Transcription and Mismatch Repair in the Mechanism of Action of the Anticancer Drug Cisplatin

by

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ABSTRACT

cis-Diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) is a powerful cytotoxin and anticancer therapeutic, used most effectively in the treatment of testicular and ovarian cancers. By contrast, the geometric isomer of cisplatin, *trans*-DDP, is comparatively non-toxic and fails to show significant antitumor activity. Cisplatin is believed to derive its cytotoxic effects from processes triggered by its reaction with DNA. The formation of cisplatin adducts can elicit many cellular responses, including inhibition of both DNA replication and transcription. Cisplatin DNA adducts are also specifically recognized by various proteins within the cell, and such cisplatin-damage recognition proteins have been previously suggested to play a role in the clinical efficacy of the drug. To date, however, the precise mechanism by which cisplatin lesions mediate the cytotoxic and antitumor activities of cisplatin remains elusive. The work in this dissertation evaluated two possible mechanisms by which cisplatin might exert its cytotoxic effects that had been heretofore largely unexplored.

The first aspect of this work evaluated a model wherein the differential cytotoxic and antitumor activities of cisplatin and *trans*-DDP may result from a greater ability of cisplatin DNA damage to inhibit RNA transcription. A nonreplicating plasmid harboring the β-galactosidase (β-gal) reporter gene was modified *in vitro* with either of the two platinum compounds and transfected into human or hamster cell lines. The use of cell lines both proficient and deficient in nucleotide excision repair allowed the examination of transcriptional bypass independent of excision repair for each platinum compound. A two to three fold higher level of transcription was observed in both cell lines from plasmids containing *trans*-DDP adducts as compared to plasmids modified by *cis*-DDP. This difference in transcriptional activity was not decreased in human and rodent nucleotide excision repair deficient cell lines, indicating that more efficient excision repair of the *trans*-DDP adducts was not the cause of its lower ability to block transcription. The

possibility that *trans*-DDP lesions are preferentially bypassed by RNA polymerase was examined by monitoring the elongation of β-gal mRNA on damaged templates *in vivo*. Nascent β-gal mRNA transcripts were recovered from nucleotide excision repair deficient xeroderma pigmentosum A cells transfected with platinated plasmids, and the extent of RNA synthesis was measured by using ribonuclease protection. The results showed that four-fold more *trans*-DDP than *cis*-DDP adducts were required to inhibit transcription elongation by 63%. RNA polymerase II translocated past a single, representative DNA adduct of cisplatin and *trans*-DDP *in vivo* with an efficiency of 0-16% and 60-76%, respectively. These data support the view that inhibition of transcription may contribute to the greater cytotoxicity of *cis*-DDP compared with its trans isomer.

The second aspect of this work evaluated a possible novel role of the human mismatch repair protein, hMSH2, as a cisplatin-damage recognition protein. The interaction of purified recombinant hMSH2 with DNA containing adducts of cisplatin and various cisplatin analogs was examined *in vitro* by using an electrophoretic gel mobility shift assay. The results showed that hMSH2 recognizes and binds specifically to DNA adducts of cisplatin. This protein displayed affinity for DNA modified by therapeutically effective platinum complexes, but not for that modified by clinically inactive platinum compounds such as *trans*-DDP. Recognition by hMSH2 was dictated, in part, by the major intrastrand DNA adduct formed by cisplatin. The results also show that hMSH2 is overexpressed in testicular and ovarian tissue, tissues in which tumors are best treated by cisplatin. These results complement a growing body of literature correlating mismatch repair activity with cisplatin toxicity in *Escherichia coli* and mammalian cells. Viewed together, these observations are consistent with a model whereby mismatch repair plays an active role in potentiating cisplatin DNA lesion toxicity. Further, these results may provide insight into a previously undiscovered mechanism by which tumor cells may acquire resistance to cisplatin.

Thesis supervisor: Professor John M. Essigmann

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bp	base pair(s)
β-gal	β-galactosidase
βG3	antisense RNA probe complementary to sequence located at 3′ end of β-gal mRNA
βG5	antisense RNA probe complementary to sequence located 1604 bp upstream of 3′ end of β-gal mRNA
CAT	chloramphenicol acetyltransferase
CHO	Chinese hamster ovary
cis-DDP	*cis*-diamminedichloroplatinum(II)
CMV	cytomegalovirus
CS	Cockayne's syndrome
D_0	platinum adducts per genome that reduce the percentage of β-gal enzyme activity or mRNA by 63% along the exponential portion of the dose response curve
EN	$[Pt(en)Cl_2]$
en	ethylenediamine
ERCC	excision repair cross complementing
DIEN	$[Pt(dien)Cl]^+$
dien	diethylenetriamine
HMG	high mobility group
hMutSα	heterodimer of hMSH2 and GTBP/p160
hMutSβ	heterodimer of hMSH2 and hMSH3
HSSB	human single-stranded binding protein
HPV	human papillomavirus
hUBF	human upstream binding factor
LEF-1	lymphoid enhancing factor
LTR	long terminal repeat
MEM	minimal essential medium
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
mtTFA	mitochondrial transcription factor
NMR	nuclear magnetic resonance
O^6-MeGua	O^6-methylguanine
O^4-MeT	O^4-methylthymine
PCNA	proliferating cell nuclear antigen
r_b	measured platinum-to-nucleotide ratio
SRY	the testis-determining factor encoded by the sex-determining region on the Y chromosome (*SRY*)
SSRP1	structure-specific recognition protein 1
SV40	simian virus 40
TCRF	transcription-repair coupling factor
trans-DDP	*trans*-diamminedichloroplatinum(II)

XPA	xeroderma pigmentosum complementation group A.
1,2-d(GpG)	*cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7(1),-*N*7(2)\}]
1,2-d(ApG)	*cis*-[$Pt(NH_3)_2$\{d(ApG)-*N*7(1),-*N*7(2)\}]
1,3-d(GpNpG)	*cis*- or *trans*-[$Pt(NH_3)_2$\{d(GpNpG)-*N*7(1),-*N*7(3)\}] (where N = any nucleotide)

I. INTRODUCTION

The coordination complex *cis*-diamminedichloroplatinum(II) (cisplatin or *cis*-DDP) was first synthesized in 1845, and is a classic example of compounds having a square planar structure (Lippard, 1982) (Figure 1). The biological effects of platinum complexes were discovered serendipitously by Barnett Rosenberg in 1965 during his experiments examining the effects of electrical fields on bacteria (Rosenberg et al., 1965). Rosenberg observed that growing *Escherichia coli* between charged platinum electrodes resulted in inhibition of cell division, but not cell growth. One of the agents responsible for this effect was found to be *cis*-DDP, which had been formed in the course of the experiments by electrolysis at the electrodes. The marked effects of cisplatin on bacterial cell division suggested that cisplatin could have potential value as an anticancer agent. Soon thereafter cisplatin was shown to have significant antitumor activity against sarcoma 180 and leukemia L1210 in mice (Rosenberg et al., 1969). Interestingly, the geometric isomer of cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP) (Figure 1), displayed no antitumor activity. Since cisplatin received FDA approval in 1979, it has proved remarkably successful as an anticancer therapeutic.

Testicular cancer has a relatively minor incidence of approximately 4.5 in 100,000 males and represents only a small fraction ($\sim$ 0.6%) of all new cancer cases in a given year (Feuer et al., 1993). Over 6,000 men in the United States, generally between the ages of 20 and 44, are diagnosed with the disease each year. Since 1973, significant improvements in survival rates for testicular cancer have led to an

overall 65% decline in mortality rate despite a 49% increase in incidence over the same time period (Feuer et al., 1993). This marked increase in cure rate has been a direct extension of the successful use of cisplatin-based chemotherapeutic regiments (Loehrer and Einhorn, 1984). Indeed, before the incorporation of cisplatin into combined therapies with vinblastine and bleomycin, few patients with advanced nonseminomatous testicular cancer were expected to survive beyond 1-2 years. According to recent estimates, testicular cancers are now 96% curable by a combination of surgery followed by chemotherapy with a regimen involving cisplatin as the principal cytotoxic agent (Feuer et al., 1993). In addition to its success in treating testicular cancer, cisplatin also displays significant activity against cancers of the ovary, cervix, bladder, lung, head and neck.

Few cytotoxic drugs are adequately selective for tumor cells, while sparing normal tissues, and cisplatin is no exception; treatment-related side effects include severe gastrointestinal toxicity, renal failure, hearing loss and peripheral nerve damage (Loehrer and Einhorn, 1984). Some of these effects have been partially alleviated by pre-hydration of patients with physiological saline and mannitol before administering cisplatin, as well as through careful dosage control (Loehrer and Einhorn, 1984). Despite such improvements, the remaining drawbacks to cisplatin therapy, including the side effects still associated with cisplatin treatment, its limited effectiveness for a subset of cancers, and acquired drug tumor resistance, underscore the ongoing need for new and novel anticancer agents. To this end, achieving a full

understanding of the mechanism of action of cisplatin will facilitate the rational design of more selective compounds that are effective against a broader range of tumor types.

Although the precise mechanism of action of cisplatin remains unclear, it is well established that cisplatin's cytotoxic effects derive from processes triggered by its reaction with DNA (Bruhn et al., 1990). Cisplatin forms a variety of DNA adducts, most of which induce significant structural distortion such as bending and unwinding of the DNA helix. Cisplatin DNA adducts are capable of blocking DNA synthesis (Pinto and Lippard, 1985; Alazard et al., 1982; Salles et al., 1983), and it has been widely postulated that cisplatin, like many chemotherapeutic agents, exerts its antitumor effects through the inhibition of DNA synthesis in rapidly dividing cancer cells. However, DNA damage caused by the therapeutically ineffective isomer of cisplatin, *trans*-DDP, has been found to be equally effective as that of cisplatin at inhibiting DNA replication (Harder et al., 1976; Bernges and Holler, 1988; Heiger-Bernays et al., 1990; Salles et al., 1983; Ciccarelli et al., 1985). Moreover, simple inhibition of DNA synthesis cannot explain the organotropic specificity of cisplatin. These observations suggest that additional mechanisms apart from the inhibition of DNA replication may also be at work. The goal of this thesis work was to explore two alternative modes of cytotoxicity for cisplatin. In this work, the geometric isomer *trans*-DDP, which lacks antitumor activity, was used as a comparitor to calibrate the relative importance of specific interactions of cisplatin

DNA adducts with molecules within the cell.

The first goal of this thesis was to evaluate a model wherein the differential cytotoxic and antitumor activities of *cis*- and *trans*-DDP may result from a greater ability of *cis*-DDP DNA damage to inhibit RNA transcription. Supporting this hypothesis are results from a study comparing the effects of *cis*- and *trans*-DDP adducts on RNA polymerase processivity *in vitro*. DNA adducts formed by *cis*-DDP were found to be a complete block to transcription elongation by *Escherichia coli* or wheat germ RNA polymerases; by contrast, a major DNA adduct formed by *trans*-DDP was effectively bypassed by these RNA polymerases (Corda et al., 1991; Corda et al., 1993; Brabec and Leng, 1993). A study of the effects of *cis*- and *trans*-DDP on transcription *in vivo* has demonstrated that *cis*-DDP can inhibit initiation of transcription by preventing the binding of a transcription factor to its cognate promoter; *cis*-DDP was found to reduce the changes in nucleosomal organization required for transcription factor access, but *trans*-DDP did not (Mymryk et al., 1995). Unfortunately, no direct comparison between the two isomers can be made from this study because the relative number of platinum DNA adducts formed in cells after treatment with each platinum isomer was not determined. Prior to this thesis work, an examination of the inhibition of transcription elongation by platinum DNA adducts *in vivo* was distinctly lacking. The results of the first part of this dissertation show that RNA polymerase II translocates past a single, representative DNA adduct of *trans*-DDP with a minimum 5-fold greater efficiency than that of *cis*-DDP *in vivo*

(Mello et al., 1995). These results support the view that inhibition of transcription may contribute to the cytotoxic activity of cisplatin.

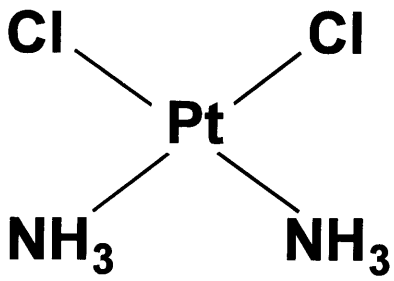
The second goal of this thesis work was to evaluate the possible role of the human mismatch repair protein hMSH2 in the mechanism of action of cisplatin. Long-patch mismatch repair is a pathway responsible for the correction of mismatched bases arising as a result of replication errors and recombination events (Modrich, 1991). The rationale for investigating a possible connection between mismatch repair proteins and cisplatin stems from several independent observations made with respect to mismatch repair in the literature. One such observation was made in early studies carried out by Martin Marinus and colleagues investigating mismatch repair in *E. coli* (Karran and Marinus, 1982; Fram et al., 1985). In this work *dam*$^-$ strains were shown to be hypersensitive to the cytotoxic effects of both the methylating agent *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and cisplatin. Interestingly, additional mutations that inactivate mismatch repair activity (*mutL*, *mutS*) in these cells were found to abrogate hypersensitivity to both agents. These results suggested that mismatch repair plays an active role in sensitizing *E. coli* cells to the cytotoxic effects of both MNNG and cisplatin. More recently these observations for MNNG were extended to higher eukaryotes; mammalian cell lines defective in mismatch repair were found to exhibit resistance to methylating agents (Kat et al., 1993; Branch et al., 1993; Branch et al., 1995; Aquilina et al., 1995). Taken together, these observations suggested that mismatch repair proteins could be involved in the mechanism of

cisplatin toxicity in mammalian cells.

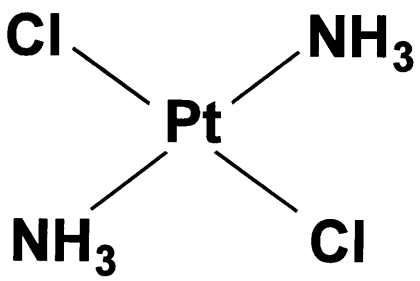
A second rationale for these studies came from the existing knowledge of a family of cellular proteins that bind specifically to cisplatin DNA adducts (Bruhn et al., 1992; Toney et al., 1989; Pil and Lippard, 1992; Lieberman et al., 1992). This protein family shares a common DNA binding motif with the non-histone chromosomal protein high mobility group 1 (HMG1). HMG-box proteins possess both sequence- and structure-specific DNA binding properties that include an affinity for bent DNA structures, such as four-way junction and cruciform DNA (Bianchi et al., 1992; Ferrari et al., 1992; Lilley, 1992). HMG box protein binding can also induce a dramatic bend in linear DNA sequences (Ferrari et al., 1992; Giese et al., 1992; Pil et al., 1993). It is therefore believed that duplex DNA bending and unwinding induced by cisplatin adducts (Bellon and Lippard, 1990; Bellon et al., 1991) provide the structural signal for HMG box protein recognition (Donahue et al., 1990; Bruhn et al., 1992). An intriguing observation made for both *Saccharomyces cerevisiae* and human MSH2 was that these proteins, in addition to recognizing single base mismatches, display an affinity for insertion/deletion mispairs and palindromic loops containing up to 14 extrahelical bases (Alani et al., 1995; Fishel et al., 1994a). Such palindromic loops create DNA structures highly reminiscent of cruciform DNA recognized by HMG proteins. These observations suggested that hMSH2, like HMG-box proteins, might recognize structural distortions induced by cisplatin DNA adducts.

Several models have been proposed and tested to explain the possible involvement of HMG-box proteins in the mechanism of action of cisplatin (see literature survey). To date, however, these models have failed to explain an important feature of cisplatin's pharmacological effects, namely its organotropic specificity for testicular and, to a lesser extent, ovarian tumors. Correlations between the expression levels of HMG-box proteins and those tissues best treated by cisplatin have not been described. An interesting observation in the literature, therefore, was that testicular tissue displays elevated levels of hMSH2 mRNA (Wilson et al., 1995). This reported overexpression provided a third motivation to explore potential interactions between cisplatin modified DNA and hMSH2. The work in the second part of this dissertation demonstrates that hMSH2 binds selectively to the DNA adducts of platinum based drugs that show clinical efficacy or potential. This work also shows that hMSH2 is overexpressed in both testis and ovary at the protein level (Mello et al., 1996). These results are consistent with a role for hMSH2 in the antitumor activity and organotropism of this important anticancer drug.

Figure 1: Chemical structures of the anticancer drug cisplatin and its therapeutically ineffective geometric isomer *trans*-DDP.



cis-DDP



trans-DDP

II. LITERATURE SURVEY

The success of cisplatin in the cure of testicular cancer has stimulated much interest in its biochemical mechanism(s) of action. To date, the precise mechanism by which cisplatin kills tumor cells remains elusive. Cisplatin can react with various macromolecules inside the cell, including DNA, RNA and proteins. It is widely believed, however, that cisplatin kills cells by processes triggered as a result of its reaction with DNA (Bruhn et al., 1990). Several lines of evidence strongly support this view. First, an examination of the number of platinum atoms bound to DNA, RNA and protein molecules in HeLa cells treated with cisplatin at its mean lethal dose indicates that relatively few protein molecules (one platinum adduct per 3-10,000 protein molecules) and RNA molecules (one platinum adduct per 10-1000 RNA molecules) are damaged by cisplatin compared to DNA molecules (nine platinum adducts per molecule) (Akaboshi et al., 1992). A study by Pascoe and Roberts found similar results (Pascoe and Roberts, 1974). Furthermore, it is reasoned that any of these damaged protein or RNA molecules, in contrast to that of DNA, are expendable and easily replaced. It could be argued, however, that some low abundance regulator of a critical event, such as control of apoptosis, may be a target of cisplatin. It is worth keeping an open mind to such options, although to date no data support them. The second line of evidence stems from several studies in which patients with different types of malignancies undergoing cisplatin therapy were monitored for levels of cisplatin adducts formed in their peripheral blood leukocytes. From these trials it was found that high levels of cisplatin adducts in the DNA of these patients correlates

extremely well with a favorable response to cisplatin treatment (Reed et al., 1987; Reed et al., 1988; Reed et al., 1993). It is difficult to build a strong case on the basis of such correlative evidence, however, because some non-DNA target not measured may have shown equal or better correlation.

The most compelling evidence supporting DNA as the target of cisplatin is the body of evidence showing that mammalian cells with a deficiency in nucleotide excision repair, which as a consequence accumulate great numbers of cisplatin DNA adducts, are much more sensitive to the drug than wild type cells (Hoy et al., 1985; Chu and Berg, 1987; Dijt et al., 1988). This evidence strongly supports the view that the cytotoxic effects of cisplatin derive primarily from its coordination to DNA. Significant effort has thus focused on the nature of that DNA damage, as well as how cells react to and process cisplatin lesions.

A. DNA adducts formed by *cis*- and *trans*-DDP

Cisplatin is not very reactive in the bloodstream, where the chloride concentration is high ($\sim$100 mM). Upon diffusion into a cell, however, the low concentration of chloride ($\sim$3 mM) facilitates hydrolysis of the two chloride ligands yielding a positively charged, bifunctional electrophilic derivative. This aquated species reacts rapidly with DNA primarily at the N7 position of purine bases to form monofunctional adducts; subsequent reaction with a second nucleophile yields

bifunctional intrastrand and interstrand crosslinks, as well as a small proportion of DNA-protein crosslinks (Bruhn et al., 1990) (summarized in Figure 2). The half-life of the monofunctional species *in vitro* is 4.1 hours, as determined by kinetics studies using ^{195}Pt-NMR (Bancroft et al., 1990). The primary DNA adducts formed by *cis*-DDP upon treatment of DNA *in vitro* include the intrastrand 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG) crosslinks (where N is any nucleotide) (Figure 3), representing approximately 65%, 25% and 8% of the total adducts formed, respectively (Eastman, 1983; Fichtinger-Schepman et al., 1985). Interstrand crosslinks are formed between two guanines in opposing strands at d(GpC) / d(GpC) sites (Eastman, 1985; Zou et al., 1994), although at a much lower frequency of $\leq$2% (Bruhn et al., 1990). Monofunctional adducts account for less than 1% of the total adduct profile (Fichtinger-Schepman et al., 1985). The examination of platinum adducts found in the DNA of mammalian cells grown in culture, or of circulating leukocytes of patients, following treatment with the drug reveal adduct profiles extremely similar to that found *in vitro* (Plooy et al., 1985; Fichtinger-Schepman et al., 1987).

The trans isomer of cisplatin, *trans*-DDP, is at least 20-fold less cytotoxic than cisplatin (Pascoe and Roberts, 1974) and is ineffective against tumors. This platinum compound also binds, although not exclusively, through the N7 position of purine bases in DNA, and forms a variety of monofunctional (Eastman and Barry, 1987) and bifunctional adducts (Eastman et al., 1988) (summarized in Figure 1).

Kinetic studies using ^{195}Pt-NMR determined the half-life of the *trans*-DDP monofunctional species *in vitro* to be 5.1 hours (Bancroft et al., 1990). The trans isomer is stereochemically hindered from forming the 1,2-intrastand crosslinks that comprise the majority of adducts formed by *cis*-DDP (Cohen et al., 1980; Pinto and Lippard, 1985) and instead forms predominantly intrastrand crosslinks at bases separated by one or more intervening nucleotides (Figure 2). Unfortunately, the *trans*-DDP adduct spectrum is not as well characterized as that of cisplatin. Reaction of *trans*-DDP with single stranded DNA and subsequent analysis of enzymatic digestion products reveals bifunctional crosslinks with the connectivities dG-Pt-dG (60%), dG-Pt-dA (35%) and dG-Pt-dC (5%) (Eastman et al., 1988); consistent with these findings are the results of replication mapping studies suggesting a preference for d(GpNpG) sequences (Pinto and Lippard, 1985). Bifunctional adducts formed by *trans*-DDP in duplex DNA exhibit a different distribution from that observed in single stranded DNA: dG-Pt-dC, dG-Pt-dG and dG-Pt-dA represent 50%, 40% and 10% of the total crosslinks, respectively (Eastman et al., 1988). A distinction between intrastrand adducts at nonadjacent bases and interstrand crosslinks could not be made by the methods used in this experiment. However, the preferential formation of dG-Pt-dC in duplex DNA suggests that it derived, at least in part, from an interstrand crosslink. More recently, interstrand adducts of *trans*-DDP have been characterized; the crosslinks indeed occur at complementary guanine and cytosine bases, and are estimated to comprise up to 20% of the total adduct profile (Eastman et al., 1988; Brabec and Leng, 1993).

Leng and colleagues have made the observation that the 1,3-d(GpApG) crosslink of *trans*-DDP present in a single stranded oligonucleotide undergoes isomerization to the interstrand crosslink when the oligonucleotide is paired with its complementary strand (Dalbies et al., 1994). This finding suggests that 1,3-intrastrand crosslinks of the trans isomer, at least in some sequence contexts, may be unstable. Isomerization of the 1,3-d(GpCpG) *trans*-DDP adduct present in a single stranded oligonucleotide to a 1,4-d(CpGpCpG) intrastrand adduct has also been observed (Comess et al., 1990). Interestingly, Leng and colleagues also report recently that when duplex DNA fragments globally modified with *trans*-DDP were examined to detect possible intrastrand crosslinks using the 3′ - 5′ exonuclease activity of T4 DNA polymerase, virtually no such crosslinks were detected (Boudvillain et al., 1995). The level of *trans*-DDP modification used in this experiment was extremely low, however, and would very likely have been below the limits of detection of their experimental system. The adduct profile formed in genomic DNA following treatment of cells with *trans*-DDP has not been examined.

Structural distortions induced to DNA by the various platinum adducts discussed above have been studied by numerous methods including polyacrylamide gel electrophoresis, chemical probes, nuclear magnetic resonance (NMR) and X-ray crystallography. Gel electrophoretic mobility studies of DNA fragments containing site-specific cisplatin adducts have demonstrated that the 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpTpG) intrastrand crosslinks each impart a directed bend to the DNA helix of

approximately 34° towards the major groove (Bellon and Lippard, 1990). The 1,2-d(GpG) and 1,2-d(ApG) adducts also induce local unwinding of the duplex by 13°, while the 1,3-d(GpTpG) crosslink causes unwinding of 23° (Bellon et al., 1991). Reactivity studies with chemical probes reveal that in the case of the 1,3-d(GpTpG) crosslink, base pairing at the 5′ G and T is disrupted, while the 3′ G base pairing is unperturbed. For both the 1,2-d(ApG) and 1,3-d(GpTpG) crosslinks, the DNA helix is more distorted at the 5′ side of the adduct (Marrot and Leng, 1989; Anin and Leng, 1990).

Analysis by X-ray crystallography of cisplatin coordinated to the dinucleotide d(GpG) has shown that the base stacking of the coordinated guanines is disrupted (Sherman et al., 1985). Very recently, an X-ray structure of the 1,2-d(GpG) adduct present in a duplex dodecamer has been solved (Takahara et al., 1995). This structure revealed that the local minor groove width is widened significantly (9.2-11.2 Å vs. the normal 5.7 Å of normal B-DNA) and, consistent with the earlier crystallographic study, that the guanine bases are destacked. The conformation of the deoxyribose at the 5′ and 3′ G nucleotide is C3′-endo and C2′-endo, respectively. Most striking was the finding of an abrupt change from B-type DNA to one resembling an A-type DNA architecture that extends well beyond the platinum lesion; it is possible that this unique structure was influenced by crystal packing. The structure of the 1,2-d(GpG) adduct in a 8-bp duplex has also been determined recently by NMR spectroscopy (Yang et al., 1995). The NMR structure indicates slightly

greater bending ($\sim 58^{\circ}$) and unwinding (21°) than was found by gel mobility studies, confirms that the minor groove is widened at the lesion site, and, in contrast to the crystal structure, indicates that the 5′ G base pair is disrupted. Conformations of the sugars are consistent with the crystal structure. Interestingly, in this same study it is reported that the 1,2-d(GpG) intrastrand adduct undergoes slow isomerization to an interstrand crosslink in the presence of chloride ion. Whether this reaction may be promoted *in vivo* by other nucleophilic biological ligands is not known.

Gel electrophoretic mobility studies of the 1,3-d(GpTpG) intrastrand crosslink of *trans*-DDP indicate that the lesion imparts a nondirected bend, or point of flexibility, to the DNA helix resembling a hinge joint (Bellon and Lippard, 1990; Bellon et al., 1991). This is in contrast to the rigid bend induced by each of the intrastrand adducts of cisplatin. Unwinding induced in plasmid DNA by global *trans*-DDP modification is reported to be $\sim 9^{\circ}$. Interestingly, monofunctional adducts of the cisplatin analog [Pt(dien)Cl]Cl, which has only one labile chloride and thus can coordinate to only one nucleophile, unwind the helix by $\sim 6^{\circ}$ (Keck and Lippard, 1992). In a separate study, global *cis*-DDP modification was found to be twice as effective at unwinding supercoiled plasmid DNA as *trans*-DDP damage (Scovell and Collart, 1985). Thus *trans*-DDP intrastrand crosslinks appear to induce less unwinding and rigid bending than their cisplatin counterparts.

The structural consequences of *cis*- and *trans*-DDP interstrand crosslinks to

DNA have also been examined. Both interstrand crosslinks appear to distort the DNA helix over a larger area than the intrastrand 1,2-d(GpG) cisplatin adduct (Schwartz and Leng, 1994). Electrophoretic mobility studies and reactivity assays using several different chemical probes indicate that a *trans*-DDP interstrand crosslink at a G/C base pair bends the helix towards the major groove by ~ 26°, unwinds it by ~ 12°, and suggest that the platinum coordinated nucleotides remain base paired (Brabec et al., 1993). Similar studies examining a cisplatin interstrand crosslink at a d(GpC)/d(GpC) site reveal a bend angle of 55° and unwinding by 79° (Sip et al., 1992; Malinge et al., 1994). A structure of this same adduct was recently determined by NMR, confirming that both cytosine bases are extrahelical, and that a localized change of helix from right- to left-handed at the site of the lesion results in a net unwinding of 87° that spans approximately four bases. A very unexpected finding, however, is that the cisplatin bridge actually lies in the minor groove, and imparts a 20° bend to the helix towards the minor groove (Huang et al., 1995). This minor groove positioning of the platinum bridge is in complete contrast to that seen for cisplatin intrastrand adducts and, if accurate, emphasizes the structural differences between interstrand and intrastrand cisplatin adducts.

The fact that *trans*-DDP cannot form 1,2-intrastrand crosslinks, which comprise greater than 90% of all adducts formed by *cis*-DDP, has led to the proposal that the 1,2-intrastrand adducts are responsible for the therapeutic activity of cisplatin. Recent findings that cellular proteins that recognize damaged DNA architectural

features that correlate with therapeutic efficacy bind specifically to these 1,2-intrastrand crosslinks lends support to this view. By similar reasoning, *cis*-DDP interstrand crosslinks are not thought to be responsible for the mechanism of cisplatin toxicity owing to the fact that the trans isomer forms significantly greater levels of this type of adduct. Such arguments rely on the assumption that *cis*- and *trans*-DDP interstrand crosslinks and/or 1,3-intrastrand crosslinks are structurally similar, while the discussion above clearly illustrates that this is not the case. Thus, until a mechanism of action is fully elucidated, all cisplatin adducts formally remain potential candidates for the specific lesion(s) that mediates the cytotoxic and therapeutic activities of the drug.

B. Cellular responses to cisplatin

1. Effects on DNA replication

Although the precise biochemical mechanism by which *cis*-DDP selectively kills cancer cells is not fully known, the DNA adducts of this platinum compound block both replication and transcription. It has been postulated, and it is indeed a widely held belief, that inhibition of DNA replication represents the primary mechanism by which cisplatin exerts its antitumor effects. Replication inhibition by cisplatin lesions is thought to slow or block cell division, therein providing a trigger for cell death. According to this hypothesis cisplatin would, importantly, have more

pronounced effects on rapidly dividing cells, such as tumor cells. Extensive efforts have thus focused on studying the effects of cisplatin and its trans isomer on DNA replication both *in vitro* and *in vivo*.

Globally modified single-stranded DNA templates have been used in studies examining the ability of *E. coli* DNA polymerase I to carry out second strand synthesis on platinated templates *in vitro*. *cis*-DDP adducts were found to block DNA polymerase I efficiently, and at sequences consistent with known sites of adduction (Pinto and Lippard, 1985; Villani et al., 1988). *trans*-DDP adducts also blocked well (Pinto and Lippard, 1985), while monofunctional adducts of cisplatin or the cisplatin analog [Pt(dien)Cl]Cl did not block the polymerase (Pinto and Lippard, 1985; Hoffmann et al., 1989). Strong inhibition by cisplatin modification has also been observed with eukaryotic enzymes. *cis*-DDP bifunctional adducts, but not monofunctional adducts, blocked purified calf thymus DNA polymerase α efficiently, and at numerous sites along the template that again correlated with preferential sites of adduct formation (Villani et al., 1988; Hoffmann et al., 1989). Using partially purified calf thymus DNA polymerase α and polymerase β, the modification level of *cis*- and *trans*-DDP that reduced template activity by 50% was found to differ by ~ 1.5, indicating that the two compounds inhibit DNA synthesis similarly (Harder et al., 1976). The two platinum isomers were also compared using an SV40-based *in vitro* replication system in which DNA synthesis was carried out by DNA polymerases present in HeLa or human embryonic kidney 293 cell-free extracts; in this system

additional cellular factors that might aid polymerases in translocating past DNA damage would presumably be present. *cis*- and *trans*-DDP adducts present on double stranded plasmid templates at an $r_b = 9 \times 10^{-4}$ both inhibited replication by 95%, while the same level of [Pt(dien)Cl]Cl monofunctional adducts produced 20% inhibition (Heiger-Bernays et al., 1990). These studies suggest that the two platinum isomers are equally effective at inhibiting DNA polymerases *in vitro*.

