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Mutagenesis in monkey cells of a vector containing a single d(GPG) *cis*-diamminedichloroplatinum(II) adduct placed on codon 13 of the human *H-ras* proto-oncogene

M.J.Pillaire, A.Margot[1], G.Villani, A.Sarasin[1], M.Defais and A.Gentil[1,*]
Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, 205 route de Narbonne, 31077 Toulouse cedex and [1]Laboratoire de Génétique Moléculaire, UPR 42, Institut de Recherches Scientifiques sur le Cancer, BP 8, 94801 Villejuif, France

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ABSTRACT

Cisplatin (*cis*-{$Pt(NH_3)_2Cl_2$}) is a widely used antitumor agent whose mutagenic activity raises the possibility of the induction of secondary cancer as a result of treatment. Mutation of the proto-oncogene *H-ras* is found in more than 30% of all human tumors, where it has been postulated to contribute to the initiation and progression of human cancers. Activating mutations in the *H-ras* gene are predominantly single-base substitutions, most frequently at codons 12, 13 and 61. In the present work we have studied the mutational spectra induced by a single *cis*-{$Pt(NH_3)_2d(GpG)$} adduct, the most frequent DNA crosslink formed by cisplatin. We have constructed a 25-mer-Pt oligonucleotide singly modified at codon 13 (GGT) within the human *H-ras* DNA sequence and we have inserted it into a single-stranded SV40-based shuttle vector able to replicate in simian COS7 cells. After replication in the mammalian host, vectors were extracted, amplified in bacteria and DNA from 124 randomly chosen colonies was sequenced. The observed mutation frequency was 21%. Base substitutions were the most frequent modification. 92% of the mutagenic events occurred at one or both of the platinated guanines of codon 13. The single G→T transversion accounted for 65% of the total mutations scored. All single base substitutions were located at the G in the 3′ position showing, for the first time, that the guanine at the 3′ side of a *cis*-{$Pt(NH_3)_2d(GpG)$} adduct may be a preferential site for cisplatin induced mutations. The substitution G→T at this position of the codon 13 of the *H-ras* proto-oncogene is known to induce the oncogenic properties of the $p21^{ras}$ protein.

INTRODUCTION

cis-Diamminedichloroplatinum(II) (cisplatin or *cis*-DDP) is a drug that is frequently used in cancer chemotherapy, mainly for the treatment of testicular and ovarian cancers (1). The compound reacts covalently with DNA and intrastrand crosslinks are the most prevalent damages, in particular the *cis*-{$Pt(NH_3)_2d(GpG)$} adduct which accounts for at least 50% of the total lesions (for reviewes 2, 3). It has been shown that a lesion at the d(GpG) sequence can interfere with *in vitro* DNA replication and transcription (4–10), and with DNA unwinding catalyzed by helicases (11–13). Furthemore, this adduct seems to be poorly repaired by eukaryotic cellular extracts (14, 15). It has been proposed that the *cis*-{$Pt(NH_3)_2d(GpG)$} adduct is the main lesion responsible for the cytotoxicity of cisplatin (16).

Cisplatin treatment may have several undesirable side effects, including tumorogenesis (which has been observed in both rats and mice) and mutagenesis (reported in both eukaryotic and prokaryotic cells) (17, 18, for review 19). In order to evaluate the risks for patients treated with this antitumor drug to develop secondary tumors and to avoid or to miniminize their impact, we need to understand the mechanism by which such complications develop. Studies of the spectrum of mutations induced by *cis*-DDP could contribute to this goal. Indeed, point mutations in specific genes, such as some proto-oncogenes or tumor supressor genes, are believed to be the initiating tumorogenic event. The majority of human cancers exhibit point mutations in the p53 tumor suppressor gene (20) and/or in specific codons of the *ras* gene family. The more mutagenic a drug is, the greater are its chances of producing secondary tumors.

