CELLULAR RESPONSES TO PLATINUM-BASED ANTICANCER DRUGS

by

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Science
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This dissertation describes the genetic effects of platinum DNA adducts in *Escherichia coli* from two perspectives. First, the genotoxicities and mutagenicities of the three major DNA adducts formed by *cis*-diamminedichloroplatinum(II) (*cis*-DDP), *cis*-\([Pt(NH_3)_2(d(GpG))](G^*G^*)\), *cis*-\([Pt(NH_3)_2(d(ApG))](A^*G^*)\), and *cis*-\([Pt(NH_3)_2(d(GpNpG))](G^*NG)^*\), were investigated. These studies were conducted by using viral genomes in which specific nucleotides in the natural sequence were replaced with the aforementioned DNA adducts. The results of the site specific evaluation of these adducts suggested principles applicable to the design and evaluation of novel anticancer platinum-based therapeutic agents. Accordingly, the second aspect of this work was the genotoxic and mutagenic evaluation of *cis*-ammine(cyclohexylamine)dichloroplatinum(II) (ACDP), a metabolite of the platinum(IV) compound, *cis*,*trans*,*cis*-ammine(cyclohexylamine)dibutyrato-dichloroplatinum(IV) (ACDDP), now undergoing clinical trials to determine its efficacy as an anticancer drug.

The first aspect of this work, the site specific evaluation of the *cis*-DDP *G*^*G*^*, *A*^*G*^*, and *G*^*T*G^* adducts, was done by incorporating oligodeoxynucleotide 24-mers, containing these lesions, into M13mp7L2 derived ss genomes. Both the platinum modified and unmodified, ss genomes were transfected into *E. coli* DL7 cells that had been, or had not been, irradiated with ultraviolet light in order to induce the SOS response. The genotoxicities of the adducts were determined by using the M13 plaque forming assay. Genomes containing the *cis*-DDP *G*^*G*^*, *A*^*G*^*, and *G*^*G*^* adducts had survival levels of 5.2 ± 1.2%, 21.6 ± 2.6%, 13.5 ± 2.5%, respectively, compared to unmodified control genomes. Upon SOS induction of the host *E. coli* cells, the survival of genomes containing the *cis*-DDP *G*^*G*^* and *A*^*G*^* adducts rose significantly to 30.8 ± 5.4% and 32.4 ± 4.9%, respectively. By contrast, the survival of genomes containing the *cis*-DDP *G*^*T*G^* adduct did not increase upon SOS induction, remaining at 14.4 ± 3.7%.

Mutations attributable to *cis*-DDP were observed only for modified genomes replicated in SOS induced *E. coli* DL7 cells. The *cis*-DDP *G*^*G*^* and *A*^*G*^* adducts produced highly targeted mutations at the 5' modified base, with the predominant mutation consisting of G → T and A → T transversions for the two adducts, respectively. A → G transitions also arose from the *cis*-DDP *A*^*G*^* adduct as well as a low level of tandem mutations from both adducts affecting the 5' modified base as well as the adjacent 5' base. The *cis*-DDP *A*^*G*^* adduct was considerably more mutagenic than the *G*^*G*^* adduct, with a mutation frequency of 6.0% compared to 1.4% for the latter adduct. In accordance with the lack of an SOS-dependent increase in survival, the *cis*-DDP *G*^*T*G^* adduct also was not mutagenic.
A drawback of current platinum based chemotherapy is the potential carcinogenicity of cis-DDP. The site specific results suggest a strategy for reducing the mutagenicity, and hence the potential carcinogenicity, of platinum compounds. The high mutagenicity of the A*G* adduct suggests that, if a platinum compound directed against the formation of this highly mutagenic DNA lesion, its overall mutagenicity would be decreased. ACDP is a compound that meets this criterion; its cyclohexyl ring causes an orientational isomerism which, upon binding to DNA, reduces the number of A*G* adducts threefold as compared with cis-DDP DNA binding. ACDP, therefore, was predicted to be less mutagenic than cis-DDP. This hypothesis was tested by comparing the genotoxicities and mutagenicities of cis-DDP and ACDP DNA adducts in E. coli. Both compounds displayed similar levels of genotoxicity in a bacteriophage M13mp18 plaque-forming assay, with survival for genomes platinated by either drug increasing by threefold in cells pretreated with uv irradiation to induce the SOS functions of the host. The mutagenicity of ACDP was lower than that of cis-DDP in the lacZ′ α-complementation forward mutational assay and was also SOS-dependent. The mutational spectra for both drugs were similar; G → T transversions at d(GpG) sites were the most common mutations while G → A transitions and A → T transversions, many at d(ApG), d(GpNpG), and d(GpG) sites, were also well represented. Adduct mapping experiments revealed excellent correlation between the location of DNA lesions and the sites of mutations, confirming that the induced mutations were a consequence of the platinum adducts. Analysis of the distribution of mutations suggested that there were no sequence-dependent mutation hotspots; mutagenesis was random throughout the lacZ′ region of the M13mp18 bacteriophage genome.

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The primary, and perhaps only, reason that I came to MIT was to obtain an education. Accordingly, I would like to thank all the people who contributed to my learning process. First and foremost, I am grateful for my faculty advisor, John Essigmann who provided me with the research project described in this dissertation, financial support, and an enjoyable, stimulating, yet highly professional, work environment. During my time at MIT, I’ve probably learned more from other members of the Essigmann laboratory than from anyone else. Amongst this group of people, the greatest contributor to the practical aspects of my education was my mentor, the relatively benevolent Lisa Naser Bradley, who taught me everything there was to know about working in a laboratory. Other laboratory members, especially Ashis Basu, Mike Wood, Tim Connors, Dan Treiber, and Lisa Bailey have also provided invaluable technical advice during numerous helpful discussions. Additionally, I would like to thank our collaborator, Stephen Lippard, and his laboratory, for important intellectual contributions and expert technical assistance. Finally I would like to express my appreciation for the faculty members I’ve had the privilege of interacting with, and learning from, especially the members of my thesis committee, Steven Tannenbaum, Bill Orme-Johnson, and Joanne Stubbe.

As I’ve found out, the graduate school experience involves more practical work than actual theoretical learning. At times, laboratory work can seem tedious and apparently unrewarding. Therefore, I gratefully acknowledge the friendship and encouragement from my family and labmates that helped me to persevere and ultimately experience the massive emotional, physical, intellectual, and potential financial, rewards of successful completion of this dissertation. From a practical perspective, the excellent technical assistance received from Wendy Rowell and Jennifer Wilson greatly facilitated my work and helped me graduate with (relatively) great alacrity. A special thanks is due Brian Donahue and Jill Mello who valiantly played crucial roles in battling the heartless MIT parking bureaucracy by helping me to obtain, collectively, four parking permits. Finally, a most special thanks to Marjie Solomon for critical reading of this manuscript, thereby enabling it to become what it is today.
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<tr>
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<th>Description</th>
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<tr>
<td>AAF</td>
<td>acetylaminofluorene</td>
</tr>
<tr>
<td>ACDDP</td>
<td>(cis,trans,cis)-aminocyclohexylamine dibutyrochloroplatinum(IV)</td>
</tr>
<tr>
<td>ACDP</td>
<td>(cis)-aminocyclohexylamine dichloroplatinum(II)</td>
</tr>
<tr>
<td>A-DNA</td>
<td>DNA in the A conformation</td>
</tr>
<tr>
<td>AF</td>
<td>aminofluorene</td>
</tr>
<tr>
<td>A<em>G</em></td>
<td>(cis-)([Pt(NH_3)_2{}d(GpG)-N7(1),-N7(2)}]</td>
</tr>
<tr>
<td>ANLL</td>
<td>acute non-lymphocytic leukemia</td>
</tr>
<tr>
<td>AP</td>
<td>apurinic/apyrimidinic</td>
</tr>
<tr>
<td>ApaL Pt</td>
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</tr>
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</tr>
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<td>64-mer oligodeoxynucleotide used as a scaffold in the construction of the (G<em>G</em>) containing genome</td>
</tr>
<tr>
<td>B-DNA</td>
<td>DNA in its biologically predominant B conformation</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAP</td>
<td>catabolite activator protein</td>
</tr>
<tr>
<td>carboplatin</td>
<td>diaminocyclobutaneplatin(II)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>dA or A</td>
<td>deoxyadenosine</td>
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<tr>
<td>cis-DDP</td>
<td>(cis)-diaminedichloroplatinum(II)</td>
</tr>
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<td>deoxycytosine</td>
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<tr>
<td>dG or G</td>
<td>deoxyguanosine</td>
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<tr>
<td>D_0</td>
<td>dose required for 50% mortality</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
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<tr>
<td>dT or T</td>
<td>thymidine</td>
</tr>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<td>EDTA</td>
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</tr>
<tr>
<td>EthBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FA</td>
<td>Fanconi’s anemia</td>
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<tr>
<td>form I</td>
<td>supercoiled DNA</td>
</tr>
<tr>
<td>form I(_o)</td>
<td>covalently closed circular ds DNA</td>
</tr>
<tr>
<td>form II</td>
<td>nicked circular ds DNA</td>
</tr>
<tr>
<td>form III</td>
<td>linear ds DNA</td>
</tr>
<tr>
<td>G*</td>
<td>platinum monoadduct</td>
</tr>
<tr>
<td>G<em>G</em></td>
<td>(cis-)([Pt(NH_3)_2{}d(GpG)-N7(1),-N7(2)}]</td>
</tr>
<tr>
<td>G<em>NG</em></td>
<td>(cis-)([Pt(NH_3)_2{}d(GpNgG)-N7(1),-N7(3)}] where (N = A, C) or (T)</td>
</tr>
<tr>
<td>G<em>TG</em></td>
<td>(cis-)([Pt(NH_3)_2{}d(GpTpG)-N7(1),-N7(3)}]</td>
</tr>
<tr>
<td>HMG</td>
<td>high mobility group</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>hUBF</td>
<td>human upstream binding factor</td>
</tr>
<tr>
<td>iproplatin</td>
<td>(cis,trans,cis)-dichlorobisdihydroxy(isopropylamine)platinum(IV)</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-(\beta)-D-galactoside</td>
</tr>
<tr>
<td>J/m^2</td>
<td>joules per meter squared</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolt</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MF</td>
<td>mutation frequency</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MT</td>
<td>metallothionein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol, average MW = 8000</td>
</tr>
<tr>
<td>Phage</td>
<td>M13 bacteriophage</td>
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cis-diaminocyclohexanedichloroplatinum(II)
cis-chlorodihydroethylenetriamineplatinum(II)
dichloroethylenediamineplatinum(II)
replicative form
ribonucleic acid
ribosomal RNA
Saccharomyces cerevisiae
Salmonella typhimurium
cis-DDP modified Sca 24 oligodeoxynucleotide
24-mer oligodeoxynucleotide containing the Sca I site
64-mer oligodeoxynucleotide used as a scaffold in the construction of the A*G* containing genome
single stranded
cis-DDP modified Stu 24 oligodeoxynucleotide
24-mer oligodeoxynucleotide containing the Stu I site
64-mer oligodeoxynucleotide used as a scaffold in the construction of the G*G* containing genome
80 mM Tris, 0.57% glacial acetic acid, 0.2 mM Na₂EDTA
89 mM Tris, 89 mM boric acid, 0.2 mM Na₂EDTA
10 mM Tris-HCl (pH 8.0), 1 mM EDTA
tris(hydroxymethyl)aminomethane hydrochloride
trans-diamminedichloroplatinum(II)
transfer RNA
ultraviolet
5-bromo-4-chloro-3-indolyl-β-D-galactoside
xeroderma pigmentosum
yeast-tryptone media
DNA in the Z conformation
I. INTRODUCTION
The genotoxic properties of chemicals that bind to DNA and selectively kill rapidly dividing cells by inhibition of replication or transcription are exploited in cancer chemotherapy. One of the most effective anticancer drugs believed to act by this mechanism is cis-diamminedichloroplatinum(II) (cis-DDP, Figure 1). cis-DDP cures over 95% of testicular cancer cases and is also used in the treatment of cancers of the head, neck, lung, stomach, esophagus, and urogenital tissues. Despite being one of the most widely used chemotherapeutic agents of the last decade, cis-DDP has several significant liabilities. Dose-limiting toxicity, acquired and intrinsic resistance, and cumbersome intravenous administration decrease the clinical efficacy of cis-DDP. In recent years intense effort has been devoted to the design and development of platinum-based drugs that have diminished toxicity, are effective against cis-DDP resistant tumors and can be administered orally. Conversely, a drawback of platinum-based chemotherapy that has not yet been addressed adequately is the suspected human carcinogenicity of cis-DDP. Accordingly, this thesis explores what attributes of platinum compounds contribute to their mutagenicity, and implied carcinogenicity, with the goal of elucidating principles that will facilitate the design and evaluation of less mutagenic, and therefore potentially less carcinogenic, drugs.

cis-DDP is a proven carcinogen in the mouse and rat (Leopold et al., 1979). As has been shown for many electrophilic carcinogens, cis-DDP is also a mutagen in bacterial (Beck & Brubaker, 1975; Benedict et al., 1977) and in mammalian (Johnson, N. P. et al., 1980) cell systems. Mutation is believed to be one of the multiple steps that results in neoplastic transformation of cells (Weinberg, 1989). Therefore, the appearance of second malignancies in patients treated with cis-DDP is not surprising (Johnson, D. C. et al, 1980; Stewart & Wilkinson, 1981; Meadet et al., 1983; Redman et al., 1984; Redman et
al., 1985; Bassett and Weiss, 1986), fueling speculation that these cancers may have resulted from cis-DDP treatment. The incidence of second cancers is highly specific. Acute non-lymphocytic leukemia (ANLL) occurred in 95% of cis-DDP treated patients suffering a second cancer, at a 50 fold higher rate of incidence than expected (Ratain et al., 1987; Nichols et al., 1990). It should be noted that, because cis-DDP typically is administered in conjunction with a battery of other chemotherapeutic agents, some of which are also suspected carcinogens, its carcinogenicity in humans is implied but not firmly established (Greene, 1992).

Several key questions arise as to how cis-DDP induces the mutations that presumably engender the cancer phenotype. Are all of its multiple DNA adducts mutagenic or is the mutagenic activity of the drug attributable only to one, or to a subset, of its adducts? Are the lesions that kill cells different from those that cause mutations and, if so, could this observation be exploited in the development of a safer therapeutic regimen? In order to address these questions, methodologies have been developed that enable one to situate a single DNA lesion at a specific site within a viral or plasmid genome (Green et al., 1984). The resulting singly modified genomes can be used to determine the mutation frequency and specificity of each adduct, and to what extent the adduct compromises the viability of the genome. By using these techniques to determine the relative contributions of each cis-DDP adduct to the total mutagenicity and genotoxicity of the compound, it may be possible to identify those lesions that are principally genotoxic and those that are primarily premutagenic. This information could then be used for the design or evaluation of new platinum based drugs; ideally a new drug would maximize the number of genotoxic lesions and minimize the proportion of premutagenic lesions (Bradley, 1991).
This dissertation reports studies of the effects on survival and mutagenesis of the three major cis-DDP DNA adducts in Escherichia coli. For this purpose, M13mp7L2 derived ss viral genomes, each containing a single cis-DDP A*G*, G*G*, or G*TG* adduct at a unique site, were constructed. This work affords significant insights into the biochemical and molecular processing of platinum DNA adducts. In more general terms, the extensive use of the lacZ' mutational assay also elucidates the molecular mechanisms eliciting mutagenicity in E. coli.

As mentioned, a practical application of this work would be the design or evaluation of new platinum based drugs. Therefore, in conjunction with the site specific studies, the comparative DNA binding, genotoxic, and mutagenic effects of cis-DDP and cis-ammine(cyclohexylamine)dichloro platinum(II) (ACDP, Figure 1) were determined by replicating drug-modified M13mp18 RF genomes in E. coli.

ACDP is the major metabolite of cis,trans,cis-ammine(cyclohexylamine)dibutyryatodichloroplatinum(IV), (ACDDP, Figure 1) a promising new platinum-based anticancer drug now in clinical trials. ACDDP is an orally active platinum (IV) compound undergoing testing as a less toxic analogue of cis-DDP that is chemotherapeutically active against resistant tumors. Its inclusion in this study is motivated by the prediction that this drug will be less mutagenic than cis-DDP. This prediction is based on the DNA-binding spectrum of ACDP combined with the results of previous site-specific studies on platinum DNA adducts. Hartwig and Lippard (1992) showed that ACDP binds to DNA in a manner similar to cis-DDP but with the notable difference that ACDP formed 3 fold fewer intrastrand crosslinks at d(ApG) sites. Considering that the cis-DDP A*G* adduct was determined to be 4-5 fold more mutagenic than either the G*G* or G*TG* adducts, ACDP is predicted to have an overall mutagenicity lower than that of cis-DDP. This hypothesis was
experimentally tested and confirmed by comparing the two drugs in the lacZ' β-galactosidase α-complementation forward mutational assay in E. coli. Simultaneously, the genotoxicity of the various platinum DNA adducts was studied to ensure that a diminution of mutagenicity was achieved while retaining a level of lethality necessary to retain the chemotherapeutic effectiveness of these compounds.
Figure 1. Structures of platinum compounds discussed in this dissertation.
II. LITERATURE SURVEY
This section briefly reviews what is known about the formation and structural effects of platinum DNA adducts and the subsequent mutagenic and genotoxic effects of these adducts in a prokaryotic system. Limitations of current platinum-based chemotherapy are outlined, demonstrating the need for new drugs. Platinum(IV) ammine/amine dicarboxylates, a class of promising new drugs, are described, followed by a discussion of ACDP, the biologically active metabolite of ACDDP, a platinum(IV) drug now undergoing evaluation in clinical trials.

A. Platinum-DNA Interactions

DNA is considered to be the principal target of cis-DDP in vivo. When mammalian cells are treated with cis-DDP, inhibition of replication occurs preferentially over inhibition of transcription and translation (Harder & Rosenberg, 1970; Howle & Gale, 1970). Pascoe and Roberts (1974) have shown that pharmacologically relevant doses of cis-DDP given to HeLa cells result in more platinum bound per molecule of DNA than RNA or protein. At the 37% survival level (i.e. the survival that corresponds to one lethal event per cell), the DNA contained 22 adducts, whereas only 1 in 8 mRNA, 1 in 30 rRNA, and 1 in 1500 tRNA or protein molecules were modified. Similarly, Akaboshi et al. (1992) reported platinum adduct levels of 1 per 3-10,000 protein molecules, and 1 per 10 - 1,000 RNA molecules, while each DNA molecule had more than 9 adducts present for D₀ values of 50% in HeLa S-3 cells. Considering that a large excess of unmodified RNA and protein molecules remain in cells undergoing cis-DDP induced mortality, it is reasonable that this drug effects cytotoxicity through its interactions with DNA.

'Portions of the literature survey are adapted (with permission) from Bradley (1991).
1. Mode of Cytotoxicity Induced by cis-DDP Modified DNA

The type and distribution of cis-DDP adducts in mammalian cells (Plooy et al., 1985a; Akaboshi et al., 1992; Kusumoto et al., 1993) and in cancer patients receiving platinum therapy (Fichtinger-Schepman et al., 1987; Fichtinger-Schepman et al., 1990) is similar to that determined in vitro. The large size and complexity of the mammalian genome, however, has hindered the study of the mechanisms of cytotoxicity, as well as the mutagenicity and repair, of cis-DDP adducts in these systems. Nevertheless, a variety of models has been proposed to account for the chemotherapeutic effectiveness of cis-DDP. cis-DDP has been proposed to kill cells by apoptosis, with sensitivity to this drug only occurring at certain points in the cell cycle (Evans & Dive, 1993; Krishnaswamy & Dewey, 1993).

Many early studies focused on the levels of platinum interstrand crosslinking of DNA as the factor responsible for the cytotoxicity of cis-DDP (Connors et al., 1979; Zwelling & Kohn, 1979). Interstrand crosslinks, however, are a minor component of the adduct spectrum of cis-DDP, comprising <5% of the total binding in vitro and in vivo. Moreover, there have been conflicting reports about the correlation between the levels of interstrand crosslinks and biological response (see Pinto & Lippard, 1985a, for a review). A significant piece of evidence refuting the importance of interstrand crosslinks is the chemotherapeutic ineffectiveness of trans-DDP, despite the fact that this geometric isomer of cis-DDP forms twice as many interstrand crosslinks (Brabec & Leng, 1993; Decoville et al., 1993). Intrastrand crosslinks, however, that are formed by cis-DDP and related compounds are strongly correlated with the effectiveness of certain platinum drugs.
Of note are the 1,2 intrastrand crosslinks formed by cis-DDP at adjacent nucleotides that, due to steric constraints, are not formed by the trans-DDP isomer, suggesting that 1,2 adducts may be responsible for the chemotherapeutic effectiveness of the former compound. The predominant 1,2 intrastrand crosslinks formed by cis-DDP are the G*G* and A*G* adducts. A class of HMG-box containing proteins has been reported that bind specifically to these two adducts (Toney et al., 1989; Donahue et al., 1990; Pil & Lippard, 1992; Brown et al., 1993). A potential consequence of the binding of these proteins to cis-DDP adducts is that bound adducts are shielded from repair thereby allowing them to persist in the genome to mediate their cytotoxicity. Indeed, the cis-DDP G*G* adduct is not repaired in human cell extracts, implying that it may be important in mediating the chemotherapeutic effects of this drug (Szymkowski et al., 1992). Alternatively, these proteins, which include important cellular regulatory proteins such as the rRNA transcription factor hUBF, may bind to cis-DDP G*G* and A*G* adducts. Once bound, the proteins are prevented from performing their normal cellular functions ultimately leading to cell death (X. Zhai & D. Treiber, personal communication). The importance of this group of proteins in mediating the chemotherapeutic effects of cis-DDP remains unclear, however, as a recent report shows no correlation between the levels of such proteins and the in vitro sensitivity of tumor cells to cis-DDP (Bissett et al., 1993). Notwithstanding the current lack of mechanistic insight into how cis-DDP DNA adducts mediate their cytotoxic effects, evidence remains strong that cis-DDP DNA adducts are in some way responsible for the therapeutic activity of this drug. Accordingly, platinum-DNA adducts will be discussed in detail below. An underlying knowledge of platinum-DNA interactions is necessary to understand the genotoxic and mutagenic effects of cis-DDP reported in this dissertation.
2. Platinum-DNA Adducts.

cis-DDP is a bifunctional electrophilic compound that reacts with DNA to form a variety of intra- and interstrand crosslinks (reviewed in Sherman & Lippard, 1987). The principal adducts are the cis-[Pt(NH$_3$)$_2$(d(GpG))] and cis-[Pt(NH$_3$)$_2$(d(ApG))] intrastrand crosslinks (referred to as G*G* and A*G*, respectively, Figure 2); minor adducts include cis-[Pt(NH$_3$)$_2$(d(GpNpG))] intrastrand crosslinks (G*NG*, where N is any intervening nucleotide), interstrand crosslinks, and monoaadducts (G*). In each of these adducts, the N7 atoms of the purine bases have replaced the chloride ligands in the cis-DDP square plane. The G*G* intrastrand crosslink constitutes 50-65% of the cis-DDP adducts formed in DNA in vitro, with the next most abundant adduct, A*G*, comprising approximately 25% of the total (Eastman, 1983; Fichtinger-Schepman et al., 1985b).

trans-Diamminedichloroplatinum(II) (trans-DDP) is the geometric isomer of cis-DDP. It also binds to DNA to produce intra- and interstrand crosslinks at the N7 positions of purine bases, although these adducts have not been as well characterized as those of cis-DDP. Binding studies with oligodeoxynucleotides have shown trans-DDP to form principally 1,3-intrastrand adducts (Eastman et al., 1988). Because this compound is clinically inactive, the formation and removal of its DNA adducts have often been compared to those of cis-DDP with the goal of explaining the potent biological activity of the latter agent.

Studies of structure-activity relationships among various platinum compounds have presented a compelling case that the cis geometry of the chloride ligands is a key feature for biological activity. For example, the monofunctional platinum compound chlorodiethylenetriamine-platinum(II) ([Pt(dien)Cl]$^+$) (Figure 1), whose major adduct occurs at
the N7 position of guanine (Johnson, N. P. et al., 1982), does not exhibit antitumor activity. Clinically active platinum compounds include cis-dichloroethylenediamine-platinum(II) ([Pt(en)Cl₂]), cis-diaminocyclohexanedichloroplatinum(II) ([Pt(dach)Cl₂]), and diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin) (Figure 1). It is noteworthy that these drugs, like cis-DDP, have ligands bound in a cis geometry. At least one of these compounds, [Pt(en)Cl₂], forms an adduct spectrum that is very similar to that of cis-DDP (Eastman, 1983; Eastman, 1986). Due to the apparent requirement of bifunctionality and a cis geometry for clinical activity, and the fact that for stereochemical reasons trans-DDP can form neither the G*G* nor A*G* adducts in which adjacent bases in the same strand are crosslinked (Table 1), it is believed that the 1,2-crosslinks are responsible for the unique biological activities of cis-DDP and the other clinically active drugs (Pinto & Lippard, 1985a). The therapeutic ineffectiveness of trans-DDP has been used to justify the contention that 1,3 intra- and interstrand crosslinks are not important in the cytotoxicity mediated by cis-DDP. Recent results, however, indicate that 1,3 intra- and interstrand crosslinks formed by cis-DDP have significantly different structural characteristics than those formed by trans-DDP. The possibility remains, therefore, that the cis-DDP 1,3 intra- or interstrand crosslinks may be therapeutically relevant lesions.

1 Both the cis- and trans-DDP interstrand crosslinks predominately form at d(GpC)/d(GpC) sites. The cis-DDP adduct forms between the guanines offset by 1 base on the adjacent strands whereas the interstrand crosslink formed by trans-DDP is between a guanine residue and the cytosine directly opposite (Brabec & Leng, 1993; Decoville et al., 1993). Considering the 1,3 intrastrand crosslinks, the trans-DDP G*NG* 1,3 adduct unwinds the DNA duplex by 9-10° and imposes a flexible hinge joint bending on the helix whereas the comparable cis-DDP lesion unwinds the DNA by 23° and produces a directed bend (toward the major groove) in the DNA helix (Bellon et al., 1991; Keck & Lippard, 1992; Zou et al., 1993). These structural differences allow differential processing of 1,3 cis- and trans-DDP DNA lesions by various polymerases (Corda et al., 1993). In conclusion, care should be exercised in concluding that cis-DDP 1,3 intra- and interstrand crosslinks are therapeutically unimportant based on analogy with the trans-DDP results.
As mentioned above, cis-DDP binds to DNA at the N7 position of the purine bases. In general, other N7 purine adducts (as formed by aflatoxin B₁ or by alkylating agents) destabilize the glycosidic bond, resulting in the release of the adducted base. Apurinic (AP) sites are generated in DNA by this process. AP sites are SOS dependent premutagenic lesions, and it has been postulated that they serve as a common intermediate in the pathway leading to mutagenesis by many chemical and physical agents (Schaaper & Loeb, 1981). The facile generation of an AP site, leading to a mutation, is unlikely to be the cause of cis-DDP induced mutations. cis-DDP DNA adducts actually stabilize the glycosidic bond and are very resistant to treatments that induce depurination (Royer-Pokora et al., 1981; Forsti et al., 1986). They can, however, be removed from DNA in vitro by cyanide or thiourea treatment, which reverses most adducts by breaking the coordinate bonds between the platinum atom and the bases (Bauer et al., 1978; Lippard & Hoeschele, 1979; Filipski et al., 1979). One cannot rule out the possibility that some powerful intracellular nucleophile might act similarly in vivo, although there is no evidence for such a "repair" mechanism at present.

Mapping experiments utilizing exonuclease III digestion of (Tullius & Lippard, 1981; Royer-Pokora et al., 1981) and replication blockage by (Pinto & Lippard, 1985b; Hemminiki & Thilly, 1988; Villani et al., 1988; Bubley et al., 1991; Murray et al., 1992) platinum modified DNA demonstrate that for cis-DDP, enzyme blockage occurs at guanine bases and at oligo (dG) sequences. trans-DDP treated DNA also poses a block to replication and exonuclease III digestion, but exhibits less of a sequence specificity with some indication of blockage at d(GpNpG) sequences. The ability of these adducts to interfere with normal enzymatic activity suggests that there is some aspect of the structure of these adducts that may account for their biological activity.
3. Structural Effects of Platinum-DNA Adducts

a. Macroscopic effects on the structure of platinum modified DNA

Many studies have focused on defining the detailed structural and physical properties of platinum DNA adducts. The binding of both cis- and trans-DDP to DNA in vitro shortens and unwinds the helix (Cohen et al., 1979). In addition, spectroscopic studies have shown that the binding of platinum compounds to DNA disrupts normal base stacking, and results in a decreased melting temperature of the DNA (reviewed by Sherman & Lippard, 1987). Studies with enzymatic probes sensitive to the structure of DNA have also indicated that platinated DNA has an abnormal structure. Single-strand specific nucleases can digest duplex DNA platinated with either cis-DDP or trans-DDP (Mong et al., 1981; Scovell & Capponi, 1982; Scovell & Capponi, 1984). Restriction endonuclease digestion experiments with globally platinated DNA suggest that the presence of a cis-DDP adduct within three base-pairs of a recognition sequence can inhibit recognition and digestion by the enzyme (Cohen et al., 1980; Ushay et al., 1981).

Antibodies that recognize each of the four DNA nucleosides have been used as probes to define the extent of cis-DDP perturbation of DNA structure (Sundquist et al., 1986). The antibodies recognize denatured DNA, but do not bind to native duplex DNA. DNA treated with increasing amounts of cis-DDP bind increasingly more antibodies to dC, and to a lesser extent dA, and dT, but not those elicited against dG. DNA treated with very low levels of the trans compound was able to bind all four antibodies, whereas [Pt(dien)Cl]⁺ treated DNA was not recognized by the antibodies. These results suggest that there is disruption of base pairing upon binding of bifunctional platinum drugs, and that this disruption is greater for trans-DDP than cis-DDP.
An altered and destabilized DNA structure that may still accommodate some form of hydrogen bonding between modified nucleotides and their complements is consistent with the physical and biochemical data presented above. It has been suggested that the high levels of platination (1 platinum adduct in 5-10 nucleotides) required for single strand nuclease digestion may lead to cooperative destabilization of the helix, resulting in single stranded regions that would not ordinarily be present in platinated DNA (Scovell & Capponi, 1982; den Hartog et al., 1985a). This cooperativity could also explain the nonlinear increase in anti nucleoside-antibody binding to DNA treated with increasing amounts of cis-DDP (Sundquist et al., 1986); alternatively, while the antibodies cannot bind to native duplex DNA, they may be able to bind to a distorted duplex DNA, and thus binding may be an indication either of distorted or single stranded regions of DNA.

As mentioned, many of the early biochemical and physical characterization studies of cis-DDP modified DNA were performed at levels of drug of up to 1 platinum adduct per 5-10 nucleotides. Evidence is accumulating that the chemotherapeutic response to cis-DDP is elicited from much lower levels of drug bound to DNA. Reed et al. (1993) report that patients successfully treated with cis-DDP typically accrue 1 adduct per 10-20,000 nucleotides of their genome. This finding suggests that single adducts, and not the cooperative effects of two or more closely spaced lesions, are the biologically relevant species in eliciting the cytotoxicity of cis-DDP. Accordingly, the structural effects of single cis-DDP adducts is addressed below.

b. Structural effects of single platinum DNA adducts

X-ray diffraction studies of the single stranded dinucleotide cis-[Pt(NH)₃{d(pGpG)}] show that platinum binding destacks the adjacent
guanine bases, resulting in a dihedral angle between the ring planes of 76-87° (Sherman et al., 1985). In the crystal form, the 3' deoxyribose is in the conformationally more flexible C(2')-endo configuration that is typical of the B-DNA architecture; the deoxyribose of the 5'-linked nucleotide, by contrast, is in the more rigid C(3')-endo geometry characteristic of A-DNA (Sherman et al., 1985).

The crystal structure of a single stranded dinucleotide is not necessarily reflective of the structure of such lesions in larger DNA contexts. NMR analysis of the cis-DDP modified d(GpG) dinucleotide is consistent with the crystal structure result depicting a severe kink (den Hartog et al., 1982). The G*G* adduct in the d(CpGpG) trinucleotide, however, appears to be less disruptive, with the bases able to adopt a stacking conformation consistent with B-DNA despite the distortions imposed by platinum binding (den Hartog et al., 1985b). Indeed, the structural repercussions of the G*G* adduct become ameliorated as the lesion is incorporated into oligodeoxynucleotides of increasing length. For example, small oligodeoxynucleotides up to six bases in length containing a single G*G* crosslink will not form duplex structures (Caradonna et al., 1982; Sherman & Lippard, 1987). Singly modified decamer sequences containing the G*G* crosslink will form duplexes, however, indicating that the structural distortion of a G*G* crosslink is fairly well localized (den Hartog et al., 1984; van Hemelryck et al., 1984; den Hartog et al., 1985b). NMR studies on these decamer sequences do not conclusively demonstrate the presence of hydrogen bonding occurring at the platinum modified guanines, however, it cannot be ruled out at present. Each study suggests that the NMR data is consistent with the presence of a kink or bend induced in the oligodeoxynucleotide by the adduct.

c. Platinum induced perturbation of duplex DNA
The structural perturbation of DNA upon cis-DDP binding has been evaluated in the context of native duplex DNA. Gel electrophoretic mobility assays have been used to determine cis-DDP-induced bend angles in DNA. By using duplex oligomers containing G*G*, A*G*, or G*NG* adducts, it was shown that each adduct imparts a directed bend in the double helix, with bend angles calculated to be 32-35° for all three adducts (Bellon & Lippard, 1990). The direction of the bend of the G*G* adduct is toward the major groove (Rice et al., 1988); it is expected that the other adducts also have bends directed toward the major groove. By contrast, 1,3 intrastrand crosslinks formed by trans-DDP consist of flexible hinge joints that allow the DNA to bend alternately toward either the major or minor grooves (Bellon et al., 1991). Unwinding of the DNA helix also occurs upon cis-DDP binding. The cis-DDP G*G* and A*G* adducts both unwind the helix by 13° while the G*NG* adduct unwinds the helix by 23°; trans-DDP unwinds the helix by 9° (Bellon et al., 1991; Keck & Lippard, 1992). The unwinding of the DNA duplex appears to be more important than drug-induced bending in determining the biological responses to platinum-modified DNA (Bellon et al., 1991).

The chemical reactivity of DNA bases to compounds that are sensitive to the structure of DNA has provided enhanced resolution to the picture of the local distortion induced by single cis-DDP-DNA adducts (Marrot & Leng, 1989; Schwartz et al., 1989; Anin & Leng, 1990). These data suggest that the helix is distorted to a greater extent on the 5' side of A*G* and G*G* adducts than on the 3' side, but that this asymmetrical distortion does not result in local denaturation in the area of the lesion. These distortions differ for the two adducts, however, as demonstrated by their different patterns of chemical reactivity, with the A*G* adduct showing evidence of more distortion than the G*G* adduct.
In contrast to the cis-DDP 1,2 intrastrand crosslinks, where, despite some distortion, the modified nucleotides retain base stacking (den Hartog et al., 1985b) and base pairing abilities (van Hemelryck et al., 1984), 1,3 crosslinks are more severely distorted. The structure of the cis-DDP G*CG* adduct has been studied by NMR (den Hartog et al., 1983; Marcelis et al., 1983). This lesion does not exhibit characteristics consistent with normal base stacking, instead the intervening cytosine is destacked, in effect "bulged out" or "turned away" from the chelated guanines. The 1,3 intrastrand crosslinks at d(GpTpG) and d(GpApG) sequences\(^1\) appear to adopt similar conformations. The disruption of hydrogen bonding occurring upon the destacking of the intervening nucleotide is reflected in the thermal stability of the DNA duplex. The melting temperatures of ds decamers containing any of the cis-DDP 1,3 adducts (G*AG*, G*CG*, or G*TG*) are decreased by \(\pm 30^\circ\text{C}\). This result suggests that these adducts are structurally similar (Urata et al., 1992). Interestingly, the presence of a T opposite the destacked central nucleotide stabilized the duplex form of DNA containing any of the 1,3 adducts (Urata et al., 1992).

Destacking of the central nucleotide, similar to that observed for the cis-DDP G*CG* adduct, occurs for trans-DDP adducts. The structures of the G*TG* (van der Veer et al., 1986a), G*CG* (Gibson & Lippard, 1986), and G*AG* (Lepre et al., 1990) adducts have all revealed destacking. The similarities in destacking exhibited by cis- and trans-DDP 1,3 intrastand crosslinks, however, is insufficient to explain the biological effects of these adducts. To give two examples, trans-DDP adducts are easily bypassed by DNA polymerases (Comess et al., 1992;

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\(^1\)The G*GG* adduct is not expected to form. The N7 of the central guanine is the most nucleophilic site in the d(GpGpG) trinucleotide sequence and reacts first with cis-DDP to form a monoadduct. The monoadduct then closes to a 1,2 intrastrand crosslink, preferentially binding, at least in the sequence context studied, to the 3' guanine residue (Yohannes et al., 1993).
Corda et al., 1993) while cis-DDP adducts are not (Comess et al., 1992). Additionally, 1,3 adducts formed by cis acting platinum compounds are good substrates for UvrABC excision repair (Page et al., 1990) while the comparable trans lesions do not appear to be repaired by this enzyme (Popoff et al., 1987). As mentioned, cis- and trans-DDP 1,3 intrastrand crosslinks bend and unwind the DNA duplex differently, likely accounting for their different biological activities.

In summary, the cis-DDP intrastrand crosslinks induce bends in duplex DNA toward the major groove of approximately 32-35°. Unwinding of the helix is more severe, at 23°, for 1,3 cis-DDP intrastrand crosslinks than for either the A*G* or G*G* adducts, both of which unwind the DNA duplex by 13°. Base-pairing probably remains intact for the A*G* and G*G* adducts, albeit in a distorted manner, and this distortion is greater on the 5’ side of the adduct than on the 3’ side. 1,3 Intrastrand crosslinks disrupt the DNA duplex more severely, with the central base destacking from the helix.

