Mutagenicity and Genotoxicity of the Major DNA Adduct of the Antitumor Drug *cis*-Diamminedichloroplatinum(II)†

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ABSTRACT: The mutagenicity and genotoxicity of *cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7(1),-*N*7(2)\}] (G*G*), the major DNA adduct of the antitumor drug cisplatin, has been investigated in *Escherichia coli*. A duplex bacteriophage M13 genome was constructed to contain the G*G* adduct at a specific site in the (–) strand. The singly platinated duplex genome exhibited a survival of 22% relative to that of the unplatinated control genomes, and this value rose to 38% in cells treated with ultraviolet light to induce the SOS response. Singly platinated single-stranded genomes were also produced. Replication of the single- and double-stranded genomes in vivo yielded SOS-dependent, targeted mutations at frequencies of 1.3% and 0.16%, respectively. The mutagenic specificity of G*G* in both single- and double-stranded DNA was striking in that 80–90% of the mutations occurred at the 5′-platinated G. Approximately 80% of the mutations were G → T transversions at that site. A model of mutagenesis is presented to explain this mutational specificity with respect to current understanding of platinum–DNA adduct structure.

cis-Diamminedichloroplatinum(II) (*cis*-DDP)[1] is a highly effective drug used mainly in the treatment for ovarian and testicular tumors (Loehrer & Einhorn, 1984). The biological effects of *cis*-DDP are believed to arise from covalent binding of the drug to DNA (Bruhn et al., 1990). The most abundant DNA adducts are the *cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7(1),-*N*7(2)\}] (G*G*), *cis*-[$Pt(NH_3)_2$\{d(ApG)-*N*7(1),-*N*7(2)\}] (A*G*), and *cis*-[$Pt(NH_3)_2$\{d(GpNpG)-*N*7(1),-N7(3)\}] intrastrand cross-links, accounting collectively for >90% of the drug molecules bound to DNA (Fichtinger-Schepman et al., 1985; Eastman, 1983). Minor adducts include interstrand cross-links, protein–DNA cross-links, and *cis*-DDP–DNA monoadducts. *cis*-DDP inhibits DNA replication in vivo and in vitro in bacterial and mammalian systems (Pinto & Lippard, 1985a,b; Villani et al., 1988; Ciccarelli et al., 1985); this property is widely believed to contribute to the antitumor effects of the drug.

The biological consequences of *cis*-DDP are unfortunately not limited to the selective killing of tumor cells. *cis*-DDP is carcinogenic in the mouse and rat (Leopold et al., 1981). Moreover, the appearance of second malignancies in patients treated with *cis*-DDP has fueled speculation that the drug may have induced these tumors (Hennings et al., 1990; Greene, 1992). Given the strong mechanistic link between mutagenesis and carcinogenesis, it is not surprising that *cis*-DDP is a mutagen in mammalian cells (Johnson et al., 1980) and in bacteria (Beck & Brubaker, 1975; Benedict et al., 1977). In the latter, mutagenicity is dependent upon SOS induction (Fram et al., 1985).

The exact nature of the mutations of *cis*-DDP has been studied in several forward mutation assays but with conflicting results. By using the endogenous *Escherichia coli lacI* gene as the target, Brouwer et al. (1981) found that the mutations arising from *cis*-DDP treatment to cells are primarily GC → TA or GC → AT substitutions at GpApG and GpCpG sequences. In a different study, the tet^R gene of pBR322 was treated in vitro with *cis*-DDP and then transferred for replication into *E. coli* cells that had been pretreated with UV to induce the SOS response (Burnouf et al., 1987). In contrast to the results of Brouwer et al. (1981), most (>90%) of the mutations detected in this assay occur at ApG and GpG sequences, with AT → TA transversions being the predominant mutation. *cis*-DDP-induced mutations in the adenine phosphoribosyltransferase gene of Chinese hamster ovary cells have also been investigated (de Boer & Glickman, 1989). In this mammalian cell line, base substitutions at GC base pairs predominate, with the majority occurring at ApGpG and GpApG sequences. Similar results recently have been observed by Bubley et al. (1991) and Cariello et al. (1992).

Anticancer drugs should show toxicity, ideally with specificity toward cancer cells, but they should display low, if any, mutagenic and carcinogenic activities. The motivation for the present study was to evaluate the relative toxic and mutagenic potentials of the individual DNA adducts of *cis*-DDP. Recently, the mutagenicity of the A*G* adduct of *cis*-DDP, which represents up to 25% of all adducts formed, was investigated by Fuchs and colleagues (Burnouf et al., 1990). They found that the adduct induces mainly A → T transversions at a frequency of 1–2% in duplex DNA in SOS-induced *E. coli*. The toxic effects of the adduct were not investigated. The work presented here has examined the genotoxicity, mutation frequency, and mutation specificity of the major DNA adduct of *cis*-DDP, the G*G* intrastrand cross-link, which accounts for approximately 65% of total drug binding.

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); G*G*, *cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7(1),-*N*7(2)\}]; A*G*, *cis*-[$Pt(NH_3)_2$\{d(ApG)-*N*7(1),-*N*7(2)\}]; T4 EndoV, bacteriophage T4 endonuclease V; 12-T, d(TCTAGGCCTTCT); Pt-12-T, d(TCTAG*G*CCTTCT); ss, single stranded; ds, double stranded; LB, light blue; AP, apurinic/apyrimidinic.