The pattern of DNA mutations produced by cisplatin has been previously examined in prokaryotic and eukaryotic systems, such as in the *lacI* gene of *Escherichia coli* (21), in the tetracycline resistance gene of *E.coli* (22), in the Chinese hamster ovary *adenine phosphoribosyl transferase* gene (23), in the yeast *sup4-O* gene (24), in the *supF* gene of a shuttle vector transfected into normal and XP human cells (25), and in exon 3 of the endogenous human *hypoxanthine guanine phosphoribosyl transferase* gene (26). In all these studies the DNA was randomly modified by cisplatin and the presence of several DNA lesions makes quantitative analysis of the mutation spectrum difficult. Base pair substitutions were mainly encountered, in particular at d(ApG) or d(GpG) sequences previously described as preferential sites

*To whom correspondence should be addressed.

for cisplatin interactions. Some of these studies reported mutational hot spots (24–26) while others did not (22, 23). Mutational spectra in *E.coli* have also been determined after infection by single-stranded DNA containing a specific *cis*-DDP DNA adduct, either *cis*-{$Pt(NH_3)_2d(ApG)$} or *cis*-{$Pt(NH_3)_2d(GpG)$} (27, 28). It was found that mutagenic processing of the lesion requires induction of the SOS system; base pair substitutions were observed at the 5′ side of the lesion, among which transversions AT→TA (27) and GC→TA (28) were the most prevalent. Bradley *et al* (28) have shown that in the singly platinated single-stranded vector the mutation frequency was 5 times higher than in the double-stranded vector. Since single-stranded DNA carrying *cis*-DDP lesion(s) cannot be processed by excision repair enzymes, a translesion synthesis must occur in order to produce fully replicated double-stranded vector. Such translesion synthesis is believed to be responsible for the observed base pair substitutions induced by the platination of both single-stranded and double-stranded DNA.

In vivo point mutations resulting from the replication through a cisplatin bifunctional cross-link might lead to the activation of proto-oncogenes and therefore participate in the establishment of secondary cancer. Mutant *ras* genes have frequently been detected in tumors and tumor cells and activation of *ras* gene products are thought to be involved in tumor initiation and/or progression. In addition, activated *ras* gene may participate in the establisment of a resistant phenotype to cisplatin (29–31). Base substitutions have been most frequently observed in mutation hot spots at codons 12, 13, 59 and 61 (32, 33).

In this work we have examined the mutation spectra induced by a single *cis*-{$Pt(NH_3)_2d(GpG)$} adduct located at the sequence d(GGT) of the codon 13 of *H-ras* gene (34). The modified DNA sequence was introduced into a single-stranded SV40-based shuttle vector which transiently replicates in simian COS7 cells (35). We found that G→T transversion were the most commonly encountered mutations. All mutations were targeted at the lesion site and were preferentially located at the 3′ side of the bifunctional adduct.

MATERIALS AND METHODS

Cells and bacteria

Monkey COS7 cells were grown in 90 mm Petri dishes in Dulbecco's modified Eagle medium supplemented with 7% fetal calf serum in a 5% CO_2 humidified atmosphere. Mutant plasmids were isolated and selected using *E.coli* DH5α (*recA1*, *hsdR17*(r_K-, m_K+)) bacteria on LB medium. JM 105 bacteria were used to produce single-stranded DNA.

Plasmid

The plasmid pS189, a generous gift from Dr.Seidman, has been described previously (36). The replication origins of both SV40 and the miniplasmid P AN7 (ori P) allow replication of the vector in mammalian cells and in bacteria respectively. Selection of transformed bacteria is based upon the ampicillin resistance gene carried by the plasmid. M13 phage replication is active in this vector (37) and allows production of single-stranded plasmid DNA using the M13 KO7 helper phage as described in Analects (Pharmacia).

