MUTAGENESIS AND GENOTOXICITY OF THE MAJOR DNA ADDUCT OF CIS-DIAMMINEDICHLOROPLATINUM(II)

by

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ABSTRACT

This work investigated the genetic effects of the major DNA adduct formed by the anticancer drug *cis*-diamminedichloroplatinum(II). A duplex Escherichia coli bacteriophage M13 genome was constructed containing a single *cis*-[$Pt(NH_3)_2\{d(GpG)\}$] intrastrand crosslink. The duplex dodecamer d(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was ligated into the HincII site of M13mp18 to produce an insertion mutant containing a unique StuI restriction enzyme cleavage site. A genome with a 12-base gap in the minus strand was produced by hybridizing HincII-linearized M13mp18 duplex DNA with the single stranded (ss) circular DNA of the 12-base insertion mutant (M13-12A). The platinated dodecamer d(TCTAG^GCCTTCT) was phosphorylated in the presence of [γ-^{32}P]ATP with bacteriophage T4 polynucleotide kinase and incorporated into the 12-base gap of the heteroduplex, thus situating the adduct specifically within the StuI site in the minus strand of the genome. Approximately 80% of the gapped duplexes incorporated a dodecanucleotide in the ligation reaction. Of these, approximately half did so with the dodecanucleotide covalently joined to the genome at both 5′ and 3′ termini. The remainder of the molecules had ligated at only one of the two sites. The site of incorporation of the dodecamer was mapped to the expected region of the genome by restriction endonuclease digestion. The *cis*-[$Pt(NH_3)_2\{d(GpG)\}$] crosslink completely inhibited StuI cleavage, which was fully restored following incubation of the platinated genome with cyanide to remove platinum as $[Pt(CN)_4]^{2-}$.

In order to study the biological effects of the adduct in ss DNA, modifications were made to the aforementioned protocol to produce a singly adducted ss genome. Specifically, a heteroduplex was formed between HincII-linearized M13mp18 duplex DNA and UV treated ss (+) strand M13-12A. The modified dodecanucleotide described above was introduced into the resultant (-) strand gap by ligation, producing a singly adducted ds genome harboring UV photoproducts in the strand opposite that containing the adduct. These genomes were then treated in vitro with phage T4 endonuclease V, which cleaves DNA at the site of UV induced pyrimidine dimers. Following denaturation by heating, a site-specifically modified ss genome was produced.

The singly adducted ss genome was introduced into wild type Escherichia coli cells by electroporation. The mutation frequency of site-directed base substitution mutations in cells induced for SOS processing was 1.3%, compared with a background mutation frequency of <0.01% in non-SOS induced cells. When the double stranded (ds) singly adducted precursor genomes (those containing biologically inactivating pyrimidine dimers in the (+) strand, and not treated with T4 endonuclease V) were similarly transfected into SOS induced E. coli cells, the mutation frequency was found to be 0.2%. Sequencing analysis revealed that the induced mutations were highly specific. Upon transfection of the ss and ds singly adducted genomes into SOS-induced wild type E. coli, 89% and 74% of the mutations, respectively, were G→T transversions at the 5'-adducted G. No induced mutations were detected in the absence of the expression of the SOS response, nor when either the ss or ds genomes were transfected into cells defective in excision repair by virtue of a missense mutation in the *uvrA* gene.

Thesis supervisor: Professor John M. Essigmann

Title: Professor of Toxicology and Professor of Chemistry

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First and foremost, I dedicate this dissertation to my parents. Their constant love and support through my long academic career have made this all possible.

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high enough for me to continue the tradition! I want to thank everyone for not complaining too much about my playing the Grateful Dead in the lab, and I particularly want to thank the Dead themselves for creating such music that gave me the spiritual renewal I so desperately needed during the years here. John showed great patience every time the Dead came to New England, and I'm sorry I never got him to a show. I even forgive Brian for kidnapping my Dead tapes and demanding brownies for their return; he's gotten much mileage out of that! "Mr. Snotface" must also be remembered, as he epitomizes the spirit of creativity that is characteristic of the lab. I also want to thank my squash partners for providing another outlet for my frustrations, particularly Mike Keck who occasionally "lets" me beat him, and who has been a good friend and an excellent repository for all manner of baked goods (probably the reason for his good nature on the squash court). The support people at MIT have also made the more mundane parts of work here more fun: Steve and Ralph in the stockroom, nighttime Frank from E-18, Brian at the pool, and Frank who delivers packages and good humor to us now.

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"Once in a while you get shown the light
In the strangest of places if you look at it right."

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAF	acetylaminofluorene
AF	aminofluorene
A^G	*cis*-[$Pt(NH_3)_2${d(ApG)}]
AP	apurinic/apyrimidinic
bp	base pair
BSA	bovine serum albumin
carboplatin	diammine(1,1-cyclobutane-dicarboxylato)platinum(II)
CHO	Chinese hamster ovary
CIP	calf intestinal phosphatase
ds	double stranded
cis-DDP	*cis*-diamminedichloroplatinum(II)
E. coli	Escherichia coli
EDTA	disodium salt of ethylenediaminetetraacetic acid
EthBr	ethidium bromide
FA	Fanconi's anemia
form I	supercoiled DNA
form I_o	covalently closed circular ds DNA
form II	nicked circular ds DNA
form III	linear ds DNA
G*	platinum monoadduct
G^G	*cis*-[$Pt(NH_3)_2${d(GpG)}]
G*NG*	*cis*-[$Pt(NH_3)_2${d(GpNpG)}]
IPTG	isopropylthio-β-D-galactoside
LB	Luria broth
nt	nucleotide
[Pt(dach)Cl_2]	diaminocyclohexanedichloroplatinum(II)
$[Pt(dien)Cl]^+$	chlorodiethylenetriamineplatinum(II)
[Pt(en)Cl_2]	dichloroethylenediamineplatinum(II)
RF	replicative form
S. typhimurium	Salmonella typhimurium
ss	single stranded
TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
trans-DDP	*trans*-diamminedichloroplatinum(II)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XP	xeroderma pigmentosum

I. Introduction

In cancer chemotherapy, the genotoxic properties of chemicals are exploited by the application of drugs that bind to DNA and selectively kill rapidly dividing cells by inhibition of replication or transcription. One of the most effective antitumor drugs to date is *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (Figure 1), which is used to treat cancers of the head, neck, and urogenital tissues. This bifunctional electrophilic compound reacts with DNA to form a variety of intra- and interstrand crosslinks. Several lines of evidence have correlated the presence of *cis*-DDP-DNA adducts with parameters directly or indirectly associated with the antitumor effectiveness of the drug. First, *cis*-DDP inhibits DNA replication in vivo in bacterial and mammalian cells (reviewed in Pinto & Lippard, 1985a), and in vitro (Pinto & Lippard, 1985b; Villani et al., 1988). Second, antibodies that selectively recognize the major *cis*-DDP-DNA crosslinks interact with the peripheral blood cell DNA of patients treated with *cis*-DDP to extents that closely parallel the clinically observed response to drug treatment (Poirier et al., 1985; Reed et al., 1987).

The biological effects of *cis*-DDP are not limited to the selective killing of tumor cells. *cis*-DDP has been found to be carcinogenic in the mouse and rat (Leopold et al., 1979). Moreover, the appearance of second malignancies in patients treated with *cis*-DDP has been reported (Johnson, D. C. et al, 1980; Stewart & Wilkinson, 1981; Mead et al., 1983; Redman et al., 1984; Redman et al., 1985; Bassett and Weiss, 1986), fueling speculation that these tumors may have resulted from *cis*-DDP treatment. 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).

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 goals, 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 adducted 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 lesions 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 mimimize the proportion of premutagenic lesions.

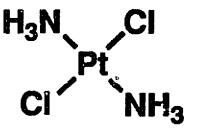
This dissertation reports studies of the effects on survival and mutagenesis of the major DNA adduct of *cis*-DDP in Escherichia coli. For

this purpose, an M13mp18 derived viral genome containing a single *cis*-DDP adduct at a unique site was constructed. The research presented here is part of a larger project that is a collaboration between Professor John M. Essigmann in whose laboratory the methodology for studying single adduct mutagenesis was developed, and Professor Stephen J. Lippard whose expertise is in the field of platinum-DNA chemistry and platinum adduct repair. As such, I shall indicate throughout the dissertation those contributions made by members of both laboratories to this work.

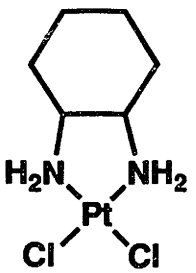
Figure 1. Structures and abbreviated names of the platinum compounds referred to in the text.

PLATINUM COMPOUNDS

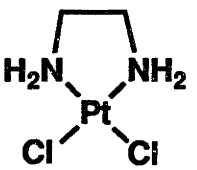
cis-DDP



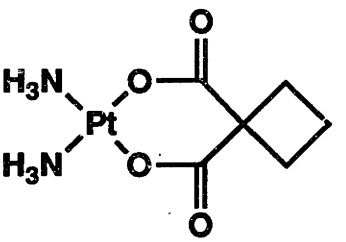
trans-DDP



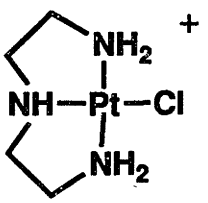
$[Pt(dach)Cl_2]$



$[Pt(en)Cl_2]$



Carboplatin



$[Pt(dien)Cl]^+$

II. Literature Survey

This section reviews what is known about the formation and processing of platinum-DNA adducts in E. coli, with a brief summary of what is known to date about these topics with respect to mammalian cells.

A. Reaction of Platinum Compounds with DNA.

1. 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 (hereafter 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 monoadducts (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 (Fichtinger-Schepman et al., 1985b; Eastman, 1983).

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

oligonucleotides have shown it 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 monofuctional platinum compound chlorodiethylenetriamineplatinum(II) ([Pt(dien)Cl]Cl) (Figure 1), whose major adduct occurs at the N7 position of guanine (Johnson et al., 1982), does not exhibit antitumor activity. Clinically active platinum compounds include dichloroethylenediamine-platinum(II) ([Pt(en)Cl_2]), diaminocyclohexanedichloroplatinum(II) ([Pt(dach)Cl_2]), and diammine(1,1-cyclobutane-dicarboxylato)platinum(II) (carboplatin) (Figure 1), and it is noteworthy that these drugs, like *cis*-DDP have ligands bound in a cis geometry. At least one of these compounds, [Pt(en)Cl_2], 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 <u>adjacent</u> 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).

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 B_1 or by alkylating agents) destablize 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; Villani et al., 1988) platinated 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 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.

2. The Effects of Platinum Adducts on DNA Structure.

The binding of both *cis*- and *trans*-DDP to DNA in vitro shortens and unwinds the helix (Cohen et al., 1979). Many studies have focused on defining the detailed structural and physical properties of *cis*-DDP adducts; 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 (determined indirectly) 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 C, and to a lesser

extent A, and T, but not those elicited against G. DNA treated with very low levels of the trans compound was able to bind all four antibodies, whereas [Pt(dien)Cl]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.

Studies with site-specifically platinated oligonucleotides have also provided information about the structure of *cis*-DDP modified DNA. Small oligonucleotides 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., 1985). NMR studies on these decamer sequences do not conclusively demonstrate the presence of hydrogen bonding occuring at the adducted guanines, however, it cannot be ruled out at present. Each group suggests that the NMR data is consistent with the presence of a kink or bend induced in the oligonucleotide by the adduct.

An altered and destabilized DNA structure that may still accomodate some form of hydrogen bonding between adducted nucleotides and their complements is consistent with the physical and biochemical data presented above. It has been suggested that the high levels of platinination (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., 1985). This cooperativity could also explain the nonlinear increase in antinucleoside-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.

X-ray diffraction studies of the single stranded dinucleotide *cis*-[$Pt(NH_3)_2${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).

As the crystal structure of a single stranded dinucleotide is not necessarily reflective of the structure of native duplex DNA, gel electrophoretic mobility assays have been used to determine *cis*-DDP induced bend angles in DNA. Using duplex oligomers containing G^G, A^G, or G*TG* 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 GG adduct is toward the major groove (Rice et al., 1988); it is expected that

the other adduct-directed bends are also toward the major groove, but this has not yet been established.

Recently, the chemical reactivity of DNA bases to compounds that are sensitive to the structure of DNA has provided additionally detailed 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 summary, the *cis*-[$Pt(NH_3)_2$(d(GpG))] intrastrand crosslink induces a bend in duplex DNA toward the major groove of approximately 32-35°. Base-pairing probably remains intact, albeit in a distorted manner, and this distortion is greater on the 5' side of the adduct than on the 3' side.

B. Effects of Platinum Compounds on Bacterial Cells.

1. Repair of Platinum Adducts in E. coli.

E. coli has several responses, both constitutive and inducible, to the damage of its genome. To date the principal path implicated in *cis*-DDP repair, and the best understood biochemically, is the excision repair system. I shall discuss the details of this system below, as well as give

evidence for the possible involvement of other repair pathways in countering platinum damage.

a. Excision Repair.

trans-DDP is toxic but not lethal to wild type and *uvr*$^-$ cells, as evidenced by a lag, but not an inhibition, in growth of *trans*-DDP treated cells (Beck et al., 1985). In contrast, E. coli *uvr* mutants are much more susceptible to the killing effects of *cis*-DDP than wild type cells, suggesting that *uvr*-mediated excision is involved in the repair of DNA-damage caused by this agent (Beck & Brubaker, 1973; Beck et al., 1985). Nucleotide excision repair in E. coli is a virtually error free system that is able to recognize a wide variety of substrates. In general these substrates are bulky or helix distorting adducts. Lesions considered to have more subtle effects on DNA architecture, such as AP sites and O^6-methylguanine, are also substrates for the UvrABC system, although in each of these specific cases the UvrABC system likely serves to backup other more quantitatively important repair processes. In depth reviews on excision repair are available (Sancar & Sancar, 1988; van Houten, 1990); I shall present here the generally accepted mechanism of action, although it should be noted that there are presently disagreements as to some of the details of the system (Grossman & Yeung, 1990; Selby and Sancar, 1990).

Damage recognition involves the *uvrA* and *uvrB* gene products, which combine in a $UvrA_2UvrB_1$ complex that either binds to DNA at the site of a lesion, or binds to a region of undamaged DNA and translocates along the helix to a site of damage. UvrC, the endonucleolytic subunit, binds to the

protein-DNA lesion complex and incises the strand of damaged DNA seven to eight nucleotides 5′ to the lesion and three to four nucleotides 3′ to the lesion. The helicase UvrD and DNA polymerase I are required for enzyme turnover and release of the oligonucleotide containing the damage. DNA polymerase I fills in the resulting gap, and DNA ligase seals the nicks to complete the restoration of the DNA duplex.

The *uvrABC* genes have been cloned, overly-expressed, and the encoded proteins have been isolated to homogeneity for in vitro biochemical experiments. These studies with the purified UvrABC proteins and randomly platinated DNA have shown that *cis*-DDP-DNA adducts are indeed substrates for nucleotide excision repair, as evidenced by the nicking of supercoiled *cis*-DDP treated DNA. The sites of cleavage have been mapped to eight nucleotides 5′ and four nucleotides 3′ to GpG sequences (Beck et al, 1985), which are major sites of *cis*-DDP binding. The authors were unable to conclude from these experiments on randomly adducted DNA whether or not UvrABC acts on the other *cis*-DDP-DNA adducts. In additional studies, the same workers observed that UvrABC can nick supercoiled *trans*-DDP-modified DNA, although no specific cleavage pattern different from the control (unmodified DNA) was evident.

UvrABC has been shown to be active on DNAs modified by two other therapeutically active platinum compounds having a cis geometry: $[Pt(en)Cl_2]$ and $[Pt(dach)Cl_2]$ (Page et al., 1990). Using substrates constructed to contain a single $[Pt(en)Cl_2]$ or $[Pt(dach)Cl_2]$ adduct in the context of a duplex 43-mer, it was demonstrated that the rate and extent of cleavage by UvrABC is adduct dependent, and that the reactivities are in

the order of G*NG* > G* > A^G > G^G. [$Pt(en)Cl_2$] binds to DNA to produce a spectrum of adducts similar to that of *cis*-DDP (Eastman, 1983; Eastman, 1986), and it is expected that *cis*-DDP adducts would be processed in the same manner by UvrABC.

b. Other Repair Systems for Platinum Adducts.

Cells that are compromised for postreplication mismatch repair show an enhanced sensitivity to *cis*-DDP (Fram et al., 1985). Adenine-methylase, encoded by the E. coli *dam* gene, is the enzyme responsible for the methylation of both A's in the symmetric GATC sequences of duplex DNA. It is also active at hemi-methylated duplex GATC sites as would be found in newly synthesized DNA. There is believed to be a lag between the synthesis of DNA and its methylation, during which time the mismatch repair proteins (MutH, MutL, and MutS) work in concert with other proteins to correct mismatched base pairs formed by the replication apparatus. The hemi-methylated state of the DNA immediately following replication enables discrimination between parental and daughter strands, such that the methylated parental strand is used as the template in repair. E. coli deficient in adenine-methylase (*dam*$^-$) are more sensitive to the killing effects of *cis*-DDP, while a *dam*$^-$ *mut*$^-$ double mutant is as resistant as wild type cells, suggesting that abortive mismatch repair may be responsible for the increased cytotoxicity of the drug to the *dam*$^-$ mutant (Fram et al., 1985). Removal of platinum adducts from *dam*$^-$ cells was considerably slower than in wild type cells, suggesting that the methylase itself may play an active role in the repair process.

The *recA* gene product plays many roles in E. coli. It is involved in genetic recombination, recombinational repair, and is the cellular signal protein that responds to DNA damage, thereby controlling the SOS response of the cell. Mutants in the *recA* gene are very sensitive to the killing effects of *cis*-DDP (but much less so to *trans*-DDP; Beck & Brubaker, 1973) and, as will be discussed in the next section, this hypersensitivity is expected owing to the role this protein plays in the SOS response. However, studies of *recA* mutants have shown that the protein's recombinational abilities are also important in the repair of *cis*-DDP adducts (Jarosik & Beck, 1984).

The existence of a repair pathway for *cis*-DDP lesions that does not involve excision repair was postulated by Germanier et al. (1984) from their results with cell strains that differ in their response to *cis*-DDP and UV light. The nature of the repair system, however, could not be determined from the experiments.

2. The SOS Response of E. coli.

The "SOS response" to DNA damage is characterized by enhanced selective DNA repair activities, enhanced mutagenesis, inhibition of cell division, and prophage induction. This diverse array of physiological responses is the result of the expression of an inducible set of genes that are under the control of the RecA and LexA proteins. The pathway has been extensively reviewed (Witkin, 1976; Little & Mount, 1982; Walker, 1984; Battista et al., 1990; Echols & Goodman, 1990), and only the salient features will be presented here.

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 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. The cleaved form of LexA can no longer function as a repressor, and this allows for the expression of the genes of the network. The genes involved bind LexA to different extents, enabling the differential activation of specific genes as is warrented 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.

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 in the 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. Another mechanism of control exists in the system at the level of the post-translational modification of the umuD

protein. RecA* mediates the autodigestion of UmuD (Burckhardt et al., 1988) in a manner analogous to that of LexA, and it is the truncated form of UmuD that plays an active role in mutagenesis (Nohmi et al., 1988). Experiments suggest that RecA* may even play a third as yet unknown role in mutagenic processing (Nohmi et al., 1988). The exact mechanism of UmuD,C-mediated mutagenesis is unclear at this time, although several possibilities have been put forward. 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.

A wide range of DNA damaging agents can induce the SOS response in E. coli, including UV radiation, methyl methanesulfonate, 4-nitroquinolone-1-oxide, and aflatoxin B_1. *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.

3. Mutagenesis by *cis*-DDP in Bacteria.

a. Mutagenesis in Salmonella typhimurium.

cis-DDP mutagenesis has been investigated using the S. typhimurium reversion assays developed by Ames and co-workers (Ames et al., 1973; McCann et al., 1975). The pattern of reversion shows that *cis*-DDP induces base pair substitutions but only in the presence of the plasmid pKM101 (Monti-Bragadin et al., 1975; Beck and Fisch, 1980). This plasmid encodes the MucA,B proteins, which are naturally occurring plasmid counterparts of the UmuD,C proteins of E. coli which play essential roles in SOS mutagenesis. Neither of these studies detect reversion in the strains sensitive to frame-shift mutagens, although there has been a single report to the contrary (Andersen, 1979). In all cases, *trans*-DDP is less toxic and less mutagenic in these cells (Beck and Fisch, 1980). [Pt(dach)Cl_2] is also mutagenic in these systems, but less so than *cis*-DDP (Leopold et al., 1981).

b. 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 demonstrate that *cis*-DDP mutagenesis is abolished in a *umuD,C*$^-$ background.

c. Mutation Spectra in E. coli.

The exact nature of the mutations of *cis*-DDP in E. coli has been studied in several forward mutation assays but with conflicting results. 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 GpApG and 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 ApG and GpG sequences in an assay in which the *tet*^{R} gene of pBR322 was treated in vitro with *cis*-DDP and then transferred for replication in vivo into E. coli cells that had been pre-treated with UV to induce the SOS response. 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.

d. The Role of UvrA and UvrB in Mutagenesis in E. coli.

The *uvrA* and *uvrB* gene products also seem to 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 UmuD,C, since UmuD,C 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). This is unlikely, however, to be the mechanism of mutagenesis of *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 to *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 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. The sites of *cis*-DDP-induced mutations can be correlated with known sites of platinum adduction, although the sites of preferred chemical reaction differ from those at which mutations arise most frequently.

C. Effects of Platinum Compounds on Mammalian Cells.

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 & 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 contains 22 adducts, whereas only 1 in 1500 protein molecules are adducted. *cis*-DDP treated DNA substrates have been shown to inhibit human DNA polymerases in vitro (Harder et al., 1976), and more recently, 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).

Although the type and distribution of *cis*-DDP adducts in mammalian cells (Plooy et al., 1985a) 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 have hindered the study of the mechanisms of mutagenicity and repair of *cis*-DDP adducts in these systems. Nevertheless, a variety of studies have demonstrated that both *cis*- and *trans*-DDP-DNA adducts are removed or repaired in mammalian cells.

Many early studies focused on the levels of platinum induced interstrand crosslinking of DNA. Although both compounds do form these lesions, they are a minor component of the adduct spectrum of each, comprising <1% 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).

1. Repair of Platinum Adducts in Mammalian Cells.

Fravell & Roberts (1979) were the first to demonstrate the quantitative loss of *cis*-DDP adducts from stationary and exponentially growing cells by direct measurement of platinum bound to genomic DNA. Host cell reactivation assays have been used to demonstrate that *cis*-DDP-DNA adducts are repaired from viral genomes transfected into various mammalian cell lines (Poll et al., 1984; Chu & Berg, 1987; Sheibani et al., 1989;

Maynard et al., 1989; Chao et al., 1990); unfortunately, the effect of the trans isomer has not been studied in these systems. An in vitro assay has recently been developed (Wood et al., 1988) that measures repair synthesis on damaged templates by extracts of human cells. It has been used to demonstrate repair of *cis*-DDP and *trans*-DDP adducts by whole cell extracts of normal human fibroblasts (Hansson & Wood, 1989). In this study, the trans isomer was repaired to a slightly greater extent than the cis isomer. Similarly, [Pt(dach)Cl_2]-DNA adducts have been shown to be repaired by whole cell extracts of HeLa cells (Sibghat-Ullah et al., 1989).

