide position. Hybridization analysis of the positive blue clones with these three probes allowed us to determine the exact base change that occurred at the eighth nucleotide. The predicted nature of the mutations was confirmed by sequencing the plasmids extracted from clones chosen on the basis of their hybridization profile. Among 54 mutant plasmids sequenced, all were found to be targeted to the site of the adduct, thus confirming an observation made in other single-adduct mutagenesis studies (3, 6, 40). More precisely, all mutant plasmids had a base substitution involving the adenine residue of the *cis*DDP{d(ApG)} adduct, as expected from previous mutation spectrum studies (23). Also in agreement with previous observations, *cis*-platinum-induced mutagenesis depends strictly upon induction of the host-cell SOS system (23). In uninduced cells, mutation frequency with plasmid p_{uv}9Pt was estimated as $<0.2 \times 10^{-3}$ (Table 1). When the cells are SOS induced, mutation frequency rises by >2 orders of magnitude (Table 1).

Average efficiency of conversion of a *cis*DDP{d(ApG)} adduct into a mutation [mutation cross section (41)] can be estimated from our previously published mutation spectrum (23) at equal to 10^{-2}. The mutation cross section determined in this study with a single *cis*DDP{d(ApG)} adduct was $\approx 1.4\text{–}2 \times 10^{-2}$ (as deduced from Table 1), a value agreeing well with the previous estimation. We stress that these mutation cross section values were determined in excision-repair-proficient strains (ref. 23, this study) and that a different mutation cross section value can be expected for the same adduct in a repair-deficient strain.

In SOS-induced cells, ≈80% of the mutants recovered with both constructions p9Pt and p_{uv}9Pt exhibits a single A → T transversion, involving the adenine residue of the *cis*DDP{d(ApG)} adduct (Table 2). A second class of single base-pair substitution mutants involving the same adenine residue are A → G transitions (10%). The remaining mutants (≈10%) are tandem base-pair substitutions involving the adenine residue of the *cis*DDP{d(ApG)} adduct and the thymine residue immediately 5′ to the adenine residue. These



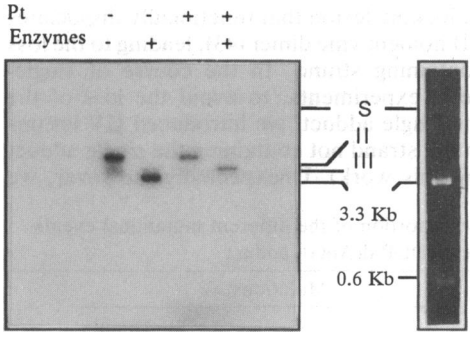


FIG. 2. Biochemical analysis of the reconstructed plasmids. The exact position of the platinum adduct in the purified plasmids was assessed by a double-restriction assay. Vectors were digested by both *Cla* I and *Spe* I restriction enzymes (1 hr at 37°C) (*Left*), and the products were analyzed on 1.4% agarose gel without ethidium bromide (*Right*). *Cla* I enzyme was chosen so that the ^{32}P-labeled 5′ end of the 9-mer remained in the large fragment of the double-restricted plasmid. After migration (2 hr, 70 V), the gel was stained with ethidium bromide, dried, and autoradiographed. The autoradiogram shows the migration profile of plasmids p9 and p9Pt. In the absence of enzymes, unmodified or platinated plasmids migrated as a single 3.9-kilobase (kb) band corresponding to circular form II DNA. Treatment of unmodified plasmids with *Spe* I/*Cla* I gave a single radioactive band of 3.3 kb; a second band of 0.6 kb was seen under UV light (far right). Restriction of p9Pt with *Spe* I/*Cla* I yielded a single 3.9-kb band of linear DNA (form III). Loss of the *Spe* I restriction site is attributed to *cis*DDP{d(ApG)} adduct on the d(ApG) sequence.

Table 1. Mutation frequency induced by a single *cis*DDP{d(ApG)} adduct

	Plasmids			
	p9	$p_{uv}9$	p9Pt	$p_{uv}9Pt$
− SOS	<0.6 (0/1661)	<1.06 (0/936)	<1.02 (0/976)	<0.17 (0/5747)
+ SOS (60 J/m²)	ND	ND	20 (214/10,472)	13.8 (498/36,000)

Mutation frequencies are expressed per 10^3 transformants. The number of mutant clones over total number of transformants are in parentheses. Constructions were introduced into W71/18 *E. coli* cells by transformation. To induce the SOS system, cells were irradiated before transformation at a UV dose of 50–70 J/m². Some blue colonies contained the starting pEMBL plasmid; these plasmids were apparently carried over during construction and represent background noise in phenotypic selection of mutants. Typical frequency of this background was $\approx 5 \times 10^{-3}$. ND, not determined.

mutants were detected on the basis of a weak hybridization profile with any of the three probes. Among 10 double mutants sequenced, all had an A → T transversion, the second base change being a T → C transition (nine cases) or a T → A transversion (one case). The mutational specificity determined in this work is in good agreement with and extends the data derived from the mutation spectrum analysis (23).