The studies described above using globally modified DNA indicate that several different platinum adducts block DNA replication. Site-specifically modified DNAs have been useful in assessing the relative abilities of individual platinum lesions to inhibit DNA polymerases *in vitro*. Comess and colleagues examined primer elongation on site-specifically platinated templates by the four enzymes *E. coli* DNA polymerase I, *E. coli* DNA polymerase III holoenzyme, bacteriophage T7 polymerase and T4 polymerase (Comess et al., 1992). Translesion synthesis was observed for each platinum adduct examined. Cisplatin intrastrand adducts inhibited DNA polymerases with the relative efficiencies: 1,2-d(GpG) > 1,2-d(ApG) > 1,3-d(GpCpG), with an average bypass efficiency of ~10%. The *trans*-DDP 1,4-d(CpGpCpG) crosslink was found to be a poor block to DNA synthesis. Although this differential inhibition by *cis*- and *trans*-DDP appears at odds with results from studies using globally modified templates, it is important to note that a 1,4-d(CpGpCpG) crosslink is not likely representative of the adducts formed by *trans*-DDP.

Site-specifically platinated DNA templates have also been employed in studies with eukaryotic enzymes. Purified calf thymus DNA polymerase ϵ is completely inhibited by a cisplatin 1,2-d(GpG) crosslink, as is the 3′ - 5′ proofreading exonuclease activity of the polymerase (Huang et al., 1993). Progression of DNA polymerase ϵ was also blocked by monofunctional cisplatin adducts on guanines. A more recent study has compared the effect of a 1,2-d(GpG) cisplatin crosslink, which was positioned on codon 13 within the human protooncogene *HRAS* sequence, on four of the five known eukaryotic DNA polymerases. In an earlier study, the 1,2-d(GpG) crosslink positioned in this same sequence in an SV40 based shuttle vector was efficiently replicated in monkey COS-7 cells, leading to mutations at the lesion site (Pillaire et al., 1994). Results revealed that DNA polymerases α, γ and ϵ are completely blocked at the site of the lesion, whereas DNA polymerase β is able to bypass the adduct efficiently (Hoffmann et al., 1995). Moreover, DNA polymerase β is able to initiate elongation directly opposite the lesion and to compete at the replication fork with the other enzymes when stalled at the lesion. Interestingly, the crystal structure of DNA polymerase β shows structural similarity to *E. coli* DNA polymerase I (Pelletier et al., 1994; Sawaya et al., 1994), which also bypasses the 1,2-d(GpG) adduct (*vide supra*).

The ability of platinum adducts to inhibit DNA synthesis has also been examined extensively *in vivo*. Treatment of mouse lymphoma cells grown in culture with *cis*- or *trans*-DDP revealed equal inhibition of DNA replication by the two

compounds when serum is absent from the media. It is noteworthy that in the presence of serum, sulfur-containing molecules selectively sequester *trans*-DDP in the medium, thereby significantly reducing DNA synthesis inhibition by this compound (Uchida et al., 1986). In a separate study, treatment of a L1210 leukemia cell line with platinum compounds shows that 50% inhibition of DNA synthesis was achieved when 1.8 x 10^{-4}, 2.4 x 10^{-4} and 80 x 10^{-4} platinum atoms are bound/nucleotide for *cis*-DDP, *trans*-DDP and [Pt(dien)Cl]Cl, respectively (Salles et al., 1983). Thus, a quantitative inhibition of DNA synthesis *in vivo* does not appear to correlate with the cytotoxic and antitumor activities of *cis*- and *trans*-DDP. Supporting this view are results from a study by Ciccarelli and coworkers in which simian virus 40 (SV40) was used as an *in vivo* model of chromatin. These authors showed that treatment of SV40-infected African green monkey CV-1 cells with *cis*- or *trans*-DDP caused equal inhibition of DNA synthesis when equal numbers of platinum adducts were present on the DNA (Ciccarelli et al., 1985). Results, however, from a subsequent study directly contradict these findings (Roberts and Friedlos, 1987). This discrepancy may reflect a technical difference in experimental design (Bruhn et al., 1990).

2. Effects on RNA transcription

The inhibition of DNA synthesis effected by *trans*-DDP both *in vitro* and in cultured cells suggests that the antitumor activity of cisplatin is not derived solely from its ability to inhibit DNA replication. Another way in which cisplatin may exert

its cytotoxic effects is through inhibition of transcription. RNA synthesis, like DNA replication, is more critical for a rapidly dividing tumor cell than for a stationary cell (Mauck and Green, 1973). The effects of *cis*- and *trans*-DDP on RNA synthesis have thus been examined, albeit not as extensively as DNA synthesis, both *in vitro* and *in vivo*.

Prior to the present work, the most definitive examination of the effects of *cis*- and *trans*-DDP on transcription focused on the blocking of RNA polymerases *in vitro*. Duplex DNAs containing site-specific platinum adducts were multimerized and then used as templates in transcription reactions catalyzed by either *E. coli* RNA polymerase or the eukaryotic wheat germ RNA polymerase II. The intrastrand 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpTpG) adducts of *cis*-DDP as well as the interstrand crosslink formed by either compound irreversibly blocked elongation of nascent RNA molecules by both polymerases. By contrast, the intrastrand 1,3-d(GpTpG) adduct of the trans isomer and the monofunctional adduct of [Pt(dien)Cl]Cl could be bypassed by RNA polymerases allowing elongation of the nascent RNA (Corda et al., 1991; Corda et al., 1993; Brabec and Leng, 1993). In response to intrastrand adducts, transcription stopped directly opposite the lesion, whereas elongation was blocked several nucleotides before the transcription complex reached an interstrand crosslink. Significantly, inhibition of the polymerase was only observed when a platinum lesion was present on the transcribed strand. In these same studies, the ability of RNA polymerase to add a single nucleoside triphosphate to a dinucleotide primer directly

opposite a cisplatin lesion was examined. None of the platinum adducts were an absolute block to this priming activity (Corda et al., 1992; Corda et al., 1993). The 1,2-d(GpG) cisplatin adduct impeded the single-step addition reaction more effectively than the 1,2-d(ApG) adduct, indicating that the polymerases could distinguish between the two structurally similar crosslinks (Corda et al., 1992). This differential inhibition was attributed to a lower affinity of the polymerase for the 1,2-d(GpG) adduct containing template, as the apparent K_m of the enzyme was increased by $\sim$ 5-fold for this substrate compared to that containing the 1,2-d(ApG) adduct. Taken together, these results indicate that platinum lesions may not only provide a physical block to the progression of RNA polymerases, but may also alter the properties of the transcription complex through the distortions they introduce to the DNA duplex.

In addition to blocking RNA polymerase processivity, cisplatin adducts can also inhibit transcription at the level of initiation. Direct evidence for this conclusion comes from a recent study in which *cis*-DDP treatment of cells inhibited binding of a transcription factor, NF1, to the mouse mammary tumor virus promoter present on a transiently introduced template (Mymryk et al., 1995). In these same studies, *cis*-DDP reduced the changes in nucleosomal organization required for transcription factor access, but *trans*-DDP did not. The relative number of DNA adducts formed in cells after treatment with each platinum isomer was not determined in this experiment, and thus no direct comparisons between the two compounds can be made in this regard.

The inhibition by cisplatin of bulk RNA synthesis in cultured human cells and in murine tumor cells *in vivo* has been examined. Total RNA and mRNA production is markedly inhibited by cisplatin, although not to the same degree as DNA synthesis (Harder and Rosenberg, 1970; Howle and Gale, 1970; Ganeva et al., 1990). The effect of cisplatin on individual gene expression has also been monitored. A panel of chimeric marker genes, where the promoter and the reporter gene were independently varied, were transiently introduced into monkey CV-1 cells, and the cells were then treated with cisplatin or *trans*-DDP. Strong differential inhibition of gene expression is observed at pharmacologically relevant doses of the drug, and the greatest inhibition correlates with the strongest promoters (Evans and Gralla, 1992a). These differential effects were not observed for *trans*-DDP, which was only weakly inhibitory to transcription from all promoters examined. Inhibition of gene expression was greater for longer genes, which likely reflects the greater number of potential sites for cisplatin modification. In similar studies carried out in human cells, cisplatin caused a surprising induction of gene expression from certain promoters, including the HIV long terminal repeat (LTR) sequence, and inhibited gene expression from others (Evans and Gralla, 1992b). Cisplatin-stimulated expression from the HIV-LTR promoter (Zoumpourlis et al., 1990) and from the human *c-myc* promoter (Spandidos et al., 1991) has been reported by others. As the mechanism responsible for this stimulation was not investigated, it remains unclear whether it was a direct result of cisplatin modification of the gene, or was an indirect consequence of a general cellular response, such as induction of transcription regulatory factors, to cisplatin

treatment.

The observations discussed above support the possibility that inhibition of RNA synthesis may contribute to the selective cytotoxic and antitumor activities of cisplatin. Although bulk RNA synthesis is less effected by cisplatin than DNA synthesis, it is reasonable to speculate that even in the absence of measurable reductions in RNA synthesis, changes induced in the delicate balance of cellular gene expression by cisplatin could be detrimental to a cell. Of relevance in this context are studies showing that *cis*-DDP treatment of L1210 cells causes arrest in the G2 phase of the cell cycle, and that the arrested cells subsequently undergo apoptosis (Sorenson and Eastman, 1988a; Sorenson et al., 1990). It has been proposed, therefore, that cisplatin DNA adducts may trigger apoptosis by inhibiting either overall gene expression, or a critical gene required for passage to mitosis (Sorenson and Eastman, 1988b).

3. Repair of platinum DNA adducts

Considering that platinum DNA adducts mediate cisplatin cytotoxicity, whether it be through inhibition of replication, transcription, or by some other mechanism, the repair of platinum lesions is an important way for a cell to increase its probability for survival. Indeed, studies in *E. coli* have demonstrated that strains deficient in Uvr(A)BC excision repair (*uvr*A, *uvr*B, *uvr*C) and recombinational repair (*lex*1, *rec*A,

*rec*B, *rec*C) are hypersensitive to cisplatin toxicity (Beck and Brubaker, 1973; Alazard et al., 1982). Such strains are also hypersensitive to *trans*-DDP at high doses, indicating that both repair pathways play a role in repair of *trans*-DDP damage as well (Alazard et al., 1982). Survival of cisplatin modified plasmids was greater in *rec*A mutant strains compared with *uvr*B mutant strains, indicating that the *rec*A-dependent pathway plays a minor role in cisplatin repair (Husain et al., 1985; Popoff et al., 1987). In these same studies the induction of the SOS response, which serves to increase expression of excision repair proteins, increased survival of *cis*- but not *trans*-DDP modified plasmid. This result suggested that DNA adducts of the two isomers may, to some extent, be repaired differently in *E. coli*.

Excision repair acts on a broad range of DNA damage, including bulky adducts formed by psoralen, benzo[a]pyrene, and UV light. The mechanism of Uvr(A)BC excision repair in *E. coli* has been well characterized (Sancar, 1996). In this system a dimer of UvrA in complex with UvrB binds to the site of damage, followed by an ATP-dependent conformational change that leads to dissociation of UvrA and the formation of a stable UvrB-DNA complex. UvrC is then recruited and together, UvrB and UvrC incise the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to the cisplatin adduct (Beck et al., 1985). UvrD (helicase II) subsequently facilitates release of the 12-13 nucleotide fragment, DNA polymerase I fills in the gap, and the remaining nick is sealed by ligation. This so called "excinuclease" activity of Uvr(A)BC on platinum DNA damage has been

examined *in vitro*. Plasmid DNA globally modified with *cis*- or *trans*-DDP was a substrate for incision by the Uvr(A)BC excinuclease, although the excinuclease was more active on *cis*-DDP modified plasmids (Beck et al., 1985; Popoff et al., 1987). The relative activity of Uvr(A)BC for individual platinum adducts was examined using substrates containing site-specific adducts of [Pt(dien)Cl]Cl; the relative rates of excision by Uvr(A)BC were found to be in the order 1,3-d(GpNpG) > monofunctional > 1,2-d(ApG) > 1,2-d(GpG) (Page et al., 1990). In a more recent study using site-specific adducts of cisplatin, the 1,2-d(GpG) crosslink was reported to be incised 3.5-fold more efficiently than a 1,3-d(GpCpG) crosslink (Visse et al., 1994). This inconsistency may be a reflection of the different platinum complexes used in the two studies.

In mammalian cells, as in *E. coli*, nucleotide excision repair is believed to be the primary mechanism for repair of platinum damage. Excision repair in mammalian cells is far more complex than in *E. coli*; 13-16 polypeptides are required for the excision step, and a total of ~30 polypeptides are involved in the entire repair process (Sancar, 1996). The mechanism of mammalian excision repair is rapidly being elucidated, and was recently the subject of several excellent reviews (Wood, 1996; Sancar, 1996). The autosomally recessive inherited disorder xeroderma pigmentosum (XP) is caused by defects in nucleotide excision repair and is characterized by extreme UV sensitivity and a high predisposition to skin cancers. Mammalian excision repair genes include those that complement the seven XP complementation

groups A through G, as well as the *ERCC* (excision repair cross-complementing) genes. As in *E. coli*, the basic mechanism involves recognition, dual incisions on the damaged strand, excision of an oligomer and resynthesis through the resulting gap. Specifically, XPA protein in complex with human single-stranded binding protein (HSSB) is responsible for damage recognition. Recognition signals for recruitment of TFIIH, a protein complex that contains both XPB and XPD and has dual functions in transcription and excision repair; the helicase activity of TFIIH is thought to open the duplex at the lesion site. XPG and the ERCC1-XPF heterodimer make incisions 3-9 phosphodiester bonds 3′ and 16-25 phosphodiester bonds 5′ to the lesion, respectively, yielding a repair patch ~25-30 nucleotides long. DNA polymerase δ or ϵ carries out the gap-filling repair synthesis with the aid of proliferating cell nuclear antigen (PCNA), an accessory factor that likely assists in initiation of synthesis at the 3′-OH of the gap. Very recently, mammalian excision repair has been reconstituted *in vitro* with purified components (Aboussekhra et al., 1995), a step that will undoubtedly facilitate further elucidation of this complex mechanism.

As was found for excision repair deficient *E. coli* mutants, mammalian cells deficient in excision repair are hypersensitive to cisplatin (Poll et al., 1984; Hoy et al., 1985; Dijt et al., 1988). XPA cells are three- to four-fold more sensitive to cisplatin compared to normal repair proficient cells as measured by the dose required to reduce survival to 37% of control untreated cells (Poll et al., 1984; Dijt et al., 1988). A nucleotide excision repair-deficient rodent cell line UV5 is similarly three-

fold hypersensitive to cisplatin, while the rodent UV20 cell line, which is deficient in the *ERCC1* gene, is 50-fold more sensitive to cisplatin than wild type cells as measured by the lowest concentration of drug that produced measurable loss in survival (Hoy et al., 1985). Studies monitoring repair indirectly through the reactivation of a cisplatin modified reporter gene transfected into mammalian cells found less gene reactivation in excision repair deficient cells than in normal cells, suggesting that less efficient repair of cisplatin adducts occurred (Chu and Berg, 1987; Sheibani et al., 1989). Experiments in which the level of cisplatin DNA adducts present in the genomic DNA of cisplatin treated cells was monitored directly over time has provided further demonstration that XPA cells are indeed deficient in repair of cisplatin intrastrand adducts (Dijt et al., 1988). The rodent UV20 cell line defective in the *ERCC1* gene was less efficient than normal cells at removal of the minor interstrand crosslink (Meyn et al., 1982), a result consistent with the possible dual role for this gene product in recombinational repair (Sancar, 1996). The rate of nucleotide excision repair of cisplatin adducts in mammalian cells has also been examined. Global removal of intrastrand adducts in repair proficient cells occurs most rapidly in the first 4-6 hours after treatment, followed by slower removal over time, and repair kinetics for the individual crosslinks 1,2-d(GpG), -d(ApG) and 1,3-d(GpNpG) (where N is any nucleotide) were found to be similar (Eastman and Schulte, 1988; Dijt et al., 1988). Excision repair deficient XPA cells are lacking in this fast repair process, although some slow repair is detected over a 24 h time period (Dijt et al., 1988).

The relative repair efficiency of cisplatin and *trans*-DDP DNA damage in mammalian cells has been examined in several studies. *In vitro* excision repair assays carried out on globally damaged plasmid DNA using human cell extracts demonstrated that both cisplatin and *trans*-DDP DNA damage stimulated repair synthesis. Extracts of XP cells were deficient in repair synthesis for either compound, indicating that excision repair operates on DNA adducts formed by both platinum isomers (Hansson and Wood, 1989; Hansson et al., 1990). Interestingly, greater repair synthesis was stimulated by *trans*-DDP modified DNA in this *in vitro* system (Hansson and Wood, 1989). A study examining the inhibitory effects of platinum DNA adducts on DNA replication *in vitro* found that pre-incubation of platinum modified substrates with cell extracts resulted in a 30% restoration of DNA replication for *trans*-DDP modified template but not for cisplatin damaged DNA (Heiger-Bernays et al., 1990). These results suggested that DNA adducts of *trans*-DDP may be preferentially repaired over those of cisplatin, and it has been postulated that *trans*-DDP may be less cytotoxic for this reason. Studies *in vivo*, however, have yielded conflicting views. A study in which levels of *cis*- and *trans*-DDP adducts in monkey cells following treatment with either compound were monitored over time found results suggesting that *trans*-DDP adducts are preferentially repaired (Ciccarelli et al., 1985). Later studies by Roberts and Friedlos challenged that conclusion, proposing that the appearance of differential repair in the Ciccarelli report was possibly caused by the different reactivities of the two isomers toward cellular DNA, and by the subsequent greater inhibitory effects of *cis*-DDP compared with *trans*-DDP

adducts to DNA synthesis and cell growth (Roberts and Friedlos, 1987). The Roberts study, however, was carried out in an environment in which the nature of the platinum compounds interacting with cells was uncertain and, as a consequence, the two studies may not be directly comparable (Sherman and Lippard, 1987). Whether *cis*- and *trans*-DDP DNA adducts are differentially repaired remains unresolved.

The repair of individual DNA adducts formed by cisplatin has also been examined. Results from an early *in vitro* excision repair assay indicated that repair synthesis carried out on cisplatin modified DNA resulted from removal of the minor adducts of cisplatin (Calsou et al., 1992). Consistent with this prediction, the 1,3-d(GpTpG) cisplatin adduct positioned site-specifically in a circular DNA duplex stimulated repair synthesis in an *in vitro* excision repair assay mediated by human cell extracts, while no repair activity was detected for the major cisplatin 1,2-d(GpG) adduct (Szymkowski et al., 1992). In independent studies, excision by human cell extracts of the 1,2-d(GpG) as well as the 1,2-d(ApG) cisplatin crosslink was observed; consistent with the previous report, however, the cisplatin 1,3-d(GpTpG) intrastrand adduct was excised most efficiently (Huang et al., 1994; Zamble et al., 1996). A site-specific interstrand cisplatin crosslink was not excised by the human excinuclease in this *in vitro* system. A reconstituted excision repair system containing highly purified repair components yielded similar results to those obtained with human cell extracts (Zamble et al., 1996). Further analysis of the human excinuclease activity *in vitro* revealed incision at the 16th phosphodiester bond 5′ to the adduct and

at the 9th phosphodiester bond 3′ to the cisplatin lesion, resulting in an excised oligomer 26 nucleotides in length (Moggs et al., 1996). Taken together, these findings suggest that structural distortions may determine the relative excision repair rates of various cisplatin adducts. Moreover, they suggest that inefficient repair of the major 1,2-d(GpG) intrastrand adduct may contribute to the antitumor activity of cisplatin.

Less than a decade ago it was revealed that transcribed DNA is repaired more efficiently than nontranscribed DNA, and that this effect is predominantly due to preferential repair of the transcribed strand (Mellon et al., 1987). This phenomenon, known as transcription-coupled repair, is the subject of recent reviews (Drapkin et al., 1994; Friedberg, 1996). The major factor contributing to this process is believed to be the pausing of RNA polymerase II at inhibitory lesions in DNA. In *E. coli*, the transcription-repair coupling factor (TRCF) mediates such repair by specifically recognizing the stalled polymerase and recruiting the $UvrA_2B$ complex to the site of damage (Selby and Sancar, 1993). In humans, the *CSA* and *CSB* gene products are necessary for transcription-coupled repair, and a defect in either gene results in Cockayne's syndrome (CS) (Hanawalt, 1994). Although the mechanism of transcription-coupled repair in humans is not known, it is believed that CSB may perform a function analogous to TRCF in *E. coli*. Interestingly, some mutations in XPB and XPD, proteins that play dual roles in transcription and repair as components of the TFIIH complex, can also give rise to CS.

Several studies have demonstrated that cisplatin damage is a substrate for transcription-coupled repair. Cisplatin intrastrand adducts are repaired more efficiently from actively transcribed genes than from transcriptionally silent regions of the genome and, moreover, from the transcribed strand as compared with the nontranscribed strand (Jones et al., 1991; May et al., 1993; Larminat et al., 1993). More efficient repair of cisplatin interstrand crosslinks from an actively transcribed gene was observed when cells were treated with low doses of the drug (Larminat et al., 1993). It is noteworthy that in these studies cleavage by Uvr(A)BC excinuclease was used to monitor the presence of cisplatin intrastrand adducts in DNA. Hence it remains possible that only those intrastrand adducts efficiently detected by the enzyme are in fact substrates for this repair process. A novel technique for gene-specific detection of cisplatin adducts was recently described that may prove useful in future studies examining this phenomenon (Rampino and Bohr, 1994).

4. Recognition by cellular proteins

a. Identification of cisplatin damage recognition proteins

Research has more recently focused on a class of eukaryotic proteins that binds specifically to cisplatin modified DNA. Such proteins were initially discovered in studies aimed at isolating DNA repair enzymes, a logical approach in that damage recognition is reasonably the initial step in repair. Initial studies using cisplatin

modified DNA probes in gel mobility shift assays revealed several factors in human cell extracts that specifically recognized cisplatin modified DNA (Chu and Chang, 1988; Donahue et al., 1990; Chao et al., 1991; Andrews and Jones, 1991). Complementary southwestern analysis experiments indicated that such cisplatin damage recognition proteins included both high molecular weight (90-100 kDa) and low molecular weight ($\sim$30 kDa) species (Toney et al., 1989; Chao et al., 1991; Andrews and Jones, 1991). Interestingly, DNA modified by *trans*-DDP or [Pt(dien)Cl]Cl was not bound by these factors, suggesting that the cis geometry was required for recognition (Toney et al., 1989; Donahue et al., 1990). As a means to identify such proteins, Toney and colleagues employed cisplatin modified DNA to screen a human B-cell cDNA library (Toney et al., 1989). This approach led to the isolation of a full length clone encoding an 81 kDa protein, which was named structure-specific recognition protein 1 (SSRP1) for lack of a known function. The predicted amino acid sequence revealed a 75 amino acid region with 47% homology to a similar region in the abundant 24.5-kDa chromosomal protein termed high-mobility-group protein 1 (HMG1) (Bruhn et al., 1992). It has since been revealed that SSRP1 is the homolog of a mouse protein, T160, which binds to recombination signal sequences required for V(D)J recombination (Shirakata et al., 1991). A formal link, however, to immunological processes has not yet been established.

The discovery that SSRP1 contained this highly conserved region, commonly referred to as the HMG-box or -domain, suggested that HMG1 would also recognize

cisplatin modified DNA. Indeed, subsequent studies showed that both HMG1 and HMG2 bound to cisplatin modified DNA, and that a mixture of HMG1 and HMG2 were in fact responsible for the ~30 kDa species observed previously in southwestern blots (Pil and Lippard, 1992; Hughes et al., 1992). Purified recombinant rat HMG1 bound specifically to cisplatin intrastrand 1,2-d(GpG) and 1,2-d(ApG) crosslinks (K_d = 3.7 x 10^{-7} M *vs.* 3.2 x 10^{-5} M for unmodified DNA), but lacked specificity for 1,3-d(GpTpG) cisplatin adducts (Pil and Lippard, 1992). Consistent with previous observations, HMG1 displayed no affinity for DNA damaged by the therapeutically ineffective analogs *trans*-DDP or [Pt(dien)Cl]Cl. Thus the 1,2-intrastrand crosslinks formed exclusively by therapeutically active platinum compounds are preferentially recognized by HMG1. Interestingly, it was reported recently that HMG1 also binds to the minor cisplatin interstrand crosslink, but not to that formed by *trans*-DDP (Kasparkova and Brabec, 1995), further underscoring the selectivity of HMG1 for cisplatin DNA adducts. The HMG-box domain is believed to mediate the recognition of cisplatin-modified DNA. Strongly supporting this view, an isolated HMG-box domain of HMG1 was found to bind the 1,2-d(GpG) cisplatin adduct with an affinity equal to that of HMG1 itself (Chow et al., 1995).

The HMG-box is in fact a novel signature DNA binding motif that defines a large and rapidly growing family of proteins (Baxevanis and Landsman, 1995). In addition to SSRP1 and HMG1, HMG-box proteins that have been shown to bind cisplatin modified DNA currently include human upstream binding factor (hUBF)

(Treiber et al., 1994), testis determining factor (SRY) (E. Trimmer, manuscript in preparation), mitochondrial transcription factor (mtTFA) (Chow et al., 1995), the *S. cerevisiae* transcriptional regulator Ixr1 (Brown et al., 1993), and the isolated HMG domains from HMG1, mSRY and lymphoid enhancing factor (LEF-1) (Chow et al., 1994). Many HMG-box proteins, such as hUBF, LEF-1, SRY and mtTFA, are specific regulators of transcription; others, such as HMG1, have as yet undefined functions, although roles for HMG1 in DNA replication, gene transcription and in modulating chromatin structure have been suggested (Einck and Bustin, 1985). There is no indication to date that HMG-box proteins are involved in repair processes, suggesting that any roles these proteins might play in cisplatin cytotoxicity are likely to be unrelated to DNA repair.

While no HMG-box proteins have been implicated in DNA repair, additional non-HMG-box proteins that bind to cisplatin modified DNA have been identified, some of which do have roles in repair. Such proteins include a 125-kDa UV-damage recognition protein identified for its ability to recognize both UV and cisplatin DNA damage (Chu and Chang, 1988), human single-stranded binding protein (HSSB or RPA) (Clugston et al., 1992), and human Ku autoantigen (Turchi and Henkels, 1996). Recognition of cisplatin damage by HSSB/RPA is consistent with this protein's role in the incision step of nucleotide excision repair. The UV-damage recognition protein was named XPE protein owing to its specific absence in a subset of XPE cell lines (Chu and Chang, 1990; Keeney et al., 1992; Kataoka and Fujiwara, 1991). XPE is

also believed to be involved in excision repair, although its precise role remains unclear (Sancar, 1996). Ku protein has been implicated in double-strand break repair and V(D)J recombination (Finnie et al., 1995). The relevance of Ku recognizing cisplatin DNA damage is unknown, although it is conceivable that the structure of cisplatin damaged DNA resembles an intermediate in the immunoglobulin gene rearrangement.

b. Structural basis for recognition by HMG-box proteins

Why do HMG-box proteins recognize cisplatin DNA adducts? All HMG-box proteins have the common feature of binding to DNA involved in structural deformations (Lilley, 1992). For example, HMG1 binds to cruciform structures and unwinds negatively supercoiled DNA (Bianchi et al., 1989), and the HMG domains of SRY and HMG1 bind to four-way junction DNA (Ferrari et al., 1992; Bianchi et al., 1992). Hence the duplex DNA bending and unwinding induced by cisplatin adducts likely provide the structural signal for HMG box protein recognition (Donahue et al., 1990; Bruhn et al., 1992). In addition to recognizing pre-bent structures, HMG-box proteins can induce dramatic bends in linear DNA sequences, as has been shown for SRY, HMG1 and LEF-1 (Ferrari et al., 1992; Pil et al., 1993; Giese et al., 1992). Indeed, several HMG-box proteins, upon binding to DNA that contained a site-specific 1,2-d(GpG) adduct, were shown to cause further DNA distortion, resulting in a 50-90° bend centered in the vicinity of the platinum crosslink (Chow et al., 1994).

Thus the bending afforded by a cisplatin adduct may facilitate the conversion of duplex DNA into the preferred binding configuration of an HMG-domain protein (Chow et al., 1995).

Recently, solution structures of the HMG-domains from several HMG-domain proteins have been determined by NMR spectroscopy. The HMG domain B from HMG1 in the absence of DNA was found to be made up of three α helices that form an L shape, with an 80° angle between the two arms that is defined by a cluster of conserved aromatic residues (Read et al., 1993; Weir et al., 1993). A structure of the SRY-HMG domain bound to its putative DNA target sequence found an analogous L-shaped configuration (Werner et al., 1995). DNA binding occurred on the concave surface of the L, and DNA contacts were made within a significantly widened minor groove. The DNA helix was severely underwound, bent by $\sim$70-80° towards the major groove, and the DNA structure displayed features intermediate between those of A- and B-DNA. A very similar structure was found for the LEF-1 HMG domain complexed with its cognate DNA (Love et al., 1995). This novel structure induced in DNA upon binding by these HMG domains is strikingly similar to the structure solved for a duplex DNA dodecamer containing a 1,2-d(GpG) cisplatin adduct (*vide supra*) (Takahara et al., 1995). Hence these studies reinforce the notion that DNA distortions induced by cisplatin adducts resemble the naturally occurring binding sites and binding configurations preferred by this class of proteins.

c. Roles for cisplatin damage recognition proteins

The selective affinity of HMG proteins for the DNA adducts of therapeutically active platinum compounds (Toney et al., 1989; Pil and Lippard, 1992) suggests a role for such proteins in potentiating cisplatin cytotoxicity. Several models to explain the possible involvement of HMG box proteins in the mechanism of action of cisplatin have been proposed. One model suggests that binding of these proteins to cisplatin DNA adducts prevents access to the lesions by DNA repair enzymes; decreased repair of cisplatin DNA adducts would result in persistence of the signal that triggers cell death. Supporting this hypothesis are results from *in vitro* excision repair assays. Preincubation of HMG1 or mtTFA with substrates containing site-specific cisplatin adducts effectively block the 1,2-d(GpG), but not the 1,3-d(GpTpG), adduct from repair by the human excinuclease present in cell extracts (Huang et al., 1994). Similar inhibition by HMG1 was observed when repair was mediated by a reconstituted system containing highly purified human repair factors (Zamble et al., 1996). Further evidence was obtained by using a yeast strain defective in the HMG-box protein Ixr1. Interruption of the *IXR1* gene desensitized yeast cells by two-fold to cisplatin; by contrast, no differential sensitivity was observed to *trans*-DDP or UV light (Brown et al., 1993). Resistance of the *ixr1* strain was associated with a three-fold decrease in accumulation of cisplatin DNA adducts. Further, an examination of the effects of Ixr1 on cisplatin sensitivity in various excision repair backgrounds revealed a dependence upon a functional excision repair recognition complex

(McA'Nulty and Lippard, 1996), suggesting that Ixr1 blocked the cisplatin adducts from the repair machinery. Taken together, these studies support the view that HMG proteins may potentiate cisplatin cytotoxicity by binding to cisplatin DNA lesions, thereby facilitating their persistence in DNA.