Human cell lines derived from patients with genetic disorders that are believed to arise from a deficiency in DNA repair have been used to study the processing of *cis*-DDP adducts. Xeroderma pigmentosum (XP) cells lines (which comprise at least nine complementation groups) are believed to be deficient in the incision step of DNA excision repair (Hanawalt & Sarasin, 1986). Cells derived from patients with Fanconi's anemia (FA) are very sensitive to DNA cross-linking reagents (Hanawalt & Sarasin, 1986). Both types of cells are sensitive to *cis*-DDP treatment (Fravel et al., 1978; Poll et al., 1984; Plooy et al., 1985b). Of the two cell lines, only XP cells are sensitive to UV light, a treatment that does not induce interstrand DNA crosslinks, and only FA cells are sensitive to mitomycin C, a classic DNA crosslinking reagent. Since both cell lines are sensitive to *cis*-DDP, this suggests that both intrastrand and interstrand crosslinks formed by *cis*-DDP are involved in cytotoxicity, and that these two types of adducts may be repaired by different processes.

The repair of *cis*-DDP adducts in XP cells has been investigated using the two assays described above. Host cell reactivation assays have shown that unlike normal cells, XP cells cannot restore the transcription of genes in platinated vectors (Chu & Berg, 1987). Similarly, extracts made from XP cell lines cannot support repair synthesis on [Pt(dach)Cl_2] treated DNA (Sibghat-Ullah et al., 1989) nor on *cis*-DDP treated DNA (Hansson & Wood, 1989; Hansson et al., 1990). If, however, the adducted DNA is pretreated with UvrABC, the proteins involved in excision repair in E. coli (vide supra), then the XP cell extracts can utilize *cis*-DDP treated DNA as a substrate for repair synthesis (Hansson et al., 1990), confirming that the XP cell repair deficiency is in the incision step of nucleotide excision repair. These studies demonstrate that DNA excision repair in mammalian cells is one avenue of repair for *cis*-DDP-DNA adducts.

Several studies have compared the repair and inhibition of replication of *cis*- and *trans*-DDP in mammalian systems. Ciccarelli and coworkers (1985) studied SV40 infected CV-1 cells treated with *cis*- or *trans*-DDP in culture. Although fourteen times more of the trans compound in the medium is required to inhibit SV40 replication to the same extent as *cis*-DDP, the two compounds are equally effective in blocking replication on a per adduct basis. Time course studies show that while *cis*-DDP-DNA binding increases steadily with increasing incubation times, the trans adduct levels peak initially and then fall to a low level. This phenomenon is ascribed to preferential repair of the trans isomer. A similar study conducted in Chinese hamster ovary (CHO) cells by Roberts & Friedlos (1987) did not detect this time dependent difference in *cis*- and *trans*-DDP adduct levels;

the reasons for this discrepancy are unknown, but may be due to differences in experimental protocol, or to an inherent difference in the cell types employed.

Recently, an SV40 based in vitro replication system, utilizing cytosolic extracts of human cell lines supplemented with exogenous viral T-antigen, has been used to study *cis*- and *trans*-DDP adducted vectors (Heiger-Bernays et al., 1990). As in the previous study, *cis*-DDP and *trans*-DDP are shown to inhibit replication to the same extent at equal levels of binding to DNA. If, however, the adducted vectors are preincubated with the cytosolic extracts before replication is allowed to commence (by the addition of the viral T-antigen), then the trans-adducted but not the cis-adducted templates are able to support increased replication. In order to test if this is a consequence of repair occurring during the preincubation, the same extracts were used in the in vitro repair assay described above (Wood et al., 1988). Indeed, a greater amount of repair synthesis is detected for the *trans*-DDP treated vectors. These results are consistent with the findings of Ciccarelli et al. (1985) indicating the presence of a repair function in mammalian cells that is selectively active on *trans*-DDP treated DNA. It may be this repair activity that is responsible for the differential chemotherapeutic effectiveness of the two isomers.

Antibodies that recognize each of the four major *cis*-DDP-DNA adducts (G^G, A^G, G*, and G-Pt-G) have recently been produced (Fichtinger-Schepman et al., 1985a; Fichtinger-Schepman et al., 1987). Their availability has made it possible to follow the fate of specific adducts in DNA after

treatment of *cis*-DDP either to cells in culture or to human cancer patients. Plooy and coworkers (1985a) have demonstrated the formation and subsequent rapid removal of G^G intrastrand crosslinks in CHO cells in the 24 hours following treatment by *cis*-DDP. The formation and repair of *cis*-DDP adducts in normal human fibroblasts has been compared to that in XP and FA cell lines over a 24 hour period (Dijt et al., 1988). The distribution of the four adducts is roughly the same for all three cell types, and again is very similar to that obtained by in vitro treatment of DNA, with G^G being the most abundant adduct. Initial adduct levels are similar for the normal and XP cells, while the adduct levels were approximately 50% greater in FA cells. The kinetics of removal in the normal and FA cell lines are similar: an initial rapid decrease in all four adduct levels, followed by a period of very slow or no repair. By contrast, the XP cells exhibited a slow but constant repair of all adducts throughout the repair period. Interestingly, the adduct distribution profile remained the same in all three cell types throughout the repair period; i.e., there was no evidence of preferential repair of one type of adduct over another, indicating that the repair systems involved are capable of removing all four types of platinum damage detectable by the antibodies. The observation that the XP cells do not exhibit the initial rapid removal of adducts suggests that excision repair is responsible for this phase of repair in the other two cell lines. Moreover, the demonstration that XP cells exhibit repair at all suggests the existence of a second repair pathway in addition to excision repair.

Using the same antibodies, the in vivo formation and removal of specific *cis*-DDP adducts has also been demonstrated in peripheral white blood cells collected from patients after receiving platinum based chemotherapy (Fichtinger-Schepman et al., 1987; Fichtinger-Schepman et al., 1990). Although there is a strong interindividual variability with respect to the total level of adducts formed, the distribution of adducts again is the same as that found by in vitro treatment of DNA. A rapid removal of adducts during the 24 hours post-treatment is also observed, demonstrating that *cis*-DDP DNA adducts can be repaired in humans. A tentative correlation was found to exist between the initial formation of high adduct levels and complete patient response, in agreement with the results of Poirier and coworkers (Poirier et al., 1985; Reed et al., 1987).

Recently, studies have been undertaken to isolate the proteins involved in repair of platinum treated DNA. Assuming that one aspect of repair involves protein recognition of and binding to damaged DNA, gel mobility shift assays have been employed to identify the presence of Pt-DNA binding proteins in mammalian cells. Chu & Chang (1988) have identified factors in mammalian cell extracts that recognize DNA damaged by *cis*-DDP. This factor does not recognize DNA damaged by UV light, and it is present in XP cells. Concurrently, Toney et al. (1989) demonstrated the presence of a factor in HeLa cells that binds specifically to DNA fragments treated with the antitumor compounds *cis*-DDP, $[Pt(en)Cl_2]$,and $[Pt(dach)Cl_2]$ but not to DNA treated with *trans*-DDP, nor the monofunctional compound [Pt(dien)Cl]Cl. Further investigations show that the factor binds specifically to G^G and A^G adducts, and not to G*TG* or monofunctional G*

adducts (Donahue et al., 1990). The role that this protein plays in the cell has yet to be identified.

2. Mutagenesis by *cis*-DDP in Eukaryotic Cells.

cis-DDP is mutagenic in mammalian cells (Johnson, N. P. et al., 1980), and its mutation spectrum has been studied in two 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 G·C base pairs. Dipurine sequences are also involved in 86% of the induced base pair substitutions, with the majority of the substitutions occurring at GpG and GpA sequences.

cis-DDP induced mutations in the adenine phosphoribosyltransferase (*aprt*) gene of CHO cells have also been investigated (de Boer & Glickman, 1989). Substitutions at G-C base pairs also predominate, with the majority occurring at ApGpG and 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 ApG sequences in which a mutation is detectable and, in the latter study, mutations at 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 antitumor drug.

D. Site Specific Mutagenesis.

The multiplicity of DNA adducts formed upon treatment of cells with *cis*-DDP complicates the identification of lesions responsible for the genotoxic and mutagenic effects of the drug. For *cis*-DDP and other drugs that act on 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.

Experiments with randomly adducted 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 adducted 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 adduction. Mutation spectrum studies can implicate lesions likely to be involved in mutagenesis, but none of these assays can definitively ascribe a specific event to a specific adduct.

Recent advances in chemical synthetic techniques and molecular biology 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.

There are several methods for producing a site specifically modified genome, and each requires the synthesis of an oligodeoxynucleotide that contains the modification to be studied. The three most common oligonucleotide 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 oligonucleotide, (2) chemical or physical modification of a preformed oligonucleotide, and (3) enzymatic synthesis, in which DNA or RNA polymerases are used to incorporate modified nucleotides into the oligonucleotide. The total synthetic approach is 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 oligonucleotide with a DNA damaging agent may yield products too numerous to allow this approach to be used effectively.

Modified oligonucleotides can be incorporated into the genomes of viruses or plasmids in several ways. In one common method (Figure 3a), the oligonucleotide is annealed to the single stranded genome of a viral vector. The 3' terminus of the oligonucleotide 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. In a second method (Figure 3b), a duplex genome is constructed in which one strand contains a gap opposite a sequence that is complementary to that of the modified oligonucleotide to be used. The oligonucleotide is annealed to the gapped duplex genome, and the nicks on either side of the oligonucleotide are covalently joined by DNA ligase.

The modified oligonucleotide used for the work presented in this dissertation was prepared by the second method described above, i.e., by allowing an unmodified dodecanucleotide to react with *cis*-DDP. The sequence chosen, and the rationale for its selection, will be presented in the next chapter. The oligonucleotide was situated in a viral genome context using the gapped duplex method (Figure 3b). The vector chosen for this work is described in the next section.

E. M13 as a Vector for Site Specific Mutagenesis.

The vector used to study the mutagenesis induced by specific *cis*-DDP adducts is the M13 system developed by Messing (1983). 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).

1. Life Cycle of M13.

M13 is a single stranded (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 4), 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 subsequently 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 M13 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 region into which DNA can be inserted without adverse effects to phage viabilty. Messing (1983) took advantage of both of these features for the development of a series of M13 molecular cloning vectors.

2. M13 Cloning Vectors.

Useful molecular cloning vectors require a method for detecting inserts, and the system developed by Messing utilizes the marker enzyme β-galactosidase. β-Galactosidase is the product of the *lacZ* gene, which is part of the *lac* operon. The gene can be transcribed as two fragments, the alpha fragment encoding the N-terminal region of the protein, and the corresponding omega fragment. Neither fragment alone is sufficient for enzymatic activity; however, the two truncated proteins can associate to produce an active enzyme, a process termed alpha complementation (Figure 5).

The M13mp series of vectors constructed by Messing contains the portions of the *lacZ* gene encoding the operator region and the alpha fragment inserted into the intergenic region of the M13 genome. A contiguous series of restriction endonuclease recognition sites unique to the M13 genome (called the "polylinker") into which foreign DNA can be inserted, is situated in-frame in the beginning of the coding sequence of the alpha fragment. The host cells, in which the *lac* operon has been deleted from the chromosome, carry the operator region and the sequences encoding the omega fragment on their F factor plasmid. Thus when M13mp phage are plated with host cells in the presence of isopropylthio-β-D-galactoside (IPTG), a gratuitous inducer of the operon, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), a chromogenic substrate of β-galactosidase, alpha complementation produces an active enzyme that cleaves X-gal, which results in a deep blue plaque color. Large insertions of DNA into the polylinker region destroy alpha complementation, and thus the recombinant phage can be identified because they produce colorless plaques when plated with IPTG and X-gal. Colorless plaques also result if termination codons are inserted in-frame, or if insertions or deletions disrupt the reading frame of the alpha fragment.

The M13mp system is particularly well suited for genetic manipulation. M13 DNA can be isolated in large quantities in either single or double stranded form, and either single or double stranded DNA can be introduced viably into cells by transformation procedures. The presence of the polylinker region allows for small changes to be made and propagated efficiently in the genome.

Table 1.

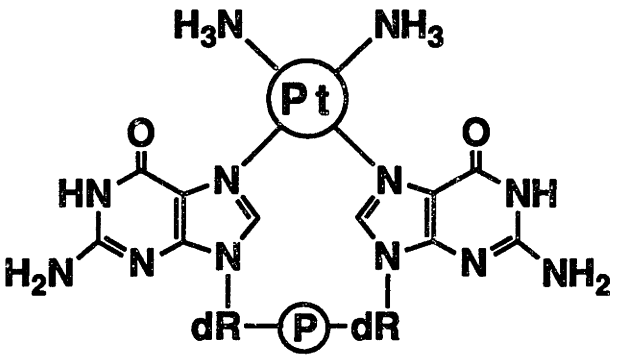
DNA adducts formed by platinum compounds

Adduct	*cis*-DDP	*trans*-DDP[a]	[Pt(dien)Cl]Cl[a]
DNA monofunctional adducts			
dG	3[b]	yes	yes
DNA intrastrand crosslinks			
d(GpG)	65	no	no
d(ApG)	25	no	no
dGpXpG)	<8	yes	no
DNA interstrand crosslinks			
	<1	yes	no
DNA-protein crosslinks			
	<1	yes	no

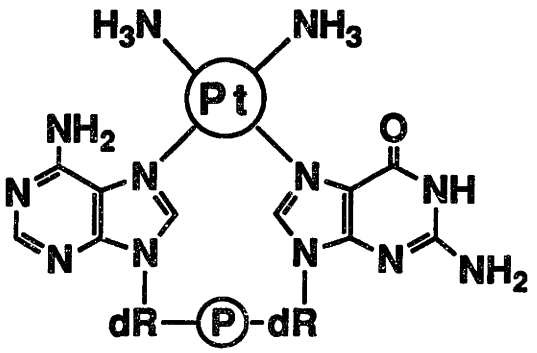
[a]The DNA adducts formed by trans-DDP and [Pt(dien)Cl]Cl have not been quantitated.

[b] Numbers indicate percentage of each adduct as compared to total platinum bound. Cases in which reliable quantitative data are not available are indicated as "yes" and "no".

Figure 2. The two major adducts formed by reaction of *cis*-DDP and DNA.


H3N
NH3
Pt
O
HN
N
H2N
N
N
dR
P
dR
O
N
NH
N
NH2

5' -GpG- 3'


H3N
NH3
Pt
NH2
N
N
N
dR
P
dR
O
N
NH
N
NH2

5' -ApG- 3'

Figure 3. Two of the common methods employed to produce site-specifically modified genomes. A. Primer extension method. B. Gapped heteroduplex method.

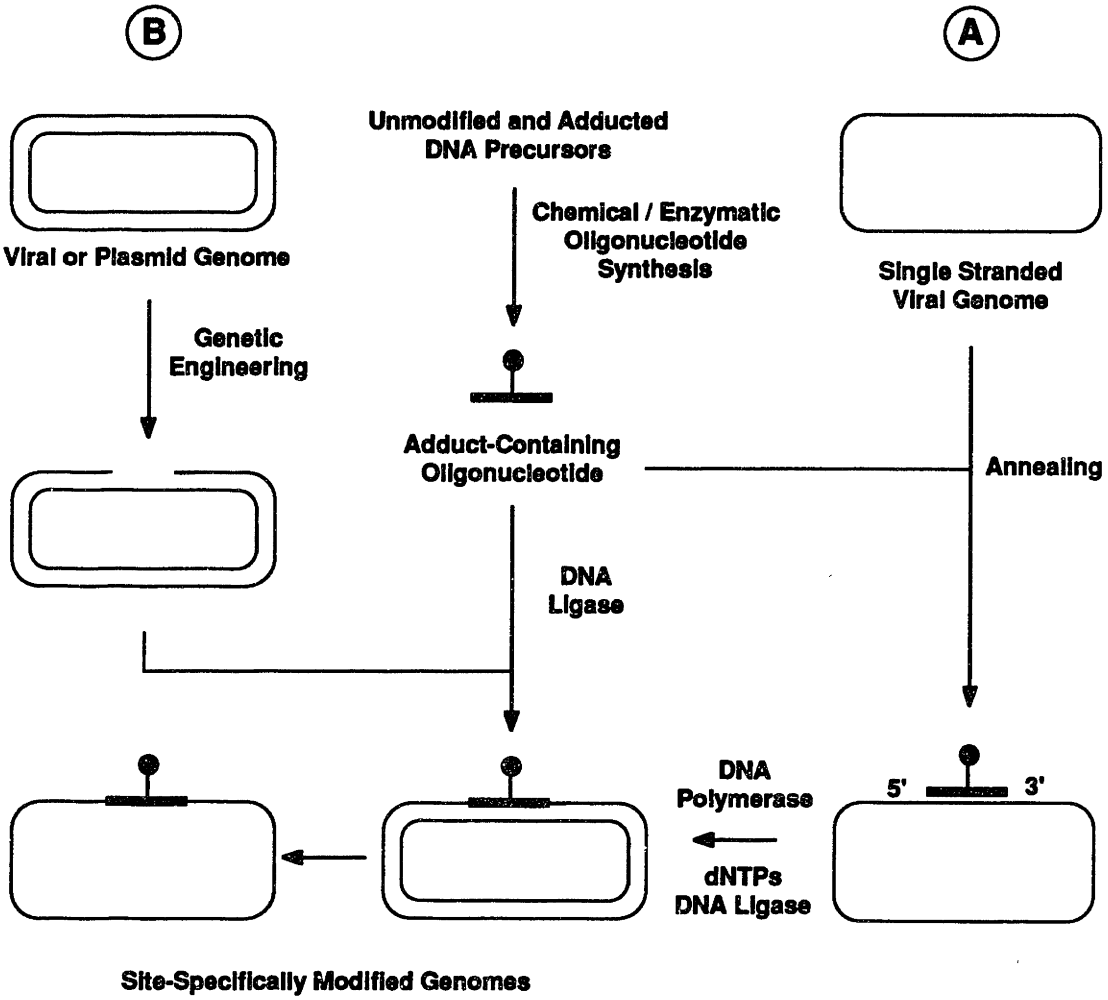

B
A
Unmodified and Adducted
DNA Precursors
Chemical / Enzymatic
Oligonucleotide
Synthesis
Viral or Plasmid Genome
Single Stranded
Viral Genome
Genetic
Engineering
Adduct-Containing
Oligonucleotide
Annealing
DNA
Ligase
DNA
Polymerase
5'
3'
dNTPs
DNA Ligase
Site-Specifically Modified Genomes

Figure 4. Outline of the rolling circle method of replication of bacteriophage M13, as described in the text.

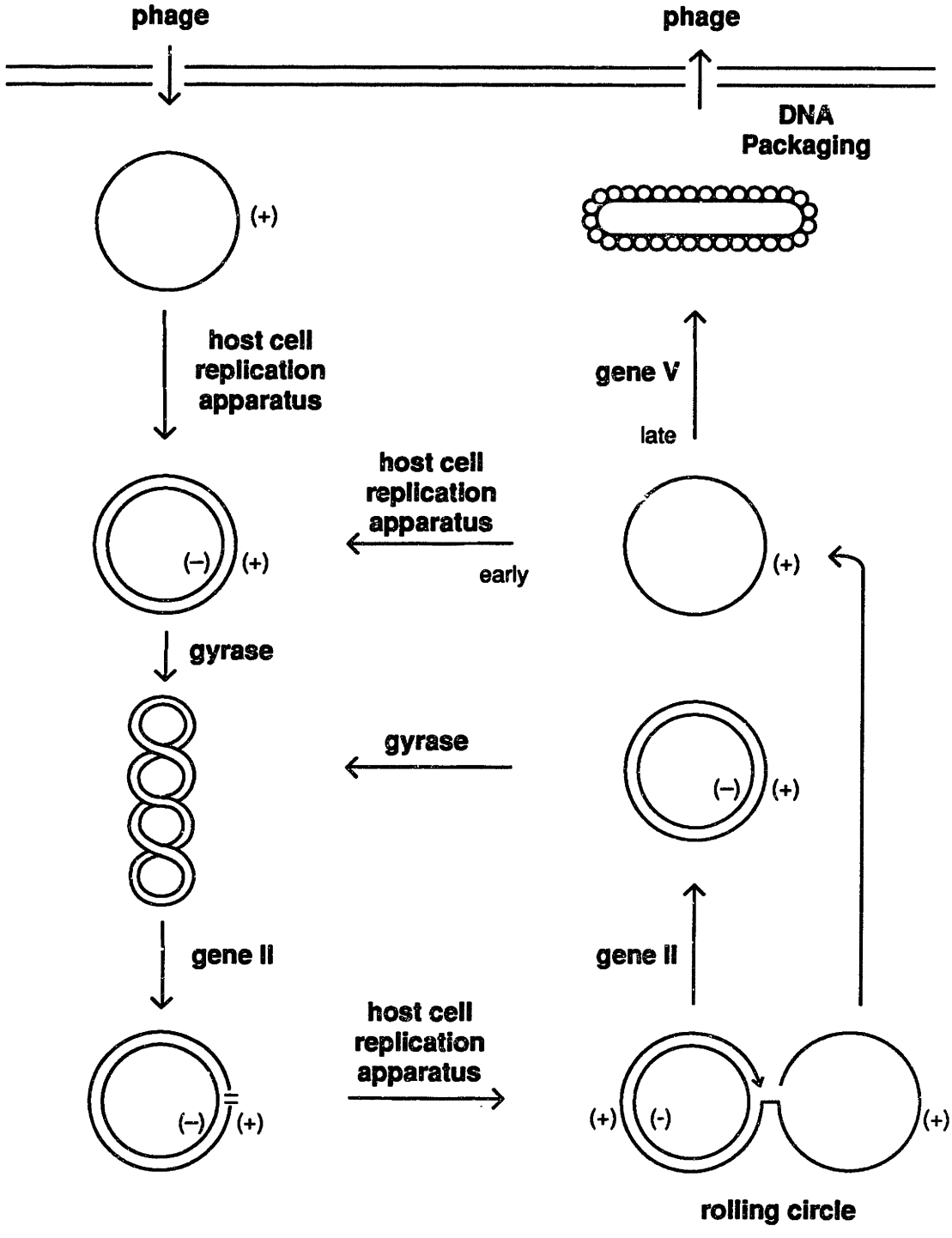
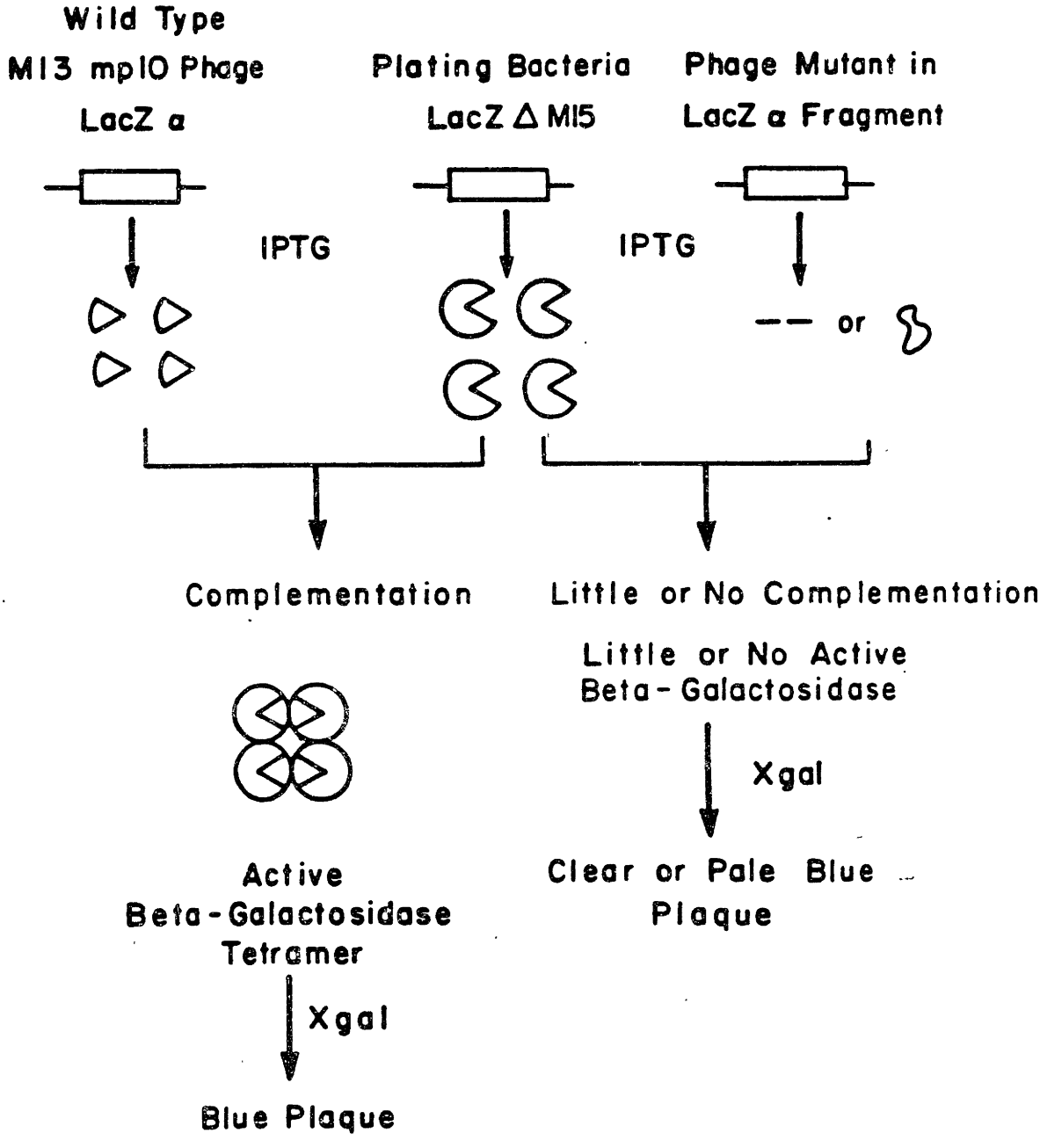

phage
phage
DNA
Packaging
(+)
host cell
replication
apparatus
gene V
late
host cell
replication
apparatus
early
(−)
(+)
(+)
gyrase
gyrase
(−)
(+)
gene II
gene II
host cell
replication
apparatus
(−)
(+)
(+)
(-)
(+)
rolling circle

Figure 5. Schematic of alpha complementation between the M13 encoded LacZ alpha fragment and the host cell encoded LacZ omega fragment, as described in the text (after Lasko, 1986).