The single-adduct mutagenesis study presented here is characterized by two principal features. (*i*) Mutations only occur at the 5′ base of the *cis*DDP{d(ApG)} diadduct—i.e., the adenine residue. Recall that the present assay can detect all base changes occurring at the guanine residue except for G → A transitions. Moreover, if a mutation had occurred on the 3′ guanine, it would have been detected by a weak hybridization profile. We note that in the mutational spectrum no mutants involving the 3′ base of any potential *cis*DDP{d(ApG)} adducts were found (23). (*ii*) There is a strong specificity for the induction of A → T transversions. Finally, a good correlation exists between the biological data presented here and structural data obtained by others (see below).

The Specific *cis*DDP Adduct-Induced Mutagenesis Displays No Strand-Loss Phenomenon. Previous work in our laboratory showed that a plasmid, bearing an average of two to three *N*-acetylaminofluorene adducts in one strand only of a double-stranded molecule, undergoes the specific loss of the adduct-containing strand (42). It was suggested that, in the course of the replication process, an *N*-acetylaminofluorene adduct acts as a blocking lesion that functionally dissociates the polymerase III holoenzyme dimer (43), leading to the loss of the adduct-containing strand. In the course of single-adduct mutagenesis experiments, to avoid the loss of the strand bearing the single adduct, we introduced UV lesions at random within the strand not containing the single adduct of interest (ref. 6, this work). Unexpectedly, however, we found here that the mutation frequency and specificity are identical, whether or not the vector bears UV lesions in the complementary strand (Tables 1 and 2), suggesting that unlike *N*-acetylaminofluorene adducts, the *cis*DDP{d(ApG)} adduct is not blocking the replication fork and, therefore, not triggering the strand-loss phenomenon. However, this contrasts with *in vitro* DNA synthesis experiments showing that *cis*DDP{d(GpG)} and, to a lesser extent, *cis*DDP{d(ApG)} adducts block *E. coli* DNA polymerase I and different eukaryotic DNA polymerases (44–46). It is obvious that *E. coli* DNA polymerase I does not accurately reflect the *in vivo* situation where replication is performed by the holoenzyme DNA polymerase III complex under SOS-induced conditions (47).

Table 2. Relative proportion of the different mutational events induced by a single *cis*DDP{d(ApG)} adduct

	Mutations, %		
	A → T	A → G	Double mutation
p9Pt	83 (1.7)	10 (0.2)	7 (0.14)
$p_{uv}9Pt$	76 (1)	10 (0.14)	14 (0.19)

Relative percentage of each type of mutation is given for both platinum-containing constructs. Terms in parentheses indicate the absolute percentage of mutant clones of each class. Double-mutational events refer to tandem base-pair substitutions involving the adenine residue and the thymine residue located immediately 5′. These mutants, in most cases, were TpA → CpT changes.

Analysis of the *cis*DDP{d(ApG)}-Induced Mutations in View of Structural Data. Specificity of mutagenesis induced by a given mutagen is thought to be closely related to the structure of the premutagenic lesion and to the deformation undergone by the modified DNA sequence. Different studies have focused on the determination of the structure of the *cis*DDP-{d(GpG)} lesion. NMR studies (10–13), molecular mechanic calculations (48, 49), and analysis in solution (12, 50) favored the formation, upon chelation of adjacent guanines, of a kinked or bent structure in DNA. Analysis of NMR data reveals an asymmetric structure for the *cis*DDP{d(GpG)} adduct: the two guanines are in an anti–anti configuration, and the 5′ nucleoside has a (C3′-endo) conformation; meanwhile, the 3′ is 70% in a (C2′-endo) conformation (13, 51, 52). A similar conclusion has also been reached from crystallographic analysis of a *cis*DDP-modified dinucleotide d(GpG) (19). Limited data are available concerning the structure of the *cis*DDP{d(ApG)} lesion. At least at the dinucleotide level, the structure of both d(GpG) and d(ApG) adducts is similar (33, 34). Chemical probes analysis (50, 53) shows that the local distortion of DNA concerns the 5′ side of the d(ApG) adduct, whereas the 3′ guanine is still paired. This is confirmed by the NMR analysis of the d(CTCA*G*CCTC)-(GAGGCTGAG) duplex platinated on the bases marked with asterisks (C.G., E. Guittet, J. Igolen, J. Y. Lallemand, and J.C.C., unpublished work). Retardation of migration of a *cis*DDP{d(ApG)}-bearing oligonucleotide also suggests that this lesion bends DNA (53). Moreover, this adduct, when formed in the sequence YAG, appears more disruptive than when formed in RAG (50). In identifying the *cis*DDP{d(ApG)} adduct by enzymatic digestion, we found that neither P1 nuclease nor calf spleen phosphodiesterase could hydrolyze the phosphodiester bond between thymine and adenine on the 5′ side of the chelate. Others have observed the same (21, 22), although Eastman (22) found that the undigested bond is located on the 3′ side of the lesion. We have no explanation for this discrepancy. The resistance to phosphodiesterase processing reflects an altered conformation of the backbone, which, in our case, is located 5′ to the adduct.

The above observations correlate well with our mutation data (ref. 23, this work), showing that mutation at d(ApG) sites always occurs at the 5′ base of the adduct (adenine residue) or for a tandem base substitution, at both the adenine residue and the adjacent 5′ base. It has been observed (54) that an adenine residue is preferentially inserted across a noninformative lesion. In this context the high specificity of mutagenesis at the d(ApG) site (90% of A → T transversion) might be explicable by considering the platinum-modified adenine base as noninformational.

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