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MATERIALS AND METHODS

Materials. Enzymes were purchased from Boehringer-Mannheim, unless otherwise indicated. Bacteriophage T4 endonuclease V (T4 EndoV) was kindly provided by R. S. Lloyd [Vanderbilt University; for a detailed description of this enzyme see Higgins and Lloyd (1987)]. The *E. coli* cell lines used were as follows: DL7 (AB1157; *lacΔU169*, *uvr*$^+$ (Lasko et al., 1988); DL6 (AB1886; *lacΔU169*, *uvrA* (Lasko et al., 1988); GW5100 (JM103, P1$^-$, from G. Walker, MIT); MM294A (*lac*$^+$), from K. Backman, Biotechnia International. The oligonucleotide d(TCTAGGCCTTCT) (12-T) was purchased from Operon Technologies, Inc. (Alemeda, CA). The synthesis and characterization of d(TCTAG*G*CCTTCT) (Pt-12-T) containing the G*G* cross-link were described previously (Naser et al., 1988).

*Construction of a Duplex M13 Genome Containing a Single G*G* Adduct.* A heteroduplex M13 genome containing a 12-base gap in the (–) strand was produced by hybridizing *Hin*dII-linearized M13mp18 RF DNA with the viral (+) strand of UV-irradiated M13-12A (Naser et al., 1988). The latter genome was prepared originally from M13mp18 by the insertion of the dodecanucleotide d(AGAAGGCCTAGA) into the center of the unique *Hin*dII site (Naser et al., 1988). M13-12A (+) strand DNA, at a concentration of 300 μg/mL in 10 mM sodium phosphate buffer (pH 7) containing $AgNO_3$ (r = 0.5; Ag^+/PO_4-DNA), was UV irradiated at 1500 J/m^2 in 50-μL droplets on a Parafilm-lined Petri dish by using a 15-W General Electric germicidal lamp, at an average dose rate of 1.1 J/(m^2·s). To remove Ag^+, the DNA was first dialyzed against H_2O. NaCl was then added to a final concentration of 0.5 M, and the solution was stirred in the dark overnight. The AgCl precipitate was removed by centrifugation, and the DNA (M13-12A-UV) in the supernatant was dialyzed against 10 mM Tris-HCl and 1 mM EDTA (pH 8) overnight.

Dodecanucleotides 12-T and Pt-12-T labeled at their 5′ termini with ^{32}P were inserted into gapped duplex genomes as described (Naser et al., 1988) to produce 12T/UV and Pt12T/UV, respectively (Figure 1).

*Synthesis of a Single-Stranded M13 Genome Containing a Single G*G* Adduct.* For experiments in which single-stranded (ss) singly platinated DNA was transfected into cells, the 12T/UV and Pt12T/UV double-stranded (ds) genomes were incubated with T4 EndoV (Figure 1) in the presence of 10 mM Tris-HCl (pH 8), 50 mM EDTA (pH 8), 100 mM NaCl, and 1 mg/mL bovine serum albumin at 37 °C for 1 h. The samples were extracted once with an equal volume of chloroform/phenol [1:1, saturated with 0.1 M Tris-HCl (pH 8)] and then added to 1 mL of 10 mM Tris-HCl and 1 mM EDTA (pH 8) and simultaneously desalted and concentrated by using a Centricon-10 microconcentrator (Amicon). Immediately before cell transformation, the DNA was denatured to produce ss genomes in 10-μL aliquots by heating at 100 °C for 2 min followed by rapid cooling on ice to prevent renaturation (Figure 1).

Transformation of E. coli with Bacteriophage M13 Genomes. E. coli DL7 and DL6 cells were grown in Luria-Bertani broth (Maniatis et al., 1982) to a cell density of approximately 1 × 10^8 cells/mL, and half of each culture was exposed to UV fluences of 50 and 12 J/m^2, respectively, as described in Lasko et al. (1988), to induce the SOS response. In each experiment, the 12T/UV and Pt12T/UV genomes, in either ss or ds form, were introduced into the cells by the process of electroporation, as described by Wood et al. (1990), by using a Bio-Rad Gene Pulser. The electroporation field

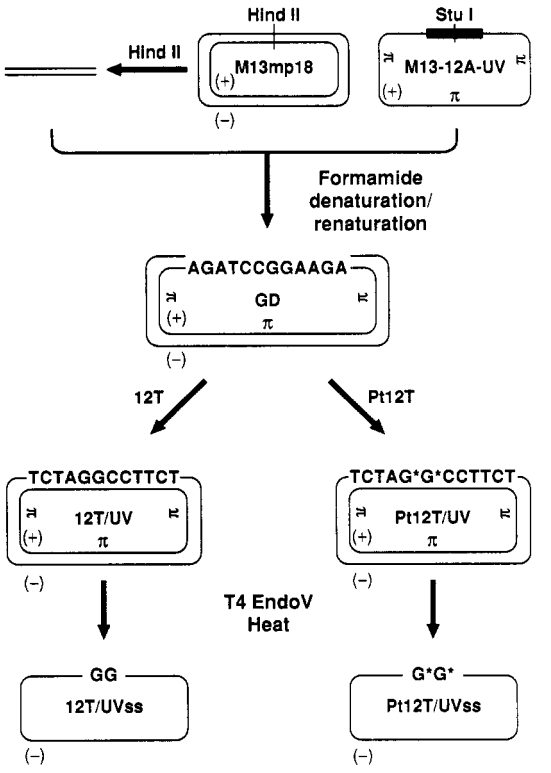

FIGURE 1: Construction of the ds and ss forms of the genomes 12T/UV and Pt12T/UV, as described in Naser et al. (1988) and the text; π denotes UV-induced photoproducts.

strengths used were those determined empirically to be optimal for cell survival and transformation efficiency for each cell type and were as follows: DL7(–UV), 12.5 kV/cm; DL7(+UV), 8.0 kV/cm; DL6(–UV), 12.5 kV/cm; DL6(+UV), 10.0 kV/cm. A portion of each transformation mixture was plated immediately to allow for enumeration of infective centers, and the remainder was incubated at 37 °C for 2–3 h, after which time progeny phage were isolated. Both transformed cells and progeny phage were plated with GW5100 cells on B-broth plates in B-broth soft agar containing isopropyl thio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (Messing, 1983).