In addition to the consequences of localized structural perturbation of the DNA architecture that results from single platinum adducts, cis-DDP’s chemotherapeutic effectiveness also has been proposed to result from a larger disruption of the DNA duplex. These models are based on the targeted binding of cis-DDP at certain nucleotide sequences (Hemminki & Thilly, 1988; Jones et al., 1991). Such binding might result in multiple adducts being formed in close proximity to each other, allowing the cooperative distortion of DNA by multiple adducts. Regions thought to be affected in this manner are alternating d(GpT)ₙ and d(GpC)ₙ stretches (Johnson et al., 1992). These sequences are almost always located in the 5’ promotor region of "house-keeping genes" and often in tissue specific gene promoters. cis-DDP modification converts and stabilizes these sequences into the Z-DNA conformation,
putatively inhibiting transcription of these essential genes ultimately leading to cell death (Johnson et al., 1992). Another model is based on the demonstration that cis-DDP modification disrupts the ordered structure of oriented DNA fibers in vitro. This result was interpreted to imply that cis-DDP could disrupt the orderly packing of DNA in the chromosome with dire ramifications for the survival of a cell subjected to such an insult (Rampino & Johnson, 1991; Rampino, 1992). A final model is based on the ability of the G*G* cis-DDP adduct to form and stabilize cruciform and hairpin structures. These structures are substrates for the aforementioned HMG-box proteins that bind specifically to cis-DDP modified DNA and may play a role in the chemotherapeutic response to this drug (Yohannes et al., 1993).

4. Replication Blockage Effects of Platinum DNA Adducts

The therapeutic effects of cis-DDP are attributed, at least in part, to the ability of platinum DNA adducts to block DNA replication and transcription. cis-DDP treated DNA substrates have been shown to inhibit human DNA polymerases in vitro (Harder et al., 1976), and the sites of termination of eukaryotic DNA polymerase α have been mapped to oligo d(G)_n sequences in the cis-DDP treated template (Villani et al., 1988). More recently, an in vitro translesion synthesis assay demonstrated that cis-DDP adducts were effective blocks to DNA replication. The G*G* adduct was the most inhibitory (with as little as 2% replication bypass) and the G*NG* adduct was the least inhibitory (as much as 25% replication bypass) (Comess et al., 1992). Similarly, transcription elongation of DNA containing either A*G* or G*G* cis-DDP adducts was blocked at the site of the lesion (Corda et al., 1991).

The ability of cis-DDP DNA adducts to block polymerases is derived from the structural distortion these lesions impose on the DNA
architecture rather than from the presence of the platinum moiety itself. Monofunctional platinum adducts do not bend or unwind the DNA helix to the extent that bifunctional crosslinks do, neither do they provide a block to polymerases. In contrast, the cis-DDP G*G* and A*G* adducts inhibit a variety of polymerases, allowing translesion synthesis at a frequency less than 10% (Comess et al., 1992). Interestingly, the thymine-thymine cyclobutane dimer, a lesion with structural characteristics similar to the cis-DDP G*G* and A*G* adducts, is also a potent inhibitor of DNA replication (LeClerc et al., 1991). Facile bypass of trans-DDP 1,3 intrastrand crosslinks (Comess et al., 1992; Corda et al., 1992) is attributable to one of two factors. First, trans-DDP 1,3 intrastrand crosslinks can interconvert with the 1,4 form (Comess et al., 1990), presumably by means of a monofunctional intermediate. A polymerase stalled at such a lesion could reinitiate replication while the adduct is in the monofunctional form and not effective at blocking DNA synthesis. Alternately, the flexible hinge joint of a trans-DDP adduct could allow the lesion to adopt a bypassable conformation allowing a stalled polymerase to resume DNA replication.
Table 1. Comparison of the adducts formed by platinum(II) compounds.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>cis-DDP</th>
<th>trans-DDP</th>
<th>[Pt(dien)Cl]Cla</th>
</tr>
</thead>
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<tr>
<td><strong>DNA monofunctional adducts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dG*</td>
<td>3⁰</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td><strong>DNA intrastrand crosslinks</strong></td>
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</tr>
<tr>
<td>d(G<em>pG</em>)</td>
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<td>no</td>
</tr>
<tr>
<td>d(A<em>pG</em>)</td>
<td>20-30%</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>d(G<em>pNpG</em>)</td>
<td>&lt; 10%</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>DNA interstrand crosslinks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d(G<em>pC)/(G</em>pC)</td>
<td>&lt; 5%</td>
<td>?</td>
<td>no</td>
</tr>
<tr>
<td>d(G<em>pC)/(GpC</em>)</td>
<td>?</td>
<td>5-20%</td>
<td>no</td>
</tr>
<tr>
<td><strong>DNA protein crosslinks</strong></td>
<td>&lt; 1%</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

a. The majority of DNA adducts formed by trans-DDP and [Pt(dien)Cl]Cl has not been quantitated.

b. Numbers indicate the percentage of each adduct compared to the total binding spectrum. In cases where accurate numbers are not available, the ability to form adducts is indicated by "yes" or "no".
**Figure 2.** *cis*-DDP G*G*, A*G*, and G*TG* adducts. Only the chemical composition of the *cis*-DDP DNA adducts are depicted. Structural conformations are discussed at length in the text and are diagrammed in the literature referenced therein.
B. Cellular Effects of Platinum Anticancer compounds

1. Genotoxicity of Platinum-DNA Adducts

The genotoxicity of platinum DNA adducts is well established in a variety of biological systems and is expected based on the in vitro experiments that demonstrated cis-DDP adducts to be potent blocks to DNA polymerases. Early work by Rosenberg and colleagues identified inhibition of DNA synthesis as the mechanism by which cis-DDP reduced the growth of E. coli (Rosenberg et al., 1967; Harder & Rosenberg, 1970). DNA adducts formed by \([\text{Pt(dach)}\text{Cl}_2]\), a chemotherapeutically active cis-DDP analogue, are genotoxic in a plasmid-based assay (Husain et al., 1985). The level of genotoxicity of these adducts, 5.5 adducts per lethal hit, is comparable to that of DNA lesions formed by benzo(a)pyrene-7,8-dihydrodiol-9,10-oxide (BPDE) or uv photoproducts (Mizusawa et al., 1981; Strike & Roberts, 1981). Different platinum DNA adducts exhibit variable levels of genotoxicity. In an SV40-based in vitro replication system utilizing HeLa cell extracts, bifunctional cis-DDP adducts inhibited DNA replication 30 fold more effectively than the monofunctional DNA adducts formed by the therapeutically ineffective compound \([\text{Pt(dien)}\text{Cl}]^+\) (Heiger-Bernays et al., 1990). A site-specifically located G*G* cis-DDP adduct in M13mp18 DNA reduced DNA replication by 78% in E. coli (Bradley et al., 1993). In addition to inhibition of DNA synthesis, cis-DDP exhibits other genotoxic effects including chromosomal abnormalities (Kliesch & Adler, 1987) and sister chromatid exchanges (Vogel et al., 1991).¹

¹It should be noted, however, that gross chromosomal abnormalities and sister chromatid exchanges may be a result of the transformed phenotype of carcinoma cells and not a direct consequence of cis-DDP treatment.
2. Mutagenesis of *cis-DDP* in *E. coli*

a. Mutagenesis in *E. coli*.

*cis-DDP* induced mutagenesis was first demonstrated in *E. coli* by Beck & Brubaker (1975). *cis-DDP* is a base-pair substitution mutagen by virtue of its ability to revert 2-aminopurine and N-methyl-N' -nitro-N-nitrosoguanidine induced mutations and, conversely, by the ability of these mutagens to revert *cis-DDP* induced mutations. As expected, the frameshift mutagen ICR-191 is unable to revert any *cis-DDP* mutants. *cis-DDP* mutagenesis is also dependent upon LexA (Venturini & Monti-Bragadin, 1978) and RecA activities (Konishi et al., 1981), in particular the recombinational capabilities of RecA (Jarosik & Beck, 1984). Mutagenic dependence on LexA activity, and the ability of the mutagenesis enhancing plasmid pKM101 to increase the level of *cis-DDP* induced mutants in various cell types (Venturini & Monti-Bragadin, 1978; Jarosik & Beck, 1984) indicates that SOS processing of *cis-DDP* lesions is required for mutagenesis. Direct evidence for SOS involvement in mutagenic processing has been provided by Fram et al. (1985), who demonstrated that *cis-DDP* mutagenesis is abolished in a *umuDC* background.

b. Mutational specificity of *cis-DDP* in *E. coli*.

The exact nature of the mutations of *cis-DDP* in *E. coli* has been studied in several forward mutational assays but with conflicting results. By using the endogenous *E. coli lacI* gene as the genetic target, Brouwer et al. (1981) found that the mutations arising from *cis-DDP* treatment to cells were primarily at d(GpApG) and d(GpCpG) sequences. No mutations were detected with the trans compound. In this work, no specific treatment was used to induce SOS other than the drug
treatment itself. By contrast, Burnouf et al. (1987) found that the majority (>90%) of mutations occurred at d(ApG) and d(GpG) sequences in the tet gene of cis-DDP modified pBR322 that was replicated in SOS induced E. coli cells. The differences between the results of these two studies could be due to differences in the mode of DNA damage (in the former study intact cells were treated, whereas in the latter DNA was modified and then introduced into the host for mutation fixation), to differences in the range of mutations detectable in the respective genetic systems, or to the state of SOS induction of the cells in which the mutations were fixed. More recently, the mutagenicity of cis-DDP G*G* (Bradley et al., 1993) and A*G* (Burnouf et al., 1990) adducts has been determined site specifically. The results of these studies are analyzed in detail in comparison with the site specific analysis of the cis-DDP G*G*, A*G*, and G*TG* adducts done in the present work (see Discussion).

3. Mechanism of cis-DDP Mutagenesis in Prokaryotes

a. SOS dependence of mutagenesis

A wide range of DNA damaging agents can induce the SOS response in E. coli, including uv radiation, methylmethanesulfonate, 4-nitroquinolone-1-oxide, and aflatoxin B1. cis-DDP also induces the SOS response, as evidenced by its ability to stimulate the filamentous growth of bacteria (Rosenberg et al., 1967), to induce prophage from lysogenic bacteria (Reslova, 1971), and to induce increased cellular levels of RecA protein (Salles & Lesca, 1982). As with many of the treatments mentioned, cis-DDP is also an SOS-dependent mutagen; this property will be described in detail in the next section. Thus the state of SOS induction is important to any mutagenesis studies of cis-DDP in E. coli. The SOS response of E. coli, therefore, will be
examined in detail below.

The SOS response to DNA damage is characterized by enhanced DNA repair activities, enhanced mutagenesis, inhibition of cell division, and prophage induction.\(^1\) This diverse array of physiological responses result from the expression of an inducible set of genes that are under the control of the RecA and LexA proteins. Most of the literature on the SOS response describes the UvrABC exinuclease complex, which efficiently repairs a wide array of bulky lesions in DNA (Sancar & Rupp, 1983), including adducts formed by cis-DDP (Brouwer et al., 1988). This pathway has been extensively reviewed (Witkin, 1976; Little & Mount, 1982; Walker, 1984; Battista et al., 1990; Echols & Goodman, 1990; Lin & Sancar, 1992; Altshuler, 1993; Grossman & Thiagalingam, 1993; Sancar & Tang, 1993). The work done for this dissertation does not directly address the repair of platinum-DNA adducts.\(^2\) This discussion, therefore, will emphasize those aspects of the SOS response that engender the mutagenic processing of damaged DNA.

Under physiologically normal cellular conditions, a repressor protein, LexA, is bound to the operator regions of the at least 17-20 genes thought to comprise the SOS regulon. The SOS network is activated when a cell is exposed to a DNA damaging agent. The inducing signal is

\(^1\)The SOS response has been most extensively studied in *E. coli* and, unless otherwise stated, this discussion describes the SOS response in *E. coli*. It should be noted that related gram-negative bacteria mount very similar responses to DNA damage. In addition, gram-positive bacteria, exemplified by *Bacillus subtilis* also exhibit SOS-like responses when confronted with DNA damage (Cheo et al., 1993) and it is postulated that eukaryotes also have similar responses to DNA damage (Ho et al., 1993).

\(^2\)The possible role of DNA repair by UvrABC in mediating the observed SOS-dependent enhanced survival of platinum drug modified M13 bacteriophage genomes replicated in *E. coli* is briefly addressed in the dissertation in the experiments comparing the survival of cis-DDP modified DNA transfected into either *E. coli* DL7 (wt) or DL6 (uvrA) cells (see Figure 11).
generally believed to be single stranded regions of DNA exposed as a result of replication arrest at sites of DNA damage. In response, the normal form of RecA is converted to an activated form, RecA*, which then associates with and promotes the autolytic cleavage of LexA (reviewed by Little, 1993). The cleaved form of LexA can no longer function as a repressor, enabling the expression of the genes of the network. The genes involved bind LexA to different extents, allowing the differential activation of specific genes as is warranted by the magnitude of the threat to the host. Therefore, low levels of inducing signal will result in only some genes being turned on, while higher levels are required to induce the system fully. As the DNA is repaired, the inducing signal decreases, RecA* levels drop, the pool of uncleaved LexA increases, and the genes of the network are in turn repressed.

b. The role of UmuDC and RecA in SOS mutagenesis

The phenotypic responses of the SOS system are the result of the expression of the genes of this regulon. Generally, the genes involved in excision repair are most easily induced. As indicated previously, excision repair is an error free process, and it seems reasonable that these genes would be involved as an early response to damage. If damage levels are high, however, and the inducing signal persists, then the umuD,C genes are expressed. These genes appear to play a key role in the error-prone bypass of replication blocking lesions.

Early studies of *E. coli* deficient in the induction of mutations by ultraviolet light implicated mutations at three chromosomal loci that encode the *umuA*, *umuB*, and *umuC* genes. The *umuA* and *umuB* gene products were determined to be the LexA and RecA proteins, respectively (Kato & Shinoura, 1977; Steinborn, 1978). The *umuC* locus was demonstrated to encode two polypeptides, the UmuD and UmuC proteins, both of which are
required for uv and chemical induced mutagenesis (Bagg et al., 1981; Elledge & Walker, 1983). This operon is under the coordinated control of the recA and lexA gene products (Kitagawa et al., 1985). Uninduced levels of umuDC expression yield about 180 molecules of UmuD and undetectable amounts of UmuC. After the LexA repressor protein is inactivated by RecA* cleavage, levels of UmuD increase to 2500 and UmuC to 200 molecules per cell (Woodgate & Ennis, 1991). The disparity in UmuD and UmuC synthesis is likely to be a result of inefficient translational coupling at a one-base overlap between the UmuD and UmuC cistrons (Perry et al., 1985; Woodgate & Sedgwick, 1992).

There are some reports that a low level of chemical and uv induced mutagenesis is possible without induction of the SOS response (Bridges & Woodgate, 1984; 1985; Fix, 1993). In most cases, however, two features of the SOS response are required for mutagenic processing of bulky lesions in DNA. First, SOS induction is necessary to achieve an adequate level of UmuC protein. UmuC is not present in non-SOS induced E. coli because (i) UmuC synthesis is inefficient compared to UmuD and (ii), because UmuC is very susceptible to proteolytic digestion, with a half life of only 6 min unless it is part of a multiprotein complex (Donnelly & Walker, 1989). The chaperone proteins encoded by the groEL and groES loci extend the half life of UmuC to 17 min. UmuC is even more refractory to proteolysis in the presence of high (i.e. SOS-induced) levels of UmuD; UmuC in a UmuD2C complex has a tₜ = 100 min (Battista et al., 1990; Donnelly & Walker, 1992).

A second reason that SOS induction is required for chemical and uv

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¹The tₜ = 100 is in the presence of GroEL and GroES proteins; without these chaperone proteins, a UmuD₂C complex has a tₜ = 50-60 min. E. coli groE mutants have greatly suppressed umuDC-dependent mutability. This decrease may simply be due to the shorter half life of UmuC in groE mutants. Alternately, GroES and GroEL may play a more direct role in UmuDC mediated mutagenesis (Donnelly & Walker, 1989; 1992).
induced mutagenesis in E. coli is that the DNA damage-activated form of the RecA protein is required for this process. Although RecA is present in high levels in non-SOS induced cells (Sommer et al., 1993), the proteolytically cleaved, activated form of this protein, RecA*, plays 3 essential roles in UmuDC mediated mutagenesis. Initially RecA* promotes cleavage of the lexA protein, thereby derepressing the expression of the umuDC operon (Bagg et al., 1981; Nohmi et al., 1988) and allowing the production of large amounts of the UmuD and UmuC proteins (Woodgate & Ennis, 1991). In the presence of excess UmuD, UmuD and UmuC associate into a UmuD2C complex. This complex is inactive until UmuD is posttranslationally modified by proteolytic cleavage between Cys-24 and Gly-25 by RecA* (Burckhardt et al., 1988; Nohmi et al., 1988). UmuD is cut in its monomeric form; its C-terminal fragment, designated as UmuD', is able to displace UmuD in the UmuD2C complex in an equilibrium reaction: UmuD2C \rightleftharpoons UmuD'DC \rightleftharpoons Umu D'2C. The UmuD'2C complex is the active species in chemical and uv induced mutagenesis (Woodgate et al., 1989; Bailone et al., 1991a; Donnelly & Walker, 1992). A third essential role of RecA* in SOS induced mutagenesis involves direct intervention in UmuD'2C mediated bypass of a bulky DNA lesion. The exact nature of the direct intervention of RecA* in effecting mutagenic translesion synthesis is undetermined. Possibilities include RecA* binding to ss DNA near a polymerase blocked by a DNA lesion, thereby stabilizing the replication fork until UmuD'2C can locate and rescue the stalled polymerase. Alternately, RecA* may play an active role in directing the UmuD'2C complex to the site of DNA lesion (Sweasy et al., 1990; Rajagopalan et al., 1992; Frank et al., 1993).

The protein complex comprised of RecA*, UmuD'2C, and PolIII, which is responsible for SOS induced mutagenesis in E. coli, has been termed a "mutasome" (Rajagopalan et al., 1992). The exact mechanism of mutasome mediated mutagenesis remains unclear. Several mechanisms, however, have
been proposed to explain low-fidelity replication past bulky DNA lesions, such as cis-DDP adducts. These include (1) inhibition of the 3'-5' proofreading function of DNA polymerase III, (2) stimulation of lesion bypass once a nucleotide has been inserted opposite a site of damage, and (3) facilitated reinitiation of the polymerase after it has dissociated from the site of blockage.

c. The role of UvrA and UvrB

In most cases, induction of the umuDC operon is sufficient for uv and chemical induced mutagenesis in E. coli (Sommer et al., 1993). There is some evidence, however, that the uvrA and uvrB gene products also play a role in cis-DDP mutagenesis. In general, chemical and physical agents producing lesions that are substrates for excision repair show high lethality in excision repair defective cells. The same repair defective cells are almost invariably much more susceptible to
the mutagenic effects of the treatment, as with uv mutagenesis (Glickman, 1983). cis-DDP appears to be an exception to this rule. Several studies have shown that mutation induction by cis-DDP in cells carrying a mutant uvrA allele is greatly decreased (Konishi et al., 1981; Brouwer et al., 1988), even in the presence of the plasmid pKM101 (Venturini & Monti-Bragadin, 1978); it is noteworthy, however, that there has been one report to the contrary (Cunningham et al., 1981). Cells deleted for uvrB are immutable by cis-DDP in an assay using the E. coli lacI gene as the target (Brouwer et al., 1981). The same experiment done in the presence of pKM101 gives the same result (Brouwer et al., 1983). There exists a uvrB5 mutant of E. coli that is defective for excision repair and is sensitive to the killing effects of cis-DDP. Unlike the uvr mutants described above, this mutant can support cis-DDP induced mutagenesis (Brouwer et al., 1988). It possesses a -1 frameshift within the uvrB5 gene and produces a truncated protein.
consisting of the 113 N-terminal amino acids of the wild type UvrB protein and a 43 amino acid tail encoded downstream from the -1 frameshift. Deletion studies of a plasmid carrying the uvrB5 gene demonstrate that mutation induction mediated by UvrB5 is due to the 113 amino acids present in the wild type gene. Deletion of the uvrC gene does not compromise the mutagenicity of cis-DDP (Brouwer et al., 1988). These results suggest that the uvrA and uvrB gene products are performing an active role in cis-DDP induced mutagenesis. This role differs from that of UmuDC, since UmuDC activity is also required for mutagenesis.

It is not unprecedented that uvrABC gene products are required for mutation induction. Interstrand crosslinking reagents such as mitomycin C and psoralen are not mutagenic in uvr backgrounds. In these cases it is the normal excision repair activity that is apparently responsible for converting the lethal crosslinks formed by these agents to mutagenic monoadducts (Murray, 1979). A similar mechanism, however, is unlikely to apply to cis-DDP because the frequency of cis-DDP interstrand crosslinks is very low (Pinto & Lippard, 1985a), and the mutability of uvrC strains indicates that excision repair itself is not involved.

A possibly related phenomenon occurs with arylamine compounds. AAF-DNA adducts can block DNA synthesis, induce SOS functions, and are lethal in uvrA, uvrB, and uvrC strains. Aminofluorene (AF) DNA adducts, by contrast, are not blocks to replication, do not induce SOS, and only uvrC strains are sensitive to the killing effects of the compound. It

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'Early reports indicated that interstrand crosslinks comprise ≤ 1% of all cis-DDP DNA adducts. More recent results suggest that a much higher proportion of all adducts, up to 5-10%, are interstrand crosslinks (Brabec & Leng, 1993; Decoville et al., 1993). These adducts, nevertheless, can be considered infrequent compared to the G*G* (~65%) and A*G* (~25%) intrastrand crosslinks, both of which have been demonstrated to be mutagenic.
has been postulated that in the absence of UvrC, UvrA and UvrB can bind to AF-DNA adducts producing a non-bypassable lethal complex (van Houten, 1990). It is possible that the binding of UvrA and UvrB to sites of adduction by cis-DDP plays a role in platinum induced mutagenesis.

van Houten (1990) has compiled genetic evidence suggesting that UvrA, UvrB, and UvrD perform some function that is essential for viability in the absence of DNA polymerase I. Double mutants of polA, which encodes DNA polymerase I, and any of these genes are lethal, but a polA uvrC double mutant is not, indicating that this putative role is different from excision repair activity.

In summary, cis-DDP is clearly an SOS dependent base-pair substitution mutagen in E. coli. As will be shown in this dissertation, sites of cis-DDP-induced mutations can be correlated with sites of platinum modification, although not all sites of preferred chemical reaction are those at which mutations arise most frequently.

4. umuDC-like Mutagenesis in Other Species

Mutagenic responses involving umu-like genes and proteins have been detected in many other bacterial species, making it clear that umu-like genes are widely distributed (Woodgate & Sedgwick, 1992; Ho et al., 1993). An SOS-regulated umuDC operon has been characterized in Salmonella typhimurium; the proteins encoded by this operon (umuDCm) are highly homologous, both structurally and functionally, to their

1*S. typhimurium commonly also have an additional umuDC-like operon, samAB, encoded on the 60-MDa cryptic plasmid, pSLT. Interestingly, despite the homology of SamA and SamB to UmuD and UmuC the former proteins are not able to restore mutability to umuDC cells (Nohmi et al., 1992).
counterparts in *E. coli* (Smith et al., 1990; Thomas et al., 1990). UmuDC homologues are also encoded by a number of plasmids. The most well known example is pKM101 (Monti-Bragadin et al., 1975; Beck and Fisch, 1980). This plasmid encodes the MucAB proteins, which are naturally occurring plasmid counterparts of the UmuDC proteins of *E. coli* which play essential roles in SOS mutagenesis. The MucAB proteins, when expressed in *S. typhimurium*, form the basis of the reversion assays developed by Ames and coworkers (Ames et al., 1973; McCann et al., 1975) and have been used to assess the mutagenicity of a wide range of suspected environmental mutagens and other compounds, such as cis-DDP and other platinum drugs (Beck & Fisch, 1980; Leopold et al., 1981).

The MucAB proteins are more efficient at inducing mutagenesis than the UmuDC proteins. UV and chemical mutagenesis is increased in both *E. coli* and *S. typhimurium* upon expression of the *mucAB* genes (Blanco et al., 1986). Additional plasmids have been identified that carry *umuDC*-like genes that are capable of restoring induced mutability in *E. coli umuDC* mutants. The plasmid TP110, to give one example, encodes a 16 kD protein with significant homology to UmuD and MucA as well as a 46 kD protein homologous to UmuC and MucB (Lodwick et al., 1990). *umuDC*-like genes are not limited to prokaryotes. Proteins necessary for the mutagenic processing of DNA lesions induced by uv light and chemicals

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1The *umuDC* gene products, for example, have 71% amino acid homology to the *E. coli* UmuD and UmuC proteins. The primary function of UmuDC-like proteins has been proposed to be the mediated bypass of replication blocking lesions in DNA and associated mutagenicity is a non-essential secondary effect. Different homologues have greatly varying abilities to impart induced mutabilities to their cellular hosts. For example, the *umuDC* gene products are weakly mutable, and the *samAB* gene products are immutable compared to their *umuDC* and *mucAB* counterparts (Koch et al., 1992; Nohmi et al., 1992). It remains unclear why some of these proteins should impart mutability to their host cells. Inducible mutagenesis has been described as a form of inducible evolution for cells in stressful environments. The benefits of such a system, however, are minimal and therefore are present (*umuDC* and *mucAB*), weakly present (*umuDC*), or not present (*samAB*) as a secondary effect of a system designed to provide the primary benefit of bypass of replication blocking DNA lesions (Woodgate & Sedgwick, 1992).
are being identified in eukaryotes. For example, the REV1 mutant of
Saccharomyces cerevisiae is non-mutable when exposed to DNA-damaging
agents that are known SOS-dependent mutagens in E. coli and are also
mutagenic in wild-type S. cerevisiae (Larimer et al., 1989). It is
anticipated that any species which is mutated by agents such as uv light
will have a cellular response to DNA damage that involves proteins that
have umuDC-like functions (Woodgate & Sedgewick, 1992). Evidence for
such proteins is beginning to emerge for the mutagenic processing of uv
irradiated DNA in humans (Thomas & Kunkel, 1993).

5. Mutagenicity and Genotoxicity of cis-DDP in Eukaryotic Cells

Cis-DDP is mutagenic in mammalian cells (Wiencke et al., 1979;
Johnson, N. P. et al., 1980), and its mutation spectrum has been studied
in at least three eukaryotic systems. One analysis was carried out on
the SUP4-o gene of Saccharomyces cerevisiae (Mis & Kunz, 1990). Single
base-pair substitutions predominate (72% of the total mutations), and
86% of those occur at GC base pairs. Dipurine sequences are also
involved in 86% of the induced base pair substitutions, with the
majority of the substitutions occurring at d(GpG) and d(GpA) sequences.
Comparable results were obtained for cis-DDP induced mutations in exon 3
of the human hypoxanthine guanine phosphoribosyltransferase gene in B-
lymphoblasts. The majority of mutations was attributable to G → T and
A → T transversions at the 5’ base of d(GpG) and d(ApG) sequences,
respectively (Cariello et al., 1992). Finally, cis-DDP and carboplatin
induced mutations in the adeninephosphoribosyltransferase (aprt) gene of
CHO cells have also been investigated (de Boer & Glickman, 1989; 1992).
The mutations induced are very similar for both drugs. Substitutions at
G-C base pairs also predominate, with the majority occurring at d(ApGpG)
and d(GpApG) sequences. The role of A*G* adducts in mutagenesis in these
studies is ambiguous partly because in the former study there are
relatively few d(ApG) sequences in which a mutation is detectable and, in the latter study, mutations at d(ApGpG) sequences cannot be ascribed with certainty to the presence of either an A*G* or G*G* adduct.

Taken together, the results of studies in mammalian cells and in human cancer patients thus far indicate that both cis- and trans-DDP DNA adducts are substrates for repair, and that the trans isomer may be repaired to a greater extent than the cis compound. Excision repair is involved in cis-DDP adduct removal and may be the activity responsible for the initial rapid phase of adduct removal following treatment. There are also indications that a second avenue of repair may exist. In all cases, the G*G* adduct is the most abundantly formed, and its prevalence is maintained during and after repair. Results suggest that in both mammalian and bacterial cells, the G*G* and A*G* adducts of cis-DDP play important roles in the genotoxic and mutagenic activity of this anticancer drug.
C. Platinum (IV) Anticancer Drugs

1. The Need for New Platinum Drugs

*cis*-DDP is a potent genotoxin and a highly effective anticancer drug. It is a key component of drug therapies for the treatment of testicular, ovarian, bladder, head and neck, and lung cancers. Cisplatin based regimens also show limited activity against malignant lymphomas and esophageal and stomach cancers (Greene, 1992). Although one of the most successful cancer chemotherapeutic agents yet developed, *cis*-DDP has several significant liabilities the foremost of which is acute toxicity to the host at clinically used doses of drug. The short term effects include nausea and vomiting (Krakoff, 1979; von Hoff et al., 1979), whereas longer term cumulative exposure can cause severe nephrotoxicity and neuropathy. Many patients also experience gastrointestinal toxicity, ototoxicity, hypomagnesemia, cardiotoxicity, myelosuppression, anaphylactic reactions, cerebral herniation, or vascular toxicity (von Hoff et al., 1979; Walker et al., 1988; Hamers et al., 1991; Icli et al., 1993). The clinical efficacy of *cis*-DDP is further diminished by both intrinsic and acquired resistance in tumor cells. An additional drawback is that *cis*-DDP requires intravenous administration, necessitating costly and time consuming hospital or clinical visits.

There has been considerable success in reducing the most severe toxicities associated with *cis*-DDP chemotherapy. Nephrotoxicity can be suppressed by administration of nucleophilic sulfur compounds (Basinger et al., 1992). Neuropathy and other toxicities have been reduced by the development of *cis*-diammine-1,1-cyclobutanedicarboxylate (carboplatin, Figure 1), a less toxic second generation platinum drug. Unfortunately carboplatin, also a platinum(II) compound, is not effective against *cis*-
DDP resistant tumors and also requires intravenous administration. These disadvantages, inherent with current platinum chemotherapy, have led to an intense search for third generation platinum drugs.

The appearance of second malignancies in patients treated with cis-DDP is another important concern associated with all platinum based therapeutic regimens. cis-DDP is a mutagen in mammalian cells (Wienke et al., 1979; Johnson et al., 1980) and, upon SOS-induction, also in bacteria (Beck & Brubaker, 1975; Benedict et al., 1977). The strong mechanistic link between mutagenesis and carcinogenesis implies that cis-DDP is a carcinogen in humans as it is in rodents (Leopold et al., 1981). This relationship, however, has not been established conclusively for cis-DDP because the drug is typically administered in conjunction with a battery of other anticancer drugs, some of which are suspected human carcinogens (Greene, 1992). Although unproven, the strong possibility that cis-DDP is carcinogenic suggests the desirability for evaluating the mutagenicity, and implied carcinogenicity, of all new platinum drugs. One of the major aspects of this thesis, therefore, was a comparison of the mutagenicity and mutational spectra of ACDP and cis-DDP by using the lacZ' β-galactosidase α-complementation forward mutational assay in E. coli.

2. Platinum(IV) Ammine/amine Dicarboxylates

A class of platinum(IV) complexes, ammine/amine platinum(IV) dicarboxylates, has recently been introduced in an attempt to overcome some of the drawbacks of cis-DDP and other related platinum(II) analogues (Figure 1) (Kelland et al., 1992b). Because platinum(IV) compounds are orally absorbed, they may afford an equally effective treatment regimen with fewer toxic side effects (McKeage et al., 1993) and greater ease of administration. Moreover, these compounds have been
found to be active against cis-DDP resistant cell lines. To give two examples, platinum(IV) dicarboxylates exhibit 25 fold enhanced cytotoxicity against resistant human cervical squamous cell carcinoma cell lines (Mellish et al., 1993) and up to 2 orders of magnitude of enhanced cytotoxicity against cis-DDP resistant human ovarian carcinoma cell lines (Kelland et al., 1992b). The latter finding alone could have profound clinical implications for the 18,000 patients diagnosed with ovarian cancer each year in the United States, two thirds of whom are projected to ultimately die of their disease (Silverburg & Lubera, 1989). Of even broader scope, platinum(IV) drugs also are effective against lung cancer cell lines possessing acquired resistance to chemotherapeutic agents including cis-DDP (Twentyman et al., 1992). One ammine/amine platinum(IV) dicarboxylate, cis,trans,cis-ammine(cyclohexylamine)dibutyratodichloroplatinum(IV) (ACDDP, Figure 1) has recently been introduced in clinical trials. A similar orally active platinum(IV) compound, cis,trans,cis-ammine(cyclohexylamine)diacetatodichloroplatinum(IV), also shows activity against resistant tumor cell lines in vitro and resistant tumors in mice and is poised to enter clinical trials (Kelland et al., 1993). Considering that a major driving force leading to the development of platinum(IV) drugs was overcoming cellular resistance, it behooves us to take a detailed look at the mechanisms responsible for resistance to platinum (II) drugs.

3. cis-DDP Resistance in Tumor Cells

Resistance to platinum(II) compounds is multifactoral, involving one or more of an array of possible mechanisms including intracellular detoxification by glutathione (Godwin et al., 1992) or metallothionein.
(Pattanaik et al., 1992), expression of oncogenes\(^1\) (Isonishi et al., 1991), or increased repair of DNA platinum adducts (Eastman & Schulte, 1988; Kelland et al., 1992a; Zhen et al., 1992). The enhanced efficacy of ACDDP, the parent compound of ACDP, could be derived from its ability to circumvent one or more of these modes of resistance. For example, the effectiveness of ACDDP could arise from the ability of some its DNA-reactive platinum(II) metabolites, other than ACDP, to form novel DNA adducts not possible with cis-DDP or ACDP. These lesions, unlike cis-DDP or ACDP adducts, could be refractory to repair, persist in the cell's genomic DNA, and thereby mediate ACDDP's enhanced cytotoxicity. This method of overcoming resistance probably does not apply to ACDDP, however, as a human carcinoma cell line, CH1cisR, has been described that retains resistance even to platinum(IV) ammine/amine dicarboxylates, apparently as a consequence of facile DNA repair (Kelland et al., 1992a).

Response to cis-DDP treatment in cancer patients as well as sensitivity of tumor cells grown in culture to platinum compounds are both correlated to high levels of platinum adducts on the genomic DNA of the host (Reed et al., 1990; 1993; Gorodetsky et al., 1993). In contrast, DNAs from patients ineffectively treated by platinum therapy and from resistant cell lines both have low levels of platinum adducts. The diminished levels platinum DNA adducts could be a consequence of (i) cytoplasmic sequestering of platinum compounds preventing them from interacting with the chromosome or (ii) facile or enhanced repair of platinum DNA adducts.

\(^1\)The expression of many oncogenes, however, does not confer cis-DDP resistance (Perez et al., 1993). Nevertheless the link between cellular growth-regulating protein and cis-DDP sensitivity remains intriguing considering that testis cancer (against which cis-DDP is > 95% effective) is one of the few types of cancer that is not characterized by occurrence of mutations in the p53 protein (Peng et al., 1993).
a. Intracellular sequestration and conjugation of cis-DDP

A number of mechanisms has been proposed for the reaction of intracellular components with cis-DDP that render the drug inactive to DNA binding. For example, ribonucleotide triphosphates, particularly ATP and GTP, can form complexes with cis-DDP and shield the drug, preventing it from binding to cellular constituents, including its therapeutic target, DNA. It is suggested that differences in ribonucleotide levels from cell to cell (e.g. rapidly dividing tumor cell have high levels) may partially explain cis-DDP resistance (Seki et al., 1993). Considerably more evidence, however, implicates overexpression of metallothionein and glutathione as causitive factors in cis-DDP resistance as discussed below.

Metallothionein (MT) is a low molecular weight (6-7 kDa), cysteine-rich protein that shows a high affinity for metals including zinc, copper, cadmium, mercury, and platinum. cis-DDP binds to MT with loss of its ammine ligands, suggesting that it is bound tetracoordinately. Each MT protein binds ~10 platinum molecules; overall, a significant fraction of intracellular platinum can be bound to MT (Pattanaik et al., 1992). Overexpression of MT in previously sensitive Chinese hamster ovary cells renders them resistant to cis-DDP (Koropatnik & Pearson, 1993). Furthermore, bladder tumor cells have been described that derive cis-DDP resistance from increased synthesis of MT (Satoh et al., 1993). An increase in MT synthesis in tumor cells may be linked to oncogene expression. To give an example, the increased expression of MT in mouse NIH 3T3 cells is correlated with the expression of the c-Ha-ras oncogene (Ischishi et al., 1991).

Glutathione is a tripeptide containing a nucleophilic sulfur derived from a cysteine residue. Glutathione plays a significant role
in resistance to a variety of drugs and in the detoxification of electrophilic compounds, therefore it is not surprising that it also has been implicated in cis-DDP resistance. The cytotoxicity of cis-DDP is enhanced in glutathione deficient cells (Ishikawa & Ali-Osman, 1993) and decreased in cells that overexpress glutathione. Human ovarian carcinoma cell lines that overexpress glutathione by 13-50 fold are 30-1000 fold more resistant to killing by cis-DDP (Godwin et al., 1992). Up to 60% of the total intracellular platinum in cis-DDP treated resistant L1210 murine leukemia cells has been shown to be bound to glutathione in a 2:1 glutathione:drug stoichiometric ratio. The glutathione-cis-DDP conjugate can be effluxed from a cell in ATP-dependent transport across the plasma membrane (Ishikawa & Ali-Osman, 1993). The resulting reduction in the intracellular accumulation of drug is one mechanism that cells employ to effect cis-DDP resistance (Timmer-Bosscha et al., 1992).

b. cis-DDP Resistance mediated by enhanced DNA repair

Sequestering of cis-DDP by metallothionein or its conjugation with glutathione and subsequent efflux from the cell are hypothesized to form a "front line" defense to prevent DNA damage. Enhanced DNA repair may be a compensatory mechanism for cells that cannot utilize these mechanisms to avoid formation of cis-DDP lesions in their genomes (Dabholkar et al., 1992). Indeed, many cis-DDP resistant cells, including L1210 murine leukemia cells (Eastman & Shulte, 1988), human ovarian carcinoma cells (Parker et al., 1991), human cervical carcinoma cells (Nishikawa et al., 1992) and Xeroderma pigmentosum and Fanconi’s

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1Eastman and Shulte (1988) recognized that resistance in L1210 murine leukemia cells was at least bifunctional as not all of the observed resistance could be explained by increased DNA repair. These cells were later found to also have enhanced levels of glutathione that provided an additional level of drug resistance (Ishikawa & Ali-Osman, 1993), as mentioned above.
Amenia cells (Zhen et al., 1993) have significantly greater rates of cis-DDP DNA adduct repair than their drug sensitive counterparts.