A second model stems from the identification of hUBF, a critical regulator of ribosomal RNA synthesis, as one of the HMG box proteins that recognizes cisplatin adducts. hUBF binds to a cisplatin 1,2-d(GpG) adduct ($K_{d(app)}$ = 60 pM) and to its cognate rRNA promoter sequence ($K_{d(app)}$ = 18 pM) with comparable affinities, leading to the proposal that cisplatin adducts may act as molecular decoys for the transcription factor *in vivo* (Treiber et al., 1994). Thus by a "transcription factor hijacking" mechanism, cisplatin may deplete cells of a necessary resource for growth. Supporting this model, levels of cisplatin adducts well below that found in cells of patients effectively antagonized hUBF-promoter interactions in footprinting assays (Treiber et al., 1994). Moreover, cisplatin modified DNA successfully inhibited transcription from the hUBF promoter in a fully reconstituted *in vitro* transcription system, while DNA modified by *trans*-DDP did not (X.Q. Zhai, manuscript in preparation). By contrast, *in vivo* experiments testing the hijacking model with the transcriptional regulator Ixr1 in yeast found that Ixr1 was not titrated away from its promoter, Cox5b, by cisplatin adducts following treatment with the drug (McA'Nulty et al., 1996). It is possible, however, that the relatively low affinity (K_d = 2.4 x 10^{-7} M) and modest specificity (~ 10-fold over unmodified DNA) of Ixr1 for a cisplatin

adduct (McA'Nulty et al., 1996) render it a poor candidate for the hijacking mechanism.

Yet a third way in which cellular proteins that associate with cisplatin DNA damage might affect cisplatin cytotoxicity is by initiating the repair of cisplatin lesions. As already mentioned, no HMG-box proteins have been implicated in DNA repair. Other cisplatin recognition proteins, including HSSB and XPE, do have roles in repair. Interestingly, XPE protein was reported to be present at 5-fold increased levels in some cisplatin resistant tumor cell lines, cells in which increased repair of cisplatin damage was also found (Chu and Chang, 1990). An $\sim$130 kDa cisplatin damage recognition protein was likewise found to be overexpressed in a cisplatin resistant cell line, and was inducible upon cisplatin treatment (Chao et al., 1991); although the species was not identified, the size and behavior of the protein suggest it may be XPE protein. A recent study reports that cisplatin treatment of human cells caused induction of a protein that binds selectively to UV-damage, and that this cisplatin-responsive induction is higher in cisplatin resistant cells than in normal cell lines (Vaisman and Chaney, 1995). These observations suggest that such cell lines were resistant by virtue of an increased capacity to repair cisplatin damage. This subject is discussed further in the next section. By contrast, there has been only one report to date of increased levels of HMG-box proteins (HMG1 and 2) in cisplatin resistant cells (Billings et al., 1994). Moreover, no human HMG-box proteins other than SRY (Goldmacher et al., 1986) are known to be differentially expressed in

testicular tissue. It is noted, however, that a mouse HMG-box protein SOX5 has been shown to be overexpressed in testicular tissue (Denny et al., 1992) and a testis-specific mouse HMG-box (tsHMG) protein has been identified (Boissonneault and Lau, 1993). Also of relevance, levels of a rapidly migrating HMG2 subtype have been reported to be significantly elevated in rat testicular tissue as compared to 15 other rat tissues (Bucci et al., 1985). The physiological relevance of HMG-box protein recognition of cisplatin damage awaits correlations between levels of these proteins in human cells/tissues and sensitivities to the drug.

5. Resistance to cisplatin

The development of resistance to cisplatin is a significant problem in cisplatin-based chemotherapy, representing the major factor responsible for treatment failure. There are two types of cisplatin resistance, the first being an acquired phenomenon that arises following exposure to the drug, and the second being an intrinsic resistance of a patient's tumor to cisplatin from the very beginning of treatment. Numerous cisplatin resistant cell lines, most of which were developed by repeated or continuous exposure to the drug in culture, have been valuable in the elucidation of the mechanisms of cisplatin resistance. Tumor cells made resistant *in vivo* have also been studied, and although far less available, may more faithfully represent the clinical situation. For example, cells treated in culture with cisplatin often acquire a much higher levels of resistance than that of tumors *in vivo* (the latter are generally on the

order of 2-3-fold more resistant) (Enns and Howell, 1991), implying that mechanisms may be operative that are not directly relevant to the clinical situation. Another caveat to examining resistance mechanisms in any cell line is that some mechanisms may operate only at the level of the organism, and not at the cellular level. This is illustrated in a study in which a murine mammary tumor acquired resistance to cisplatin *in vivo*, but when cells from the tumor were cultured *in vitro* they were no longer resistant to the drug (Teicher et al., 1990). Interestingly, when the tumor was implanted into other host animals, it was capable of altering cisplatin pharmacokinetics, suggesting that the tumor secreted hormonal substances that produced a change in drug distribution. In a subsequent study, the resistant phenotype of these *in vivo*-derived tumor cells was rescued in culture when the cells were grown as three-dimensional multicellular tumor spheroids that more closely mimic *in vivo*-like conditions (Kobayashi et al., 1993). The mechanism underlying this multicellular resistance remains to be established.

Despite the above caveats, studies of cisplatin resistant cell lines have revealed numerous mechanisms that contribute to this phenomenon, including 1) reduction in cellular accumulation; 2) elevated levels of molecules that sequester cisplatin, such as glutathione and metallothionein; 3) increased capacity to repair cisplatin DNA adducts; and 4) changes in expression of regulatory proteins, such as oncogenes and tumor supressors (Andrews and Howell, 1990; Scanlon et al., 1991; Timmer-Bosscha et al., 1992). Resistance is generally believed to be multifactorial, and thus one or

more of these mechanisms may be operative in a particular resistant tumor or cell line.

a. Cisplatin accumulation

Given that DNA adducts are believed to mediate cisplatin cytotoxicity, limiting the number of cisplatin adducts formed in DNA is a logical way that cells can decrease their susceptibility to the drug. One mechanism by which cells may achieve this end is through limited drug uptake. Decreased accumulation of cisplatin has been consistently observed in resistant cell lines (Andrews and Howell, 1990; Scanlon et al., 1991), although these decreases are generally small, even when levels of resistance are high. Changes in accumulation have in some studies been specifically attributed to a decrease in influx of cisplatin, as decreased accumulation could be observed almost immediately after treatment (Waud, 1987; Hromas et al., 1987). Despite the possible importance to cisplatin resistance, the mechanism underlying these changes in accumulation remains unknown. In fact, the mechanism by which cisplatin enters a cell, whether through passive diffusion or active membrane transport, is also a matter of debate, and was recently the subject of review (Gately and Howell, 1993).

b. Sequestration by sulfur-containing molecules

A second mechanism by which cells may limit the formation of cisplatin adducts is by increasing levels of sulfur-rich molecules that can sequester cisplatin before it reaches DNA. Such molecules include metallothionein and glutathione. Metallothionein and glutathione have both been shown to interact with cisplatin in cells, and in either case a significant proportion of cytoplasmic cisplatin (25% and 60%, respectively) was found to be complexed by the molecule (Sharma and Edwards, 1983; Ishikawa and Ali-Osman, 1993). Studies examining levels of metallothionein and glutathione in normal and cisplatin resistant cell lines, however, indicate that these molecules are overexpressed in some but not all resistant cells.

Metallothioneins function in the detoxification of heavy metals, such as cadmium and mercury. A ten-fold overexpression of metallothionein in mouse cells generated four-fold resistance to cisplatin, directly demonstrating that metallothionein can affect cisplatin toxicity (Kelley et al., 1988). Supporting this notion, cells containing elevated levels of metallothionein and demonstrating resistance to cadmium were cross-resistant to cisplatin (Andrews et al., 1987; Bakka et al., 1981). Moreover, tumor cell lines that acquired resistance to cisplatin *in vitro* overexpress metallothionein (Kelley et al., 1988). Levels of metallothionein mRNA also correlate with resistance in human lung cancer cell lines (Kasahara et al., 1991). However, in other cell lines, levels of metallothionein do not correlate with resistance (Sheibani et

al., 1989) and in one instance elevated levels of metallothionein are actually associated with an increase in sensitivity to cisplatin (Koropatnick and Pearson, 1993).

In the case of glutathione, similar observations have been made. Increasing glutathione levels in a normal lung cell line by 50% causes a 1.4-fold decrease in cisplatin sensitivity, demonstrating that glutathione also can alter cisplatin sensitivity (Russo et al., 1986). Examination of ovarian tumor cell lines made 30-100-fold resistant to cisplatin in culture revealed a 13-50-fold increase in glutathione levels compared to their cisplatin-sensitive parent cell line (Godwin et al., 1992). Cell lines displaying more physiologically relevant levels of cisplatin resistance (six-fold) have also been shown to contain elevated glutathione levels (three-fold) (Hospers et al., 1988) and depletion of glutathione with the inhibitor buthionine sulfoximine sensitizes both normal and resistant cells to cisplatin (Hamilton et al., 1985). However, as is the case for metallothioneine, many resistant cell lines display no changes in glutathione levels (Chao et al., 1992; Kelley et al., 1988). Hence there appears to be some, but variable, correlation between cisplatin resistance and elevated glutathione or metallothionein levels.

c. DNA repair capacity

An alternative strategy that cells may use to reduce levels of cisplatin DNA adducts, and thus acquire resistance to the drug, is to increase their capacity to repair

cisplatin damage. Levels of repair in normal and cisplatin resistant cell lines have been extensively examined (Chu, 1994; Zamble and Lippard, 1995). In several studies, ovarian cancer cell lines made resistant to cisplatin in culture are 2-fold more efficient at repairing cisplatin damage in cellular DNA (Masuda et al., 1988; Masuda et al., 1990) as well as in transfected plasmid DNA (Parker et al., 1991). Examination of a testicular tumor cell line and a 5.6-fold cisplatin-resistant variant derived *in vitro* revealed that total cisplatin adducts are removed from DNA significantly faster in the resistant cells than in the parent line (Kelland et al., 1992). Cell lines that display an intrinsic resistance to cisplatin also have increased levels of cisplatin-damage repair (Scanlon et al., 1991; Dabholkar et al., 1992b). Increases in cisplatin adduct repair have been observed in resistant variants of the murine leukemia L1210/0 cell line (Eastman and Schulte, 1988; Sheibani et al., 1989); it has subsequently been revealed, however, that the apparent increased repair capacity was actually due to the spontaneous reversion in the resistant cells of a previously unknown nucleotide excision repair defect for the XPG gene in the parental cell line (Vilpo et al., 1995). Indeed, repair is not always associated with resistance (Tashiro and Sato, 1992). In one resistant cell line cisplatin repair is actually found to be increased (Hill et al., 1992).

Possible correlations between cisplatin repair and resistance have been examined in tumor tissue as well. Levels of repair capacity in tumor biopsies from untreated ovarian cancer patients were measured using an *in vitro* repair assay and

found to vary by up to 10-fold; this differential capacity for cisplatin adduct repair was postulated to be responsible for the variability in clinical response to cisplatin therapy (Jones et al., 1994). In another study a 2.8-fold increase in repair of cisplatin damage was found in cells from tumor tissue obtained after failed cisplatin therapy compared to cells from the same tumor before treatment (Ali-Osman et al., 1994). Interestingly, cisplatin resistance and increased repair in this tumor tissue was associated with elevated levels of DNA polymerase β (Pol β), a common observation for cisplatin resistant cell lines (Scanlon et al., 1991). Although Pol β is not thought to be involved in DNA repair synthesis, it has been reported to be the only human DNA polymerase capable of efficient replication past a cisplatin 1,2-d(GpG) adduct (Hoffmann et al., 1995). Thus increased expression of Pol β may help cells to tolerate cisplatin damage by facilitating replicative bypass of the DNA lesions. Expression levels of proteins involved in excision repair are also correlated with cisplatin resistance. In tumor tissue from ovarian cancer patients, mRNA of the human nucleotide excision repair gene ERCC1 is higher in tissue from patients that are clinically resistant to the drug (Dabholkar et al., 1992a). Elevated levels of the repair-associated protein proliferating cell nuclear antigen (PCNA) are reported in cisplatin resistant murine leukemia cell lines (Haneda et al., 1991). As noted in the preceding section, XPE protein has also been found at increased levels in cisplatin resistant cell lines (Chu and Chang, 1990). Enhanced DNA repair thus appears to be a common strategy that cells use to reduce their susceptibility to the drug.

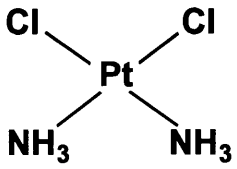
d. Effects of regulatory proteins

More recent research into cisplatin resistance has focused on the expression of regulatory proteins, such as tumor suppressors and oncogenes. Increased expression levels of the protooncogenes *ras*, *fos* and *myc* all correlate with cisplatin resistance (Scanlon et al., 1991). In addition, correlations between resistance and elevated levels of the *p53* tumor suppressor gene product have been reported (Davol et al., 1996). The *p53* gene encodes a nuclear phosphoprotein that, through a cascade of events, activates G1 cell cycle arrest in response to some forms of DNA damage, thereby allowing the cell more time to repair its DNA before entering S phase (Ko and Prives, 1996; Gottlieb and Oren, 1996). In addition, it is thought that p53 may actually induce excision repair. Enhanced levels of p53 could thus confer cisplatin resistance by increasing time for repair of cisplatin DNA damage; conversely, inactivation of p53 may sensitize cells to cisplatin. Consistent with this view, inactivation of p53 in human fibroblasts by acute expression of human papillomavirus (HPV), which binds to p53 and targets it for degradation via a ubiquitin-dependent pathway, rendered the cells 6-9-fold more sensitive to cisplatin (Mirakhur et al., 1996). Similarly, disruption of p53 in human MCF7 breast cancer cells by using HPV or a dominant-negative mutant p53 gene sensitizes cells to cisplatin, and this sensitization is associated with a decreased ability of the cells to repair cisplatin-damaged plasmid DNA (Fan et al., 1995). Further, mouse embryonal fibroblasts lacking functional p53 (p53−/−) were found to be hypersensitive to cisplatin

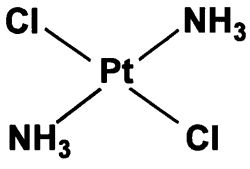
compared to fibroblasts homozygous (p53+/+) or heterozygous (p53+/−) for wild type p53.

In addition to its role in the G1-checkpoint, p53 can also activate programmed cell death, or apoptosis, in response to certain forms of DNA damage, and this pathway can often override the G1-checkpoint response (Thompson, 1995; White, 1996). p53 is in fact required for efficient execution of the apoptotic response induced by various anticancer drugs (Lowe et al., 1993). Through this pathway, disruption of p53 could lead to resistance to cisplatin and other DNA damaging agents. Evidence that p53 can affect cisplatin cytotoxicity via this pathway also exists. Adenovirus-mediated transfer of the wild type p53 gene into monolayers or multicellular spheroid tumor cultures of lung cancer cell lines with a homozygous deletion of p53 markedly increases sensitivity of the cells to cisplatin (Fujiwara et al., 1994). Further, a relationship between p53 and the development of cisplatin resistance in ovarian carcinoma cell lines in culture has been demonstrated; these cisplatin resistant cells were shown to harbor two mutant p53 alleles and displayed reduced susceptibility to cisplatin-induced apoptosis (Parego et al., 1996). Interestingly, testicular germ cell tumors, which are exquisitely sensitive to cisplatin therapy, are unusual in that they rarely exhibit p53 mutations (Peng et al., 1993). These observations suggest that the preferential sensitivity of testicular tumors to cisplatin could, in part, be owed to their predominantly wild type p53 genotype.

Figure 2: DNA adduct profiles of cisplatin and *trans*-DDP. Numbers indicate the percentage of each adduct compared to the total binding spectrum. In cases where accurate numbers are not available, the ability to form adducts is indicated by "yes" or "no".



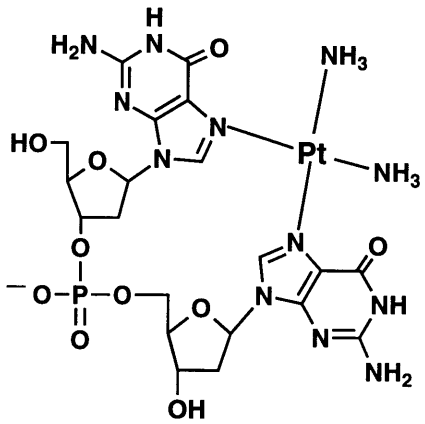
cis-DDP



trans-DDP

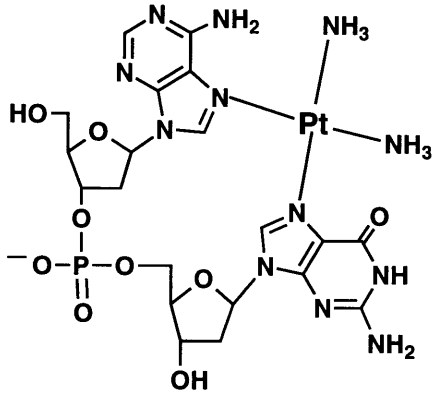
Monofunctional adducts		
dG	yes	yes
Intrastrand crosslinks		
d(GpG)	65%	No
d(ApG)	25%	No
d(GpNpG)	6%	~40%
Interstrand crosslinks		
d(G*pC)/(G*pC)	2%	?
d(G*pC)/(GpC*)	?	~20%
DNA-protein crosslinks	yes	yes

Figure 3: The chemical composition of the cisplatin 1,2-d(GpG), -d(ApG), and 1,3-d(GpTpG) adducts. Structural conformations of these adducts are discussed in the text and are illustrated in references therein.



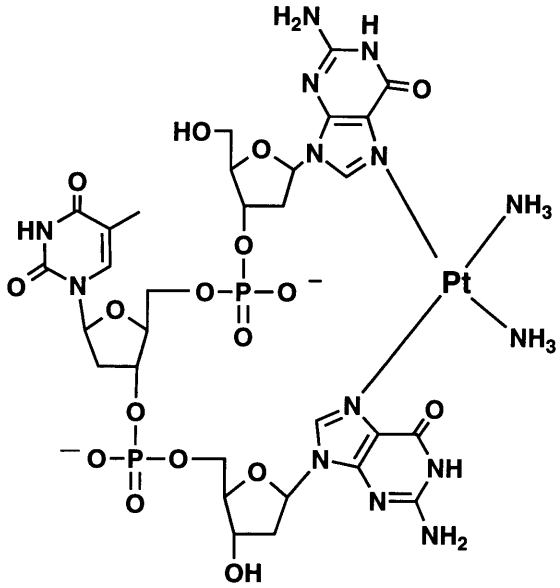
cis-[Pt(NH$_3$)$_2${d(GpG)}]

(1,2-d(GpG))



cis-[Pt(NH$_3$)$_2${d(ApG)}]

(1,2-d(ApG))



cis-[Pt(NH$_3$)$_2${d(GpTpG)}]

(1,3-d(GpTpG))

III. DNA ADDUCTS OF CISPLATIN AND ITS TRANS ISOMER INHIBIT RNA POLYMERASE II DIFFERENTIALLY *IN VIVO*

A. Introduction

The observation that *trans*-DDP is equally effective at inhibiting DNA synthesis as *cis*-DDP (Harder et al., 1976; Bernges and Holler, 1988; Heiger-Bernays et al., 1990; Salles et al., 1983; Ciccarelli et al., 1985) suggests that mechanisms other than the blocking of DNA replication may contribute to the cytotoxic activity of this anticancer drug. One model to explain the differential cytotoxicities of these two platinum complexes is that *cis*-DDP adducts may more effectively inhibit transcription. The goal of the work summarized in this chapter was to compare directly the effects of *cis*- and *trans*-DDP DNA adducts on RNA synthesis in mammalian cells. A nonreplicating transient expression vector harboring a prokaryotic reporter gene under the transcriptional control of a eukaryotic promoter was modified *in vitro* to defined levels with either platinum compound and transfected into human or rodent cell lines. The relative inhibition of RNA synthesis by the two platinum compounds was evaluated by 1) measuring levels of β-galactosidase enzyme activity in extracts made from the transfected cells and 2) recovering nascent β-gal mRNA transcripts and directly monitoring transcription elongation through a specific region of the gene by using ribonuclease protection analysis. In order to eliminate the variable of excision repair from the experimental system, experiments were carried out in both nucleotide excision repair proficient and deficient cell lines of both human and rodent origin, thus allowing examination of transcriptional bypass independently of excision repair for each platinum compound. Together, these studies facilitated an

estimation of the efficiency with which RNA polymerase II translocates past a single, representative DNA adduct of *cis*- or *trans*-DDP during transcription elongation *in vivo*.

The approach adopted in this study afforded two distinct advantages over alternative strategies wherein cells are directly treated with platinum compounds and the effects on endogenous gene expression are monitored. First, one can easily control and precisely quantify the number of platinum adducts introduced into DNA by either platinum isomer prior to transfection. Second, one can monitor the effects of platinum adducts on transcription without the confounding effects of platinum damage to other cellular processes, such as inhibition of DNA replication, ribonucleotide reduction and induction of apoptosis. This latter point is significant, considering the complexity of cellular responses elicited upon cisplatin treatment. Moreover, it is believed that cellular processing of plasmid DNA accurately reflects that of chromosomal DNA, as evidenced by accurate predictions of the nucleotide excision repair deficiencies of certain cell lines (Protic-Sabljic and Kraemer, 1985; Athas et al., 1991). These analyses were made by monitoring repair of UV damage in nonreplicating transient expression vectors.

B. Materials and Methods

1. Cells and Culture Conditions

HeLa cells (P. Sharp, MIT) were maintained in suspension culture in Joklik-modified minimal essential medium (MEM) (Gibco), supplemented with 5% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. HeLa cells were maintained at a cell density of 5 x 10^4 - 5 x 10^5 cells/ml and had a doubling time of approximately 24 h. The Chinese hamster ovary (CHO) cells designated AA8 and UV20, obtained from the American Type Culture Collection, were maintained in suspension culture in α-MEM (Gibco) supplemented with 10% fetal bovine serum, penicillin and streptomycin. UV20 was derived by mutation of the AA8 cells (Thompson et al., 1981) and belongs to complementation group 1 (formerly group 2) (Collins, 1993). CHO cells were maintained at a cell density of 3 x 10^4 - 4 x 10^5 cells/ml and had a doubling time of approximately 12-15 h. Human lymphoblastoid cell lines transformed with Epstein-Barr virus were obtained from the NIGMS Human Genetic Mutant Cell Repository. Cell line GM02250D is from a donor homozygous for xeroderma pigmentosum belonging to complementation group A (XPA) and GM05567 is from the proband's apparently unaffected, but possibly heterozygous, brother. Cells were maintained in suspension culture in RPMI 1640 medium (Gibco) supplemented with 15% fetal bovine serum, penicillin and streptomycin, at a cell density of 2 x 10^5 - 1.5 x 10^6 cells/ml, and had a doubling time of approximately 24

h.

2. Plasmids

All plasmids used were incompetent for replication but allowed for the transient expression of a prokaryotic reporter gene in mammalian cells. CMV-β-gal (7330 bp; provided by R. Tepper, Harvard Medical School) contains the prokaryotic β-galactosidase (β-gal) gene inserted between the eukaryotic cytomegalovirus (CMV) promoter and simian virus 40 (SV40) splice and polyadenylation sequence. PSV2-CAT (5003 bp) harbors the bacterial chloramphenicol acetyltransferase (CAT) gene flanked by the SV40 early promoter and splice and polyadenylation sequence (Gorman et al., 1982). pcDNA3-CAT (6230 bp; Invitrogen) contains the CAT gene between the CMV promoter and the bovine growth hormone polyadenylation sequence. All plasmids were isolated from *E. coli* DH5α cultures by using the maxi- or mega-plasmid purification kits of Qiagen, Inc.

Platination reactions were carried out in 3 mM NaCl, 1 mM Na_2HPO_4, pH 7.4, with 200 μg/ml DNA and appropriate platinum compound/DNA molar ratios by incubating at 37°C for 16 h. Unreacted platinum compound was removed by dialysis (14 h) against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. To serve as an unmodified control, plasmid DNA was subjected to the same treatment in the absence of platinum compound. Levels of platinum modification were determined by flameless atomic

absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95 graphite furnace.

3. Transfections

Platinated DNA was transfected into cells by electroporation with a BTX Electro Cell Manipulator 600. Electroporation conditions for each cell line were first extensively optimized by independently varying the voltage and capacitance settings; conditions were chosen that yielded the greatest levels of β-gal expression per μg of protein in cell extracts made from the transfected cells. Using optimized electroporation conditions, duplicate samples of DNA at each platinum level were transfected into cells. Specifically, HeLa cells in logarithmic growth (2-4 x 10^5 cells/ml) were concentrated by centrifugation and resuspended at 1.4 x 10^7 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Plasmid DNA (20 μg) and 5 x 10^6 cells (350 μl) were mixed, transferred to a sterile 0.2 cm electroporation cuvette, and a single electrical pulse was immediately delivered to the cells at 245 V, 1150 μF and 13 Ω. Similarly, XPA and normal human lymphoblasts (5-8 x 10^5 cells/ml) were transfected by mixing 40 μg of plasmid DNA with 2 x 10^7 cells (350 μl) and electroporating at 125 V, 2700 μF and 72 Ω in RPMI supplemented with 15% fetal bovine serum, penicillin and streptomycin. CHO cells (1-2 x 10^5 cells/ml) were transfected by mixing 5 x 10^6 cells (350 μl) in α-MEM supplemented with 10% fetal bovine serum, penicillin and

streptomycin with 40 μg of DNA and electroporating at 165 V, 1150 μF and 13 Ω. Following 10 min at room temperature, transfected cells were transferred to 10 ml of prewarmed complete medium and incubated in a 10 cm culture dish at 37°C for 15, 24 or 48 h (see Results). For RNase protection assays, XPA lymphoblasts were cotransfected with 32 μg of modified CMV-β-gal and 8 μg of pcDNA3-CAT and cells were harvested 4 h after transfection.

4. Transient Expression Assays

Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.8. Cells were lysed by three cycles of freeze-thaw, and the lysate was centrifuged (14,000g) for 15 min at 4°C. The supernatant from cells transfected with CMV-β-gal was assayed for β-gal activity by the method of Eustice et al. (Eustice et al., 1991), with slight modifications. Up to 40 μl of cell lysate was assayed in a 200 μl solution containing 9 mM $MgCl_2$, 102 mM β-mercaptoethanol, 80 mM Na_2HPO_4, pH 7.3, and 9 mM chlorophenol red β-D-galactopyranoside (Boehringer-Mannheim). Assay mixtures were incubated for a minimum of 30 min at 37°C, stopped by the addition of 300 μl of 0.5 M EDTA, and the absorbance at 570 nm measured with a Beckman DU-65 spectrophotometer. Cell extracts prepared from mock-transfected cells served as negative controls. The protein concentration of cell extracts was determined by the Bio-Rad microassay with bovine serum albumin as the protein standard (Bradford,

1976). β-Gal activity for each sample was normalized to the amount of protein present and is expressed as a percent of the activity determined for an unmodified control. Percent β-gal activity for duplicate samples at each platinum level were graphed as a function of platinum adducts/genome. Curves exponentially fitted to the data were used to determine the platinum level effecting a 63% reduction in β-gal activity (D_0).

In experiments where Hela cells were transfected with PSV2-CAT, the amount of CAT protein present in cell extracts was measured by using an enzyme immunoassay for CAT (CAT-ELISA, Boehringer Mannheim) according to the protocol provided by the supplier. Briefly, up to 40 μg of cell extract was placed in microtiter wells that had been prebound with CAT antibodies. CAT protein bound to the wells was subsequently hybridized with a second CAT antibody conjugated to digoxigenin (Anti-CAT-DIG, 2 μg/ml), and finally with an antibody to digoxigenin conjugated to peroxidase (Anti-DIG-POD, 150 mU/ml). The amount of peroxidase enzyme present in each well was determined by using ABTS^R-substrate, a chromophoric substrate to peroxidase. The absorbance of samples in the microtiter wells was measured at 405 nm in a Titertek^R Multiskan 96-well plate reader (Eflab). The amount of CAT (pg) (determined using a prepared standard curve) was normalized for the amount of protein present and reported as a percent of unmodified control.

5. RNA Isolation

Total RNA was isolated by a modification of the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Cells were lysed with 0.5 ml of RNAzol^{TM}B (Biotecx Laboratories), 50 μl of chloroform was added, and the lysate was centrifuged (14,000g) for 15 min. RNA was purified from the aqueous phase with 0.5 volumes of isopropyl alcohol and 0.05 volumes of RNA Tack^{TM} Resin (Biotecx). After two washes with 75% ethanol (v/v), RNA was eluted from the resin with 100 μl H_2O. To remove possible contaminating plasmid DNA, all RNA samples were treated with RNase-free DNase (Boehringer Mannheim) for 20 min at room temperature.

6. Probes

A 215 bp DNA sequence that begins 233 bp and terminates 18 bp 5′ to the stop codon of the β-gal gene was PCR amplified and then inserted downstream of, and in opposite orientation to, the T7 promoter of pGEM-3zf(-) (Promega). This pGEM-3zf-β-gal3 construct was linearized with Pst I, and served as template for the synthesis of a ^{32}P-labeled antisense RNA probe complementary to the 3′ end of the β-gal mRNA (βG3; 215 nucleotide protected fragment). Linearized transcription vector pTri-β-gal (Ambion) served as the template for a second β-Gal antisense RNA probe complementary to an internal sequence extending from base 934 to 1235 of the 3072

bp of the β-gal gene (βG5; 301 nucleotide protected fragment). Antisense RNA probes for CAT (152 nucleotide protected fragment) and for human β-actin (127 nucleotide protected fragment) were synthesized from the linearized vectors pTri-CAT and pTri-β-Actin-125-Human (Ambion), respectively. All antisense radiolabeled RNA probes were transcribed *in vitro* by using Ambion MAXIscript™. T7 RNA polymerase (10 U) was incubated with pGEM-3zf-β-gal3 (1 μg), 500 μM ATP, GTP and TTP, and 5 μM (α-^{32}P)UTP (800 Ci/mmol, 50 μCi) (Amersham) in a 20 μl reaction at 37°C for 1 h. This reaction produced a 246 nucleotide transcription product containing the 215 nucleotide sequence complementary to the β-Gal gene. Template DNA was removed by adding RNase-free DNase (2 U) and incubating at 37°C for an additional 15 min. pTRI-CAT and pTRI-B-Gal were similarly transcribed with T7 and SP6 RNA polymerases, yielding 241 and 344 nucleotide transcription products, respectively. pTri-B-Actin-125-Human was transcribed by using SP6 RNA polymerase yielding a transcription product of 218 nucleotides, except that the reaction was carried out in the presence of an additional 150 μM unlabeled UTP in order to produce a lower specific activity probe. All probes were immediately purified by electrophoresis on an 8 M urea 5% polyacrylamide gel, and were stored at -20°C.