*Effect of G*G* on Survival.* The relative survival of the Pt12T/UV genome compared to 12T/UV was assayed by transfecting equal amounts of DNA (as determined by UV fluorescence of the DNA electrophoresed in an agarose gel in the presence of ethidium bromide) into DL7 cells, induced or not for the SOS response. The full volume of each transformation mixture was plated immediately following electroporation, and survival was determined on the basis of the number of infective centers produced.

Mutant Selection Protocol. The mutation frequency is defined as the fraction of progeny phage that was mutant. Since this fraction was expected to be small, it was necessary to isolate the mutants from a large pool of mainly wild-type progeny. Mutant selection was accomplished by taking advantage of the original placement of the adduct in the unique *Stu*I restriction endonuclease recognition site. Induced mutants were refractory to cleavage by this enzyme, whereas wild-type progeny were linearized and rendered effectively nonviable. Double-stranded DNA prepared from cells infected with the progeny phage was digested with *Stu*I, which cleaves progeny with the wild-type sequence at the original site of adduct formation, and *Hin*dII, which cleaves DNA from the contaminating parental M13mp18 DNA (Figure 1). After transformation by the $CaCl_2$ procedure (Maniatis et al., 1982)

into MM294A cells, the progeny phage were isolated as described above. Rounds of mutant selection were repeated 2–4 times, as required, until all of the wild-type DNA was eliminated. Single-stranded DNA derived from individual plaques isolated after the last round was sequenced according to the method of Sanger (Sanger et al., 1977).

Mutation Frequency Determination. The sequence context of the adduct was such that both wild-type and mutant progeny exhibited a blue plaque color phenotype. Approximately 0.1–1% of the progeny showed a light blue (LB) plaque color. These mutants also appeared in the controls and apparently were induced by the genetic engineering manipulation of the M13 genome.[2] We took advantage of this phenotypically distinct class of phage and used it as an internal standard to normalize variations in DNA recovery, and hence phage yield, through the requisite rounds of selection for G*G*-induced mutants. The LB phage are genetically homogeneous as determined by DNA sequence analysis, are easily distinguished from the other plaque colors observed, and are not a substrate for either of the selection enzymes. Since the adduct-induced mutants by definition are also not substrates for the selective enzymes, the ratio of mutant blue plaques to LB plaques after the initial transformation should remain constant during the rounds of selection. After the last round of selection, the blue and LB plaques were enumerated to determine their relative frequencies. Single blue plaques were then isolated, and ss DNA was prepared and sequenced to identify the mutation. A sufficient number of blue plaques (18–32) was sequenced to determine the proportion of G*G*-induced mutants in the blue plaque population. The mutation frequency of the adduct was calculated by using the equation:

$$\mathrm{MF} = (M/B \text{ sequenced}) \times (B/\mathrm{LB}) \times (\mathrm{LB}_0/T) \times 100$$

where MF is the mutation frequency in the initial progeny phage pool, M is the number of adduct-induced mutants detected after the last round of selection as determined by DNA sequencing, B sequenced is the total number of blue plaques determined by DNA sequencing after the last round of selection (where B sequenced = M + genetic engineering-induced mutations), and LB is the number of light blue plaques after the last round of selection. LB_0 and T are the number of light blue plaques and the total number of plaques, respectively, in the initial progeny phage pool (i.e., the population that has not yet undergone selection).

A reconstruction experiment was performed in which it was demonstrated that a "model" mutant DNA (the mutant described in footnote 2) was neither selected for nor selected against during two rounds of passage through the *E. coli* host (data not shown).

RESULTS

Production of Site-Specifically Modified Genomes. In general, single replication-inhibiting adducts situated in duplex DNA have been observed to be weakly if at all toxic and mutagenic. This phenomenon is artifactual due in part to biased replication in favor of the unmodified DNA strand (Koffel-Schwartz et al., 1987). Two approaches have been taken to circumvent this problem. The first is to situate single adducts in a ss phage genome (Loechler et al., 1984). This method reduces the opportunity for adduct repair, eliminates the option for preferential replication of the unplatinated DNA strand, and thus is likely to give mutation frequency and survival reduction values that approach the inherent properties of the lesion. The second approach, which more closely models the physiologically normal state, involves the introduction of biologically inactivating lesions such as UV photoproducts or uracil residues into a duplex genome, specifically into the DNA strand not containing the adduct. Such lesions prevent the production of progeny from the unplatinated strand (Burnouf et al., 1990). In the present study, we have developed a system whereby the genetic effects of the adduct can be evaluated in *both* ss and ds DNA. The use of the duplex construct (Figure 1) allowed us to study the adduct in a ds form that presumably could be repaired, thus facilitating evaluation of the effect of DNA repair on survival and mutagenesis. The use of UV photoproducts as the strand-inactivating lesions had the added value of enabling the synthesis of a singly platinated, ss circular genome following T4 EndoV[3] treatment and removal of the incised opposing strand by heat denaturation (Figure 1). The photoinduced cyclobutane type thymine–thymine cross-links are in vitro substrates for T4 EndoV [a pyrimidine dimer DNA–glycosylase and an apyrimidinic/apurinic (AP) endonuclease; Haseltine et al., (1980)].

Survival of cis-DDP-Modified Genomes. Experiments were performed to establish the relative survival of the 12T/UV and Pt12T/UV ds genomes in DL7 cells. Survival of the ds genome containing the G*G* cross-link compared to the unplatinated controls was 22% in SOS-uninduced cells[4] and rose to 38% in the SOS-induced cells. Each survival value was calculated on the basis of 23 separate transformations; relative standard deviations were approximately ±5%. Previously, we showed that the survival of a ss genome containing the G*G* adduct is approximately 10% that of the control (Naser et al., 1988).