Repair of cis-DDP DNA adducts has not yet been well characterized in eukaryotic cells. Early indications, however, are that this repair is inducible. Burkle et al. (1993) report that cis-DDP induces poly(ADP-ribosyl)ation and subsequent activation of nuclear proteins implicated in DNA repair. Furthermore, repair of cis-DDP lesions is gene and strand specific (Jones et al., 1991; Zhen et al., 1992; Stevnsner et al., 1993).

4. Circumvention of cis-DDP Resistance by Platinum(IV) Drugs

The mechanism by which platinum(IV) drugs are able to circumvent cis-DDP resistance does not appear to involve alteration of glutathione or metallothionein synthesis or decreased DNA repair (Kelland et al., 1992a; Mistry et al., 1992; Twentyman et al., 1992). Instead, platinum(IV) drugs overcome cellular resistance to platinum(II) compounds by a mechanism that is just now becoming apparent as the mode of cis-DDP transport into cells is elucidated. Generally it has been supposed that cis-DDP enters cells by passive diffusion and initially achieves similar levels in all cells. Differences in drug sensitivity of various cells were attributed to the aforementioned cytoplasmic sequestration of cis-DDP or facile repair of DNA lesions. The low levels of drug observed in some resistant cell lines was regarded as a consequence of increased efflux of the drug. Evidence for the model in which cis-DDP enters cells by passive diffusion, which would allow similar levels of drug initially to accumulate in both cis-DDP resistant

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1Clearance of cis-DDP from some resistant cells can be mediated by efflux of cis-DDP-glutathione conjugate across the plasma membrane by active transport, as previously discussed.
and sensitive cells, was compelling. This evidence included the observations that accumulation is not saturable, is proportional to extracellular drug concentration, and is not inhibited by structural analogues of cis-DDP (reviewed by Gately & Howell, 1993).

In conflict with the passive diffusion model, however, was the observation that cis-DDP, a relatively hydrophilic compound, is effectively blocked by lipid membranes (Melvik et al., 1986), suggesting that carrier mediated or active transport must play a role in the cellular uptake of platinum(II) drugs (Andrews & Howell, 1990). Recently, considerable evidence has been amassed to support protein-mediated trans-membrane transport of cis-DDP (Gately & Howell, 1993; Morikage et al., 1993). The exact nature of the cis-DDP transporter has not yet been described but it appears to involve membrane-associated tubulin (Christen et al., 1993). The fact that cis-DDP transport is protein mediated implies that cells may effect resistance by down-regulating the uptake of drug.¹

Platinum(IV) compounds such as ACDDP are at least 5 orders of magnitude more lipophilic than cis-DDP (Kelland et al., 1992b), a characteristic that allows their passive diffusion through lipid plasma membranes thereby facilitating entry into cells that fail to accumulate cis-DDP. To give an example, when 41McisR human ovarian carcinoma cells are treated with platinum(IV) compounds, the intracellular concentration

¹A speculative mechanism has been proposed to explain how the uptake of cis-DDP may be down regulated. Briefly, cis-DDP, being an eukaryotic mutagen, could mutate the carrier protein to an inactive form thereby abolishing transport of the drug into the cell (Gately & Howell, 1993). Evidence consistent with this model is the observation that a gated channel protein is overexpressed on the membrane of cis-DDP resistant murine lymphoma cell lines. This overexpression is postulated to be an attempt to compensate for the functional loss of the channel (if it had indeed been mutationally inactivated) which, in addition to cis-DDP transport also plays other cellular roles (Kawai et al., 1990; Gately & Howell, 1993).
of drug reaches 100-fold higher levels than when the cells are dosed with either cis-DDP or ACDP (Kelland et al., 1992a). As a consequence of the higher intracellular drug concentration resulting from ACDDP treatment, more DNA adducts form than when the cells are treated with either platinum(II) compound. Assuming that the similarities between cis-DDP and ACDP observed in the bacterial systems employed in this thesis are predictive of their genetic effects in an eukaryotic system, the platinum(II) metabolic derivatives of ACDDP, including ACDP, form DNA adducts in tumor cells that have the same biological consequences as cis-DDP DNA adducts. Therefore, it is plausible that the increased number of DNA adducts that forms when cells are treated with the platinum(IV) dicarboxylates (Kelland et al., 1992a), and not the chemical properties of these lesions, that is responsible for the greater sensitivity of tumor cells to ACDDP.

5. ACDP, a Cellular Metabolite of ACDDP, Forms DNA Adducts

a. DNA binding properties of platinum(IV) drugs

Once inside a cell, platinum(II) compounds such as cis-DDP lose their chloride ions through hydrolysis covalently bind to DNA (Lippard, 1978) forming a variety of stable adducts (Eastman, 1983; Fichtinger-Scheppman et al., 1985). cis-DDP adducts inhibit DNA replication in vitro (Pinto & Lippard, 1985b; Comess et al., 1992), and in vivo both in bacterial (Alazard et al., 1982) and mammalian (Ciccarelli et al., 1985; Heiger-Bernays et al., 1990) cells. This inhibition of DNA replication results in cytotoxicity that is generally accepted to contribute to the antitumor effects of cis-DDP. Platinum(IV) compounds, on the other hand, are much more inert to ligand substitution reactions (Hartley, 1973) and are expected to form DNA adducts slowly, if at all (Kelland et al., 1992b). Platinum(IV)
dicarboxylates, such as ACDDP, therefore, most likely function as prodrugs that are reduced intracellularly, possibly by cellular components such as protein sulfhydryls, glutathione or ascorbic acid (Eastman, 1987; Gibbons et al., 1989), to form reactive platinum(II) species (Blatter et al., 1984; van der Veer et al., 1986b). At least six platinum(II) metabolites of ACDDP are produced in this manner (Kelland et al., 1992b). The major metabolite in humans is cis-ammine(cyclo-hexylamine)dichloroplatinum(II) (ACDP).¹

ACDP binds to DNA in vitro to form a spectrum of adducts similar to those formed by cis-DDP but with the notable exception that three fold fewer adducts form at d(ApG) sites and about double the number at d(GpNpG) sites (Hartwig & Lippard, 1992). Because these adducts block DNA replication in vitro (Hartwig & Lippard, 1992), it seems likely that ACDDP ultimately manifests its cytotoxic (and potentially chemotherapeutic) effects by the same mechanisms employed by cis-DDP. The toxicities of the drugs are examined in this thesis in vivo by assessing the genotoxic effects of both cis-DDP and ACDP DNA adducts in bacteriophage M13mp18 RF genomes in E. coli.

The genetic effects of DNA adducts formed by cis-DDP and ACDP are influenced by two factors. First, the molecular processing of DNA modified by both compounds is probably determined by structural distortions of the duplex upon binding a square planar platinum(II) compound (Bellon et al., 1991). Such compounds usually bind DNA at similar nucleotide sequences, forming mainly 1,2-intrastrand crosslinks, which lead to similar bending and unwinding of the duplex helix (Lippard et al., 1983; Page et al., 1990; Hartwig & Lippard, 1992). Therefore, ACDP also is likely to be the major intracellular metabolite of cis,trans,cis-ammine(cyclohexylamine)diacetatodichloro-platinum(IV), another platinum(IV) compound with clinical aspirations (Kelland et al., 1993).
structural deformation of the DNA duplex is a major factor contributing to the genotoxicity and mutagenicity of platinum DNA adducts, the genetic effects of cis-DDP and ACDP would be expected to be very similar. A second factor that could influence the biological effects of platinum(II) compounds is the nature of the ligands attached to platinum. In particular, the cyclohexyl ring of ACDP, a moiety absent in cis-DDP, was predicted to have a significant impact on the molecular and biological effects of this compound, as discussed below.

b. Mutagenic implications of the orientational isomerization exhibited by ACDP DNA adducts

Unlike cis-DDP which is symmetrical, ACDP is asymmetrical owing to its cyclohexyl ring. The asymmetry of the ACDP complex gives rise to an interesting orientational isomerism when the complex forms 1,2-intrastrand crosslinks with DNA (Hartwig & Lippard, 1992). As illustrated in Figure 3 (a), with d(GpG) adducts the cyclohexylamine ligand can point either toward the 5' or the 3' guanosine residue; both orientational isomers form upon binding of the complex to DNA. It is noteworthy that one of the isomers (II) potentially forms a favorable hydrogen bond between the cyclohexylamine moiety of the drug and the O6 of guanine. With the d(ApG) adduct, by contrast, the analogous isomer (IV, Figure 3 (b)) probably does not form, apparently because the cyclohexylamine moiety sterically clashes with the exocyclic amino group of the 5' adenine (Hartwig & Lippard, 1992). Thus the ACDP drug forms fewer adducts at d(ApG) sites than cis-DDP. While cis-DDP forms an adduct spectrum comprised of 65% G*G*, 25% A*G*, and 10% G*NG* adducts the ACDP spectrum has approximately 58% G*G*, 8% A*G*, and 18% G*NG* adducts, respectively. Because the ACDP spectrum has threefold fewer highly mutagenic A*G* platinum adducts (this work, Burnouf et al., 1990; Bradley et al., 1993), it is predicted that the overall mutagenicity of
this compound would be lower than that of cis-DDP.
Figure 3. Orientational isomerism of DNA adducts formed by ACDP at (a) d(GpG) and (b) d(ApG) sequences. ACDP can bind to d(GpG) sequences with the cyclohexylamine moiety oriented in either the 3' (I) or 5' (II) direction. In contrast, the 3' orientational isomer (III) preferentially forms at d(ApG) sequences as the 5' isomer (IV) forms an unfavorable steric interaction between the cyclohexylamine moiety of ACDP and the exocyclic amino group of the 5' adenine residue (adapted from Hartwig & Lippard, 1992).
(a) d(GpG) ACDP adducts:

I

II

Favorable H-bonding interaction

(b) d(ApG) ACDP adducts:

III

IV

Unfavorable steric interaction
D. Background of Experimental Systems Used in this Work

1. Site Specifically Situated versus Randomly Distributed DNA Adducts

Experiments with randomly modified DNA can provide insights into the nature of the lesions most likely to play important roles in genotoxicity and mutagenicity. *In vivo* survival assays with randomly modified vectors allow one to determine the total number of adducts required for a lethal event, and *in vitro* polymerase blockage experiments can correlate sites of termination with putative sites of modification. Therefore studies with randomly modified DNA, such as those performed for cis-DDP and ACDP in this thesis, can implicate lesions likely to be involved in mutagenesis. Such assays, nevertheless, cannot definitively ascribe a specific genetic event to a specific DNA adduct. Chemical synthetic and molecular biologic techniques have made it possible to construct biologically active singly modified genomes (Fowler et al., 1982; Green et al., 1984; Loechler et al., 1984; reviewed in Basu & Essigmann, 1988). These tools can be used to address the questions of what effect a specific adduct has on DNA replication and mutagenesis, and what role DNA repair plays in modulating those effects.

As mentioned, the multiplicity of DNA adducts formed upon treatment of cells with DNA damaging agents complicates the identification of lesions responsible for the genotoxic and mutagenic effects of the drug. For cis-DDP, ACDP, or other drugs that form multiple lesions when binding to DNA, it is highly desirable to assess the contribution of each DNA adduct to overall biological activity. As mentioned previously, if the genotoxic and mutagenic activity can be defined for each adduct, it may be possible to use the information to guide the development of safer and more effective drugs. A major focus of this
ythesis was to perform a site specific comparison of the mutagenicity and genotoxicity of the three major DNA adducts formed by cis-DDP.

a. Site specific modification of oligonucleotides

There are several methods for producing a site specifically modified genome, and each requires the synthesis of an oligodeoxynucleotide which contains a chemically modified nucleotide. The three most common oligodeoxynucleotide synthetic strategies are: (1) total synthesis, which involves the production of a modified protected monomer that is used in the solution or solid phase synthesis of the oligodeoxynucleotide, (2) chemical or physical modification of a preformed oligodeoxynucleotide, and (3) enzymatic synthesis, in which DNA or RNA polymerases are used to incorporate modified nucleotides into the oligodeoxynucleotide. The total synthetic approach is often preferred because it enables both the facile production of sufficient amounts of material for full structural characterization, and it allows for the placement of the adduct in any desired sequence context. The choice of a method, however, depends on the chemical nature and stability of the adduct or lesion to be studied. For example, some adducts are unstable under the conditions required for total synthesis, making this approach unfeasible with current technology. Similarly, the treatment of a preformed unmodified oligodeoxynucleotide with a DNA damaging agent may yield products too numerous to allow this approach to be used effectively.

The modified oligodeoxynucleotides used for the work presented in this dissertation were prepared by the second method described above, i.e., by allowing an unmodified oligodeoxynucleotide to react with cis-DDP. The sequences chosen, and the rationale for their selection, will be presented in the next (Materials and Methods) section. The
oligodeoxynucleotides were situated in a viral genome context by using the methodology mentioned below and described in detail in the Materials and Methods section.

b. Methods for constructing singly modified genomes

Modified oligodeoxynucleotides can be incorporated into the genomes of viruses or plasmids in several ways. In one common method (Figure 4 (a)), the oligodeoxynucleotide is annealed to the single stranded genome of a viral vector. The 3’ terminus of the oligodeoxynucleotide then acts as a primer for complementary strand synthesis by a DNA polymerase. DNA ligase is used to seal the nick resulting from complete complementary strand synthesis. This method has been used to construct genomes containing site specifically situated thymine dimers (Huang et al., 1992), psoralen DNA adducts (Kodadek & Gamper, 1988), and the cis-DDP G*G* adduct (Szymkowski et al., 1992). A factor that limited the usefulness of this approach in the present work is the secondary structure present in M13 genomic DNA. M13 DNA assumes secondary structure of sufficient stability to prevent efficient replication of the M13 genome (Reckmann et al., 1985). The holoenzyme form of the T4 polymerase can be used to overcome secondary structure problems associated with M13 replication (Kodadek & Gamper, 1988; Szymkowski et al., 1992). Unfortunately the proteins comprising the T4 DNA polymerase holoenzyme are not easily available in their native forms, further limiting the usefulness of this method. An additional drawback of constructing singly modified genomes by using this approach is the ds nature of the construct produced. As discussed below, M13 ds genomes are susceptible to strand replication bias effects that prevent a single replication blocking adduct from being replicated during DNA synthesis. Ideally, M13-derived ss genomes are preferred substrates for site specific studies of replication blocking lesions.
In a second method used to construct singly modified genomes (Figure 4 (b)), a duplex genome is constructed in which one strand contains a gap opposite a sequence that is complementary to that of the modified oligodeoxynucleotide to be used. The modified oligodeoxynucleotide is annealed to the gapped duplex genome, and the nicks on either side of the oligodeoxynucleotide are covalently joined by DNA ligase. This method can be adapted to produce ss genomes by engineering a non-ligatable nick in the strand not containing the adduct. This strand can then be removed by heat denaturation. To give two examples, this technique has been used to study arylamine adducts (Reid et al., 1990) and 8-hydroxyguanine residues (Wood et al., 1990). In some cases, yet another method is preferred for the construction of a ss singly modified genome. This third technique, used in the present work, involves the digestion and removal of the hairpin region of the M13mp7L2 ss genome with EcoR I, followed by annealing and ligation of the modified oligodeoxynucleotide into a gap formed with the aid of a scaffold oligodeoxynucleotide, as outlined in Figure 4 (c).

2. M13 Bacteriophage

The vector used to study the mutagenesis induced by specific cis-DDP adducts is the M13 system developed by Messing (1983) and adapted for use in site specific genome construction by C. Lawrence and coworkers (Banerjee et al., 1988; LeClerc et al., 1991). In addition to the site specific genetic analysis of the cis-DDP G*G*, A*G*, and G*TG* adducts, the same M13 bacteriophage system, with minor variations, was used for a comparative assessment of the DNA binding spectra, genotoxicities, and mutagenicities of cis-DDP and ACDP. The following is a short description of the biology of the bacteriophage M13, and of the relevant modifications made in the phage genome to facilitate its
use as a cloning vector (Meyer & Geider, 1982; Messing, 1983; Zinder & Horiuchi, 1985) and vehicle for the lacZ’ mutational assay.

a. Life cycle and replication of M13

M13 is a ss F-specific filamentous bacteriophage that infects E. coli harboring an F factor plasmid. During infection, the rod shaped phage adsorbs to the F pilus of the host and the coat proteins are removed as the circular (+) strand of DNA enters the cell. The phage DNA is the template for synthesis of the complementary DNA (-) strand by the host cell replication apparatus, producing a double stranded replicative form (RF) molecule. The M13 RF serves as a template for replication, by a rolling circle mechanism (Figure 5), and for transcription of mRNA species encoding viral proteins.

The protein product of the M13 gene II is required for rolling circle replication. It cleaves the (+) strand at a specific site, and the 3’ terminus thereby produced then acts as the primer for replication by the host cell machinery. The 5’ end of the (+) strand is displaced as the apparatus moves around the (-) strand template. Once the replication apparatus has returned to the origin, termination occurs when the displaced (+) strand is cleaved and its 5’ and 3’ ends subsequently are ligated to form a genome-length ss circle. The double stranded RF is sealed and supercoiled, and can again serve as a substrate for gene II and the replication apparatus. Early in infection, the (+) strands formed are replicated to produce RF molecules. Later in the infection process (after approximately 15-20 min), the cellular levels of the product of viral gene V, a single stranded DNA binding protein, are high enough that all of the newly displaced (+) strands are sequestered by the protein, and are thus unable to enter the replication cycle. In this way a steady state level
of 100-200 molecules of RF are maintained in the cell. The gene V protein-coated (+) strands are translocated to the cell membrane where the viral coat proteins are imbedded, and the DNA is packaged and finally extruded from the cell. M13 does not lyse its host; the "plaques" seen when the phage are plated are actually areas of slowly growing infected cells.

Because the M13 bacteriophage is not packaged into preformed capsids, but rather is encapsidated as it emerges from the cell, DNA of almost any length can be packaged. The M13 genome also contains a small intergenic "polylinker cloning" region into which DNA can be inserted without adverse effects to phage viability. Messing (1983) took advantage of both of these features for the development of a series of M13 molecular cloning vectors.

b. Strand bias effects

The viability of bacteriophage M13 genomes in either the ds or ss forms leads to an interesting, but noisome, phenomenon known as strand bias. Strand bias effects arise from the ability of either strand of M13 to give rise to progeny independent of the other strand. A consequence of this mode of replication, is that a replication blocking lesion in either strand can render that strand inactive, and direct the formation of all progeny from the other, undamaged strand. A replication blocking lesion in a ds M13 genome, therefore, would not be replicatively bypassed and its genetic effects would not be exhibited. This liability can be avoided by constructing a ss genome containing the DNA lesion; with no undamaged strand present to serve as a template for replication, the adduct must be replicated for the M13 genome to be viable.
3. The M13 lacZ' Forward Mutational Assay

a. Forward mutational assays

Forward mutational assays in non-essential genes have the ability to detect a wide range of single-base substitutions, as well as other classes of mutagenic events such as insertions and deletions at a large number of sites. The high degree of sensitivity afforded by a forward mutational assay was essential in order to detect subtle differences that might exist in the mutational spectra of the two very similar compounds, cis-DDP and ACDP, studied in this work. The lacZ' forward mutational assay utilized in this study has been employed by others to examine the mutagenicity of a variety of DNA damaging agents including UV irradiation (LeClerc & Istock, 1982; LeClerc et al., 1984), singlet oxygen (Decuyper-Debergh et al., 1987), oxygen radicals (Reid & Loeb, 1992), aflatoxin (Sahasrabudhe et al., 1989), N-acetoxy-N-acetyl-2-aminofluorene (Gupta et al., 1991), ionizing radiation (Ayaki et al., 1986; Hoebee et al., 1989), 4-aminobiphenyl (Lasko et al., 1988), and apurinic/apyrimidinic (AP) sites (Kunkel, 1984). A survey of these studies revealed that the lacZ' forward mutational assay is a sensitive and broad target for the detection of mutations, with 141 single-base substitutions detected at 98 different sites along with additional additions and deletions.

b. Molecular basis of lacZ' mutational assay

The lacZ' forward mutational assay is based on a series of proteins that have β-galactosidase activity. Mutations that disrupt the β-galactosidase activity of these proteins are phenotypically identified colorimetrically in the M13 plaque forming assay. Wild-type β-galactosidase encoded by the lacZ gene of the E. coli lac operon is a
116,353 dalton, 1023 amino acid residue protein (Kalnins et al., 1983). 
β-galactosidase is enzymatically active as a tetramer. Certain strains of E. coli are chromosomally deleted of the lac operon but carry the F' lacIZ M15 episome that codes for production of the M15 protein. M15 is a naturally occurring mutant of β-galactosidase lacking amino acid residues 11-41. The M15 protein exists as dimers in solution but are unable to form tetramers, and despite have a strong substrate binding site, lacks enzymatic activity (Langely et al., 1975).

The M15 protein attains β-galactosidase activity in a process known as α-complementation. Complementation, by definition, is the restoration of biological activity by the non-covalent interaction of two (or more) different proteins. In this case, α-complementation involves two polypeptides, an α-acceptor and an α-donor as shown in Figure 6. When the M15 protein functions as the α-acceptor, the α-donor can be any of several peptide fragments containing the amino acids 11-41 that are deleted from M15; for example peptide fragments 3-92 or 3-41 of wild-type β-galactosidase (Welpy et al., 1981b). The β-donor utilized in the M13 lacZ' mutational assay is supplied by the lacZ' polypeptide encoded by the modified M13 bacteriophage genome created by Messing et al. (1983). The lac regulatory region and the first 146 amino acid residues of the lacZ gene, produced by Hind II restriction of the E. coli lac operon DNA, was inserted into the major intergenic region of the M13 genome. The peptide coding region has been further elongated by the addition of a polylinker cloning region in the "mp" series of M13. For example, M13mp18, the construct used in the comparison of the genetic effects of cis-DDP and ACDP DNA adducts in this dissertation, contains an additional 18 amino acid residues so that the entire lacZ' peptide fragment is 164 residues in length.

The M13 lacZ' polypeptide fragment, comprised of 164 amino acid
residues, is considerably longer than required for the α-complementation process. As shown in Figure 7, the lacZ' polypeptide can be divided into three regions, the N-terminal overlap, the M15 host deletion, and the C-terminal overlap. It is well established that the overlapping regions are not essential for α-complementation because the polypeptide encoded by these DNA sequences is duplicated in the M15 α-acceptor protein. The polypeptide chain required to attain β-galactosidase activity, therefore, can be supplied by either the M15 α-acceptor protein or the M13 lacZ' α-donor polypeptide (Zabin, 1982; Welpy et al., 1981). The significance of these regions of the M13 lacZ' DNA sequence to the mutational assay is that the N- and C-terminal overlapping regions (Figure 7) are not expected to be as sensitive to the detection of mutations as the lacZ' DNA sequence coding for the amino acids deleted from the M15 α-acceptor protein. In addition, the lacZ' regulatory region is also expected to give rise to detectable mutations. Mutations in the ribosome binding site, for example, influence the production of mRNA, resulting in expression of activity ranging from 1 to 130% of wild-type, depending on the mutation (Guillerez, et al., 1991).
Figure 4. General methods used to construct site specifically modified M13 based bacteriophage genomes. (a) A modified oligodeoxynucleotide is annealed to a circular ss DNA template and acts as a primer for synthesis of a complementary strand by T4 DNA polymerase. T4 DNA ligase covalently seals the newly synthesized strand, resulting in a singly modified, covalently closed circular (Form I\textsubscript{c}) ds genome. (b) A modified oligodeoxynucleotide is annealed into a "gapped heteroduplex" genomic construct and its ends are covalently linked to the shorter of the two genomic strands by using T4 DNA ligase. (c) In a variation of the method outlined in (b), a modified oligodeoxynucleotide is annealed into a "gap" created by the annealing of a "scaffold" oligodeoxynucleotide into an EcoR I linearized M13mp7L2 ss genome. Once again, the modified oligodeoxynucleotide is covalently joined to the genomic DNA by using T4 DNA ligase. Details of all three methods are described in the references given in the text, in addition, the last method (c) was used to construct the singly modified cis-DDP G*G*, A*G*, and G*TG* containing genomes used in this dissertation and is described in detail in the Materials and Methods section.
a. T4 DNA replication complex → dNTP's → T4 DNA ligase

b. Anneal modified oligodeoxynucleotide → T4 DNA ligase

c. Anneal modified oligodeoxynucleotide → T4 DNA ligase
Figure 5. Outline of the life cycle of the M13 bacteriophage (from Bradley, 1991). The natural infection of an E. coli cell by the ss viral form of the M13 bacteriophage genome is shown. It should be noted that, upon introduction into a cell by artificial methods (such as CaCl$_2$ transformation or electroporation), both the viral (ss) and replicative (ds) forms of the M13 genome are viable. Both forms of DNA were used in this work; ss for the site specific analysis of the cis-DDP G*G*, A*G*, and G*TG* DNA adducts and ds for the comparison of the genetic effects of DNA adducts formed by cis-DDP and ACDP.
phage

host cell replication apparatus

gyrase

gene II

host cell replication apparatus

rolling circle

DNA Packaging

gene V

late

early
Figure 6. Molecular basis of the lacZ' mutational assay. The lacZ' α-complementation process restores β-galactosidase activity to the M15 protein. When complemented with the wild type lacZ' polypeptide, the M15 protein tetramerizes and attains β-galactosidase activity that is observed phenotypically by the cleavage of the X-gal prochromophore resulting in the formation of blue plaques when plated with E. coli GW5100 cells in the plaque forming assay. If the DNA sequence has been mutated, the lacZ' polypeptide might not be synthesized (due to a nonsense mutation in the coding region or by disruption of the regulatory region upstream of the coding sequence) resulting in colorless plaques. Alternately, the lacZ' polypeptide might be synthesized, but be functionally compromised, resulting, once again, in no β-galactosidase activity (colorless plaques) or, in some cases, diminished activity (light blue plaques). The lacZ' region of the M13mp18 genome is depicted in greater detail in Figure 7.
Wild type \textit{lacZ}' M13mp18 DNA sequence

\[ \text{IPTG} \]

Wild type \textit{lacZ}' polypeptide

\[ + \]

\text{M15 }\alpha\text{-acceptor protein}

\[ \text{\(\alpha\)-complementation} \]

\[ \beta\text{-galactosidase activity} \]

Blue plaques

Mutated \textit{lacZ}' M13mp18 DNA sequence

\[ \text{IPTG} \]

\text{lacZ}' polypeptide is defective or absent

\[ + \]

Little or no \(\alpha\)-complementation

\[ \beta\text{-galactosidase activity} \]

Colorless or light blue plaques
Figure 7. Functional regions of the lacZ' DNA sequence of the M13mp18 genome. Mutations can be detected in a 370 bp region of the lacZ' sequence of the M13mp18 genome. This DNA sequence can be divided into two regions: The regulatory region, containing the CAP binding site, -35 promoter, operator, -10 promoter, and ribosome binding site, and the lacZ' polypeptide coding sequence (See Figure 18 for more detail on the exact DNA sequences involved). The lacZ' coding sequence can be divided into 3 subregions: The N-terminal overlapping region, the M15 deletion region, and the C-terminal overlapping region. Only the portion of the lacZ' polypeptide (shown as the gray pie wedge) corresponding to the amino acids deleted from the M15 α-acceptor protein is required to effect α-complementation. The N- and C-terminal overlapping regions of the lacZ' polypeptide are duplicated in the M15 protein and are not necessary for β-galactosidase activity. As will be described (see Discussion and Figure 20) the overlapping regions may in fact provide steric constraints that prevent optimal complementation and result in diminished enzymatic activity.
lacZ' DNA sequence of the M13mp18 bacteriophage genome:

Regulatory region | N-terminal overlap | M15 deletion | C-terminal overlap | 3'

5' → lacZ' polypeptide

N → C

Protein folding (conformational changes)

N-terminal overlapping region

α-complementation

M15 α-acceptor protein

lacZ' polypeptide

C-terminal overlapping region

Tetramer formation

β-galactosidase activity
III. MATERIALS AND METHODS
A. Materials

Restriction endonucleases (except for Stu I and Sca I, from Boehringer-Mannheim), T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Phosphatases and DNAses were from Sigma. The E. coli cell lines used were: DL7 (AB1157; lacA169, uvr+), DL6 (DL7; uvrA) (Lasko et al., 1988) and GW5100 (JM103, P1, from G. Walker, MIT). Bacterial culture media was from Difco Laboratory. M13mp7L2 bacteriophage genomes were a gift of C. W. Lawrence (U. of Rochester). Sequencing reagents were obtained from United States Biochemical Corporation (Sequenase) or New England Biolabs (CircumVent). The oligodeoxynucleotides used as sequencing primers were purchased from New England Biolabs or synthesized by the Biopolymers Lab, MIT and purified by denaturing polyacrylamide gel electrophoresis (PAGE).

Laboratory chemicals and reagents were purchased from Mallinckrodt, with the following exceptions. [α-32P]dATP (600 Ci/mmol) was purchased from Amersham, [γ-32P]ATP (6,000 Ci/mmol) was purchased from New England Nuclear (DuPont). Acrylamide:bis-acrylamide (19:1), TEMED, and ultrapure™ phenol was purchased from BioRad. TRIS was from Boehringer-Mannheim. IPTG and X-gal were purchased from Gold Biotechnology (St. Louis, MO) or Biosynth International (Skokie, IL). cis-DDP was purchased from Sigma and purified as described (Raudaschl et al., 1983) and ACDP was synthesized as described (Hartwig & Lippard, 1992). DNA purification systems were from the QIAGEN or Promega corporations. Oligodeoxynucleotides used in the construction of the singly cis-DDP modified genomes were synthesized by using Applied Biosystems reagents and a Model 381 DNA synthesizer.
B. SOS-Induction of *E. coli* DL6 and DL7 Cells

The mutability of many bulky DNA adducts is an SOS dependent phenomenon in *E. coli*. Since this work involved the study of such lesions, it was essential to determine the conditions required to induce the SOS response in the *E. coli* DL6 (uvrA) or DL7 (wt) cells that served as hosts for the replication of platinum drug modified genomes. SOS induction was achieved by irradiating the *E. coli* cells with uv light to damage their chromosomal DNA. A recovery period allowed for the expression of SOS proteins in response to this DNA damage. Platinum modified M13mp18 genomes were transfected into the uv irradiated *E. coli* cells. The occurrence of platinum induced mutations indicated that the SOS response had been induced; this section describes the optimization of conditions required for the induction of the SOS response in *E. coli* DL6 and DL7 cells.

1. *cis-DDP* Modification of M13mp18 Bacteriophage Genomes

Purified *cis-DDP* was dissolved in TE by incubating at 37°C for 1 hour with frequent mixing. The purity and concentration of the drug solution was determined by the uv-vis spectrum from 220-570 nm. Purity was assessed by comparing the observed $A_{301}/A_{246}$ and $A_{301}/A_{348}$ ratios to the expected values of 4.9 and 5.3, respectively. The molar concentration of the *cis-DDP* solution was determined by the OD value at 301 nm with $\varepsilon = 131$ (Raudaschl et al., 1983). Modification of M13mp18 RF DNA was achieved by incubating 5.2 pmole of DNA with 0, 5, 10, 15, 20, 50, and 100 fold molar excesses of *cis-DDP* in a 500 µl solution of 3 mM NaCl, 0.5 mM Na$_2$HPO$_4$ and 0.5 mM NaH$_2$PO$_4$ (pH 7.2) for 18 hours at 37°C. Platination reactions were terminated by the addition of NaCl to a final concentration of 0.5 M. Unbound *cis-DDP* was removed by ethanol precipitation of the DNA followed by two 80% ethanol washes. The amount
of DNA recovered after precipitation and resuspension in TE buffer was quantitated by $A_{260}$ OD readings and the levels of cis-DDP bound to DNA ($r_b$ values) were determined by flameless atomic absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95 graphite furnace.

2. Transformation of E. coli with cis-DDP Modified M13mp18 Genomes

E. coli DL6 and DL7 cells were grown in 100 ml batches in Luria-Bertani broth (Maniatis et al., 1989) to a cell density of approximately $10^8$ cells/ml. Bacterial cells from each 100 ml culture were harvested by centrifugation at 4000 g for 5 min at 4°C, resuspended in 100 ml of chilled water, and recentrifuged at 8000 g for 10 min. The cells were then resuspended in 40 ml of water and a final centrifugation was done at 13000 g for 10 min. The cells were resuspended in water to give a final volume of 1.0 ml and kept on ice until needed. Unmodified genomes as well as the M13mp18 RF DNA modified with various levels (5.9, 11, 14, 20, 32, and 72 adducts per genome) of cis-DDP were used to transform the prepared E. coli cultures by the process of electroporation. The cell suspension (120 μl, about $10^9$ viable cells) was mixed with 10 μl (10 ng) of DNA and transferred to a prechilled electroporation cuvette. Cells were electroporated in a BTX Electro Cell Manipulator 600 at 50 μF, 129 Ω, and 11.5 kV/cm. One ml of SOC media (Hanahan, 1985) was mixed with the cell suspension following electroporation. A portion of the transformed bacteria was plated immediately in the presence of E. coli GW5100 cells, isopropylthio-β-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) (Messing, 1983) to obtain independent infective center plaques that were counted to obtain survival data. The remainder of the transformed bacteria was incubated for 2 hours at 37°C to allow for phage replication followed by centrifugation at 17000 g for 10 min to pellet the cells. The phage-containing supernatant was stored at 4°C and later plated with E. coli
GW5100 cells, IPTG and X-gal to obtain mutational frequency data.

3. UV Irradiation of *E. coli* DL7 Cells

*E. coli* DL7 cells\(^1\) were grown in 100 ml batches in LB media to a cell density of approximately 10\(^8\) cells/ml. Cells from each 100 ml culture were harvested by centrifugation at 4000 g for 5 min and resuspended in 50 ml of 4°C 10 mM MgSO\(_4\) and maintained on ice. The cell suspension (25 ml) was pipetted into 150 mm diameter petri dishes. The cells were exposed to uv irradiation at 1.1 - 1.2 J/m\(^2\) with gentle shaking over a range of fluences from 5 to 80 J/m\(^2\). Further handling of the uv irradiated cells was done under yellow lights to avoid reversal of the uv-induced damage in their chromosomal DNA by photoreactivation. An equal volume of 2x LB media, prewarmed to 37°C, was added to the cells immediately after uv irradiation. The cells were incubated for 40 min at 37°C to allow for expression of SOS proteins. Aliquots of cells were plated on LB plates before and after irradiation to determine the rate of mortality caused by the uv treatment. Subsequent preparation for electroporation was done as described above for the non-uv irradiated cells. M13mp18 RF DNA modified with 32.1 cis-DDP adducts per genome, a level of modification determined to both allow the detection of mutations and provide an adequate level of survival, as described above, was electroporated into the *E. coli* DL7 cells (at 11.0 kV/cm) that had been uv irradiated at fluences between 5 and 80 J/m\(^2\).

\(^1\)These experiments were originally intended to be done in both *E. coli* DL6 (uvrA) and DL7 (wt) cell lines. However, due to technical limitations (to be discussed in the Results section), only the wild-type DL7 cells were used in subsequent experiments.
C. Site Specific Comparison of *cis*-DDP DNA Adducts

1. Preparation of *cis*-DDP Modified Oligodeoxynucleotides

a. Design of oligodeoxynucleotide sequence

The sequences of the oligodeoxynucleotides used in the site specific comparison of the *cis*-DDP G*G*, A*G*, and G*TG* adducts were chosen based on the following criteria:

i. The platinum binding site was chosen to be located in the recognition site for a restriction endonuclease. The structural distortion imposed on duplex DNA prevents restriction endonucleases from digesting platinum-modified recognition sites. This feature is of aid in the characterization of genetically engineered bacteriophage genomes constructed with these oligodeoxynucleotides since platinum modified genomes are refractory to digestion while their unmodified counterparts remain susceptible to digestion.

ii. The restriction sites must be chosen to contain either the d(GpG), d(ApG), or d(GpNpG) nucleotide sequence to allow formation of the G*G*, A*G*, and G*NG* adducts, respectively.

iii. Drug induced mutations that occur within a restriction site render the site refractory to digestion, a feature that is essential for the selection of mutants. Restriction sites added by genetic engineering, however, must not occur elsewhere in the M13mp18 genome so that the selected sequences will form unique restriction sites, and allow selection for mutants, as will be discussed further below. The restriction sites selected were Stu I, d(AGGCCT), Sca I, d(AGTACT), and ApaL I, d(GTGCAC) for formation of the G*G*, A*G*, and G*NG* adducts,
respectively. One necessary drawback of this approach is its failure to detect nontargeted mutations that fall outside the restriction site.

iv. A step in the construction of singly modified genomes involves annealing the cis-DDP modified oligodeoxynucleotides to complementary DNA templates. The significant structural distortion caused by platinum binding to DNA requires that flanking regions be added to each side of the drug-targeted restriction site to facilitate the annealing process. The flanking regions must avoid nucleotide sequences (i.e. purines) that compete for platinum binding; therefore, d(TCT) repeats were chosen.