7. Ribonuclease Protection Assay

Assays were carried out by using the RPA II kit (Ambion) according to the

protocol of the supplier. Briefly, total RNA (20 μg) was hybridized simultaneously with the CAT (2 x 10^5 cpm), β-actin (1 x 10^5 cpm) and either βG5 or βG3 RNA probes (3.5 x 10^5 cpm) at 45°C for 14 h. Hybridization mixtures were then digested with 1000 units/ml RNase T1 by incubating at 37°C for 30 min. Following the simultaneous inactivation of the RNase T1 and ethanol precipitation of the protected radiolabeled RNA probes, the protected fragments were separated on an 8 M urea 5% polyacrylamide gel. Quantitative analysis was performed by using a PhosphorImager (Molecular Dynamics).

8. Calculation of Bypass Efficiencies

Bypass efficiencies of RNA polymerase II for a single representative DNA adduct of either *cis*- or *trans*-DDP were calculated as follows. The gene inactivation target was considered to include either the CMV promoter and transcribed strand of the β-gal coding sequence and SV40 splice/polyadenylation sequence (5120 nucleotides of 14976 total nucleotides in plasmid) or alternatively only the transcribed strand of the coding and polyadenylation sequence (3936 of 14976 total nucleotides). The D_{0T}, or number of platinum adducts required within this putative gene inactivation target to produce one lethal hit (reduce β-gal enzyme activity to 37% of the unmodified control), were calculated by multiplication of the previously determined D_0 values (Table 1) by the fraction of the total plasmid that was the gene inactivation target. All D_{0T} values were assumed to follow a Poisson distribution.

The following polynomial equation, in which each term describes the individual contributions of each population of adducts described by the Poisson distribution to the total transcription blocking observed, was then solved iteratively:

$$0.37 = (a \times X^0) + (b \times X^1) + (c \times X^2) + (d \times X^3) + (e \times X^4) + (f \times X^5) \ldots$$

where for a given D_{0T} value, the Poisson distribution would predict that fraction *a* of the platinum modified DNA population would contain no adducts, *b* would contain one adduct, *c* would contain two adducts, *d* would contain three adducts, and so on; 0.37 refers to the fraction of transcriptional activity at which D_{0T} was determined; X represents the bypass frequency of RNA polymerase for a single representative adduct of either platinum isomer. This calculation of bypass efficiency was carried out twice, where the gene inactivation target sequence was considered either to include, or not, the promoter region of the gene; the two resulting values for bypass efficiency represent a maximum and minimum estimation, respectively, of the actual bypass efficiency. Bypass efficiencies were similarly calculated for the experiment where ribonuclease protection analysis was used to monitor directly the inhibition of transcription elongation. In this case, however, the gene inactivation target was considered to be the 1604 nucleotide sequence flanked by the two antisense RNA probes. D_0 values were determined from the curves exponentially fitted to the data.

C. Results

1. Host cell reactivation of *cis*- and *trans*-DDP damaged plasmids in HeLa cells

The relative effects of *cis*- and *trans*-DDP DNA adducts on transcription were examined by using the host cell reactivation assay. HeLa cells were transfected with *cis*- or *trans*-DDP damaged CMV-β-gal plasmid DNA and, after 24 h, β-gal activities and total protein content were determined. Relative to the β-gal activity from unmodified plasmid, which was defined as 100%, β-gal expression decreased exponentially with increasing platinum damage (Figure 4). A reference point used for comparison of the dose response curves is D_0, or the level of DNA damage sufficient to reduce the β-gal activity to 37% of its original value along the exponential portion of the curve (Protic-Sabljic and Kraemer, 1985). Two-fold more *trans*-DDP damage (D_0 = 40 adducts/genome; r_b= 0.0027) than *cis*-DDP damage (D_0 = 21 adducts/genome; r_b= 0.0014) was required to produce an equivalent reduction in β-gal activity. Three testable models could explain the differential reactivation of plasmids modified by *cis*- and *trans*-DDP: (1) translesion synthesis by RNA polymerase may be more efficient on DNA modified by *trans*-DDP than *cis*-DDP; (2) *trans*-DDP adducts may be more rapidly repaired than *cis*-DDP adducts; and (3) there may be preferential formation of *cis*-DDP adducts compared with *trans*-DDP adducts within the β-gal gene.

In order to determine whether the differential reactivation effects observed in Figure 4 were a consequence of different distributions of *cis*- and *trans*-DDP DNA adducts, the reactivation assay was carried out in HeLa cells using a second plasmid, PSV2-CAT. PSV2-CAT differs from CMV-β-gal in that it contains different potential gene-inactivating target (i.e., promoter, coding, and splice/polyadenylation sequences) as well as non-target sequences (Figure 5). With PSV2-CAT, the D_0 value for *trans*-DDP was 38 adducts/genome (r_b= 0.0038), as compared to 19 adducts/genome for *cis*-DDP (r_b= 0.0019). This two-fold difference in D_0 values was identical to that observed in the CMV-β-gal system. These observations argued against the possibility that the different levels of gene expression from *cis*- and *trans*-DDP modified DNA is a consequence of different platinum levels within the transcribed reporter gene.

2. Impact of nucleotide excision repair status on reactivation of platinated DNA in human cell lines

In order to assess the possible contribution of excision repair to the observed greater reactivation of *trans*-DDP-modified DNA, the host cell reactivation assay was carried out in nucleotide excision repair deficient XPA and apparently normal, consanguineous human lymphoblasts. Measurement of β-gal activity 24 h ($\sim$ one cell doubling time) after transfection of both cell lines with *cis*- or *trans*-DDP damaged plasmid demonstrated the expected reduction in activity with increasing platinum

damage (Figure 6). XPA cells exhibited a steeper dose response than did repair proficient cells. The four-fold differential in D_0 values found for XPA and normal cells (Table 1) is comparable to differences previously observed for *cis*-DDP (Poll et al., 1984; Chu and Berg, 1987) and for UV damage (Lehmann and Oomen, 1985; Protic-Sabljic and Kraemer, 1985; Athas et al., 1991). A comparison of the two platinum compounds in normal lymphoblasts revealed a 1.6-fold greater D_0 value for *trans*- compared to *cis*-DDP, consistent with our results obtained in HeLa cells. Experiments in XPA cells, in which platinum adducts cannot be removed by excision repair, found an even larger differential of 2.7-fold between *cis*- and *trans*-DDP. If excision repair were contributing to the differential reactivation of *cis*- and *trans*-DDP damaged DNA observed in normal cells, one would have expected to find similar D_0 values for *cis*- and *trans*-DDP in excision repair deficient cells.

3. Impact of nucleotide excision repair status on reactivation of platinated DNA in rodent cell lines

Additional experiments were performed to assess whether the observed differential effects of *cis*- and *trans*-DDP on transcription might be species or cell line specific. CHO repair proficient AA8 cells and excision repair deficient UV20 cells were transfected in parallel with *cis*- or *trans*-DDP damaged CMV-β-gal DNA, and β-gal activity was measured 15 h ($\sim$ 1 cell doubling time) after transfection. As shown in Figure 7, the transcriptional activity from platinated DNAs decreased exponentially

with increasing dose. A ~ 3.5 fold enhanced reactivation of *trans*-DDP damaged plasmid was found in either cell line (Table 1). Interestingly, D_0 values obtained in AA8 cells were virtually identical to those found in UV20 cells (Table 1), suggesting that little or no repair of either platinum isomer, within the detection limits of this assay, had yet occurred in the repair proficient AA8 cells. It is interesting that no repair was apparent by this assay after one doubling time in CHO cells, whereas significant levels were observed in human lymphoblasts after 24 h, approximating one doubling time for these cells. This distinction may reflect a less efficient or rapid DNA nucleotide excision repair system in rodent cells as compared to human cells.

β-gal activity was next measured in either cell line 48 h after transfection. In excision repair deficient UV20 cells, D_0 values found for *cis*- and *trans*-DDP at 48 h were identical to those found at 15 h. This result illustrates the repair deficient phenotype of UV20 cells and supports the view that excision repair is the primary mechanism of removal of platinum DNA adducts, although the action of other repair pathways on the platinum adducts before 15 h cannot be excluded. In contrast, in repair proficient AA8 cells, D_0 values for *cis*- and *trans*-DDP increased significantly and proportionately from 15 to 48 h, indicating excision repair of the adducts of both isomers. A comparison at 48 h of the two isomers in repair proficient cells revealed a 3.2-fold larger D_0 for *trans*-DDP than for *cis*-DDP; in the absence of repair this difference was 4.3-fold. This trend in the changes of D_0 values obtained in CHO cells at 48 h paralleled closely that observed in human cells. Taken together, our

results in CHO and human cells indicate that excision repair of *trans*-DDP lesions was not contributing to the greater reactivation of *trans*-DDP damaged DNA in these studies.

4. Effects of platinum DNA adducts on transcription elongation

Experiments were carried out to examine whether platinum DNA adducts were inhibiting β-gal gene expression by blocking RNA polymerase processivity *in vivo*. Blocking of mRNA synthesis within a defined region of the β-gal gene was measured directly by ribonuclease protection analysis. Excision repair deficient XPA cells were used in order to eliminate, as far as possible, any differences in excision repair as a variable in the experiment. XPA cells were cotransfected with *cis*- or *trans*-DDP modified CMV-β-gal and a second unmodified plasmid, pcDNA3-CAT, to control for transfection efficiency. Total RNA was isolated four h after transfection of the cells in further effort to ensure that the effects of transcription, and not leaky excision repair, were being monitored. Northern analysis verified that total RNA samples were free of contaminating plasmid DNA (data not shown). Total RNA was hybridized simultaneously with antisense RNA probes for CAT, constitutively expressed β-actin, and one of two sequences within β-gal mRNA. One β-gal probe, βG3, complementary to a sequence located at the immediate 3′ end of β-gal mRNA (Figure 8A), was used to measure only full length mRNA transcript. A second probe, βG5, was complementary to a sequence located 1604 bp upstream from the

βG3 sequence (Figure 8A). βG5 detected full length transcripts as well as those prematurely terminated due to the presence of platinum adducts within the 1604 bp sequence between βG3 and βG5. RNAs from the same sample but hybridized with either βG3 or βG5 were run in adjacent lanes. A representative autoradiograph is shown in Figure 8B.

Hybridizations with βG3 showed that amounts of full length transcript decreased exponentially with increasing platinum modification (Figure 8B, *B* lanes). Curves generated from these data exhibit a three fold differential in D_0 values for *cis*- and *trans*-DDP (curves not shown), in agreement with that found in previous experiments with XPA cells where β-gal enzyme activity was measured. Hybridizations with βG5 (Figure 8B, *A* lanes) also showed a decrease in β-gal mRNA transcript with increasing number of *cis*-DDP adducts. By contrast, similar levels of *trans*-DDP adducts had relatively little effect on mRNA levels detected by this probe. Data obtained from these two antisense RNA probes were used to calculate the fraction of total β-gal mRNA (βG5) that was successfully transcribed through the 1604 bp region to yield full length transcript (βG3). Studies *in vitro* indicate that only platinum adducts present on the transcribed strand of DNA effectively block RNA polymerase processivity (Corda et al., 1991). The fraction βG3/βG5 therefore provides a measure of the blocking effected by platinum adducts on the transcribed strand of the 1604 bp region. Curves generated from these data (Figure 9) revealed that 1.2 *cis*-DDP adducts were required in this 1604 nucleotide sequence to reduce

full length transcript to 37% of that synthesized from unmodified plasmid. At the levels of modification examined for *trans*-DDP, full length transcript never fell below 56%. The slope of the dose-response curve predicts, however, that ~ 5 *trans*-DDP adducts would be necessary to effect 37% inhibition. These results indicate that *cis*-DDP DNA adducts block RNA polymerase processivity more effectively than *trans*-DDP adducts *in vivo* by at least 4-fold.

D. Discussion

1. Differential inhibition of RNA transcription by *cis*- and *trans*-DDP

The effects of the anticancer drug *cis*-DDP and its geometric isomer *trans*-DDP on RNA synthesis were investigated *in vivo* by using a nonreplicating transient expression vector in a host cell reactivation system. Transfection of HeLa cells with *cis*- or *trans*-DDP damaged plasmid revealed a two fold greater expression of the template modified by *trans*-DDP than by *cis*-DDP (Figure 4,5). These data suggested that DNA adducts of *cis*-DDP were more inhibitory to transcription than adducts formed by *trans*-DDP. Alternatively, the possibility existed that more efficient repair of *trans*-DDP adducts compared with *cis*-DDP adducts could have contributed to our observations. The latter observation would be consistent with the suggestion of a previously published report (Ciccarelli et al., 1985; Heiger-Bernays et al., 1990).

Nucleotide excision repair is believed to be the main process by which platinum adducts are removed from DNA. More specifically, *cis*-DDP adducts are known substrates for transcription-coupled excision repair (Jones et al., 1991; May et al., 1993), of relevance in that the assay used in this study monitors adducts present in an actively transcribed gene. Cells from patients with the autosomal recessive disease XP belonging to complementation group A are defective for XPAC (XPA complementing) protein, which binds *cis*-DDP damaged DNA and functions as a key component in recognition of DNA damage during repair (Jones and Wood, 1993). XPA cells are thus deficient in the incision step of excision repair (Cleaver, 1968; Friedberg, 1985). It has been demonstrated that XPA cells are deficient in the repair of *cis*-DDP (Chu and Berg, 1987; Dijt et al., 1988; Hansson et al., 1990) and *trans*-DDP DNA adducts (Hansson et al., 1990), as well as in the transcription-coupled repair of *cis*-DDP (Zhen et al., 1993) and UV (Evans et al., 1993) damage. Our experiments in XPA cells (Figure 6; Table 1) showed that the differential in D_0 values for *trans*-DDP compared to *cis*-DDP (2.7-fold) was enhanced relative to that found in a consanguineous normal cell line (1.6-fold). This result suggested that preferential excision repair of *trans*-DDP was <u>not</u> the cause of the enhanced expression found for *trans*-DDP damaged DNA.

Experiments carried out in CHO excision repair deficient UV20 cells (complementation group 1, formerly group 2) gave remarkably similar results (Figure 7; Table 1). The CHO group 1 repair defect is partially complemented by the human

ERCC1 gene (van Duin et al., 1988; Larminat and Bohr, 1994). The ERCC1 gene product, by comparison with its homologous protein Rad 10 in *S. cerevisiae* (van Duin et al., 1986), is believed to function in the 5′ incision step of excision repair as well as in mitotic recombination (Bardwell et al., 1994). This latter point is relevant in that interstrand crosslinks may require recombinational repair for their removal from DNA. CHO group 1 repair deficient cell lines have an increased sensitivity to both *cis*- and *trans*-DDP (Hoy et al., 1985), are deficient in the removal of interstrand (Meyn et al., 1982) as well as intrastrand *cis*-DDP cross-links (Larminat and Bohr, 1994), and have been shown to be deficient in the repair of *cis*-DDP adducts present in an actively transcribed gene (Larminat and Bohr, 1994). Our experiments showed that the differential in D_0 values for *trans*-DDP compared to *cis*-DDP was, again, slightly greater in the repair deficient UV20 (4.3-fold) than in the parental AA8 (3.7-fold) cell lines. Further, significant repair of both isomers was observed, as operationally defined by this assay, in the time that elapsed between 15 h and 48 h after transfection in AA8 cells. By contrast, no repair of either isomer was evident during the same time period in the UV20 cells. The most straightforward explanation of these data is that excision repair is the primary mechanism of platinum adduct removal for both platinum isomers. The formal possibility exists that *trans*-DDP lesions could be removed by an additional process in excision repair deficient cells; to date, however, there is no evidence of such a process. The same differential between *cis*- and *trans*-DDP was found at 15 h and 48 h, further supporting the view that excision repair did not contribute to the enhanced transcriptional activity from

trans-DDP damaged templates. Taken together, the results from human and rodent repair deficient cell lines thus suggested that *cis*-DDP adducts inhibited transcription more efficiently than adducts of *trans*-DDP *in vivo*.

A statistical analysis of the data obtained in excision repair deficient cells, assuming that repair by another putative process is negligible, allowed the calculation of an approximate efficiency of translesion synthesis by RNA polymerase. Inhibition of transcription by platinum adducts could be caused by the impeded translocation of RNA polymerase II along the transcribed strand of the DNA template (Corda et al., 1991). Gene inactivation could also be mediated at the level of initiation through the inhibition of transcription factor binding (Mymryk et al., 1995). In this analysis the gene inactivation target sequence within CMV-β-gal was therefore considered to include the double stranded promoter region as well as the transcribed strand of the coding and splice/polyadenylation sequence. This analysis revealed that, on average, 1.2 *cis*-DDP DNA adducts within this putative target were required to reduce β-gal activity by 63% (D_{0T}) in XPA cells. In contrast, at least 3.3 DNA adducts of *trans*-DDP were necessary to produce the same level of inhibition. If gene inactivation at the promoter was small and can be ignored, the D_{0T} values obtained in repair deficient cell lines can then be used to calculate an approximate bypass efficiency of RNA polymerase for both *cis*- and *trans*-DDP. This calculation was accomplished by using the Poisson distribution of D_{0T} for either isomer. Inclusion of the promoter in the target sequence that ultimately determined D_{0T} may or may not be a valid assumption.

By carrying out calculations for both situations, however, an upper and lower limit for the bypass rates of each isomer could be established. The bypass frequency by RNA polymerase for a single DNA adduct of *trans*-DDP was determined to be in the range of 60-76%, whereas the rate of bypass of a single *cis*-DDP adduct was calculated to be between 0-17% (summarized in Table 2). The D_{0T} for *cis*-DDP of ~ 1 indicates, assuming a Poisson distribution of adducts, that the observed gene expression from *cis*-DDP damaged template originated almost entirely from a DNA population that contained no platinum adducts within the target sequence.

The analysis detailed above relied on the assumption that adducts in the promoter region contributed negligibly to gene inactivation in our experiments. If adducts in the promoter region were important, then our putative bypass efficiencies may have been underestimated. This possibility led to a direct comparison of the relative abilities of *cis*- and *trans*-DDP DNA adducts to block transcription elongation. RNA synthesis through a specific portion of the β-gal coding sequence *in vivo* was monitored by ribonuclease protection analysis (Figure 8A,B). Results of this experiment revealed that 1.2 *cis*-DDP adducts, on average, were required in the transcribed strand of the monitored region to inhibit transcription elongation by 63% (Figure 9). The slope of the dose-response curve for *trans*-DDP predicts that $\sim$ 5 adducts would be necessary to produce equivalent inhibition. These data, obtained by the direct examination of transcript elongation (Table 2), were consistent with those found by measuring overall β-gal expression, suggesting that *cis*-DDP adducts

mediated transcriptional inactivation in our system primarily at the level of the coding region. The data further suggested that *cis*- and *trans*-DDP adducts block RNA polymerase II *in vivo* differentially by a minimum of 4-5 fold. It is noteworthy that the CMV promoter represents only 23% of the total gene inactivating target sequence in CMV-β-gal; the relative size of the promoter compared to the coding sequence, therefore, may be the reason for the observed minimal contributions derived from adducts within the promoter.

2. Molecular basis for transcription inhibition by platinum DNA adducts

It has long been known that DNA damage has an inhibitory effect on transcription (Sauerbier and Hercules, 1978). Bifunctional alkylating agents such as chlorambucil, melphalan, nitrogen mustard (Pieper et al., 1989), and sulfur mustard (Masta et al., 1996) are known to inhibit transcription. DNA adducts formed by acetylaminofluorene (Donahue et al., 1996) and thymine glycol, a lesion arising from ionizing radiation and spontaneous oxidative damage, also block RNA polymerase II *in vitro* (Htun and Johnston, 1992). Cyclobutane pyrimidine dimers, the predominant DNA lesions induced by UV radiation, are strong blocks to rat RNA polymerase II during transcription elongation when present on the transcribed strand *in vitro* (Donahue et al., 1994). *In vivo* studies of UV damage using the host cell reactivation assay report that 1 UV lesion in a ~ 2 kb target was sufficient to reduce gene expression by 63% in XPA fibroblasts (Protic-Sabljic and Kraemer, 1985). Our

results that 1.2 *cis*-DDP adducts were required to effect an equivalent inhibition in XPA lymphoblasts suggested that a single *cis*-DDP adduct presented an equivalent block to transcription elongation as one pyrimidine dimer.

The results outlined in this chapter are in agreement with previous *in vitro* studies examining the effects of specific DNA adducts formed by *cis*- and *trans*-DDP on transcription elongation by purified eukaryotic RNA polymerases (summarized in Table 3). Results of such studies show that each of the three predominant intrastrand adducts formed by *cis*-DDP, including the 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpTpG) cross-links, as well as the interstrand cross-link at a d(GC) site, provide absolute blocks to RNA polymerases when present on the transcribed strand (Corda et al., 1991; Corda et al., 1993). Such adducts comprise ~ 97% of the total adduct spectrum of *cis*-DDP (Bruhn et al., 1990). Our range of bypass rates (0-17%) for *cis*-DDP *in vivo* are thus consistent with results from these *in vitro* studies. By contrast, *in vitro* studies with *trans*-DDP have found that the intrastrand 1,3-d(GpTpG) adduct of *trans*-DDP, unlike that formed by *cis*-DDP, does not provide an absolute block to RNA polymerase *in vitro* (Corda et al., 1993). Although the *trans*-DDP adduct spectrum has not been as well characterized as that of *cis*-DDP, the 1,3-d(GpNpG) adduct could account for ~ 40% of the total adducts formed by the trans isomer (Eastman et al., 1988). Our results (bypass efficiencies of 60-76%) thus suggest that this lesion, and the bulk of the remaining intrastrand adducts formed by *trans*-DDP, are bypassed *in vivo*. The *trans*-DDP interstrand cross-link, which blocks

transcription elongation effectively *in vitro* (Brabec and Leng, 1993) and represents up to 20% of the total *in vitro* adduct spectrum (Eastman et al., 1988; Brabec and Leng, 1993), could be responsible for a significant portion of the ~ 30% blocking efficiency observed in this work.

Why might intrastrand DNA adducts of *cis*-DDP be more effective blocks to RNA polymerase processivity than those of *trans*-DDP? An explanation may lie in the structural differences between the intrastrand adducts of the two compounds. The 1,2-d(GpG) *cis*-DDP adduct induces a directed bend of 34° towards the major groove (Bellon and Lippard, 1990; Yang et al., 1995; Takahara et al., 1995), and unwinds the helix by 13° (Bellon et al., 1991); both the 1,2-d(ApG) and 1,3-d(GpTpG) *cis*-DDP adducts induce similar DNA bending (34°) and unwinding (-13° and -23°, respectively) (Bellon and Lippard, 1990; Bellon et al., 1991). By contrast, the 1,3-d(GpTpG) *trans*-DDP adduct imparts a nondirected bend to the DNA helix, forming a hinge joint (Bellon and Lippard, 1990; Bellon et al., 1991). It is possible that increased flexibility as a structural element of platinum-DNA adducts may allow for more facile translesion synthesis through the site of damage. The recent crystal structure of the 1,2-d(GpG) adduct in a duplex dodecamer reveals that the DNA helix surrounding the lesion undergoes an abrupt change from B-type to an A-type DNA architecture (Takahara et al., 1995). If this unique structure occurs *in vivo*, it is possible that the transcription complex senses and is halted by such a change in DNA structure. Alternatively, it is possible that monofunctional *trans*-DDP adducts

transiently formed during the described rearrangements of the 1,3-d(GpNpG) *trans*-DDP adduct to a 1,4-d(CpGpCpG) adduct (Comess et al., 1990) and to an interstrand crosslink (Dalbies et al., 1994) provide greater opportunity for bypass by the polymerase.

It is additionally interesting that *cis*- and *trans*-DDP adducts differentially affected the translocation of RNA polymerases, while no significant differences are observed in blocking of DNA polymerases. It is reasonable to speculate that enzyme fidelity may be less crucial for RNA polymerase as compared to DNA polymerase. An error that is incorporated during DNA replication would have potentially devastating long-term consequences, whereas reduction in transcriptional fidelity would be expected to be more tolerable. Upon arrest of RNA polymerase II at transcriptional pause sites, it is known that the transcription factor SII can induce nascent transcript cleavage and, in some cases, shortening of the transcript at the 3′ end. These events apparently give the polymerase the opportunity to reelongate the nascent RNA chain through the pause site (Reines, 1994). In this way the eukaryotic transcription complex appears to possess a mechanism that increases the number of chances a polymerase molecule has of bypassing the block.

3. Implications regarding the relative repair of *cis*- and *trans*-DDP adducts

Differential transcriptional inhibition as demonstrated in this report is one of

several effects that may act in concert to bring about the different toxicities of *cis*- and *trans*-DDP. For example, incubation of DNA templates modified by *cis*- or *trans*-DDP with mammalian cell free extracts results in enhanced repair of *trans*-DDP damaged templates over those modified with *cis*-DDP, suggesting that preferential repair may explain, at least in part, the lower toxicity of *trans*-DDP (Hansson and Wood, 1989; Heiger-Bernays et al., 1990). Studies *in vivo*, however, have yielded conflicting views that may be attributable to technical differences in experimental design[1] (Ciccarelli et al., 1985; Roberts and Friedlos, 1987). Interestingly, the results of the present study indicate that, in the system used, excision repair of *cis*- and *trans*-DDP adducts occurs to an equal extent. This conclusion is supported by the relatively proportionate increase in D_0 values for *cis*- and *trans*-DDP when determined in excision repair deficient and then repair proficient cell lines, both of human and rodent origin (Table 1).

The apparent discrepancy between the similar repair of *cis*- and *trans*-DDP observed in the present study and the evidence for preferential repair of *trans*-DDP in some of the literature can be explained in several ways. First, one aforementioned possibility is that the trans isomer is repaired by a pathway in addition to nucleotide excision repair. Second, the possibility exists that a population of *trans*-DDP adducts may be completely uninhibitory to transcription, and therefore would be undetectable by the host cell reactivation assay. If this adduct population were repaired

[1] See section B, part 3 of literature survey for further discussion on this point.

preferentially, then this repair would go undetected as well. These non-inhibitory *trans*-DDP lesions could include specific intrastrand crosslinks, monofunctional adducts, or perhaps transient monofunctional adducts that exist during the described rearrangement from 1,3-intrastrand to 1,4-intrastrand crosslinks of *trans*-DDP (Comess et al., 1990). It should be noted, however, that under the conditions of our platination reactions, few monofunctional adducts should persist (Bancroft et al., 1990).

Another possible explanation for the apparently equal repair of *cis*- and *trans*-DDP in the present study involves the fact that cellular DNA repair is heterogeneous. DNA repair is more efficient in the transcribed strand of expressed genes compared to the nontranscribed strand or unexpressed DNA sequences (Hanawalt, 1994). Models for this phenomenon depict RNA polymerase, stalled at a DNA lesion, facilitating the direction of repair to the transcribed strand of the gene. On this basis it is speculated that strand-specific repair may be related to the degree of transcriptional blocking, or to the degree of chromatin distortion, invoked by the lesion (May et al., 1993). If this view is valid, then a body of evidence (Corda et al., 1993; Mymryk et al., 1995; Mello et al., 1995) suggests that *cis*-DDP may be a better substrate for transcription-coupled repair than *trans*-DDP. cis-DDP adducts are known substrates for transcription-coupled repair both in human (Evans et al., 1993) and rodent cells (Jones et al., 1991; May et al., 1993). The host cell reactivation system used in the present work detects repair of transcription-blocking adducts in the transcribed strand

of the active β-gal gene, and therefore it is likely that this system specifically monitors transcription-coupled strand-specific repair. Previous studies that suggested preferential repair of *trans*-DDP were monitoring overall genome repair (Ciccarelli et al., 1985) or repair by cell extracts of unexpressed DNA sequences (Heiger-Bernays et al., 1990). Thus, although *trans*-DDP may in fact be preferentially repaired in the overall genome, transcription-coupled repair of the transcription blocking *cis*-DDP adducts on the transcribed strand of the active β-gal gene may be compensating for that preferential repair in our system. In this context it should be noted that neither the XPA nor the UV20 repair deficient phenotype used in these studies demonstrate transcription-coupled repair of UV or *cis*-DDP damage (Zhen et al., 1993; Evans et al., 1993; Larminat and Bohr, 1994), and therefore transcription-coupled repair could not have influenced the difference in gene inactivation by *cis*- and *trans*-DDP found in these cell lines.

4. Transcription inhibition as a mediator of cisplatin cytotoxicity

The results outlined in this chapter are consistent with the view that inhibition of transcription may play a role in the cytotoxicity of *cis*-DDP. With regard specifically to the antitumor activity of *cis*-DDP, RNA synthesis is more critical for a rapidly dividing tumor cell than for a stationary cell (Mauck and Green, 1973). Sorenson and colleagues have shown that *cis*-DDP induced cell-cycle arrest in the G2 phase and that the arrested cells proceeded either to recover or, at higher cisplatin

doses, to die via the apoptotic pathway (Sorenson and Eastman, 1988a; Sorenson et al., 1990). Numerous studies have subsequently demonstrated G2 cell-cycle arrest in response to cisplatin treatment (Piacentini et al., 1993; Evans and Dive, 1993; Shinomiya et al., 1994). Sorenson and colleagues proposed that platinum-DNA adducts may trigger apoptosis through a general disruption of gene expression, or by specific inhibition of a gene necessary for mitosis (Sorenson and Eastman, 1988b). Studies testing this hypothesis have yet to be described in the literature.

Supporting the notion that inhibition of gene transcription may be a trigger for apoptosis are results of a recent study examining induction of apoptosis by UV damage. Ljungman *et al.* found that Cockayne's syndrome cells, which are specifically defective in the specialized transcription-coupled repair pathway but are proficient for overall genomic repair, undergo UV induced apoptosis at much lower doses of UV light than normal cells. A comparison of normal and XPC cells, the latter of which are proficient in transcription-coupled repair but are defective in genomic repair, showed that they were induced to undergo apoptosis at identical doses of UV light (Ljungman and Zhang, 1996). Induction of apoptosis in this panel of cell lines correlates with induction of p53 and the inhibition of total and poly(A) mRNA synthesis. These results imply that it is not the number of UV lesions in bulk DNA, but rather the presence and persistence of UV lesions in the transcribed strand of actively transcribed genes, that are critical for induction of apoptosis by UV damage. The authors propose that blocking of RNA polymerase by UV lesions elicits a signal

that, through a cascade of events involving induction of p53, triggers execution of the apoptotic pathway. Given that UV damage and cisplatin are similarly effective at inhibiting RNA polymerase *in vivo* (1849 Protic-Sabljic 1985; this work) and *in vitro* (842 Selby 1990; 1162,1837), these recent findings support Sorenson and Eastman's original hypothesis (Sorenson and Eastman, 1988b) that blocking of RNA transcription by cisplatin may provide a trigger for apoptosis. If inhibition of RNA transcription does play a role in cisplatin-induced apoptosis, the present studies suggest that *trans*-DDP would be much less likely to trigger this pathway.

E. Conclusions and Future Experiments

The focus of the work summarized in this chapter was to compare directly the effects of DNA adducts formed by cisplatin and *trans*-DDP on RNA synthesis in mammalian cells. The results demonstrate that both platinum complexes are capable of inhibiting transcription *in vivo*, but that DNA adducts of *cis*-DDP, on average, are a minimum of 4-5-fold more efficient at blocking transcription elongation by RNA polymerase II *in vivo* than those of *trans*-DDP. The bypass efficiency of RNA polymerase II for a single DNA adduct of *trans*-DDP was calculated to be 60-76%, whereas the rate of bypass of a single *cis*-DDP adduct was found to be 0-17%. These results suggest that inhibition of transcription may contribute to the significantly greater cytotoxicity of cisplatin compared with its therapeutically ineffective trans isomer.