*Mutational Frequency of the G*G* Adduct.* Mutation frequencies for the G*G* adduct present in both ss and ds genomes were determined in *E. coli* DL7 cells (Table I). In all experiments, the cross-link only induced mutations in SOS-induced cells. The mutation frequencies of the platinated ss

[2] The origin of genetic engineering mutants has been discussed in Loechler et al. (1984) and McKay et al. (1992). In the present study, we strongly suspect on the basis of DNA sequencing that most of the genetic engineering mutants were due to a 3′ exonuclease activity that contaminated the *Hin*dII preparation used to linearize the M13mp18 genome (see Figure 1). In one case, the removal of the "C" residue in the third position of this site gave the mutation with the light blue plaque color phenotype, which was used as an internal standard. We also observed colorless plaques at a slightly higher frequency; we believe these were caused by more extensive exonuclease activity, based again upon DNA sequence analysis.

[3] It was essential that complete denaturation was achieved, and this goal was accomplished by producing a high level of endonuclease-sensitive photoproducts within the viral genome. The need for such a high level of UV damage was evidenced by the observation that UV fluences of 180–1080 J/m^2 failed to render all of the ss-derived DNA sensitive to the enzyme (data not shown). This problem was overcome by treating the M13-12A ss DNA with UV in the presence of Ag^+, which greatly increases the rate of thymine dimer formation (Rahn, 1983; Rahn & Landry, 1973). When this method was used, the strand of the genomes containing photoproducts was indeed cleaved to completion by T4 EndoV. In control experiments not presented here, it was established that *cis*-DDP–DNA adducts were not a substrate for T4 EndoV, that genomes having UV-induced photoproducts in only one strand of a ds genome were sensitive to T4 EndoV, and that only the strand containing the photoproducts in these genomes was cleaved by the enzyme.

[4] We note, however, that the electroporated material contained SOS-inducing UV-damaged DNA and that *cis*-DDP adducts are known to induce the SOS response in *E. coli* (Reslova, 1971; Salles & Lesca, 1982). Hence, we cannot guarantee that the cells in our experiment were truly SOS-uninduced. We also add, however, that the mutation frequency in our "SOS-uninduced" cells was much lower than in the SOS-induced cells. This observation argues that if SOS is on in the "SOS-uninduced" cells, its level of expression is probably far below maximal levels.

Table I: Mutation Frequencies (%) of a Single G*G* Adduct in Single-Stranded and Double-Stranded Bacteriophage Vectors Replicated in *E. coli* DL7 Cells[a]

	genomes			
	12T/UV		Pt12T/UV	
experiment	–SOS	+SOS	–SOS	+SOS
1-ss	<0.02	<0.04	<0.01	1.81
		<0.03	<0.01	0.86
2-ds	nd	nd	<0.001	0.11
				0.31
3-ds	nd	0.03	nd	0.21
				0.20

[a] ss and ds indicate that vectors were introduced into cells in single- and double-stranded form, respectively; nd, not determined.

vector from two independent replicas (experiment 1) were 0.86% and 1.8%; $\bar{x}$ = 1.3%; typically in such an experiment, 10^5–10^6 transformants would be generated. The mutation frequencies of control unplatinated genomes or of the platinated genomes introduced into SOS-uninduced cells were almost 2 orders of magnitude lower, which is a value close to the limit of sensitivity of the mutation analysis protocol. In two independent experiments (experiments 2 and 3), the average mutation frequencies obtained for the G*G* adduct present in a ds genome were 0.21% and 0.20%, respectively. It is noteworthy that these values are 5–10-fold lower than those for the comparable ss genomes. This difference could be due in part to the impact of DNA repair in protecting against the toxic effects of the G*G* adduct. *cis*-DDP adducts are known to be substrates for UvrABC excision repair (Sancar & Rupp, 1983; Beck et al., 1985), and the involvement of the mismatch repair system has also been implicated (Fram et al., 1985). The inability of either of these systems to act on ss DNA probably accounts for the higher mutation frequency of the G*G* adduct in ss genomes.

All of the experiments presented in Table I were performed in parallel in DL6 cells (data not shown), which are defective in excision repair owing to a mutant *uvrA* allele (Lasko et al., 1988). No mutations were detected in these cells either with or without SOS induction. Consistent with this finding, it has been reported that the gene products of *uvrB* (Brouwer et al., 1981) and *uvrA* but not *uvrC* (Brouwer et al., 1988) are necessary for *cis*-DDP mutagenesis. The possible role these two proteins play in the mutagenic process has yet to be defined.

*Mutational Specificity of the G*G* Adduct.* The mutations fixed in SOS-induced cells exhibited a remarkable specificity (Table II). For the ss genomes, 94% of the mutations were G → T transversions at the 5′-adducted G. Of the mutants recovered from the ds genomes, 84% (experiment 2) and 82% (experiment 3) of the total mutations in each experiment issued from the 5′-platinated G. Again, most of the mutations from the ds genomes were G → T transversions (Table II). Additional mutations occurred at low but detectable frequencies. The two other observed base pair substitutions at the 5′-adducted G were detected only in experiment 2: G → C (12.5%) and G → A (6%). In only two cases was a mutation at the 3′-adducted G detected [G → C (3%, experiment 2) and G → T (4.5%, experiment 3)]. There were three examples of double mutations encompassing the 5′-adducted G and the A immediately 5′ to it: ApG → TpT (12.5%, experiment 2) and ApG → CpT (6%, experiment 1; 4.5%, experiment 3), and two of the mutants had base substitutions located at the A immediately 5′ to the site of adduction: A → G (4.5%), A → T (4.5%), experiment 3.