Considering the above criteria, the following oligodeoxynucleotide sequences were selected (containing the indicated restriction sites):

- d(TCT TCT TCT AGG CCT TCT TCT TCT) - Stu I
- d(TCT TCT TCT CTA GTA CTC TCT TCT) - Sca I
- d(TCT TCT TCT GTG CAC TCT TCT TCT) - ApaL I

where the restriction sites are shown in bold and the potential sites for platinum binding are underlined. The nomenclature used to describe these oligodeoxynucleotides is Stu 24, Sca 24, and ApaL 24 for the unmodified samples and Stu Pt, Sca Pt, and ApaL Pt for their platinated counterparts.

b. Synthesis of Platinated Oligodeoxynucleotides

i. Synthesis of unmodified oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 381 DNA synthesis machine on a 10 μmole scale by using β-cyanoethyl phosphoramidite chemistry (reagents from Applied Biological
Sciences). After synthesis, oligodeoxynucleotides were deprotected by incubating for 16 hr at 55°C in concentrated ammonium hydroxide followed by vacuum drying in a Savant speed-vac system. Oligodeoxynucleotides were purified by polyacrylamide gel electrophoresis (PAGE). Approximately 3.6 mg of crude material was resuspended in 0.5 ml 50% formamide, heated for 2 min at 90°C and run on a denaturing 35 x 43 x 0.15 cm 20% polyacrylamide gel. The gel was run at 1200 V for 9-12 hr until the bromphenol blue dye migrated to the bottom of the gel. Full length material, approximately comigrating with xylene cyanol dye, was identified by uv shadowing on a fluorescent TLC plate and excised from the gel. DNA was removed from the gel slices by the process of electroelution by using AMICON’s Centrieluter apparatus with Centricon-3 concentration devices. Following electroelution, TBE buffer and traces of urea were removed from the samples by performing five washes with TE (by performing repeated 2.0 ml to 100 μl concentration centrifugations in Centricon-3s at 5000g). Purified oligodeoxynucleotides were quantitated by taking OD readings at 260 nm and the DNA concentrations were determined from the extinction values of \( \epsilon = 211,600 \) for Stu 24, \( \epsilon = 215,300 \) for Sca 24, and \( \epsilon = 211,600 \) for ApaL 24. The yield after purification was typically 30-35%. Purified material was aliquoted and stored at -80°C until needed.

ii. Platination of oligodeoxynucleotides

cis-DDP was dissolved in TE to a concentration of about 2 mM. A 1.5 fold molar excess of cis-DDP was incubated with 50 μM Stu 24, Sca 24 or ApaL 24 in TE at pH 7.4. Nitric acid was added to the Stu 24 sample to reduce the pH to 5.2 in order to protonate the N7 position of the adenine residue in the d(AGGCCT) Stu I recognition site. Such a protonation directs selective modification of the d(GpG) site. Reactions were allowed to proceed for 16-20 hr at 37°C. Typically 50-
70% of oligodeoxynucleotide was platinated. Reaction products were PAGE purified as described in the previous section.

The purity of the 24-mers was monitored by 5′ phosphorylation of the oligodeoxynucleotides in conjunction with analytical PAGE. Phosphorylation was done in a 30 μl reaction by using 20 units of T4 polynucleotide kinase with 0.05 μg DNA and 25 μCi γ-32P ATP and the reaction buffer supplied with the enzyme. The reaction mixture was incubated at 37°C for 45 min followed by heat inactivation of the enzyme at 68°C for 15 min. Two μl of the phosphorylation reaction (with 2 μl of formamide dye) was run on a denaturing 35 x 43 x 0.04 cm 20% polyacrylamide gel at 1500 V until the xylene cyanole dye migrated to within 5 cm of the bottom. The purity of the oligodeoxynucleotides was determined by PhosphorImager analysis.

iii. Characterization of platinated oligodeoxynucleotides

Putatively cis-DDP modified oligodeoxynucleotides migrate slower during PAGE than do their unmodified counterparts. Platinated oligodeoxynucleotides can be treated with cyanide ions to remove the cis-DDP DNA adducts as Pt(CN)₄. Subsequent to cyanide treatment, the deplatinated oligodeoxynucleotide should comigrate with unmodified material, thereby confirming that the retarded migration was indeed due to the presence of a platinum-DNA adduct. Cyanide treatment of Stu Pt, Sca Pt, and ApaL Pt was done by incubating 0.02 μg of 5′ γ-32P labeled oligodeoxynucleotide in 0.3 M NaCN (pH=9.0) solution for 36 hours at 37°C (Schwartz et al., 1990).

The cyanide treated oligodeoxynucleotides were run on denaturing 20% polyacrylamide gels with unmodified controls to confirm the removal (and therefore the prior existence) of the cis-DDP adduct. Once the
presence of a cis-DDP adduct was confirmed, further characterization was
done by enzymatically digesting the oligodeoxynucleotides to their
constituent deoxynucleosides. Digestion was done by combining 1.0 OD
unit of DNA in 50 mM NaOAc (pH=5.5), 20 mM MgCl₂ with 4,000 units of
dNAse I (Sigma) and 10 units P₁ nuclease in 125 µl and incubating for 16
hours at 37°C. Then, to dephosphorylate the monomeric deoxynucleotides,
25 µl 1.0 M TRIS (pH=9.0), 10 µl alkaline phosphatase (~10 units, Sigma)
and 1.0 µl calf intestinal phosphatase (~24 units, Boehringer Mannheim)
were added and incubation was continued for 4 hours. Deoxynucleosides
were resolved by reverse phase HPLC by using an Ultramex 5 micron 4 mm x
25 cm analytical column. Under these HPLC conditions, the nucleoside
standard dC eluted at 11.3 min, dG at 16.6 min, dT at 17.8 min and dA at
21.9 min on a 0-20% acetonitrile gradient over 40 min in 0.1 M ammonium
acetate buffer at a flow rate of 1 ml/min. A Pt(dG)₂(NH₃)₂ standard
(from S. Bellon, MIT) eluted at 19.0 min. The identity of the
deoxynucleosides obtained from the digestion of the 24-mers was
determined by comparison of their elution time with that of
deoxynucleoside standards. The identity of the peaks was confirmed
further by spectral analysis by using a Hewlett-Packard 1040A (diode
array) Detection System. The relative abundance of each deoxynucleoside
was determined from the integration of peak areas, normalized by the
relative extinction coefficients of 15,400, 7,300, 11,700, and 8,800 for
dA, dC, dG, and dT, respectively.

2. Preparation of cis-DDP Modified Genomes

Bacteriophage M13-derived ss genomes containing the cis-DDP G*G*,
A*G*, and G*TG* adducts were constructed based on methodology described
by Banerjee et al. (1988) and outlined in Figure 8. Briefly, M13mp7L2
ss DNA was digested with the restriction endonuclease EcoR I to remove a
42 nucleotide hairpin structure, thereby creating a linearized ss
genome. Scaffold oligodeoxynucleotides, 64 bases in length, were annealed to the linearized genomes resulting in a 24 nucleotide "gap" complementary to the platinated or unmodified 24-mers. 5’-Phosphorylated 24-mers were annealed to their complementary sequence in this gap and T4 DNA ligase was utilized to covalently incorporate the platinated, or unmodified, oligodeoxynucleotide 24-mers into the M13 ss genomes.

a. Scaffold oligodeoxynucleotides

Scaffold oligodeoxynucleotides were synthesized by the MIT Biopolymers Laboratory and gel purified as previously described with the exception that a 15%, instead of 20%, gel was used. The sequences of the scaffold 64-mers are (underlined regions are complementary to the indicated 24-mers, the flanking 20 nucleotides are complementary to either end of the linearized M13mp7L2 ss genomes):

**Stu 64** (complementary to the Stu 24 and Stu Pt oligodeoxynucleotides):

5’- AAA ACG ACG GCC AGT GAA TTA GAA GAA GAC ACT GAA TCA TGG TCA TAG C -3’

**Sca 64** (complementary to the Sca 24 and Sca Pt oligodeoxynucleotides):

5’- AAA ACG ACG GCC AGT GAA TTA GAA GAG AGT ACT AGA GAA GAC ACT GAA TCA TGG TCA TAG C -3’

**ApaL 64** (complementary to the ApaL 24 and ApaL Pt oligodeoxynucleotides):

5’- AAA ACG ACG GCC AGT GAA TTA GAA GAA GAG TGC ACA GAA GAA GAC ACT GAA TCA TGG TCA TAG C -3’

b. Preparation of linearized M13mp7L2 ss genomes
M13mp7L2 phage were propagated in E. coli GW5100 cells grown in 2x YT media and isolated by PEG/NaCl precipitation as described by Yamamoto & Alberts (1970). Single stranded DNA was obtained from the phage by multiple phenol:chloroform extractions followed by a chloroform extraction. The M13mp7L2 DNA was purified further by elution through a hydroxylapatite (Biorad DNA grade, batch 35354) column in buffer that was 76 mM in Na$_2$HPO$_4$ and 76 mM in NaH$_2$PO$_4$ followed by dialysis into TE with 4 buffer changes. One hundred and thirty pmole (312 µmole) M13mp7L2 ss DNA was digested by incubating with 4000 units of EcoR I for 2 hours at 23°C in 50 mM NaCl, 100 mM TRIS (7.5), 5 mM MgCl$_2$ and 100 µg/ml BSA. The extent of digestion was determined by running 0.5 µg of SS DNA on an agarose gel capable of resolving the circular and linear forms of M13 viral genomes (Gel conditions: 1.0% agarose, run in TBE buffer at 3 V/cm for 4-5 hours and then stained in 1.0 µg/ml ethidium bromide solution for 0.5 hour).

c. Phosphorylation of oligodeoxynucleotide 24-mers

The Stu 24, Stu Pt, Sca 24, Sca Pt, ApaL 24, and ApaL Pt oligodeoxynucleotides were 5’ phosphorylated by using T4 polynucleotide kinase. Phosphorylation conditions were 280 pmole oligodeoxynucleotide, 1.0 mM ATP, 1x kinase buffer supplied with the enzyme and 30 units of enzyme in 50 µl incubated for 45 min at 37°C followed by heat inactivation of the enzyme at 68°C for 15 min. The extent of phosphorylation was not determined for these particular reactions because the reaction conditions used previously had been demonstrated to result in complete phosphorylation.

d. Annealing and ligation reactions

Scaffold oligodeoxynucleotides were annealed to the EcoR I
restricted linear M13mp7L2 ss genomes by combining 5.6 pmole of the bacteriophage genome with 560 pmole (a 100 fold excess) of either Stu 64, Sca 64, or ApaL 64 in a volume of 75 µl. Annealing was effected by heating at 80°C for 15 min, incubating at 65°C for 30 min followed by cooling to 16°C over 5 hours. The extent of annealing was analyzed by running 1 µg aliquots on an agarose gel as described above. Seven replicas of each of the Stu 64, Sca 64, and ApaL 64 scaffold oligodeoxynucleotide-genome mixtures were processed. One replica of each set of seven served as a no oligodeoxynucleotide control, 0.56 nmoles (a 100 fold excess) of the appropriate unmodified 24-mers were added to 3 replicas, and 0.56 nmoles of the respective platinated 24-mers were added to the remaining 3 replicas. The phosphorylated 24-mers were covalently ligated into the ss genomes by incubating with 1000 units of T4 DNA ligase (New England Biolabs) per sample in the buffer supplied with the enzyme for 16 hours at 16°C.

An alternate approach to genome construction was to add 11.2 pmole (a 2 fold excess) of the appropriate 24-mer to the scaffold oligodeoxynucleotide-genome mixtures before the annealing process. The advantages of this methodology was that a much smaller excess of 24-mer (2 fold vs. 100 fold) oligodeoxynucleotide could be used and the entire annealing process was completed in a one step reaction. Accordingly,

'The advantages of using a greatly reduced amount of oligodeoxynucleotide are twofold. First, from a practical perspective, it is considerably easier to construct and characterize modified oligodeoxynucleotide to be used in a 2 fold excess compared to the amount required for a 100 fold excess. More importantly, purer genomes can be constructed. To explain, if the oligodeoxynucleotide contained a 1% impurity, there would be sufficient amount of this contaminant to ligate into all of the genomes if this material was used in a 100 fold excess and it ligated into the genome much more efficiently than the modified oligodeoxynucleotide. By contrast, if a 2 fold excess was used with a 1% impurity, the maximum amount of impurity incorporated into the genome would be 2%. My suspicions with regard to this latter point, the purity of the resultant site specifically modified genome, have been confirmed by another laboratory colleague, Elisabeth Bailey, who is working with oligodeoxynucleotides containing aflatoxin adducts (E. Bailey, personal communication).
Stu 24, Stu Pt, Sca 24, Sca Pt, ApaL 24, or ApaL Pt oligodeoxynucleotides were added in a volume of 20 μl and annealing was performed as before.

e. Characterization of genetically engineered genomes

The genetically engineered, singly cis-DDP modified M13mp7L2 ss genomes were characterized by heat denaturation to remove the scaffold oligodeoxynucleotide. Heating at 90°C for 2 min to remove the scaffold resulted in a linear ss genome if either a 24-mer oligodeoxynucleotide had not been ligated into its complementary "gap" or if it had only been covalently attached at either its 5′ or 3′ end. Such linear M13 ss genomes are biologically inactive. Conversely, if the 24-mer had been ligated at both its 5′ and 3′ termini, a completely covalently closed circular genome is formed that should remain circular after heat treatment. These circular genomes were distinguished from the incompletely ligated, linear genomes by agarose gel electrophoresis (as described above) to provide an estimate of the ligation efficiency.

3. Genotoxicities of cis-DDP Modified Genomes

The genotoxicities of the cis-DDP G*G*, A*G*, and G*TG* adducts were determined by transfecting the Stu Pt, Sca Pt, and ApaL Pt M13mp7L2-derived genomes into bacterial cells in parallel with the unmodified Stu 24, Sca 24, and ApaL 24 control genomes. Survival was determined by counting the relative number of infective centers formed by cis-DDP modified, compared to unmodified, genomes, in the M13 plaque forming assay.

Transfection of the site-specifically cis-DDP modified genomes into E. coli DL7 cells was done as previously described with minor
differences. Immediately before electroporation 40 ng of each genome was diluted to 100 μl in water and heated at 90°C for 2 min to remove the scaffold and maintained on ice to prevent its reassociation. One hundred μl of cells was added to the heat treated DNA and the resulting mixture was electroporated. Infective centers were plated to obtain survival values that represented the genotoxicity of each adduct.

Survival was determined for genomes that underwent replication in both SOS induced and non-SOS induced E. coli DL7 cells. Progeny phage were grown and processed, as described below, to determine the mutagenicity of each adduct.

4. Mutagenicity of Site-Specifically Modified Genomes

The mutagenicities of the cis-DDP G*G*, A*G*, and G*TG* adducts were determined by analysis of progeny phage obtained from the transfections, as described in the previous section, of the cis-DDP site specifically modified genomes into E. coli DL7 cells (see Figure 9). Progeny phage from multiple transfection samples were pooled and used to produce RF DNA. The RF DNA was digested with the appropriate restriction enzyme to enrich for genomes that had putatively platinum induced mutations within the Stu I, Sca I, or ApaL I restriction sites resulting from the cis-DDP G*G*, A*G*, or G*TG* adducts, respectively. The digested DNA was transfected into E. coli DL7 cells in parallel with uncut buffer control DNA (steps 3A and 3B, respectively, of Figure 9). Putative mutation frequencies were obtained by counting infective center plaques. Only mutant DNAs in the digested samples should remain uncut

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1The relative amounts of cis-DDP modified and unmodified genomes were determined by uv florescence of the DNA electrophoresed in an agarose gel in the presence of ethidium bromide as described by Bradley et al. (1993). This method of quantitation had previously been estimated to be accurate within 5%, which implies that between 38 and 42 ng of DNA would have been used in each replicate, a range of variability that is negligible in view of the actual survival differences observed between unmodified and platinum modified genomes (see Results section).
and produce infective center plaques. By contrast, the DNA in the buffer control samples should be 100% viable. The relative number of plaques from the digested samples compared to the buffer control samples represent putative mutation frequencies. The actual mutation frequencies were obtained by sequence analysis of putative mutants. The details of this process, used to determine the fraction of progeny that is mutant, is described in greater detail below.

a. RF DNA preparations from progeny phage

Progeny phage from the transfection of cis-DDP G*G*, A*G*, and G*TG* modified genomes as well as their unmodified counterparts into E. coli DL7 cells were used to infect GW5100 cells to produce RF M13 genomic DNA. A single GW5100 colony was grown to saturation (about 14 hours) in 2x YT media. One ml of the cell culture was added to 100 ml of 2x YT media followed by a 0.5 hour incubation at 37°C. Approximately 10⁶ phage representing each of 30-32 independent transfections were combined for each of the Stu 24, Stu Pt, Sca 24, Sca Pt, ApaL 24 and ApaL Pt M13 ss genomes that had been replicated in SOS-induced E. coli DL7 cells. Similarly, phage were combined from 18-20 replicates for each of the genetically engineered M13 ss genomes that had been replicated in non-SOS induced cells. The pooled phage from each of these samples was added to the pregrown 100 ml GW5100 culture and incubated for 2.5 hours. An additional 100 ml of 2x YT media was added and incubation was continued for another 2.5 hours. RF DNA was isolated from the cells by using the QIAGEN ‘midi’ plasmid purification system. Approximately 100 µg of DNA was obtained from each preparation. Typically the RF DNA was contaminated with fragmented chromosomal E. coli DNA, ss M13 DNA and a ~800 bp deletion mutant that necessitated further purification.
RF DNA was purified by agarose gel electrophoresis. One half of each preparation (~50 μg DNA in 0.5 ml TE, 0.1 ml 40% sucrose loading dye) was run on a 200 ml (prepared in a 12.6 x 20.0 cm gel support) 0.8% agarose gel with 0.5 μg/ml ethidium bromide in TAE buffer at 3 V/cm for 4 hour. Covalently closed circular RF DNA migrated as supercoiled material and was excised from the gel upon visualization with uv light. The DNA was electroeluted from the gel into Centricon-100 devices and desalted by performing five washes with TE (by repeated 2.0 ml to 100 μl centrifugation concentrations at 1000g in Centricon-100s). The DNA was quantitated by taking $A_{260}$ OD readings, typically a yield of 10 μg was obtained from each agarose gel.

b. Restriction digestion of M13 bacteriophage RF DNA

Gel purified RF DNA was digested with either Stu I (G*G* and GG genomes), Sca I (A*G* and AG genomes), or ApaL I (G*TG* or GTG genomes). Digestion conditions were 1.0 μg RF DNA, 10 units enzyme, and 5.0 μl of the 10x buffer supplied with the enzyme in a total volume of 50 μl. Digestion was done by incubating for 2 hours at 37°C. In parallel, buffer control samples, identical in composition but lacking enzyme, were incubated. Digests were performed in quadruplicate on RF DNA originating from each type of the site-specifically constructed genomes that had been replicated in either SOS induced or non-SOS induced cells. The extent of digestion was estimated by using agarose gel electrophoresis.

c. Determination of mutation frequency

Putative mutation frequencies engendered by the cis-DDP G*G*, A*G*, and G*TG* adducts were determined by parallel transfection of digested and buffer control RF DNA into E. coli DL7 cells. Typically
five ng of DNA per sample was transfected by the electroporation methodology previously described. Infective center plaques were counted to determine the relative survival of the digested and uncut DNA samples. In the restriction digested samples, only DNA with a mutation in the restriction site should remain uncut and be capable of producing infective center plaques. To ensure that these plaques did indeed represent mutants, plaques were picked and used to produce ss DNA samples that were sequenced by using the dideoxy chain termination method described by Sanger et al. (1977) with either primer P6285 or P6331 shown in Table 2.

d. Determination of mutational specificity

The putative mutation frequencies determined by the relative survival of digested and uncut DNA samples included any mutations that occurred in the 6 bp restriction sites recognized by the Stu I, Sca I, or ApaL I restriction endonucleases. To ascribe mutations to the cis-DDP G*G*, A*G*, or G*TG* adducts specifically, it was necessary to compile comprehensive mutational spectra for each of the restriction sites from both platinated and unmodified genomes replicated in both SOS induced and non-SOS induced E. coli DL7 cells. Preliminary DNA sequencing analysis revealed the presence of wild type and parental M13mp7L2 genomes. Further enrichment for mutants, therefore, was desirable and entailed a second round of RF DNA preparations and restriction endonuclease digestions. Progeny phage from the initial transfection of digested DNA samples into E. coli DL7 cells was used to obtain RF DNA by using the QIAGEN plasmid purification system as before. The second round of restriction digests was done with a greater enzyme:DNA ratio than initially used in order to eliminate any residual wild-type genomes. EcoR I was included in the reaction to digest the contaminating parental M13mp7L2 genomes. Reaction conditions were: 250
ng RF DNA, 25-30 units of Stu I, Sca I, or ApaL I as appropriate, and 5.0 μl of 10x buffer supplied with the enzyme in a volume of 50 μl. Incubation at 37°C was done for 1 hour, and then 5 M NaCl was added to a concentration of 125 mM along with 20 units of EcoR I. Incubation was continued for another hour. The extent of digestion was estimated by agarose gel electrophoresis. Transformation of digested DNA and buffer controls into E. coli DL7 cells was done by electroporation as before. Single stranded DNA grown from infective center plaques was sequenced to determine mutations.
Table 2. Primers used for sequencing of mutant DNAs and adduct mapping of platinated genomes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6027</td>
<td>CCC GCG CGT TGG CCG ATT C</td>
</tr>
<tr>
<td>M6086</td>
<td>GCG GGC AGT GAG CGC AAC GC</td>
</tr>
<tr>
<td>M6209</td>
<td>AAC AGC TAT GAC CAT G</td>
</tr>
<tr>
<td>M6306</td>
<td>CAA CGT CGT GAC TGG G</td>
</tr>
<tr>
<td>P6285</td>
<td>GTA AAA CGA CGG CCA GT</td>
</tr>
<tr>
<td>P6331</td>
<td>AAG TTG GGT AAC GCC</td>
</tr>
<tr>
<td>P6507</td>
<td>CAG TAT CGG CCT CAG G</td>
</tr>
<tr>
<td>P6527</td>
<td>GTT TGA GGG GAC GAC</td>
</tr>
<tr>
<td>P6704</td>
<td>CCA ATA GGA AAC GCC ATC</td>
</tr>
</tbody>
</table>

a. M designates primers complementary to the (-) strand, P for those complementary to the (+) strand; the number designates the 5’ nucleotide position that the primer anneals to in the M13mp18 RF genome.
Figure 8. Method used to construct singly cis-DDP modified M13 ss genomes based on methodology developed by C. Lawrence (Banerjee et al., 1988; LeClerc et al., 1991). The cis-DDP G*G*, A*G*, and G*TG* adducts (collectively represented as "Pt" in the diagram), along with unmodified controls, were introduced into M13 ss genomes by using this method, as described in greater detail in the text. The figure shows the alternate approaches used in genome construction: A two step process (bottom) involving preannealing the scaffold 64-mer followed by annealing and ligation of the 24-mer or a one step (top) annealing reaction of both the 64-mer and 24-mer followed by a ligation reaction.
EcoR I recognition site

EcoR I Digest

M13mp7L2 ss DNA

"Scaffold" 64mer oligodeoxynucleotide (2 fold excess)

1. Anneal
2. Ligate

Singly-modified M13 ss genome

"Gap" complementary to platinated (or unmodified) 24mer oligodeoxynucleotide

Platinated or unmodified 24 mer oligodeoxynucleotide (100 fold excess)

Ligate
Figure 9. Method of mutational analysis. Singly cis-DDP modified M13 ss genomes were replicated in E. coli cells, progeny phage were isolated and used to produce RF DNA. In the diagram, 1/4 (25%) of the RF DNA molecules contain a mutation in the restriction site (bold) used for mutational analysis. The putative mutation frequency, in this example, after restriction digest, transfection into E. coli, and enumeration of infective center plaques would be determined to be 25%. Sequence analysis of the undigested RF molecules, in this example, revealed that 1/5 (20%) of the putative mutants is a genuine cis-DDP induced event. The overall mutation frequency in this example is 5% (i.e. 25% x 20%).

*Wild type progeny are assumed to arise because of enzymatic inefficiency at step 3A.

**Other non-specific mutant progeny are assumed to be either induced spontaneously or by the genetic engineering manipulations used. These mutations generally are present at frequencies < 0.1%. 
1. Replication in *E. coli*  
2. RF DNA preparations from pooled progeny phage  
3A. Restriction digest with selection enzyme  
3B. Buffer control for restriction digest  
4. Transfect into *E. coli*  
5. Count infective center plaques to determine relative survival  
6. Sequence analysis  

**25%** (Putative Mutation Frequency)  
- wild type*
- large deletion**
- small deletion**
- random base pair mutation**
- platinum specific mutation

**100%**

Actual mutation frequency = putative mutation frequency x fraction of sequenced samples that are platinum induced mutants, in this case

MF = (25%) x (1/5) = 5%
D. Comparison of the Mutagenicities, Genotoxicities, and Adduct Distributions of *cis*-DDP and ACDP DNA Adducts

1. Random Platination of Bacteriophage M13mp18 RF Genomes

Platination of M13mp18 RF DNA was achieved by incubating 25 μg of DNA in 500 μl solutions of 3 mM NaCl, 0.5 mM Na₂HPO₄, and 0.5 mM NaH₂PO₄ (pH 7.2) with either *cis*-DDP or ACDP for 16 hours at 37°C. In addition, M13mp18 RF DNA was incubated under these conditions, but with neither drug present, to serve as an unmodified control. The platination reactions were terminated by the addition of NaCl to a final concentration of 0.5 M. Unbound platinum was removed by ethanol precipitating the DNA and washing twice with 80% ethanol. Levels of platinum bound to DNA were determined by flameless atomic absorption spectroscopy.

2. Transfection of Platinated M13mp18 RF Genomes

The *cis*-DDP and ACDP modified M13mp18 genomes were transfected into *E. coli* DL7 cells by the electroporation methodology previously described. Typically, 10 ng of platinated or unmodified RF DNA was transfected into SOS induced or non-SOS induced cells. A portion of the transformed bacteria was plated immediately in the presence of *E. coli* GW5100 cells, IPTG, and X-gal to obtain infective center plaques that were counted to determine survival or were isolated and used to produce ss DNA that was subjected to sequencing analysis. The remainder of the bacteria was incubated for 2 hours at 37°C to allow for phage replication. The resultant progeny phage were stored at 4°C and later plated to obtain mutation frequency data.
3. Assay of β-Galactosidase Activity

Ten ml cultures of E. coli GW5100 (2 X 10⁹ cells) were infected with 10⁶ wild-type or mutant M13mp18 phage and incubated for 0.5 hour at 37°C. IPTG was added to a final concentration of 2 mM and incubation continued for 60 min. Cell extracts were prepared by the method of Wickner et al. (1972). β-Galactosidase activity was measured by using the ONPG (o-nitrophenol-β-D-galactopyranoside) assay described in Maniatis et al. (1989) or in an assay employing CPRG (chlorophenol red β-D-galactopyranoside) as the chromophore (Eustice et al., 1992).

4. Identification and Sequencing of Mutants

Mutations that led to the disruption of the α-complementation process in the lacZ' mutational assay gave rise to phenotypically distinct plaques in the M13 plaque forming assay. Mutant plaques were distinguished from wild-type plaques by the intensity of the blue chromophore produced by β-galactosidase cleavage of X-gal. Putative mutants were replated to verify phenotypic purity by a published method (Kunkel, 1984). Single-stranded DNA derived from mutant plaques was isolated and sequenced according to the method of Sanger et al. (1977) by using primers P6331 and P6507 (Table 2). Mutations that occurred more than once in an individual electroporation sample were not scored to ensure that each mutant represented an independent event.

5. Replication Mapping of cis-DDP and ACDP DNA Adducts

Replication mapping of DNA adduct experiments were done by using Vent₉™(exo') DNA polymerase (New England Biolabs) with five M13mp18 RF DNA samples (unmodified, cis-DDP at 12 and 35 adducts per genome, and ACDP at 6.6 and 45 adducts per genome) and the primers shown in Table 2.
Typical reaction conditions were 0.10 pmole RF template DNA, 3.0 pmole primer, 20 μCi [α-35S]dATP, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl (pH 8.8), 5 mM MgSO4, 0.1% Triton X, 60 μM dATP, 300 μM dCTP, dGTP, and dTTP, with 5 units of Vent<sup>®</sup>(exo') DNA polymerase. Reactions were carried out in a Coy TempCycler II thermalcycler with a 2 min 95°C denaturation step, a 1 min 55°C annealing step, a 1 min 72°C primer extension followed by a final denaturation step at 95°C. In parallel, unmodified M13mp18 RF DNA was sequenced by using the CircumVent™ (New England Biolabs) thermal cycle dideoxy DNA sequencing methodology. The DNA fragments generated in these reactions were separated by using denaturing PAGE and quantitatively analyzed with a Molecular Dynamics PhosphorImager. The values for the stop sites were normalized by the method described in Figure 10 to take into account the additional [α-35S]dATP molecules incorporated into longer fragments.
Figure 10. Method for quantitation of stop sites used in the replication mapping of cis-DDP and ACDP DNA adducts. (a) M13mp18 RF genomes were digested with the restriction enzymes shown to generate a set of linearized DNA fragments. A mixture, containing equimolar ratios of each digestion product, was prepared and subjected to primer extension reactions with each of the primers shown (and listed in Table 2) to generate several sets of primer extended fragments of defined length (based on the distance from the primer to restriction site). The fragments generated by each primer were separated by denaturing PAGE and quantitated by use of a Molecular Dynamics PhosphorImager. (b) The intensity of stop sites increased as a function of distance from the primer due to additional incorporation of [α-35S]dATP. Although this increase in intensity is a function of the thymidine content of the DNA strand undergoing replication (the radiolabeled dATP is incorporated opposite T’s), a linear curve \( y = -0.118 + 5.50 \times 10^3 x \), \( R^2 = 0.886 \) by Cricket Graph (Cricket Software) fit the data well and was used to normalize the intensity of the restriction mapping stop sites as a function of their distance from primer.
(a)

EcoRI (6231)

BanII (6237)

XbaI (6258)

HindII (6264)

PstI (6270)

SphI (6276)

BglII (6431)

M13mp18 RF DNA
Relative intensity of stop site

Distance from primer

0 100 200 300

(b)
IV. RESULTS
A. SOS Induction of E. coli DL7 cells

1. Transformation of E. coli with cis-DDP modified M13mp18 RF Genomes

M13mp18 RF genomes that were incubated with cis-DDP were analyzed by atomic absorption spectroscopy to determine the level of drug that bound to the DNA. Platinum levels of 0, 5.9, 11, 14, 20, 32, and 72 adducts per genome were determined for RF DNA that had been incubated with 0, 5, 10, 15, 20, 50, and 100 fold molar excesses of cis-DDP, respectively. The plaque-forming ability of these genomes, when replicated in either E. coli DL6 or DL7 cells, decreased in a dose dependent manner as the level of cis-DDP adducts increased Figure 11). In the repair proficient E. coli DL7 cells, a modification level of 32 adducts per genome reduced survival to about 0.1% compared to unmodified genomes. This level of survival corresponded to approximately 100,000 independent infective centers per 10 ng of cis-DDP modified RF DNA. A sample of this size was adequate to obtain statistically significant mutation data; therefore, these genomes were used in the determination of the uv dose necessary to induce the SOS response in E. coli DL7 cells. Not surprisingly, the genotoxicity of cis-DDP modified genomes was much greater in the excision repair deficient E. coli DL6 (uvrA) cells, as evidenced by the data shown in Figure 11.

2. UV Irradiation of E. coli DL7 Cells

UV irradiation of E. coli DL7 cells resulted in a dose-dependent

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'Initially, these experiments were planned to be done with both E. coli DL7 and DL6 cells. A number of technical factors made it difficult to continue experimentation with the repair deficient E. coli DL6 cells. First, cis-DDP modified genomes were so lethal in these cells that it was unlikely that at levels of modification allowing for survival >1% that there would be any lesions in the 370 bp lacZ' region of M13mp18 where mutations are detected. The chance of detecting mutations was
decrease in survival. Only ~5% of the cells were viable after uv irradiation with a fluence of 80 J/m^2 when treated by using the experimental conditions described (Lasko et al., 1988; Bradley, 1991). Survival increased significantly (Figure 12) when the cells were kept chilled on ice until immediately before uv irradiation and prewarmed (37°C) 2x LB was added promptly after irradiation. M13mp18 RF DNA modified with 32 cis-DDP adducts per genome was transfected into E. coli DL7 cells that had been uv irradiated at various fluences up to 80 J/m^2 and subsequently allowed to recover for 40 min to express SOS proteins. Progeny phage were plated and mutants were phenotypically identified (by using the lacZ' mutational assay that is described in detail elsewhere in this dissertation). The mutation frequency as a function of uv dose to the host E. coli DL7 cells is given in Figure 13. The mutation frequency increased in an exponential-like manner up to ~30 J/m^2 and remained relatively constant to ~60 J/m^2. Due to the variability of the data it was unclear if the apparent increase in the mutation frequency between 60 and 80 J/m^2 were a real result; regardless, the low cell survival at 80 J/m^2 (see Figure 12) precluded the use of cells experiencing such high levels of irradiation. Practically, a fluence of > 30 J/m^2 was sufficient to achieve an adequate level of mutagenesis while a fluence of < 40 J/m^2 maintained high cell survival (Figure 12). Accordingly, subsequent experiments utilized fluences of between 35 and 40 J/m^2 to SOS induce E. coli DL7 cells.

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further diminished by reports that UvrA, which is defective in E. coli DL6 cells, is required for the mutability of cis-DDP DNA adducts. Furthermore, induction of the SOS response required uv doses to the host cell of <5 J/m^2, fluences too low accurately reproduce (the apparatus used for uv irradiating the cells was accurate within 2 J/m^2, which is a significant error range compared to 5 J/m^2, but is manageable compared to the higher doses of >30 J/m^2 used with the wild-type cells). These factors made it improbable that a mutagenic evaluation of cis-DDP adducts could be done successfully in the E. coli DL6 cells. Nevertheless, the comparison of survival of cis-DDP modified genomes in these two cell lines (Figure 11) offers insights into the molecular processing of these adducts, as addressed in the Discussion section.
Figure 11. Genotoxicity of cis-DDP modified DNA replicated in host E. coli with various levels of repair proficiency. Survival of M13mp18 RF genomes, modified with increasing levels of cis-DDP, was determined in the M13 plaque forming assay. E. coli DL6 (uvrA) cells showed a genotoxicity of 1.2 adducts/lethal hit, the comparable value for repair proficient E. coli DL7 cells was 4.6 adducts/lethal hit. Upon induction of the SOS response in the wild type cells (DL7), survival increased, requiring 10 adducts/lethal hit.
Figure 12. Survival of *E. coli* DL7 cells after uv irradiation.

Survival decreased in a dose-dependent, linear manner for cells that were uv irradiated by using previously described methodology (Lasko et al., 1988; Bradley, 1991). Optimization of the uv irradiation process (as described in the text) resulted in enhanced survival at low levels of uv irradiation (up to ~40 J/m²). Above 40 J/m² cell survival decreased more rapidly (the curve approximating this response on the diagram is a computer generated 3rd order polynomial having no physical significance).
Cell survival (%) after UV irradiation

uv dose (J/m²)

Methodology after Lasko et al. (1988)
Modified methodology (see text)
Figure 13. Mutation frequency as a function of uv dose given to host E. coli DL7 cells. M13mp18 RF genomes modified with an average of 32 cis-DDP DNA adducts were transfected into bacterial cells uv irradiated at the indicated fluences up to 80 J/m². The data were approximated by a 3rd order polynomial determined by Cricket Graph (Cricket Software; y = 0.000x³ - 0.052x² + 2.511x + 0.597). The mutation frequency increased in an exponential-like manner up to ~30 J/m² and remained constant to ~60 J/m². Due to the variability of the data it was unclear if the apparent increase in the mutation frequency between 60 and 80 J/m² were a real result; regardless, the low cell survival at 80 J/m² (see Figure 12) precluded the use of cells experiencing such high levels of uv irradiation. Practically, a fluence of >30 J/m² was sufficient to achieve an adequate level of mutagenesis while a fluence of <40 J/m² maintained high cell survival (Figure 12). Accordingly, subsequent uv irradiation was done at fluences of 35-40 J/m².
B. Comparison of the Genotoxicities and Mutagenicities of the \textit{cis-DDP} \textit{G*G*}, \textit{A*G*}, and \textit{G*TG*} DNA Adducts

1. Preparation of \textit{cis-DDP} Modified Oligodeoxynucleotides

The Stu 24, Sca 24, ApaL 24 unmodified and the Stu Pt, Sca Pt, and ApaL Pt \textit{cis-DDP} modified oligodeoxynucleotides were characterized after synthesis and gel purification. Greater than 99.7\% of 5\' $\gamma$-$^{32}$P labeled Stu 24, Sca 24, and ApaL 24 unmodified oligodeoxynucleotides ran as a single band on a denaturing 20\% polyacrylamide gel (determined by PhosphorImager analysis) indicating that these samples were of high purity. The migration of the \textit{cis-DDP} modified oligodeoxynucleotides was retarded by about 6\% on a 20\% denaturing polyacrylamide gel, resulting in a separation of 2 cm between platinum modified and unmodified samples run on a 35 x 43 cm gel. This degree of separation allowed for efficient gel purification of the modified oligodeoxynucleotides. Stu Pt was >99.7\% pure, Sca Pt about 99.4\% homogenous, while ApaL Pt was purified to approximately 98\% homogeneity. None of the platinated 24-mers were contaminated by a detectable amount (i.e. <0.05\% by PhosphorImage quantitation) of unmodified material. The ~2\% contamination in the ApaL Pt sample migrated within 2 mm of the putatively G*TG* modified oligodeoxynucleotide during denaturing PAGE indicating that this material probably was \textit{cis-DDP} modified at sites other than G*TG*. This contaminant also coeluted under a variety of HPLC conditions making it practically impossible to purify ApaL Pt to greater than 98\% homogeneity. It is interesting to note that these impurities disappeared upon cyanide treatment of ApaL Pt, confirming that they were a consequence of \textit{cis-DDP} modification, possibly as monoadducts and 1,2- or 1,4-intrastrand crosslinks. The identity of this contaminant is explored further below.
The purified oligodeoxynucleotides were characterized further by HPLC analysis of their constituent monodeoxynucleosides. The base composition analysis revealed that all of the 24-mers were of the expected composition. A detailed description of the base analysis will be included in this report only for the ApaL 24 and ApaL Pt oligodeoxynucleotides, although all oligodeoxynucleotide 24-mers were similarly analyzed.