Wild Type
M13 mp10 Phage
LacZ α
Plating Bacteria
LacZ Δ M15
Phage Mutant in
LacZ α Fragment
IPTG
IPTG
or
Complementation
Little or No Complementation
Little or No Active
Beta-Galactosidase
Xgal
Clear or Pale Blue
Plaque
Active
Beta-Galactosidase
Tetramer
Xgal
Blue Plaque

III. Construction and Characterization of a Duplex Viral Genome Containing the Major DNA Adduct of *cis*-DDP Built into a Specific Site

A. Introduction

In order to understand at the molecular level the toxic and mutagenic effects of *cis*-DDP treated DNA *in vivo*, I have constructed a duplex genome containing a single *cis*-[$Pt(NH_3)_2$(d(GpG))] intrastrand crosslink at a specific site. This chapter describes in detail the construction and characterization of the site-specifically platinated genome. Portions of this chapter have appeared as Pinto et al. (1986), Naser et al. (1988a), and Naser et al. (1988b).

B. Materials and Methods

1. Materials

All enzymes, except where noted, were purchased from New England Biolabs. The cell lines used were the following: *E. coli* GW5100 (JM103 $P1^-$, from Graham Walker, MIT), *E. coli* DL7 (AB1157 *lac* Δ*U169*, from Dana Lasko, MIT), and *E. coli* MM294A (*lac*$^+$, from K. Backman, Biotechnia International).

The oligonucleotides used in this work, d(AGAAGGCCTAGA) and d(TCTAGGCCTTCT), were synthesized using the phosphotriester method by Dr. Ann Pinto in the laboratory of Dr. S. J. Lippard. The synthesis and detailed characterization of the dodecanucleotide d(TCTAGGCCTTCT)

containing the *cis*-[$Pt(NH_3)_2$(d(GpG))] crosslink was performed by Dr. Pinto and is described in Naser et al. (1988a).

2. Construction of Precursor Genomes.

RF and ss DNAs were prepared as described by Lasko et al. (1987). A blunt-end ligation reaction between 1 μg of HincII-linearized M13mp18 DNA and a 450-fold molar excess of the unphosphorylated duplex d(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was carried out in the presence of 66 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 15 mM dithiothreitol, 1 mM ATP, and 800 units of bacteriophage T4 DNA ligase in a reaction volume of 20 μl at 16 °C overnight. The ligation products were then electrophoresed through a 1% low-melting point agarose gel containing 0.5 μg/ml ethidium bromide (EthBr). The band migrating at the position of a linearized genome was excised, and the DNA isolated by heating and phenol extraction (Maniatis et al., 1982). This DNA was phosphorylated in the presence of 1 mM ATP, 66 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 15 mM dithiothreitol, and 40 units of T4 polynucleotide kinase, in a total volume of 10 μl at 37 °C for 45 min. The reaction volume was brought to 20 μl maintaining the same ATP and buffer concentrations, and 800 units of T4 DNA ligase were added to catalyze intramolecular ligation. The ligation products were precipitated with EtOH, resuspended, and treated with HincII to linearize any residual starting material. E. coli MM294A cells made competent by $CaCl_2$ treatment were transformed with the restriction digest mixture (Maniatis et al., 1982). Two to three hr after the heat-shock treatment, cells were pelletted by centrifugation, leaving the progeny phage in the supernatant. Phage were screened for the presence of the dodecanucleotide and its

orientation by sequencing according to the method of Sanger et al. (1977). The sequencing primer was the M13 pentadecamer primer from New England Biolabs; the polymerase was DNA polymerase I (Klenow fragment, Boehringer Mannheim Biochemicals). This method produced the two insertion genomes: M13-12A and M13-12T.

3. Nomenclature.

d(TCTAGGCCTTCT) and d(AGAAGGCCTAGA) are abbreviated 12-T and 12-A, respectively. Pt-12-T is 12-T containing the *cis*-[$Pt(NH_3)_2$(d(GpG))] crosslink. M13-12A and M13-12T are the products of insertion of 12-A and 12-T, respectively, into the plus strand of M13mp18 at the HincII site. M13-12A-Pt(-) and M13-12A-u(-) are M13-12A with Pt-12-T and 12-T, respectively, built into the minus strand.

4. Insertion of Platinated and Unplatinated Dodecanucleotides into the (-) Strand of Duplex Genomes.

Gapped duplex formation, using formamide denaturation, and purification were performed as described by Lasko et al. (1987). Briefly, hydroxylapatite-purified M13-12A ss viral DNA and HincII-linearized M13mp18 RF were hybridized at a 20:1 molar ratio of ss (+) to linearized (-) strand to produce a 12-base gap specifically in the (-) strand.

Phosphorylation of Pt-12-T and 12-T prior to ligation into the gapped duplexes was carried out according to the method of Green et al. (1984) on 126 pmol of the dodecanucleotide, in the presence of 200 μCi γ-[^{32}P]-ATP (3000 Ci/mmol, Amersham). Unlabeled ATP was added to the reaction to bring

the ATP:dodecamer ratio to 10:1. HPLC conditions employed to determine the extent of phosphorylation of the dodecamers were as follows: a C-18 reversed phase column, and a gradient of 15-45% B over 30 min, where A = 0.1 M NH_4OAc (pH 6.5) and B = 1:1 A:CH_3CN.

The ligation was performed on 1 μg of gapped duplex DNA, with a 600:1 molar excess of ^{32}P-phosphorylated dodecanucleotide in a reaction volume of 40-80 μl using conditions described in Green et al. (1984), without the addition of bovine serum albumin. T4 DNA ligase was added to the reaction mixture twice in 800-unit portions, and the reaction was allowed to proceed for 6 or more hr. Excess ATP and dodecanucleotide were separated from higher molecular weight ligation products by drop dialysis (Silhavy et al., 1984) against 1 l TE buffer for 1-3 hr, followed by Sepharose CL 4B chromatography using TE containing 100 mM NaCl as eluant. Three-drop fractions were collected and the radioactivity quantitated by Cerenkov counting. Peak fractions eluting at and around the void volume were pooled and stored at 4 °C; these fractions contained the genomes that had incorporated Pt-12-T and 12-T, designated M13-12A-Pt(-) and M13-12A-u(-) respectively.

5. Characterization of Site-Specifically Adducted Genomes.

The following methods were employed to study M13-12A-Pt(-) and M13-12A-u(-). Restriction digests were typically performed on 300-1000 cpm of the ligation products using the three-buffer system of Maniatis et al. (1982), with the addition of 0.1 μg of unlabeled M13-12A RF DNA as an internal control. Agarose dye gels contained 0.8% agarose and 0.5 μg/ml

EthBr. In all cases digestion was complete as judged by visualization of carrier M13-12A RF DNA by EthBr fluorescence. Autoradiography was performed overnight at room temperature.

Removal of the platinum adduct as $[Pt(CN)_4]^{2-}$ was accomplished by incubating the ligation products for 3 hr at 37 °C in the presence of 0.3 M NaCN (pH 8.0). The pH of the cyanide solution was adjusted with HCl in a fume hood.

Ligation efficiency was determined by a densitometric method. A portion of each ligation mixture was electrophoresed through an agarose dye gel. The gel was photographed using Type 55 Polaroid Positive/Negative Land Film, dried, and then autoradiographed using pre-flashed Kodak X-O-Mat AR film at room temperature. The lanes on the photographic negative and the autoradiogram were scanned by using a Bio-Rad Model 620 Video Densitometer, and the relative amounts of nicked circular (form II) and covalently closed circular (form I_o) material for each lane were compared between the negative and the autoradiogram.

C. Results

1. Construction of Precursor Genomes.

The duplex dodecamer d(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was inserted into the unique HincII site of M13mp18 by a blunt-end ligation procedure (see Figure 6) to produce vectors denoted as M13-12A and M13-12T. The excess of dodecamer used in the first ligation reaction produced linear M13mp18 molecules flanked at each end by a duplex dodecamer having only one strand

covalently attached via the 5'-phosphoryl groups to the linear genome (note the nicks in Figure 6). The heating step employed during the isolation of these linear DNA molecules from the agarose gel also served to denature non-covalently-associated dodecamer strands, resulting in genome-length molecules with 12-base overhanging ends. It should be noted that, owing to the non-self-complementary nature of the dodecamer sequences, the inserts can assume two different orientations at either end of the linear genome; consequently, only half of the resulting molecules have complementary overhanging ends. Only genomes with complementary ends can participate in an intramolecular ligation reaction. The genomes produced by this protocol were sensitive to StuI cleavage and refractory to HincII, indicating correct placement of the inserts. Insert orientation was established by DNA sequencing. Both orientations, M13-12A and M13-12T, were found in the first ten insert genomes sequenced.

2. Preparation and Characterization of the ds Genome Containing the Pt-DNA Adduct Specifically in the (-) Strand.

Figure 7 outlines the strategy employed to situate Pt-12-T and 12-T within a gap in a heteroduplex genome. The phosphorylation efficiency of both Pt-12-T and 12-T was approximately 95%. The ATP:dodecamer ratio used in the polynucleotide kinase reaction optimized both the amount of label incorporated and the extent of phosphorylation, as determined by HPLC analysis (data not shown). Under the HPLC conditions employed, the phosphorylated dodecamer, whether platinated or unplatinated, eluted approximately one min before its corresponding unphosphorylated form. Ligations using 1 μg of gapped duplexes typically yielded 5-7 x 10^4 cpm of

high molecular weight material. The products of one such ligation, M13-12A-Pt(-) and M13-12A-u(-), are shown in Figure 8 ("a" lanes).

Incorporation of a ^{32}P radiolabel at the fifth phosphate 5′ to the site of platination in the dodecamer (Figure 7) facilitated characterization of the adducted genome. Electrophoresis of the site-specifically platinated DNA through a 0.8% agarose gel containing EthBr and subsequent autoradiography revealed two bands, corresponding to form I_o (covalently closed, relaxed circular) and form II (nicked circular) DNA (Figure 8). The presence of form I_o DNA indicates that ligation into the 12-base gap occurred at both the 5′- and 3′-ends of the oligonucleotide. Form II DNA can arise from incomplete ligation or the presence of nicks elsewhere in the genome. To determine whether the dodecamer had ligated into the correct area of the genome, the position of the ^{32}P label was physically mapped using restriction endonucleases. Digestion of M13-12A-Pt(-) with restriction enzymes BglII, FokI, and PvuII (Figure 8) localized the ^{32}P to the region around the former HincII site, indicating that ligation had not occurred elsewhere on the genome.

Digestion of M13-12A-u(-) with StuI yielded linear DNA, whereas M13-12A-Pt(-) was insensitive to cleavage (Figure 8, "b" lanes). This result suggests the presence of the platinum adduct within the recognition sequence of the enzyme; other studies have shown that *cis*-DDP platinated DNA inhibits digestion by other restriction enzymes (Ushay et al., 1981). The platinum adduct did not directly inhibit the activity of the enzyme, as shown by the complete digestion of the non-radioactive, unplatinated M13-12A RF DNA added as an internal control (data not shown). Incubation

of M13-12A-Pt(-) with cyanide released the platinum from DNA, and rendered the molecule cleavable by StuI (Figure 8, "c" lanes). These data, combined with NMR pH titration data obtained on the platinated dodecanucleotide (vide infra), strongly indicate the presence of the G^G adduct within the StuI restriction site in the M13-12A-Pt(-) genome.

3. Determination of the Extent of Ligation at the 5′ and 3′ Ends of the Dodecamer.

On the basis of the foregoing experiments, it was concluded that specific ligation of the platinated dodecamer into the 12-base gap had occurred at the desired position within the genome. Next it was determined whether the dodecanucleotide had ligated at both its 5′ and 3′ termini. Ligation products migrated as both form I_o and form II DNAs in an agarose dye gel (Figure 8, "a" lanes); both forms were visible in the dye gel itself and on the corresponding autoradiogram. Bands appearing on the autoradiogram represent DNA that has, by virtue of the ^{32}P radiolabel, incorporated a dodecamer and is therefore denoted M13-12A-Pt/u(-). Form I_o DNA is relaxed, covalently closed circular DNA that co-migrates with the supercoiled genome in an agarose dye gel. The presence of labeled form I_o DNA indicates conclusively that the dodecanucleotide ligated into the genome at both ends. The form II (nicked circular) DNA visible in the autoradiogram could have arisen either from incomplete dodecamer ligation or from random nicks present elsewhere in the genome. These two possibilities were distinguished by the following experiment.

The M13-12A-Pt(-) and M13-12A-u(-) ligation products were digested with the enzyme PvuII to produce a 334-base ds fragment containing the dodecanucleotide (Figure 9). The fragment was denatured with formamide and heat, and electrophoresed on a denaturing 5% polyacrylamide gel, which was then autoradiographed. The presence of a single band corresponding to the 334-nt ss fragment indicates ligation occured on both sides of the dodecanucleotide. Ligation exclusively at the 5' terminus of the dodecamer leaves a nick on the 3' side such that the radiolabel would be contained within a 223-nt ss fragment (Figure 9a). Conversely, if ligation occurred only on the 3' terminus, the radiolabel would migrate with a 123-nt fragment. The results in Figure 9b show that in both the platinated and unmodified genomes, approximately half of the label is associated with a 334-nt fragment, and the remainder is equally distributed between the 123- and 223-nt fragments. Therefore, in half of the genomes the dodecamer ligated on both the 5' and 3' sides. In the other half, ligation occurred only on one side of the dodecamer.

4. Determination of Ligation Efficiency.

Although, by definition, all DNA visible in the autoradiogram has incorporated labeled dodecamer, the same need not be true for DNA visible in the agarose dye gel (see Figure 10). If the ligation efficiency, defined as the percent of gapped duplexes having a dodecamer (either fully or partially ligated) incorporated into the 12-base gap, were less than 100%, then residual gapped duplexes would appear as unlabeled form II DNA. Densitometry was used tc determine the ligation efficiency. That is, it

determined whether all DNA visible in the form II band in the agarose dye gel had incorporated a dodecamer.

The method relies on the assumption that all DNA visible as form I_o in the agarose dye gel is M13-12A-Pt(-)/u(-) (Figure 10). Work in our laboratory has shown that a ligation event can occur between the 5' and 3' ends of the (-) strand of a four- or six-base gapped duplex molecule (termed "gapped duplex ligation"), and that the resultant DNA migrates with form I_o in an agarose dye gel (E.L. Loechler and A.K. Basu, unpublished observations). To test whether such a ligation occurred with a 12-base gap, a mock ligation using only gapped duplex DNA and no dodecamer was carried out, and the products electrophoresed through an agarose dye gel. No form I_o material was detected, even when the gel was intentionally overloaded (data not shown). It is concluded, therefore, that ligation does not detectably occur across the 12-base gap. This result thus establishes that all DNA appearing as form I_o in the dye gel is M13-12A-Pt(-)/u(-). Therefore, form I_o bands visible in the dye gel and autoradiogram represent identical DNA molecules.

To determine if form II DNA in the agarose dye gel bands were solely M13-12A-Pt(-)/u(-) or a mixture of ligation products and residual gapped duplexes, relative band intensities for forms II and I_o in corresponding lanes in the agarose gel and autoradiogram were determined by densitometry and normalized such that the intensities of form II + form I_o = 100 in each lane (Figure 10). The ratio of band intensities of form II and form I_o in the autoradiogram (c/d using the designations in Figure 10) defines the ratio of these forms of M13-12A-Pt(-)/u(-), both labeled and unlabeled, in

the ligation products. Therefore, this same ratio should obtain for DNA present in the agarose dye gel if the ligation efficiency were 100% (i.e., a/b = c/d). Any increase in the form II:form I_o ratio for the agarose dye gel versus the autoradiogram would be due to excess form II DNA present in the former, which could only result from the presence of residual gapped duplex DNA. Denoting this excess by x, gives (a-x)/b = c/d. Since x is the percent of gapped duplexes that did not incorporate a dodecamer, then 100 - x is the ligation efficiency. Using this method it was shown that the ligation efficiency for both M13-12A-Pt(-) and M13-12A-u(-) was ≈80% (average of three determinations).

D. Discussion

Construction of viral genomes containing the major *cis*-DDP-DNA adduct, the *cis*-[$Pt(NH_3)_2$(d(GpG))] intrastrand crosslink, was accomplished by ligation of a dodecanucleotide containing the adduct at a specific site into a gapped duplex M13 genome. A dodecanucleotide was chosen for several reasons. Insertion of the 12-base sequence into the cloning region of M13mp18 preserves the *lacZ* reading frame, ensuring a functional β-galactosidase protein. It was anticipated that a short (four to six base) platinated oligonucleotide, similar to those previously employed by others (Green et al.; 1984, Lasko et al., 1987; Basu et al., 1987), might not efficiently ligate into a gapped genome because of the severe structural distortions imposed on the DNA by *cis*-DDP binding. These structural changes are evidenced by helix unwinding (Cohen et al.,1979), reduction in the degree of Watson-Crick base pairing (den Hartog et al., 1984; van Hemelryck et al., 1984; Sundquist et al., 1986), and the 90° dihedral angle

between the coordinated guanine bases in a *cis*-[$Pt(NH_3)_2$(d(pGpG))] crosslink in a dinucleotide (Sherman et al., 1985). A 12-base oligonucleotide was chosen to provide the structural insulation believed necessary for ligation. The sequence was designed to position the *cis*-[$Pt(NH_3)_2$(d(GpG))] target centrally within the dodecamer, and to provide a restriction site (StuI) unique to the M13-12A genome. Location of the adduct within the StuI site affords a powerful tool for characterizing the platinated genome, and for mutant selection in mutagenicity studies. Pyrimidines were selected to flank the StuI sequence to minimize the number of platination targets. Both GpG and ApG sequences are potential platinum binding sites in the dodecamer employed, but an A^G adduct was not anticipated since *cis*-[$Pt(NH_3)_2$(d(GpG))] was the only product found in the reaction of *cis*-DDP with $[d(AGGCCT)]_2$ (Caradonna et al., 1982), and was the major product of the reaction with d(TCTAGGCCTTCT). The *cis*-[$Pt(NH_3)_2$(d(GpG))] G(N7)-G(N7) structure of the platinated dodecamer was established by pH-dependent NMR studies (Naser et al., 1988a); thus, a chemically well-characterized platinated dodecanucleotide of high purity was thus provided by Dr. Pinto for use in preparing site-specifically modified genomes.

Ligation of platinated and unmodified dodecamers into the 12-base gap was demonstrated by the experiments presented in Figure 8 and Pinto et al. (1986). The *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct is located in the recognition sequence for the restriction enzyme StuI and its presence inhibited cleavage by this enzyme. This result is consistent with an earlier study of BamHI cleavage of globally platinated pBR322 DNA in which it was

estimated that restriction enzyme activity would be inhibited ±3 basepairs from the site of a platinum crosslink (Ushay et al., 1981). In both studies, it was established that inhibition was due to the platinum adduct because removal of the adduct with cyanide restored the sensitivity of the genome to endonuclease cleavage. The ability to remove completely the platinum adduct by cyanide reversal is analogous to the enzymatic removal of O^6-methylguanine by O^6-methylguanine-methyltransferase (Green et al., 1984; Couto et al., 1986). This reversibility provides a tool for directly correlating adduct presence with biological activity.

Interestingly, both platinated and unplatinated dodecamers ligated into the gapped duplexes with similar, high efficiencies. The extent of ligation on either end of the dodecamers was also similar; however, only half of the incorporated oligonucleotide formed covalent bonds at both termini. This result is in contrast to previous results from our laboratory using tetranucleotides containing O^6-methylguanine or *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl, and a hexanucleotide containing $1,N^6$-ethenoadenine (Green et al., 1984; Lasko et al., 1987; Basu et al., 1987). In each case the adducted oligonucleotide ligated into gapped genomes completely (i.e., on both the 5′ and 3′ ends), albeit with overall efficiencies less than half of that of the corresponding unmodified oligonucleotide, a result attributed to the presence of the adduct. The reason for incomplete ligation of 12-T and Pt-12-T at their 5′ and 3′ termini is unknown.

The disrupting effect of a single *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct on duplex DNA stability was further demonstrated by gradient denaturing gel

electrophoresis. A singly platinated restriction fragment was demonstrated to melt at a lower denaturant concentration than did the corresponding unmodified fragment (Naser et al., 1988a). These data are consistent with the failure of StuI to cleave the genome, as described above. It is noteworthy, however, that disruption of DNA structure was not so severe as to prohibit DNA restriction at sites farther removed from the adduct, nor ligation of the platinated oligonucleotide into the genome.