Table II: Specificity and Frequency Distribution of Mutations of the Major DNA Adduct of *cis*-DDP in Single-Stranded and Double-Stranded DNA

EXPERIMENT	1 - ss		2 - ds		3 - ds	
	#	%	#	%	#	%
TT	——		4/32	12.5	——	
CT	1/18	6.0			1/22	4.5
(–) 5′ — A G* G* C C T — 3′						
C	——		1/32	3.0	——	
T	——		——		1/22	4.5
T	17/18	94.0	21/32	66.0	18/22	82.0
C	——		4/32	12.5	——	
A	——		2/32	6.0	——	
G	——		——		1/22	4.5
T	——		——		1/22	4.5
MF (SOS⁺):	$\bar{X}$ = 1.3%		$\bar{X}$ = 0.13%		$\bar{X}$ = 0.21%	

[a] Experiments 1, 2 and 3 are data from ss and ds DNAs, respectively. The sequence indicated is the recognition sequence of *StuI* in the adduct-containing (–) strand of the genomes. Data of two separate transformations were combined. G*G* indicates the site of the adduct; #, the number of each mutation over the total number of mutant blue plaques sequenced after the last round of selection; %, the percentage of each type of mutation with respect to the total number of mutations; –, the displayed mutation was not observed.

DISCUSSION

The multiplicity of damaged nucleotides formed in the genomes of cells treated with a genotoxin hampers our ability to correlate biological end points with specific DNA adducts. The recent development of techniques enabling the construction of site-specifically modified genomes has provided a means to address this problem [see Basu and Essigmann (1988) for a review]. The goal of this work was to determine which DNA adduct(s) of *cis*-DDP kill cells and which adduct(s) give rise to mutations. The latter may correlate with the formation of secondary tumors observed among cancer patients treated with the drug (Green, 1992). As the first step in this process, we have inserted the major DNA adduct of *cis*-DDP into a bacteriophage genome at a specific site and have used the genome both to measure the killing potential of a ds genome replicated in *E. coli* and to determine the amount and type of mutations directed by the adduct in ss and ds DNA.

The first issue addressed experimentally was that of genotoxicity. The survival of a single G*G* adduct situated in the (–) strand of a ds M13 genome, in which the opposite strand contained UV photoproducts, was 22% in physiologically normal *E. coli* DL7 cells. Survival almost doubled when the cells were SOS-induced. This increase in genome survival in SOS-induced cells was expected on the basis of the knowledge that *cis*-DDP forms replication blocking lesions in vitro (Pinto & Lippard, 1985b; Villani et al., 1988) and is an SOS-dependent mutagen (Fram et al., 1985). A similar phenomenon has been reported for single *cis-syn*- and *trans-syn*-cyclobutane dimers situated in ss M13 genomes (Banerjee et al., 1988, 1990). This increased survival is most likely due to the increased bypass of the replication blocking lesions by DNA polymerase, increased DNA repair, or a combination of the two processes. In earlier work (Naser et al., 1988), we examined the survival of a ss genome containing a single G*G* adduct. The adduct was situated in the (+) strand of the M13 genome, and the survival was approximately 10% in wild-type *E. coli* (DL7) cells that were not induced for the SOS response. The survival results for the platinated vectors in SOS-uninduced cells from the present work and those

previously reported are comparable. We note, however, that the survival value for G*G* in duplex DNA (approximately 22%) is lower than that reported previously in Naser et al. (1988), where we used a different experimental system. As proposed in our earlier study, we suspect that the adduct in duplex DNA causes an artifactual asymmetric replication in favor of the unplatinated strand. The results presented here support that notion.

The slightly higher survival value for G*G* in ds DNA (22%) as compared to ss DNA (10%) may be due to the better repair of the adduct in duplex DNA or to preferential repair of the (–) strand (Mellon & Hanawalt, 1989; Selby & Sancar, 1991), which is the transcribed strand in the M13 vector (Messing, 1983). There are no studies comparable to this one that have directly evaluated the in vivo genotoxicity of the A*G* adduct of *cis*-DDP, although the mutagenic properties of this lesion have been studied (Burnouf et al., 1990). Recent work studying the in vitro replication past single site-specifically situated *cis*-DDP adducts (G*G*, A*G*, and G*CG*) has shown that G*G* is more effective at inhibiting replication by several polymerases, including the *E. coli* polymerase III holoenzyme (Comess et al., 1992).

The mutation frequency data on the G*G* cross-link are presented in Table I. It is clear that induction of the SOS response is required for mutagenesis, within the detection limits of our assay. The mutation frequency was found to be almost 5-fold lower in ds than in ss DNA. A qualitatively similar observation has been made for other adducts, for example, O^6-methylguanine (Loechler et al., 1984; Essigmann et al., 1986). In the present work the duplex genomes contained biologically inactivating lesions in the strand opposite to the adduct, so that differences in mutation frequency between ss and ds genomes are probably due to the more facile repair of the adduct in the latter. In this regard it is noteworthy that the adduct in the ds vector was situated in the transcribed strand, which is known to be preferentially repaired in some mammalian and bacterial systems (Mellon & Hanawalt, 1989).

A strikingly high degree of specificity of mutagenesis was observed for the G*G* adduct (Table II). Combining all the data, 86% of the mutations occurred at the 5′-platinated G, and 78% of the total were G → T transversions at that site. The qualitative features of mutagenesis by this adduct are in accord with one of the major types of mutation produced by cisplatin in SOS-induced bacterial cells (Burnouf et al., 1987) and in human B lymphoblasts treated in vitro (Cariello et al., 1992). The G → T mutational specificity is consistent with a model of mutagenesis in which the *E. coli* polymerase, as its default option, inserts an adenine opposite a noninstructive lesion (Strauss et al., 1982; Schaaper et al., 1983). The classic example of such a noninformational lesion, the apurinic/apyrimidinic (AP) site, has been studied in both random and site-specific mutagenesis assays (Kunkel, 1984; Lawrence et al., 1990). The results of these earlier studies show that between 60% and 75% of the induced mutants result from the insertion of an adenine opposite an AP site. The higher frequency of adenine incorporation opposite the *cis*-DDP adduct, as compared to adenine insertion opposite an AP site, may indicate that there is some misinformational, and not purely noninformational, character to the platinum adduct.