Unmodified ApaL 24 oligodeoxynucleotide was digested in triplicate and analyzed by using reverse-phase HPLC. Peaks were identified by spectral analysis and comparison to monodeoxynucleoside standards. The area of the peaks corresponding to each deoxynucleoside was integrated and normalized based on its extinction coefficient, and then further normalized based on the most abundant base, dT. The following data are from the 3 ApaL 24 digests:

<table>
<thead>
<tr>
<th>Base</th>
<th>Expected</th>
<th>ApaL 24 Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>dC</td>
<td>8</td>
<td>7.75 ± 0.13</td>
</tr>
<tr>
<td>dG</td>
<td>2</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>dT</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>dA</td>
<td>1</td>
<td>1.15 ± 0.10</td>
</tr>
</tbody>
</table>

The ApaL 24 oligodeoxynucleotide is of the expected composition when values are rounded to the nearest integer. ApaL Pt should be identical to ApaL 24 after treatment with cyanide to remove the cis-DDP G*TG* adduct. This premise is supported by the observations that cyanide treated ApaL Pt coelutes with ApaL 24 on reverse phase HPLC and comigrates under denaturing polyacrylamide gel electrophoresis. Further evidence that the two species are identical is derived from the digestion and HPLC analysis of cyanide treated ApaL Pt (designated ApaL Pt(CN)):
The base composition values for the cyanide treated sample, ApaL Pt(CN), are within the values previously established for the unmodified ApaL 24 sample (previous page and right column above). These data support the finding that the retarded mobility of cis-DDP treated ApaL 24 during gel electrophoresis is due to the presence of a platinum adduct that was removed during the cyanide treatment. The identity of this putative G*TG* adduct was confirmed by HPLC analysis of the digestion products of ApaL Pt:

<table>
<thead>
<tr>
<th>Base</th>
<th>Expected</th>
<th>ApaL Pt (CN)</th>
<th>ApaL 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>dC</td>
<td>8</td>
<td>7.72</td>
<td>7.75 ± 0.13</td>
</tr>
<tr>
<td>dG</td>
<td>2</td>
<td>1.56</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>dT</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>dA</td>
<td>1</td>
<td>1.08</td>
<td>1.15 ± 0.096</td>
</tr>
</tbody>
</table>

The disappearance of dG corresponded to the appearance of a new peak that coeluted with a Pt(dG)₂(NH₃)₂ standard (from S. Bellon, MIT), indicating that most of the ApaL Pt oligodeoxynucleotide contained the desired G*TG* cis-DDP adduct. The presence of a small dG peak, however, reinforced the PAGE observation that this oligodeoxynucleotide contained ~ 2% impurity (i.e. not all of the dG was cis-DDP modified, allowing some monomeric dG to form. No monomeric dG, and therefore no dG peak, would be expected from a sample containing 100% G*TG*). The observation that there was slightly less than expected dC and dA (0.04 for dC and 0.02 for dA based on the ApaL Pt data compared to the ApaL 24 data)
suggested the nature of the impurity in the ApaL Pt oligodeoxynucleotide. The "missing" dC and dA could be due to the presence of cis-DDP 1,2 G*C*, 1,4 G*TGC*, and 1,3 G*CA* intrastrand crosslinks.

The composition of the 2% impurity contaminating the ApaL Pt oligonucleotide was explored by polyacrylamide gel purification of material running close (within 5 mm) to the band containing the desired G*TG* adduct. This material (denoted as ApaL Pt'), after 5' γ-32P labeling, ran as a smear on an analytical gel, suggesting that it is a mixture of several cis-DDP intrastrand crosslinks. Digestion and HPLC analysis showed significant, but less than expected, modification at dG positions:

<table>
<thead>
<tr>
<th>Base</th>
<th>Expected</th>
<th>ApaL Pt'</th>
<th>ApaL 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>dC</td>
<td>8</td>
<td>6.94</td>
<td>7.75 ± 0.13</td>
</tr>
<tr>
<td>dG</td>
<td>0</td>
<td>0.39</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>dT</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>dA</td>
<td>1</td>
<td>0.78</td>
<td>1.15 ± 0.10</td>
</tr>
</tbody>
</table>

In parallel with the appearance of unmodified dG (0.39), 0.81 fewer dC's and 0.38 fewer dA's were observed than expected based on the previous analysis of ApaL 24. This analysis is consistent with the previous hypothesis that the observed impurities are 1,2 G*C*, 1,4 G*TGC*, and 1,3 G*CA* cis-DDP adducts.

The Stu 24, Stu Pt, Sca 24, and Sca Pt oligodeoxynucleotides were subjected to similar (but less extensive due to the absence of detectable levels of impurities) analysis and determined to be of the desired base and adduct composition. Even further confirmation that these samples were of the intended composition was obtained, somewhat
indirectly, after the oligodeoxynucleotides were incorporated into M13-derived SS genomes. Replication of these genomes in E. coli resulted in progeny that were subjected to DNA sequencing. In all cases the progeny contained sequences corresponding to the putative oligodeoxynucleotide sequences, once again confirming the composition of the cis-DDP modified and unmodified oligodeoxynucleotide 24-mers.

2. Preparation of Singly cis-DDP Modified M13 SS Genomes

Bacteriophage M13-derived ss genomes containing the cis-DDP G*G*, A*G*, and G*TG* adducts were constructed based on the methodology outlined in Figure 8. Scaffold oligodeoxynucleotides were purified to >95% homogeneity, with only a small amount of contaminating 63-mer and a trace of 62-mer present. Based on previous work (Banerjee et al., 1988) this level of purity was within acceptable limits to allow the scaffold oligodeoxynucleotides to be utilized for genome construction.

EcoR I digestion of M13mp7L2 ss genomes resulted in complete conversion of circular DNA to the linear form as determined by agarose gel electrophoresis. Annealing of the Stu 64, Sca 64 or ApaL 64 scaffold oligodeoxynucleotides to the linearized ss genomes restored about 50% of the material to the circular form. This circular material apparently had both ends of the scaffold annealed to the respective ends of the linear genomes with a 24 nucleotide gap complementary to the 24-mers in between. 5’-Phosphorylated Stu 24, Stu Pt, Sca 24, Sca Pt, ApaL 24 and ApaL Pt were ligated into genomes containing their respective complementary sequences. The ligation reactions were determined to be highly efficient, with essentially all of the 50% of the material that was in the circular form after the scaffold/genome annealing process remaining circular even after the scaffold oligodeoxynucleotides had been removed by heat denaturation. These genomic constructs could only
remain in the circular form if the 24-mers had been covalently incorporated into the linear genomes at both their 5' and 3' termini. In contrast, all of the originally circular material was converted back to linear form after heat denaturation of the scaffold in samples that were subjected to ligation conditions, but without 24-mer present. This control ensured that ligation across the 24 nucleotide gap did not occur under the conditions used, ensuring that the 5' and 3' ends of the linearized M13mp7L2 genomes did not religate.

A test transformation was performed to demonstrate that linear M13 ss genomes were biologically inactive and therefore incapable of producing progeny in E. coli DL7 cells. Portions of the ligation reactions done without 24-mer present were linearized by heat denaturation and transfected into E. coli DL7 cells in parallel with control samples comprised of DNA from samples where the ligation was done with unmodified 24-mer present. Infective center plaques were counted to ascertain the relative biological activity of the linear and circular genomes. The linear genomes formed only 0.05-0.2% as many plaques as the unmodified circular genomes. DNA sequence analysis showed that the plaques from this putatively linear DNA arose from a low level of contaminating parental M13mp7L2 genomes that remained uncut during and EcoR I digest and undetected during the agarose gel electrophoresis that had been used to assess the extent of the EcoR I digestion. No progeny were found that were derived from the EcoR I linearized form of M13mp7L2 DNA. From the number of plaques sequenced, combined with the survival of the linearized DNA, it was estimated that the linear ss genomes had a plaque forming ability at least 10^5 fold lower than the circular form of the genome. These results demonstrated that the genetically engineered genomes could be used without further purification, since only the desired product, a circular genome with a 24-mer covalently ligated at both ends, was biologically viable.
3. Genotoxicities of Singly cis-DDP Modified M13 SS Genomes

The genotoxicities of the cis-DDP G*G*, A*G*, and G*TG* adducts were determined by transfecting the Stu Pt, Sca Pt, and ApaL Pt M13 derived ss genomes\(^1\) into \textit{E. coli} DL7 cells in parallel with equal amounts of the unmodified Stu 24, Sca 24, and ApaL 24 genomes. Initially, survival values were determined separately for genomes made with either a 2-fold or 100-fold excess of the 24-mer oligodeoxynucleotides (See Figure 8). Results from these experiments, done in non SOS induced \textit{E. coli} DL7 cells reveal no differences between genomes constructed by either methodology (Figure 14).\(^2\) Therefore, no further distinction was made between results obtained by using genomes constructed by either method. Survival was also determined for the cis-DDP modified and control genomes replicated in SOS induced cells. Results for toxicities under SOS conditions, compared with the data from the non SOS induced samples, are given in Figure 15. Without SOS induction, the G*G* adduct was the most genotoxic lesion with a survival of only 5.16 \(\pm\) 1.15\% compared to unmodified genomes. Genomes containing the G*TG* adduct had a 2.5 fold higher survival at 13.5 \(\pm\) 2.5\% while survival for A*G* containing genomes was even higher at 21.6 \(\pm\) 2.6\%.

Upon SOS induction, survival for genomes with G*G* and A*G* \textit{cis}-DDP adducts increased to about the same absolute level, 30.8 \(\pm\) 5.4\% and 32.4

---

\(^1\)The nomenclature of the site-specifically \textit{cis}-DDP modified genomes and their unplatinated counterparts is as defined in the list of abbreviations.

\(^2\)As previously mentioned, if the platinated 24-mer oligodeoxynucleotides were contaminated with unmodified 24-mers, the unmodified material might be incorporated in the M13 genomes more efficiently than the platinated 24-mers. To give an example, if an 100-fold excess of 24-mer was used, a 1\% unmodified contaminant theoretically could be incorporated into 100\% of the genomes, but only into 2\% if the genomes if a 2-fold excess of 24-mer was used. Since no differences were observed between genomes made by either method, however, it is reasonable to assume that the \textit{cis}-DDP modified genomes were not contaminated with unplatinated material. This conclusion is consistent with the analysis that showed that the platinated 24-mers contained \(<\)0.03\% of unmodified material.
± 4.9%, respectively. The relative increase in survival, however, was much higher for the G*G* adduct (-6 fold) than for the A*G* adduct (0.5 fold). Interestingly, survival was not enhanced upon SOS induction for the G*TG* adduct, remaining at 14.4 ± 3.7%, a value not significantly above the 13.5 ± 2.5% survival seen for replication in non-SOS induced cells.

4. Mutagenicities of Singly cis-DDP Modified M13 SS Genomes

The mutation frequencies of the cis-DDP G*G*, A*G*, or G*TG* adducts situated in genomes replicated in either SOS induced or non-SOS induced E. coli DL7 cells were determined in parallel with unmodified control genomes. Putative mutation frequencies, determined after the restriction digest mutation selection scheme (Figure 9), are listed in Table 3. As anticipated from the results of previous studies, the highest mutation frequencies were obtained from cis-DDP modified genomes replicated in SOS induced cells. The putative mutation frequencies for cis-DDP modified genomes were much lower when replicated in non-SOS induced cells, as was the situation for unmodified genomes replicated in either SOS induced or non-SOS induced cells.

Only samples that contained a single (or in some cases tandem) base changes in the six nucleotide restriction sites were scored as putative mutants. Samples not scored as putative cis-DDP induced mutants fit into one of five categories:

a. M13mp7L2 contaminating parental genomes from incomplete EcoR I digestion

b. "Wild type" genomes corresponding to the sequence of the genetically engineered constructs
c. Large (−800 bp) deletions of a specific composition

d. Variable, mid size (−30–80 bp) deletions, generally lacking the entire sequence corresponding to the 24-mer and varying lengths of the adjacent DNA sequence

e. A specific 15 bp deletion consisting of the restriction site and one of the flanking d(TCT TCT TCT) sequences

The parental M13mp7L2 and "wild type" genomes were present at frequencies up to 1%, the various deletions were less abundant, occurring at frequencies less than 0.1%.

The identification of a large number of mutants, from both cis-DDP modified and unmodified samples, was required to determine which mutations were platinum induced as compared to those arising spontaneously. To facilitate the identification of mutants, a second round of selection was done to enrich for the desired base pair mutations occurring within one of the Stu I, Sca I, or ApaL I restriction sites. The RF DNA preparations, restriction digestions, transfections and sequencing of infective center plaques was done as in the initial selection process with a modification of the restriction digest step. EcoR I was added to the digestion reactions to remove contaminating M13mp7L2 genomes. A larger excess of the selection enzyme (Stu I, Sca I, or ApaL I) was used to ensure complete elimination of any residual wild type genomes. Sequencing after this second round of selection yielded from 90% to over 99%, depending on the sample, of the desired restriction site mutants. The remainder of the samples was the large (c, above) or 15 bp (e, above) deletion mutants. These genomes lacked the requisite restriction sites and were refractory to cutting and, therefore, could not be selected against during the digestion
reactions. By contrast, the mid-sized (d, above) deletion mutants retained at least one EcoR I site\(^1\) and were eliminated along with the residual wild type and parental M13mp7L2 genomes.

The relative abundance of the various mutations seen in the Stu I, Sca I, or ApaL I restriction sites are given in Table 4 a, b, and c, respectively. Genomes containing either cis-DDP G*G* or A*G* adducts that were replicated in SOS induced cells experienced significantly enhanced frequencies of specific mutations that occurred at the site of cis-DDP modification. For example, 75% of the 81 mutants identified in the G*G* spectrum (Table 4 (a)) were G → T transversions at the 5' dG of the d(AGGCCT) sequence suggesting that these mutations were a consequence of cis-DDP modification. Additionally, tandem mutation of the d(ApG) nucleotides of the Stu I recognition sequence to d(TpT) and d(CpT) was adduct specific, albeit at a much lower frequency. The combined cis-DDP induced mutations occurred at a frequency of 1.39% (Table 5). Similarly, mutations occurring in A*G* genomes replicated in SOS induced cells were highly targeted, with 65% of the 100 mutants identified consisting of A → T transversions at the 5' base of the A*G* adduct (Table 4 (b)). Also similar to G*G*, a tandem d(TpA) → d(CpT) double mutation was adduct specific; it only appeared in cis-DDP modified genomes replicated in SOS induced cells. Less definitive is the A → G transition that is 2.7 fold more prevalent in the modified samples (at 16%) than in the unmodified samples (at 5.9%). Considering the A → G transition to be platinum specific,\(^2\) the A*G* adduct produced

---

\(^1\)The presence of the EcoR I site indicated that these deletion mutants were progeny of undigested M13mp7L2 parental genomes (a, above).

\(^2\)An A → G transition arising at the 5' nucleotide of the cis-DDP A*G* was also observed at approximately the same relative abundance in another site specific study (Burnouf et al., 1990 - see Discussion for detail). It should be noted that if the 5' nucleotide of a cis-DDP 1,2 intrastrand crosslink does have a propensity to induce N → G mutations, these mutations would not be observed for the G*G* adduct (i.e. G → G is not a mutation) and would only show up in the mutational spectra of
a 5.98% mutation frequency. The data summarizing cis-DDP induced mutations are given in Table 5.

By contrast to the mutational spectra induced by the cis-DDP A*G* and G*G* containing genomes, the mutational spectrum arising genomes containing the cis-DDP G*TG* adduct that had been replicated in SOS induced E. coli DL7 cells showed no targeted mutations. As can be seen from Table 4 (c), the most abundant mutation, 23% of the total 143 samples analyzed, was a C → T transition at the 5′ dC of the d(GTGCAC) sequence. This mutation was even more prevalent (at 29%) in the unmodified spectrum, suggesting that it was not cis-DDP induced. Additionally, no mutations occurred at the cis-DDP modified dGs at a rate significantly higher than in the control mutational spectra. It is clear that the cis-DDP G*TG* adduct is not mutagenic under cellular conditions that induce high mutation frequencies for the cis-DDP G*G* and A*G* adducts.

Control experiments were done in which platinated genomes were replicated in non-SOS induced E. coli DL7 cells. In addition, unmodified genomes were replicated in both SOS induced or non-SOS induced cells. The results of these experiments (Table 4 a, b, and c, bottom three panels) revealed mutations that were broadly distributed with no apparent pattern that would indicate that the observed mutations were induced by the cis-DDP DNA adducts. The platinated samples did not experience a significantly greater occurrence of mutations at the bases modified with cis-DDP than at any other base within the restriction sites. In fact, the platinated and unmodified genomes produced similar mutational patterns, suggesting that these background mutations (occurring at the frequencies indicated in Table 5) were likely a

A*G*. Considering these two factors, this A → G transversion was deduced to be a legitimate platinum induced mutation.
consequence of the genetic engineering processes used to construct the singly modified genomes. These control experiments also confirmed that *cis*-DDP mutagenesis is an SOS dependent process in *E. coli*. 
Table 3. Putative mutation frequencies induced by cis-DDP G*G*, A*G*, or G*TG* adducts

<table>
<thead>
<tr>
<th>Restriction Site(^a)</th>
<th>cis-DDP modified</th>
<th>SOS induced</th>
<th>MF(^b) after digestion</th>
<th>Mutants/Total(^c)</th>
<th>Adjusted MF(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGCCT</td>
<td>+</td>
<td>+</td>
<td>5.1 %</td>
<td>11/32</td>
<td>1.7 %</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>1.4 %</td>
<td>8/32</td>
<td>0.35%</td>
</tr>
<tr>
<td>Stu I</td>
<td>+</td>
<td>-</td>
<td>0.75%</td>
<td>19/56</td>
<td>0.25%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0.12%</td>
<td>15/38</td>
<td>0.05%</td>
</tr>
<tr>
<td>AGTACT</td>
<td>+</td>
<td>+</td>
<td>9.1 %</td>
<td>24/32</td>
<td>6.8 %</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>1.3 %</td>
<td>2/32</td>
<td>0.08%</td>
</tr>
<tr>
<td>Sca I</td>
<td>+</td>
<td>-</td>
<td>1.3 %</td>
<td>5/32</td>
<td>0.20%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>1.2 %</td>
<td>0/30</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>GTGCAC</td>
<td>+</td>
<td>+</td>
<td>4.1 %</td>
<td>41/128</td>
<td>1.3 %</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>2.9 %</td>
<td>4/32</td>
<td>0.36%</td>
</tr>
<tr>
<td>Apal I</td>
<td>+</td>
<td>-</td>
<td>0.89%</td>
<td>25/32</td>
<td>0.70%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0.88%</td>
<td>26/32</td>
<td>0.72%</td>
</tr>
</tbody>
</table>

\(^a\) The restriction recognition sites containing the three cis-DDP DNA adducts (underlined) are shown.

\(^b\) After digestion with the appropriate restriction enzyme only mutated sequences should be viable and give rise to progeny, the proportion of these species are given in relation to undigested controls. In theory, these values would be the platinum induced mutation frequencies (MF), but, in practice they may include nonspecific mutations or wild type sequences that escaped selection (for a more detailed discussion, see text and Figure 9).

\(^c\) Putative mutants from \(^b\) were sequenced. The numbers given are the number of mutants per total number of independent replicas analyzed by sequencing.

\(^d\) The adjusted MF is equal to the putative mutation frequency from \(^b\) multiplied by the fraction of mutants detected in \(^c\). It should be noted that the adjusted MF includes all single (and double) base pair changes detected in the restriction sites. Mutation analysis was performed as described in Table 4 and Table 5 to determine cis-DDP induced mutations.
Table 4(a). Relative abundance of mutations induced by the cis-DDP G*G* adduct.a

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>A</th>
<th>G</th>
<th>G</th>
<th>C</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOS(+)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.9</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.2</td>
<td>2.5</td>
<td>2.5</td>
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<tr>
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</tr>
<tr>
<td>T</td>
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<td>75</td>
<td>2.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Δ</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>other</td>
<td>T(C)-T</td>
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<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOS(-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
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<td></td>
<td>4.8</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.8</td>
<td>9.5</td>
<td>1.6</td>
<td>3.2</td>
<td>4.8</td>
<td></td>
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</tr>
<tr>
<td>G</td>
<td>1.6</td>
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<td>4.8</td>
<td>4.8</td>
<td></td>
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</tr>
<tr>
<td>T</td>
<td>4.8</td>
<td>4.8</td>
<td>7.9</td>
<td>4.8</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ</td>
<td>3.2</td>
<td>9.5</td>
<td>9.5</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unmod.</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>A</td>
<td>16</td>
<td>5.3</td>
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</tr>
<tr>
<td>C</td>
<td>32</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>5.3</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Δ</td>
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<td></td>
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<td></td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

- A: Adenine
- C: Cytosine
- G: Guanine
- T: Thymine
- Δ: Delta
- SOS(+): Single-strand breaks
- SOS(-): Double-strand breaks
- Pt’d: Pretreatment
- Unmod.: Unmodified
- other: Other variations
- n: Number of experiments

a: Modified from the original data.
Table 4 (b). Relative abundance of mutations induced by the \textit{cis}-DDP A$^*$G$^*$ adduct.

\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & T & A & G & T & A & C & T \\ \hline
\text{SOS(+) A} & 1.0 & & & 1.0 & & & \\ \hline
\text{Pt'd C} & 4.0 & & & 1.0 & & & \\ \hline
\text{G} & 16 & & & & & & \\ \hline
\text{(n=100) T} & 65 & & & 4.0 & & & \\ \hline
\text{\Delta} & & & & & & 1.0 & \\ \hline
\text{other} & & & & & C-T & 7.0 & \\ \hline
\text{SOS(+) A} & 14 & 3.9 & 2.0 & 5.9 & & & \\ \hline
\text{Unmod. C} & 5.9 & 18 & 5.9 & 3.9 & & & \\ \hline
\text{G} & 5.9 & & 3.9 & 2.0 & & & \\ \hline
\text{(n=51) T} & 14 & & 2.0 & & & & \\ \hline
\text{\Delta} & 5.9 & & & 2.0 & & & \\ \hline
\text{insertion} & & & & & 2.0 & 2.0 & \\ \hline
\text{SOS(-) A} & 7.9 & & 7.9 & & & & \\ \hline
\text{Pt'd C} & 5.3 & 7.9 & & 11 & & & \\ \hline
\text{G} & 5.3 & & 7.9 & & & & \\ \hline
\text{(n=38) T} & 5.3 & & 7.9 & & & & \\ \hline
\text{\Delta} & 11 & 2.6 & 7.9 & 5.3 & 18 & & \\ \hline
\text{other} & & & & & & & \\ \hline
\text{SOS(-) A} & 29 & & & & & & \\ \hline
\text{Unmod. C} & 14 & 29 & & & & & \\ \hline
\text{G} & & & & & & & \\ \hline
\text{(n=7) T} & & & & & & & \\ \hline
\text{\Delta} & & & & & 29 & & \\ \hline
\text{other} & & & & & & & \\ \hline
\end{tabular}
Table 4 (c). Relative abundance of mutations induced by the cis-DDP G*TG* adduct.

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>G</th>
<th>T</th>
<th>G</th>
<th>C</th>
<th>A</th>
<th>C</th>
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<th>C</th>
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<tbody>
<tr>
<td>SOS(+) A</td>
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<td>2.1</td>
<td>5.6</td>
<td>7.7</td>
<td>0.7</td>
<td></td>
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<tr>
<td>Pt’d   C</td>
<td>3.5</td>
<td>1.4</td>
<td>3.5</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G</td>
<td>2.1</td>
<td>0.7</td>
<td>4.2</td>
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<tr>
<td>(n=143) T</td>
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<tr>
<td>Δ</td>
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<td>4.2</td>
<td>2.8</td>
<td>0.7</td>
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<td>T-T</td>
<td>2.8</td>
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<td></td>
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<tr>
<td>SOS(+) A</td>
<td>5.8</td>
<td>2.9</td>
<td>5.8</td>
<td>1.0</td>
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<tr>
<td>Unmod. C</td>
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<tr>
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<td>1.9</td>
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<td>(n=104) T</td>
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<tr>
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</tr>
<tr>
<td>SOS(-) A</td>
<td>32</td>
<td>12</td>
<td>4.0</td>
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</tr>
<tr>
<td>Pt’d   C</td>
<td>4.0</td>
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<tr>
<td>G</td>
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</tr>
<tr>
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<td>4.0</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOS(-) A</td>
<td>19</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmod. C</td>
<td>7.7</td>
<td>12</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=26) T</td>
<td>27</td>
<td>23</td>
<td></td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. The target DNA sequence, with or without cis-DDP modification at the underlined bases, is given in large type across the top of the table. Mutations observed in this DNA sequence after replication in SOS induced or non induced cells are indicated in the boxes below the sequence. The
numbers given are the relative proportion of each mutation observed, given as a percentage of "n" (where n = the total of all mutations determined for each panel).
b. Both the d(GpC) and d(CpA) sequences were mutated to d(TpT) at the indicated frequencies.
c. Both the d(GpC) and d(CpA) sequences were mutated to d(TpT) at the indicated frequencies.
Table 5. Mutations induced by the cis-DDP G*G*, A*G*, and G*TG* adducts

\[
\begin{array}{cccc}
A & G* & G* & C & C & T \\
\downarrow & & & & & \\
T & 1.31 \% & & & & \\
T--T & 0.044 \% & & & & \\
C--T & 0.044 \% & & & & \\
Total: & 1.39 \% & & & & \\
\end{array}
\]

\[
\begin{array}{cccc}
T & A* & G* & T & A & C \\
\downarrow & & & & & \\
T & 4.42 \% & & & & \\
G & 1.09 \% & & & & \\
C--T & 0.48 \% & & & & \\
Total: & 5.98 \% & & & & \\
\end{array}
\]

\[
\begin{array}{cccc}
G* & T & G* & C & A & C \\
\downarrow & & & & & \\
\text{No cis-DDP induced} & & & & & \\
\text{mutants observed} & & & & & \\
\end{array}
\]

\[
\begin{array}{cccc}
\text{Background Mutation Frequencies} & & & \\
\text{SOS(+) Platinated:} & 0.35\% & & \\
\text{Unmodified:} & 0.35\% & & \\
\text{SOS(-) Platinated:} & 0.25\% & & \\
\text{Unmodified:} & 0.05\% & & \\
\end{array}
\]

a. Mutations were determined to be cis-DDP induced by analysis of the mutational spectra presented in Table 4. Mutations that occurred at a significantly higher frequency at or near the site of platinum modification in the SOS(+) cis-DDP modified samples compared to the unmodified and SOS(-) samples were deemed to be drug induced. The remainder of the mutations were nonspecific events comprised the data indicated under "Background Mutation Frequencies."
Figure 14. Survival of singly cis-DDP modified M13 ss genomes in non-SOS induced *E. coli* DL7 cells. The survival of genomes constructed with either a 100-fold or 2-fold excess of 24-mer oligodeoxynucleotide was determined by using the M13 plaque forming assay. The values given, relative to unmodified control genomes and including 11-12 replicas per data point, indicate that no differences exist between genomes made by either method. In subsequent experiments no distinction is made between either type of genome.
Survival (%) relative to unmodified genomes

- Genomes constructed with a 100-fold excess of 24 mer oligodeoxynucleotide
- Genomes constructed with a 2-fold excess of 24 mer oligodeoxynucleotide

cis-DDP DNA adduct

G*G*  A*G*  G*TG*
**Figure 15.** Survival of singly cis-DDP modified M13 ss genomes in SOS induced, or non induced, *E. coli* DL7 cells. Survival values were determined in the plaque forming assay and are relative to unmodified control genomes. The data for the non SOS induced cells are pooled values of the results shown in Figure 14 with 23-24 replicas per sample. The data from the SOS induced samples is the average of 40-42 replicas.
cis-DDP DNA adduct
C. Comparison of the Mutagenicities, Genotoxicities and Adduct Distributions of cis-DDP and ACDP DNA Adducts

1. Survival of cis-DDP and ACDP Modified M13mp18 RF Genomes

A key goal of this thesis work was to compare the genotoxicity of cis-DDP and ACDP. Accordingly, M13mp18 RF DNA was modified with both platinum compounds. Atomic absorption spectroscopy indicated that almost all the drug present in solution bound to the M13mp18 RF DNA in the 16 hour platinum reactions. Adduct levels of the cis-DDP and ACDP treated M13mp18 RF genomes are given in Table 6. In a plaque forming assay, transformation efficiencies of E. coli DL7 with unmodified M13mp18 RF DNA were 1-4 x 10^9 transformants/µg DNA for non-SOS induced samples and slightly lower (from 1-5 x 10^8 transformants/µg DNA) for SOS induced samples. Increasing numbers of DNA adducts reduced the number of infective centers in a dose-dependent manner for genomes modified with either platinum compound (Figure 16). cis-DDP appeared to be slightly more genotoxic than ACDP, averaging 3.33 adducts per lethal hit compared to 5.22 adducts per lethal hit for ACDP (Table 7). When the host DL7 cells were pretreated with UV irradiation to induce the SOS response, survival relative to unmodified genomes increased approximately threefold for DNA treated with either drug.

2. Identification of Mutants

¹In an earlier experiment, cis-DDP was slightly less toxic than in the present experiment. The earlier value of 4.61 adducts/lethal hit (see Figure DL7/DL6) for cis-DDP modified genomes replicated in non SOS induced cells was very close to the genotoxicity of ACDP reported here. Interestingly, the earlier genotoxicity of 10.1 adducts/lethal hit for cis-DDP in SOS induced cells is essentially identical to the present value of 10.3 adducts/lethal hit.
The mutational assay used in this study is based on the ability of the lacZ' peptide fragment encoded by the M13mp18 viral genome to serve as an α-donor to complement the host bacterial M15 protein, the α-acceptor, to produce β-galactosidase activity in a process known as α-complementation (LeClerc et al., 1984). With X-gal used as the prochromophore, β-galactosidase activity arising from the lacZ' α-complementation process results in blue plaques when M13mp18 bacteriophage are plated with IPTG, X-gal and E. coli GW5100 cells. Mutations in the M13mp18 lacZ' regulatory or peptide coding regions that affect α-complementation give rise to phenotypically distinct plaques. The colors of mutant plaques observed in this study ranged along a continuum from having no detectable β-galactosidase activity (colorless plaques) to slightly less than wild-type activity (light blue plaques) as observed previously (LeClerc et al., 1984; Decuyper-Debergh et al., 1987). Interestingly, a few mutants exhibited much greater than wild-type β-galactosidase activity and formed intensely dark blue plaques. Quantitation of their β-galactosidase activity revealed up to 60 fold enhancement of enzyme activity over wild-type (Table 8).

3. Determination of Mutation Frequency

The mutation frequency was determined by dividing the sum of colorless, light blue and dark blue mutant plaques by the total number of plaques. The mutation frequencies for replicates showed high standard error margins (±35%) making it necessary to repeat the mutation frequency experiments several times to enable statistically valid comparisons between the two drugs (Table 9). Mutagenesis was SOS-dependent for both platinum drugs. Under the appropriate SOS conditions the mutation frequency increased in an adduct dependent manner for both compounds, with cis-DDP being approximately twofold more mutagenic than ACDP (Figure 17).
4. *LacZ'* Mutational Spectra of *cis*-DDP and ACDP Modified DNA

Mutations in the *lacZ'* DNA sequence of M13mp18 which had been identified by phenotypically distinct plaques were determined by DNA sequencing. A total of 303 mutants was identified by sequencing, 142 from ACDP-modified DNA, 115 from *cis*-DDP-modified DNA, and 46 from unmodified control genomes. Most of the mutations induced by either drug were single base substitutions with a smaller number of single base deletions or insertions, as shown in Figure 18. Spontaneous mutations arising from unmodified DNA are shown in Figure 19. A few samples had multiple substitutions or large deletions or both; these mutants are described in Table 10. A summary of all mutations by type is given in Table 11. The amino acid changes resulting from mutations in the DNA sequence corresponding to the coding region for the *lacZ'* polypeptide are listed in Table 12.

Mutational spectra for the platinum compounds were derived from genomes with an average of 1.85 *cis*-DDP or 2.36 ACDP adducts in the 370 nucleotide segment of the *lacZ'* DNA sequence that was the target for detectable mutations (Figure 18). At these adduct levels, the mutation frequency was approximately eight and five fold higher than the spontaneous mutation frequency for *cis*-DDP and ACDP modified genomes, respectively (Figure 17). The induced spectra, therefore, would be expected to be comprised of 10-20% spontaneous mutants. Nevertheless, the spontaneous mutational spectrum was significantly different than that induced by either platinum drug. Considering single base changes, the induced spectra had twice as many transversions as transitions in contrast to the spontaneous spectrum, which had almost a 3:1 ratio of transitions to transversions. CG → TA transitions comprised almost half of the spontaneous mutations. In addition, the spontaneous spectrum had relatively more deletions and multiple base changes than the induced
spectra. Seven of the 46 spontaneous mutations were two specific large deletions; from position 5970 to 6172 and from 6250 to 6396. The 6250-6396 deletion comprises the same region that is absent from the M15 \( \alpha \)-acceptor protein; similar mutations have been observed in other \( \text{lacZ}' \) mutational assays and are attributable to recombination with the F' episome of the host bacterium (LeClerc et al., 1984). The 5970-6172 deletion is also possibly due to a recombination event suggesting that these large deletions, although observed in the drug induced spectra (at much lower frequencies) are the result of spontaneous events and not a consequence of the cis-DDP or ACDP DNA adducts.

5. Replication Mapping of DNA Adducts

The secondary structure of double-stranded M13mp18 RF genomes required that adduct mapping experiments be performed by using one thermal cycle with Vent\(_R\)\(^\text{TM}(\text{exo})\) DNA polymerase (see Discussion). The signal from single cycle reactions was considerably weaker than from the multicycle reactions employed by others who have used this methodology (Bubley et al., 1991; Murray et al., 1992). Fragments up to 150 bases in length were difficult to visualize by autoradiography on x-ray film, but could be seen after exposure to a phosphorimaging plate (Amemiya & Miyahara, 1988) followed by analysis with a Molecular Dynamics PhosphorImager. Fragments longer than 200 bases gave stronger signals, but were difficult to align at base resolution on the concurrently run sequencing ladder. These restrictions required that the \( \text{lacZ}' \) region be mapped by using primers offset by 100 to 150 nucleotides, generating at least two sets of fragments for every nucleotide position. Fragments having fewer than 150 bases could be aligned precisely with the sequencing ladder and were used to determine location of the termination sites. Longer fragments corresponding to the same termination sites, but generated by a more distant primer, were used to determine the
intensity of the stop site by PhosphorImager quantitation. The pattern of termination sites was very similar for DNA modified at either low (11.6 adducts per genome for cis-DDP or 6.6 adducts per genome for ACDP) or high (35.4 or 45.0 adducts per genome, respectively) levels. The major difference was that the genomes containing fewer adducts gave rise to a greater amount of high molecular weight material corresponding to fragments of more than 500 nucleotides in length. Conversely, the genomes platinated with the higher levels of drug produced almost no fragments above 400 bases in length. Consequently shorter fragments were more abundant and gave enhanced signal intensity; therefore, only the highly modified samples were subjected to rigorous quantitation. The resulting adduct distributions are graphically displayed in Figure 18.

The adduct mapping experiments showed that DNA modification predominantly occurred at d(GpG) sites, especially at runs of three or more G’s and, to a lesser extent, at d(ApGpG) sites, for both drugs. Weaker termination sites were detected at d(ApG), d(GpNpG), and a variety of other sites. PhosphorImager quantitation of strong termination sites yielded accurate information on the relative abundance of G*G* and, to a lesser extent, A*G* and G*NG*, adducts. Unfortunately, two factors complicated the quantitative evaluation of weak termination sites. First, the limitation of the single cycle methodology used in the adduct mapping experiments resulted in the weaker binding sites giving signals barely above background, making quantitation of some of these sites difficult. The second, and more intractable, obstacle was the observation that many potential adduct formation sites overlap. The problem is exemplified by the (+) strand sequence from 6395 to 6398: 5’-d(GpApGpG)-3’. This sequence has the potential to form an A*G*, G*NG*, or G*G* adduct. DNA polymerases can halt when encountering the first (3’) nucleotide of a platinum adduct,
but do so more often at the second (5') nucleotide or even at the nucleotide 5' to the adduct (Comess et al., 1992). Consequently, the uncertainty of where the adduct would form in this sequence, coupled with the variability of where the polymerase would stop when encountering a given adduct, leads to the conclusion that a stop site corresponding to the adenine at position 6396, for example, could be attributed to any one of the three possible adducts.
Table 6. Adduct levels of genomes modified with cis-DDP or ACDP

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pt adducts per M13mp18 RF genome</th>
<th>Adducts per lacZ' mutational target</th>
<th>Bound drug to nucleotide ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>6.2</td>
<td>0.33</td>
<td>0.41 x 10^3</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>0.61</td>
<td>0.80 x 10^3</td>
</tr>
<tr>
<td></td>
<td>14.2</td>
<td>0.75</td>
<td>0.98 x 10^3</td>
</tr>
<tr>
<td></td>
<td>19.8</td>
<td>1.04</td>
<td>1.37 x 10^3</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>1.24</td>
<td>1.68 x 10^3</td>
</tr>
<tr>
<td></td>
<td>29.5</td>
<td>1.54</td>
<td>2.02 x 10^3</td>
</tr>
<tr>
<td></td>
<td>35.4</td>
<td>1.85</td>
<td>2.44 x 10^3</td>
</tr>
<tr>
<td></td>
<td>61.6</td>
<td>3.22</td>
<td>4.25 x 10^3</td>
</tr>
<tr>
<td>ACDP</td>
<td>0.64</td>
<td>0.03</td>
<td>0.04 x 10^3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.13</td>
<td>0.17 x 10^3</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>0.35</td>
<td>0.46 x 10^3</td>
</tr>
<tr>
<td></td>
<td>17.6</td>
<td>0.92</td>
<td>1.21 x 10^3</td>
</tr>
<tr>
<td></td>
<td>23.4</td>
<td>1.23</td>
<td>1.61 x 10^3</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>2.36</td>
<td>3.10 x 10^3</td>
</tr>
<tr>
<td></td>
<td>85.7</td>
<td>4.49</td>
<td>5.90 x 10^3</td>
</tr>
</tbody>
</table>
Table 7. Comparative survival for M13mp18 RF DNA modified with either cis-DDP or ACDP.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pt adducts per M13mp18 RF genome</th>
<th>Survival(%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOS(-)</td>
<td>SOS(+)</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>23.1</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>14.6 ± 9.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.8 ± 19.8</td>
</tr>
<tr>
<td></td>
<td>14.2</td>
<td>4.10 ± 2.1</td>
<td>7.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>19.8</td>
<td>2.5 ± 1.6</td>
<td>19.7 ± 13.3</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>4.4 ± 2.6</td>
<td>17.0 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>29.5</td>
<td>0.7 ± 0.33</td>
<td>5.0 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>35.4</td>
<td>1.1 ± 0.51</td>
<td>5.5 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>61.6</td>
<td>0.02 ± 0.01</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adducts per lethal hit:&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.33 ± 2.04</td>
<td>10.3 ± 4.77</td>
<td></td>
</tr>
<tr>
<td>ACDP</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.64</td>
<td>93.4</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>35.7 ± 14.2</td>
<td>59.3 ± 20.5</td>
</tr>
<tr>
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<td>6.6</td>
<td>23.5 ± 11.5</td>
<td>49.9 ± 15.4</td>
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<tr>
<td></td>
<td>17.6</td>
<td>9.4 ± 4.2</td>
<td>20.2 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>23.4</td>
<td>3.1 ± 1.6</td>
<td>12.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>0.8 ± 0.6</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>85.7</td>
<td>-----</td>
<td>0.2</td>
</tr>
<tr>
<td>Adducts per lethal hit:</td>
<td>5.22 ± 2.97</td>
<td>16.3 ± 5.71</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Survival values were determined relative to unmodified M13mp18 RF genomes.
<sup>b</sup> Error margins are given for the 95% confidence level (s.d. x 1.96).
<sup>c</sup> Adducts per lethal hit calculated for 37% survival based on curve fitting by Cricket Graph. Equations for curve fit lines are for cis-DDP, SOS(-): y=0.58(10<sup>-0.05x</sup>), R²=0.951; SOS(+): y=0.99(10<sup>-0.04x</sup>), R²=0.899; for ACDP, SOS(-): y=0.64(10<sup>-0.06x</sup>), R²=0.942; SOS(+): y=0.74(10<sup>-0.03x</sup>), R²=0.986.
Table 8. Location, mutation and enhanced β-galactosidase activity of dark blue mutants.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Position</th>
<th>Number observed</th>
<th>Fold β-gal enhancement&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Consequence for peptide structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC → TA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6219</td>
<td>1</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Met → Ile (1st coding residue)</td>
</tr>
<tr>
<td>CG deletion</td>
<td>6416-6418</td>
<td>1</td>
<td>n.d.</td>
<td>frameshift → causing stop codon @6434</td>
</tr>
<tr>
<td>GC → TA</td>
<td>6418</td>
<td>1</td>
<td>47.6</td>
<td>Gln → stop codon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>44.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td>GC → TA</td>
<td>6421</td>
<td>1</td>
<td>n.d.</td>
<td>Gln → stop codon</td>
</tr>
<tr>
<td>GC → TA</td>
<td>5442</td>
<td>1</td>
<td>58.1</td>
<td>Glu → stop codon</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>55.2</td>
<td></td>
</tr>
<tr>
<td>GC → AT</td>
<td>6459</td>
<td>1</td>
<td>8.5</td>
<td>Trp → stop codon</td>
</tr>
<tr>
<td>GC → TA</td>
<td>6472</td>
<td>1</td>
<td>n.d.</td>
<td>Glu → stop codon</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enhancement is expressed relative to the average activity determined for 6 independent wild type samples.