Figure 6. Scheme for insertion of dodecanucleotide d(TCTAGGCCTTCT)·d(AGAAGGCCTAGA) into the HincII site of M13mp18 to produce the insertion mutant M13-12A as described in the text; a second insertion mutant, M13-12T, was also produced (not shown here), in which the duplex dodecanucleotide was ligated in the opposite orientation. Relevant restriction enzyme recognition sites are indicated on the M13-12A map.

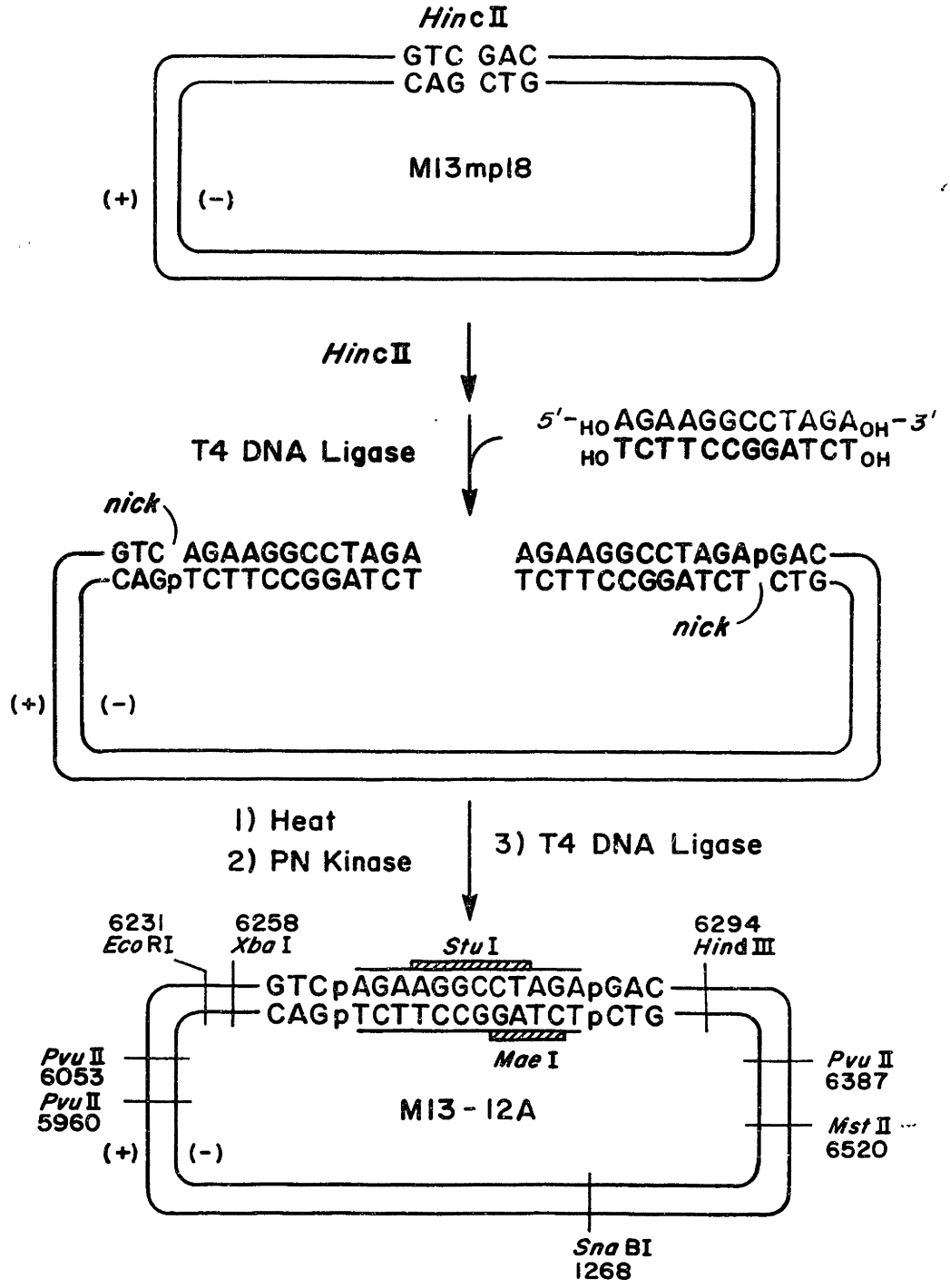

HincII
GTC GAC
CAG CTG
M13mp18
(+)
(-)
HincII
T4 DNA Ligase
5'-HO AGAAGGCCTAGAOH-3'
HO TCTTCCGGATCTOH
nick
GTC AGAAGGCCTAGA
CAGpTCTTCCGGATCT
AGAAGGCCTAGApGAC
TCTTCCGGATCT CTG
nick
(+)
(-)
1) Heat
2) PN Kinase
3) T4 DNA Ligase
6231
EcoRI
6258
XbaI
StuI
6294
HindIII
GTCpAGAAGGCCTAGApGAC
CAGpTCTTCCGGATCTpCTG
MaeI
PvuII
6053
PvuII
5960
M13-12A
PvuII
6387
MstII
6520
(+)
(-)
SnaBI
1268

Figure 7. Scheme for gapped heteroduplex synthesis and subsequent ligation of the adducted dodecanucleotide to produce the site-specifically platinated ds genome M13-12A-Pt(-), as described in the text.

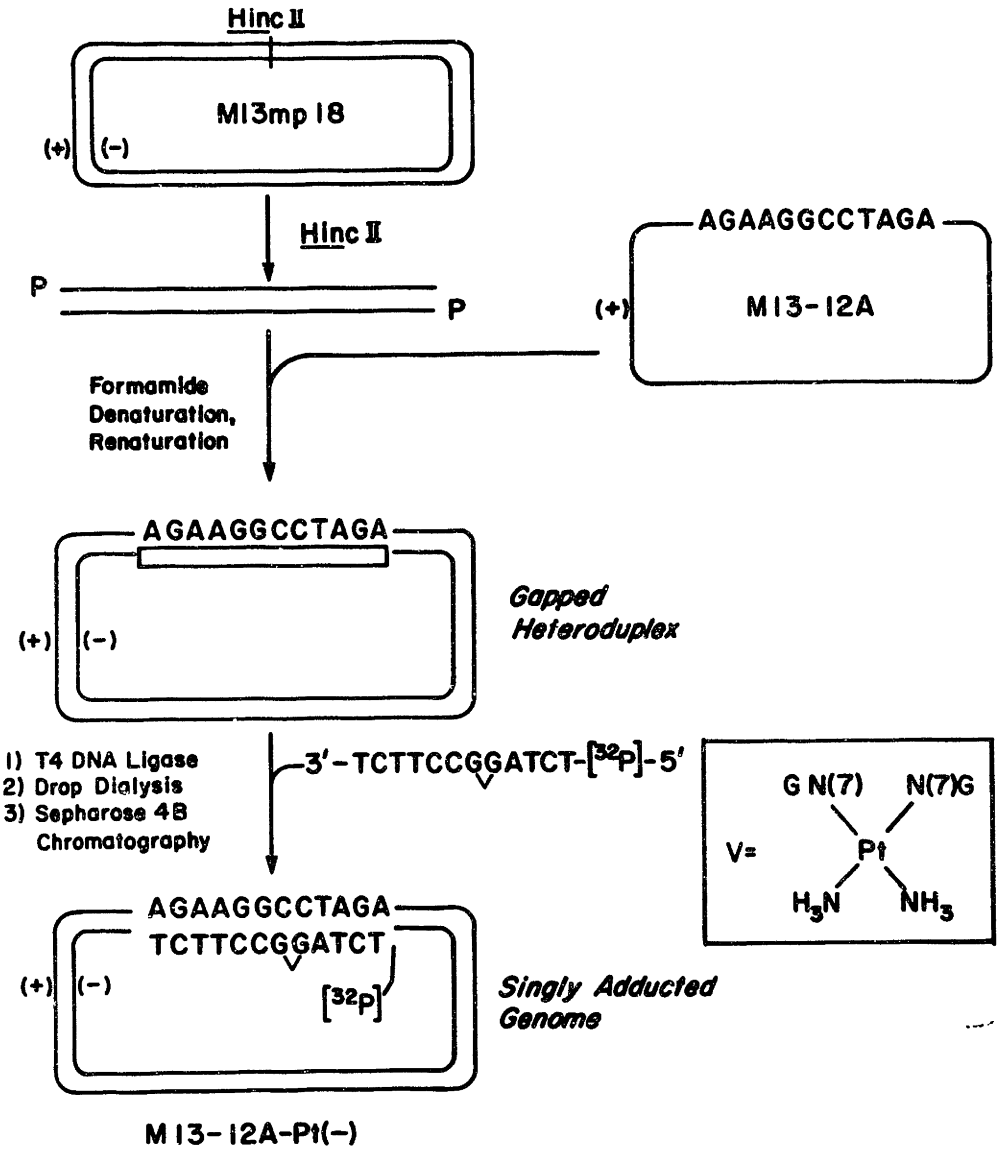

Hinc II
M13mp 18
(+)
(-)
Hinc II
P
P
AGAAGGCCTAGA
(+)
M13-12A
Formamide
Denaturation,
Renaturation
AGAAGGCCTAGA
(+)
(-)
Gapped
Heteroduplex
1) T4 DNA Ligase
2) Drop Dialysis
3) Sepharose 4B
Chromatography
3'-TCTTCCGGATCT-[32P]-5'
V=
G N(7)
N(7)G
Pt
H3N
NH3
AGAAGGCCTAGA
TCTTCCGGATCT
(+)
(-)
[32P]
Singly Adducted
Genome
M13-12A-Pt(-)

Figure 8. Characterization of the M13-12A-Pt(-) and M13-12A-u(-) genomes by restriction endonuclease digestion. Autoradiogram of a 0.8% agarose gel containing 0.5 μg/mL EthBr. Lanes a-e display material digested with nothing, StuI, BglII, FokI, or PvuII, respectively. Lanes f and g display cyanide treated M13-12A-Pt(-) and M13-12A-u(-) followed by digestion with StuI. Unlabeled M13-12A RF (0.1 μg) was added to each digestion as an internal standard. Digestion of this standard DNA was judged to be complete in all cases following visualization by EthBr fluoresence. The shaded box in the genome map contains the postition of the ^{32}P label and, where present, the platinum atom.

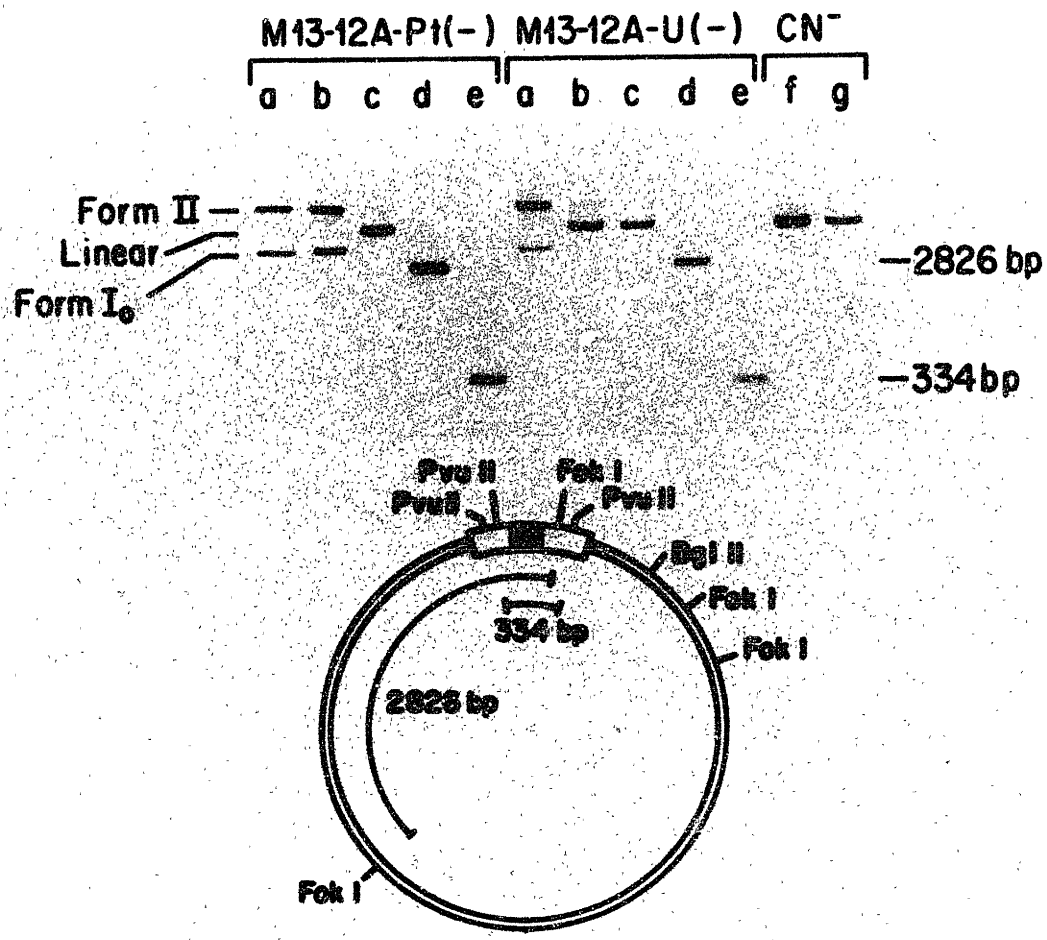
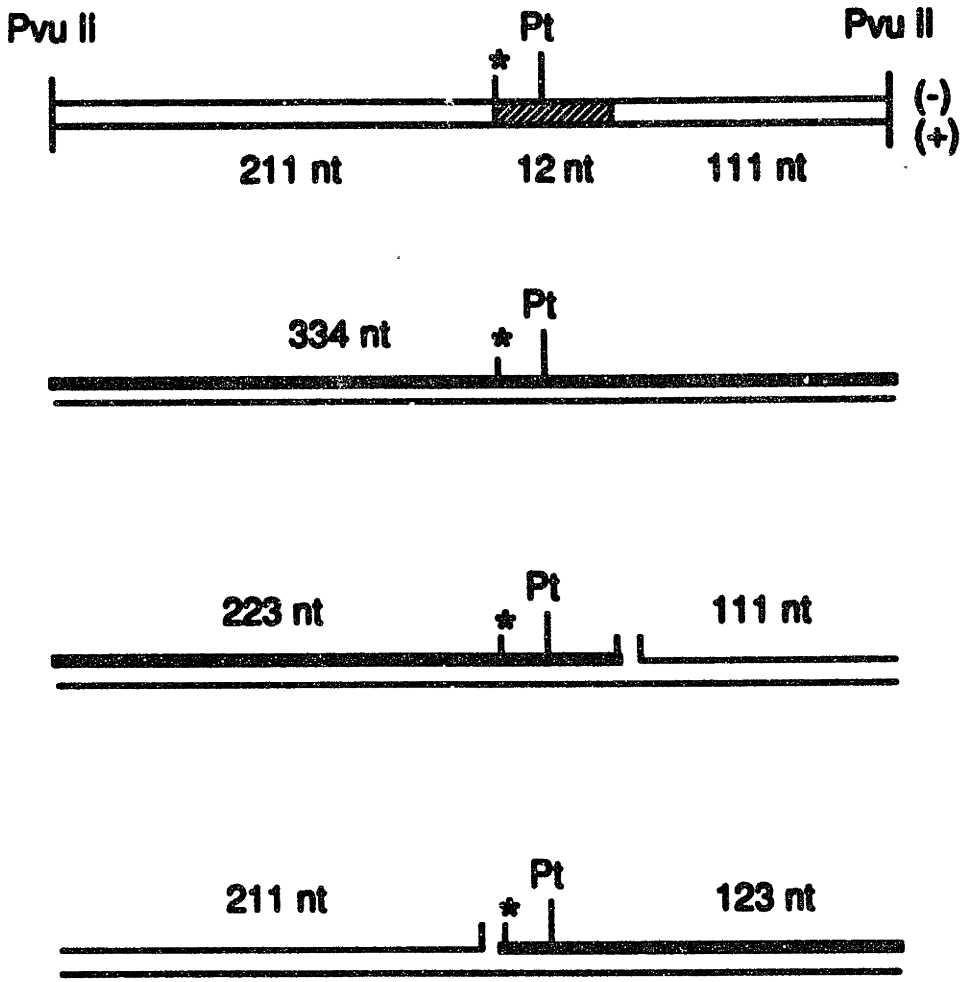

M13-12A-P1(-)
M13-12A-U(-)
CN⁻
a b c d e a b c d e f g
Form II
Linear
Form I$_o$
—2826 bp
—334 bp
Pvu II
Pvu II
Fok I
Pvu II
Bgl II
Fok I
Fok I
334 bp
2826 bp
Fok I

Figure 9. Determination of the extent of ligation at 5′ and 3′ termini of the platinated and unmodified dodecanucleotides into the gapped duplex M13-12A genome. Frame a: Map of the 344-bp PvuII fragment encompassing the inserted dodecanucleotide. Bold lines in the lower three maps denote ss fragment sizes produced by ligation at both the 5′ and 3′ termini, on the 5′ terminus only, or on the 3′ terminus only. Pt denotes the site of platinum binding, if present; *, the site of the ^{32}P label; and nt, nucleotide. Frame b: Autoradiogram of a 5% denaturing polyacrylamide gel. Ligation products were digested with PvuII, denatured with formamide and heat, and electrophoresed. Unlabeled lanes are ss molecular weight standards.

a


Pvu II
Pt
Pvu II
(-)
(+)
211 nt
12 nt
111 nt
334 nt
Pt
223 nt
Pt
111 nt
211 nt
Pt
123 nt

b

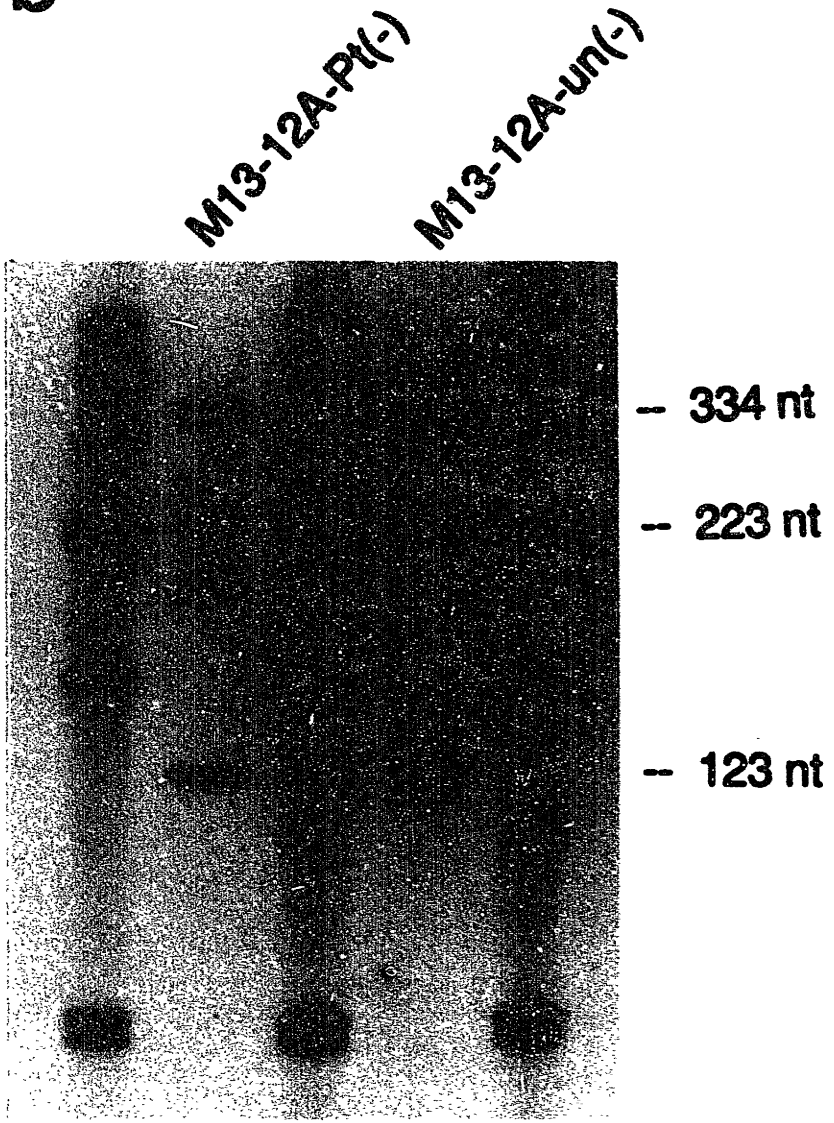
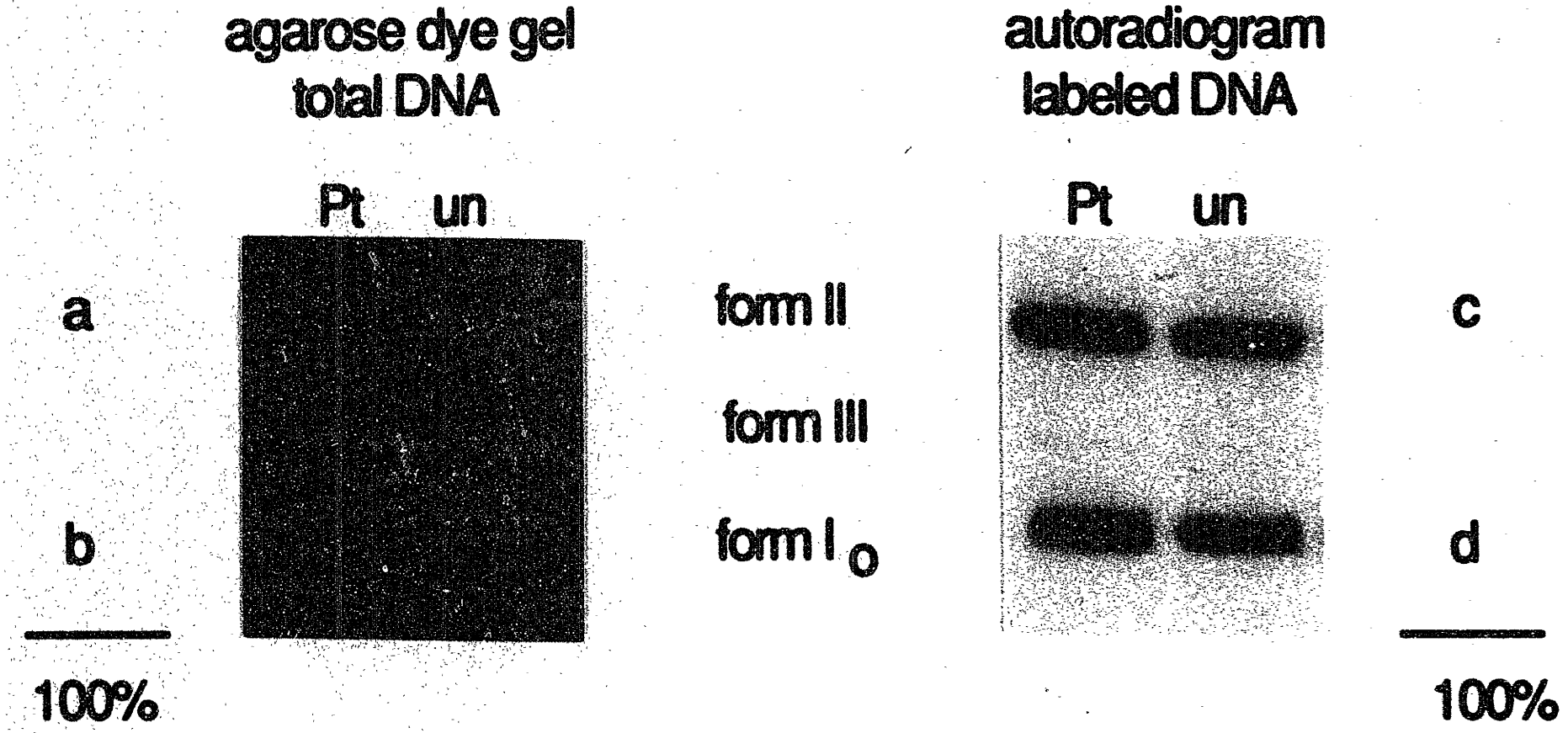

M13-12A-Pt(-)
M13-12A-un(-)
334 nt
223 nt
123 nt

Figure 10. Agarose dye gel and corresponding autoradiogram used for ligation efficiency determinations, as described in the text. Pt is M13-12A-Pt(-), and un is M13-12A-u(-).


agarose dye gel
total DNA
Pt
un
a
b
100%
form II
form III
form I o
autoradiogram
labeled DNA
Pt
un
c
d
100%

IV. Construction and Characterization of a Single Stranded Viral Genome Containing the Major DNA Adduct of *cis*-DDP Built into a Specific Site.