One surprising finding was the fewer than expected number of G → A transitions induced by the G*G* adduct. On the basis of the work of Burnouf et al. (1987) and Cariello et al. (1992), we expected that this mutation would be observed more frequently in our study than it was (Table II). We speculate that the sequence context we studied may not have been optimal for induction of this transition or that some as yet unidentified DNA adduct of cisplatin may induce the G → A change.

The genetic effects of a single A*G* adduct have recently been investigated (Burnouf et al., 1990). The results indicate that the mutation frequency of the adduct is 1–2% in SOS-induced cells when present in a ds plasmid genome. As in the present study, inactivating UV photoproducts were placed in the DNA strand opposite the adduct. The mutational specificity of the A*G* adduct correlates well with the results presented here for G*G*: 90% of the A*G* directed mutations occur at the 5′-adducted base, and 80% of the total mutations are transversions; the remainder are A → G transitions. Double mutations involving the platinated adenine and the base immediately 5′ to it are also observed at a low frequency. These results help to explain the mutational spectrum of *cis*-DDP (Burnouf et al., 1987) which showed that *cis*-DDP-directed mutations occur at ApG and GpG sequences, with a strong preference seen for mutations occurring at the 5′ base. It is noteworthy that the present finding of a mutation frequency of 0.2% for the G*G* adduct in ds DNA is 5–10-fold lower than that observed for the A*G* adduct. From the mutation spectrum of *cis*-DDP (Burnouf et al., 1987), it was estimated that the A*G* adduct would be approximately 5 times more mutagenic than the G*G* adduct. Our results, taken together with those of Fuchs and colleagues (Burnouf et al., 1990), are consistent with that prediction. We caution, however, that the genetic effect of the A*G* adduct was analyzed in a plasmid-based system, whereas G*G* was studied in a viral vector. The optimal comparison requires evaluation of the two adducts for mutagenicity in the same system.

The asymmetric disposition of mutagenesis on the 5′ side of the G*G* adduct is also in accord with the known molecular architecture at the lesion site [reviewed in Lepre and Lippard (1990)]. The binding of *cis*-DDP to DNA significantly shortens and unwinds the DNA helix (Cohen et al., 1979). X-ray diffraction analysis of the *single-stranded* dinucleotide, *cis*-[Pt(NH$_3$)$_2$\{d(pGpG)\}], reveals that platinum binding destacks the adjacent guanine bases, resulting in a dihedral angle between the ring planes of 76–87° (Sherman et al., 1985). Gel electrophoretic mobility assays on homogeneous oligonucleotides containing G*G* or A*G* adducts show that both, when present in *duplex* DNA, bend the helix by 32–35° (Bellon & Lippard, 1990) and, at least for the G*G* adduct, this bend is in the direction of the major groove (Rice et al., 1988). NMR spectroscopic data indicate that the platinated guanines of the G*G* adduct may still be hydrogen bonded with their complementary cytosines (den Hartog et al., 1984, 1985; van Hemelryck et al., 1984). Studies of the chemical reactivity of the bases surrounding a single platinum adduct also suggest that the guanines may still be base paired. The pattern of reactivity, however, indicates that the helix is distorted in the area of the adduct and that this distortion is more pronounced on the 5′ side of the lesion (Marrot & Leng, 1989; Schwartz et al., 1989; Anin & Leng, 1990).

Taken together, most of the data on the molecular architecture of the G*G* adduct predict that polymerases would have more difficulty traversing the 5′ nucleotide than the 3′ nucleotide. The model that best fits the data (Figure 2) shows the 3′ nucleotide of the adduct easily bypassed with accurate nucleotide insertion. By contrast the 5′-platinated nucleotide, with its more distorted structure, acts as the primary misinformational site. This model based on in vivo results is

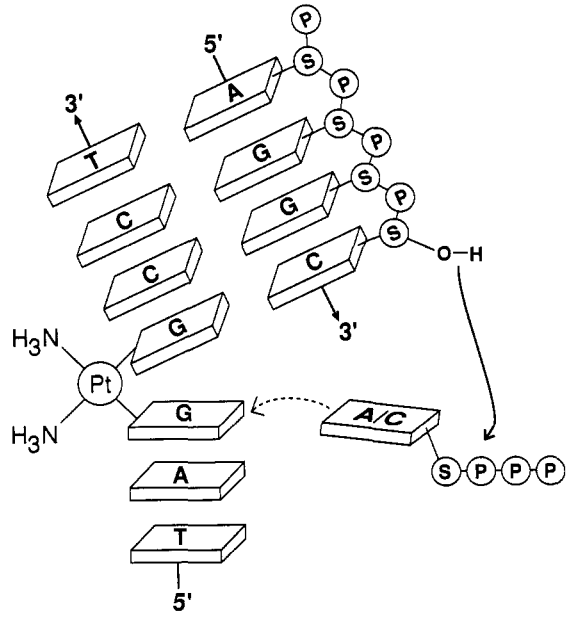

FIGURE 2: Schematic of replication bypass of the G*G* adduct. The 3′-platinated G is in the C(2′)-endo conformation and the 5′-adducted G is in the C(3′)-endo conformation (Sherman et al., 1985). The dihedral angle between the platinated guanines is approximately 32° (Bellon & Lippard, 1990), and the unwinding angle induced by the adduct is 13° (Bellon et al., 1991).