<sup>b</sup> The first nucleotide of each pair is derived from the viral (+) strand, the second from the (-) strand of the M13mp18 RF genome.

<sup>c</sup> Not determined.
Table 9. Mutation frequency of cis-DDP and ACDP modified M13mp18 RF genomes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Adducts per(^a) lacZ(^b)' target</th>
<th>Number of experiments</th>
<th>Mutants per total plaques</th>
<th>Mutation frequency(x10(^4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
<td>18</td>
<td>113/205,565</td>
<td>5.5 ± 2.5(^b)</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>0.61</td>
<td>6</td>
<td>153/82,914</td>
<td>18.5 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>6</td>
<td>213/67,134</td>
<td>31.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>1.24</td>
<td>6</td>
<td>240/81,005</td>
<td>29.6 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>1.54</td>
<td>6</td>
<td>208/56,088</td>
<td>37.1 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>1.85</td>
<td>8</td>
<td>359/84,882</td>
<td>42.3 ± 9.9</td>
</tr>
<tr>
<td>ACDP</td>
<td>0.13</td>
<td>13</td>
<td>172/165,827</td>
<td>10.4 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>13</td>
<td>206/176,929</td>
<td>11.8 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>15</td>
<td>180/108,357</td>
<td>16.6 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>1.23</td>
<td>17</td>
<td>198/97,990</td>
<td>20.2 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>2.36</td>
<td>16</td>
<td>446/180,544</td>
<td>24.7 ± 8.6</td>
</tr>
</tbody>
</table>

\(^a\) The number given represents the average number of adducts located in the 370 nucleotide DNA sequence of the lacZ\(^b\)' region of M13mp18 that was the target for the detection of mutations.

\(^b\) Error margins are given for the 95% confidence level (s.d. x 1.96)
Table 10. Miscellaneous spontaneous and induced lacZ' mutations

<table>
<thead>
<tr>
<th>Identity</th>
<th>Number</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous</strong></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Large deletion (5970-6172)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Large deletion (6250-6396)</td>
</tr>
<tr>
<td><strong>cis-DDP</strong></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Large deletion (5970-6172)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Large deletion (6250-6396)</td>
</tr>
</tbody>
</table>
|              | 1      | 6248-6251: d(CpCpGpG) → d(CpApG)\(^a\)  
(del C or G, with C or G → A) |
|              | 1      | 6463-6467: d(CpCpGpG) → d(CpApG)\(^a\)  
(del C or G, with C or G → A) |
| **ACDP**     | 4      |                  |
|              | 1      | Large deletion (6250-6396)  |
|              | 1      | 6338-6340: Deletion of 2 (CG)'s  
polylinker region deleted, replaced  
with sections of the other strand  
(totaling the same number of bases  
that were deleted) |
|              | 1      | 6463-6467: d(CpCpGpG) → d(CpApG)\(^a\)  
(del C or G, with C or G → A) |

\(^a\) Only the sequence for the viral (+) strand is shown although the mutation could be derived from either strand.
Table 11. *LacZ'* mutational spectra of cis-DDP and ACDP modified and unmodified M13mp18 RF DNA listed by types of mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>cis-DDP</th>
<th>ACDP</th>
<th>Spontaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transversions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC → TA</td>
<td>22</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>CG → AT</td>
<td>23</td>
<td>23</td>
<td>2.2</td>
</tr>
<tr>
<td>AT → TA</td>
<td>0.9</td>
<td>4.2</td>
<td>---</td>
</tr>
<tr>
<td>TA → AT</td>
<td>13</td>
<td>8.5</td>
<td>---</td>
</tr>
<tr>
<td>GC → CG</td>
<td>1.7</td>
<td>0.7</td>
<td>4.3</td>
</tr>
<tr>
<td>CG → GC</td>
<td>0.9</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>AT → CG</td>
<td></td>
<td>0.7</td>
<td>---</td>
</tr>
<tr>
<td>TA → GC</td>
<td></td>
<td>1.4</td>
<td>---</td>
</tr>
<tr>
<td>Transitions</td>
<td>32</td>
<td>28</td>
<td>54</td>
</tr>
<tr>
<td>CG → TA</td>
<td>20</td>
<td>17</td>
<td>43</td>
</tr>
<tr>
<td>GC → AT</td>
<td>8.7</td>
<td>4.2</td>
<td>2.2</td>
</tr>
<tr>
<td>TA → CG</td>
<td>2.6</td>
<td>4.9</td>
<td>2.2</td>
</tr>
<tr>
<td>AT → GC</td>
<td>0.9</td>
<td>1.4</td>
<td>6.5</td>
</tr>
<tr>
<td>Other mutations</td>
<td>7</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Single base insert.</td>
<td>0.9</td>
<td>4.9</td>
<td>---</td>
</tr>
<tr>
<td>Single base delet.</td>
<td>0.9</td>
<td>4.9</td>
<td>11</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>5.2</td>
<td>2.8</td>
<td>15</td>
</tr>
</tbody>
</table>

a. Numbers given are percentages of the total 115 cis-DDP, 142 ACDP and 46 spontaneous mutations identified.
b. The first nucleotide of each pair is derived from the viral (+) strand, the second from the (-) strand of the M13mp18 RF genome. For the induced mutations, the sequence context of the mutation, combined with the adduct mapping data showing the location of the platinum DNA adducts, allowed assignment of the mutation to one strand or the other as shown in Figure 18.
c. Miscellaneous mutations are listed in Table 10.
Table 12. Amino acid changes in the lacZ' peptide fragment that result from transition or transversion mutations

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>a. a. change</th>
<th>Position</th>
<th>Mutation</th>
<th>a. a. change</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal overlapping region</td>
<td></td>
<td></td>
<td>31 a. a. residue M15 deletion (cont)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6218 (2) b</td>
<td>TA → GC c</td>
<td>Met → Arg</td>
<td>6365 (2)</td>
<td>CG → TA</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>6219 (3)</td>
<td>GC → TA</td>
<td>Met → Ile</td>
<td>6365 (2)</td>
<td>CG → AT</td>
<td>Pro → His</td>
</tr>
<tr>
<td>6236 (2)</td>
<td>CG → AT</td>
<td>Ser → stop</td>
<td>6367 (1)</td>
<td>CG → AT</td>
<td>Pro → Thr</td>
</tr>
<tr>
<td>6237 (3)</td>
<td>GC → TA</td>
<td>none d (Ser)</td>
<td>6368 (2)</td>
<td>CG → TA</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>6242 (2)</td>
<td>CG → AT</td>
<td>Ser → stop</td>
<td>6371 (2)</td>
<td>TA → CG</td>
<td>Phe → Ser</td>
</tr>
<tr>
<td>6245 (2)</td>
<td>TA → AT</td>
<td>Val → Glu</td>
<td>6372 (3)</td>
<td>CG → AT</td>
<td>Phe → Leu</td>
</tr>
<tr>
<td>6247/6249</td>
<td>CG → AT</td>
<td>Pro → Thr</td>
<td>6376 (1)</td>
<td>AT → TA</td>
<td>Ser → Cys</td>
</tr>
<tr>
<td>6258 (3)</td>
<td>TA → AT</td>
<td>none d (Pro)</td>
<td>6377 (2)</td>
<td>GC → TA</td>
<td>Ser → Ile</td>
</tr>
<tr>
<td>6261 (3)</td>
<td>AT → TA</td>
<td>none d (Leu)</td>
<td>6377 (2)</td>
<td>GC → AT</td>
<td>Ser → Asn</td>
</tr>
<tr>
<td>6262 (1)</td>
<td>GC → TA</td>
<td>Glu → stop</td>
<td>6378 (3)</td>
<td>CG → AT</td>
<td>Ser → Arg</td>
</tr>
<tr>
<td>6266 (2)</td>
<td>GC → AT</td>
<td>Ser → stop</td>
<td>6379 (1)</td>
<td>TA → AT</td>
<td>Trp → Arg</td>
</tr>
<tr>
<td>6270 (3)</td>
<td>CG → AT</td>
<td>none d (Thr)</td>
<td>6380 (2)</td>
<td>GC → AT</td>
<td>Trp → stop</td>
</tr>
<tr>
<td>6293 (2)</td>
<td>TA → CG</td>
<td>Leu → Pro</td>
<td>6380 (2)</td>
<td>GC → TA</td>
<td>Trp → Leu</td>
</tr>
<tr>
<td>31 a. a. residue M15 deletion</td>
<td></td>
<td></td>
<td>6314 (2)</td>
<td>GC → AT</td>
<td>Arg → His</td>
</tr>
<tr>
<td>6305 (2)</td>
<td>TA → AT</td>
<td>Leu → stop</td>
<td>6382 (1)</td>
<td>CG → AT</td>
<td>Arg → Ser</td>
</tr>
<tr>
<td>6307 (1)</td>
<td>CG → TA</td>
<td>Glu → stop</td>
<td>6383 (2)</td>
<td>GC → TA</td>
<td>Arg → Leu</td>
</tr>
<tr>
<td>6310 (1)</td>
<td>CG → TA</td>
<td>Arg → Cys</td>
<td>6383 (2)</td>
<td>GC → AT</td>
<td>Arg → His</td>
</tr>
<tr>
<td>6311 (2)</td>
<td>GC → AT</td>
<td>Arg → His</td>
<td>6391 (1)</td>
<td>GC → TA</td>
<td>Glu → stop</td>
</tr>
<tr>
<td>6313 (1)</td>
<td>CG → TA</td>
<td>Arg → Cys</td>
<td>6394 (1)</td>
<td>GC → TA</td>
<td>Glu → stop</td>
</tr>
<tr>
<td>6313 (1)</td>
<td>CG → AT</td>
<td>Arg → Ser</td>
<td>6395 (2)</td>
<td>AT → TA</td>
<td>Glu → Val</td>
</tr>
<tr>
<td>6314 (2)</td>
<td>GC → AT</td>
<td>Arg → His</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6314 (2)</td>
<td>GC → TA</td>
<td>Arg → Leu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6316 (1)</td>
<td>GC → TA</td>
<td>Asp → Tyr</td>
<td>6397 (1)</td>
<td>GC → TA</td>
<td>Ala → Ser</td>
</tr>
<tr>
<td>6317 (2)</td>
<td>AT → GC</td>
<td>Asp → Gly</td>
<td>6398 (2)</td>
<td>CG → TA</td>
<td>Ala → Val</td>
</tr>
<tr>
<td>6317 (2)</td>
<td>AT → TA</td>
<td>Asp → Val</td>
<td>6400 (1)</td>
<td>CG → AT</td>
<td>Arg → Ser</td>
</tr>
<tr>
<td>6319 (1)</td>
<td>TA → AT</td>
<td>Trp → Arg</td>
<td>6400 (1)</td>
<td>CG → TA</td>
<td>Arg → Cys</td>
</tr>
<tr>
<td>6319 (1)</td>
<td>TA → CG</td>
<td>Trp → Arg</td>
<td>6401 (2)</td>
<td>GC → AT</td>
<td>Arg → His</td>
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<tr>
<td>6320 (2)</td>
<td>GC → CG</td>
<td>Trp → Ser</td>
<td>6413 (2)</td>
<td>CG → AT</td>
<td>Pro → His</td>
</tr>
<tr>
<td>6320 (2)</td>
<td>GC → AT</td>
<td>Trp → stop</td>
<td>6416 (2)</td>
<td>CG → TA</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td>6320 (2)</td>
<td>GC → TA</td>
<td>Trp → Leu</td>
<td>6416 (2)</td>
<td>CG → AT</td>
<td>Ser → Tyr</td>
</tr>
<tr>
<td>6321 (3)</td>
<td>GC → TA</td>
<td>Trp → Cys</td>
<td>6418 (1)</td>
<td>CG → TA</td>
<td>Gln → stop</td>
</tr>
<tr>
<td>6321 (3)</td>
<td>GC → AT</td>
<td>Trp → stop</td>
<td>6421 (1)</td>
<td>CG → TA</td>
<td>Gln → stop</td>
</tr>
<tr>
<td>6322 (1)</td>
<td>GC → TA</td>
<td>Glu → stop</td>
<td>6427 (1)</td>
<td>CG → TA</td>
<td>Arg → Cys</td>
</tr>
<tr>
<td>6327 (3)</td>
<td>CG → AT</td>
<td>Asn → Lys</td>
<td>6434 (2)</td>
<td>TA → AT</td>
<td>Leu → Gln</td>
</tr>
<tr>
<td>6340 (1)</td>
<td>CG → TA</td>
<td>Gln → stop</td>
<td>6436 (1)</td>
<td>AT → TA</td>
<td>Asn → Tyr</td>
</tr>
<tr>
<td>6340 (1)</td>
<td>GC → AT</td>
<td>Gln → Lys</td>
<td>6438 (3)</td>
<td>TA → AT</td>
<td>Asn → Lys</td>
</tr>
<tr>
<td>6344 (2)</td>
<td>TA → CG</td>
<td>Leu → Pro</td>
<td>6439 (1)</td>
<td>GC → TA</td>
<td>Gly → Cys</td>
</tr>
<tr>
<td>6344 (2)</td>
<td>TA → GC</td>
<td>Leu → Arg</td>
<td>6440 (2)</td>
<td>GC → TA</td>
<td>Gly → Val</td>
</tr>
<tr>
<td>6347 (2)</td>
<td>AT → TA</td>
<td>Asn → Ile</td>
<td>6442 (1)</td>
<td>GC → TA</td>
<td>Glu → stop</td>
</tr>
<tr>
<td>6347 (2)</td>
<td>AT → CG</td>
<td>Asn → Thr</td>
<td>6447 (3)</td>
<td>GC → TA</td>
<td>Trp → Cys</td>
</tr>
<tr>
<td>6349 (1)</td>
<td>CG → AT</td>
<td>Arg → Ser</td>
<td>6451 (1)</td>
<td>TA → CG</td>
<td>Phe → Leu</td>
</tr>
<tr>
<td>6350 (2)</td>
<td>GC → TA</td>
<td>Arg → Leu</td>
<td>6459 (3)</td>
<td>GC → AT</td>
<td>Trp → stop</td>
</tr>
<tr>
<td>6350 (2)</td>
<td>GC → AT</td>
<td>Leu → His</td>
<td>6464 (1)</td>
<td>CG → TA</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>6352 (1)</td>
<td>CG → TA</td>
<td>Leu → Phe</td>
<td>6464 (1)</td>
<td>CG → AT</td>
<td>Pro → Gln</td>
</tr>
<tr>
<td>6353 (2)</td>
<td>TA → AT</td>
<td>Leu → His</td>
<td>6469 (1)</td>
<td>CG → TA</td>
<td>Pro → Ser</td>
</tr>
<tr>
<td>6353 (2)</td>
<td>TA → CG</td>
<td>Leu → Pro</td>
<td>6469 (1)</td>
<td>CG → AT</td>
<td>Pro → Thr</td>
</tr>
<tr>
<td>6356 (2)</td>
<td>CG → GC</td>
<td>Ala → Gly</td>
<td>6470 (2)</td>
<td>CG → AT</td>
<td>Pro → Gln</td>
</tr>
<tr>
<td>6359 (2)</td>
<td>CG → TA</td>
<td>Ala → Val</td>
<td>6472 (1)</td>
<td>GC → TA</td>
<td>Gln → stop</td>
</tr>
<tr>
<td>6360 (3)</td>
<td>AT → GC</td>
<td>none d (Ala)</td>
<td>6482 (2)</td>
<td>CG → TA</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>6361 (1)</td>
<td>CG → TA</td>
<td>His → Tyr</td>
<td>6482 (2)</td>
<td>CG → AT</td>
<td>Pro → Gln</td>
</tr>
</tbody>
</table>

C-terminal overlapping region | | |
| 6359 (2) | CG → TA | Ala → Val | 6472 (1) | GC → TA | Gln → stop |
| 6360 (3) | AT → GC | none d (Ala) | 6482 (2) | CG → TA | Pro → Leu |
| 6361 (1) | CG → TA | His → Tyr | 6482 (2) | CG → AT | Pro → Gln |
Table 12. (cont)
a. a. a. designates amino acid.
b. Numbers in parentheses are codon positions.
c. The first nucleotide of each pair is derived from the (+) strand, the second from the (-) strand of the M13mp18 RF genome.
d. Neutral mutations that do not lead to an amino acid change; each of these samples, with the exception of the TA → AT transversion at position 6258, had phenotypically detectable missense mutations elsewhere.
e. A double mutant with this base change at both the 1st and 3rd codon.
Figure 16. Survival of cis-DDP and ACDP modified M13mp18 RF genomes in E. coli DL7 cells. Survival was determined by using the M13 plaque forming assay for cells either not treated (a) or treated (b) with uv irradiation to induce the SOS response. Survival values were determined relative to unmodified M13mp18 RF genomes; each data point represents the average of 6-9 replicas. Error margins are given for the 95% confidence level (s. d. x 1.96). Adduct/lethal hit (37% survival) values were calculated from curve fit lines generated by Cricket Graph (Cricket Software).
Figure 17. Mutation frequency as a function of adduct level in the 370 nucleotide DNA sequence of M13mp18 RF genomes that forms the target for detectable mutations in the lacZ' region of cis-DDP (●) or ACDP (■) modified M13mp18 RF genomes. The error bars represent 95% confidence intervals (s.d. x 1.96).
Adducts per 370 nucleotide lacZ' mutational target region of the M13mp18 genome
Figure 18. Mutation and DNA binding spectra for the lacZ' region of M13mp18 RF DNA. The lacZ' DNA sequence is represented in four segments: (a) upstream regulatory region; (b) 29 residue lacZ' peptide coding sequence that forms the N-terminal overlap with the M15 α-acceptor protein; (c) lacZ' peptide coding sequence corresponding to the 31 residue (93 bp) deletion in M15; and (d) 30 residues of the lacZ' peptide coding sequence that forms part of the 54 residue C-terminal overlap with M15. Bars indicate relative adduct levels, mutations are designated by letters, arrows are intended to provide visual clarity in defining the location of mutations occurring at sites without measurable adducts. Mutations were assigned to the (+) or (-) strand based on adduct position and known sites of platinum induced mutations. Mutations potentially attributable to adducts in either strand, or not clearly arising from either strand, are indicated in bold italic form. Adducts and mutations representing the cis-DDP spectra are shown above the strands, and the ACDP spectra are located below the strands. Deletions are indicated by (Δ), insertions by (+); underlined sequences represent any one of the possible bases that was deleted when a deletion mutation occurred at runs of identical nucleotides. Adduct distributions were not determined for positions >6450 in the (+) strand. The T-T at positions 6247-6249 indicates a d(GpGpG) → d(TpGpT) double mutation.
CAP binding site
-35 promoter
operator
-10 promoter
ribosome binding site

lacZ' coding sequence - (N-terminal overlap)
polylinker cloning region

163
Figure 19. Spontaneous mutational spectra for single base changes. Underlined sequences represent any one of the possible base pair deleted when deletion occurred at runs of identical nucleotides. Deletions are indicated by (Δ). Other spontaneous mutations are described in Table 11.
V. DISCUSSION
This dissertation describes the comparative genotoxicity and mutagenicity of platinum-DNA adducts from two perspectives. First, cis-DDP G*G*, A*G*, and G*TG* adducts site-specifically situated in M13-derived ss viral genomes exhibited different relative genotoxicities and mutagenicities when replicated in *E. coli* DL7 cells. The differential mutagenicities of the various cis-DDP DNA adducts suggested strategies for the design of novel platinum drugs that would be less mutagenic than those currently in use. For example, the A*G* adduct, which is 4-5 fold more mutagenic than the G*G* adduct, should be minimized in the DNA binding spectra of future drugs. Accordingly, the second aspect of the work described in this dissertation involved the evaluation of ACDP. ACDP is a metabolite of ACCDP, a promising platinum(IV) drug, which has DNA binding properties similar to cis-DDP with the notable exception that it forms three-fold fewer adducts at d(ApG) sites. The less frequent occurrence of the more highly mutagenic A*G* adduct suggested that the overall mutagenicity of ACDP would be lower than that of cis-DDP. This hypothesis was experimentally tested and confirmed.

A. Genotoxicities of the G*G*, A*G*, and G*TG* Adducts

1. Comparison to Previous Results

The genotoxicities of the cis-DDP A*G* and G*NG* adducts have not been determined previously. The genotoxicity of the G*G* adduct, however, has been studied in *E. coli*. Bradley et al. (1993) report a survival level of 22% for this adduct located in M13mp18 genomes replicated in non-SOS induced *E. coli* DL7 cells. This value was considerably higher than the 5.2% survival observed under the same biological conditions in the present work. The discrepancy in survival can be attributed to differences in the genomes employed in each study. The previous work utilized a G*G* adduct situated in the (-) strand of
replicative form (RF) M13mp18 bacteriophage genomes. The complementary (+) strand had been subjected to uv irradiation to damage the DNA. The resulting thymine-thymine cyclobutane dimers served to inhibit DNA replication. Since the damaged (+) strand could no longer serve as a template for replication, DNA synthesis of the adduct containing (-) strand was ensured, thereby circumventing the strand bias effects inherent in M13 replication.¹ The presence of the (+) strand, however, was detrimental insofar as it provided a ds DNA template amenable to repair by the UvrABC complex (UvrABC is the cellular system that is primarily responsible for the repair of cis-DDP adducts in E. coli). It was likely, therefore, that some of the G*G* adducts situated in the ds genomes used by Bradley et al. (1993) were repaired. This hypothesis is consistent with the location of the G*G* in the (-) strand. Before M13mp18 RF DNA can be replicated in E. coli, a number of viral genes, all encoded by the (-) strand, must be transcribed. Strand specific DNA repair in E. coli is linked to transcription (Mellon & Hanawalt, 1989; Selby & Sancar, 1991; Kinnala & Brash, 1992; Oller et al., 1992) and shown to be applicable to cis-DDP DNA adducts in the M13 bacteriophage system (K. Yarema, unpublished data) suggesting that the G*G* adduct in the (-) strand of M13mp18 RF genomes would be susceptible to repair by the UvrABC repair complex. Conversely, such repair is not possible in the ss genomes used in this thesis work. Without UvrABC mediated repair, it is reasonable that the survival of genomes containing the G*G* was fourfold lower in the current system.

2. Comparative Genotoxities of cis-DDP adducts

¹As a result of the modified rolling circle replication of M13 genomes (Figure M13), a replication blocking lesion in either strand of a M13 RF genome can render that strand inactive by directing preferential replication of the other, undamaged, strand. All progeny, therefore, would be derived from the undamaged strand and the adduct containing strand would remain unreplicated and the genetic effects of the DNA lesion would not be observed.
The viability of M13-derived ss genomes containing any of the cis-DDP adducts was significantly reduced compared to unmodified genomes. The cis-DDP A*G*, G*TG*, and G*G* adducts reduced the plaque-forming ability of M13 ss genomes replicated in non-SOS induced *E. coli* DL7 cells by 79%, 87%, and 95% respectively (Figure 15). While each of these adducts can be qualitatively considered to be an effective deterrent to replication, the quantitative differences in survival are statistically significant and warrant further discussion. The factors most likely to account for adduct to adduct variability are (i) differential ability of the adducts to be repaired or (ii) differential ability of the adducts to be bypassed by DNA polymerases.

a. Differential repair

cis-DDP DNA adducts are primarily repaired by the UvrABC repair complex in *E. coli* (Husain et al., 1985). Although the UvrABC excision repair system is under regulatory control of the inducible SOS system, significant levels of UvrA, UvrB, and UvrC proteins (20, 200, and 10 per cell, respectively (van Houten, 1990)) are present in non-SOS induced cells allowing for repair of infrequently occurring DNA lesions. The cis-DDP lesions introduced into *E. coli* by transfection of singly-modified M13-derived genomes fit into this category. The possibility exists, therefore, that the cis-DDP G*G*, A*G*, and G*TG* adducts are repaired at different rates by the UvrABC system, thereby accounting for the observed differences in survival of the genomes containing these lesions. Several lines of evidence, however, suggest that this situation does not apply. First, UvrABC is not expected to be active on ss DNA (van Houten, 1990) because a ds template is required for the tracking mechanism employed by the UvrAB proteins to locate DNA lesions (Grossman & Thiagalingam, 1993). Furthermore, no structural basis exists to account for differential recognition of these adducts by
UvrABC that would explain the observed differences in survival (the structural implication of UvrABC recognition of the G*G*, A*G*, and G*TG* adducts are discussed in detail in the next section). The G*NG* adduct imposes more local distortion of the DNA duplex than the A*G* or G*G* adducts and might be expected, if anything, to be better recognized by UvrABC (Sancar & Sancar, 1988). Indeed, the G*NG* adduct formed by the cis-DDP analogue cis-diaminocyclohexanedichloroplatinum(II) ([Pt(dach)Cl₂], Figure 1) is a much better substrate for UvrABC than either the A*G* or G*G* adducts (Page et al., 1990). These results are in clear conflict with the relative survival of the singly-modified genomes observed in this thesis work, implying that UvrABC mediated repair does not account for the survival differences experienced by the cis-DDP G*G*, A*G*, and G*TG* adducts.

Repair of cis-DDP adducts that elude processing by UvrABC has been reported (Germanier et al., 1984). Mismatch repair (Fram et al., 1985) and recombinational repair (Husain et al., 1985) have been implicated as secondary repair systems for cis-DDP modified DNA. Both of these modes of repair operate post-replicatively and, unlike UvrABC, are therefore expected to be able to operate on lesions in ss DNA, such as the cis-DDP DNA adducts located in the genomes employed in this work. It is not clear, however, from what is known about mismatch and recombinational DNA repair how these mechanisms could account for the survival differences observed between the cis-DDP A*G*, G*G*, and G*TG* adducts.

Recombinational repair occurs when a polymerase encounters certain replication-blocking DNA lesions. The polymerase stops replication and reinitiates about 1000 bases beyond the adduct, thus generating a ss gap containing the DNA lesion. This post-replication gap is filled in by RecA protein, which transfers the complementary strand from a sister
duplex into the gap (Sancar & Sancar, 1988). Similarly, mismatch repair involves post-replicative processing of DNA, requiring that the lesion first must be successfully bypassed during DNA synthesis (reviewed by Modrich, 1987). These mechanisms appear to balance each other and might be expected to diminish differences between adducts. For example, adducts that are easily bypassed might be better candidates for mismatch repair whereas adducts more effective at blocking DNA replication would be more likely to produce the ~1000 nucleotide gaps processed by the recombinational repair system. Few details, however, are known about the processing of cis-DDP adducts by either of these systems and it is unclear how either system could discriminate between cis-DDP G*G*, A*G*, and G*CG* adducts to produce the differential plaque-forming abilities of these adducts situated in M13-derived ss genomes.

b. Translesion synthesis

Although cis-DDP DNA adducts potently inhibit DNA synthesis, they are not absolute blocks to DNA replication. E. coli polymerase I has been shown to bypass cis-DDP in vitro, with G*G* adducts in M13 ss genomes more inhibitory to replication the A*G* adducts (Hoffman et al., 1989). This result was not quantitatively analyzed, but it is qualitatively in agreement with the 4 fold higher survival of A*G* containing genomes compared to G*G* containing genomes in E. coli DL7 cells utilized in the present experiment. Comess et al. (1992) utilized site-specifically situated adducts in M13 genomes to investigate the ability of cis-DDP G*G*, A*G*, and G*CG* to inhibit DNA replication in vitro. Frequency of replication bypass of the cis-DDP G*G*, A*G*, and G*CG* adducts by E. coli polymerase III was 6%, 9%, and 4%, respectively. The value of 6% compared well with the 5% survival of the G*G* containing genome replicated, presumably also by Pol III, in E. coli DL7 cells. These results are consistent with the interpretation
that the G\*G* adduct was not repaired in vivo and survival was entirely due to translesion synthesis. The in vivo survival of the A\*G* and G*TG* adducts, at 21% and 13%, respectively, were significantly higher than the 9% and 4% observed in the in vitro assay.

The quantitative discrepancies between the in vivo and in vitro results could be due to the A\*G* and G*TG* adducts being repaired by some poorly understood mechanism while the G\*G* adduct remained unrepaired. Alternately, repair may be completely inoperative and translesion synthesis may have been the sole mechanism to account for survival of the various cis-DDP modified genomes. In the latter case, the in vitro replication conditions used by Comess et al. (1992) may have been sufficiently different than the in vivo conditions employed in the present work to account for differences in the in vitro and in vivo results. For example, the frequency of translesion synthesis of cis-DDP adducts is strongly dependent on salt and nucleotide concentrations (K. Yarema, unpublished observations), which could be significantly different inside a cell than in vitro. Furthermore, intracellular translesional synthesis could be aided by accessory proteins absent from the in vitro experiments. Notwithstanding these caveats, the relative in vitro replication bypass results are quantitatively consistent with the in vivo results obtained in E. coli DL7 cells, suggesting that bypass of the cis-DDP G\*G*, A\*G*, and G*TG* adducts plays a major role in determining their genotoxicities.

3. Mode of SOS-Dependent Survival Increase

Survival of M13-derived ss genomes containing either a G\*G* or A\*G* cis-DDP adduct increased significantly when replicated in SOS induced E. coli DL7 cells. The observed survival increase is attributable to three possible causes: (1) repair of the adduct by the
UvrABC excision complex, (2) repair by some other mechanism, or (3) to enhanced bypass of the lesion. Enhanced bypass of the adducts is the favored explanation as there is considerable evidence to suggest that cis-DDP adducts would not be repaired in M13-derived ss genomes. These possibilities will be discussed below, leading to the conclusion that the SOS dependent increased survival is best explained by increased translesion synthesis past cis-DDP adducts during DNA replication.

a. Enhanced survival is not due to repair by UvrABC

There is considerable evidence that the UvrABC excision repair complex, the major system responsible for repair of cis-DDP adduct in E. coli, is not responsible for the SOS-dependent increase in survival experienced by genomes containing the G*G* and A*G* adducts. In addition to the factors discussed above, the anticipated lack of repair of these adducts is supported further by the experimental observation that the G*TG* cis-DDP adduct does not experience an SOS-dependent increase in survival similar to the G*G* and A*G* adducts. In the unlikely event that UvrABC excision repair was responsible for the observed SOS-dependent increase in survival\(^1\), the G*TG* adduct should also be affected as it is expected to be a substrate for these enzymes based on two factors. First, DNA adducts formed by trans-DDP, most of

\(^1\)The constitutive levels of the UvrABC proteins, 20, 200, and 10 per cell respectively, proficiently repair low levels of cis-DDP DNA adducts. This conclusion is based on the data shown in Figure DL7/DL6. Cells without UvrABC repair (E. coli DL6) show a 3.7 fold greater sensitivity to cis-DDP than repair proficient (E. coli DL7) cells (1.24 and 4.60 adducts/lethal hit, respectively). Upon SOS induction of the wild-type cells, survival increased, but only 2.2 fold, to 10.1 adducts/lethal hit. The SOS dependent increase in repair is conceivably a consequence of the increased level of the UvrA and UvrB proteins (to 200 and 1000 per cell, respectively; UvrC remains at 10 per cell), but is more likely due to other SOS dependent responses, such as the induction of the umuDC genes, as discussed in the text. This is especially the case for the singly modified genomes. It is difficult to imagine a single cis-DDP DNA adduct overwhelming the constitutive levels of the UvrABC proteins such that an increase in these proteins would have a significant increase in survival such as seen in Figure SSS.
which are 1,3-intrastrand crosslinks (i.e. G*NG*) are repaired in E. coli, possibly by UvrABC. It should be noted that G*NG* cis- and trans-DDP are structurally dissimilar (see below), a factor that could explain the discrimination against the recognition of cis-DDP G*NG* adducts by UvrABC. A structural analysis of the various cis-DDP adducts compared to the entire spectrum of UvrABC recognized DNA damage, however, reveals that G*NG* cis-DDP are similar to other damages recognized by this repair apparatus. The thymine-thymine cyclobutane dimer is the prototype of a large group of bulky DNA adducts formed by a diverse group of DNA damaging agents that are repaired by UvrABC (Grossman et al., 1988). Distortion of duplex DNA by thymine dimers is similar to that caused by cis-DDP DNA adducts:

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Helix Unwinding</th>
<th>Helix Bending</th>
</tr>
</thead>
<tbody>
<tr>
<td>T*T (uv dimer)</td>
<td>9°</td>
<td>32° (major groove)</td>
</tr>
<tr>
<td>G<em>G</em> (cis-DDP)</td>
<td>13°</td>
<td>32-35° (major groove)</td>
</tr>
<tr>
<td>A<em>G</em> (cis-DDP)</td>
<td>13°</td>
<td>32-35° (major groove?)</td>
</tr>
<tr>
<td>G<em>NG</em> (cis-DDP)</td>
<td>23°</td>
<td>30-35° (?)</td>
</tr>
<tr>
<td>G<em>NG</em> (trans-DDP)</td>
<td>9-10°</td>
<td>30-35° (hinge joint)</td>
</tr>
</tbody>
</table>

As can be seen, cis-DDP G*NG* adducts impose structural distortion on DNA very similar to that of thymine dimers and the cis-DDP G*G* and A*G* adducts with the exception of significantly greater unwinding of the DNA.

Repair of trans-DDP DNA adducts by the UvrABC complex is inferred from experiments in which trans-DDP modified M13mp18 RF (ds) genomes were transfected into wild-type (DL7) or uvrA (DL6) E. coli cells. The plaque forming ability of these genomes was about an order of magnitude greater (and similar to cis-DDP modified DNA) in the wild type cells indicating that the trans-DDP adducts were being efficiently repaired by UvrABC (K. Yarema, unpublished results). There have been reports to the contrary, however, that trans-DDP adducts are not repaired, or repaired with greatly reduced efficiency by uvrABC. There are also indications that these adducts are repaired by systems other than UvrABC in E. coli (Beck et al., 1985; Alazard et al., 1982; Popoff et al., 1987).
helix (23° vs. 9° or 13°). UvrABC, however, recognizes a spectrum of DNA lesions that unwind the DNA helix anywhere from 6° to 88° (van Houten, 1990). Another structural determinant of UvrABC recognition is the bending of the DNA helix. A model of damage recognition involves uvrABC detection of lesions that direct DNA bending toward the major groove (van Houten, 1990). The direction of DNA bending imposed by the cis-DDP G*NG* adducts has not yet been determined, if the bend is toward the minor groove, the ability of this lesion to be repaired could be compromised. A recent model requires that the structure of the minor groove proximal to the lesion closely mimics normal DNA to facilitate UvrA assisted binding of UvrB into the minor groove (Visse et al., 1991; Williams & Gao, 1992). Considering the precedent of the [Pt(dach)Cl₂] G*NG* adduct (Page et al., 1990), it is most probable, however, that the cis-DDP G*NG* adduct is bent toward the major groove and is recognized by UvrABC. Based on this structural analysis, the cis-G*TG* adduct should have been a substrate for UvrABC excision repair and shown a survival increase similar to the cis-DDP G*G* and A*G* adducts if excision repair was a factor.

b. Alternate mechanisms for repair of cis-DDP adducts

Repair of cis-DDP DNA adducts by mechanisms other than the primary system of UvrABC excision repair has been reported (Germanier et al., 1984). As mentioned above, both mismatch repair (Fram et al., 1985) and post replication recombinational repair (Husain et al., 1985) have been implicated as secondary repair systems for cis-DDP modified DNA.