A. Introduction.

The previous Chapter detailed the construction and characterization of a singly adducted ds genome that was to be used in experiments to determine the mutation frequency, specificity, and genotoxicity of the *cis*-[$Pt(NH_3)_2(d(GpG))$] adduct. While this work was underway, it was becoming increasingly obvious to workers in the site specific mutagenesis field that single adducts in duplex DNA induced few mutations. This is thought to be due either to the rapid repair of the single adduct in ds DNA, to a bias against the replication of the adducted strand of singly adducted duplex genomes, or to both.

The effects that repair and double- versus single-strandedness of the vector can have on single adduct mutation frequency is illustrated by studies on O^6-methylguanine. A duplex vector containing a single O^6-methylguanine adduct transformed into wild type E. coli shows a mutation frequency of only 0.04% (Essigmann et al., 1986). When the O^6-methylguanine lesion is present in ss DNA, by contrast, the mutation frequency increases by an order of magnitude to 0.4% (Loechler et al., 1984). When the vectors are introduced into cells depleted of the enzyme responsible for repairing this adduct, the mutation frequency of the duplex vectors increases approximately 200-fold, and that of the ss vectors increases 50-fold.

These results emphasize that DNA repair can have a profound effect on the mutation frequency of single adducts in site specifically modified DNA. It is noteworthy that the repair system responsible for removal of O^6-methylguanine lesions in DNA is active on both double and single stranded DNA (Lindahl, 1982). Nucleotide excision repair, which is believed to be the major pathway of repair of *cis*-DDP adducts in E. coli, requires a ds DNA substrate (A. Sancar, personal communication). Hence, it is expected that a platinum adduct present in ss form probably would not be removed before it is encountered by the replication apparatus.

As illustrated by the work with O^6-methylguanine, even when repair systems are compromised, the mutation frequency of the ds vectors is less than that for the ss vectors (8% versus 20%, respectively). Work from the laboratory of R. P. P. Fuchs indicates that there may be a strand bias in the replication of duplex genomes carrying adducts on only one strand (Koffel-Schwartz et al., 1987). Heteroduplexes of pBR322 were constructed such that one strand encodes tetracycline resistance and the opposing strand contained a single base mutation that rendered its progeny sensitive to the same drug. If the strand coding for tet^R carries lesions in its DNA, then the progeny arising from the introduction of the heteroduplexes into cells are predominantly tet^S. Conversely, if the tet^S strand carries the lesions, the progeny are predominantly tet^R. If neither strand is adducted, then tet^R and tet^S progeny occur with equal frequency. The cause of the observed bias is as yet unknown, although, it is conceivable that it may simply be that replication is slowed or halted on the adducted strand while the unadducted strand can be replicated without hinderance, resulting

in a greater proportion of progeny from the latter. This issue is of major concern since it has been shown that *cis*-DDP-DNA adducts can block replication (Pinto & Lippard, 1985b).

In order to obviate the possible problems of repair and strand-bias in replication, I decided that I should study *cis*-[$Pt(NH_3)_2$(d(GpG))] mutagenesis in ss DNA. This required the development of a method for producing singly adducted ss genomes. The first two methods I attempted were unsuccessful for various reasons; they are described in Appendix I.

The successful method, outlined in Figures 11 and 12, was developed in part to capitalize on gapped heteroduplex production by the formamide method described in the previous Chapter (which efficiently forms heteroduplexes between ss circular and ds linear DNAs, Figure 7). This procedure also makes it possible to situate replication inhibitory lesions on the DNA strand opposite that containing the platinum adduct. This step renders the opposing strand biologically inactive when the genome is transfected into cells. It was also desirable that these lesions be the substrate for enzymes that would recognize and cleave phosphodiester bonds in the opposing strand in vitro. Cleavage of the opposing strand enables it to be removed by denaturation, yielding as a major product a singly adducted, ss circular genome.

There were two candidates for the opposing strand lesions that could be used for strand inactivation. The approach I elected <u>not</u> to take involved the incorporation of uracil into the ss DNA in place of thymine (by growing the phage in *dut*$^-$ *ung*$^-$ mutant E. coli; Kunkel, 1985). The

uracilated DNA can be treated in vitro first with uracil DNA glycosylase and subsequently with an apyrimidinic/apurinic endonuclease to produce the desired nicked strand. The strand-inactivating lesion I used was the UV-induced thymine dimer, which is a substrate for T4 endonuclease V (a pyrimidine dimer DNA-glycosylase and an apyrimidinic/apurinic endonuclease; Haseltine et al., 1980; Figure 13). I chose to use thymine dimers since they have been employed for strand inactivation in similar studies by others (Burnouf et al., 1989; Burnouf et al., 1990), and purified T4 endonuclease V was available to me courtesy of Dr. R. S. Lloyd (Higgins & Lloyd, 1987). The details of the construction and characterization of these genomes are the subject of this Chapter.

B. Materials and Methods.

1. Materials.

All enzymes were purchased from Boehringer-Mannheim, unless otherwise indicated[1]. T4 endonuclease V was kindly provided by Dr. R. S. Lloyd (Vanderbilt University). The cell lines and the M13 derived genomes used were the same as described in the previous Chapter. M13mp18 DNA platinated at a drug/nucleotide ratio of 0.002 was prepared by Dr. Judith Burstyn in the laboratory of Dr. S. J. Lippard.

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1. The previous work utilized the restriction endonuclease HincII, purchased from New England Biolabs. In this and subsequent work, the enzyme HindII, from Boehringer-Mannheim was used. HincII and HindII recognize the same sequence and cleave the DNA at the same site, however, I have found HindII to be a more efficient enzyme with less nonspecific endonuclease contamination.

The oligonucleotide d(TCTAGCCCTTCT) used in this work was purchased from Operon Technologies, Inc. (Alemeda, CA). The synthesis and characterization of d(TCTAGGCCTTCT) containing the *cis*-[$Pt(NH_3)_2$(d(GpG))] crosslink was performed by Dr. Burstyn essentially as described (Naser et al., 1988a).

2. Irradiation of DNA.

RF and ss DNAs were UV irradiated at various fluences in TE in 50 μl droplets on a parafilm lined petri dish using a 15 W General Electric germicidal lamp, at an average dose rate of 1.1 J/m^2 as measured by an Ultraviolet Products radiometer. Irradiation of DNA in the presence of silver was performed by combining ss DNA and Ag^+ in the form of $AgNO_3$ to obtain an r value ([Ag]/[PO_4-DNA]) of 0.5, in 0.01 M PO_4 at a final DNA concentration of 300 μg/mL. The DNA was irradiated at 1500 J/m^2 in 50 μL droplets. To remove the silver, the DNA was first dialysed against H_2O, NaCl was added to a final concentration of 0.5 M, and the solution was stirred in the dark overnight to remove the remaining Ag^+ as AgCl. The precipitate was pelleted by centrifugation and the DNA in the supernatant dialysed against TE overnight.

3. T4 Endonuclease V Reaction Conditions.

DNA (0.1-1.0 μg) was incubated with T4 endonuclease V (1 μl of a 0.02 mg/ml solution of enzyme in TE and 100 μg/ml BSA) in the presence of 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 50 mM NaCl, and 1 mg/mL BSA at 37°C for 1 hr. EDTA was present to prevent any nonspecific endonuclease activity; EDTA has no effect on T4 endonuclease V activity.

4. General Methods.

Standard gel electrophoresis was through a 0.8% agarose gel containing 0.5 μg/ml EthBr, and the DNA visualized by EthBr fluoresence. When it was necessary to distinguish between ss circular and ss linear forms of DNA, electrophoresis was performed in the cold through an agarose gel (1% agarose, 50 mM NaCl, and 1 mM EDTA (pH 8.0)), in a denaturing running buffer of 30 mM NaOH, and 1 mM EDTA (pH 8.0). The gel was soaked in the denaturing buffer for at least 30 min prior to use, and the DNA electrophoresed at 75-100 V for 15-20 hr.

Gapped duplex formation and ligation reactions were performed as described in the previous Chapter.

C. Results and Discussion.

Before making the site specifically modified genomes, it was necessary to demonstrate that the method for producing the ss genomes was feasible. To test the system, experiments were performed to demonstrate the following:

1. Platinum-DNA Adducts are not a Substrate for T4 Endonuclease V.

When UV treated plasmid DNA is allowed to react with T4 endonuclease V, the DNA is nicked at the sites of UV dimers, converting form I (supercoiled) DNA to form II (nicked circular) DNA. The two forms are easily distinguished following electrophoresis on a standard agarose gel. M13mp18 DNA randomly modified with *cis*-DDP, at a drug/nucleotide ratio of 0.002, was incubated with T4 endonuclease V, and the reaction products were

electrophoresed. The results showed that none of the form I platinated DNA was nicked by the enzyme, and therefore it was concluded that platinum-DNA adducts are not a substrate for T4 endonuclease V.

2. UV Irradiated Single Stranded DNA can Efficiently Produce Gapped Heteroduplexes.

UV treated ss M13mp18 DNA was hybridized to form a heteroduplex with HincII-linearized ds M13mp18 DNA. Gel electrophoresis of the products revealed the formation of form II DNA, demonstrating that heteroduplex formation did indeed occur, producing a hemi-dimerized product (i.e., duplex genomes with multiple thymine dimers in the (+) strand), with a nick in the (-) strand. The form II band on the gel was not as well resolved as when non-irradiated ss DNA was used; indeed, the UV irradiated ss DNA itself runs slightly slower than non-irradiated ss DNA on a gel. Heteroduplex formation did go to completion, however, as evidenced by the small amount of linear DNA present after the reaction (see previous Chapter).

3. Hemi-thymine-dimerized DNA is a Substrate for T4 Endonuclease V.

The hemi-dimerized "nicked duplexes" produced above (so-named because the (-) strand contains a nick, not a gap) were treated with T4 DNA ligase and ATP to seal the nick. The covalently closed circular (form I_o) DNA produced by this reaction co-migrated with form I DNA on an agarose dye gel. This ligated DNA was then treated with T4 endonuclease V. The results of gel electrophoresis of the products demonstrated that the hemi-

dimerized covalently closed circular DNA was a substrate for the enzyme, as evidenced by the efficient conversion of form I_o DNA to form II.

4. Only the Dimerized Strand is Cleaved by T4 Endonuclease V.

When hemi-dimerized nicked duplex DNA (as defined above, not treated with ligase) is electrophoresed in a denaturing agarose gel, the ss circular DNA (containing the thymine dimers) migrates slightly slower than the ss linear DNA, which does not contain thymine dimers, allowing separation. Nicked duplex DNA was treated with T4 endonuclease V, and the DNA then electrophoresed through a denaturing gel. The results showed that only the ss circular DNA was digested by the enzyme, as evidenced by decreased intensity of the ss circular band in the gel.

5. All the Dimerized Strands are Cleaved by T4 Endonuclease V.

It was essential that all the dimerized strands be cleaved by the T4 endonuclease V to allow for complete denaturation. To achieve this objective, a high level of thymine dimers per genome was required. The need for such a high level is evidenced by the observation that UV fluences of 180-1080 J/m^2 failed to render all of the ss derived DNA sensitive to T4 endonuclease V. The problem was that, at a certain fluence, the number of thymine dimers per genome reaches a steady state level, that is, unfortunately, below that needed for my experiment. It was possible, however, to increase the thymine dimer per genome level by UV treating the DNA in the presence of Ag^+, which greatly increases the rate of thymine dimer formation (approximately 37% of the thymines are expected to be in the form of dimers; Rahn, 1983; Rahn & Landry, 1973). When this method was

used, the hemi-thymine dimerized genomes were indeed cleaved to completion by T4 endonuclease V, as evidenced by the total disappearance of the ss circular band in denaturing agarose gels.

When all of the above conditions were met, gapped heteroduplex M13 genomes were made using the UV/Ag^+ treated ss DNA (GD(T^T)); control (unmodified) and platinated dodecamers were ligated into the 12-base gaps of the gapped heteroduplexes as described previously (Naser et al., 1988a) to produce ligation products (see Figure 3). These were:

1. 12T/T^T: A duplex genome containing unmodified dodecamer (12T) in the (-) strand and thymine dimers (T^T) in its complement,

2. Pt12T/T^T: The corresponding genome containing the platinated dodecamer (Pt12T) in the (-) strand and T^T in the (+) strand.

The genome ligation products were characterized by digestion with StuI (the restriction enzyme in whose recognition site the platinum adduct was situated). As expected on the basis of my earlier studies, the unplatinated genomes cut to completion with StuI, and the genomes containing the single platinum adduct were completely refractory to StuI digestion. Varying amounts of each ligation product were electrophoresed through agarose gels and visually inspected in order to ensure that equal amounts of DNA were used in the experiments described in the next Chapter in which the adduct was evaluated biologically.

Figure 11. Scheme for gapped heteroduplex synthesis and subsequent ligation of the adducted or unadducted dodecanucleotide to produce the ds genomes Pt12T/T^T or 12T/T^T, which contain biologically inactivating UV induced pyrimidine dimers in the strand opposite that containing the adduct, as described in the text.

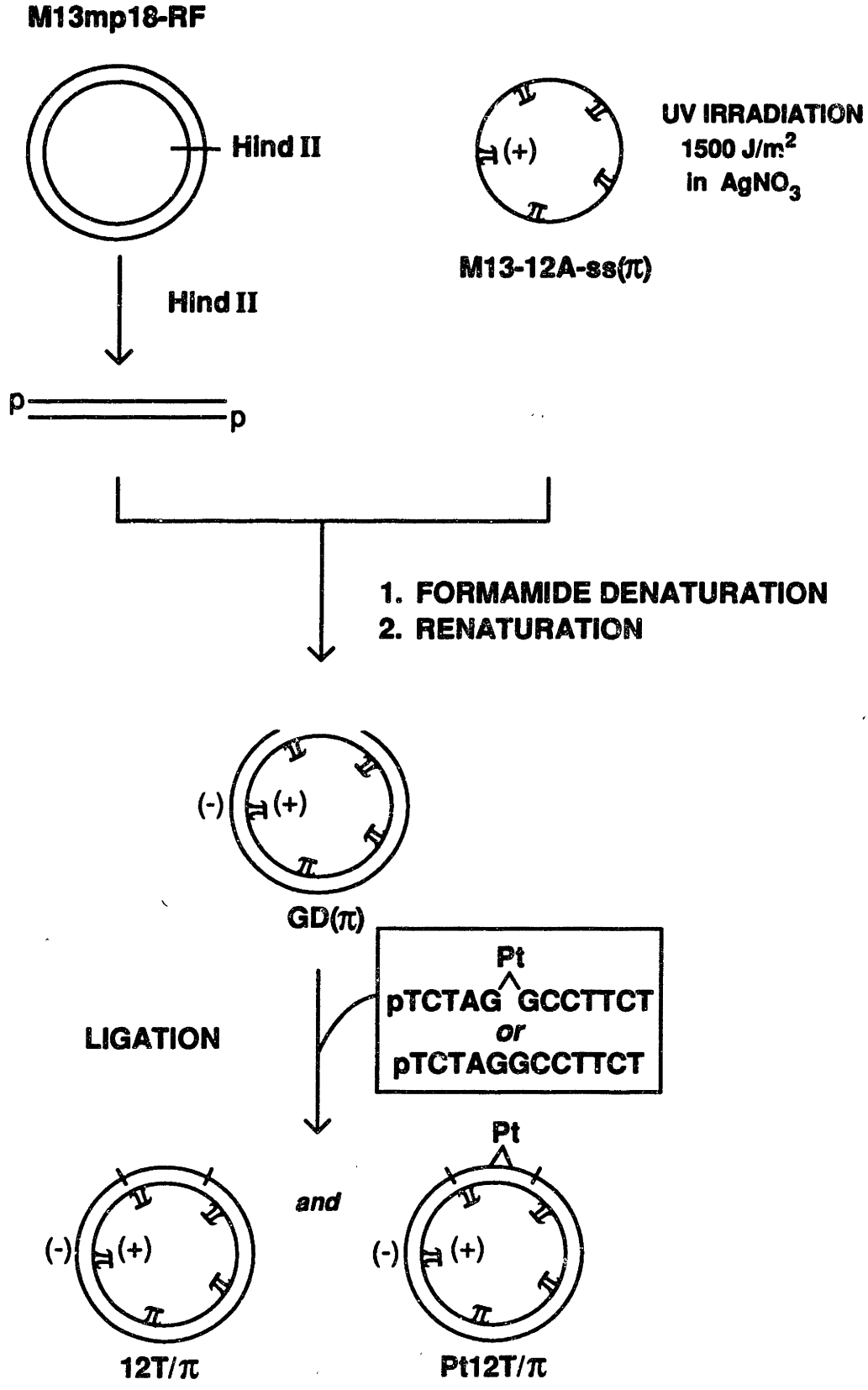

M13mp18-RF
Hind II
Hind II
p
p
M13-12A-ss(π)
UV IRRADIATION
1500 J/m2
in AgNO3
1. FORMAMIDE DENATURATION
2. RENATURATION
(-)
(+)
GD(π)
LIGATION
Pt
pTCTAG GCCTTCT
or
pTCTAGGCCTTCT
and
Pt
12T/π
Pt12T/π

Figure 12. Scheme for producing ss genomes by T4 endonuclease V treatment and subsequent heat denaturation of the singly adducted ds genome Pt12T/T^T and the unmodified control genome 12T/T^T.

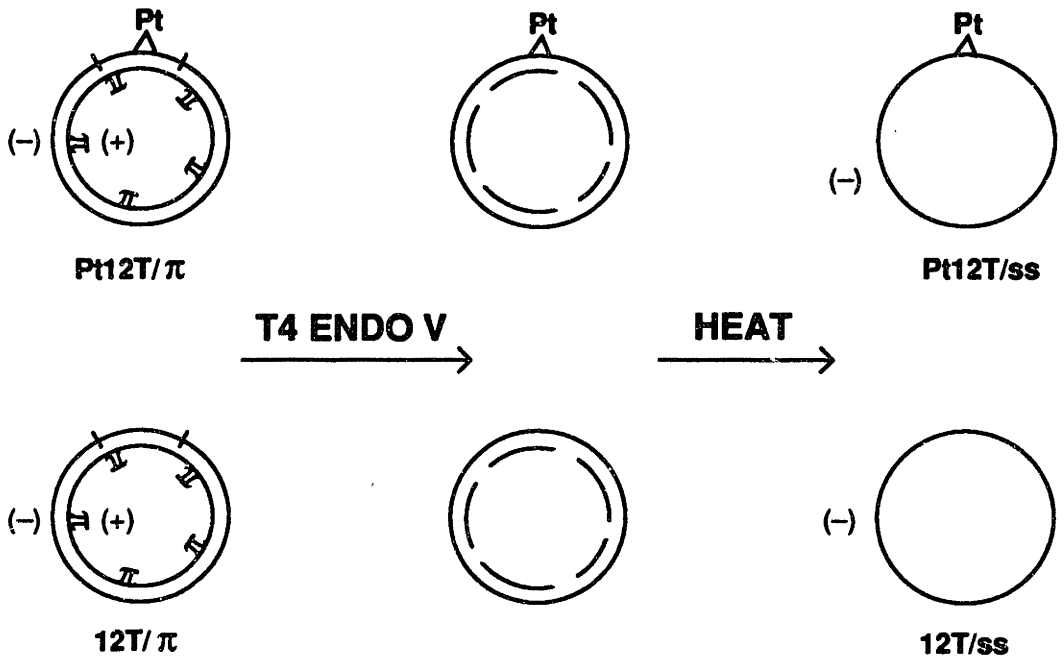
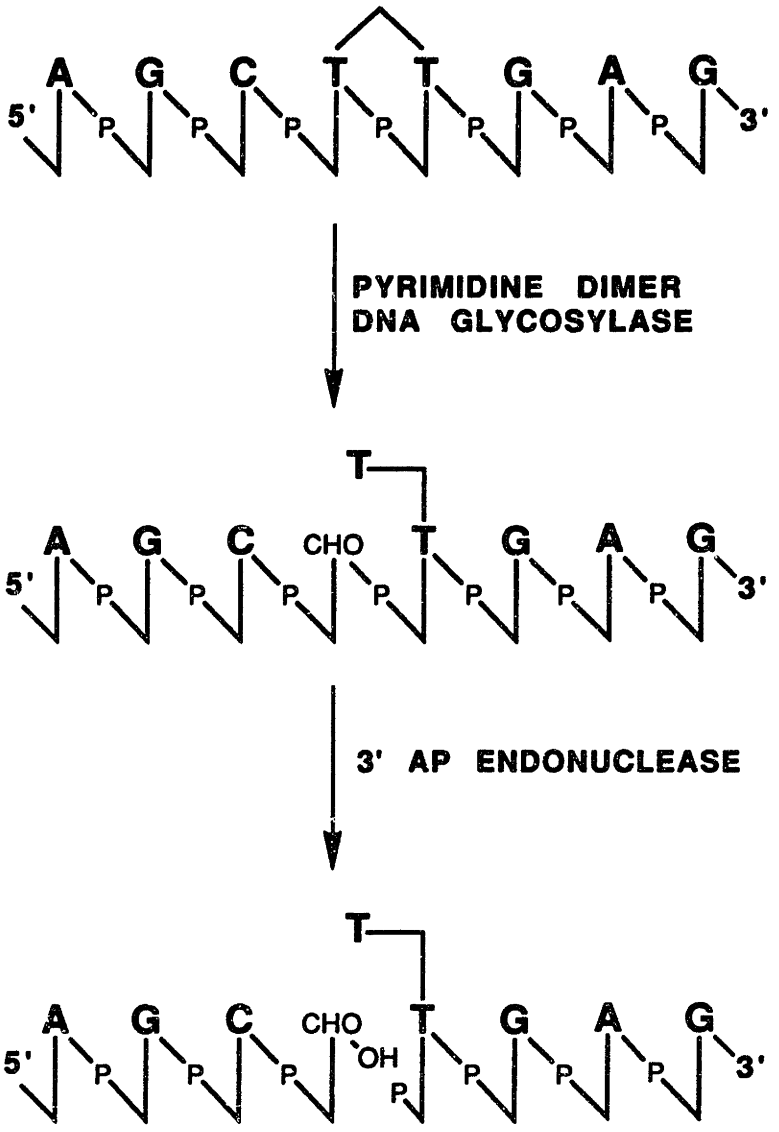

Pt
(–)
(+)
Pt12T/π
Pt
Pt
(–)
Pt12T/ss
T4 ENDO V
HEAT
(–)
(+)
12T/π
(–)
12T/ss

Figure 13. Mechanism of action of T4 endonuclease V (after Friedberg, 1984).