Oligonucleotide synthesis

The oligonucleotide 14-, 22- and 50-mers shown in Figure 1, were synthesized on Cyclone Plus DNA synthesizer from

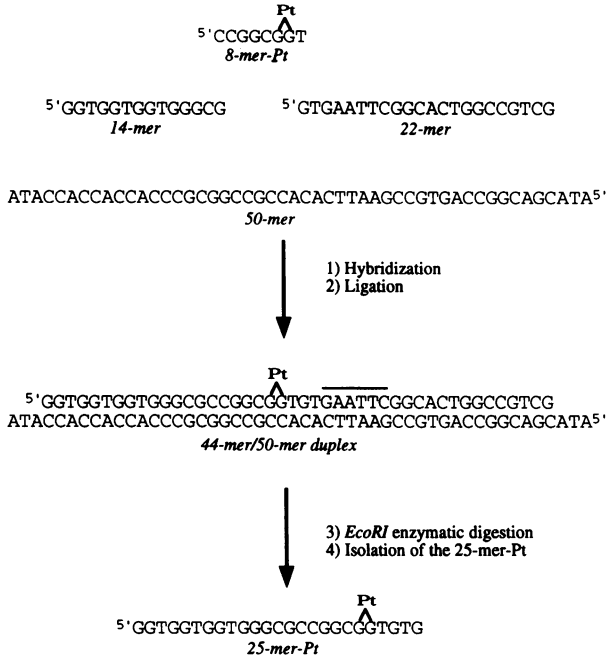

Figure 1. Steps in the purification of the 25-mer-Pt oligonucleotide. The same procedure was used to purified the unmodified 25-mer. The sequence of the oligonucleotides reproduces codons 7 to 14 of the human *H-ras* proto-oncogene. Pt: cisplatin bifunctionnal adduct. Solid line indicates the *Eco*RI site present on the duplex.

MilliGen/Biosearch (Novato, CA) and purified as previously described (34). Briefly, each oligonucleotide was isolated by electrophoresis on 20% polyacrylamide gel, eluted in TEN buffer (1 mM EDTA, 300 mM NaCl and 10 mM Tris–HCl pH 8.5), and filtered through a Sep-Pack C18 cartridge (Waters) for desalting. The oligonucleotides were kept in eionized water and the concentration of each solution was determined according to its extinction coefficient (38). Octamers 8-mer (unplatinated) and 8-mer-Pt (platinated) used in this study, were those previously purified and named 'unpt' and 'oligomer d' respectively (34). 20-mer and 62-mer oligonucleotides used for the construction of the single-stranded vector were synthesized using an Applied Biosystem 380B DNA synthesizer. All concentrations of DNA are in units of moles oligonucleotide.

5′ Phosphorylation of oligonucleotides

The purifed oligonucleotides (8-mer, 8-mer-Pt, 22-mer), at a final concentration of 25 μM, were phosphorylated by incubating 250 pmoles of DNA per unit of T4 polynucleotide kinase (New England Biolabs) and 150 μM ATP in the buffer supplied by the manufacturer, at 37°C for 30 min. Then the same quantity of enzyme and ATP were added and the incubation continued for an additional 15 min. HPLC analysis of the phosphorylated 8-mer and the 8-mer-Pt confirmed that they were quantitatively phosphorylated under these experimental conditions (data not shown). When labeling was just used to check the purity, 8 pmoles oligonucleotides were labeled with 1 unit of T4 polynucleotide kinase and $\gamma^{32}P$ ATP (Amersham) in a final volume of 20 μl and incubated at 37°C for 30 min. The reaction was quenched by heating at 65°C for 10 min to inactivate the enzyme.

Construction and purification of the 25-mer and the 25-mer-Pt

The overall construction is depicted in Figure 1. Experimental details of individual steps are described below.

Annealing of the oligonucleotides. 1.8 molar excess of the 14-mer, 50-mer and 5′ phosphorylated 22-mer were mixed with either 8 μM of the 5′ phosphorylated 8-mer or 8 μM of the 5′ phosphorylated 8-mer-Pt in the T4 DNA ligase buffer (50 mM Tris−HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, from Eurogentec). The solution was heated at 90°C for 5 min and then allowed to cool to room temperature. The 8-mer-Pt was stable under these conditions judging from electrophoresis mobility (34).

Ligation of the oligonucleotides. The samples were diluted to 0.7 μM and incubated at 37°C before adding the T4 DNA ligase; 10 Units of T4 DNA ligase were added per 5′ termini ligation site, and further incubated 1 h at 37°C. The solution was heated at 65 °C for 10 min and the ligated material was then ethanol-precipitated, resuspended in deionized water and passed through a G-50 Nick Spin Column (Pharmacia).