The thymine cyclobutane dimer lesion and the G*G* and A*G* cis-DDP adducts all have bends directed toward the major groove, as is the situation for most DNA lesions recognized by the UvrABC repair complex for which this information has been determined. In addition, several compounds, such as G*NG* trans-DDP adducts are recognized that have hinge joints in which the bend is flexible, being directed toward the major groove at least part of the time (van Houten, 1990).
Neither of these repair systems has been reported to be SOS-dependent, suggesting that the SOS-dependent increase in survival for G*G* and A*G* adduct containing genomes replicated in *E. coli* DL7 is not due to repair by any known mechanism. SOS-dependent bypass of the replication blocking *cis-DDP* lesions, therefore, is the probable explanation for the SOS dependent survival experienced by the *cis-DDP* G*G* and A*G* adducts.

c. SOS-dependent bypass of *cis-DDP* adducts.

The SOS inducible proteins umuC and umuD' act in concert with DNA polymerase III and recA* (and possibly other proteins) to allow the polymerase to bypass lesions that block DNA replication in *E. coli* (Bridges & Woodgate, 1984). The exact mechanism of translesion synthesis remains elusive (Woodgate & Sedgwick, 1992). UmuD' and UmuC, however, are known to associate with a polymerase stalled at the site of DNA damage, unlike UvrA2B which tracks along ds DNA until a damaged site is reached (Grossman & Thiagalingam, 1993). UmuDC-mediated translesion synthesis, therefore, is equally effective regardless whether the original DNA substrate was ss or ds. It appears likely that the SOS-dependent increase in survival for M13-derived ss genomes containing the *cis-DDP* G*G* and A*G* adducts is related to UmuDC mediated bypass of the lesions. If this hypothesis is correct, the lack of a survival increase for the G*TG* adduct would suggest that this lesion should not be mutagenic. This prediction is based on the lack of participation of the umuDC gene products in translesion synthesis past the *cis-DDP* G*TG* adduct (and their implied participation in bypass of the *cis-DDP* G*G* and A*G* adducts). Since UmuDC mediated bypass is required for *cis-DDP* mutagenesis, this model predicts that the *cis-DDP* G*TG* adduct would not be mutagenic. This prediction was experimentally tested and verified, as discussed in the next section of this dissertation.
B. Mutagenicities of the G*G*, A*G*, and G*TG* Adducts

1. SOS-Dependence of Mutagenesis

The mutability of cis-DDP DNA adducts in E. coli is absolutely dependent on the induction of the SOS response. Accordingly, no adduct-specific mutations were observed for genomes containing any of the cis-DDP G*G*, A*G*, or G*TG* adducts replicated in non-SOS induced E. coli DL7 cells. Unlike some SOS-dependent functions, such as UvrABC excision repair, UmuDC activity, which includes error prone bypass of DNA lesions, is not detected in non-SOS induced cells. This lack of activity is expected considering that UmuC is not present (or present at undetectably low levels) in non-SOS induced cells. Furthermore, although UmuD is present at moderate levels (~180 molecules/cell, Woodgate & Ennis, 1991), it only becomes active upon cleavage to its truncated UmuD' form by RecA* (Burckhardt et al., 1988; Nohmi et al., 1988). RecA* is also hypothesized to play a direct role in the mutagenic bypass of bulky lesions such as cis-DDP adducts (Sweasy et al., 1990) and is only present in SOS induced cells. Considering that these several proteins are all required for the mutagenicity of cis-DDP adducts, it is not surprising that cis-DDP induced mutations were not observed in non-SOS induced E. coli DL7 cells.

By contrast to the results for the non-SOS induced E. coli DL7 cells, highly targeted cis-DDP induced mutations were observed for the G*G* and A*G* adducts in SOS induced cells. The occurrence of drug induced mutations associated with the cis-DDP G*G* and A*G* adducts correlated with the enhanced survival of the genomes containing these

\[\text{1The cellular regulation and intermolecular interactions required for UmuDC mediated mutagenicity are reviewed in depth in the Literature Survey.}\]
adducts in the plaque forming assay, as discussed above. Conversely, genomes containing the G*TG* adduct did not experience an increase in survival, neither did they give rise to adduct-induced mutations. These results are consistent with the hypothesis that the UmuD' and UmuC proteins are responsible for both the survival increase and mutagenicity experienced by the cis-DDP G*G* and A*G* adducts in the SOS-induced E. coli DL7 cells.

2. Mutational Specificity of cis-DDP Adducts

a. Comparison to previous results

The mutational specificities of the cis-DDP G*G* and A*G* adducts have both been determined, albeit in different systems employing site-specifically modified vectors. Each adduct, when incorporated into genomes and replicated in SOS induced E. coli, produced highly targeted mutations at the 5' base of the cis-DDP modified dinucleotide sequence. Furthermore, the nature of the mutations was similar for each adduct. About 75% of G*G* mutations were G → T transversions (Bradley et al., 1993) and about 80% of A*G* mutations were comparable A → T transversions (Burnouf et al., 1990). In both cases tandem mutations affecting the 5' modified base as well as the nucleotide 5' to the adduct site were observed at low frequencies. The mutational specificities of the G*G* and A*G* adducts located in the M13-derived ss genomes used in the present work are remarkably similar to these previous results, both with regard to the actual G → T and A → T transversions observed at the 5' nucleotide of the G*G* and A*G* adducts, respectively, but also with regard to the relative abundance of each of these predominant mutations. Even minor mutations, such as the tandem mutants, and the A → G transition experienced by the A*G* adduct mirrored previous results (Burnouf et al., 1990).
b. The *cis*-DDP G*TG* adduct is not mutagenic

The mutagenicity of the *cis*-DDP G*NG* adduct has not been previously determined, although, assays utilizing randomly modified DNA have suggested that this lesion is mutagenic (Brouwer et al., 1981; 1982). In contrast to the reported putative mutagenicity of the G*NG* adduct, and unlike the G*G* and A*G* adducts, the G*TG* adduct was not mutagenic in this study. Several lines of evidence indicate that this lack of mutagenicity is a real result and not an unfortunate byproduct of suboptimal experimental routines. First, M13 ss genomes containing the *cis*-DDP G*TG* adduct were replicated in SOS induced *E. coli* DL7 cells under the same conditions that resulted in the mutagenic processing of the *cis*-DDP G*G* and A*G* adducts. The mutagenicity of the 1,2 adducts implied that the host cells were adequately SOS induced to elicit a mutagenic response from *cis*-DDP DNA adducts. Furthermore, the lack of mutability of G*TG* corresponded to the lack of a SOS-dependent increase in survival for this adduct in the plaque forming assay. These results are consistent with the mechanistic explanation that the G*TG* is not a substrate for UmuDC-mediated translesional synthesis, an event that is required both for the induction of mutations and a SOS dependent survival increase, as previously discussed.

There are several possible explanations for the discrepancies between the current lack of mutability of the G*TG* adduct and previously observed mutations induced at d(GpNpG) sequences by *cis*-DDP. Many of the putative G*NG* induced mutations observed by Brouwer et al. (1981; 1982) occurred at d(GpApG) sequences. Considering that the A*G* adduct is expected to form more readily than the G*AG* lesion, mutations arising at these latter sequences might be a consequence of a 1,2 A*G* crosslink rather than the 1,3 G*AG* crosslink. This explanation, however, is likely not valid because most mutations occurred at the 5′ G
in the d(GpNpG) sequence; if these mutations had resulted from a A*G* adduct, the site specific experiments indicate that A, not the 5' G, should be the site of mutation. The lacZ' mutational assay used in this thesis work provides additional evidence that G*NG* adducts are mutagenic. As will be discussed later, G*NG* adducts were implicated as premutagenic lesions.

Another explanation for the apparent discrepancies in the mutability of the G*NG* adducts may be that, while the cis-DDP G*TG* adduct may not be mutable, G*AG* and G*CG* adducts are able to produce mutations, implying that the cis-DDP G*TG* adduct is a poor model for the study of platinum G*NG* adducts in general. While this hypothesis cannot be ruled out at present, it remains unclear why the G*AG* and G*CG* adducts would be mutable and the cis-DDP G*TG* adduct not. As previously discussed, the three cis-DDP 1,3 intrastrand crosslinks are structurally similar and, from this perspective, might be considered to have equivalent mutagenic potentials. Finally, these adducts may or may not be premutagenic lesions dependent on their local sequence contexts. Again, this is unlikely. The flanking region for both the G*G* and A*G* adducts was similar to that of the G*TG* adduct, and both of the former adducts were mutagenic. Furthermore, as will be discussed in an upcoming section regarding the comparative mutagenicities of cis-DDP and ACDP, platinum induced mutagenesis does not appear to be sequence dependent in the lacZ' region of the M13 genome.

c. Mutational frequencies of the cis-DDP G*G* and A*G* adducts

Early indications that the cis-DDP A*G* adduct was more mutagenic (approximately 5 fold) than its G*G* counterpart (Burnouf et al., 1987) were supported by the site specific studies which reported a mutation frequency of 0.2% for the cis-DDP G*G* adduct (Bradley et al., 1993) and
~2% for the cis-DDP A*G* adduct (Burnouf et al., 1990). There were two important caveats regarding the hypothesis that the A*G* adduct is the more mutagenic lesion. First, the work by Burnouf et al. (1987) involved randomly modified DNA. The sites of platinum binding to the DNA were not determined which made it impossible to know if apparent mutational hotspots were the result of a sequence being more mutagenic or simply a consequence of more platinum binding to sites that produced more mutations. These limitations were overcome by the site-specific studies. Nevertheless important differences in experimental techniques remained that made comparison of results difficult. The A*G* adduct was studied in a plasmid (Burnouf et al., 1990) while the G*G* adduct was incorporated into a viral (M13) genome (Bradley et al., 1993). Differences in the replication and cellular processing of these constructs limited the comparative analysis of the mutagenicity of these adducts. Furthermore, the cis-DDP G*NG* adduct had not been studied site specifically so its contribution to the overall mutagenicity of cis-DDP remained unknown. Accordingly, a major focus of this thesis work was to determine the mutagenicity of all three adducts under identical conditions in the same biological system.

The cis-DDP A*G* adduct was slightly more than fourfold more mutagenic than the cis-DDP G*G* adduct (6.0% vs 1.4%) in singly modified M13-derived ss genomes replicated in SOS induced E. coli DL7 cells. Interestingly, this result is in good agreement, albeit at the low end, of the prediction that the A*G* adduct would be 5-10 fold more mutagenic than the G*G* adduct. Despite the agreement in the relative mutagenicities of the G*G* and A*G* determined in previous studies, a comparison of the various studies illustrate that these adducts, when replicated under different conditions, do have different mutagenic potentials. For example, the absolute mutation frequencies observed for adducts located in ss genomes in this study were much (4-10 fold) higher
than the mutation frequencies observed in the previous studies (Burnouf et al., 1990; Bradley et al., 1993). A plausible explanation for the different mutability of the cis-DDP adducts in different systems is differential repair of the lesions. Adducts located in ds genomes (Burnouf et al., 1990; Bradley et al., 1993) uniformly experienced lower mutagenicities than when located in ss genomes (this work). These results suggest that DNA repair systems remove the adducts from the ds substrates, thus ameliorating their mutagenic potential. In ss genomes, however, the major repair mechanism, UvrABC, for cis-DDP adducts is inoperative, allowing the damage to persist and give rise to a higher level of mutations.

d. Structural determinants of mutagenicity

The specificity of mutagenesis induced by a mutagen is a consequence of the structure of the premutagenic lesion. To give an example, the prototypic replication blocking, SOS dependent, premutagenic lesion, the thymine-thymine cyclobutane dimer, is most severely distorted at the 3' base (Hruska et al., 1975). Selective distortion of the 3' base is suggested as the explanation that the mutations arising from thymine dimers are highly targeted to the 3' modified base, with >90% of all induced mutations occurring at this site (Banerjee et al., 1988; LeClerc et al., 1991). Therefore the result that the mutations induced by the cis-DDP G*G* and A*G* adducts were targeted to the 5' base of the adduct site with a high specificity is consistent with the structural data, depicted in Figure 20 as well as discussed in detail in the Literature Survey and briefly below, that the 5' base of a cis-DDP modified dinucleotide suffers the greatest distortion upon platinum binding.

The cis-DDP chelated d(GpG) sequence has been extensively

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characterized as a dinucleotide (den Hartog et al., 1982; Sherman et al., 1985), in longer DNA sequences (Caradonna & Lippard, 1982; den Hartog et al., 1983; 1985b), as well as in duplex DNA (van Hemelryck et al., 1984; Bellon et al., 1991). The 3' modified G is relatively unperturbed from its normal B-DNA conformation. Conversely, the 5' modified G is distorted, experiencing an altered orientation that induces a bend between the planes of the two modified nucleotides. Based on this structural information, the result that mutations were targeted to the 5' G was anticipated. The structural perturbation imposed on the DNA duplex by 1,2 intrastrand binding is localized, with distortion restricted to the sequence in close proximity to the lesion. The DNA is more perturbed 5' to the adduct than it is in the 3' direction. Once again, it is not surprising that while cis-DDP induced mutations were overwhelming located at the 5' position of the adduct site, the only other induced mutations were tandem mutations involving the same 5' modified base as well as its neighboring 5' nucleotide.

The mutations induced by the cis-DDP A*G* adduct were qualitatively almost identical to those from the comparable G*G* adduct. A*G* induced mutations were highly specific to the 5' modified base with A → T transversions comprising the predominant mutation. In addition, tandem mutations involving the modified adenine residue and its 5' neighboring base were also observed for the A*G* adduct. The only difference in the mutational specificity of the A*G* adduct compared to the G*G* adduct was the occurrence of A → G transitions at the 5' modified base (of course, comparable, i.e. G → G, mutations are not possible for G*G* adducts). The A → G transitions occurred less frequently than the predominant A → T transversions, results that are consistent with previous observations (Burnouf et al., 1990; Bradley et al., 1993). The structure of the cis-DDP A*G* adduct has not been as well characterized as the cis-DDP G*G* adduct has been. From what is
known, however, the nature of the structural perturbations imposed on DNA by both adducts is similar (van Hemelryck et al., 1984; Dijt et al., 1989; Urata et al., 1992) accounting for the comparable mutational specificities exhibited by each lesion. The extent of structural distortion caused by binding at d(ApG) sites, however, is greater than that caused by binding to d(GpG) sites (Marrot & Leng, 1989; Schwartz et al., 1989; Anin & Leng, 1990). The greater structural distortion imposed by cis-DDP binding at d(ApG) sites compared to d(G*G) sites, could account for the former lesion's fourfold higher mutagenicity as compared to the cis-DDP G*G* adduct.

The A → G transitions derived from A*G* adducts notwithstanding, cis-DDP induced purine → thymine transversions with a high specificity. This pattern of mutability is consistent with an SOS dependent premutagenic lesion exhibiting its effects by a noninformational, rather than a misinformational, mechanism. Misinformational lesions mediate their mutagenicity by incorrect base pairing during DNA replication and are generally independent of the SOS response. By contrast, for replication blocking lesions such as apurinic/apyrimidinic (AP) sites, the structure of the DNA lesion precludes base pairing, rendering the lesion "noninformational." Such lesions are only mutagenic when a stalled polymerase is rescued by UmuDC facilitated translesion synthesis. Most often during UmuDC mediated translesion synthesis, an adenine residue is inserted opposite a noninformational lesion,\(^1\) resulting in the insertion of thymine in the place of the original lesion upon subsequent rounds of DNA replication, thus fixing the N → T

\(^1\)Adenines are preferentially inserted opposite noninformational lesions. The basis for this preference is hypothesized to be that the primary DNA damage suffered by prokaryocytes would be from ultraviolet radiation producing thymine-thymine cyclobutane dimers in the genome. An adenine residue inserted opposite such a lesion would result in accurate DNA replication, since a thymine would be incorporated in subsequent rounds of DNA replication, correctly replacing the dimerized thymine residues.
mutation. It is hypothesized that the structural perturbation suffered by the 5' base of the cis-DDP G*G* or A*G* adducts render the 5' base noninformational, leading to the observed A → T or G → T transversion mutations, respectively. It should be noted that, although cis-DDP DNA adducts do act as noninformational lesions as evidenced by the occurrence of drug induced mutations, in the vast majority of bypass events (98.6% and 94.0% for the G*G* and A*G* adducts, respectively) correct base pairing does occur, resulting in nonmutagenic DNA synthesis. This relatively high rate of fidelity is likely possible because the base pairing region of cis-DDP modified nucleotides is not affected by platinum binding. Interestingly, even the G*TG* adduct, despite the more severe disruption of the DNA duplex effected by the destacking of its central nucleotide, was also replicated nonmutagenically. Indeed, mutagenic replication of the cis-DDP G*TG* adduct was not observed at all (presumably because the structure of this lesion prevented its recognition by UmuDC).

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1The extent of fidelity for DNA synthesis past DNA lesions in E. coli varies greatly. Very low fidelity occurs during SOS dependent bypass of the thymine-thymine pyrimidine-pyrimidone (6-4) uv photoproduct. This process is only 9% accurate (MF = 91%, LeClerc et al., 1991). Bypass of the uv thymine-thymine cyclobutane dimer occurs with higher fidelity, being 93% accurate (MF = 7%) (Banerjee et al., 1989). The latter example is comparable to the 94% accuracy observed for the bypass of the cis-DDP A*G* adduct in the present work (incidentally studied in the same M13-derived ss genome as the cyclobutane dimer). Nevertheless these rates, or even that of the less mutagenic G*G* adduct (98.6% accuracy in translesion bypass) must be considered to be very low in fidelity compared to normal replication of DNA, a process in which unmodified nucleotides are replicated more accurately by 7-9 orders of magnitude.
Figure 20. Molecular basis for the targeting of mutations to the 5’ base of a bifunctional cis-DDP adduct. As discussed in the text, the 3’ modified base is relatively unperturbed from normal B-DNA structure and consequently suffers few, if any, induced mutations. The 5’ modified base, however, is significantly distorted and is believed to be able to function as a noninformational lesion. Adenosine residues are preferentially inserted opposite the 5’ modified base. In subsequent rounds of replication, T’s are inserted opposite the A’s, thereby fixing the G → T transversion mutations for the G*G* adduct as shown (similar rationale explains A → T transversions targeted to the 5’ base of the A*G* adduct).
C. Comparison of the Genotoxicities and Mutagenicities of cis-DDP and ACDP DNA Adducts

1. Survival

The plaque-forming ability of M13mp18 RF DNA modified with either cis-DDP or ACDP was reduced in a dose dependent manner, suggesting that the DNA adducts formed by both compounds are capable of blocking DNA synthesis in E. coli. Genotoxicities, 3.3 and 5.2 adducts per lethal hit for cis-DDP and ACDP, respectively, are comparable to the lethality of DNA damage formed by benzo(a)pyrene-7,8-dihydrodiol-9.10-oxide (BPDE), uv irradiation, or cis-1,2-diaminocyclohexyldichloroplatinum(II) ([Pt(DACH)Cl₂], Figure 1). The DNA lesions of these compounds, all processed by the SOS system in E. coli, had genotoxicities of 3, 5.54, or 5.5 adducts per lethal hit, respectively (Mizusawa et al., 1981; Strike & Roberts, 1981; Husain et al., 1985) in a variety of plasmid systems. Furthermore, the in vivo results obtained in the present study are in agreement with previous work employing in vitro primer extension methodology that revealed cis-DDP and ACDP adducts block DNA replication to a similar extent, with cis-DDP slightly more inhibitory (Hartwig & Lippard, 1992).

Pretreatment of the host E. coli cells with uv irradiation to induce the SOS response increased the survival of M13mp18 genomes modified with either drug approximately threefold in the plaque forming assay. An SOS-dependent increase in survival for ds DNA modified with cis-DDP is attributable to repair of the lesions by UvrABC (Beck et al., 1985; Husain et al., 1985) or to increased bypass of the adduct, mediated by the umuDC and recA protein complexes (Elledge & Walker, 1983; Bridges & Woodgate, 1984; Rajagopalan et al., 1992). The SOS
dependent survival increase for ACDP closely paralleled that seen for cis-DDP indicating that E. coli processes both types of DNA adducts in a similar manner. Furthermore, since the processivity of eukaryotic, as well as prokaryotic, polymerases is decreased by platinum DNA adducts (Villani et al., 1988; Corda et al., 1991; Comess et al., 1992) the similar genotoxicities of cis-DDP and ACDP in this bacterial system might predict their relative lethalities in eukaryotic cells. This hypothesis is supported by the recent finding that ACDP is about as cytotoxic as cis-DDP and in human ovarian carcinoma cell lines (Kelland et al., 1992b).

2. Comparative mutation frequencies of cis-DDP and ACDP

a. Comparison to other mutational assays

Since cis-DDP is an established mutagen in several bacterial and mammalian systems, its mutagenicity in the lacZ’ mutational assay was anticipated. Other platinum compounds are also mutagens; carboplatin, for example, produces mutations similar to those induced by cis-DDP in the APRT gene of Chinese hamster ovary cells (de Boer & Glickman, 1992). Although other platinum drugs are mutagenic, many are quantitatively less mutagenic than cis-DDP. Carboplatin and cis,trans,cis-dichlorobis-dihydroxy(isopropylamine)platinum(IV) (iproplatin, Figure 1), to give two examples, are considerably less mutagenic than cis-DDP at equitoxic doses in V79 lung fibroblasts and BHK21/C13 cells (Chibber & Ord, 1992). The mutagenicity of ACDP falls into this latter category, being lower than cis-DDP in the present lacZ’ mutational assay. ACDP, like cis-DDP, was found to be an SOS-dependent mutagen in E. coli; mutagenesis by such

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1It should be noted that ACDDP, and not its DNA-reactive metabolite ACDP, that has significant therapeutic advantages over cis-DDP owing to the former compound’s pharmacological properties.
agents is not a passive process but requires specific processing by a cellular system. As discussed, the SOS system consists of a family of inducible proteins including at least three (umuD', umuC, and recA*) that, along with PolIII, form a "mutasome" at the site of a DNA adduct to aid in error prone, and thus mutagenic, bypass of the lesion (Bagg et al., 1981; Bridges & Woodgate, 1985; Bailone et al., 1991b). Compounds forming DNA adducts in E. coli requiring the specific intervention of these proteins to induce mutations are often mutagenic in eukaryotes (Walker, 1984; Ho et al., 1993). It is reasonable, therefore, to expect that ACDP will also be less mutagenic in human cells, a feature that, if realized, would be especially attractive in supporting the clinical potential of ACDDP, its parent compound, as an anticancer drug.

b. Mechanistic rationale for differences in the mutation frequencies of cis-DDP and ACDP

The spectra of adducts formed by cis-DDP and ACDP suggest a molecular mechanism responsible for their relative mutagenicities. The major adduct formed by both compounds occurs at d(GpG) sites; approximately 65% of the total adducts for cis-DDP and 54% for ACDP occur at these sites. The A*G* adduct, however, is about 3 times more prevalent in cis-DDP adduct spectrum than for ACDP (25% vs 8%; Hartwig & Lippard, 1992). A molecular rationale has been developed to explain the diminished ability of ACDP to form the A*G* adduct. The asymmetry of the ACDP complex gives rise to an orientational isomerism when the complex forms 1,2 intrastrand crosslinks with DNA (Hartwig & Lippard, 1992). As illustrated in Figure 3, with d(GpG) adducts, the cyclohexyl ligand can point either toward the 5' or 3' guanine residue and hence both orientational isomers form upon binding to DNA. It is noteworthy that one of the isomers (II) potentially forms a favorable hydrogen bond between the cyclohexylamine moiety of the drug and the N6 of guanine.
Formation of the analogous isomer (IV, Figure 3) does not occur, apparently because the cyclohexylamine moiety sterically clashes with the exocyclic amino group of the 5' adenine. As a consequence, the ACDP drug forms fewer adducts at d(ApG) sites (Hartwig & Lippard, 1992).

Site specific mutagenic studies have suggested that the A*G* adduct is 5-10 times more mutagenic than the G*G* adduct (Burnouf et al., 1990; Bradley et al., 1993). Work done for this thesis confirmed that the A*G* adduct was four to five fold more mutagenic than the G*G* adduct. When formed, the A*G* adduct appears to be a dominant contributor to the mutational spectra of platinum compounds. Therefore, because the highly mutagenic A*G* adduct is more abundant in the cis-DDP adduct spectrum, the finding that the overall mutagenicity of cis-DDP would be higher than for ACDP is understandable. Also in agreement with the observed lower mutagenicity effected by ACDP was the finding that the G*TG* adduct was not mutagenic. Therefore even though G*NG* adducts are twice as abundant in the ACDP spectrum compared to the cis-DDP adduct spectrum (18% vs 8-10%), these adducts are incapable of contributing to the potential mutagenicity of ACDP.

There are two caveats, however, to this mechanistic rationale used to account for the relative mutability of the DNA lesions formed by cis-DDP and ACDP. First, it is not clear that the cis-DDP G*TG* adduct is representative of all platinum 1,3 intrastrand crosslinks. As discussed previously, some mutational assays, including the present one, suggest that 1,3 adducts might be mutagenic. Such studies, however, employed randomly modified DNA and are not as definitive as the site specific evaluation of the cis-DDP G*TG* adduct, which showed that this lesion was not mutagenic in the context evaluated. The G*CG* and G*AG* adducts have not been studied site specifically, and could be mutable, thereby accounting for the apparent mutagenicity of G*NG* adducts. Since the
G*TG*, G*CG*, and G*AG* adducts appear to be structurally similar, it is difficult to understand from a structural perspective how the G*CG* and G*AG* adducts could engender a different level of mutagenicity than the G*TG* adduct. Nevertheless, if the G*CG* and G*AG* adducts, unlike the G*TG* adduct, are mutagenic and comprise a significant proportion of the 18% of the G*NG* adducts formed by ACDP, the mutagenicity of this compound would be higher than otherwise anticipated.

Second, the mutagenic potentials of the cis-DDP G*G*, A*G*, and G*TG* adducts have been assumed to reflect the mutagenic potential of the equivalent adducts formed by ACDP. The accuracy of this assumption is dependent on two factors that could influence the genetic effects of the DNA adducts formed by cis-DDP and ACDP. First, the structural distortion imposed on the DNA architecture by platination (Bellon et al., 1991) could be the primary influence affecting the molecular processing of DNA modified by these compounds. Many platinum(II) compounds bind DNA at similar nucleotide sequences, forming mainly 1,2- and 1,3- intrastrand crosslinks, which effect similar bending and unwinding of the DNA duplex (Lippard et al., 1983; Page et al., 1990; Hartwig & Lippard, 1992). If structural deformation of the DNA helix is the major factor contributing to the genotoxicities and mutagenicities of platinum DNA adducts, the effects of cis-DDP and ACDP would be expected to be very similar. Conversely, if the genetic effects of cis-DDP and ACDP DNA adducts are a consequence of the ligands attached to the platinum adduct, the cyclohexyl ring of ACDP, a moiety lacking in cis-DDP, the two compounds should elicit different biological effects.

The results of this study suggest that both of the aforementioned factors play a role in the mutagenic activity of platinum-DNA adducts. The levels of genotoxicity and types of mutations arising from both cis-DDP and ACDP were very similar indicating the importance of the
structural deformation of DNA on these processes. On the other hand, the influence of the cyclohexyl ring of ACDP was manifest through its ability to direct the formation of potentially less mutagenic adducts resulting in a lower mutation frequency as compared to that of cis-DDP.

c. Comparison of mutation frequencies determined from the lacZ' and the site specific studies

A practical application of the site specific determination of the mutagenicities of the cis-DDP G*G*, A*G*, and G*TG* adducts is the evaluation of new platinum drugs. As discussed above, it appears possible that the overall mutagenic potential of a platinum compound can be extrapolated from the site specific analysis of individual DNA adducts. To reinforce the validity of this approach further, a quantitative evaluation of the results from the site specific and random mutagenesis assays was done. From the results of the site specific experiments, an "average" cis-DDP DNA adduct would be predicted to have a mutagenicity of 2.41%:

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Relative abundance x MF</th>
<th>Normalized MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>G<em>G</em></td>
<td>(0.65) x (1.4%)</td>
<td>0.91%</td>
</tr>
<tr>
<td>A<em>G</em></td>
<td>(0.25) x (6.0%)</td>
<td>1.50%</td>
</tr>
<tr>
<td>G<em>NG</em></td>
<td>(0.10) x (0.0%)</td>
<td>0.00%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2.41%</td>
</tr>
</tbody>
</table>

Assuming that ACDP adducts have similar mutagenicities as the comparable adducts formed by cis-DDP, an "average" ACDP DNA adduct should have a mutagenicity of 1.29%:
Based on these data, cis-DDP should be 1.87 fold (2.41% vs 1.29%) more mutagenic than ACPD. From Figure 17, at an average of 1 adduct in the 370 bp fragment of M13mp18 genomes in which mutations are detectable in the lacZ' mutational assay, cis-DDP had a mutagenicity of 0.27%, the comparable value for ACPD was 0.16%. cis-DDP was 1.7 fold (0.27%/0.16%) more mutagenic than ACPD, a value that closely agrees with the value of 1.89 fold predicted from the site specific results. Clearly, the site specific approach to the evaluation of the different DNA adducts formed by a mutagen has validity in predicting the overall mutability of the compound. Accordingly, as the mechanism for platinum binding to DNA is understood in increasing detail (Bloemink et al., 1992; Hartwig & Lippard, 1992; Bernges & Holler, 1992; Green et al., 1992), an important factor in the design of new platinum drugs should be the selection of substituents that direct the formation of the less mutagenic, and hence safer, DNA adducts.

d. The absolute mutation frequency differs between singly and randomly cis-DDP modified genomes

The mutation frequencies for both cis-DDP and ACPD are much lower in the work utilizing randomly modified DNA than was observed in the site specific studies. As discussed elsewhere, this apparent difference in mutability could be a consequence of utilizing ds DNA in the random study. The randomly modified ds DNA was replicated in repair proficient E. coli known to repair platinum DNA lesions efficiently. By contrast,
the site specific results were obtained by using ss DNA for which repair would not be expected. The platinum DNA adducts, therefore, could persist in the genome and give rise to a much higher level of mutagenicity.

An alternate explanation for the different mutation frequencies is that different methods were used to detect mutations. The selection of mutants occurring in the singly cis-DDP modified M13 ss genomes was based on the ability of a mutation to block cutting at a restriction site. This method, in theory, should allow 100% of the mutations that occurred within the six base restriction site to be detected. By contrast, the selection of mutants occurring from randomly cis-DDP or ACDP modified M13mp18 RF DNA was indirect. Mutations observed in the lacZ' mutational assay rely on amino acid changes that functionally alter β-galactosidase activity which, in turn is phenotypically detected. Considering that the genetic code is degenerate, some mutations do not result in amino acid substitutions and remain silent. Other mutations, although effecting amino acid changes, do not alter β-galactosidase activity sufficiently to be phenotypically observed. A large number of mutants, therefore, can go undetected in the lacZ' mutational assay. Interestingly, by accounting for these factors, as discussed below, the results of the two studies agree fairly well.

The mutation frequency varied from about 0.3 to 0.5% for the randomly modified genomes (1.85 or 2.36 adducts per 370 nucleotide mutation target sequence for cis-DDP and ACDP, respectively) used in the lacZ' mutational assay. Four mutations not giving rise to amino acid changes were found in samples that had mutations elsewhere that enabled their detection in the lacZ' mutational assay (see Table 12, and further discussion in the next section). Assuming that similar level of mutations occurred in all platinum modified genomes, but remained
undetected for the reasons discussed above, the actual mutation frequency of these genomes would be increased by 1.32% (i.e. 4 neutral mutations/303 total samples sequenced) to give a total frequency between 1.62 and 1.82% implying that only 19 to 27% of all mutations that actually occurred were detected. This range of detection is close to the 35% of base substitution mutations predicted to be observed based on theoretical considerations of the degeneracy of the genetic code and the phenotypic consequences of amino acid substitutions in protein function (Stark et al., 1979). Of greater relevance, these adjusted mutation frequencies are comparable to the values of 1.29% and 2.41% for ACDP and cis-DDP, respectively, predicted above.

3. Features of the LacZ' Forward Mutational Assay

The mutational spectra of cis-DDP and ACDP obtained in the present study confirms the utility of the lacZ' mutational assay to detect a wide range of mutations as discussed in the literature survey section. One hundred and eleven different single-base substitutions occurring at 85 sites were detected. Fifty-eight of these mutations were unique to this study. Combined with the data from the other studies previously surveyed, the lacZ' mutation assay now has the capacity to detect at least 199 different single-base substitutions at 130 sites as well as single base frameshifts and large deletions. The extreme sensitivity of the lacZ' mutational assay enables it to avoid biases inherent in other mutational assays.

Approximately 80% of the observed mutations were in the DNA sequence coding for the lacZ' peptide fragment (Figure 18). Missense mutations comprised about 80% of this group along with about 15% nonsense mutations (Table 12). Interestingly, five neutral mutations, at the wobble (3rd) position of serine, proline, leucine, threonine, and
alanine codons were found. Four of the five samples had second, apparently unrelated, missense mutations at other sites that probably were responsible for the serendipitous detection of the neutral mutations. No other mutation was found in the sample with the neutral change in the proline codon, however, despite sequencing the lacZ' DNA sequence from positions 6010 to 6695 (a range considerably larger than the 370 bp sequence in which all of the other mutations were observed). Neutral mutations that affect α-complementation previously have been described for valine (Decuyper-Debergh et al., 1987), proline and alanine (Sambamurti et al., 1988), and leucine (Hoebee et al., 1991) codons. The latter group speculates that these mutations interfere with correct transcription or translation.

4. Dark Blue plaques

Approximately 98% of mutants identified in the lacZ' mutational assay had reduced or abolished β-galactosidase activity. An infrequently occurring class of mutations, however, had significantly enhanced enzymatic activity as described in Table 8. An interesting feature of this group is that they all, with one exception, have mutations that form a stop codon in the C-terminal overlap region. Furthermore, every stop codon detected in the C-terminal overlap region gave rise to a mutated genome that produced the phenotypically observed dark blue plaques (Figure 18).

1The one exception is a mutant that had the initiating methionine residue changed to an isoleucine. Despite lacking the initial methionine, the lacZ' polypeptide was nevertheless synthesized as evidenced by β-galactosidase activity. Peptide synthesis could have been initiated at the third residue, which is also a methionine, or possibly further downstream. Either way, a truncated N-terminal overlapping region would be generated and the same mechanistic explanation given for enhanced β-galactosidase activity (in the text and Figure 21) in the mutants with truncated C-terminal polypeptides would apply.
Presumably, the peptide encoded by the C-terminal overlap region interferes with the association of the subunits of the M15 α-acceptor protein and lacZ' polypeptide α-donor during complementation. Optimal tetramerization of the polypeptide subunits is prevented, resulting in reduced β-galactosidase activity (Figure 21 (a)). "Wild-type" β-galactosidase activity in the M13 system, therefore, is lower than the activity theoretically possible from the molar equivalents of enzyme present during the plaque forming assay. In mutants with enhanced β-galactosidase activity, the truncated peptide in the C-terminal overlap region is hypothesized to allow more efficient tetramer formation with a resultant increase in enzymatic activity (Figure 21 (b)), perhaps approaching the level of activity of the natural form of β-galactosidase (Figure 21 (c)). The M112 α-complementation system provides a precedent for this hypothesis. M112 is an inactive β-galactosidase mutant, similar to M15 in that it forms dimers but not tetramers, but only lacks nine residues from the amino acid sequence 23-31. Under α-complementation conditions, M112 can reach a level of activity many times higher than is possible with the M15 protein, as a result of more efficient tetramer formation of the M112 polypeptide subunits during α-complementation (Welpy et al., 1981a; Zabin, 1982).

5. Mutational Spectra of cis-DDP and ACDP Modified DNA

The mutagenicity of cis-DDP DNA adducts has been investigated in a number of assays, with varying results. The most definitive mutagenesis studies have employed site specifically modified genomes containing the two major cis-DDP DNA adducts. About 80% of the mutations caused by an A*G* adduct in pEMBL8 plasmid replicated in E. coli were A → T mutations at the 5' A of the d(ApG) pair (Burnouf et al., 1990). The cis-DDP G*G* adduct located in an M13mp18 RF genome and replicated in E. coli produced remarkably similar mutations, with greater than 80% G → T
mutations at the 5' G (Bradley et al., 1993). The site specific studies previously described in this dissertation confirm the results of Burnouf et al. (1990) and Bradley et al. (1993) and provide the additional result that the cis-DDP G*TG* adduct is not mutagenic under conditions that rendered the cis-DDP G*G* and G*G* adducts mutable. As might be expected from the site specific mutagenesis studies, G → T or A → T mutations occurring at the 5' position of d(GpG) or d(ApG) sites, respectively, were observed in several studies using randomly platinated DNA in the tetracycline-resistance gene of pBR322 in E. coli (Burnouf et al., 1987), in exon 3 of the human hypoxanthine guanine phosphoribosyl transferase gene in B-lymphoblasts (Cariello et al., 1992), in the SUP4-o gene of Saccharomyces cerevisiae (Mis & Kunz, 1990) and in the supF gene of pZ189 in normal human fibroblasts or XP12BE cells (Bubley et al., 1991). In addition to these expected mutations, the studies by Mis & Kunz (1990) and Cariello et al. (1992) also detected significant numbers of G → A and G → C mutations at d(GpG) sites. In two other studies, utilizing the lacI gene of E. coli (Brouwer et al., 1981; 1982) or the adenine phosphoribosyl-transferase gene of Chinese hamster ovary cells (de Boer & Glickman, 1989) as the mutational target, G → T, G → A, or G → C mutations at the 5' position of d(GpApG) or d(GpCpG) sequences predominated.