A G C T T G A G
5'
3'
P
PYRIMIDINE DIMER
DNA GLYCOSYLASE
T
CHO
3' AP ENDONUCLEASE
OH

V. Site Specific Mutagenesis of the Major DNA Adduct of *cis*-DDP.

A. Introduction

The site-specifically platinated genomes described in the previous Chapter, both in ss and ds form, were transfected into E. coli cells, and the progeny phage from these experiments were analysed to determine both the mutation frequency and specificity of the G^G adduct. In this Chapter, I present the results of these experiments, and discuss these results in relation to other work in the field.

B. Materials and Methods

1. Materials.

The E. coli cell lines used were: DL7 (AB1157; *lacΔU169*, *uvr*$^+$; from D. Lasko, MIT); DL6 (AB1886; *lacΔU169*, *uvrA*; from D. Lasko, MIT); GW5100 (JM103; P1$^-$; from G. Walker, MIT); MM294 (*lac*$^+$, from K. Backman, Biotechnia International). The synthesis and characterization of the site specifically modified duplex genomes 12T/T^T and Pt12T/T^T (containing a single *cis*-[Pt(NH_3)$_2${d(GpG)}] adduct) were described in the previous chapter. T4 endonuclease V was a gift from R. S. Lloyd (Vanderbilt University).

2. T4 Endonuclease V Reaction Conditions.

For experiments in which ss singly adducted DNA was transfected into cells, the 12T/T^T and Pt12T/T^T duplex genomes were incubated with T4 endonuclease V in the presence of 10 mM Tris-HCl (pH 8.0), 50 mM EDTA

(pH 8.0), and 1 mg/mL BSA at 37 °C for 1 hr. The samples were extracted once with an equal volume of phenol saturated with Tris-HCl (pH 8.0) and then added to 1.0 mL TE and simultaneously desalted and concentrated by centrifugation in a Centricon-10 microconcentrator (Amicon). Immediately before transformation, the DNA was denatured to produce ss genomes in 10 μL aliquots by heating at 100 °C for 2 min followed by rapid cooling on ice to prevent renaturation.

3. UV Treatment of E. coli Cells to Induce the SOS Response.

E. coli DL7 cells were exposed to UV to induce the SOS response (Lasko et al., 1988). Briefly, cells from an overnight culture were diluted 1:100 in LB medium (Maniatis et al., 1982) and grown to a density of approximately 1 x 10^8 cells/mL. Cells were harvested by centrifugation at 4 °C (8000g, 10 min), and resuspended in an equal volume of ice cold 10 mM $MgSO_4$. One-half of the suspension was kept on ice, while the other half was UV irradiated to induce the SOS response. The cells to be irradiated were aliquoted into 150 mm petri dishes, on ice, in 25 mL portions, and treated with UV at 50 J/m^2 using a 15 W General Electric germicidal lamp. UV fluence was measured with an Ultraviolet Products radiometer. Cells were plated on LB plates immediately before and after the UV treatment to determine cell survival. An equal volume of 2X LB was added to both cultures, which were then incubated for 40 min at 37 °C to allow for expression of the SOS response.

E. coli DL6 cells were UV treated using the same protocol with the following modifications: cells were resuspended in fresh LB rather than

$MgSO_4$, and treated with 12 J/m^2 of UV, after which the cells were diluted with an equal volume of LB and incubated at 37 °C for 20 min.

4. Introduction of DNA into Cells by Electroporation.

In each experiment, the 12T/T^T and Pt12T/T^T genomes, in either ss or ds form, were introduced into E. coli by the process of electroporation (Wood et al., 1990). After the above 40 min incubation, the cells were centrifuged at 4 °C in 100 mL aliquots at 8000g for 10 min, resuspended in 100 mL ice cold water, and recentrifuged for 15 min. The cells were then resuspended in 40 mL water and spun again for 15 min. The supernatant was carefully aspirated from the pellet, and the cells were resuspended in 200 μL glycerol/water (10% v/v). For each transformation, 40 μL of the cell suspension was added to 10 μL of the DNA solution and then transferred to the bottom of a cold 0.2 cm Bio-Rad Gene Pulser cuvette. Cells were electroporated in a Bio-Rad Gene Pulser set at 25 μF and 2.5 kV (untreated cells) or 1.6 kV (UV treated cells), with the Pulse Controller set at 200 ohms. Immediately following electroporation, 1 mL of SOC medium (Hanahan, 1985) was added to the cell suspension. The cells were then transferred to a culture tube and a portion of each transformation mixture was plated for infective centers within 20 min of the electroporation. Another aliquot of cells was plated on LB plates before and after electroporation to determine cell survival. The remainder was incubated at 37 °C for 1.5 hr, and then centrifuged at 15,000g for 10 min to remove the cells from the progeny phage in the supernatant. Both transformed cells and progeny phage were plated with GW5100 cells on B-broth plates in B-broth soft agar containing IPTG and X-gal (Messing, 1983).

5. Mutant Selection Protocol.

Mutation frequencies were determined from the numbers of mutant phage harbored in the progeny phage pool from each transformation. Selection of mutants was accomplished by taking advantage of the original placement of the adduct in a unique recognition site for the restriction endonuclease StuI. Base pair substitution mutants in the progeny phage pool were enriched by selection with StuI (Loechler, 1984) by virtue of the fact that they are refractory to cleavage by this enzyme (Figure 14). RF DNA was prepared from the progeny phage, digested with StuI (which removes progeny with the wild type sequence at the original site of adduction) and HindII (which removes contaminating parental M13mp18 DNA), and transformed by 42 °C heat shock into $CaCl_2$-treated E. coli MM294A cells (Maniatis, 1982). The transformed cells were allowed to grow at 37 °C in 1.0 ml LB for 1.5 hr following the heat shock treatment to produce progeny phage, which were isolated as described above. Rounds of selection with StuI and HindII were repeated 2-4 times. Single stranded DNA derived from individual blue plaques isolated in the last round was sequenced by the method of Sanger et al. (1977).

C. Results.

1. Plaque Heterogeneity Following Initial Transformations.

The singly adducted genomes, 12T/T^T and Pt12T/T^T, as ss DNA produced by pretreatment of the genomes with T4 endonuclease V, or as untreated duplex DNA, were introduced into E. coli DL7 cells, which are wild type with respect to DNA repair functions. The infective centers and progeny

phage from the initial transformations of both the adducted and control genomes had three distinct plaque color phenotypes: blue, light blue (0.1-1%), and colorless (or white; 0.1-1.5%).[1] DNA from individual plaques of each color, isolated from the progeny phage, was sequenced to determine the genotype. The blue plaque DNA was expected to be a mixture of wild-type M13-12A and the putative *cis*-DDP induced base pair substitution mutants. The blue plaques sequenced were uniformly dodecamer-containing wild type M13-12A; this was not surprising as the frequency of induced mutations was expected to be low. The white and light blue plaques were induced by the genetic engineering procedures used and their DNA displayed, upon sequencing, certain characteristic mutation types (Figure 15). The white plaque DNA displayed a heterogeneous group of mutations; these genomes were either parental M13mp18 DNA that had undergone 1-base and 2-base deletions in the HindII site, or dodecamer-containing M13-12A DNA that had 1-base or 2-base deletions in the former HindII site. All of the light blue plaques sequenced were M13mp18 DNA in which the C in the third position of the HindII recognition sequence (5'-$G_1T_2C_3G_4A_5C_6$-3') was deleted (Figure 15). The light blue plaques and the white plaques not containing a dodecamer sequence (those most closely resembling an M13mp18 sequence) are believed to have arisen from blunt end ligation of linear DNA that remained in the gapped heteroduplex preparation; the deletions may have resulted from a low level of exonuclease activity in the HindII enzyme preparation. The white plaques containing a dodecamer (those most closely resembling an

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1. Most progeny had the blue plaque color phenotype. The numbers in parentheses refer to the proportion of other phenotypes observed.

M13-12A sequence) probably arose from the ligation of dodecamers into gapped heteroduplexes produced between ss circles and "nibbled" linear ds DNA.

The specific base pair deletion seen in the light blue plaques resulted in an in frame *opal* TGA codon within the mutated HindII site in the *lac*Z alpha sequence (Figure 15). The light blue phenotype was surprising because the plating strain GW5100 is an *amber* suppressor, not an *opal* suppressor strain. The -1C deletion at the third position of the HindII recognition sequence should have resulted in a white plaque phenotype such as the similar -1G deletions at the fourth position of the HindII recognition sequence shown in Figure 15. Several light blue plaques were sequenced using a primer that enabled the entire sequence of the *lac*Z coding region to be determined; none of the plaques sequenced exhibited any other mutation. The light blue phenotype remained true after several rounds of plaque purification. A similar phenomenon has been observed by Dr. E. Loechler (personal communication) using a pUC18 vector in which a -2 deletion places an *opal* stop codon in frame in the *lac*Z gene and results in a light blue plaque phenotype in an *amber* suppressor plating strain. He has also isolated a mutant that has an in frame *opal* stop codon due to a base pair substitution mutation; this also exhibits a light blue plaque phenotype. I am unable at this time to provide an explanation for this phenomenon.

2. Determination of Mutation Frequency.

As described in the Methods section, mutant selection was accomplished by sequential rounds of restriction endonuclease digestion and E. coli transformation. The presence of the light blue plaques allowed for their use as an internal control during the rounds of mutant selection: they are genetically homogeneous, they are easily distinguishable from the other plaque colors, and they are not a substrate for either of the selective enzymes. Since the platinum adduct-induced mutants by definition are also not substrates for the selective enzymes, the ratio of mutant blue plaques to light blue plaques after the initial transformation should remain the same during the rounds of selection. Thus, the ratio determined after the last round of selection should be the same as the ratio before the selection was applied.

After the last round of selection, the numbers of blue and light blue plaques were quantitated to determine their relative frequencies. Single blue plaques were then isolated and ss DNA was prepared and sequenced to identify the mutation. Sufficient numbers of blue plaques (18-24) were sequenced to determine the relative proportion of mutants in the blue plaque population. To determine the ratio of mutant blue plaques to light blue plaques at this stage, the ratio:

[(the number of mutants identified in the blue plaque population by sequencing) / (the total number of blue plaques sequenced)]

was multiplied by the ratio:

[(the number of blue plaques after the last round) / (the number of light blue plaques after the last round)]

to give the ratio:

[(mutant blue plaques) / (total light blue plaques)].

Since this ratio holds for all stages of the analysis, it can be multiplied by the ratio:

[(the number of light blue plaques in the progeny phage) / (total number of plaques in the progeny phage)]

determined after the initial transformation, to yield the mutation frequency:

[(the number of mutant blue plaques in the progeny phage) / (the total number of plaques in the progeny phage)].

More succinctly, the mutation frequency of the adduct can be calculated by using the following equation:

$$MF = (M/B_{R_n}\text{sequenced}) \times (B_{R_n}/LB_{R_n}) \times (LB_{pp}/T_{pp})$$

where MF is mutation frequency in the progeny phage pool, R_n denotes the last round of selection, M is the number of adduct induced mutants in R_n (as determined by DNA sequencing), B_{R_n} is the total number of blue plaques sequenced in R_n (where B_{R_n} sequenced = M + genetic engineering induced mutations), B is the number of blue plaques, LB is the number of light blue

plaques, T is total phage number, and pp denotes the initial progeny phage pool, i.e., the population that has not yet undergone selection.

3. Mutagenesis by *cis*-[$Pt(NH_3)_2$(d(GpG))] in a Single Stranded Phage Genome.

Mutation frequencies for the *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct present in ss genomes were determined in E. coli DL7 cells either induced or not induced for the SOS response. The mutation frequency of the platinated ss vector in SOS-induced cells from two independent replicas within the one experiment (designated T128 by my notebook nomenclature) was 0.86% and 1.8%; $\bar{x}$ = 1.3%. The mutation frequencies of unadducted genomes or of the platinated genomes introduced into SOS-uninduced cells were an order of magnitude lower, which was close to the limit of sensitivity of the mutation analysis protocol (Table 2). The mutations fixed in SOS-induced cells exhibited a remarkable specificity (Figure 16): 89% of the mutations were G→T transversions at the 5'-adducted G.

4. Mutagenesis by *cis*-[$Pt(NH_3)_2$(d(GpG))] in a Double Stranded Phage Genome.

Mutation frequencies for the *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct present in ds genomes, in which the strand opposite the adduct contained inactivating lesions, were also determined in cells either induced or not induced for the SOS response. The 12T/T^T and Pt12T/T^T genomes (not pretreated with T4 endonuclease V) were transfected into SOS-induced and uninduced E. coli DL7 cells in three independent experiments. The mutation specificity data from all three experiments are in very close agreement with each other.

The mutation frequency data in the last experiment, however, is much lower than expected from the results of the first experiments. Although the last experiment was performed on a much larger scale, we have reason to believe that it is the anomalous outlier, as will be discussed in the next section.

The mutation frequency data obtained from two experiments in which SOS-induced and uninduced DL7 cells were transformed with the 12T/T^T and Pt12T/T^T genomes in ds form (by my notebook nomenclature, transformations T123 and T126) are shown in Table 2. These transformations were independent experiments using DNA derived from two separate ligations. The mutation frequencies obtained were 0.13% and 0.2%, respectively, for the site specifically platinated genome in SOS-induced cells. These values, however, are 5-10 fold lower than for the comparable ss genomes. As can be seen from comparing Figures 16 and 17, the mutations fixed in SOS-induced cells are remarkably similar: 84% (T123) and 82% (T126) of the total mutations in each experiment occurred at the 5′ adducted G and, of those, 78% and 100% (66% and 82% of the total, respectively) were G→T transversions, the same mutation identified when the adduct was present in ss DNA. The two other detectable base pair substitutions at this position were observed only in the first experiment, [G→C (12.5%), and G→A (6%)]. In only two cases, one in each experiment, was a mutation at the 3′-adducted G detected [G→C (3%, T123), and G→T (4%, T126)]. There were two examples of double mutations encompassing the 5′-adducted G and the A immediately 5′ to it: ApG→TpT (12.5%, T123), and ApG→CpT (4%, T126), and two of the mutants had base substitutions at the 5′-A [A→G (4%), A→T (4%), T126].

5. Survival of *cis*-DDP-Adducted Genomes.

Experiments were performed to determine the relative survival of the adducted and unadducted ds genomes in SOS-induced and uninduced DL7 cells. These genomes contained biologically inactivating thymine dimers in the strand opposite that of the adduct. Data were derived using vectors that had been made from freshly prepared gapped heteroduplexes. Survival of the site specifically platinated genomes compared to the unadducted controls was 22% in uninduced cells, and rose to 38% in the SOS-induced cells (each survival value was calculated on the basis of 23 separate transformations). Mutation frequencies were also determined for one experiment (T130), and those data are presented in Table 2. The mutation frequencies in all cases were an order of magnitude lower than those obtained in the previous experiments and, as can be seen, the level of platinum-induced mutagenesis was indistinguishable from that of the control genomes. However, the spectrum of mutations induced by the platinum adducted genomes in SOS-induced cells in this experiment (Figure 18) was virtually identical to that obtained in the previous experiments.

D. Discussion.

The results of my experiments show that a single *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ adduct, whether present in ss or ds DNA, is mutagenic in wild type SOS-induced E. coli cells. Further, they show that the induced mutations are most likely to occur at the 5′ adducted G, and that they are predominantly G→T transversions. In this section I shall discuss specific

aspects of this work: survival, mutation frequency, and mutation specificity with respect to other work in the field.

1. Survival of the Singly Adducted Genomes.

During the course of this work two studies of the survival of the singly platinated genomes have been conducted. The first was reported in Naser et al. (1988a), and the results of the second study are presented here. In the earlier experiments, the survival of the singly platinated ss genome, as compared to an unplatinated control genome, was 10-12% in E. coli DL7 cells uninduced for the SOS response. The adduct was situated in the M13 (+) strand and the genomes were prepared according to the protocol of Green et al. (1984). The survival data presented here were for single G^G adducts situated in the (-) strand of ds DNA, in which the opposite strand contained biologically inactivating UV-induced pyrimidine dimers. Survival was 22% in uninduced E. coli DL7 cells and rose to 38% in SOS-induced cells. The survival results for the platinated vectors in SOS-uninduced cells from this work and those previously reported are comparable, although the survival value was slightly higher in the more recent work. It must be kept in mind, however, that the adducts were present in two different genome forms.

The increase in genome survival in SOS-induced cells was expected based upon knowledge that *cis*-DDP is a replication blocking lesion and an SOS dependent mutagen. This phenomenon has been reported for single *cis-syn* and *trans-syn* cyclobutane dimers situated in a ss M13 genome (Banerjee et al., 1988; Banerjee et al., 1990). The increased survival is most

likely due to the increased bypass of the replication blocking lesions, albeit at the expense of replication fidelity. These survival data, however, were derived from experiments that included T130, in which the mutation frequency of the G^G adduct was suspiciously low; thus, it may be that the SOS state was not optimally induced in these cells, and that the 38% value represents at best a lower limit for SOS-induced increase in survival.

2. Mutation Frequency of *cis*-[$Pt(NH_3)_2$(d(GpG))].

The observed mutation frequency of the *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct present in a ss genome was, on average, 1.3%. This mutation frequency decreased to 0.13-0.2% when the *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct was present in a ds genome. These values are within the range typically observed in vivo for most other site specifically modified genomes. When ss vectors containing a single O^6-methylguanine adduct are transformed into wild type E. coli, the mutation frequency is 0.4% (Loechler et al, 1894), and this value decreases to 0.04% when the lesion was present in ds DNA (Essigmann et al., 1986).

An example of the effect of DNA repair on single adduct mutation frequency is also afforded by O^6-methylguanine. The mutation frequency of the single adduct present in duplex DNA increases ≈200-fold when the host cells are depleted of the proteins responsible for repairing this adduct (Essigmann et al., 1986). A parallel observation is made when the O^6-methylguanine adduct is present in ss DNA; the mutation frequency of 0.4% in repair proficient cells rises to ≈20% in repair deficient cells. These

results emphasize that DNA repair can have a profound effect on the mutation frequency of single adducts in site specifically modified DNA.

As discussed in Section IV, the mutation frequency of singly adducted ds genomes is very low and this has been shown to be due in part to a strand bias in replication (Koffel-Schwartz et al., 1987). The placement of thymine dimers in the strand opposite that containing the single *cis*-DDP adduct resulted in strand inactivation in that their presence prevented progeny from being produced from that strand. The presence of the thymine dimers in the genome, however, does not preclude recognition of the platinum adduct by repair enzymes. Several repair systems have been identified that act upon platinated DNA. *cis*-DDP-DNA adducts are known to be substrates for the uvrABC excision repair system (Sancar & Rupp, 1983; Beck et al., 1985). The effect of repair by this system on the ds adducted genomes may account for the lower mutation frequency observed in the ds vectors (ss DNA is not a substrate for the uvrABC excision nuclease complex; A. Sancar, personal communication). A second pathway, the mismatch repair system, has been implicated in the response of E. coli to *cis*-DDP adducts by the results of Fram et al. (1985). Survival and the rate of platinum excision from the genome of E. coli were both decreased in cells mutant in adenine methylase (*dam*$^-$), one of the genes involved in the mismatch repair pathway. The exact mechanism by which this repair system affects platinum genotoxicity is as yet unknown. Thus there are at least two systems in E. coli that are involved in the repair of *cis*-DDP adducts when present in duplex DNA. The inability of either of these systems to

act on ss DNA may account for the higher mutation frequency of the G^G adduct when present in a ss form.

In an attempt to determine the effect of repair on G^G mutagenesis, the 12T/T^T and Pt12T/T^T vectors in ds form (Figure 11) were introduced into SOS-induced and uninduced cells defective in excision repair by virtue of a missense mutation in the *uvrA* gene (DL6 cells; Lasko et al., 1988). In these experiments, no adduct induced mutations were detected, even in the SOS-induced cells. There have been reports that *uvrB* (Brouwer et al., 1981) and *uvrA* but not *uvrC* (Brouwer et al., 1988) are necessary for *cis*-DDP mutagenesis (see Chapter 2 for a more in depth discussion). No model has been proposed for the role these two gene products may play and, to my knowledge, no other genotoxic compounds have required the involvement of these two proteins for mutagenesis. In an effort to determine if *cis*-DDP adducts present in M13 DNA are mutable in *uvrA*$^-$ cells, mutagenesis studies are being carried out with randomly platinated M13 DNA transformed into various SOS-induced or uninduced *uvr*$^-$ cell lines (K. Yarema and W. Rowell, experiments in progress).

Recently, the mutation frequency and specificity of a single *cis*-[$Pt(NH_3)_2${d(ApG)}] adduct has been investigated by Burnouf et al. (1990). Results indicate that the mutation frequency of the adduct is 1-2% in SOS-induced cells when present in a ds genome containing inactivating UV-induced thymine dimers in the strand opposite the adduct. My result of a mutation frequency of 0.2% for the G^G adduct in ds DNA is five-fold lower. From the mutation spectrum of *cis*-DDP (Burnouf et al., 1987), it is concluded that the A^G adduct is five times more mutagenic than the G^G

adduct. My results are consistent with their prediction (i.e., 5 x 0.2% = 1.0%); however it is emphasized that the genetic effect of the A^G adduct was analyzed in a plasmid based system, whereas G^G was studied in a viral vector. The optimal comparison would require that the two adducts be evaluated in the same system.

3. Mutation Specificity of *cis*-[$Pt(NH_3)_2$(d(GpG))].

The results of my experiments indicate that mutagenesis by the *cis*-[$Pt(NH_3)_2$(d(GpG))] adduct of *cis*-DDP is dependent upon the induction of the bacterial SOS system. The induced mutations show a remarkably high degree of specificity: if the data from all experiments are combined, 87% of the induced mutations occurred at the 5' adducted G, and 82% of the induced mutations were specifically G→T transversions.

The results of A^G adduct directed mutagenesis (Burnouf et al., 1990) correlate almost perfectly with the results presented for the G^G adduct in this report: 90% of the A^G directed mutations occur at the 5'-A, and 80% of the total mutations are A→T transversions; the remainder are A→G transitions. Double mutations encompassing the adducted adenine and the base immediately 5' to it were also observed at a low frequency. The results from these site specific mutagenesis studies on G^G and A^G are also in agreement with the results from a forward mutation assay conducted by Burnouf et al. (1987) who conclude that *cis*-DDP directed mutations occur at ApG and GpG sequences, known sites of platinum binding, and that a strong preference is seen for mutations occurring at the 5' base at potential sites of adduction. Mutagenesis is observed only in cells

treated with UV to induce the SOS response. The results of all of these studies are in contrast with those of Brouwer et al. (1981) in which mutations were detected at GpXpG sequences; as discussed in Chapter 1 of this dissertation, however, there are major differences between the two systems that could account for the disparate results.