in full accord with that proposed by Comess et al. (1992), who examined the template function of G*G*, A*G*, and several other adducts in vitro. In these earlier in vitro studies, we showed that nucleotide insertion opposite G*G* typically was arrested upon formation of a base pair across from the 3′ guanine of the adduct. The model is also supported by the studies of Fuchs and colleagues (Burnouf et al., 1987), who proposed that the occurrence of mutations preferentially at the 5′ base of potential platination sites in globally platinated DNA was due to facile and accurate bypass of the platinated base on the 3′ side of the dinucleotide adduct. In the present work, the occurrence of double mutations suggests that the perturbation of DNA structure extends at least one nucleotide 5′ to the adduct. No additional mutations extending further in the 5′ direction were evident in this study nor in that on A*G* (Burnouf et al., 1990), suggesting that the DNA duplex is not disrupted beyond the base immediately 5′ to the adduct. In support of this model of localized perturbation, restriction endonuclease digestions of the G*G* platinated genome demonstrate that the adduct is able to inhibit cleavage by enzymes having recognition sequences that overlap the site of platination (*StuI* and *MaeI*), with no effect on cleavage by enzymes with recognition sequences 4 or 9 bases from the adduct (Pinto et al., 1986; Comess et al., 1992).

The observation that the majority of the G*G*- and A*G*-induced mutations occur at the 5′-platinated base suggests that the 3′-platinated G, the first base encountered by the polymerase during replication, is able to transmit its genetic information correctly via the replication apparatus of the cell. Reaction of *cis*-DDP occurs at the N7 position of purines and therefore does not directly interfere with the Watson–Crick base pairing regions of the bases. It is noteworthy that the monofunctional compound [Pt(dien)Cl]Cl, which also binds to the N7 of guanine, is neither a block to replication nor is it mutagenic (Johnson et al., 1982; Pinto & Lippard, 1985b). Thus it is unlikely that the 5′ mutations are due solely to adduct formation at the N7 position, but are instead a consequence of the structural distortion of this base induced by the cross-link formation. Replication blockage, and subsequent mutagenesis, at this site could be the consequence of the bend induced between the planes of the two platinated bases, an altered orientation of the 5′ modified base, or both.

Finally, from a practical perspective, what do the data on G*G* and A*G* portend for cancer patients treated with therapeutic regimens including *cis*-DDP? If one assumes that it is valid to compare the results of this study with those of Fuchs (Burnouf et al., 1990) and, further, that the combined studies can be extrapolated from *E. coli* to man, two conclusions can be drawn. First, the G*G* DNA adduct is highly lethal and hence it alone could account for much of the cytotoxicity of *cis*-DDP. Second, both A*G* and the G*G* are mutagenic, but the mutagenic impact of the former exceeds that of the latter by 5–10-fold. With the objective of reducing the frequency of second tumors associated with *cis*-DDP treatment, it follows that minimizing the formation of the more highly mutagenic A*G* adduct would be a good criterion to use in designing new platinum anticancer drugs.

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REFERENCES

Anin, M. F., & Leng, M. (1990) *Nucleic Acids Res. 18*, 4395–4400.
Banerjee, S. K., Christensen, R. B., Lawrence, C. W., & LeClerc, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8141–8145.
Banerjee, S. K., Borden, A., Christensen, R. B., LeClerc, J. E., & Lawrence, C. W. (1990) *J. Bacteriol. 172*, 2105–2112.
Basu, A. K., & Essigmann, J. M. (1988) *Chem. Res. Toxicol. 1*, 1–18.
Beck, D. J., & Brubaker, R. R. (1975) *Mutat. Res. 27*, 181–189.
Beck, D. J., Popoff, S., Sancar, A., & Rupp, W. D. (1985) *Nucleic Acids Res. 13*, 7395–7412.
Bellon, S. F., & Lippard, S. J. (1990) *Biophys. Chem. 35*, 179–188.
Bellon, S. F., Coleman, J. H., & Lippard, S. J. (1991) *Biochemistry 30*, 8026–8035.
Benedict, W. F., Baker, M. S., Haroun, L., Choi, E., & Ames, B. N. (1977) *Cancer Res. 37*, 2209–2213.
Brouwer, J., van de Putte, P., Fichtinger-Schepman, A. M. J., & Reedijk, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7010–7014.
Brouwer, J., Vollebregt, L., & van de Putte, P. (1988) *Nucleic Acids Res. 16*, 7703–7711.
Bruhn, S. L., Toney, J. H., & Lippard, S. J. (1990) *Prog. Inorg. Chem. 38*, 477–516.
Bubley, G. J., Ashburner, B. P., & Teicher, B. A. (1991) *Mol. Carcinog. 4*, 397–406.
Burnouf, D., Daune, M., & Fuchs, R. P. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3758–3762.
Burnouf, D., Gauthier, C., Chottard, J. C., & Fuchs, R. P. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6087–6091.
Cariello, N. F., Swenberg, J. A., & Skopek, T. R. (1992) *Cancer Res. 52*, 2866–2873.
Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985) *Biochemistry 24*, 7533–7540.
Cohen, G. L., Bauer, W. R., Barton, J. K., & Lippard, S. J. (1979) *Science (Washington, D.C) 203*, 1014–1016.
Comess, K. M., Burstyn, J. N., Essigmann, J. M., & Lippard, S. J. (1992) *Biochemistry 31*, 3975–3990.
de Boer, J. G., & Glickman, B. W. (1989) *Carcinogenesis 10*, 1363–1367.