EcoRI enzymatic digestion. The ligated material, at a concentration of 2 μM, was incubated with 1 unit of enzyme per octamer at 37°C for 1h in the buffer supplied by the manufacturer (Eurogentec), and subsequently heated for 20 min at 65°C to inactivate the endonuclease. The digested material was ethanol-precipitated.

Purification of the 25-mer and the 25-mer-Pt. Purification of the resulting 25-mers was performed by electrophoresis on a 20% polyacrylamide gel, prepared in TBE followed by electrophoresis of the digested material on denaturing polyacrylamide gel (7 M formamide, 7 M urea). Alternatively a non-denaturing gel was used to separate of the 25-mers from a 32-mer (obtained following digestion of the 44-mer/50-mer duplex by *Eco*RI) by hybridizing the 32-mer to an excess of a complementary 60-mer. The gel was illuminated at 254 nm to visualise the bands, the section containing the single-stranded 25-mer or the 25-mer-Pt was excised, and DNA was eluted in TEN buffer, ethanol-precipitated, dissolved in deionized water and passed through a G-50 Nick Spin Column. The concentration of purified 25-mer oligonucleotide was determined according to its extinction coefficient ($\epsilon^{M}{}_{260\ nm}$ = 233.5 ×10^3 M^{-1}. cm^{-1}) (38).

Construction of the single-stranded vector carrying the 25-mer-Pt

Single-stranded plasmid DNA was digested with *Bgl*II enzyme (New England Biolabs) after hybridization of a 20-mer oligonucleotide creating a double-stranded region at the unique *Bgl*II site present on the plasmid (Fig 2). Linear DNA was recircularized using a 62-mer scaffold oligonucleotide carrying in the middle a sequence of 25 nucleotides complementary to the 25-mer-Pt oligonucleotide. After hybridization with the 25-mer-Pt, ligation was performed for 4 hours at 16°C with 400 units of T4 DNA ligase (New England Biolabs). The reaction products were briefly heated at 90°C and filtered through a sephacryl S-400 column to remove the 62-mer scaffold oligonucleotide. Finally, in order to ensure total removal of remaining partially double-stranded molecules, digestions with *XbaI* and *Hinc II* restriction endonucleases, whose restriction sites