The following are suggestions for future studies:

1. Using the same ribonuclease protection assay system used in the present work, an additional antisense RNA probe complementary to the sequence at the immediate 5′ end of the β-gal mRNA could be designed. This probe would detect differences specifically in nascent initiated transcripts, and thus assess the relative ability of *cis*- and *trans*-DDP to inhibit initiation from the CMV promoter.

2. Transient expression vectors containing single site-specific platinum adducts positioned either in the promoter or the gene coding region could be constructed. These plasmids could then be transfected into cells and the ribonuclease protection assay system developed in this work could be used to examine directly the abilities of individual site-specific DNA adducts of cisplatin and *trans*-DDP to block RNA polymerase II elongation, and to block initiation at the site of the promoter, *in vivo*.

3. To date, examinations of the effects of platinum DNA adducts on transcription *in vitro* have used *E. coli* RNA polymerase or the eukaryotic wheat germ RNA polymerase. Recent studies have described a system using rat RNA polymerase II and in which the effects of SII on the ability of the arrested polymerase to bypass a lesion or to carry out nascent transcript cleavage, as well as the stability of the arrested polymerase complex, could be assessed (Donahue et al., 1994). It would be interesting to examine and compare the individual adducts of *cis*- and *trans*-DDP in this system.

4. The question of whether inhibition of gene transcription by cisplatin is critical for cisplatin-induced apoptosis could be tested by comparing the induction of apoptosis by cisplatin in normal repair proficient cells, XPA cells (completely deficient in excision repair), XPC cells (deficient in overall genomic repair) and CS cells of complementation groups A or B (deficient in transcription-coupled repair). If inhibition of transcription plays a role in triggering apoptosis, CSA/B cells would be expected to be more susceptible to cisplatin-induced apoptosis than both normal and XPC cells.

5. The question of whether the host cell reactivation assay reflects overall genome repair or transcription-coupled repair of cisplatin damage could be examined by comparing the reactivation of cisplatin modified reporter genes in normal, XPA, XPC and CSA/B cell lines. If transcription-coupled repair of cisplatin damage is primarily monitored, reactivation of cisplatin modified plasmid should be relatively similar in normal and XPC cells, while reactivation in XPA and CSA/B would likewise be similar. Any differences in reactivation observed between XPA and XPC cells would reflect the action of transcription-coupled repair on the plasmid DNA.

6. The experimental system described here using RNase protection to map RNA polymerase arrest sites could be used to assess the transcriptional effects of many other DNA damaging agents.

Figure 4: Dose-dependent decrease in β-gal gene expression in HeLa cells transfected with either *cis*-DDP- or *trans*-DDP-damaged CMV-β-gal DNA. β-gal activity in cell lysates was normalized for protein content and is expressed as a percent of that found in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically $\pm 10\%$.

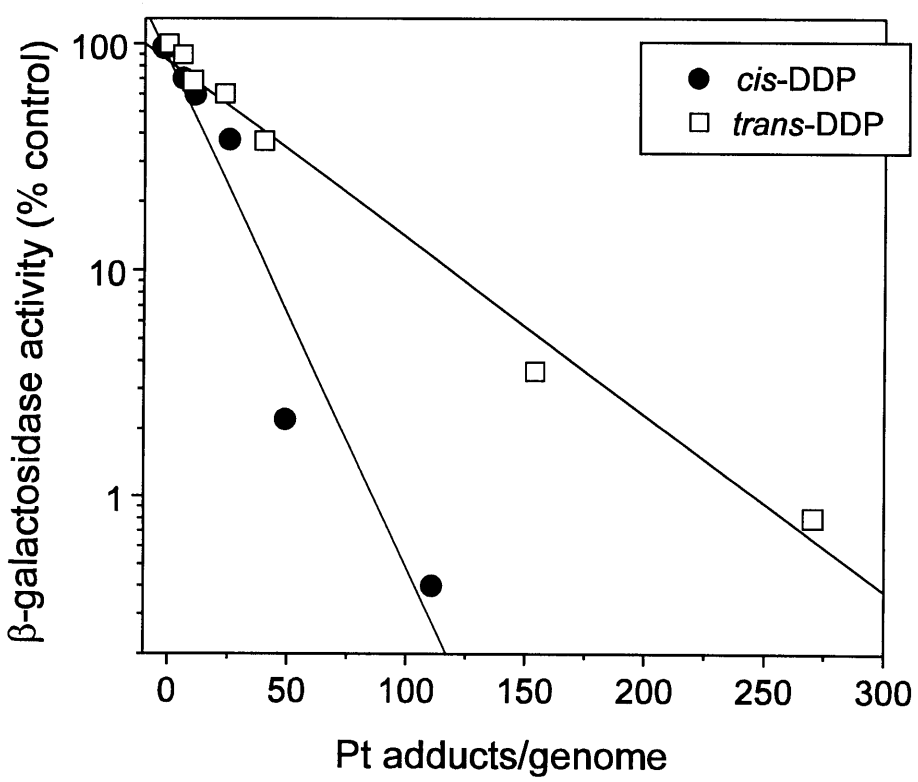

β-galactosidase activity (% control)
100
10
1
0
50
100
150
200
250
300
Pt adducts/genome
cis-DDP
trans-DDP

Figure 5: Dose-dependent decrease in CAT gene expression in HeLa cells transfected with either *cis*-DDP or *trans*-DDP-damaged PSV2-CAT DNA. Amount of CAT protein in cell lysates was normalized for total protein content and is expressed as a percent of that found in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one experiment.

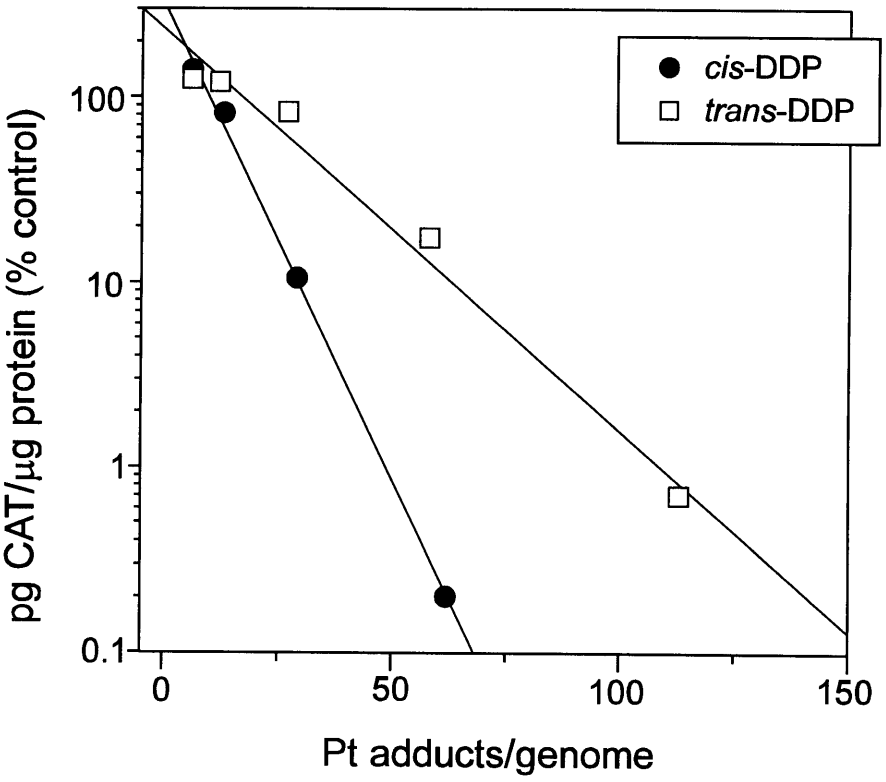

cis-DDP
trans-DDP
pg CAT/μg protein (% control)
100
10
1
0.1
0
50
100
150
Pt adducts/genome

Figure 6: Dose-dependent decrease in β-gal gene expression in repair deficient XPA (top panel) and consanguineous apparently normal (bottom panel) lymphoblastoid cell lines transfected with either *cis*-DDP- or *trans*-DDP-damaged CMV-β-gal DNA. β-Gal activity in cell lysates was normalized for protein content and is expressed as a percent of that found in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically ±10%.

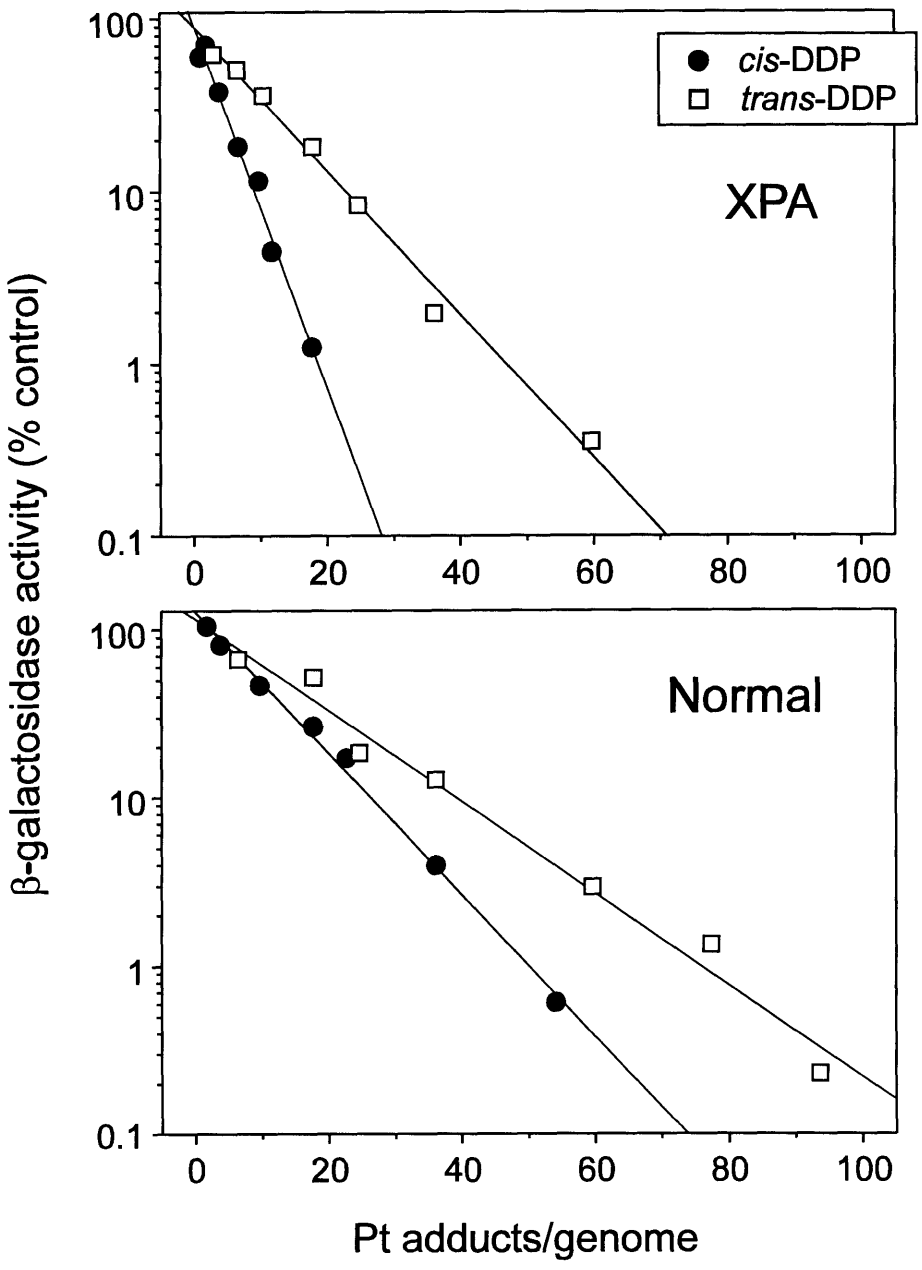

β-galactosidase activity (% control)
100
10
1
0.1
0
20
40
60
80
100
cis-DDP
trans-DDP
XPA
Normal
Pt adducts/genome

Figure 7: Dose-dependent decrease in β-gal gene expression in repair deficient (UV20, left panels) and repair proficient (AA8, right panels) CHO cell lines that had been transfected either 15 h (top panels) or 48 h (bottom panels) earlier with either *cis*-DDP- or *trans*-DDP-damaged CMV-β-gal DNA. β-Gal activity in cell lysates was normalized for protein content and is expressed as a percent of that found in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically ±10%.

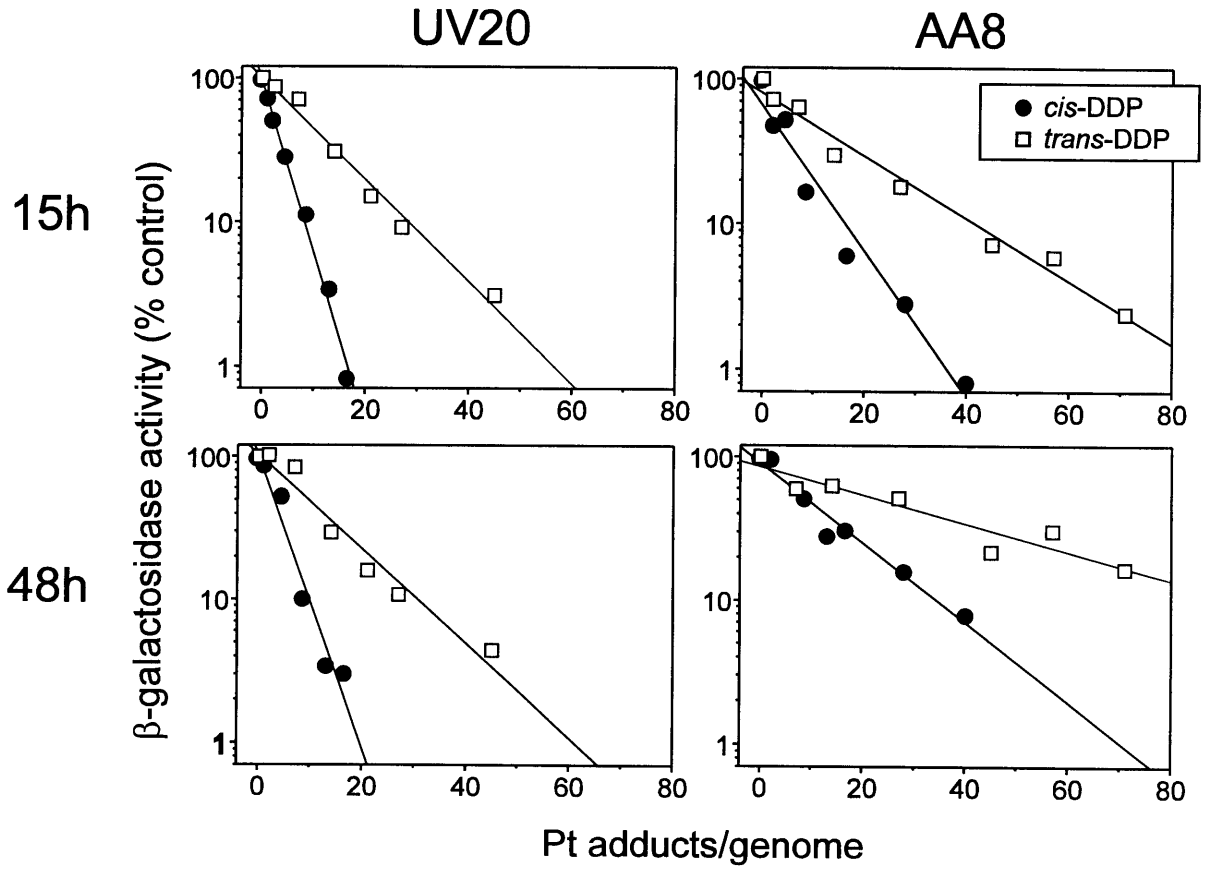

UV20
AA8
15h
48h
β-galactosidase activity (% control)
Pt adducts/genome
cis-DDP
trans-DDP
100
10
1
0
20
40
60
80

Table 1: Transcriptional inactivation (D_0) by *cis*- and *trans*-diamminedichloroplatinum(II) adducts in CMV-β-gal genomes: Comparison of repair deficient and repair proficient cell lines[a]

	D_0			
Platinum Derivative:	***cis*-DDP**		***trans*-DDP**	
DNA Repair Status:	XPA	Normal	XPA	Normal
24 h	3.6[b]	12.4	9.7	21.0
DNA Repair Status:	UV20	AA8	UV20	AA8
15 h	3.5[c]	3.6[c]	12.4[d]	13.5[d]
48 h	3.2[e]	11.0[c]	13.7[e]	35.0[c]

[a] D_0 values are the level of platinum compound in adducts per genome required to reduce expression of the β-galactosidase gene of CMV-β-gal to 37% of that of an unmodified control. D_0 values were determined as described in materials and methods. D_0 values can be converted to r_b by dividing by 14,660, the total number of nucleotides in the genome. Human lymphoblastoid lines were compared from an XP-A patient and her apparently unaffected brother at 24 h after transfection. CHO repair deficient UV20 cells were compared with parental repair proficient AA8 cells at both 15 h and 48 h after transfection.

[b] Values represent the mean of D_0 values obtained from three separate experiments that together utilized two batches of independently platinated DNAs. The relative standard deviations for percent β-galactosidase activity between experiments were typically ±10%. Typical data of one experiment are shown in Figure 6.

[c] Values represent the D_0 from one experiment.

[d] Values represent the mean of D_0 values obtained from two experiments. The relative standard deviations for percent β-galactosidase activity between experiments were typically ±10%. Typical data of one experiment are shown in Figure 7.

[e] Same as in [d], except that the values represent the mean of D_0 values obtained from three experiments.

Figure 8: Blocking of RNA polymerase by platinum adducts was examined directly by ribonuclease protection. (A) Location of two antisense RNA probes that were used to analyze transcription blocking within a 1604 bp sequence of the β-gal coding region. The probes βG5 and βG3 produced protected fragments of 301 and 215 nucleotides, respectively. (B) Total RNA was isolated from XPA cells that had been cotransfected previously with a *cis*- or *trans*-DDP damaged CMV-β-gal plasmid and a second unmodified vector pcDNA3-CAT. Ribonuclease protection assays on total RNA were carried out using antisense RNA probes for each of CAT, β-actin and β-gal as described in materials and methods. The RNA probe used for β-gal was either βG5 (*A* lanes) or βG3 (*B* lanes).

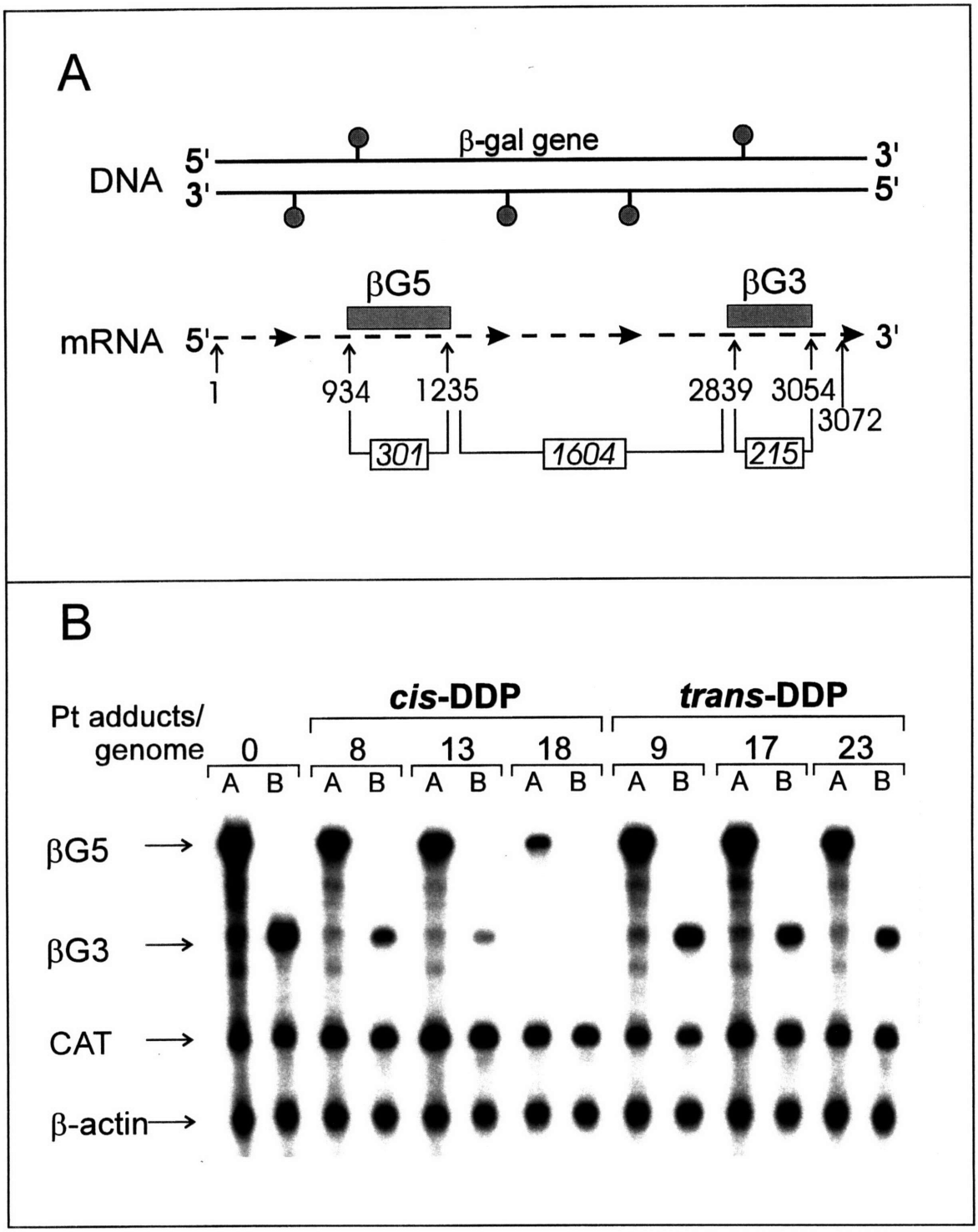

A
β-gal gene
DNA
5'
3'
3'
5'
βG5
βG3
mRNA
5'
3'
1
934
1235
2839
3054
3072
301
1604
215
B
Pt adducts/
genome
cis-DDP
trans-DDP
0
8
13
18
9
17
23
A B
βG5
βG3
CAT
β-actin

Figure 9: Quantitative analysis of ribonuclease protection assay presented in Figure 8. Levels of CAT, β-actin and β-gal mRNA protected fragments were quantitated by PhosphorImaging. The PhosphorImaging intensity of the RNase-resistant portion of each probe was normalized by correcting for the number of radioactive nucleotides. The level of β-gal mRNA in each lane was additionally normalized to the level of CAT mRNA in order to control for variations in transfection efficiency. Bands corresponding to β-actin demonstrate that equivalent amounts of total RNA were used for each lane. The fraction of β-gal mRNA successfully transcribed through the 1604 bp region to reach full length was calculated by the fraction of the normalized values βG3/βG5. This fraction is expressed as a percent of that determined for the unmodified plasmid. Percentages are presented as a function of the number of adducts present, on average, within the 1604 nucleotide sequence.

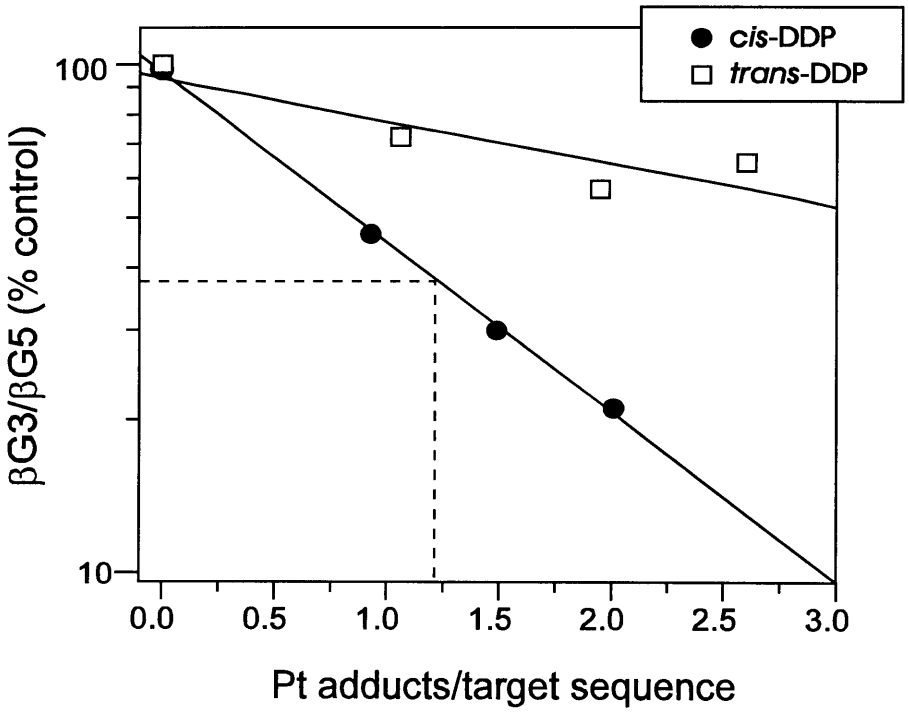

cis-DDP
trans-DDP
100
10
βG3/βG5 (% control)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Pt adducts/target sequence

Table 2: Bypass efficiency of RNA polymerase II for a single representative DNA adduct formed by *cis*- or *trans*-diamminedichloroplatinum(II)

Pt Derivative	***cis*-DDP**	***trans*-DDP**
Human XPA[a]	0-17%	60-70%
CHO-UV20[a]	0-16%	69-76%
Human XPA[b]	16%	ND

[a] RNA polymerase bypass efficiencies were determined from data obtained in transient expression assays. Bypass efficiencies were calculated using D_{0T} values (multiplication of D_0 values summarized in Table 1 by the fraction of the total DNA genome that was the gene inactivation target) as described in Materials and Methods. This calculation of bypass efficiency was carried out twice, where the gene inactivation target sequence was considered to either include, or not, the promoter region of the gene; the two resulting values for bypass efficiency are presented as a maximum and minimum estimation, respectively, of the actual bypass efficiency.

[b] Bypass efficiencies were calculated using D_{0T} values obtained in experiments analyzing transcription blocking by using the ribonuclease protection assay (Figure 8A,B). The gene inactivation target was here considered to be the 1604 nucleotide sequence monitored in the experiment. The number of platinum adducts required within the 1604 nucleotide sequence to allow only 37% full length transcript (D_{0T}) was obtained from the curves exponentially fitted to the data (Figure 9).

Table 3: Comparison of RNA polymerase II bypass efficiencies for *cis*- and *trans*-DDP DNA adducts determined *in vivo* with transcription blocking of RNA polymerases by individual platinum adducts *in vitro*

	***cis*-DDP**			***trans*-DDP**		
	Percentage of total adducts	Inhibition *in vitro*[1]	Bypass efficiency of RNA pol II *in vivo*	Percentage of total adducts	Inhibition *in vitro*[1]	Bypass efficiency of RNA pol II *in vivo*
Interstrand	~2%	+	→ 0-16%	~20%	+	→ 60-76%
Intrastrand						
GG	65%	+		NA[2]	NA	
AG	25%	+		NA	NA	
GNG	8%	+		~40%	–	

[1]Corda, Y., et al. (1991) *Biochemistry 30*, 222.
Corda, Y., et al. (1993) *Biochemistry 32*, 8582.
Brabec, V., and Leng, M. (1993) *Proc. Natl. Acad. Sci. 90*, 5345.
[2]NA, not applicable

IV. THE MISMATCH REPAIR PROTEIN hMSH2 BINDS SELECTIVELY TO DNA ADDUCTS OF CISPLATIN

A. Introduction

The goal of the work presented in this chapter was to evaluate a potential role for the human mismatch repair recognition protein hMSH2 in the mechanism of action of cisplatin. The rationale for this investigation stems from several independent observations made in the literature with respect to mismatch repair: 1) Mismatch repair activity is implicated in the toxic effects of cisplatin in *E. coli*; 2) MSH2 proteins recognize DNA structures similar to those for which HMG-box proteins display affinity; 3) hMSH2 mRNA is reported to be present at elevated levels in testicular tissue, the tissue in which tumors are best treated by cisplatin. These observations raised the question of whether mismatch repair proteins might physically interact with cisplatin DNA adducts and, in so doing, play a role in the cytotoxicity and antitumor activities of cisplatin in human cells. The work discussed in this chapter examines the interaction of purified recombinant hMSH2 with DNA globally modified by cisplatin and various other cisplatin analogs by both southwestern blot analysis and the electrophoretic gel mobility shift assay. The binding of hMSH2 to DNA containing a single site-specific 1,2-intrastrand d(GpG) cisplatin adduct was also examined. Additionally, the relative expression levels of hMSH2 protein in a panel of human tissues were examined by western analysis. The results support the view that mismatch repair may play a role in sensitization of human cells to an important anticancer drug.

B. Materials and Methods

1. Proteins and antibodies

Purified recombinant hMSH2 was provided by S. Acharya and R. Fishel (Thomas Jefferson University, Philadelphia), and was overexpressed and purified as described (Mello et al., 1996). Mouse monoclonal antibodies to hMSH2 and to α-tubulin were purchased from Oncogene Sciences. Goat anti mouse antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology, Inc. and the chemiluminescent substrate was purchased from Amersham.

2. Preparation of platinum-modified DNA probes

cis-DDP, *trans*-DDP, [Pt(en)Cl_2], and [Pt(dien)Cl]Cl were prepared as described (Watt and Cude, 1968; Dhara, 1970; Lippard et al., 1983). Restriction enzyme digestion of pSTR3 with *Cla*I and *EcoR*V yielded 162-bp and 4205-bp restriction fragments. Platination reactions of the restriction fragments were carried out in 3 mM NaCl, 1 mM Na_2HPO_4 (pH 7.4) with 100 μg/ml DNA and appropriate platinum compound/DNA molar ratios by incubating at 37°C for 16 h. Unreacted platinum compound was removed by dialysis (24 h) against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE). Levels of platinum modification were determined by flameless atomic absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95

graphite furnace. The r_b value determined for DNA modified with [Pt(dien)Cl]Cl was approximate. The 162-bp DNA probes were radiolabeled with [α-^{32}P]dCTP (6000 Ci/mmol, New England Nuclear), purified from the 4205-bp restriction fragment on native 5% polyacrylamide gels, and resuspended in TE to 5000-10000 cpm/μl.