Most platinum induced mutations detected in the present study occurred at d(ApG) or d(GpG) sites; furthermore many of these mutations were A → T or G → T transversions at the 5' base, consistent with the results of the site-specific and several of the random mutagenesis studies. Features of other cis-DDP mutational assays, such as the significant number of G → A transitions observed as well as mutations
that could have arisen only from d(GpNpG) sites\textsuperscript{1} were also represented in the cis-DDP mutational spectrum; indeed, essentially all of the mutations described for cis-DDP in the several mutational assays listed above also were observed in the present lacZ\textsuperscript{'} mutational assay. The differences in the mutagenicity previously observed for cis-DDP are more a reflection of the biases inherent in the mutational assays used, rather than an indication of different mutagenic processing of cis-DDP in the various systems. It is reasonable, therefore, to expect that the mutation spectrum obtained for ACDP in this study also would be indicative of its mutagenic potential in a range of prokaryotic and eukaryotic systems.

cis-DDP and ACDP induce similar types of mutations but there are some significant differences in their distribution (Table 11). The predominant type of mutation for both drugs was the single base substitution: 93\% of cis-DDP mutations and 87\% of ACDP mutations. The major mutation observed for both drugs was the G $\rightarrow$ T transversion arising from either the (+) or (-) DNA strands at almost identical rates. In addition, G $\rightarrow$ A and A $\rightarrow$ T mutations were well represented in each mutational spectrum (Figure 18). There were some differences for infrequently occurring mutations, but there were too few of these from which to draw meaningful conclusions. Even though the types of mutations were comparable for each platinum compound, the locations at which the mutations occurred varied. Of the 257 induced mutations, 86 (43 for each drug) were identical mutations that occurred at the same sites for both drugs; the remaining 72 cis-DDP and 99 ACDP mutations were similar in nature but occurred at different sites. The disparity

\textsuperscript{1}Unlike the occurrence of mutations expected from G$^*$G$^*$ and A$^*$G$^*$ adducts in the assays using randomly modified DNA, site specific results from the G$^*$TG$^*$ adduct indicated that this lesion should not be mutagenic. The discrepancy in these results was previously addressed (in section V.B.3) and will not be discussed further at this juncture.
in the distribution of the mutations arising from each drug may be less a reflection of the mutagenic potential of these compounds than an inadequate sample size.\(^1\)

Regardless of sample size, however, it is unlikely that the mutational spectra of the two drugs would become completely identical. Differences exist in the mutational spectra of cis-DDP and ACDP for two reasons. First, the molecular processing of cis-DDP and ACDP DNA adducts is affected, at least to some degree, by the cyclohexyl substituent of ACDP. For example, Hartwig and Lippard (1992) report different stop site preferences for polymerases encountering site-specifically prepared cis-DDP and ACDP G*G* adducts although, quantitatively, the replication blockage effected by each adduct is similar (92% for the cis-DDP G*G* adduct compared to 85-90% for the ACDP G*G* adduct (Hartig & Lippard, 1992)). The results of the present study suggest similar behavior in the mutagenicities of these adducts; the overall mutational spectra are very similar but the individual mutations sites vary. A second factor contributing to the differences in the mutational spectra are differences in the distribution of DNA adducts formed by cis-DDP and ACDP. The sequence specificity of DNA binding sites is determined by a combination of steric and hydrogen bonding effects influenced by the substituents attached to the platinum atom (Green et al., 1992). As indicated earlier, the ability of ACDP to form orientational isomers (Figure 3) could contribute to the observed diminution in highly mutagenic A*G* intrastrand crosslinks.

\(^1\)As more mutants of each drug were sequenced, the proportion of identical mutations increased. For example, after sequencing about half the mutants analyzed in this study (53 cis-DDP and 61 ACDP) only 7 (12%) mutations were identical but increased to 43 (33%) by the end of the study. It is reasonable to expect that if the sample size was large enough the distribution of the mutations would be as similar as the type of mutations given in Table 11.

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As discussed, an analysis of the types of mutations induced by cis-DDP adducts in the site specific studies suggested that these adducts functioned as noninformational premutagenic lesions. This mode of mutagenesis is consistent with the mutational results observed for the platinum-modified M13mp18 genomes utilized in the lacZ' mutational assay. The similarities in the mutational spectra of cis-DDP and ACDP provides additional support for the hypothesis that platinum DNA adducts are noninformational, rather than misinformational, lesions. The mutagenicity of both platinum compounds is similar to AP sites. A mutational spectrum determined for AP sites in an M13mp2 lacZ' mutational assay showed a pattern of mutagenesis similar to that seen for both platinum drugs in the present study (Kunkel, 1984). In Kunkel's study, 79% of spontaneous single base changes were transitions; a value that dropped to 28% for mutations induced by AP sites. Similarly, in the present study, the number of transitions decreased from 73% of single base changes in unmodified DNA to 33% in platinated DNA. The similarities between mutations caused by cis-DDP and ACDP observed here, as well as the similarities of mutational spectra for cis-DDP and carboplatin in the APRT gene of mammalian cells (de Boer & Glickman, 1992) suggest that mutations induced by platinum(II) compounds are primarily a consequence of adduct-induced bending and unwinding of the DNA helix and are not greatly influenced by any misinformational effects exerted by substituents attached to the platinum atom.

6. Correlation of Sites of Adduct Formation with Mutational Spectra

The spontaneous mutational spectrum generated from unmodified DNA was significantly different than the cis-DDP and ACDP induced spectra, indicating that specific platinum adducts, not random DNA damage, were responsible for the mutagenicity associated with the modified DNA samples. Replication mapping of adducts was done to demonstrate that
platinum adducts were present at the sites where mutations occurred to reinforce further the mechanistic link between these DNA lesions and the induced mutational events. As can be seen in Figure 18, most of the observed mutations do occur at sites of platinated nucleotides. A fraction of the mutants, (23 cis-DDP and 25 ACDP), however, occur at nucleotides that apparently are not modified; these mutants result from one of two causes. First, at many potential sites of modification, the level of platination, although detectable, was too weak to quantitate and therefore was not included in Figure 18. Such sites include the cis-DDP induced mutants at position 6147 of the (-) strand. Seven cis-DDP and 12 ACDP induced mutants fit into this category. Second, as mentioned in the Results section, approximately 12-20% of the mutants in the drug induced spectra are spontaneous in origin, and would not be expected to occur at drug modified sites. An example of this class of mutants can be observed near 6310 where similar mutations are seen in the cis-DDP, ACDP, and spontaneous mutational spectra. Considering these factors, the correlation between the sites of adduct formation and the actual the location of mutations is excellent.

The adduct mapping experiments also revealed that platinum binding in the M13mpl8 lacZ' DNA sequence is stochastically determined for the major G*G* adduct. The empirically derived adduct distribution matched the distribution predicted from the known binding preferences of these drugs (Figure 22, white and gray bars). Ideally, the adduct mapping experiments would have had sufficient sensitivity to compare the relative number and sites of minor DNA adducts (e.g. A*G* and G*NG*) formed by cis-DDP and ACDP. Unfortunately, the adduct distribution experiments did not have sufficient sensitivity to allow a rigorous comparison of the relative number and sites of these adducts. Such information would afford further insight into the hypothesis that ACDP is less mutagenic because it forms fewer of the highly mutagenic A*G*
adducts. Unfortunately the difficulty in quantifying infrequently occurring adducts coupled with the inability to determine which adduct occurred at a site capable of giving rise to more than one adduct, prevented a rigorous comparison of the relative mutagenic potentials of the A*G*, G*G*, G*NG* or other DNA adducts formed by cis-DDP and ACDP. This point notwithstanding, it is reasonable to predict that, as for the G*G* adducts, the infrequently occurring adducts also would be stochastically distributed in the lacZ' DNA sequence of the M13mp18 RF genomes. Accordingly, the threefold higher level of A*G* adducts in the cis-DDP adduct spectrum compared to the ACDP adduct spectrum (Hartwig & Lippard, 1992) would offer as attractive explanation for the higher mutagenicity of cis-DDP. An analysis of the specific adducts that produce mutations is consistent with this hypothesis. The data in Table 13 suggest that over three times as many mutations occur at d(ApG) sites in the cis-DDP mutational spectrum as in the ACDP spectrum.

The data in Table 13 includes a relatively small subset of the entire mutational spectra that only includes mutations of unambiguous origin. Mutations of ambiguous origin also can be assigned to particular nucleotide sequences assuming that the mutational spectral data obtained in this study reflect the results of previous site specific mutagenesis studies showing that mutations predominantly occur at the 5' nucleotide of either an d(ApG) or d(GpG) site (Burnouf et al., 1990; Bradley et al, 1993) as well as the work by Brouwer et al. (1981), which indicated that mutations also preferentially occur at the 5' position of d(GpNpG) sites. Primarily by using this criterion to assign mutations to particular DNA adducts (Table 14), A*G* adducts once again were shown to induce significantly fewer mutations in the ACDP spectrum than in the cis-DDP spectrum. Another interesting finding is the relatively high proportion of mutations at d(GpNpG) sites, suggesting that the G*NG* adduct, like the A*G* adduct, is more mutagenic that the
G*G* adduct (as mentioned, this observation conflicts with the nonmutability of the cis-DDP G*TG* adduct in the site specific experiments). The tentative nature of these conclusions should be emphasized, considering that the data in Table 14 are based on several assumptions. Nevertheless, it is of note that the conclusions reached from Table 14 are in agreement with the smaller, but more precise, set of data presented in Table 13.

7. Sequence Dependence of Mutagenesis

Another goal of the adduct mapping experiments was to determine whether mutations arising from cis-DDP or ACDP adducts were sequence-dependent; that is, are mutation hotspots observed in the lacZ' DNA sequence of M13mp18 RF genomes modified with the platinum drugs? Apparent mutation hotspots are commonly detected in lacZ' β-galactosidase α-complementation mutation assays (Kunkel, 1984; LeClerc et al., 1984; Decuyper-Debergh et al., 1987; Sambamurti et al., 1988; Hoebee et al., 1989; Gupta et al., 1991; McBride et al., 1991). A limitation of these previous studies, however, is that the exact sites of DNA damage were not determined. It is therefore unclear whether multiple mutations at a particular site comprise a sequence-dependent hotspot or are simply a consequence of a higher level of DNA damage at that site. In contrast, the adduct mapping data obtained in the present study allow a direct comparison between the sites of platination and sites of mutagenesis.

a. Sensitivity to mutation is related to the local function of the lacZ' sequence

The data in Figure 18 clearly show that some sites experience
significantly more mutations than other sites with similar levels of platinum binding. By contrast, several highly modified sites have few, if any, mutations. The sites of enhanced, or diminished, mutagenicity, however, are more closely correlated with the functional specificity of the lacZ' DNA sequence than with the local nucleotide composition of the DNA. As shown (Figure 21), some regions of the lacZ' DNA sequence of the M13mp18 genome, such as the CAP binding site, -35 promoter, ribosome binding site, and 93 nucleotide sequence corresponding to the deletion in the M15 α-acceptor protein are hypersensitive to mutations. Mutations occurring in these functionally critical areas are expected to have a greater chance of disrupting the α-complementation process. Such mutations are more easily detected than mutations in less crucial regions of the lacZ' sequence of M13mp18 such as the sequences between the regulatory elements in the promoter/operator region and the sequence encoding the N- and C-terminal overlapping regions of the lacZ' polypeptide. The peptide overlapping regions are duplicated in the M15 α-acceptor protein and are not critical for β-galactosidase activity (Figure 7). It is expected that most mutations in the latter segments of the lacZ' DNA sequence would have minimal, if any, impact on α-complementation and would probably go undetected, as appears to be the case in this study.

b. Distribution of missense mutations

The hypothesis that mutations occur randomly with respect to sequence context (but are correlated with site of drug modification) gains additional support from the relative distribution of missense and nonsense mutations. The level of missense mutations was closely linked to the functional importance of the lacZ' polypeptide. Only three mutations giving rise to amino acid substitutions were detected in the 29 residue N-terminal overlapping region (Figure 7, Table 12). This
region has been shown to be relatively insensitive to changes in amino acid composition (LeClerc et al., 1984; Bernges & Holler, 1992); and it is not surprising that few missense mutations were detected. The C-terminal region of the lacZ' peptide, like the N-terminal region, has a structural overlap with the M15 α-acceptor protein and, similarly, would be expected to be relatively insensitive to mutations because the functional peptide chain can be supplied by either the α-donor or α-acceptor (Welpy et al., 1981b). Although the C-terminal overlapping region is composed of 54 amino acids, detectable mutations typically have been confined to the portion of this region immediately adjacent to lacZ' DNA sequence coding for the 31 amino acid residues deleted from the M15 α-acceptor protein. In this study, no mutations were detected further than 30 residues from the sequence representing the deletion in the M15 protein. Somewhat surprisingly, missense mutations were detected at a significantly higher rate than in the N-terminal overlapping region, affecting 13 of these 30 residues. No mutations were detected in the 24 amino acid residues comprising the C-terminal end of the lacZ' peptide fragment. In contrast to the overlapping regions, the DNA sequence coding for the portion of the lacZ' polypeptide deleted from the M15 α-acceptor protein was extremely sensitive to amino acid substitutions. Fifty one different missense mutations were detected affecting 21 of the 31 amino acid residues encoded by this DNA sequence (Figure 18, Table 12).

c. Distribution of nonsense mutations

Unlike the region specific distribution of missense mutations, discussed above, approximately the same number of nonsense mutations were detected in the lacZ' DNA sequences corresponding to the 29 amino acid residue N-terminal overlapping region, the 31 amino acid region deleted from the M15 α-acceptor protein, and the 30 residue C-terminal
overlapping region of the \textit{lacZ'} polypeptide fragment. These three similarly sized DNA segments, suffered six, six, and five nonsense mutations, respectively. Each of the resulting truncated \textit{lacZ'} polypeptide fragments resulting from nonsense mutations in the C-terminal overlapping region gave rise to enhanced $\beta$-galactosidase activity (as described previously and explained in Table 8 and Figure 20).

Every nonsense mutation, because of its disruption of polypeptide synthesis, is expected to be detected in the \textit{lacZ'} mutational assay. If these mutations occurred randomly, an equal distribution would be expected in the 3 regions of the \textit{lacZ'} polypeptide; such a distribution was experimentally observed. By contrast, missense mutations, by only affecting the composition of a single amino acid, could be expected to go undetected in nonessential regions of the \textit{lacZ'} polypeptide. Therefore, even if missense mutations were randomly distributed, more would be expected to be observed in the M15 deletion region than in the overlapping regions of the \textit{lacZ'} sequence. Once again, this prediction was verified experimentally.

d. Mutations are correlated to a position within a codon

Further analysis of the \textit{lacZ'} peptide coding region indicates that observed mutations also reflect the position of the codon. More specifically, 36 mutations occurred at the first base of the codon, 47 at the second base, and only 15 at the third base (of these 15, only 10 gave rise to amino acid changes; see Table 12). The low number of mutations seen at the third, or wobble, position was expected because of the degeneracy of the genetic code. This result suggests mutations could be occurring at roughly comparable rates at all three positions but are detected more readily at the first and second positions.
The region-specific distribution of missense mutations, the constant distribution of nonsense mutations throughout the entire DNA sequence coding lacZ' polypeptide fragment, and the observation that the first and second positions of codons were subject to enhanced mutagenicity compared with the wobble position implies that mutations reflect the functional sensitivity of the regions of the M13mp18 lacZ' DNA sequence. It is concluded, therefore, that cis-DDP and ACDP are not sequence-dependent mutagens in this system. Taking into account the adduct spectra (Figure 18), mutations occur at a similar rate throughout the entire lacZ' DNA sequence of the M13mp18 genome. Although sequence dependent mutation hotspots were not found for platinum(II) adducts in the lacZ' mutation assay, such hotspots may exist for other DNA lesions in this system. Furthermore, a study in a human cells indicates that sequence dependent hotspots exist for platinum induced mutations in an eukaryotic system (Bubley et al., 1991). More study is required to determine what factors contribute to whether or not a DNA adduct will experience sequence specific mutagenesis.

e. The lack of mutational hotspots reinforces the validity of the site specific results

A major focus of the work described in this dissertation was the comparison of the relative mutagenicities of the various cis-DDP DNA adducts. The validity of comparing the relative mutation frequencies of the cis-DDP G*G*, A*G*, and G*TG* adducts in different sequence contexts^1 is uncertain. Because the local sequence context could render one adduct more mutable than another, the relative mutation frequencies,

^1The site specific cis-DDP G*G*, A*G*, and G*TG* adducts were incorporated into similar, but not identical sequences. Each adduct was flanked by identical d(TpCpT)_3 sequences, but were directly located in different restriction endonuclease restriction sites, resulting in variability in the nearest neighbor nucleotides.
in theory, could reflect the local sequence context, and not the inherent mutability, of each adduct. This argument is undermined by the multiple lines of evidence, discussed above, that indicate that cis-DDP is not a sequence dependent mutagen in the lacZ' DNA sequence of M13 genomes replicated in E. coli DL7 cells.

8. Technical Features of the Adduct Mapping Experiments

Adduct mapping experiments of the type used in this work and in many other studies are based on the premise that the DNA lesion under study is capable of efficiently blocking DNA synthesis. When primer extension reactions are performed on DNA templates containing such adducts, each lesion encountered by a polymerase will block further replication, thereby generating a set of DNA fragments corresponding to sites of platinum binding. DNA adducts formed by either cis-DDP (Comess et al., 1992) or ACDP (Hartwig & Lippard, 1992) efficiently inhibit DNA polymerases, allowing the location of these adducts to be determined in the lacZ' region of the M13mp18 RF genome. Despite the success of these experiments in locating the sites of platinum adducts and determining their relative abundance, some technical difficulties were encountered that are worthy of discussion.

Success in locating DNA binding sites of DNA adducts in adduct mapping experiments depends on the ability of the polymerase to be blocked only at sites of DNA adducts. Unfortunately, the lacZ' region of ds M13mp18 DNA has numerous restriction sites that form stable secondary structures that also serve as termination sites for polymerases. Termination sites attributable to secondary structure were of sufficient intensity to obscure termination sites arising from platinum DNA adducts when the mapping experiments were performed with Sequenase T7 DNA polymerase or T4 DNA polymerase at 37°C. Addition of
E. coli or T4 single-strand binding proteins ameliorated secondary structure termination to a degree, but did not reduce the background sufficiently to detect adduct stop sites unambiguously. Since high temperatures were expected to lessen secondary structure effects, adduct mapping was attempted by using thermal cycling methodology described recently (Bubley et al., 1991; Murray et al., 1992). This technique involves the linear amplification of modified DNA by performing primer extension reactions at high temperatures repeatedly on the same template population with a single primer. Adduct mapping by this method was attempted with unmodified, cis-DDP and ACDP modified M13mp18 RF genomes by performing high temperature linear amplification with Taq or Vent<sup>®</sup> (exo) DNA polymerases over 15 to 20 cycles. Despite expectations of lessened secondary structure at high temperatures, the same termination sites, similar to those seen with Sequenase or T4 DNA polymerase at 37°C, were observed for platinated and unmodified DNA samples despite varying the primer concentration from a 5 to 20 fold excess and the extension temperature from 55 to 82°C. When a single cycle was carried out, however, only faint secondary structure termination sites were observed for the unmodified DNA whereas strong adduct induced termination sites were observed for both the cis-DDP and ACDP modified samples.

The discrepancy in the results obtained in the single cycle and 20 cycle adduct mapping results can be explained by considering that the two populations of DNA fragments produced in each amplification cycle. One population of DNA fragments is from the secondary structure termination events. A second population results from adduct specific termination events. In successive cycles, fragments produced in previous cycles can reanneal to the M13mp18 template DNA, serve as primers, and be further extended. A fragment terminated at the site of a DNA lesion is unlikely to reanneal to another template with an adduct
at the same site; further extension of this fragment results in the loss of the adduct termination site information generated in the previous cycle(s). Conversely, all template molecules have the same secondary structure, allowing these termination sites to accumulate during each cycle. The net effect of this process is that fragments generated by secondary structure termination sites accumulate over 15 to 20 cycles, obscuring the fragments produced at adduct termination sites in the last cycle (and the few that have persisted from previous cycles). Although this methodology may be adequate for vectors free of secondary structure or other extraneous termination sites, caution is necessary when performing adduct mapping experiments by thermal cycling to ensure that the results accurately reflect the actual adduct distribution. We therefore conclude that previous studies performed by using the thermal cycling methodology should be viewed with caution.
Table 13. Assignment of mutations of unambiguous origin to specific cis-DDP or ACDP DNA adducts

<table>
<thead>
<tr>
<th>Adduct</th>
<th>cis-DDP</th>
<th></th>
<th>ACDP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
<td>Percent</td>
</tr>
<tr>
<td>A<em>G</em></td>
<td>5</td>
<td>17</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>G<em>G</em></td>
<td>4</td>
<td>14</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>G<em>NG</em></td>
<td>16</td>
<td>55</td>
<td>19</td>
<td>43</td>
</tr>
<tr>
<td>other</td>
<td>4</td>
<td>14</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>Totalb</td>
<td>29</td>
<td>100</td>
<td>44</td>
<td>100</td>
</tr>
</tbody>
</table>

a. Only mutations of unambiguous origin are included on this table. Many mutations could not be definitively assigned to a particular adduct. For example, a d(GpApGpG) sequence could accommodate a G*NG*, A*G*, or G*G* adduct, making it difficult to assign a mutation to a specific adduct.

b. The total number of mutations included in this table is a small subset of the entire number of drug induced mutations that were determined (i.e. 29 of 115 cis-DDP and 44 of 142 ACDP induced mutations). A similar analysis can be applied to the entire set of mutants by making a number of assumptions; such an analysis is presented in Table 14. It should be noted that the same conclusion that ACDP induced about 3 fewer more mutations at A*G* sites than cis-DDP did is reached from both tables despite the differences in data sets.
Table 14. Assignment of all mutations, of both ambiguous and unambiguous origin, to specific cis-DDP or ACDP DNA adducts

<table>
<thead>
<tr>
<th>Adduct</th>
<th>cis-DDP</th>
<th>ACDP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutations of unambiguous origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A<em>G</em></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>G<em>G</em></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>G<em>NG</em></td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>other</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td><strong>Mutations for which the site of origin is ambiguous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A<em>G</em> or G<em>G</em></td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>(A<em>G</em>)</td>
<td>(4)</td>
<td>(3)</td>
</tr>
<tr>
<td>(G<em>G</em>)</td>
<td>(12)</td>
<td>(20)</td>
</tr>
<tr>
<td>G<em>G</em> or G<em>NG</em></td>
<td>32</td>
<td>27</td>
</tr>
<tr>
<td>(G<em>G</em>)</td>
<td>(28)</td>
<td>(20)</td>
</tr>
<tr>
<td>(G<em>NG</em>)</td>
<td>(4)</td>
<td>(7)</td>
</tr>
<tr>
<td>A<em>G</em> or G<em>NG</em></td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>(A<em>G</em>)</td>
<td>(13)</td>
<td>(3)</td>
</tr>
<tr>
<td>(G<em>NG</em>)</td>
<td>(5)</td>
<td>(7)</td>
</tr>
<tr>
<td>A<em>G</em>, G<em>G</em>, or G<em>NG</em></td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>(A<em>G</em>)</td>
<td>(3)</td>
<td>(2)</td>
</tr>
<tr>
<td>(G<em>G</em>)</td>
<td>(9)</td>
<td>(14)</td>
</tr>
<tr>
<td>(G<em>NG</em>)</td>
<td>(1)</td>
<td>(4)</td>
</tr>
<tr>
<td><strong>Total of all mutations</strong></td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>A<em>G</em></td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>G<em>G</em></td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>G<em>NG</em></td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>other</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

a. Mutations assigned to these adducts are unambiguous (the data in this category is the same as depicted in Table 13.

b. Mutations that could be a consequence of more than one type of lesion because of the overlap of potential binding sites are assigned to a particular adduct based on the preferential site of mutation induction determined in site specific (Burnouf et al., 1990; Bradley et al., 1993) or random (Brouwer et al., 1982) studies of cis-DDP mutagenicity. When the lesion causing a mutation could not be determined by using the above criteria, the mutation was assigned to a particular adduct based on the binding preferences of the platinum compound; for example, if there were three ACDP induced mutations resulting from either an A*G* or G*NG* adduct, one was assigned to the A*G* adduct and two to the G*NG* adduct based on the 1:2 ratio of A*G* to G*NG* adducts (8% vs 18%) (Hartwig & Lippard, 1992).

c. The adducts to which mutations are assigned based on the above criteria are enumerated in parentheses.

d. Total mutations attributable to each adduct are given as percentages and are the sum of the unambiguous and assigned mutations.
Figure 21. Speculative model to account for enhanced β-galactosidase activity of phenotypically "dark blue" plaques. a. The lacZ' polypeptide encoded by M13mp18 is about 3 times larger than required for α-complementation. The excess peptide comprises the N- and C- terminal overlapping regions (Figure 7). These overlapping regions are hypothesized to interfere with the α-complementation process by sterically preventing optimal association of the β-galactosidase subunits. b. Mutants that have a truncated C-terminal overlapping region may be able to associate more efficiently during the α-complementation process, perhaps attaining a conformation similar to the natural form of β-galactosidase (c), resulting in greatly enhanced enzymatic activity (Table 8).
a. M13mp18 "wild-type" lacZ polypeptide
M15 α-acceptor protein

α-complementation

tetramerization

Reduced β-galactosidase activity due to suboptimal tetramerization

b. lacZ' polypeptide with truncated C-terminal overlapping region
M15 α-acceptor protein

α-complementation

tetramerization

The truncated C-terminal overlapping region allows more efficient tetramerization, resulting in enhanced β-galactosidase activity

c. Naturally occurring, non-complemented β-galactosidase

β-galactosidase

tetramerization

β-galactosidase activity
Figure 22. Comparison of the predicted and actual adduct distributions with the distributions of mutations in the various segments of the lacZ' DNA sequence of M13mp18 RF DNA. The predicted adduct distributions (data were pooled for both drugs for this analysis as no significant differences were discernable at the level of this analysis) were determined based on the known binding preferences of cis-DDP (65% at GpG sites, ~25% at ApG sites, ~10% at GpNpG sites and ~1% at other sites) and ACDP (54% at GpG sites, 8% at ApG sites and 10-18% at GpNpG sites) to DNA. The region referred to as "other" represents the DNA sequence between the various regulatory elements upstream of the lacZ' polypeptide coding region.
D. Application of Results to Drug Design and Evaluation

1. Relevance results from prokaryotes in the analysis of human drugs

The experiments performed for this thesis were done in a bacterial system with viral DNA but nevertheless have relevance in the design of drugs intended for use in humans. The mechanism(s) by which platinum drugs kill tumor cells are only beginning to be elucidated at the molecular level. Likewise, the induction of potentially tumorigenic mutations by platinum DNA adducts in mammalian systems is not well understood. Nevertheless, there are indications that prokaryotic and eukaryotic DNA and RNA polymerases process platinum DNA adducts in a similar manner (Villani et al., 1988; Corda et al., 1991; Comess et al., 1992). Indeed, evidence is emerging that both prokaryotes and eukaryotes have functional homologues to the *E. coli* umuDC genes which are required for the mutagenic processing of replication blocking DNA lesions (Woodgate & Sedgewick, 1992; and discussed in the Literature Survey). Therefore a comparison of the DNA lesions formed by *cis*-DDP and ACDP in a bacterial system, in which the processing of *cis*-DDP DNA adducts has been extensively studied and is understood at a molecular level, may yield clues to predict the eukaryotic response to DNA adducts formed by these platinum compounds.

DNA adducts formed by *cis*-DDP and ACDP are both processed by the SOS system of *E. coli*. It is reasonable, therefore, to expect that their relative mutagenicities described in this dissertation will reflect their relative mutagenicities in eukaryotic systems as well. The assumption that platinum compounds will elicit similar genetic responses in prokaryotes and eukaryotes has already been demonstrated for *cis*-DDP. As discussed, the mutagenicity of *cis*-DDP has been studied and shown to be similar in a variety of systems. It is anticipated that the
mutability of ACDP determined in this work will also be reflected in eukaryotes.

2. Implications of replication blockage, mutagenic and genotoxic studies for platinum-based drug design

The work done in this investigation has implications for the design of human anticancer drugs. A significant concern of platinum based therapeutic regimens is the occurrence of secondary tumors, possibly as a consequence of drug induced mutations. A major focus of the present study was to address to determine the comparative mutagenicities of DNA adducts formed by cis-DDP and ACDP. Two factors indicate that these results have validity in predicting the mutagenic potential of these compounds in humans. First, one aspect of this work, the comparative genotoxicities of cis-DDP and ACDP in the bacteriophage plaque-forming assay, is consistent with recent results in human carcinoma cell lines (Kelland et al., 1992b). Second, the mutational spectrum determined for cis-DDP encompasses mutations seen in a variety of mutational assays, including eukaryotic systems, suggesting that the spectrum for ACDP will also predict its mutagenic potential in a variety of systems, including human cells. The immediate consequence of the lower mutagenicity of ACDP compared with cis-DDP is that the parent compound of ACDP, ACDDP, may be less carcinogenic than current platinum drugs.

An analysis of the molecular characteristics shared by chemotherapeutically effective platinum agents suggests additional implications that this study may have for drug design. Platinum compounds bind to DNA to form a variety of adducts, but only those that form intrastrand crosslinks at d(ApG) or d(GpG) sites are believed to be effective anticancer drugs. A*G* and G*G* adducts induce specific
bending and unwinding of the DNA helix that is recognized by a set of HMG box-containing proteins that may, at least in part, mediate the cytotoxicity of cis-DDP in mammalian cells (Toney et al., 1989; Donahue et al., 1990; Bruhn et al., 1992; Pil & Lippard, 1992; Brown et al., 1993). This study further supports the role of structural distortion of the DNA helix, rather than the nature of the ligands attached to the platinum atom, as the critical element that mediates the molecular effects of platinum drugs. More specifically, ACDP showed comparable lethality to cis-DDP in the plaque-forming assay and elicited similar types of mutations, suggesting that these genetic events were a consequence of similar DNA distortions induced by both drugs, not the presence or absence of the cyclohexyl moiety of ACDP. Despite the obvious importance of structural distortion of the DNA in mediating cytotoxicity and genotoxicity, the ligands attached to the platinum atom also have a crucial role in determining the biological effects of these compounds. The influence of the ligands, however, is exerted before DNA binding by influencing the composition of the adduct spectrum. The cyclohexyl ring of ACDP, for example, directed a dramatic reduction of the highly mutagenic A*G* adduct compared to the number of the comparable A*G* adducts formed by cis-DDP, a compound lacking the cyclohexyl group. At the same time ACDP formed almost an equivalent level of G*G* adducts as cis-DDP, an important result considering that G*G* adducts are high toxic (Bradley et al., 1993; this work) and are refractory to repair in human cell extracts (Szymkowski et al., 1992) and may be the primary agent of cytotoxicity for these platinum compounds in eukaryotic cells. These findings demonstrate a molecular rationale for lowering the mutagenicity, and therefore the potential carcinogenicity, of platinum anticancer drugs.
VI. SUGGESTIONS FOR FUTURE RESEARCH
The objectives of the work outlined in this dissertation were twofold. The first aspect dealt with the site specific determination of the genotoxicities and mutagenicities of the \textit{cis}-DDP \textit{G*G*}, \textit{A*G*}, and \textit{G*TG*} adducts in wild type \textit{E. coli} cells. Several experiments will be outlined below that both expand the range of platinum adducts worth examining by use of site specific techniques and extend the genetic contexts of the cells in which the adducts were replicated. The second aspect of this work addressed the practical aspects of engineering new platinum drugs. This dissertation describes the relative mutagenicities of the various DNA adducts formed by platinum compounds and demonstrates a strategy to design drugs with lower mutagenic potentials. Experiments will be mentioned that continue to elucidate principles designed to facilitate the design of future generations of platinum anticancer drugs.

A. Site Specific Experiments

1. \textit{cis-} and \textit{trans}-DDP \textit{G*NG*} Adducts

The site specific studies of the \textit{cis}-DDP \textit{G*G*} and \textit{A*G*} adducts, both previously (Burnouf \textit{et al.}, 1990; Bradley \textit{et al.}, 1993) and in this work have yielded consistent, well defined results regarding the genotoxicities and mutagenicities of these adducts. Results for the \textit{cis}-DDP 1,3 intrastrand crosslinks are less definitive, however. The \textit{cis}-DDP \textit{G*TG*} adduct, based on its structural similarities to the \textit{cis}-DDP \textit{G*AG*} and \textit{G*CG*} adducts, is expected to be a good model for all three lesions. Nevertheless, a disparity exists in the results obtained for the various \textit{cis}-DDP 1,3-intrastrand crosslinks. \textit{cis}-DDP \textit{G*TG*} was shown to not be mutagenic in the site specific part of this work, however the random mutagenesis section of this thesis as well as similar studies (Brouwer \textit{et al.}, 1981; 1982) have implicated \textit{cis-G*NG*} adducts.
as premutagenic lesions. The possibility exists that the mutations observed in the lacZ' mutational assay did come from G*AG* or G*CG* adducts. A site specific evaluation of these adducts is required to determine if they, unlike cis-DDP, are mutagenic. If the cis-DDP G*AG* and G*CG* adducts are mutagenic, a more detailed structural analysis of the three 1,3 intrastrand crosslinks would be beneficial in order to reveal the subtle differences between these adducts that predicate their relative mutagenic potentials.

Additional information on the mutability of 1,3 intrastrand crosslinks could be derived from a site specific evaluation of trans-DDP G*AG*, G*CG*, and G*TG* adducts. The 1,3 intrastrand crosslinks formed by trans-DDP have structural similarities to the comparable adducts formed by cis-DDP adducts such as the destacking of the central nucleotide. Conversely, structural dissimilarities also exist between the 1,3 DNA adducts formed by cis- and trans-DDP. For example, the degree of unwinding of the DNA duplex and the directed bend of the helix by cis-DDP are different than the comparable properties shown by trans-DDP. A comparison of the respective cis- and trans-DDP G*AG*, G*CG*, and G*TG* adducts would define exactly which of these structural characteristics are important in the genotoxic and mutagenic processing of these adducts.

2. Interstrand Crosslinks

Interstrand crosslinks compose a minor fraction of the DNA binding spectrum of cis-DDP and similar platinum(II) compounds. These adducts, however, remain inadequately studied, and their genetic effects are largely uncharacterized. Despite their relative inabundance, interstrand crosslinks could play an important role in the cellular response to cis-DDP if they are disproportionately genotoxic or
The study of interstrand crosslinks is technically more challenging than the study of the intrastrand crosslinks. An interstrand crosslink, of course, would require placement in a ds genome, and therefore could not be incorporated into the type of singly modified genomes described in this work. As of yet, modified methodology, describing the high yield, efficient synthesis, and subsequent incorporation into genomes, of such adducts has not been reported but, if developed, could be used to determine the genetic effects of cis-DDP interstrand crosslinks.

3. Site Specific ACDP Adducts

The second part of this dissertation described the testing and confirmation of the prediction that ACDP had a lower mutagenicity than cis-DDP because it formed fewer of the highly mutagenic A*G* adducts. This prediction was based on the assumption that the DNA adducts formed by different platinum compound have similar biological consequences, for example an ACDP G*G* adduct and a cis-DDP adduct are equally mutable. This assumption is reasonable and furthermore, was supported by the experimental observation that ACDP was less mutagenic than cis-DDP in the lacZ' mutational assay. Nevertheless, a site specific comparison of cis-DDP and ACDP adducts would conclusively verify, or discount, this assumption.

The structural isomerism exhibited by ACDP upon DNA binding is another justification for a site specific study of ACDP DNA adducts. The cyclohexyl ring of ACDP can be oriented in either the 5' or 3' direction when bound to DNA (Figure 3); it would be interesting to determine if these isomers elicited different biological responses. If so, this result would indicate that the cyclohexyl ring, and possibly other ligands of platinum(II) compounds, are important in mediating...
genotoxic and mutagenic effects. Alternatively, if the biological effects experienced by both isomers are similar, it would indicate that the bending and unwinding of the DNA duplex are more important in effecting the cellular responses to platinum adducts. The latter outcome would further support the assumption that the site specific results obtained from cis-DDP DNA adducts are applicable to adducts formed by other platinum compounds.

B. Genetic Experiments

The different genotoxicities and mutagenicities experienced by cis-DDP DNA adducts (or not, in the case of cis-DDP G*TG*) in SOS competent, compared to non-SOS induced, E. coli cells were, in large part, attributed to UmuDC mediated translesion synthesis past these adducts. As discussed, several lines of evidence indicate that umuDC gene products do play a major role in this process but do not rule out the intervention of other systems as well. For example, post replication mismatch repair and recombinational repair could both have been involved in the cellular processing of the singly modified ss genomes containing the cis-DDP DNA adducts. E. coli mutants deficient in recombinational repair and in the various aspects of mismatch repair could be used to determine what, if any, role these systems have in eliciting the genetic effects of cis-DDP DNA adducts. In any event, the role of UmuDC could be more closely scrutinized by the use of various E. coli umuD and umuC mutants.

The study of cis-DDP could be extended to mammalian systems by using either site specifically or randomly modified shuttle vectors. The processing of cis-DDP adducts is not as well characterized in eukaryotic cells as it is in prokaryotes. Fortunately, however, many mammalian cell lines with different repair deficiencies are becoming
available, and should aid in the elucidation of the mechanisms of DNA repair in eukaryotes. More specifically, the study of cis-DDP modified DNA in these systems may yield clues that improve the therapeutic efficacy of platinum compounds.

C. Drug Design

Research devoted to the design of novel platinum drugs has been oriented toward reducing toxic side effects to the patient and overcoming drug resistance that renders platinum therapy ineffective against most tumors. This dissertation has demonstrated the feasibility of another objective that deserves to be incorporated into the design of new drugs.

Current platinum drugs are suspected human carcinogens; therefore novel drugs are also potential carcinogens. Given the strong mechanistic link between mutagenesis and carcinogenesis, it follows that new drugs should be designed to have as low mutagenicity as possible to reduce their potential carcinogenicity. This thesis has demonstrated that one way to achieve this worthy goal is to direct against the formation of highly mutagenic DNA adducts, such as A*G*. The underlying principles governing the binding of platinum compounds to DNA are beginning to be understood mechanistically (Bernges & Holler, 1992; Bloemink et al., 1992; Green et al., 1992; Hartwig & Lippard, 1992) making it possible to rationally design compounds that avoid the formation of the highly mutagenic A*G* adduct. Accordingly, such compounds should be designed and evaluated in mutational assays, either comprehensively as done for cis-DDP and ACDP in this work, or perhaps, at least initially, in quick screening assays, ideally in eukaryotic systems.
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