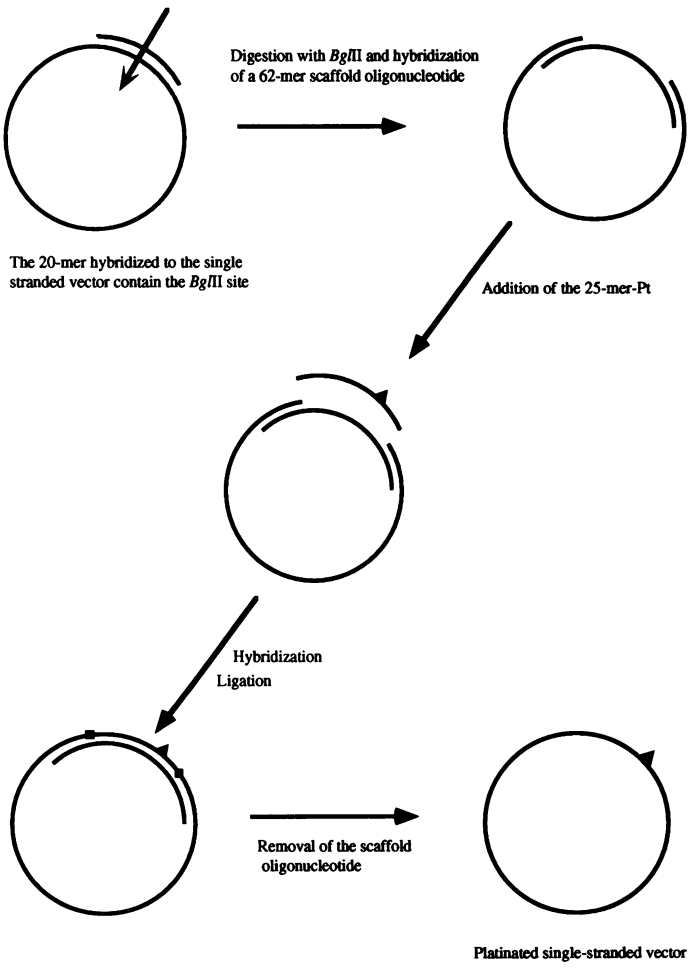

Figure 2. Construction of the single-stranded vector containing a specific cisplatin intrastrand crosslink. ▲: cisplatin bifunctional adduct.

are located in the 62-mer scaffold, were performed. The same protocol was used to construct the unmodified vector.

Mutational assay

Subconfluent COS7 cells were transfected, using the DEAE dextran method (39), with the single-stranded plasmid DNA containing the partial *H-ras* sequence, either intact or carrying a *cis*-DDP adduct in the codon 13. Three days later cells were harvested and extrachromosomal DNA was extracted using an alkaline lysis procedure (40). After digestion with the *Bgl*II restriction endonuclease to eliminate any contaminant molecules which did not contain the 25-mer insert, the DNA was shuttled into *E.coli* DH5α bacteria by Hanahan's method (41). Plasmid DNA was extracted from ampicillin resistant clones by an alkaline lysis method, and randomly chosen DNA molecules were then sequenced by the chain elongation terminating method using sequenase II kits (USB).

RESULTS

Construction of the SV40 shuttle vector containing the specific *cis*-DDP adduct

We have previously purified and characterized a platinated 8-mer-Pt oligonucleotide containing the well defined *cis*-{$Pt(NH_3)_2$d-

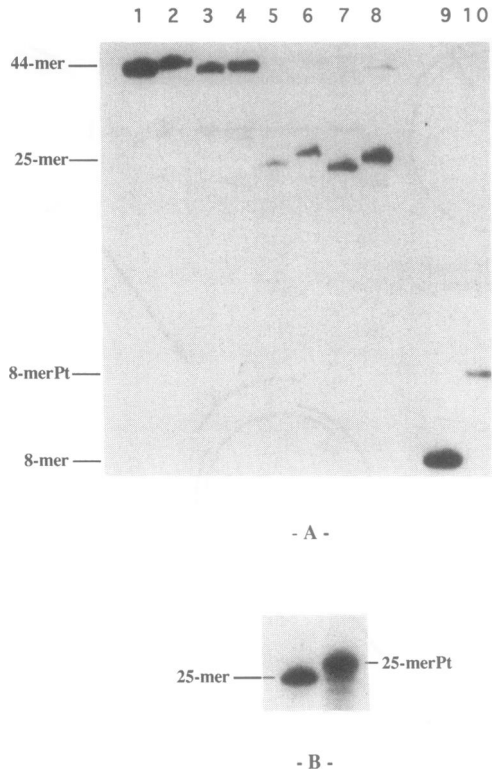

Figure 3. Denaturating polyacrylamide gel showing the products of the different steps in the construction of unplatinated and platinated 25-mers. 8-mer oligonucleotides were 5′ end labelled with γ^{32}P ATP by T4 polynucleotide kinase. **A)** Odd numbered lanes show unplatinated samples and even numbered show platinated samples. Lanes 1 and 2: ligated 44-mer oligonucleotides before ethanol precipitation. Lanes 3 and 4: ligated material after ethanol precipitation. Lanes 5 and 6: *Eco*RI digested material before ethanol precipitation, lanes 7 and 8: digested material after ethanol precipitation. Lanes 9 and 10: intact and platinated 8-mer oligonucleotides prior ligation. **B)** The purifed 25-mers were 5′ end labelled with γ^{32}P ATP and analysed on denaturating gel as described in Materials and Methods.