4. Interpretation of the Mutagenic Specificity of G^G within the Framework of Structural Information on this Adduct.

We are fortunate in that many studies have focused on defining the detailed architecture and physical properties of the G^G adduct. The binding of *cis*-DDP to DNA significantly perturbs the structure of DNA; when bound, the adduct shortens and unwinds the DNA helix (Cohen et al., 1979). The structural features of single platinum adducts have been investigated in sequences as small as dinucleotides and as large as 22-mers. X-ray diffraction studies of *cis*-[$Pt(NH_3)_2${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). 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). Gel electrophoretic mobility assays on homogeneous oligomers containing G^G or A^G adducts show that both adducts bend the helix by 32-35° (Bellon & Lippard, 1990) and, at least for the G^G adduct, this bend is in the direction of the major groove (Rice et al., 1988). NMR data indicate that the adducted guanines of the G^G adduct may still hydrogen bond with their complementary cytosines, albeit in a distorted

manner (den Hartog et al., 1984; van Hemelryck et al., 1984; den Hartog et al., 1985). Studies on the chemical reactivity of the bases surrounding a single platinum adducts also suggest that the guanines may still be base paired; however, the pattern of reactivity indicates that the helix is distorted in the area of the adduct, and that this distortion is more pronounced on the 5′ side of the adduct (Marrot & Leng, 1989; Schwartz et al., 1989; Anin & Leng, 1990).

The results of the site specific mutagenesis studies presented in this dissertation combined with those by Burnouf et al. (1990) support the view that the major structural distortion is on the 5′ side of the adduct. The majority of the mutations observed in both systems occurs at the 5′-adducted base. In the studies presented here for G^G, there were only two examples of mutations occurring at the 3′-G; there were no such mutations detected in the A^G work. Double mutations encompassing the 5′-adducted base and its immediate 5′-neighbor occurred in both systems at a low frequency (an average of 6% and 10% of the total induced mutations for the G^G and the A^G adducts, respectively). There were only two examples of mutations occurring exclusively at the base 5′ to the adduct in the G^G studies, and none in the A^G work. These data, taken together, are consistent with a model in which the 3′ base of the adduct is relatively unperturbed and, by virtue of the more flexible conformation of its sugar, it is able to stack with the base 3′ to it (see Figure 19). In this model, the 5′-adducted base is the one that is the most distorted from normal DNA structure; the occurances of double mutations suggest that this perturbation may extend to the nucleotide immediately 5′ to the adduct. No

mutations were in evidence in either study any further in the 5' direction from the adducts, suggesting that the DNA distortion does not extend beyond this base. In support of this model of localized perturbation, restriction endonuclease digestions of the G^G adducted genome demonstrate that the adduct is able to inhibit cleavage by enzymes whose recognition sequences overlap the site of adduction (StuI and MaeI) but have no effect on cleavage by enzymes whose recognition sequences are 4 or 9 bases from the site of adduction (Pinto et al., 1986).

The observation that the majority of the G^G and A^G induced mutations occurs at the 5'-adducted base suggests that the 3'-adducted G (the first base encountered by the polymerase during replication) is able to convey its genetic information correctly via the replication apparatus of the cell (Figure 19). As can be seen in Figure 2, reaction of *cis*-DDP occurs at the N7 position of purines and therefore does not disrupt the Watson-Crick base pairing regions of the bases. It is noteworthy that the monofunctional compound [Pt(dien)Cl]Cl, which binds to the N7 of guanine, is neither a block to replication nor mutagenic. Thus it is unlikely that the 5' mutations are due solely to the adduction at the N7 position, but are instead a consequence of the structural distortion of this base induced by the crosslink formation. Replication blockage, and subsequent mutagenesis, at this site could be the consequence of the bend induced between the planes of the two adducted bases, an altered orientation of the 5' adducted base, or both.

As stated previously, a block to replication is most likely the reason for the decreased survival of the singly platinated genomes compared to the

unplatinated controls. It is noteworthy, however, that there is a significant level of survival (22%) in cells not induced for the SOS response. In ds genomes, this level of survival could be attributable to repair of the adduct. Although studies designed to determine the survival of the ss adducted genomes were not carried out in a quantitative manner, qualitative results indicate that survival of these vectors is similar to the ds genomes and, in ss DNA, repair is less likely to be the reason for genome survival. It is possible that in cells not induced for SOS, the DNA polymerase is stalled at the bend induced by the platinum adduct and can then proceed when the DNA adopts a conformation that will allow for correct reading of the next base. In this regard, it is noteworthy that T_m studies on duplex oligonucleotides in which the 5' G of a G^G adduct in one strand is mispaired with the four different bases, show that the G*·C pairing is the most stable (Urata et al., 1990).

The SOS induction of the cells allows for greater lesion bypass, as evidenced by an increase in survival of the adducted genomes (38%), but this bypass occurs at the expense of fidelity of replication. G^G mutagenesis was seen only in SOS-induced cells, and the mutations were almost exclusively 5' G→T transversions. *cis*-DDP mutagenesis has been shown to be dependent upon the *umu*D,C gene products (Fram et al., 1985) and it is probably these proteins that play a significant role in the mutagenic bypass of the 5' adducted base in the G^G and A^G adducts.

The mutation specificity of the G^G and A^G adducts is consistent with the model of mutagenesis in which the E. coli polymerase inserts an adenine opposite a noninstructive lesion as its default option (Straus et al.,

1982; Schaaper et al., 1983). Random mutagenesis studies on DNA containing AP sites (Kunkel, 1984) show that 60% of the mutations result from the insertion of adenine across from the lesion. The frequency and spectrum of mutations arising from a single abasic site have recently been described (Lawrence et al., 1990). Single stranded M13 derivatives were constructed to contain a single abasic site at one of two positions. The abasic site is a true noninformational lesion in DNA and, as expected, adenine is the most frequent nucleotide incorporated opposite the site in SOS-induced cells: 50% and 77% for the two sites tested. The authors reason that if one assumes that a noninformational lesion will lead to adenine incorporation during replication 50-75% of the time, then any increase in this frequency should be due to some form of information presented by the lesion. Thus the high frequency of correct insertion of adenine opposite either a *cis-syn* or a *trans-syn* cyclobutane dimer in M13 DNA (94% and 89%, respectively; Banerjee et al., 1988; Banerjee et al., 1990) was attributed to the instructive character of the two types of cyclobutane dimers studied.

In the case of the single platinum adducts, if the template 5'-adducted base were a strictly noninformational lesion, one would expect that adenine insertion opposite the lesion to occur ≈60% of the time. The occurence of this mutation in 82% of the cases for the G^G adduct and 80% of the cases for the A^G adduct suggests that there may be other forces at work directing this mutation, and that these lesions can be termed misinformative rather than uninformative. This is not to say that a noninformational state of the lesion does not play a role in its

mutagenesis, but that it probably does not account for all of the mutations directed by these adducts.[2]

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2. The raw data used for calculating the survival values and mutation frequencies can be found in Appendix B.

Table 2.

Mutation Frequencies of a Single *cis*-[$Pt(NH_3)_2${d(GpG)}] Adduct in Single Stranded and Double Stranded Vectors

		Mutation Frequencies (%)			
		12T/T^T[b]		Pt12T/T^T[b]	
Experiment	ds/ss[a]	-SOS	+SOS	-SOS	+SOS
T128	ss	<0.02	<0.04	<0.01	1.81
			<0.03	<0.01	0.86
T123	ds	n.d.[c]	n.d.	<0.001	0.11
					0.31
T126	ds	n.d.	0.03	n.d.	0.21
					0.20
T130	ds	<0.002	0.01	0.03	0.03

[a] ds indicated genomes were transformed into cells as double stranded DNA containing UV induced photoproducts in the strand opposite that containing the adduct; ss indicates genomes were treated with T4 endonuclease V and transformed into cells as single stranded DNA

[b] Genomes are as defined in Figure 3

[c] Not determined

Figure 14. Outline of the steps involved in the sequential rounds of mutant selection.

Enrichment of Mutant Phage from the Progeny Phage Pool

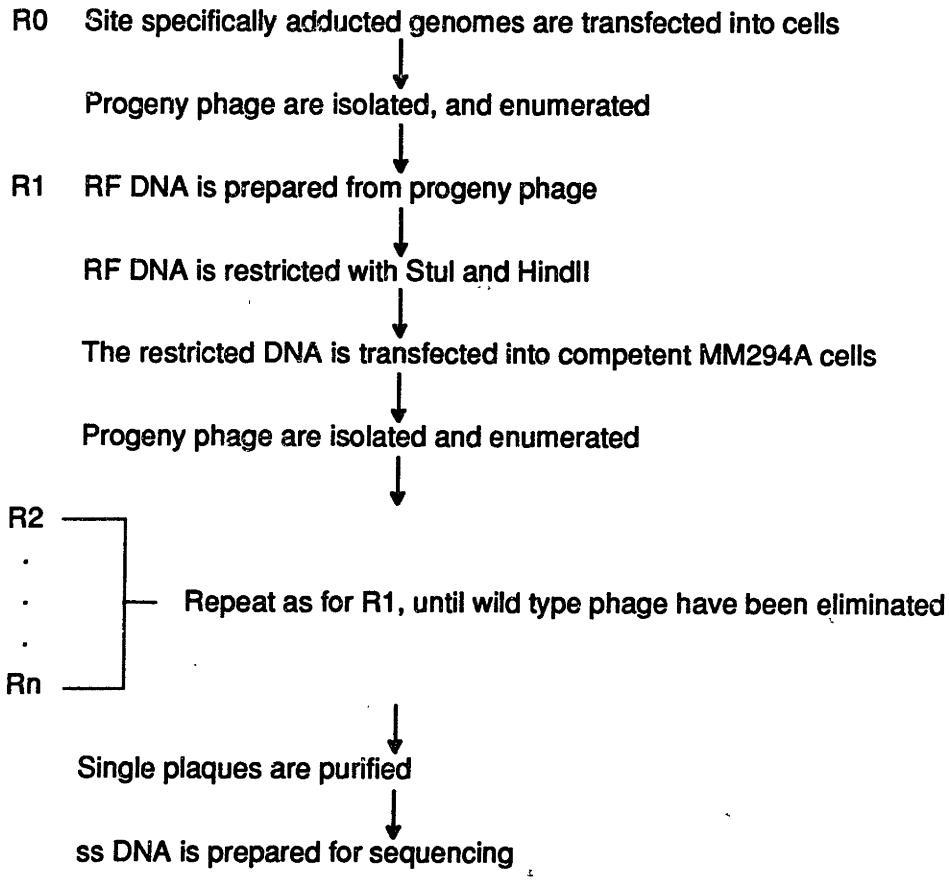


Figure 15. Genetic engineering induced mutations. The sequences shown are of the (+) strand of the HindII recognition sequence in M13mp18 (top), the (+) strand of the isolated mutants in the vicinity of the HindII site (middle), and the (+) strand of M13-12A in the vicinity of the dodecamer insert, located within the former HindII site of M13mp18 (bottom). The left column describes the phenotype of the phage harboring each sequence. Horizontal bars mark the site of restriction endonuclease recognition sequences and the dodecamer insert, as indicated; vertical bars indicate codon usage in the *Lac*Z gene; numbers indicate the positions of the bases in the HindII recognition sequence; (-), indicates deletion; __, indicates the site of a deleted base.

GENETIC ENGINEERING INDUCED MUTATIONS

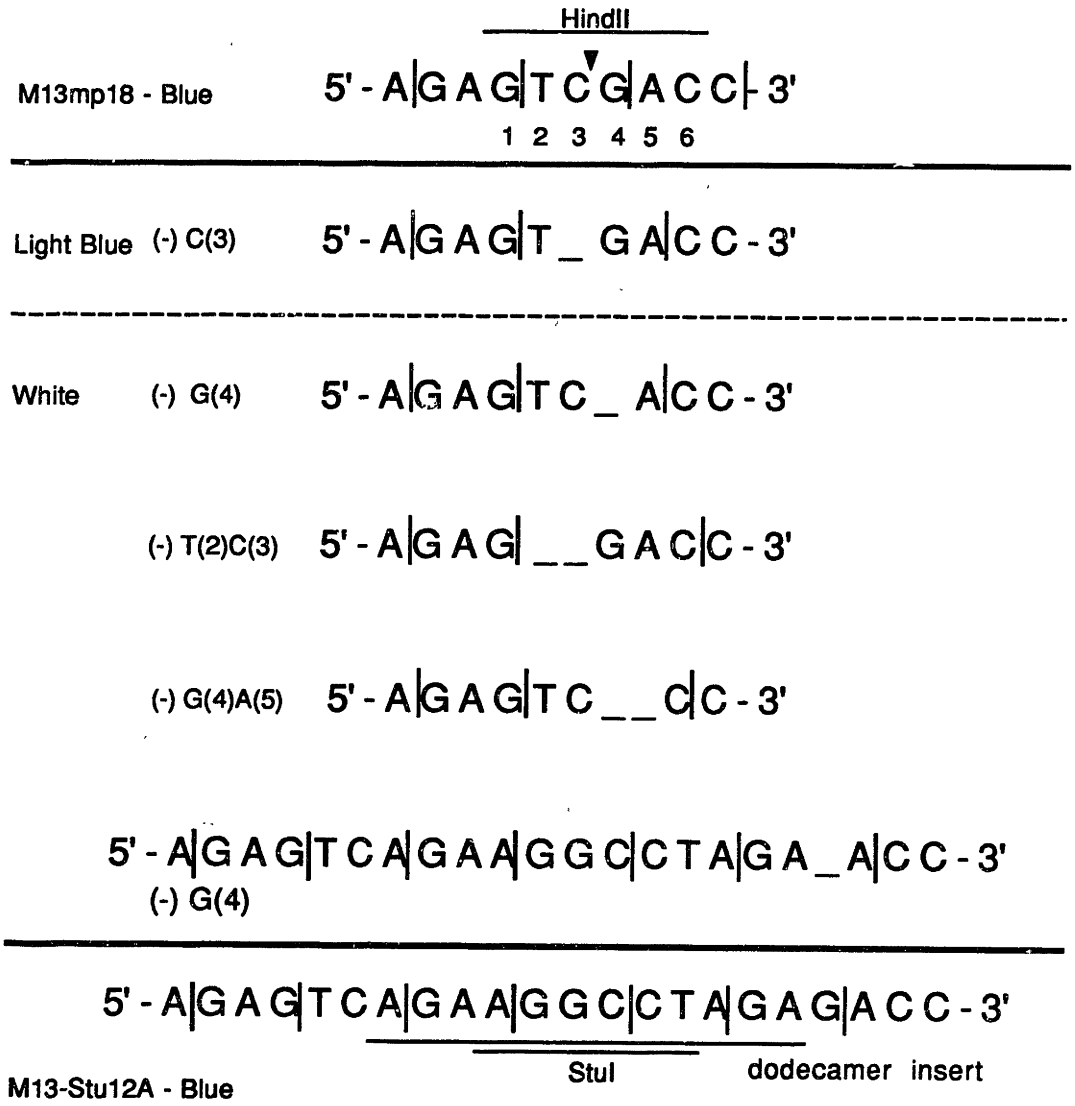


Figure 16. Specificity and frequency distribution of mutations of the major DNA adduct of *cis*-DDP in single strand DNA. The sequence indicated is the recognition sequence of StuI in the singly adducted (-) strand genome. *, data of two separate transformations combined; ^, indicates the site of the adduct; #, indicates the number of each mutation over total number of mutant blue plaques sequenced after the last round of selection; %, indicates the percentage of each type of mutation with respect to the total number of mutations; horizontal bars indicate that the displayed mutation was not observed.

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

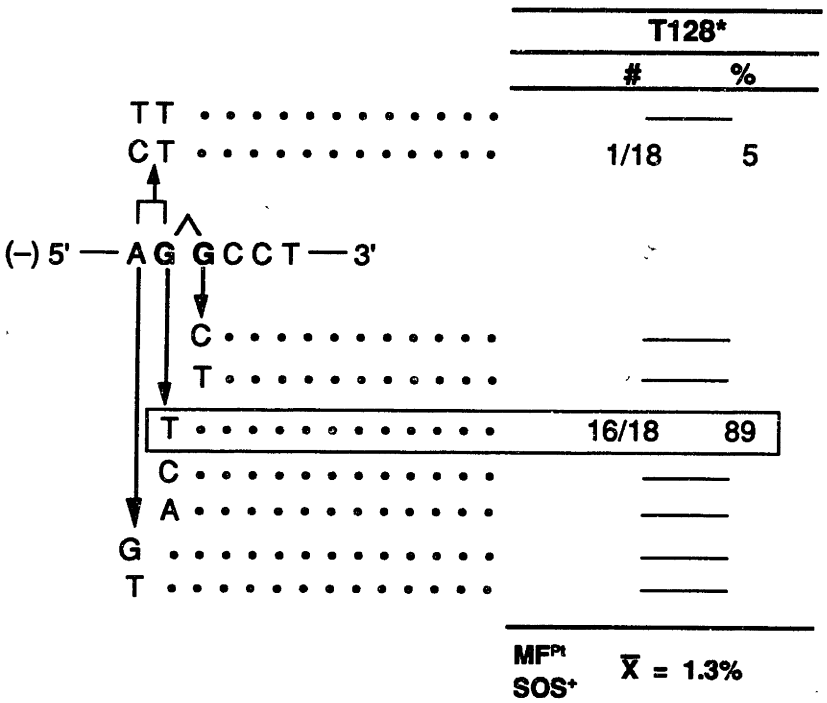
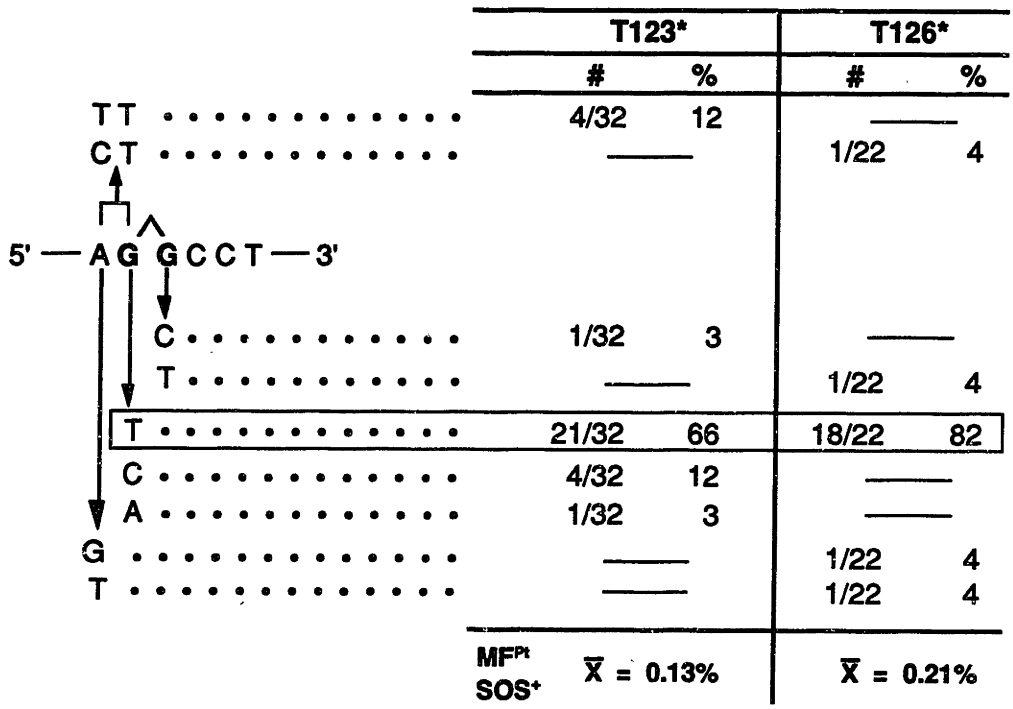


Figure 17. Specificity and frequency distribution of mutations of the major DNA adduct of *cis*-DDP in double strand DNA. Symbols are the same as described for Figure 16.

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



	T123*		T126*	
	#	%	#	%
TT	4/32	12	———	
CT	———		1/22	4
C	1/32	3	———	
T	———		1/22	4
T	21/32	66	18/22	82
C	4/32	12	———	
A	1/32	3	———	
G	———		1/22	4
T	———		1/22	4
MF^{Pt} SOS^{+}	$\bar{X}$ = 0.13%		$\bar{X}$ = 0.21%	

Figure 18. Specificity and frequency distribution of mutations of the major DNA adduct of *cis*-DDP in double strand DNA. Symbols are the same as described for Figure 16.

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

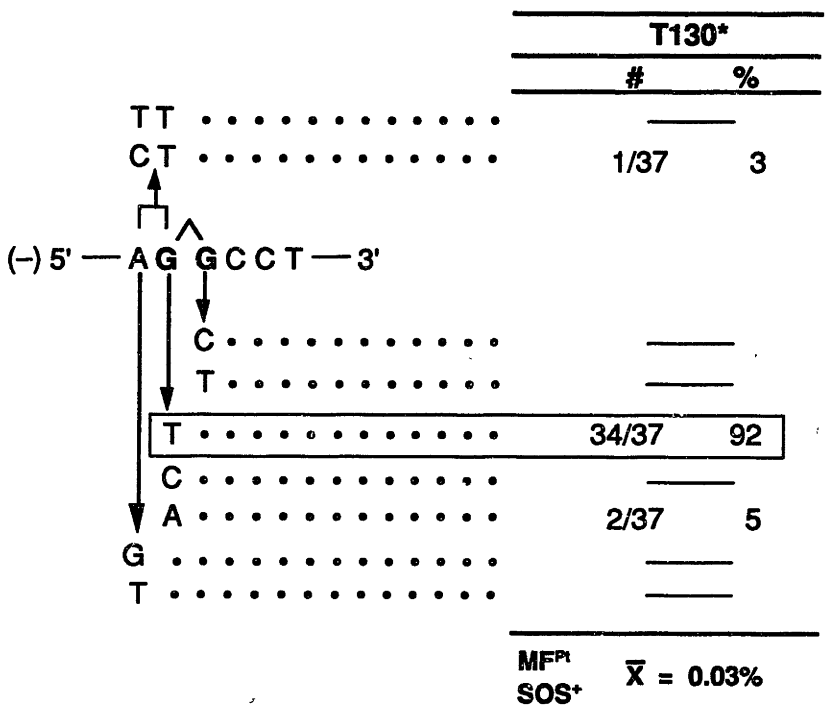
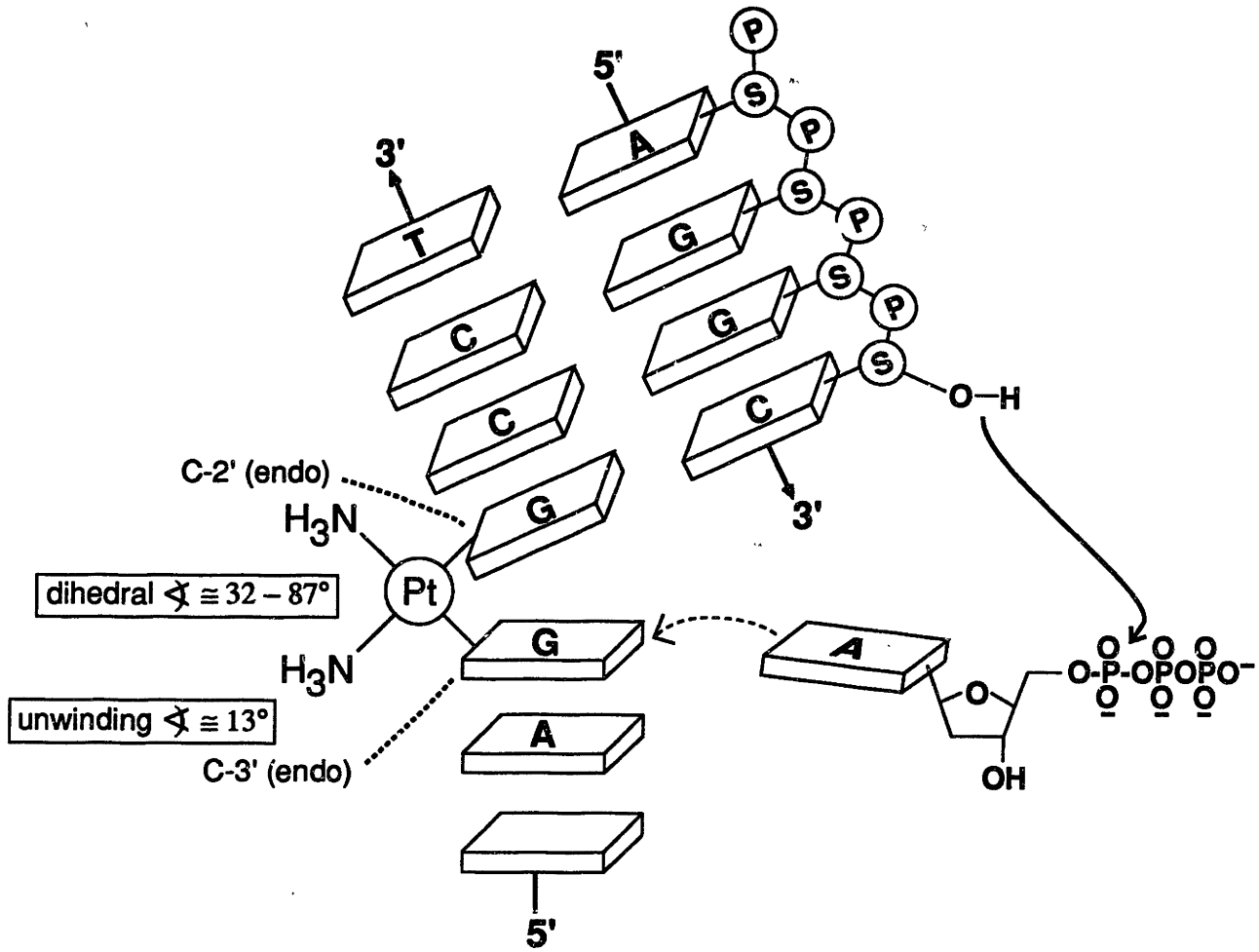


Figure 19. Schematic of replication bypass of the G^G adduct. Dihedral angle values from Bellon & Lippard (1990) and Sherman et al. (1985). Unwinding angle value for the G^G adduct from S. F. Bellon, J. H. Coleman, & S. J. Lippard, manuscript in preparation.