den Hartog, J. H. J., Altona, C., van Boom, J. H., van der Marel, G. A., Haasnoot, C. A. G., & Reedijk, J. (1984) *J. Am. Chem. Soc. 106*, 1528–1530.
den Hartog, J. H. J., Altona, C., van Boom, J. H., van der Marel, G. A., Haasnoot, C. A. G., & Reedijk, J. (1985) *J. Biomol. Struct. Dyn. 2*, 1137–1155.
Eastman, A. (1983) *Biochemistry 22*, 3927–3933.
Essigmann, J. M., Loechler, E. L., & Green, C. L. (1986) *Prog. Clin. Biol. Res. 209A*, 433–440.
Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707–713.
Fram, R. J., Cusick, P. S., Wilson, J. M., & Marinus, M. G. (1985) *Mol. Pharmacol. 28*, 51–55.
Greene, M. H. (1992) *J. Natl. Cancer Inst. 84*, 306–312.
Haseltine, W. A., Gordon, L. K., Lindan, C. P., Grafstrom, R. H., Shaper, N. L., & Grossman, L. (1980) *Nature (London) 285*, 634–641.
Hennings, H., Shores, R. A., Poirier, M. C., Reed, E., Tarone, R. E., & Yuspa, S. H. (1990) *J. Natl. Cancer Inst. 82*, 836–840.
Higgins, K. M., & Lloyd, R. S. (1987) *Mutat. Res. 183*, 117–121.
Johnson, J. P., Macquet, J. P., Wiebers, J. L., & Monsarrat, B. (1982) *Nucleic Acids Res. 10*, 5255–5271.
Johnson, N. P., Hoeschele, J. D., Rahn, R. O., O'Neill, J. P., & Hsie, A. W. (1980) *Cancer Res. 40*, 1463–1468.
Koffel-Schwartz, N., Maenhaut-Michel, G., & Fuchs, R. P. P. (1987) *J. Mol. Biol. 193*, 651–659.
Kunkel, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494–1498.
Lasko, D. D., Harvy, S. C., Malaikal, S. B., Kadlubar, F. F., & Essigmann, J. M. (1988) *J. Biol. Chem. 263*, 15429–15435.
Lawrence, C. W., Borden, A., Banerjee, S. K., & LeClerc, J. E. (1990) *Nucleic Acids Res. 18*, 2153–2157.
Leopold, W. R., Batzinger, R. P., Miller, E. C., Miller, J. A., Earhart, R. H. (1981) *Cancer Res. 41*, 4368–4377.
Lepre, C. A., & Lippard, S. J. (1990) *Nucl. Acids Mol. Biol. 4*, 9–38.
Loechler, E. L., Green, C. L., & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6271–6275.
Loehrer, S. J., & Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704–713.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marrot, L., & Leng, M. (1989) *Biochemistry 28*, 1454–1461.
McKay, W., Benasutti, M., Drouin, E., & Loechler, E. L. (1992) *Carcinogenesis 13*, 1415–1425.
Mellon, I., & Hanawalt, P. C. (1989) *Nature 342*, 95–98.
Messing, J. (1983) *Methods Enzymol. 101*, 20–78.
Naser, L. J., Pinto, A. L., Lippard, S. J., & Essigmann, J. M. (1988) *Biochemistry 27*, 4357–4367.
Pinto, A. L., & Lippard, S. J. (1985a) *Biochim. Biophys. Acta 780*, 167–180.
Pinto, A. L., & Lippard, S. J. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4616–4619.
Pinto, A. L., Naser, L. J., Essigmann, J. M., & Lippard, S. J. (1986) *J. Am. Chem. Soc. 108*, 7405–7407.
Poirier, M. C., Reed, E., Zwelling, L. A., Ozols, R. F., Litterst, C. L., & Yuspa, S. H. (1985) *Environ. Health Perspect. 62*, 89–94.
Rahn, R. O. (1983) in *DNA Repair: A Laboratory Manual of Research Procedures* (Friedberg, E., & Hanawalt, P., Eds.) Vol. 2, pp 75–85, Dekker, New York.
Rahn, R. O., & Landry, L. C. (1973) *Photochem. Photobiol. 18*, 29–38.
Reed, E., Ozols, R. F., Tarone, R., Yuspa, S. H., & Poirier, M. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5024–5028.
Reed, E., Ostchega, Y., Steinberg, S. M., Yuspa, S. H., Young, R. C., Ozols, R. F., & Poirier, M. C. (1990) *Cancer Res. 50*, 2256–2260.
Reslova, S. (1971) *Chem.-Biol. Interact. 4*, 66–70.
Rice, J. A., Crothers, D. M., Pinto, A. L., & Lippard, S. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4158–4161.
Salles, B., & Lesca, C. (1982) *Biochem. Biophys. Res. Commun. 105*, 202–208.
Sancar, A., & Rupp, W. D. (1983) *Cell (Cambridge, Mass.) 33*, 249–260.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schaaper, R. M., Kunkle, T. A., & Loeb, L. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 487–491.
Schwartz, A., Marrot, L., & Leng, M. (1989) *Biochemistry 28*, 7975–7979.
Selby, C., & Sancar, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8232–8236.
Sherman, S. E., Gibson, D., Wang, A. H. J., & Lippard, S. J. (1985) *Science (Washington, D.C) 230*, 412–417.
Strauss, B., Rabkin, S., Sagher, D., & Moore, P. (1982) *Biochemie 64*, 829–838.
van Hemelryck, B., Guittet, E., Chottard, G., Girault, J. P., Huynh-Dinh, T., Lallemand, J. Y., Igolen, J., & Chottard, J. C. (1984) *J. Am. Chem. Soc. 106*, 3037–3039.
Villani, G., Hubscher, U., & Butour, J. L. (1988) *Nucleic Acids Res. 16*, 4407–4418.
Wood, M. L., Dizdaroglu, M., Gajewski, E., & Essigmann, J. M. (1990) *Biochemistry 29*, 7024–7032.