(GpG)} adduct at the d(GGT) sequence of the codon 13 of the human *H-ras* gene (34). As an intermediate step in the insertion of the specific lesion into the SV40 shuttle vector, we have constructed a 25-mer-Pt oligonucleotide that contained the *H-ras* sequence from codon 7 to 14 and the adduct. Sequential steps in this construction are described in Fig 1. Experimental conditions used (see Materials and Methods) were optimized to insure the complete incorporation of the platinated 8-mer-Pt into the 25-mer-Pt end product. Fig 3A shows the intermediate products (either platinated or unplatinated) obtained during the construction of the 25-mers. Ligation of the platinated 8-mer-Pt is quite efficient, since no radioactivity was detected at the position of the 8-mer-Pt alone (lane 2 and 4); the faint shorter bands seen in lanes 1 to 4 represented ligation intermediates (8-mer plus 22-mer, 8-mer plus 14-mer). The efficiency of the *Eco*RI digestion leading to the platinated 25-mer-Pt product (lanes 5 and 8) was estimated to be 90% by densitometry of the autoradiography (data not shown), indicating that the lesion did not severely affect the enzyme action, although it has been reported that cisplatin lesions in the very close proximity of restriction sites can inhibit DNA cleavage by restriction enzymes (42). Electrophoretic analysis of the purified platinated 25-mer-

Table 1. Mutations induced by the single cisplatin intrastrand crosslink.

		Σ	%
single and double base substitutions	T T	1	3.8
	A	1	3.8
	T	17	65.4
	TT	1	3.8
	AT	2	7.7
	AC	2	7.7
wild type *H-ras* sequence	5'GGC GCC GGC GGT GTG AAT TCG		
single base insertion, deletion, base substitutions	▲ T	1	3.8
	0 TTC 0	1	3.8
Mutation frequency		21% (26/124)	

The sequence shown in the box is the portion of the human *H-ras* proto-oncogene used in this study, which was specifically modified on the two adjacent guanines of codon 13 (underlined sequence). Σ, number of each type of mutations found; %, percentage of each type of mutations with respect to the total number of mutations. In addition to single and double base substitutions, we have observed a) insertion of a T (▲); b) deletion of C and G on either side of the codon 12 (0) together with a triple base substitution. Results are pooled from four independent transfections.

Pt showed that its mobility was reduced when compared to the purified unplatinated 25-mer (Fig 3B), thus confirming the presence of the adduct (43). The 25-mers, either intact or platinated, were subsequently inserted into the single-stranded vector, as depicted in Fig 2 and described in Material and Methods.

Sequence analysis of the mutation induced by the *cis*-DDP adduct

The single-stranded DNA vector, either intact or containing *cis*-{Pt(NH$_3$)$_2$d(GpG)} lesion, was transfected into COS7 cells. After 72 hours at 37°C, replicated vectors were extracted from the cells (35) and digested with *BglII* to eliminate any residual replicated molecules which did not initially contain the 25-mer sequence. The DNA was then amplified in *E.coli* DH5α in order to clone individual molecules and to obtain sufficient amount of material for sequencing. The vectors to be sequenced were randomly chosen among total populations produced from 4 independent transfections performed either with platinated or unplatinated DNA. Sequencing of 124 platinated plasmids yielded a mutation frequency of 21%, and Table 1 shows the type of mutations obtained. As can be seen, the vast majority of the mutations were targeted at the two adjacent guanines of codon 13 containing the lesion. None of these targeted mutations were observed among the 105 unplatinated vectors sequenced as controls. Base substitutions were the most frequent mutations found on the platinated vectors; with respect to all mutations, transversions G→T accounted for 65% of the total mutations scored. Interestingly all the single substitutions occurred at the 3′ side of the platinum adduct, contrary to the previously reported mutagenic events induced upon replication of *cis*-{Pt(NH$_3$)$_2$d(GpG)} damaged single-stranded M13 DNA by *E.coli* (28). Each of the remaining mutations did not represent more than 8% of the total mutagenic events induced by the cisplatin adduct. Among the 105 control vectors sequenced, we found 4 independent mutations. Two of them were base substitutions (C→T and G→A), located at either the third position

of the codon 10 or at the second position of the codon 12, while the other two were deletions spanning through codons 10 or 14 plus 15. However, none of them was located at the position of the codon 13 of the *H-ras* gene.