A 100-bp DNA probe containing a single, centrally located *cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(1),*N*7(2)}] intrastrand crosslink and the analogous unmodified control were constructed as previously described (Pil and Lippard, 1992), with minor modifications. The six deoxyoligonucleotides used in the construction were as follows: A: 5'-GAG ATC GAT GGA CTA GCC AGC TGC CTT GAT ATC ACG TCA G; B: 5'-TGA TAT CAA GGC AGC TGG CTA GTC CAT CGA TCT C; C: 5'-TCG ACT GAG AAG AGA CCA GAA GGA GAC TGA CG; D: 5'-AGT ACC CGG GTA GTC AAC AGC TGG AGC GAT ATC A; E: 5'-AGT CGA TGA TAT CGC TCC AGC TGT TGA CTA CCC GGG TAC T; GG20: 5'-TCT CCT TCT GGT CTC TTC TC. Deoxyoligonucleotides were purified by polyacrylamide gel electrophoresis. The central 20-base oligonucleotide, Pt-GG20: 5'-TCT CCT TCT G*G*T CTC TTC TC, where the asterisks denote the *cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(1),*N*7(2)}] crosslink, was prepared by reacting 250 μM GG20 deoxyoligonucleotide with 1.4 molar excess of cisplatin in a reaction containing 3 mM NaCl, 1 mM Na_2HPO_4 (pH 7.4) and incubating at 37°C for 20 h. Pt-GG20 was resolved from unreacted deoxyoligonucleotide on a 7 M urea, 20% polyacrylamide gel. The GG-20 control oligonucleotide was carried through these same steps except without cisplatin

in the reactions. The presence of a platinum adduct in Pt-GG20 was demonstrated by complete cyanide reversal to an unmodified state. The presence of a bifunctional platinum adduct was confirmed by determining the molecular mass of Pt-GG20 by electrospray mass spectrometry (calculated molecular mass = 6181, observed molecular mass = 6178).

Oligonucleotides B, C, D, GG20 and Pt-GG20 were 5'-phosphorylated with ATP and T4 polynucleotide kinase. Complementary strands were individually annealed at 1:1 molar ratios (1200 pmol A and B; 1200 pmol D and E; 600 pmol C and GG20; 600 pmol C and Pt-GG20) by heating to 90°C and then cooling to room temperature over 5 h. The DNA duplexes were then mixed in four separate 300 pmol reactions, two containing the platinated Pt-GG20 duplex and two the control GG20 duplex, in 200 μl total volume containing 5 μl ligase and incubated for 12 h at 16°C. Ligated material was phenol-chloroform extracted, ethanol precipitated, resuspended in 1X TE and passed over a Sephadex G-25 QuickSpin (Boehringer Mannheim) column. Full length 100-mers were purified from unligated material by electrophoresis on a 7 M urea, 8% polyacrylamide gel, eluted in 1X TE, ethanol precipitated and resuspended in 1X TE. Following reannealing of the two complementary strands by the same protocol as above, the 100-bp duplex DNA was purified from contaminating single-stranded DNA on a native 12% polyacrylamide electrophoresis gel. Duplex DNA was eluted in 1X TE, ethanol precipitated, air dried and resuspended in 1X TE. Final yields were typically 25-42%. Probes were radiolabeled with [γ-^{32}P]ATP (6000

Ci/mmol, New England Nuclear), passed over a Sephadex G-50 Quick Spin column, and stored at -20°C.

3. Binding assays

Binding assays typically contained radiolabeled 162-bp DNA probes (present at 100-200 pM, 5000-10000 cpm) either unmodified or modified with platinum compounds, and purified hMSH2 present at 0-500 nM. Binding reactions were carried out in 15 μl reactions containing 25 mM potassium phosphate (pH 7.5), 25 mM NaCl, 0.5 mM dithiothreitol, 0.05 mM EDTA, 50 μg/ml BSA, 5% glycerol, and 50 ng of nonspecific chicken erythrocyte competitor DNA. Binding was performed at 35°C for 10 min. Samples were then loaded onto 4% (29:1; acrylamide:bis) native polyacrylamide gels containing 1X TBE (90 mM Tris (pH 8.0), 2.0 mM EDTA, 90 mM boric acid) and 5% glycerol, and electrophoresed at 4°C in 1X TBE at 25 mA for two h. Binding assays with 100-bp probes (100 pM, 8000 cpm) were similarly conducted except that 10 ng of nonspecific chicken erythrocyte competitor DNA was present in the reactions. Amounts of bound and unbound radiolabeled probe were determined by quantitative analysis of dried gels using a Molecular Dynamics PhosphorImager. The $K_{d(app)}$ was determined by a nonlinear least squares fitting of the binding data to the standard Hill equation (Creighton, 1993).

4. Competition assays

Competition assays were carried out by titrating increasing amounts of nonradiolabeled competitor DNA into binding reactions that contained constant amounts of both hMSH2 (present at 130 nM) and ^{32}P-labeled 162-bp cisplatin-modified (r_b = 0.018) DNA probe (present at 150 pM). Nonradiolabeled competitor DNA was pBR322 that was either unmodified (control) or modified with cisplatin (r_b = 0.043).

5. Western blot analysis

Nuclear and whole cell extracts from HeLa cells were prepared as described (Dignam et al., 1983). Whole cell extracts of the human embryonic carcinoma cell line N-Tera 2.D1 (Andrews et al., 1984) were kindly provided by E. Trimmer. Preprepared protein extracts from adult human tissues were obtained from Clontech. For Western blot analysis of hMSH2, 400 μg of protein from human tissues, 75 μg of protein from HeLa nuclear extract, and 10 ng of purified hMSH2 were resolved on an 8% SDS-polyacrylamide gel and electroblotted to nitrocellulose. For Western blot analysis of α-tubulin, 100 μg of protein from human tissues and 75 μg of nuclear HeLa extract were used. Blots were preincubated in 10 mM Tris, 150 mM NaCl, 0.05% Tween (1X TTBS) containing 5% milk for 30 min and washed once with 1X TTBS. The blots were incubated with a primary mouse monoclonal antibody either to

hMSH2 (Oncogene Sciences) or to α-tubulin (Oncogene Sciences) at concentrations of 0.8 μg/ml (1:133 dilution) and 0.1 μg/ml (1:1000 dilution), respectively, in 1X TTBS containing 1% milk for 2 h. Following 3 x 20 min washes with 1X TTBS, blots were incubated with a horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Inc.) at a 1:2000 dilution for 30 min. Blots were then subjected to 3 x 15 min washes with 1X TTBS and 1 x 3 min wash with 10 mM Tris, 150 mM NaCl. Antigen specific signals were visualized by using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations and blots immediately exposed to film for 10-30 min. Autoradiograms of Western blots were scanned using a Hewlett Packard Scanjet IIp and scanned images were analyzed by using the NIH Image 1.6 program. Relative intensities of hMSH2 signal were determined after normalization to the band for α-tubulin.

6. Southwestern analysis

Protein samples (75 μg of HeLa whole cell extract, nuclear extract, and N-Tera 2.D1 whole cell extract) were resolved on 5-15% gradient SDS/polyacrylamide gels and transferred to nitrocellulose membranes. The air-dried membranes were processed as previously described (Toney et al., 1989) with minor modifications. In the probing step, the labeled DNA was present at ~5 x 10^4 cpm/ml, and the nonspecific competitor poly[d(I•C)-d(I•C)] was at 5 μg/ml. The DNA probe was modified with cisplatin (r_b = 0.037), *trans*-DDP (r_b = 0.052) or was an unmodified control. Protein-DNA

complexes were detected by using a Molecular Dynamics PhosphorImager. For the accompanying Western analysis, the filter was probed with a mouse monoclonal antibody to hMSH2 (Oncogene Sciences) at 0.2 μg/ml (1:500 dilution), and antibody binding was visualized by using a chemiluminescent detection system (Bio-Rad).

C. Results

1. Examination of hMSH2 binding to cisplatin modified DNA by Southwestern analysis

Previous studies of interactions between cisplatin modified DNA and proteins present in HeLa cell-free extracts by Southwestern analysis revealed species of 97, 94 and 28 kDa (Toney et al., 1989; Treiber et al., 1994). These observations led to the eventual demonstration that the HMG domain containing proteins hUBF (97/94 kDa) and HMG-1 (28 kDa) bind specifically to cisplatin DNA adducts (Pil and Lippard, 1992; Treiber et al., 1994). In these same studies, a 105 kDa band was also detected with cisplatin modified probes, and to a much lesser extent with unmodified or *trans*-DDP modified probes (Treiber et al., 1994). The molecular weight of hMSH2, 105 kDa, suggested that this mismatch repair protein could be the 105 kDa species detected in the prior studies. Parallel Southwestern and Western blots of proteins present in HeLa nuclear and whole cell extracts were carried out in an effort to determine whether the 105 kDa species was hMSH2 (Figure 10). Whole cell extracts of the

human carcinoma cell line N-Tera 2.D1, which was derived from an embryonal carcinoma testicular tumor (Andrews et al., 1984), were also examined in this experiment for the presence and/or levels of hMSH2; embryonal carcinoma testicular tumor cell lines have been shown to be extremely sensitive to cisplatin toxicity (Oosterhuis et al., 1984).

One single band corresponding to hMSH2, which migrated as 107 kDa, appeared in the Western blot (Figure 10b). Approximately equal levels of hMSH2 were detected in the HeLa and embryonal carcinoma cell extracts. In the Southwestern blots, only two bands were detected in the 100 kDa region (Figure 10a). The more intense of these two bands appeared to correspond to the prior "105 kDa" band in that it was also detected by unmodified and *trans*-DDP modified probes; in my experiments, however, this species ran as 98 kDa. The resolution achieved apparently did not allow detection of discrete 97 and 94 kDa species corresponding to hUBF, which likely are comigrating with this intense band. A second, fainter and slower migrating species, which ran in my experiments as 107 kDa, was specifically detected by cisplatin modified DNA and appeared to migrate at the same position as hMSH2 in the Western blot. It is not clear whether this band was observed in prior studies. Although this upper band appeared to comigrate with hMSH2 in the Western blot, this in no way proves the identity of the species. In order to identify this species definitively as hMSH2, similar experiments would need to be carried out in which both purified hMSH2 protein and extracts from cells deficient in hMSH2 were

included in the Southwestern and Western analysis. Greater resolution in the 100 kDa region of the blot would also be required. These points notwithstanding, we were encouraged to look further at the possibility that hMSH2 might be a platinum adduct binding protein.

2. hMSH2 binds selectively to DNA modified by therapeutically active platinum compounds

In order to test the hypothesis that hMSH2 might recognize cisplatin-modified DNA, purified hMSH2 was used in an electrophoretic mobility shift assay with DNA globally modified by cisplatin and cisplatin analogs (Figure 11). Binding of hMSH2 protein to a radiolabeled 162-bp DNA probe containing cisplatin DNA adducts was readily observed by the retarded migration of the radiolabeled probe through the gel (Figure 12, lane 4). Under identical conditions the protein did not cause a shift of unmodified 162-bp probe (Figure 12, lane 2). Unlabeled nonspecific competitor DNA (chicken erythrocyte DNA of homogeneous length) was required in the binding reactions in order to observe discrete bands. When the synthetic polymer poly[d(I•C)-d(I•C)] was present as the nonspecific competitor DNA in these reactions, shifted bands were more diffuse and relatively little difference in binding was observed between the platinated and unmodified DNA probes. Although the cause of this behavior is unclear, it is possible that the poly[d(I•C)-d(I•C)] forms looped DNA structures that are attractive to hMSH2, and thus compete for specific binding to the

platinated probe.

A hallmark of the interaction of HMG box proteins with platinum damage is the observed selectivity for DNA adducts of therapeutically active platinum compounds (Toney et al., 1989; Pil and Lippard, 1992). It is this specificity that has suggested a role for HMG box proteins in the mechanism of action of cisplatin. In order to determine if hMSH2, which does not contain an HMG box, might display similar selectivity, the interaction of hMSH2 with DNA modified by various cisplatin analogs (Figure 11) was examined. hMSH2 bound to DNA modified by the therapeutically active platinum analog [Pt(en)Cl_2] (en, ethylenediamine) (EN; Figure 12, lane 8), although to a lesser extent than that observed for cisplatin modified DNA. By contrast, hMSH2 displayed no affinity for DNA containing adducts of the inactive platinum complexes *trans*-DDP and [Pt(dien)Cl]$^+$ (dien, diethylenetriamine) (DIEN; Figure 12, lanes 6 and 10, respectively).

Cisplatin and [Pt(en)Cl_2] differ from the two clinically inactive compounds in that the chloride ligands possess a cis geometry, enabling the formation of 1,2-intrastrand crosslinks at adjacent nucleotides. These crosslinks, formed at GG and AG sites, comprise greater than 90% of all adducts formed by cisplatin (Eastman, 1983; Fichtinger-Schepman et al., 1985). The observed hMSH2 binding results suggest that this protein may recognize either or both of the 1,2-intrastrand platinum adducts. Less binding was observed to DNA modified with [Pt(en)Cl_2] compared to that with

cisplatin. This result is interesting in that the two platinum complexes are believed to form the same spectrum of adducts (Eastman, 1983). This differential may be due in part to a slight difference in modification level of the two DNA probes (r_b = 0.018 vs. 0.012 for cisplatin and [Pt(en)Cl_2], respectively). These results may also indicate that substitution of the two ammine groups of cisplatin with a bidentate ligand (ethylenediamine) negatively affects hMSH2-cisplatin adduct interactions.

3. Characterization of the interaction between hMSH2 and cisplatin modified DNA

In order to characterize further the nature of the interaction between hMSH2 and cisplatin modified DNA, hMSH2 protein was titrated into binding reactions containing a constant concentration of cisplatin-modified DNA probe. At hMSH2 concentrations approximating half-maximal binding, two distinct bands were consistently observed (Figure 13A). The addition of increasing amounts of protein caused the complex to be increasingly retarded through the gel, presumably because multiple proteins bound to the multi-platinated probe. The binding isotherm (Figure 13B) revealed that the fraction of bound probe increased to saturation over a narrow range of hMSH2 concentrations, consistent with positive cooperative hMSH2 binding (Hill constant n_H = 2.4). The 162-bp probe used in this experiment contained an average of six platinum adducts per duplex molecule (one platinum adduct per 27 bp). The apparently cooperative binding behavior may thus be a consequence of multiple

platinum adducts situated in relatively close proximity in the duplex DNA; the binding of one hMSH2 molecule to a platinum adduct may render the subsequent binding of a second hMSH2 molecule to a nearby platinum adduct more favorable. Alternatively, hMSH2 may be binding as a dimer or some higher-ordered complex, as has been previously proposed (Fishel et al., 1994a). Generation of the binding isotherm yielded a $K_{d(app)}$ = 67 nM. Neither the active fraction of the hMSH2 preparation nor the aggregation state of the protein have been established, and thus the estimation of $K_{d(app)}$ assumes that hMSH2 binds as a monomer and that 100% of the protein is active in cisplatin-adduct binding. These considerations, coupled with the observed complex nature of hMSH2 binding to the multi-platinated probes, dictate that the $K_{d(app)}$ be considered only an approximation of the affinity of hMSH2 for a platinum adduct.

The specificity of the interaction between hMSH2 and cisplatin modified DNA was examined by competing the association between hMSH2 and [^{32}P]-platinated DNA with nonradiolabeled platinated or unmodified supercoiled plasmid DNA. Increasing concentrations of unlabeled competitor DNA, either modified with cisplatin (r_b = 0.043) or unmodified control, were titrated into binding reactions containing hMSH2 and radiolabeled platinated 162-bp probe (r_b = 0.018). A representative experiment is shown in Figure 14. A 400-fold molar excess of competitor platinum adducts was required to achieve complete competition of hMSH2 binding. An approximately five-fold greater concentration of unmodified competitor DNA compared with cisplatin modified DNA was required to reduce binding by 50% (Figure 14b). This differential

affinity is comparable to that observed when examining the binding of the *S. cerevisiae* MSH2 protein to a G-T mismatch, four-base loop, or 14-nucleotide palindromic insertion loop relative to homoduplex DNA (Alani et al., 1995). Previous studies of the interaction of hMSH2 with DNA probes containing one-base mismatches or insertion/deletion loops also found similar selectivity over homoduplex DNA (Fishel et al., 1994a; Fishel et al., 1994b).

4. hMSH2 binds to DNA containing low levels of cisplatin modification

The degree of DNA modification by cisplatin used in the above experiments, approximating one DNA adduct per 27 bp, is significantly higher than would be encountered *in vivo*. In order to examine whether hMSH2 binds to DNA containing a level of cisplatin modification more closely approximating that found in cellular DNA, binding assays were carried out with hMSH2 and 162-bp probes modified to a range of r_b levels. Discrete shifted bands that represented specific binding to a cisplatin modified DNA probe of r_b = 0.018 (Figures 12, 13, 14 and Figure 15, lane 8) were also observed when hMSH2 was included in binding reactions with DNA probes of r_b = 0.010 (Figure 15, lane 7), 0.0040 (lane 6), 0.0025 (lane 5) and 0.0012 (lane 4). The fraction of probe bound diminished proportionately as the cisplatin modification level decreased, reinforcing the specific nature of this shifted band. The appearance of an increased fraction of unshifted material was likely a reflection of an increasing population of unmodified DNA probe. The lowest modification level of r_b = 0.0012

(lane 4) corresponded to an average of 0.4 cisplatin adducts per probe molecule. The Poisson distribution predicts that 27% of this DNA population would contain a single adduct, that 6% would contain two adducts, and that the remainder of the population would be unmodified. These data thus indicated that hMSH2 is capable of binding to DNA containing a single cisplatin adduct.

5. hMSH2 binds to the 1,2-d(GpG) intrastrand crosslink

The 1,2-intrastrand d(GpG) cisplatin crosslink represents 65% of all adducts formed when this compound is allowed to react with DNA (Bruhn et al., 1990). The observation that hMSH2 binds to globally modified DNA in which a maximum of one to two cisplatin adducts were present suggested that hMSH2 may recognize this 1,2-d(GpG) crosslink. The specificity of hMSH2 for platinum compounds that uniquely form 1,2-intrastrand crosslinks also supported this view. Binding of hMSH2 to a 100-bp probe containing a single d(GpG) adduct was therefore examined. Binding assays revealed a discrete band that was specific to the platinated probe (Figure 16), demonstrating that hMSH2 recognizes the major cisplatin crosslink. However, nonspecific binding to the 100-bp probe was high in these gel shift assays, precluding any thermodynamic analysis of the interaction to be carried out with confidence. The possibility exists that the manner in which the 100 bp probe was constructed, whereby six deoxyoligonucleotides are ligated together, contributed to the observed nonspecific binding with this probe. Although great care was taken in purification of the

deoxyoligonucleotides used, any failure products present could in theory lead to internal one base extrahelical loops in the final ligated material. It is also possible that the length of the probe contributed to this nonspecific binding by providing a high ratio of nonspecific binding sites to specific sites. The presence of multiple cisplatin DNA adducts, or specific binding sites, in the 162 bp DNA probe used in the aforementioned experiments may thus have aided in the detection of specific DNA-protein complexes over nonspecific binding. Previous binding studies with human and yeast MSH2 have used DNA probes of approximately 35 bp in length (Alani et al., 1995; Fishel et al., 1994a; Fishel et al., 1994b); significant nonspecific DNA binding with the yeast protein was described (Alani et al., 1995), however, suggesting that such binding may be characteristic of MSH2 proteins. Nevertheless, the 1,2-d(GpG) adduct present in a shorter DNA probe for which ligation is not required could be useful in future gel shift studies.

6. Relative expression of hMSH2 in human tissues

The expression of hMSH2 was examined by Western analysis of protein extracts prepared from five different human tissues using hMSH2-specific monoclonal antibody. The same amount of total protein was analyzed for each tissue examined. A protein band of M_r 105,000 was observed in all five human tissues, corresponding to full length hMSH2 (Figure 17a). In addition, a lower band of ~ M_r 75,000 that was of approximately equal intensity to the full length hMSH2 band was observed in all

tissues except the ovary, where the lower band was present but faint. This lower band was seen both when using monoclonal antibody (Figure 17) or a polyclonal antibody to hMSH2 (data not shown) and was not a nonspecific signal due to secondary antibody. This protein band is believed to be a specific degradation product of hMSH2 present in these tissue extracts. As a control, these same five human tissue extracts were analyzed by Western analysis for α-tubulin expression (Figure 17b). A sharp band of M_r 50,000 corresponding to α-tubulin was observed. Relatively similar levels of α-tubulin were found in all five protein extracts, demonstrating similar total protein content for each tissue extract.

High levels of hMSH2 protein were found in both testicular and ovarian tissue, while lower levels were observed in tissue of the liver, heart, and colon. Quantitative analysis of hMSH2 specific signals and normalization to that of α-tubulin for each tissue examined indicated this enrichment of hMSH2 in testicular and ovarian tissue to be approximately five-fold. It should be noted that five-fold is likely an underestimate because the chemiluminescent signals due to hMSH2 in the testis and ovary lanes were overexposed beyond the linear range of the film in order that signals could be detected in the other tissues. The observed overexpression of hMSH2 protein in the testis is consistent with previous results from our collaborator showing that hMSH2 is overexpressed in human testicular tissue at the RNA level (Wilson et al., 1995). However, elevated levels of hMSH2 RNA were not previously found in ovarian tissues (Wilson et al., 1995), suggesting that posttranscriptional regulatory mechanisms may

be responsible for the elevated levels found in these tissues. Tumors that arise in tissues of the testis and ovary are those best treated by cisplatin (Loehrer and Einhorn, 1984). The correlation of the enrichment of hMSH2 in these tissues with tumor response to cisplatin treatment supports the possibility that the protein may play a role in sensitizing these tissues to cisplatin toxicity.

D. Discussion

1. hMSH2 binds to therapeutically active platinum DNA adducts

The work summarized in this chapter demonstrates that the human mismatch repair protein hMSH2 recognizes and binds specifically to duplex DNA containing cisplatin DNA adducts. The results also show that hMSH2 binds to DNA containing adducts of the clinically effective cisplatin analog [Pt(en)Cl_2], but not to DNA modified with the therapeutically inactive platinum complexes *trans*-DDP or [Pt(dien)Cl]$^+$. The selective affinity of hMSH2 for DNA modified with cisplatin or [Pt(en)Cl_2] suggests that this protein recognizes the 1,2-intrastrand crosslinks formed uniquely by these two compounds. Setting precedent for such specificity is HMG1, which binds selectively to cisplatin intrastrand 1,2-d(GpG) and 1,2-d(ApG) crosslinks, but lacks specificity for 1,3-d(GpNpG) crosslinks (Pil and Lippard, 1992). Although it has been shown that HMG1 also binds to the interstrand crosslink of cisplatin, but not to that formed by *trans*-DDP (Kasparkova and Brabec, 1995), the infrequency with

which the cisplatin interstrand adduct is formed (2%) (Bruhn et al., 1990) suggested that preferential recognition of this adduct in the above experiments was unlikely. An examination of the binding of hMSH2 to a series of globally modified probes representing a range of platinum modification levels showed that hMSH2 bound to DNA fragments that contained a maximum of one to two cisplatin adducts; this result supported the view that cisplatin 1,2-intrastrand crosslinks, which comprise ~ 90% of the total adduct spectrum, were responsible for hMSH2 recognition. Binding assays using a 100-bp probe containing a single, site-specific 1,2-d(GpG) crosslink (Figure 16) definitively showed that this major adduct (65%) was indeed bound by hMSH2. Further, this result demonstrated that hMSH2 is capable of binding to a single cisplatin DNA adduct. It should be noted that these data do not exclude the possibility that the less abundant 1,2-d(ApG) crosslink and/or the minor interstrand crosslink may also be recognized to some extent.

What is the basis for hMSH2 recognition of cisplatin DNA adducts? It is not yet clear how the presence of a mismatch in the DNA helix dictates recognition by mismatch repair proteins. In general, single-base mismatches induce very minor distortion to the DNA helix (Werntges et al., 1986; Bhattacharyya and Lilley, 1989; Viogt and Topal, 1990). Structural changes induced to DNA upon binding by mismatch repair proteins have also not been described. The observation, however, that both *S. cerevisiae* and human MSH2 display greater selectivity for palindromic and loop insertion mispairs compared to single-base mismatches *in vitro* (Fishel et al.,

1994a; Alani et al., 1995) suggests that these proteins can recognize distorted DNA structures. Bulges in DNA resulting from unpaired bases generate a directed kink in the helical axis (Fazakerley and Boulard, 1995). The cisplatin 1,2-d(GpG) adduct induces directed bending (34°) and local unwinding (13°) to the DNA helix (Bellon and Lippard, 1990; Bellon et al., 1991), and this structural distortion dictates recognition by HMG box proteins. Noteworthy is the structural resemblance between insertion/deletion palindromic loops for which MSH2 proteins display greatest affinity (Fishel et al., 1994a; Alani et al., 1995) and four-way junction and cruciform DNAs bound by HMG proteins (Bianchi et al., 1992; Ferrari et al., 1992; Lilley, 1992). It is thus reasonable to speculate that cisplatin adducts attract hMSH2 by distorting DNA in a manner that mimics the presence of an insertion/deletion mispair, or a favorable DNA structure that is formed during the assembly of mispair-protein complexes. Further structural studies probing the nature of hMSH2-cisplatin DNA adduct interactions are clearly warranted.

Significantly less binding was observed to DNA modified by [Pt(en)Cl_2] than by cisplatin (Figure 12), two compounds that form adducts at identical sites in DNA (Eastman, 1983). This result may be explained in part by the lower level of [Pt(en)Cl_2] modification (r_b = 0.012) compared to that of cisplatin (r_b = 0.018) present on the DNA fragments used in this experiment. Yet the observed affinities of hMSH2 for cisplatin modified DNAs of r_b= 0.018 and 0.010 (Figure 15) would not predict such a large binding differential based on the r_b alone. How else might this result be

explained? The ammine ligands of cisplatin 1,2-intrastrand adducts lie in the major groove of DNA. Substitution of the cisplatin ammines with a series of modified ethylenediamine derivatives does not affect binding by HMG1 (Pil, 1993), an observation consistent with the knowledge that HMG-box proteins bind from the minor groove side (van de Wetering and Clevers, 1992; King and Weiss, 1993; Werner et al., 1995; Love et al., 1995). The present results could indicate that the interaction of hMSH2 with platinum-modified DNA involves contact with the major groove. Alternatively, the ethylenediamine ligand may modulate DNA bending and unwinding induced by cisplatin 1,2-intrastrand adducts (Bellon and Lippard, 1990; Bellon et al., 1991; Yang et al., 1995; Takahara et al., 1995) and interstrand crosslinks (Sip et al., 1992; Malinge et al., 1994; Huang et al., 1995), or could restrict further bending that may be required upon binding by hMSH2. Interestingly, in a recent study that examined binding of mismatch repair proteins in nuclear cell extracts to DNA globally modified with either cisplatin or the analog oxaliplatin, no binding was observed to DNA adducts formed by the latter compound. Oxaliplatin, like $[Pt(en)Cl_2]$, contains a bidentate ligand (diaminocyclohexane) in place of the two ammine groups of cisplatin, thus reinforcing the view that such a substitution in some way interferes with hMSH2 binding (Fink et al., 1996).

2. Affinity and specificity of the hMSH2-cisplatin adduct interaction

The apparent dissociation constant for the binding of hMSH2 to a DNA

fragment containing an average of six cisplatin adducts was found to be 67 nM. The complex nature of binding to the multi-platinated probe in these experiments (see Results section), however, dictates that this $K_{d(app)}$ be viewed as only an approximation of the affinity of hMSH2 for a cisplatin adduct. With this caveat in mind, the estimated $K_{d(app)}$ is roughly comparable with dissociation constants determined by quantitative DNase I footprinting analysis for the binding of the *E. coli* MutS protein to the eight possible single-base mismatches; reported K_d values range from 20 nM, for binding to a G-T mismatch, to 480 nM, for binding to a C-C mispair (Su et al., 1988). To date, dissociation constants for mispair-binding with either *S. cerevisiae* or human MSH2 have not been described.

In this work, although binding by hMSH2 to a single, site-specific 1,2-d(GpG) cisplatin adduct was observed, high nonspecific binding precluded thermodynamic analysis of this interaction. It is possible that the length of the probe (100-bp) used in the gel shift experiment, and/or the way in which the probe was constructed (see results section), may have contributed to this nonspecific binding. However, significant binding to homoduplex DNA has also been described for the yeast MSH2 protein (Alani et al., 1995). In general, high nonspecific binding attributed to DNA repair proteins is not uncommon; such interactions may facilitate the linear diffusion, or tracking, of repair proteins to specific damaged sites in the DNA. Such a model has been suggested experimentally for the prokaryotic DNA repair enzyme T4 Endo V (Nickell et al., 1992). With regard specifically to MSH2, it has been postulated that a

conformation change may take place when a target site is reached that results in increased stability of the DNA-protein complex and recognition signals for interaction with other mismatch repair proteins (Alani et al., 1995). An examination of hMSH2 binding to the 1,2-d(GpG) cisplatin adduct present in a shorter DNA fragment may be useful in the future towards diminishing nonspecific binding observed in this work. The minimum DNA length required for specific hMSH2 binding has not been examined. Footprinting studies with MutS, however, indicate that protein-DNA contacts for the *E.coli* protein span approximately 20 bases surrounding the mispair (Su et al., 1988). An examination of hMSH2 binding to cisplatin adducts by footprinting methods may also be useful in future studies in that nonspecific binding is less of a factor in this technique as compared with band-shift assays.

Competition studies with hMSH2 demonstrated a five-fold greater affinity for DNA containing cisplatin adducts as compared to unmodified DNA. Previous studies with both the *S. cerevisiae* and human MSH2 proteins found a similarly modest degree of specificity for DNA containing single-base mismatches or insertion/deletion loops over homoduplex DNA (Fishel et al., 1994a; Fishel et al., 1994b; Alani et al., 1995). The selectivity of MSH2 proteins for DNA mispairs *in vitro* seems at variance with the highly efficient repair of DNA mispairs in cells. One possible explanation may be that productive repair is achieved through a tracking mechanism as noted above. Further explanation, however, may have been provided by the recent discovery that hMSH2 can be purified as a heterodimer with another human MutS homolog, GTBP/p160 (also

named hMSH6) (Palombo et al., 1995; Drummond et al., 1995). This purified complex, termed hMutSα, also displays an affinity for DNA mispairs *in vitro* (Drummond et al., 1995). Dissociation constants have not been determined, nor has the specificity of binding been described; hence direct comparisons between binding by hMSH2 and hMutSα can not yet be made. The most compelling evidence reported to date for the participation of GTBP in mismatch repair comes from studies in which tumor-derived cell lines defective in hMSH2 or GTBP were examined for mutator phenotype, microsatellite instability (Bhattacharyya et al., 1994; Papadopoulos et al., 1995), and for the capacity of extracts derived from these cells to carry out mismatch repair on a series of mispaired substrates in various sequence contexts (Drummond et al., 1995). The collective data indicate that both hMSH2 and GTBP/p160 are required for recognition of single- and one-base mispairs, but that recognition of larger loops, while requiring hMSH2, can be independent of GTBP/p160.

The observations that loop repair requires hMSH2 but not GTBP/p160 led to the suggestion that hMSH2, either alone or in complex with another protein, recognizes larger loops (Papadopoulos et al., 1995; Drummond et al., 1995; Marsischky et al., 1996). In support of the latter, two independent studies in *S. cerevisiae* revealed that MSH2 forms a heterodimer not only with MSH6, the yeast homolog of GTBP/p160, but also with yet another MutS homolog, MSH3. Results from these genetic studies suggested that the MSH2-MSH3 complex preferentially recognizes insertion/deletion loops over single-base mispairs (Marsischky et al., 1996;

Johnson et al., 1996). Supporting this view, a yeast MSH2-MSH3 heterodimer displayed low affinity for a G/T mismatch *in vitro*, but bound specifically and with increasing affinity to loops containing 2, 4, 8 and 14 extrahelical nucleotides (Habraken et al., 1996). A recent study by Palombo et al. confirmed these results with the human homologs; hMSH2 and hMSH3 were shown to form a heterodimer, which they named hMutSβ, and the purified complex displayed preferential affinity for loops of two to four bases over single base mismatches and one base loops (Palombo et al., 1996). Given that hMSH2 alone is capable of binding mispairs and insertion/deletion loops with modest specificity, it seems that GTBP/p160 and hMSH3 serve to modulate hMSH2 binding specificity, and the two heterodimers hMutSα and hMutSβ appear to complement one another in mispair recognition.