5'
3'
A
T
C
C
C
G
G
G
G
C
P
S
O–H
C-2' (endo)
H3N
Pt
H3N
dihedral ∢ ≅ 32 – 87°
unwinding ∢ ≅ 13°
C-3' (endo)
A
O
OH
O-P-OPOPO⁻
5'

VI. Concluding Remarks and Suggestions for Future Research.

The goal of the work described in this dissertation has been to determine the mutation specificity and frequency of the major DNA adduct of the antitumor drug *cis*-DDP, namely the *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand crosslink. To acheive this goal, a method was developed for the insertion of a site-specifically adducted oligonucleotide of any sequence and reasonable length into a viral genome containing a gap of equal size opposite a complementary sequence. The unique feature of this method is that the site of adduct placement is not restricted to sequences already present in the vector genome, thus enabling the genetic fate of adducts to be studied in any desired sequence context. This methodology was employed to create ss and ds site specifically modified M13-derived genomes containing a single *cis*-DDP G^G adduct. The results presented in this thesis show that a single G^G adduct, whether present in ss or ds DNA, is mutagenic in wild type SOS-induced E. coli cells. Further, they show that the induced mutations are most likely to occur at the 5' adducted G and are predominantly G→T transversions.

Based on the information presented in this dissertation, the following suggestions for future research are offered:

1. This work has resulted in the construction of an M13 based vector containing a single G^G crosslink, the major adduct of the antitumor drug *cis*-DDP, and the use of the vector to determine the genotoxicity and mutagenic frequency and specificty of the adduct. The next logical step

would be to use this system to investigate the mutagenicity and genotoxicity of the other adducts formed by reaction of *cis*-DDP with DNA; specifically the A^G and GXG intrastrand crosslinks, and the interstrand crosslink. Dodecamer sequences have been synthesized to contain the A^G and GXG adducts (by Dr. Burstyn and Kenneth Comess of the Lippard laboratory), and I have constructed the insertion genomes necessary to accomodate these adducted oligomers. Kevin Yarema of this laboratory has already begun the work on this project. By comparing the results of the survival and mutagenicity assays of the various adducts in the same system, it may be possible to rank order the adducts with respect to their killing potential and with respect to their mutagenic potential. The results of this ordering may make it possible to design a more effective drug; i.e., one that would minimize the frequency of mutagenic adducts while maximizing the frequency of lethal lesions.

2. Excision repair, post replication mismatch repair, and recombinational repair have all been implicated in the bacterial response to *cis*-DDP damage to the genome. The study of genotoxicity and mutagenicity of each of the individual adducts in bacterial cell strains defective in these forms of DNA repair would be useful for elucidating both the mechanism of repair of *cis*-DDP adducts and the effect that each type of repair has on modulating the overall genotoxicity and mutagenicity of *cis*-DDP-DNA adducts.

3. Workers in this laboratory have developed shuttle vector systems suitable for studying specific DNA lesions in mammalian cells (Ellison et al., 1989). These vectors could be adapted to study the individual adducts

of *cis*-DDP in mammalian cells that are wild type or defective in various forms of DNA repair.

4. As mentioned in the literature survey, a damage recognition protein has recently been identified in human cells that recognizes DNA treated with *cis*-DDP, specifically the G^G and A^G adducts. A gene presumably encoding this factor has been cloned. At present, the role that this protein plays in the cell is unknown. One method of studying its function would be to express the protein in bacterial cells and, using the site specifically platinated vectors, determine what effect if any its presence has on modulating the genotoxic and mutagenic effects of the individual adducts.

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Appendix A

Chapter IV of this dissertation presents a protocol employed for producing singly adducted single stranded genomes. Two other methods were also attempted, without success, and are described here for informational purposes.

The goal was to produce a gapped heteroduplex molecule that contained a nick in the strand opposite that containing the gap. Upon complete ligation of an adducted oligonucleotide into the gap (i.e., ligation occurring at both the 5′ and 3′ termini of the oligomer), a nicked circular genome (form II) would be produced. Heat or formamide denaturation, or exonucleolytic digestion, of this genome would result in a biologically active circular ss genome that could then be introduced into cells for processing. The duplex genomes described in Chapter III were unsuitable for use because they did not contain a nick in the strand opposite that harboring the adduct (Figure 7), and no method was available to introduce a nick solely, and quantitatively, into that strand. It must be reiterated here that <u>all</u> of the strands opposite that harboring the adduct must contain a nick; if not, the unnicked strand would give rise to wild type progeny whose presence would interfere with the survival and mutation frequency calculations.

The first method attempted was essentially that described by Green et al. (1984, Figure 20), and employed to produce the genomes used to obtain the survival data presented in Naser et al. (1988a). This method involved forming a heteroduplex between the ds form of the parent molecule M13mp18

linearized with HindII, and the ds form of the insert genome M13-12T linearized with BglII, and treated with calf intestinal phosphatase (CIP). As shown in Figure 20, due to the nonself-complementary nature of the dodecanucleotide sequence, only half of the gapped duplexes produced by this method can accomodate the adducted dodecanucleotide (i.e., those with a gap in the (+) strand). Due to the CIP treatment of the M13-12T derived DNA, a non-ligatable nick is present in the strand opposite that containing the gap; the nick prevents recircularization of this strand during ligation of the dodecanucleotide into the gap. Unfortunately, the gapped duplexes obtained by this method were very unstable, and virtually all of them reverted to linear material after only 24 hr. This has not been the case with gapped duplexes containing smaller sized gaps, i.e., four bases (Green et al., 1984), five bases (Wood et al., 1990), or six bases (Basu et al, 1987) in length. The basis of this instability is not understood but it may be attributable to the large gap size of twelve base units.

The second method, shown in Figure 21, was developed to take advantage of the formamide method of gapped heteroduplex formation presented in Chapter III. This method involved producing a ss linear M13mp18 genome by annealing a 30-base oligonucleotide complementary to the cloning region of the genome and treating the partial duplex with the restriction endonuclease HindII, whose recognition site was centrally located within the duplex region. This product was then mixed with BglII linearized, CIP treated duplex M13-12A in a 20:1 ratio of M13mp18(+) strand to M13-12A (-) strand. Gapped duplexes were produced by this method, but in a very low yield. Most of the structures produced were linear multimers.

Thus, the gap size was apparently too large to allow for stable gapped duplex production by the first method, and the heteroduplexing efficiency was too low by using the second method. Fortunately a third method presented in Chapter IV was successful, and is recommended for the high-yielding production of heteroduplexes containing large gaps.

Figure 20. Scheme for gapped heteroduplex synthesis by the heating method of Green et al. (1984) and subsequent ligation of the adducted oligonucleotide to produce a site-specifically platinated genome harboring the adduct in the (+) strand and a nonligatable nick in the opposite strand.

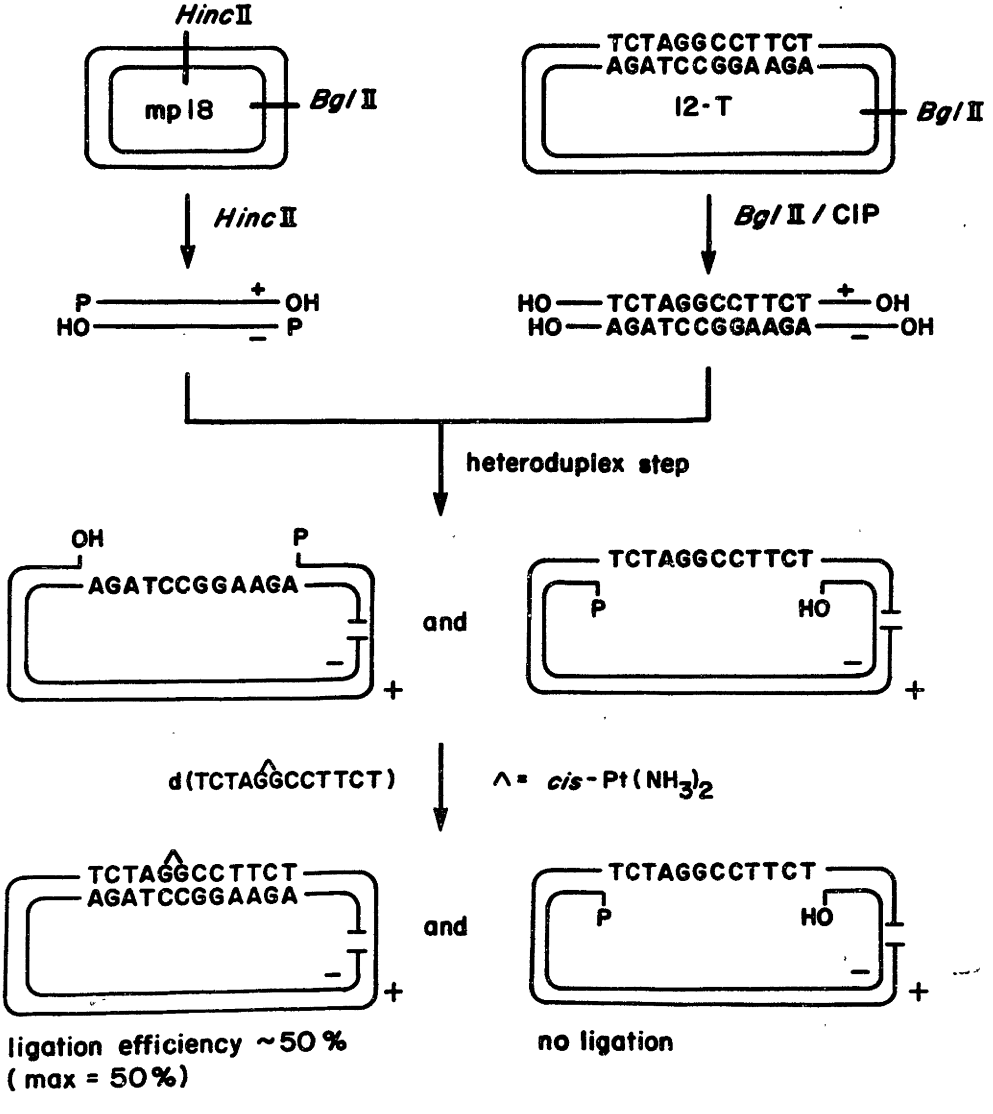
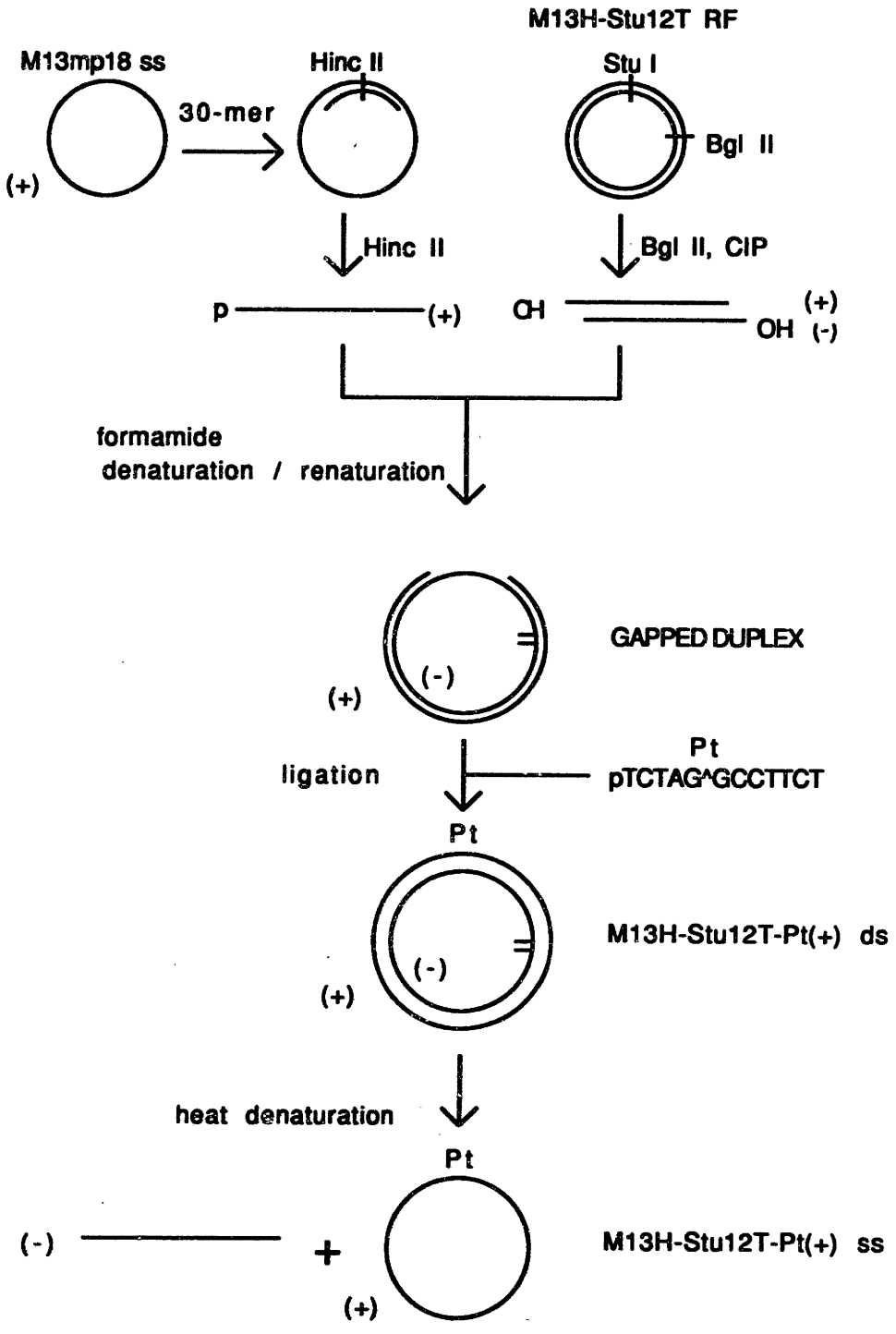

HincII
mp18
BglII
HincII
P
OH
HO
P
TCTAGGCCTTCT
AGATCCGGAAGA
12-T
BglII
BglII / CIP
HO
TCTAGGCCTTCT
OH
HO
AGATCCGGAAGA
OH
heteroduplex step
OH
P
AGATCCGGAAGA
and
TCTAGGCCTTCT
P
HO
d(TCTAGGCCTTCT)
^ = cis-Pt(NH3)2
TCTAGGCCTTCT
AGATCCGGAAGA
and
TCTAGGCCTTCT
P
HO
ligation efficiency ~50 %
(max = 50%)
no ligation

Figure 21. Scheme for gapped heteroduplex synthesis utilizing a ss linear (+) strand M13mp18 genome and a ds linear M13-12T genome to produce a site-specifically modified genome harboring the adduct in the (+) strand and a nonligatable nick in the opposite strand.


M13H-Stu12T RF
M13mp18 ss
Hinc II
Stu I
30-mer
(+)
Bgl II
Hinc II
Bgl II, CIP
p
(+)
OH
(+)
OH
(-)
formamide
denaturation / renaturation
GAPPED DUPLEX
(-)
(+)
Pt
ligation
pTCTAG^GCCTTCT
Pt
M13H-Stu12T-Pt(+) ds
(-)
(+)
heat denaturation
Pt
(-)
+
M13H-Stu12T-Pt(+) ss
(+)

Appendix B

On the following pages are the data, in table form, from which the survival and mutation frequency values were calculated in Chapter V.

Table 3.

Survival Data for the Genomes 12T/T^T and Pt/T^T Transformed into *E.coli* DL7 Cells Induced, or Not, for the SOS Response

Genome	SOS[a]	#[b]	TXF/ml ($\bar{x}$)[c]	% Survival[d]	% Survival ($\bar{x}$)
12T/T^T[e]	(+)	6	2.32 (± 0.19) x 10^6		
Pt/T^T	(+)	6	7.76 (± 0.62) x 10^5	33.4 (± 3.8)	
12T/T^T	(+)	5	1.28 (± 0.14) x 10^6		
Pt/T^T	(+)	6	5.32 (± 0.70) x 10^5	41.6 (± 7.1)	37.5 (± 5.6)
12T/T^T	(-)	8	2.48 (± 0.24) x 10^6		
Pt/T^T	(-)	7	5.08 (± 1.09) x 10^5	20.5 (± 4.8)	
12T/T^T	(-)	2	2.30 (± 0.32) x 10^6		
Pt/T^T	(-)	2	5.14 (± 0.73) x 10^5	22.4 (± 4.4)	
12T/T^T	(-)	2	1.50 (± 0.14) x 10^6		
Pt/T^T	(-)	2	3.66 (± 0.08) x 10^5	24.4 (± 2.3)	22.4 (± 4.4)

a (+) indicates cells were induced for SOS functions, (-) indicates they were not.

b # indicates the number of separate transformations used to obtain the values in the next column.

c txf/ml indicates the number of infective centers produced per ml of transformation mixture.

d % survival of the platinated genome with respect to the unplatinated control.

e genome pairs indicate transformations performed with the same cells on the same day.

Table 4.

Mutation Frequency Data - T128[a]

			1	2	3	4
Genome	SOS	Replica	mutant[b] / blue	blue[b] / light blue	light blue[c] / blue	M.F.(%)
12T/T^T -ss	(-)	1	<1/12	96/12	1/2954	<0.02
		2	n.d.			
	(+)	1	1/12	99/16	2/2736	0.04
		2	1/11	67/27	5/4069	0.03
Pt/T^T -ss	(-)	1	<1/12	59/1	<1/2457	<0.01
		2	<1/12	92/44	<1/2328	<0.01
	(+)	1	11/12	234/22	6/3231	1.81
		2	7/12	88/15	5/2539	0.86

[a] M.F. (%) value in column 4 is derived by multiplying the column values 1 x 2 x 3 by 100.

[b] numbers determined after last round of selection (Rn).

[c] numbers determined before rounds of selection were applied (Ro).

Table 5.

Mutation Frequency Data - T123[a]

Genome	SOS	Replica	mutant / blue	blue / light blue	light blue / blue	M.F.(%)
12T/T^T -ds	(-)		n.d.			
	(+)		n.d.			
Pt/T^T -ds	(-)	1	<1/16	16/356	9/1544	<0.001
	(+)	1	14/15	38/176	7/1313	0.11
		2	18/19	58/256	4/1278	0.31

[a] headings as described in Table 4.

Table 6.

Mutation Frequency Data - T126[a]

			1	2	3	4
Genome	SOS	Replica	mutant / blue	blue / light blue	light blue / blue	M.F.(%)
12T/T^T -ds	(-)		n.d.			
	(+)	1	3/12[b]	20/69	20/5141	0.03
Pt/T^T -ds	(-)		n.d.			
	(+)	1	9/12[b] 10/12[c]	30/75	14/2013	0.21[b] 0.23[c]
		2	10/12[b] 12/12[c]	85/129	13/3510	0.20[b] 0.24[c]

[a] headings as described in Table 4.

[b] values for 5' G→T transversions only.

[a] values for all site directed mutations combined.

Table 7.

Mutation Frequency Data - T130[a]

			1	2	3	4
Genome	SOS	Replica	mutant / blue	blue / light blue	light blue / blue	M.F.(%)
12T/T^T -ds	(-)	1	3/11	25/121	23/6529	0.02
		2	<1/12	5/58	17/5225	<0.002
	(+)	1	1/12	24/19	13/4543	0.03
		2	2/12	15/96	16/4241	0.01
Pt/T^T -ds	(-)	1	<1/24	92/268	31/7706	0.006
		2	3/23	220/338	23/6778	0.03
	(+)	1	20/24	63/270	11/7021	0.03
		2	18/24	39/301	18/5324	0.03

[a] headings as described in Table 4.

Biographical Sketch

I was born Lisa Jolene Naser on 1 April 1957, in San Jose, CA, the daughter of Madeline Meltvedt and Delbert Merlin Naser. I was graduated from Fox Chapel Area High School, Pittsburgh, PA, in 1975 and from the University of Idaho, Moscow, ID, in 1983, where I earned a B.S. degree *suma cum laude* in Zoology and Chemistry. In between, I attended the College of William & Mary, in Williamsburg, VA, and then moved to Idaho where I held various jobs with the U. S. Fish & Wildlife Service, the U. S. Bureau of Reclamation, and the Idaho Department of Fish & Game. I entered the Division of Toxicology in the Massachusetts Institute of Technology as a graduate student in the laboratory of my thesis advisor, Professor John M. Essigmann, in September of 1983. I was married to my longtime friend and companion, Terence Michael Bradley, in October of 1990. I received my Ph.D. degree in Toxicology in the spring of 1991.