DISCUSSION

Genotoxic agents can often form several types of lesions on the DNA. The dissection, at the molecular level, of the role of each lesion in biological processes such as replication, transcription, repair or recombination should help to understand the mechanisms by which a given genotoxic agent perturbs DNA metabolism. Recently developed techniques, enabling the introduction of a single, well defined adduct into the genome, makes such studies feasible (44).

cis-{$Pt(NH_3)_2d(GpG)$} is the most common adduct formed by the drug cisplatin on DNA. This lesion inhibits DNA replication and transcription *in vitro*, is refractory to repair by crude cell extracts (4−7, 10, 14, 15), and therefore is expected to play an important role in the processes of cytotoxicity and mutagenicity induced by the antitumoral compound.

We have measured the spectrum of mutations induced by the *cis*-{$Pt(NH_3)_2d(GpG)$} lesion placed on the two adjacent guanines of the codon 13 (GGT) of the human proto-oncogene *H-ras* following its replication in simian COS7 cells. To our knowledge, this is the first report of the types and positions of the mutations induced in mammalian cells by this well defined adduct. We have chosen the codon 13 of the *H-ras* proto-oncogene because it is a hot spot of mutations implicated in human carcinogenesis (32, 33) and includes a d(GpG) sequence which is a preferential site for *cis*-DDP binding.

Since we have previously purified and characterized a set of platinated 8-mer-Pt at both codons 12 and 13 of the proto-oncogene *H-ras* (34), we attempted to apply the same protocol to the 8-mer-Pt on the codon 12 of *H-ras* sequence. However, we were unable to obtain ligation at the 5′ side of this 8-mer-Pt (data not shown). Our ability to ligate the platinated 8-mer-Pt containing the adduct on the codon 13, d(CCGGCG*G*T), but not the one bearing the lesion on the codon 12, d(CCG*G*CGGT), might be explained by the observation that the distorsion of the double helix is more pronounced at the 5′ side of the bifunctionnal adduct than at the 3′ side (43, 45, 46) which could lead to inefficient ligation at the 5′ side of the 8-mer-Pt at the codon 12.

The 25-mer oligonucleotides either intact or platinated, were inserted into the single-stranded vector as outlined in Fig 2. We have chosen a single-stranded vector so that removal of the lesion by a nucleotide excision repair mechanism prior its replication can not occur. To insure that only the circular single-stranded DNA ligation product depicted in Fig 2 will be amplified in the cell, we digested with restriction endonucleases the single-stranded/62-mer scaffold hybrid which could have escaped our purification protocol. The linear DNA, either produced by the endonucleases digestion or by a partial ligation of the 25-mer to the vector, will be rapidly degraded in the cell (Cabral-Neto, J.B., Gentil, A., Margot, A., and Sarasin, A., unpublished results).

Several laboratories have studied the replication, both *in vivo* and *in vitro*, of single-stranded DNA containing the specific *cis*-{$Pt(NH_3)_2d(GpG)$} lesion (7, 9, 10, 28, 47). Two reports have examined the capacity of prokaryotic DNA polymerases to bypass the *cis*-DDP lesion *in vitro*. In the first, for all the enzymes tested (T7 pol, Taq pol, T4 pol, *E.coli* Klenow fragment and pol III holoenzyme) the authors found a translesion synthesis varying from 2% to 6% depending on the polymerase used (7). In the second, only the *E.coli* Klenow fragment showed the capacity to elongate past the lesion to an extent of 10% to 25%, while for the pol III holoenzyme or its α subunit, no bypass was observed (10). No detectable bypass synthesis was observed *in vitro* with the eukaryotic ϵ polymerase (9). The survival rate of the (+) strand of the M13 genome containing a unique d(GpG) adduct was approximatively 10% following its transfection into wild type *E.coli* (28, 47). In the present study we estimate that the survival of the vector bearing the *cis*-{$Pt(NH_3)_2d(GpG)$} adduct in COS7 cells was roughly of 26%, while the frequency of induced mutations was of 21%. Survival was calculated by comparing the number of the ampicillin resistant *E.coli* colonies after transfection with total DNA recovered from COS7 cells transformed with either platinated or unplatinated vector. The high rate of survival detected here for platinated vectors could be attributed to a relatively efficient bypass of the lesion by the host replicative machinery. This substantial translesion synthesis resulted in an elevated rate of mutations.