While the work presented in this chapter was In Press (Mello et al., 1996), a study of the purified heterodimer hMutSα binding to cisplatin modified DNA was reported in the literature (Duckett et al., 1996). Results from the study complement the work presented in this chapter. hMutSα was shown to bind tightly to the 1,2-d(GpG) cisplatin adduct, but little or no binding was observed to the 1,3-d(GpTpG) *trans*-DDP adduct. Although no dissociation constants describing the interaction were presented, the affinity for the 1,2-d(GpG) adduct was reported to be ~10-fold lower than that for a GT mismatch, and thus comparable to the affinity of hMutSα for various other single-base mispairs. The authors also stated that hMutSα binds poorly to the 1,2-d(ApG) and 1,3-d(GpTpG) adducts of cisplatin. Numerous experimental

differences, including the length of the platinated DNA probe, sequence context of the adduct, and nature of nonspecific competitor DNA in the binding reactions, make it difficult to make any direct comparisons between binding by hMSH2 and hMutSα to the 1,2-d(GpG) adduct. Yet it is important to note that the preferential affinity of hMutSα for the 1,2-d(GpG) cisplatin adduct is consistent with the substrate specificity displayed by hMSH2 in this Dissertation and in Mello et al. (1996). The reported affinity of hMutSα for the 1,2-d(GpG) but not the 1,2-d(ApG) adduct is intriguing, considering that the two lesions distort the DNA helix in a very similar manner (Bellon and Lippard, 1990; Bellon et al., 1991), and suggests that a very subtle difference in structure is detected by hMutSα. Nevertheless, the results obtained with hMutSα strongly support the view that mismatch repair proteins may participate in the cytotoxic response that occurs upon exposure to cisplatin. With regard to hMutSβ, I predict, based upon its preference for binding to loops over single-base mismatches, that this complex may bind to cisplatin DNA adducts with greater affinity than hMSH2 or hMutSα. Studies addressing this question may provide insight into the basis for recognition of cisplatin adducts by mismatch repair proteins.

3. Overexpression of hMSH2 in human testicular and ovarian tissues

Results discussed above demonstrate that hMSH2 preferentially binds to DNA adducts of therapeutically active platinum compounds. Similar binding specificity has been observed for HMG box proteins. To date, however, few correlations between

expression of HMG proteins and the sensitivity of tissues to cisplatin has been found. Expression of the HMG box protein SRY is required for testes formation, and thus male sex determination, in the mammalian embryo (Koopman et al., 1990). Although the expression pattern in adult tissues has not yet been carefully characterized, the SRY transcript has indeed been detected in testicular tissue, but also in other tissues at slightly lower levels, and SRY expression is reported to be very low overall (Clépet et al., 1993). The full length SRY protein binds to the 1,2-d(GpG) cisplatin adduct with a dissociation constant of 10^{-7} M and with 10-30-fold specificity over unmodified DNA; the isolated HMG-box of SRY binds to the same lesion with 100-fold greater affinity than the full length protein (E. Trimmer, manuscript in preparation). Thus, it has yet to be established whether SRY may play a role in cisplatin cytotoxicity. Although no other human HMG proteins are known to be differentially expressed in testicular tissue, the SRY-related mouse HMG-box protein SOX5 is significantly overexpressed in testicular tissue as compared to five other mouse tissues (Denny et al., 1992b) and a gene encoding a human homolog has been cloned (Denny et al., 1992a). It is also noted that a testis-specific mouse HMG-box (tsHMG) protein has been identified (Boissonneault et al., 1993). Interestingly, a rapidly migrating subtype of HMG2 has been observed to be present at elevated levels in testicular tissue of rat and thirteen other vertebrate species (Bucci et al., 1985). The mRNA for hMSH2 is overexpressed in testis (Wilson et al., 1995), a result that is extended in the present work, which showed enhanced expression at the protein level by Western analysis. Western analysis of hMSH2 protein in various human tissues in the present work

indicates that hMSH2 is also present at elevated levels in ovarian tissue. These observations are significant in that cancers occurring in testicular and ovarian tissues are those most successfully treated by cisplatin.

Between 90 and 95% of all primary testicular malignancies are germ cell tumors (Campbell and Walsh, 1986). In this regard it is noteworthy that the screening of testicular germ cell tumors for microsatellite instability has revealed no mononucleotide or dinucleotide repeat instability commonly associated with hMSH2 deficiency and colorectal cancer (Huddart et al., 1995). Approximately 20% of the tumors showed some abnormalities, predominantly in trinucleotide and tetranucleotide repeats, the significance of which is unknown. The results suggest that hMSH2 protein is functional in testicular germ cell tumors and thus support the notion that mismatch repair proteins may actively contribute to the tissue-specific antitumor activity of cisplatin.

Approximately 5×10^4 cisplatin lesions are formed in the cells of patients upon treatment with the drug (Reed et al., 1993). Unfortunately, the relative level of hMSH2 protein and cisplatin lesions in a cell is unknown because the number of hMSH2 molecules in a cell has not yet been reported. It is nevertheless clear that overexpression of this protein in specific tissues would have the effect of 1) increasing the absolute ratio of number of molecules of hMSH2 protein to cisplatin lesions within those cells, and 2) increasing the concentration of hMSH2 in the nucleus, where the

protein is localized (Leach et al., 1996). With respect to the approximated dissociation constant for the hMSH2-cisplatin adduct complex, an increase in the nuclear concentration of hMSH2, estimated to be at least five-fold in testis and ovary as compared to other tissues evaluated, could disproportionately increase the platinum lesion occupancy by hMSH2. Thus, if mismatch repair proteins can in fact mediate cisplatin lethality by any of the mechanisms discussed below, it is reasonable to predict that they might do so most effectively in the cells of testicular and ovarian tissue.

4. Models for the participation of mismatch repair proteins in cisplatin cytotoxicity

The results presented in this dissertation are the first demonstration that purified hMSH2 can bind to DNA lesions other than mispaired bases or loops. This interaction could signify the involvement of mismatch repair proteins in the removal of cisplatin adducts from DNA. This possibility is underscored by the discovery of an association between the genes involved in mismatch correction with transcription-coupled nucleotide excision repair of UV damage in both *E. coli* (Mellon and Champe, 1996) and eukaryotes (Mellon et al., 1996). Although the role of mismatch repair proteins in these processes is still unclear, it is noteworthy that nucleotide excision repair is the primary mechanism for repair of cisplatin damage and that cisplatin adducts are known substrates for transcription-coupled repair (Jones et al., 1991; May et al., 1993). No evidence exists, however, to suggest that the repair pathway normally responsible for

processing of replication errors is actually involved in the repair of cisplatin damage. Indeed, as is discussed below, the bulk of evidence available with regard to mismatch repair and cisplatin toxicity suggests otherwise, namely that mismatch repair may participate in the events leading to the cytotoxic response that occurs upon cellular exposure to the drug.

The results presented in this chapter clearly demonstrate that hMSH2 displays specificity for DNA adducts of therapeutically effective platinum complexes, results that could imply a role for hMSH2 in mediating cisplatin toxicity. In support of this notion, results from early work by Marinus and colleagues (Karran and Marinus, 1982; Fram et al., 1985) demonstrate a connection between mismatch repair and cisplatin lethality. These authors show that *dam*$^-$ strains of *E. coli*, which have decreased adenine methylase activity and thus are unable to distinguish between parental and newly replicated DNA strands, are hypersensitive both to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and to cisplatin. Significantly, the introduction of a mutation in either *mutS* or *mutL*, which inactivates mismatch repair activity in these cells, abrogates hypersensitivity to both agents (Jones and Wagner, 1981; Karran and Marinus, 1982; Fram et al., 1985). *E. coli dam*$^-$ and wild type strains are not differentially sensitive to the therapeutically inactive platinum compound *trans*-DDP.

Connections between defective mismatch repair and resistance to MNNG have

been observed in higher eukaryotes as well. Goldmacher et al. (Goldmacher et al., 1986) isolated an MNNG-resistant derivative of the human lymphoblastoid cell line TK6. This variant cell line exhibits a mutator phenotype and defective mismatch repair activity *in vitro* (Kat et al., 1993). The observed MNNG-resistant phenotype is not due to increased repair of O^6-methylguanine (O^6-MeGua), but is instead an acquired ability to tolerate the presence of the apparently toxic methylated base in DNA (Karran and Bignami, 1992). Independent examples of methylation-tolerant cell lines that are defective in mismatch repair subsequently have been reported (Branch et al., 1993; Branch et al., 1995; Aquilina et al., 1995). A model to explain this apparently paradoxical correlation of a defect in repair with the tolerance of methylation damage proposes a futile cycle of excision repair (Goldmacher et al., 1986; Karran and Marinus, 1982). In this model, O^6-MeGua lesions formed in DNA are presumed to be recognized by the mismatch repair system due to the absence of a good complementary match for the methylated base. Repair attempts at O^6-MeGua-containing base pairs would be directed at the newly synthesized strand after DNA replication; failure to find a correct base pair for O^6-MeGua would invoke a futile cycle of incision and resynthesis in the opposing strand, resulting in the accumulation of lethal DNA strand breaks. Tolerance results when a defect in mismatch repair precludes initiation of this abortive repair cycle. Supporting this hypothesis is the observation that a G-T mismatch-binding activity in cell extracts, and more recently that the purified heterodimer hMutSα, binds efficiently to O^6-MeGua-T base pairs *in vitro* (Griffin et al., 1994; Duckett et al., 1996). Moreover, a study examining the

processing of O^6-MeGua in plasmid DNA by human cell extracts demonstrates that this lesion can be processed by a pathway involving excision and resynthesis of DNA that is not associated with the O^6-MeGua-DNA methyltransferase enzyme (Karran et al., 1993).

Results outlined in this chapter, viewed together with the original observations of Marinus, suggest that a mechanism analogous to that proposed for O^6-MeGua could be operative in cellular processing of cisplatin adducts. By this model (depicted in Figure 18) the mismatch recognition protein(s), at least one of which is overexpressed in testicular and ovarian tissue, bind to cisplatin-modified bases present in the DNA. Initial recognition triggers the recruitment of other mismatch repair proteins, including hMLH1 and hPMS2, and the consequent initiation of misdirected repair attempts at sites of cisplatin damage. Again, if repair were directed to the newly synthesized strand, the cisplatin lesion responsible for recognition would be retained in the DNA, thereby initiating a futile cycle of excision and resynthesis. This processing could provide, perhaps through the accumulation of DNA strand breaks, a signal for apoptosis. In this regard it is noteworthy that DNA strand breaks have been observed in cells after treatment with cisplatin, although it is unclear whether they originated from repair activity or as a consequence of apoptosis (Sorenson and Eastman, 1988a).

The post-replicative nature of mismatch repair suggests that this mechanism

would be operative only for cisplatin lesions present in newly replicated DNA. It is noteworthy that the strand signal employed for direction of long-patch mismatch repair in mammalian cells is unknown; although the presence of nicks in DNA is capable of both stimulating and directing mismatch repair, undirected mismatch repair does occur in the absence of a strand interruption both in mammalian cells and cell extracts (Varlet et al., 1996). It is therefore conceivable that mismatch repair proteins could also attempt to process cisplatin adducts present in unreplicated DNA. It should be noted that based on current evidence, it is possible that recognition of cisplatin adducts by hMSH2 in complexed form with partner proteins would be required for this model to be operative. The recent demonstration that hMutSα binds efficiently to the 1,2-d(GpG) cisplatin adduct (Duckett et al., 1996) thus lends supports to this model.

A second and equally plausible mechanism by which hMSH2 could contribute to the therapeutic activity of cisplatin is through the formation of a complex with cisplatin adducts that is refractory to nucleotide excision repair (depicted in Figure 19). It is reasoned that slow repair of cisplatin lesions would result in selective retention of the signal that triggers cell death. This notion is supported by the observation that XPA cells, which are deficient in nucleotide excision repair, are hypersensitive to cisplatin (Chu and Berg, 1987). In principle, the involvement of other mismatch repair partner proteins would not be required for this mechanism to be operative, although the same mechanism could be proposed for the binding of hMutSα (or hMutSβ) to cisplatin lesions. The repair shielding model has previously been proposed for the role

of HMG proteins in cisplatin cytotoxicity. Indeed, evidence exists supporting the view that HMG box proteins sensitize cells to cisplatin by shielding cisplatin adducts from repair (Brown et al., 1993; Huang et al., 1994; McA'Nulty and Lippard, 1996). As noted above, however, little correlation between the levels of HMG proteins and the sensitivity of target tissues to cisplatin has been found. The discovery that hMSH2 is able to bind cisplatin adducts and is also overexpressed in testicular and ovarian tissue renders it a reasonable candidate for the repair shielding model. Further assessment of the feasibility of this model would require knowledge of both the abundance of hMSH2 in a cell and the true equilibrium dissociation constant for the protein-cisplatin adduct interaction. Future experiments could be directed at determining if hMSH2, either alone or in combination with partner proteins, shields cisplatin adducts from repair.

An alternative to the above models is that the recognition of cisplatin DNA adducts by hMSH2 could induce cell death by interface with cellular processes other than mismatch repair (depicted in Figure 20). The recognition by mismatch repair proteins of other types of DNA damage, including UV dimers (R. Fishel, unpublished data), DNA adducts formed by 2-aminofluorene and N-acetyl-2-aminofluorene (Li et al., 1996), and base pairs involving O^6-MeGua and O^4-methylthymine (O^4-MeT) (Duckett et al., 1996), has led to the suggestion that mismatch repair proteins may serve as general sensors of genetic damage (Kat et al., 1993; Koi et al., 1994). Of relevance is the recent observation that the mismatch repair deficient, alkylation

tolerant cell line HCT116, which is deficient in hMLH1, fails to arrest at the G2 checkpoint upon exposure to the base analog 6-thioguanine (Hawn et al., 1995). Similar behavior is seen with a cell line deficient in GTBP/p160 following treatment with MNNG (Goldmacher et al., 1986). It is proposed that the mismatch repair system may be involved in triggering G2 arrest in response to alkylating agents, either through the generation of DNA strand breaks, or by interacting directly or indirectly with other protein(s) responsible for the G2 checkpoint (Hawn et al., 1995). It is well established that cisplatin treatment of mammalian cells causes an arrest at G2 for several days, after which cells either recover or become apoptotic (Sorenson and Eastman, 1988a; Sorenson and Eastman, 1988b; Sorenson et al., 1990; Piacentini et al., 1993; Evans and Dive, 1993; Shinomiya et al., 1994). Thus hMSH2 recognition may be involved in cisplatin-induced cell death through the provision of a signal for G2 arrest that does not involve an excision activity associated with mismatch repair. Preliminary experiments carried out by M. Kartalou in our laboratory indicate that the mismatch repair deficient cell line LoVo, which is defective in hMSH2, fails to arrest at G2 after treatment with cisplatin, suggesting that this phenomenon may not be limited to alkylating agent damage.

The models outlined above could conceivably operate in concert to aid the organotropic cytotoxicity of cisplatin. One possible complication is the possibility that HMG box proteins, which are abundant in the cell, may interfere with the ability of hMSH2 to gain access to the cisplatin adducts. Of primary concern in this regard is

hUBF, an HMG protein that binds to cisplatin adducts three to four orders of magnitude more tightly than other known HMG proteins (Treiber et al., 1994). It is noted, however, that hUBF is localized in the nucleolus (Roussel et al., 1993) whereas hMSH2 appears to be more generally distributed in the nucleus (Leach et al., 1996). It is not anticipated, therefore, that hUBF would necessarily compete with hMSH2 for cisplatin adducts in nuclear DNA. By contrast, HMG1, which is not localized to the nucleolus, is approximately ten-fold more abundant than hUBF and binds to the cisplatin 1,2-d(GpG) and -d(ApG) adducts with a $K_{d(app)}$ = 370 nM (Pil and Lippard, 1992). Further speculation on this issue is difficult, however, as neither the abundance of hMSH2 in a cell nor the true binding constant for the interaction of hMSH2 (or hMutSα) with a cisplatin adduct is known at this time.

5. Evidence *in vivo* for a connection between human mismatch repair proteins and cisplatin cytotoxicity

A growing body of work exists implicating mismatch repair machinery as a modulator of the cytotoxic response of mammalian cells to cisplatin. The first line of evidence comes from the analysis of cisplatin resistant human cell lines. A panel of tumor cell lines selected for resistance to cisplatin following exposure to the drug were screened for the RER^+ phenotype that is associated with defects in mismatch repair. This phenotype was found to be common in the cisplatin resistant lines, and one such RER^+ cell line was examined and shown to be deficient in G-T mismatch binding

activity (Anthoney et al., 1996). In independent studies, other tumor cell lines selected for resistance to cisplatin were found to display a mutator phenotype, microsatellite instability (Aebi et al., 1996), and to contain associated defects in the mismatch repair proteins hMLH1 and hPMS2 (Aebi et al., 1996; Drummond et al., 1996). It is noteworthy that while these studies show that loss in mismatch repair can accompany cisplatin resistance, they do not in any way indicate that such repair deficiency is actively contributing to that resistance.

Complementary studies to those described above have attempted to determine whether loss of mismatch repair in itself can contribute to cisplatin resistance. Studies show that the mismatch repair deficient cell lines HCT116 and HEC59, which are defective in hMLH1 and hMSH2, respectively, are two fold resistant to cisplatin when compared to proficient sublines complemented with the chromosome carrying the respective functional gene[2] (Aebi et al., 1996; Fink et al., 1996). Similar differential sensitivity was also observed to the therapeutically effective cisplatin analog carboplatin (Fink et al., 1996). By contrast, these mismatch repair deficient and proficient cell lines are not differentially sensitive to *trans*-DDP or several other platinum complexes, including oxaliplatin (Fink et al., 1996). In this same study, gel mobility shift assays carried out with nuclear cell extracts demonstrate that, in contrast to cisplatin modified DNA, DNA modified by oxaliplatin was not recognized by

[2] The HCT116 cell line may harbor an additional defect in hMSH3 (R. Fishel, personal communication).

mismatch repair proteins present in the extracts. These results support the view that recognition of platinum DNA adducts by mismatch repair proteins has a functional consequence, namely a change in drug sensitivity. Studies carried out by M. Kartalou in our laboratory have also found that mismatch repair deficient cells are two to four fold resistant to cisplatin compared to normal cells. The cell lines used in the latter work are not isogenic, however, and hence the observed resistance cannot be definitively attributed to mismatch repair. Studies on isogenic cell lines are in progress.

The results detailed above could have far-reaching implications for the problem of acquired clinical resistance to cisplatin. A tumor that acquires resistance to cisplatin and an associated loss in mismatch repair following treatment with the drug would likely display cross-resistance to methylating, and perhaps other alkylating (e.g., *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (Tong et al., 1982)) anticancer agents. Likewise, the inactivation of the mismatch repair pathway by alkylating agent anticancer drugs may lead to tumors that are relatively refractory to treatment by cisplatin. Such observations may have profound clinical importance in determining optimum treatment regimens for cancer patients. Moreover, a further understanding of the precise role mismatch repair proteins may play in the therapeutic mechanism of cisplatin may ultimately prove useful in the design of more effective anticancer drugs.

E. Conclusions and Future Experiments

The work summarized in this chapter demonstrates a novel platinum adduct binding property of the human mismatch repair protein, hMSH2. The protein binds selectively to the adducts of platinum based drugs that show clinical efficacy or potential. The significance of the work is threefold. First, hMSH2 is overly expressed in testis and ovary, the two tissues in which tumors are best treated by cisplatin. These observations suggest that hMSH2 may contribute to the organotropism of the drug. Second, the present demonstration of an interaction between hMSH2 and cisplatin DNA adducts *in vitro* complements a growing body of literature correlating mismatch repair activity with both cisplatin and methylating agent cytotoxicity. Viewed together, these observations are consistent with a model whereby mismatch repair plays an active role enhancing DNA lesion toxicity. Moreover, because mismatch repair deficiency correlates with resistance to alkylating agents, our results may provide insight into a heretofore undiscovered mechanism by which tumor cells may acquire resistance to cisplatin. Finally, the demonstration that hMSH2 binds to DNA lesions other than single-base mismatches and insertion/deletion mispairs could imply a broader range of substrates for the mismatch repair system, and the possibility of multiple roles for mismatch recognition proteins. Based on the results of this dissertation, the following are suggested for future studies.

1. Both hMSH2 and GTBP/p160 together are presently believed to function as a

recognition complex in mismatch repair. Yet hMSH2 alone is capable of binding to mispairs and DNA damage *in vitro*. It thus remains unclear whether such recognition by hMSH2 alone occurs *in vivo*, and whether such binding may be productive. A direct comparison of the binding by hMSH2 and the heterodimer hMutSα to a cisplatin lesion, involving both thermodynamic and kinetic analysis, would provide biochemical evidence for which protein(s) are most likely to interact with cisplatin adducts *in vivo*. Information gained from these experiments could reveal the nature of the individual contributions made by GTBP/p160 to the formation of hMutSα-DNA mispair complexes.

2. As was discussed above, hMSH2 forms complexes with both GTBP/p160 and hMSH3 to form hMutSα and hMutSβ, respectively. These two heterodimers display complementary binding specificities, whereby hMutSα binds preferentially to single base and one base mispairs and hMutSβ shows greatest affinity for two base or larger insertion/deletion loops. The DNA bending and unwinding induced by cisplatin DNA adducts would suggest that hMutSβ, which binds preferentially to the subset of mispairs that create the greatest distortion to DNA, might also recognize cisplatin lesions. A comparison by gel shift assay of the binding of hMutSα and hMutSβ to the 1,2-d(GpG) adduct should be carried out to address this question. The relative binding of the two recognition complexes to the 1,2-d(ApG), 1,3-d(GpNpG) and to the interstrand crosslink would also be interesting to examine. Results of these studies would provide insight into the basis for recognition of both mispaired substrates and

cisplatin adducts by these mismatch recognition proteins. Further, they could indicate which heteroduplex would be most likely to play a role in the processing of cisplatin adducts by mismatch repair *in vivo*.

3. The structural features of DNA-protein complexes formed by hMSH2, hMutSα or hMutSβ are completely unknown. An examination of mismatch recognition protein(s) binding to DNA that is modified with platinum compounds in which the cisplatin ammines are substituted with various bulky ethylenediamine derivatives could provide information as to whether minor groove contacts are important for binding. More direct information, however, could be obtained through both DNase I footprinting and methylation protection footprinting analysis, and eventually by NMR and X-ray crystallography. Such studies could provide information regarding: 1) the extent of protein-DNA contacts surrounding the mispair or cisplatin adduct, 2) whether the mismatch repair protein(s) make primarily major or minor groove contacts, and 3) whether GTBP/p160 or hMSH3 modulates hMSH2 binding specificity through the provision of additional DNA contacts.

4. Initial cisplatin cytotoxicity assays have been carried out by M. Kartalou in the laboratory using a panel of mismatch repair deficient and proficient, but non-isogenic, cell lines; although results indicate a 2-4 fold resistance to cisplatin of mismatch repair deficient cells compared to proficient cells, definitive conclusions cannot be made owing to the different genetic backgrounds of the cells. Cytotoxicity assays should

ideally be carried out with a panel of isogenic cell lines in which each of the mismatch repair recognition proteins, hMSH2, GTBP/p160, and hMSH3 are defective. These studies will complement the binding experiments involving hMutSα and hMutSβ proposed above. The relative binding affinities of hMutSα and hMutSβ for cisplatin adducts would perhaps be reflected in the cisplatin sensitivities of cell lines defective for the corresponding proteins. For example, if hMutSβ were found to bind significantly more tightly to cisplatin adducts than hMutSα, then it would be predicted that cell lines deficient in hMSH3 or hMSH2 would be more resistant to cisplatin toxicity than cell lines deficient in GTBP/p160. Examination of isogenic cell lines deficient in other proteins required for repair, including hMLH1 and hPMS2, would indicate whether subsequent binding of these proteins to the initial recognition complex is required for the putative processing of cisplatin adducts by mismatch repair. Isogenic cell lines created in Hein te Riele's laboratory in the Netherlands will enable this work.

5. In conjunction with the toxicity assays described above, the possible role of mismatch recognition proteins in the G2 checkpoint in response to cisplatin treatment could also be addressed. Following treatment of mismatch repair proficient and deficient cell lines with cisplatin, the population of cells in each phase of the cell cycle can be examined by using flow cytometry. As noted in the discussion, preliminary experiments carried out by M. Kartalou in our laboratory indicate that the hMSH2 deficient cell line LoVo lacks the ability to arrest at G2 upon cisplatin treatment, but

that other cell lines deficient in hMLH1 or GTBP/p160 do not exhibit this same defect. As the cell lines used in this experiment are not isogenic, and controls with methylating agents have not yet been carried out, the potential role of mismatch repair in cisplatin induced G2 arrest is still unclear. The panel of isogenic cell lines discussed above will thus also prove useful in these studies.

6. A DNA repair synthesis assay using replication competent cell-free extracts has previously been used to demonstrate that O^6-MeGua lesions are processed *in vitro* by a pathway that involves the excision and T-antigen independent resynthesis of DNA; the demonstration that such processing is absent in extracts made from mismatch repair deficient cells attributes these activities to the mismatch repair machinery (Karran et al., 1993; Ceccotti et al., 1996). The proposed abortive repair mechanism for cisplatin adducts could thus be tested *in vitro* by examining whether excision and DNA synthesis activity due to mismatch repair is provoked by cisplatin modified DNA by using this same system. A key feature of this assay is that nucleotide excision repair is apparently not active in the cytoplasmic cell extracts used, thus eliminating the significant potential problem of simultaneous cisplatin adduct repair by the nucleotide excision repair machinery. It is possible that globally modified plasmid DNA could be useful for such studies, although plasmids containing single site-specific cisplatin adducts would be the ideal substrates. The preparation of such substrates has been a longstanding area of expertise in this laboratory.

7. The proposed repair shielding model could be tested *in vitro* by using either purified UvrABC proteins or mammalian cell extracts competent for repair. Preincubation of DNA substrates containing either global or site-specific DNA damage with mismatch recognition protein(s) facilitates formation of DNA-protein complexes. DNA substrates are then added to repair reactions and the effect of bound protein(s) on the repair of platinum adducts is monitored. With regard specifically to the 1,2-d(GpG) adduct, excision repair of this lesion was previously found to be inefficient in assays using mammalian cell extracts; the methods of J. Huang et al. (Huang et al., 1994) have subsequently allowed detection of such repair *in vitro* and may therefore be useful in such studies.

Figure 10: Southwestern and Western analysis of HeLa and N-Tera 2.D1 cell extracts. Parallel blots of HeLa whole cell extract (HWC), HeLa nuclear extract (HN), and N-Tera 2.D1 whole cell extract (NTer) were probed with radiolabeled cisplatin modified DNA in Southwestern analysis (A) or monoclonal antibody for hMSH2 in Western analysis (B). In Southwestern analysis, control blots of HWC and HN were probed with radiolabeled unmodified DNA or DNA modified with *trans*-DDP (A). The molecular weights of species detected in HeLa cell extract that recognize cisplatin modified DNA are noted in A. The position of hMSH2 is shown in the Western blot (B).

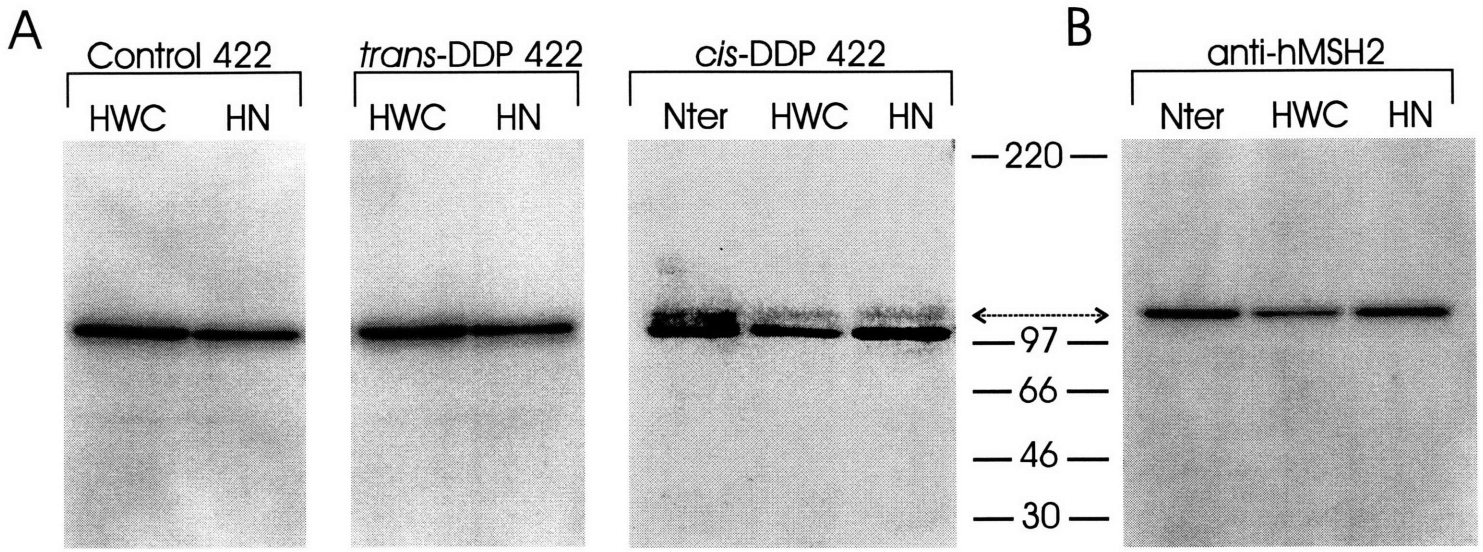

A
Control 422
HWC
HN
trans-DDP 422
HWC
HN
cis-DDP 422
Nter
HWC
HN
B
anti-hMSH2
Nter
HWC
HN
220
97
66
46
30

Figure 11: Structures of platinum compounds used in hMSH2 binding studies. Cisplatin (*cis*-DDP) and [Pt(en)Cl_2], both therapeutically active platinum complexes, have chloride ligands in the cis geometry. The trans isomer of cisplatin, *trans*-DDP, and [Pt(dien)Cl]Cl are clinically ineffective cisplatin analogs.

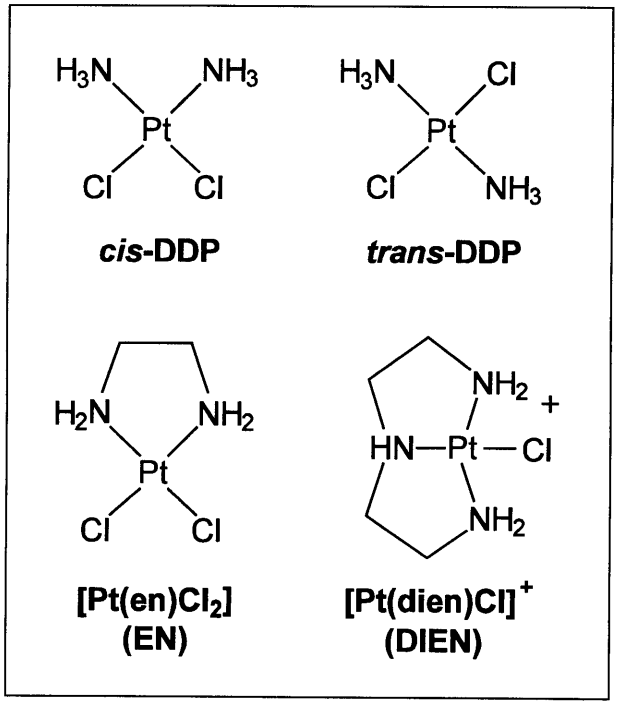

H3N
NH3
Pt
Cl
Cl
cis-DDP
H3N
Cl
Pt
Cl
NH3
trans-DDP
H2N
NH2
Pt
Cl
Cl
[Pt(en)Cl2]
(EN)
NH2
+
HN—Pt—Cl
NH2
[Pt(dien)Cl]+
(DIEN)

Figure 12: Selectivity of hMSH2 for DNA modified with therapeutically active platinum compounds. Radiolabeled 162-bp DNA probe was modified with cisplatin (lanes 3 and 4), *trans*-DDP (lanes 5 and 6), $[Pt(en)Cl_2]$ (lanes 7 and 8), or [Pt(dien)Cl]Cl (lanes 9 and 10) at drug-to-nucleotide ratios (r_b) = 0.018, 0.036, 0.012, and 0.020, respectively. Unmodified 162-bp probe appears in lanes 1 and 2. DNA probes were incubated in the absence (-) or presence (+) of hMSH2 (100 nM). Two discrete shifted bands were observed only when hMSH2 was incubated with DNA containing adducts of the therapeutically active complexes cisplatin or $[Pt(en)Cl_2]$ (lanes 4 and 8, respectively).

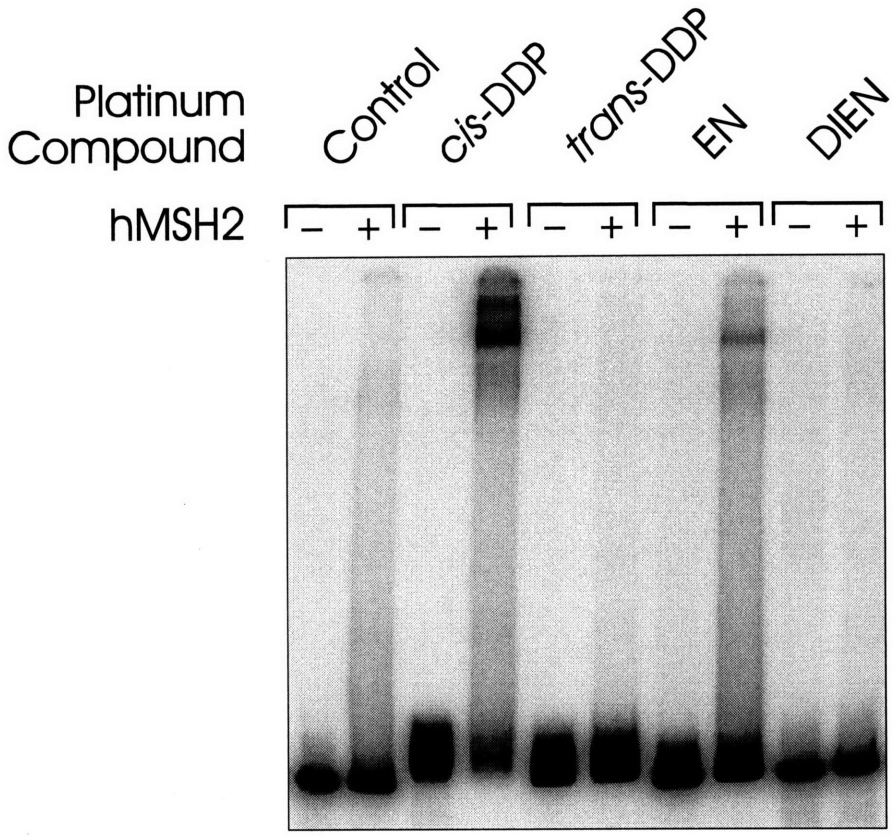

Platinum Compound
Control
c/s-DDP
trans-DDP
EN
DIEN
hMSH2
– + – + – + – + – +

Figure 13: Binding isotherm describing the interaction between hMSH2 and cisplatin-modified DNA. (A) hMSH2 protein was titrated into binding reactions containing 124 pM radiolabeled 162-bp probe that contained an average of 6 cisplatin DNA adducts (r_b = 0.018). Cisplatin-modified probe in the absence of hMSH2 is shown in lane 1. (B) The fraction of bound probe in each lane was quantitated by PhosphorImager analysis and is presented as a function of the concentration of hMSH2 present in the binding reactions. The binding curve was generated by fitting these binding data to the Hill equation.

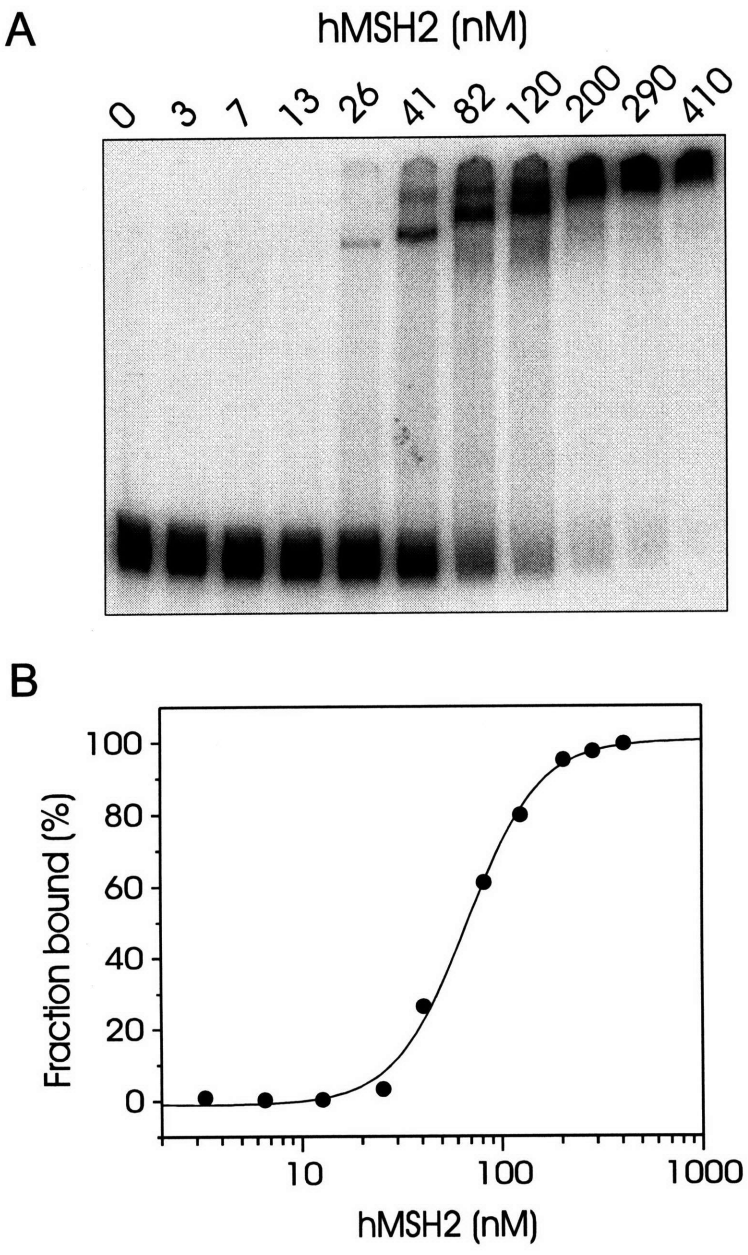

A
hMSH2 (nM)
0
3
7
13
26
41
82
120
200
290
410
B
Fraction bound (%)
100
80
60
40
20
0
10
100
1000
hMSH2 (nM)

Figure 14: Specificity of hMSH2 binding to cisplatin-modified DNA. Unlabeled duplex DNA, either cisplatin-modified or unmodified control, was used to compete the association between hMSH2 and a radiolabeled 162-bp cisplatin-modified DNA probe. (A) ^{32}P-labeled 162-bp probe modified with cisplatin (r_b = 0.018, 150 pM) was incubated in the presence of hMSH2 (100 nM) and 0-710 pM unlabeled cisplatin-modified DNA (r_b = 0.043) (left panel), or 0-6400 pM unlabeled unmodified DNA (right panel). Cisplatin-modified probe in the absence of hMSH2 is shown in lane 1 for either competitor. (B) The fraction of bound probe in each lane was quantitated by PhosphorImager analysis and is presented as a function of the concentration of competitor DNA present in the binding reactions.

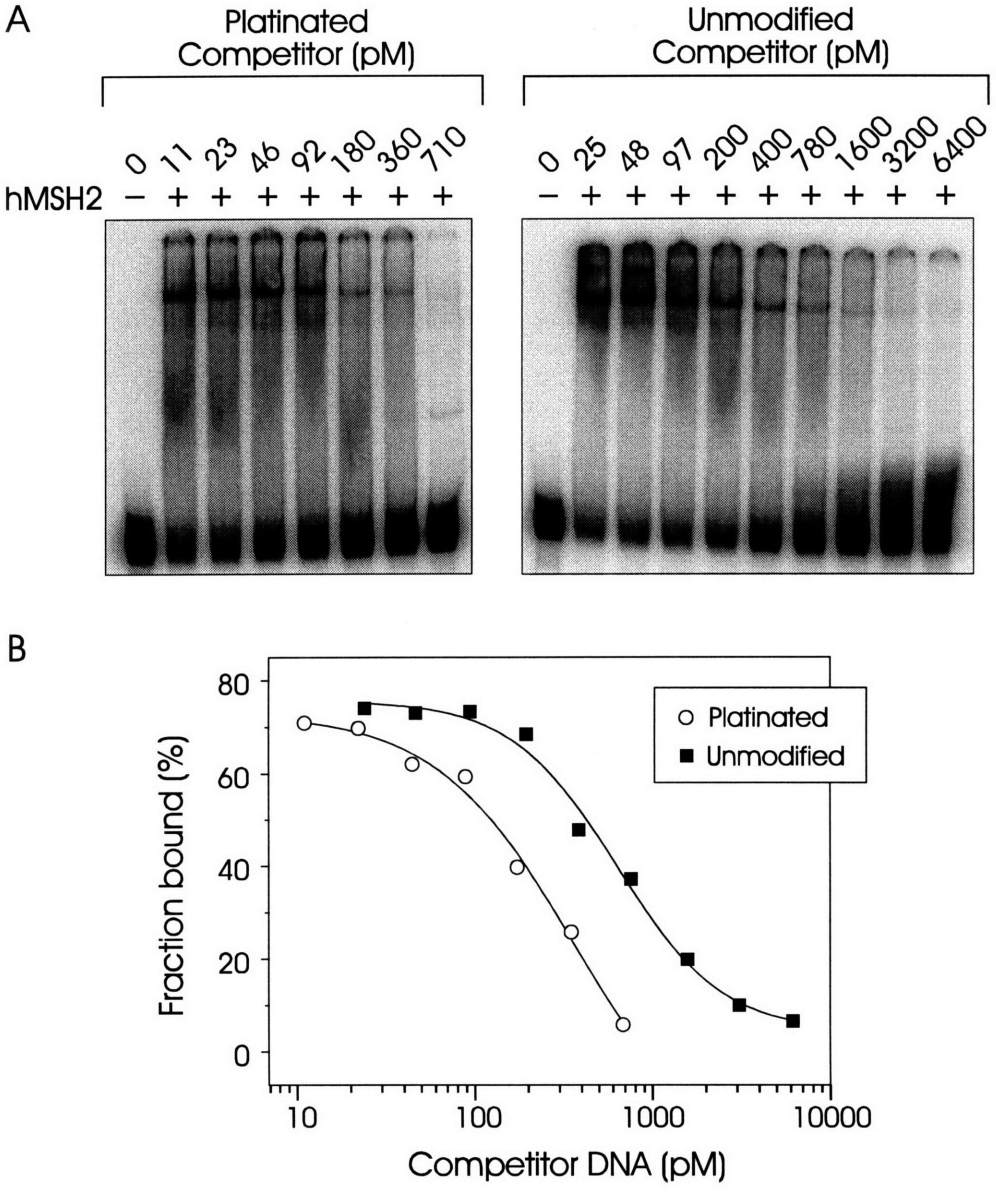

A
Platinated Competitor (pM)
Unmodified Competitor (pM)
0 11 23 46 92 180 360 710
0 25 48 97 200 400 780 1600 3200 6400
hMSH2 − + + + + + + +
− + + + + + + + + +
B
Fraction bound (%)
80
60
40
20
0
10
100
1000
10000
Competitor DNA (pM)
Platinated
Unmodified

Figure 15: Binding of hMSH2 to cisplatin-modified DNA at different bound ratios of Pt/nucleotide (r_b). Radiolabeled 162-bp probe modified with cisplatin at drug-to-nucleotide ratios of 0.0012 (lane 4), 0.0025 (lane 5), 0.0040 (lane 6), 0.010 (lane 3 and 7) and 0.018 (lane 8), corresponding to an average of 0.4, 0.8, 1.3, 3.4 and 6 cisplatin adducts per probe molecule, respectively, were incubated in the absence (-) or presence (+) of hMSH2 (130 nM). Unmodified probe appears in lanes 1 and 2. Retarded bands representing specific binding of hMSH2 to the DNA probe of r_b = 0.018 (Figures 12, 13 and 14 and Figure 15, lane 8) were also present at the lower levels of platinum modification. Specific binding diminished as the degree of cisplatin modification decreased. The broad, faster migrating band present in all lanes containing protein represents nonspecific binding to the 162-bp probe that was incompletely competed by nonspecific competitor DNA present in the binding reactions.

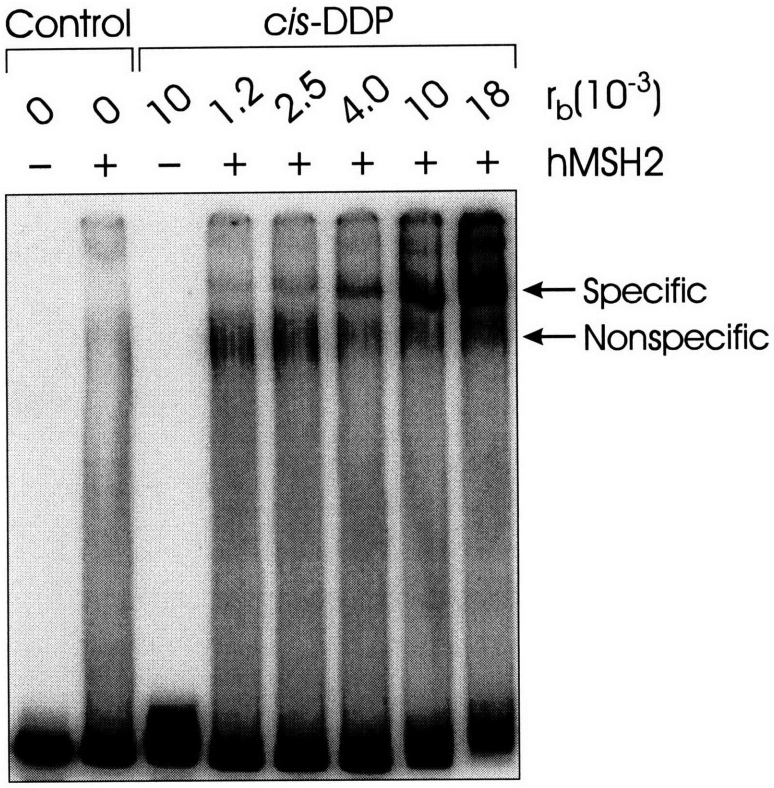

Control
cis-DDP
0 0 10 1.2 2.5 4.0 10 18 $r_b(10^{-3})$
− + − + + + + + hMSH2
← Specific
← Nonspecific

Figure 16: Binding of hMSH2 to a 100-bp probe containing a single, site-specific 1,2-d(GpG) cisplatin intrastrand crosslink. hMSH2 (160 nM) was incubated with 100-bp probe that contained the major cisplatin adduct or with its unplatinated control (100 pM). A discrete shifted band was visible that was specific for the platinated probe, while a faster migrating band representing nonspecific binding was observed with both the platinated and unplatinated probes.

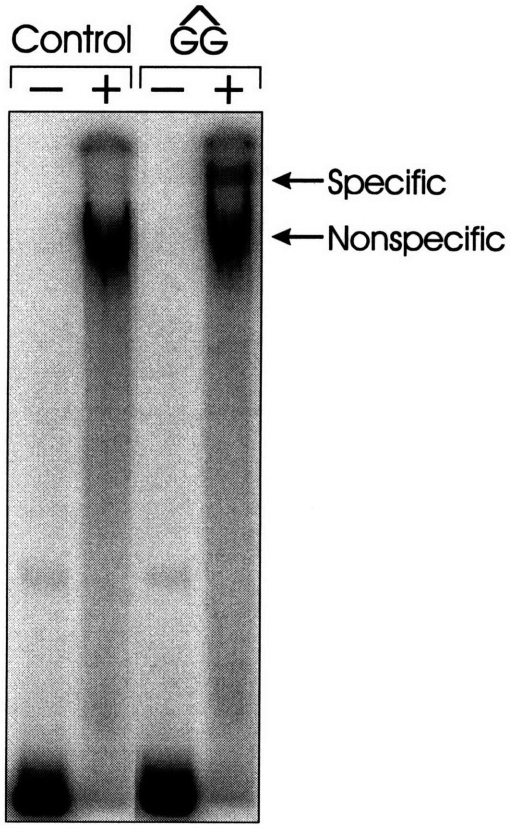

Control
GG
−
+
−
+
Specific
Nonspecific

Figure 17: Western blot analysis of hMSH2 expression in human tissue. (A) Protein extracts derived from human tissues were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with monoclonal antibody to hMSH2. Nuclear extract of HeLa cells and purified hMSH2 were included as positive controls. Lane 1, Heart; Lane 2, Liver; Lane 3, Ovary; Lane 4, Testis; Lane 5, Colon; Lane 6, HeLa nuclear extract; Lane 7, purified hMSH2 from a separate loading. The upper band corresponds to full length hMSH2 protein, M_r 105,000. The lower band is believed to be a specific degradation product of hMSH2 present in these tissue extracts. (B) Protein extracts from human tissues were blotted and probed with monoclonal antibody to α-tubulin, demonstrating similar total protein content for each tissue sample.

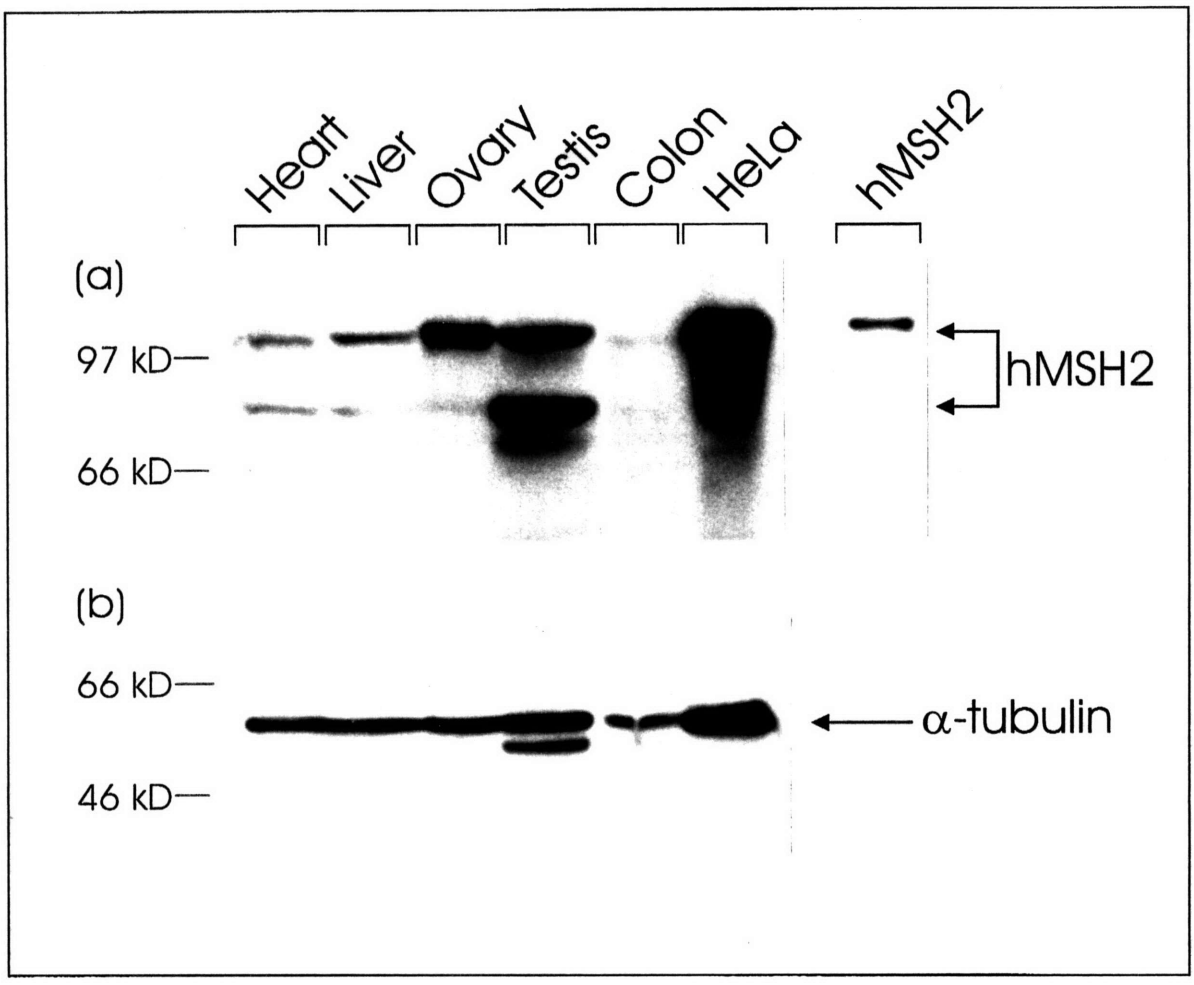

Heart
Liver
Ovary
Testis
Colon
HeLa
hMSH2
(a)
97 kD
66 kD
hMSH2
(b)
66 kD
46 kD
α-tubulin

Figure 18: The abortive repair model for the role of hMSH2 in potentiating cisplatin cytotoxicity. See text (discussion section) for details.

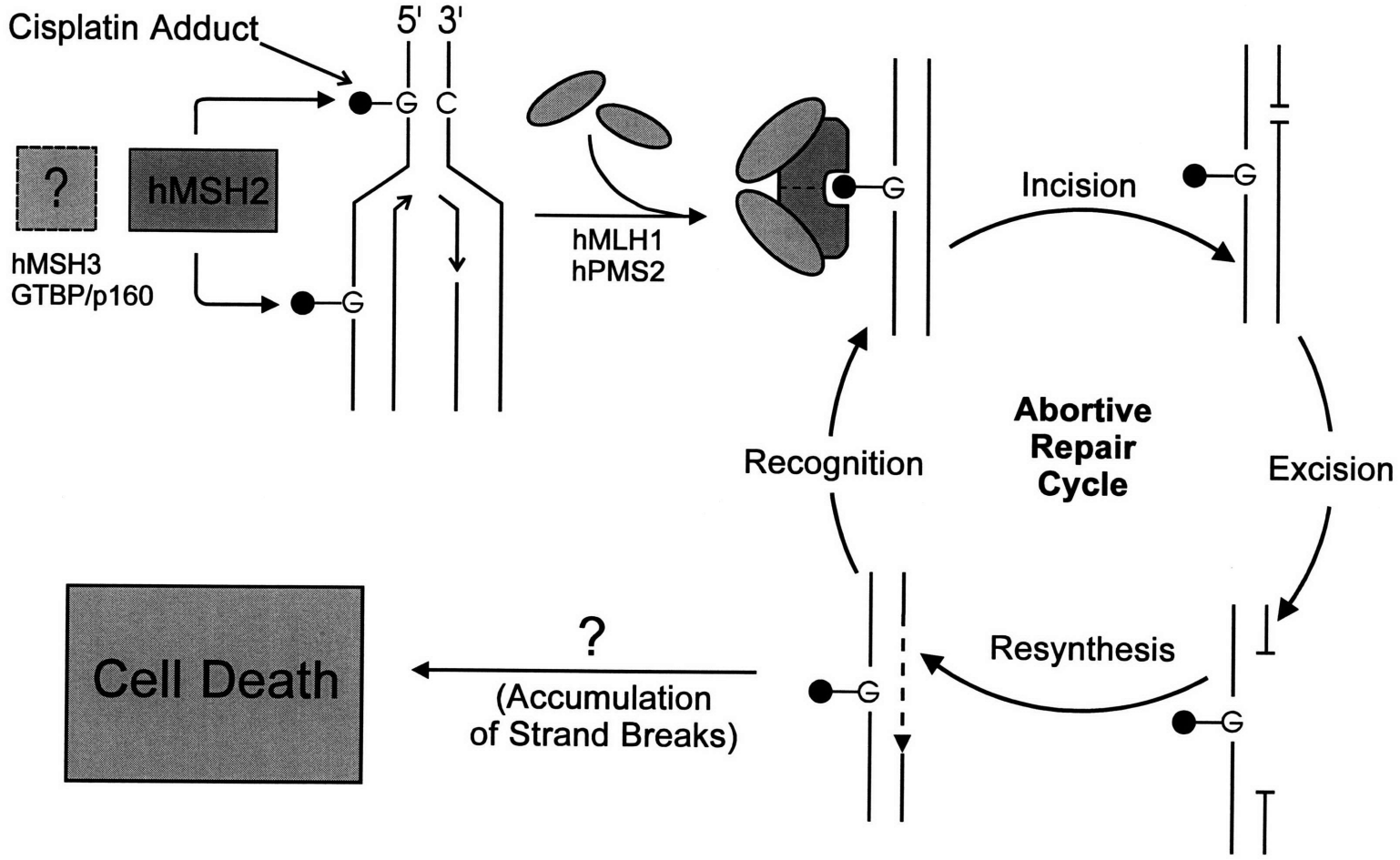

Cisplatin Adduct
5' 3'
G
C
?
hMSH2
hMSH3
GTBP/p160
G
hMLH1
hPMS2
G
Incision
G
Abortive
Repair
Cycle
Recognition
Excision
Resynthesis
G
G
?
(Accumulation
of Strand Breaks)
Cell Death

Figure 19: The repair shielding model for the participation of hMSH2 in mediating cisplatin cytotoxicity. See text (discussion section) for details.

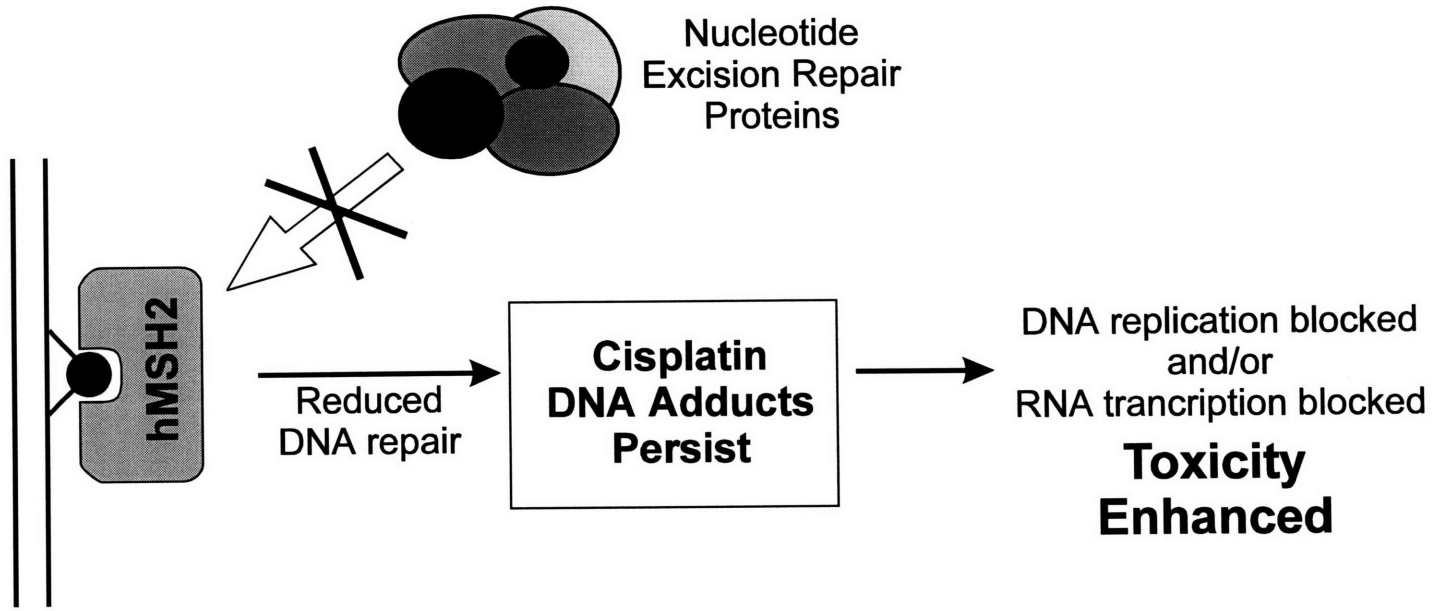

Nucleotide
Excision Repair
Proteins
hMSH2
Reduced
DNA repair
Cisplatin
DNA Adducts
Persist
DNA replication blocked
and/or
RNA trancription blocked
Toxicity
Enhanced

Figure 20: A model describing a role for hMSH2 in cisplatin toxicity in which hMSH2 acts as a general sensor of genetic damage and provides a signal resulting in arrest at the G2 cell-cycle checkpoint. See text (discussion section) for details.

hMSH2
G
G
Me–G
?
(provision
of signal)
Cell Cycle
Arrest
in G2
APOPTOSIS
Cisplatin
Aminofluorene
N-Acetyl-2-Aminofluorene
MNNG

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BIOGRAPHY

The author was born December 19, 1966 in Fall River, Massachusetts. She spent the majority of her childhood in Lakeville, Massachusetts, where she attended George R. Austin Middle School and Apponequet Regional High School. In 1984 she entered Trinity College in Hartford, CT where she majored in Chemistry. While at Trinity she spent her junior year abroad studying at the University of Durham in Durham, England. She was elected to *Phi Beta Kappa* in 1987, received the Vernon K. Krieble Chemistry Scholar award, and graduated first in her class with a B.A. degree in 1988. In the year following graduation, the author conducted research under Dr. Peter Arvan at Beth Israel Hospital in Boston, MA. She then entered graduate school in the Department of Chemistry at Columbia University in New York, NY where she earned her M.A. in chemistry in 1990. During each of the summers from 1988-1990, the author was enrolled full time at the Rhode Island School of Design in Providence, RI, completing coursework in the studio arts towards an M.A. in art education. To pursue her interest in biological chemistry, she entered the Department of Chemistry at MIT in the fall of 1990 and joined the laboratory of Dr. John Essigmann. Her doctoral research focused on the interactions of cellular proteins with DNA adducts formed by the anticancer drug cisplatin. Upon completion of her doctoral studies on December 4, 1996, she continued working in this area in Dr. Essigmann's laboratory as a postdoctoral associate.