The observed mutagenic events were highly targeted, since 92% of them were located on one or both of the guanines of the (GGT) triplet of codon 13 (Table 1). Among the detected base changes, the transversion G→T accounted for 65% of the total mutations. The appearance of this type of transversion is in agreement with the 'A rule', suggesting the preferential incorporation of a A in front of a non instructional lesion (48). Previous studies in mammlian cells, aimed at determining the sites of mutations induced by cisplatin, indicated that mutations occured at the binding site of the drug, and that base pair substitutions were the most frequent mutations. However G→T was not always the major base substitutions observed (21−28).

One interesting feature of our results is the observation that the most frequent base substitutions were situated at the 3′ side of the *cis*-{$Pt(NH_3)_2d(GpG)$} adduct (Table 1). Since the vectors sequenced were randomly chosen from the total population, we think that the location of nearly all the mutations at the 3′ side of the adduct was not the result of a selection bias. The 3′ position of the mutations contrasts with previous reports in prokaryotic systems showing that specific d(ApG) and d(GpG) *cis*-DDP crosslinks provoked mutagenic events mainly at the 5′ side of the lesions (27, 28). These authors attributed the finding that the 5′ platinated nucleotide acts as a primary misinformational site, to the observation that platinum distortion of the DNA helix is more pronounced at the 5′ side of the lesion (43, 45, 46). Cisplatin induced mutagenesis previously reported in eukaryotic systems was located at runs of purines in the DNA sequence, thus preventing a precise location of the mutagenic event either on the 3′ or the 5′ side of the putative adducts.

There are several possible explanations for the discrepancy between observed mutation sites in *E.coli* and our results. The fact that we found induced mutations almost exclusively at the 3′ side of the *cis*-{$Pt(NH_3)_2d(GpG)$} crosslink, shows that preferential mutation position is not determined only by the structure of the dinucleotide adduct. In the present study guanine residues are found in the nucleotide sequences adjacent to the *cis*-{$Pt(NH_3)_2d(GpG)$} lesion, whereas the flanking sequences in previous studies with prokaryotes have fewer purines and lack guanine (27, 28). The influence of sequence context on the site of preferential base substitution can not be demonstrated or ruled out by this limited data. Alternatively, different mutation hotspots

at the platinated dinucleotide could reflect different processing of the lesion in prokaryotes and eukaryotes. The mutation at the 3′ side of the *cis*-{$Pt(NH_3)_2d(GpG)$} lesion observed here could be the consequence of fundamental differences in translesion synthesis by *E.coli* and eukaryotic replication machineries, stressing that caution may be necessary in extrapolating mutation profiles which have been determined in prokaryotes.

The *cis*-DDP induced transversion G→T that we have observed at very high frequency at the codon 13 of *H-ras* gene will lead to a Gly→Val change in the oncogenic $p21^{ras}$ protein. This particular amino-acid substitution has been reported to be one of the key step in the activation of the proto-oncogene (32). This activation is believed to be part of the process of tumorigenicity and could contribute to secondary tumor formation as a result of cisplatin treatment or to the acquisition of a cisplatin resistant phenotype (29- 31).

The biochemical mechanisms enabling DNA synthesis past the *cis*-{$Pt(NH_3)_2d(GpG)$} lesion are a current subject of investigation. A report examining *in vitro* replication of the *H-ras* proto-oncogene by the eukaryotic polymerase α has recently been published (49). Studies of *in vitro* DNA synthesis by eukaryotic proteins acting on templates containing the specific *cis*-{$Pt(NH_3)_2d(GpG)$} crosslink on the codon 13 of *H-ras* proto-oncogene are underway in our laboratory and are expected to elucidate the molecular mechanisms of its activation.

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