THE RESPONSES OF CELLULAR PROTEINS TO CISPLATIN-DAMAGED DNA

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Deborah B. Zamble
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Signature of Author:_____
Department of Chemistry
December 17, 1998

Certified by:_____
Stephen J. Lippard
Arthur Amos Noyes Professor of Chemistry
Thesis Supervisor

Accepted by:_____
Dietmar Seyferth
Chairman, Departmental Committee on Graduate Studies

This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

John M. Essigmann
Professor of Chemistry and Toxicology
Committee Chairman

Stephen J. Lippard
Arthur Amos Noyes Chair and Professor of Chemistry
Thesis Supervisor

Jun Liu
Assistant Professor of Chemistry and Biology

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Abstract

The anticancer drug cisplatin is a small inorganic molecule that forms several types of covalent adducts on DNA. There is evidence that at least some of the consequences of this genetic damage are mediated by proteins that bind to the cisplatin-DNA cross-links or influence cellular pathways in response to the genotoxic stress. In either case, such factors can regulate the processing of the cisplatin lesions and thereby affect cellular sensitivity to the drug. Identification of these proteins and exploration of their cellular functions has implicated multiple systems including several classes of DNA repair, transcription, cell cycle and cell death responses. Chapter one reviews the current information of how components of these pathways respond to cisplatin-DNA adducts and contribute to the cytotoxic mechanism of action.

Chapters two and three explore the capacity of one cellular defense mechanism, the nucleotide excision repair pathway, to remove cisplatin-DNA damage, and factors that can modulate this activity were examined. The cisplatin intrastrand cross-links are all excised by human nucleotide excision repair, however removal of the minor interstrand cross-link was not detected. Experiments performed with a reconstituted system of purified nucleotide excision components demonstrated that structural differences among the types of cisplatin-DNA lesions affect the relative excision repair rates. Attempts to correlate the repair capacity of human tissue with cisplatin sensitivity were unsuccessful, however the use of several model systems suggest that there are cellular factors that can modulate the removal of the cisplatin-DNA adducts. Members of the HMG-domain family of proteins specifically blocked the in vitro repair of the 1,2-intrastrand cross-links, an effect that was observed with a single HMG-domain polypeptide, and in the reconstituted repair assay. These results suggest that the ability of HMG-domain proteins to recognize cisplatin-modified DNA could affect cellular removal of the lesions, and that HMG-domain proteins with a selective expression pattern could contribute to cisplatin sensitivity. In particular, the testis-specific proteins tested were the most effective at inhibiting repair, which may be relevant to the favorable clinical response of testicular tumors to cisplatin-based chemotherapy. No repair inhibition by a mismatch repair protein was detected, although binding to cisplatin-DNA adducts was observed.

Another factor that could explain the efficacy of cisplatin in treating testicular cancer is that, unlike other human neoplasms, a low frequency of mutations in the p53 tumor suppressor gene are observed in this tumor type. Previous studies have investigated the relationship between p53 status and cisplatin sensitivity, but have provided conflicting results. The p53-mediated responses in murine testicular teratocarcinoma cells exposed to cisplatin were examined in chapter four. Cisplatin exposure of cells with a wild-type p53 gene resulted in accumulation of p53 protein through posttranscriptional mechanisms; induction of p53-target genes was also observed. Drug treatment resulted in rapid apoptosis in p53-wild-type cells but not in a $p53^{-/-}$ teratocarcinoma line. In the latter cells, cisplatin exposure caused prolonged cell cycle arrest in the G_1/early S phase, accompanied by induction of the p21 gene. Clonogenic assays demonstrated that the p53 mutation did not confer resistance to cisplatin. These experiments suggest that cisplatin inhibits cellular proliferation of testicular teratocarcinoma cells by two possible mechanisms, p53-dependent apoptosis and p53-independent cell cycle arrest.

In the final chapter, the role of HMG-domain proteins in the mechanism of cisplatin cytotoxicity was further investigated. A human cervical carcinoma cell line was constructed which ectopically expressed a testis-specific protein, tsHMG, under the control of an inducible promoter. Examination of tsHMG expression and cisplatin-induced apoptosis on a cellular level revealed that the nuclear protein can modulate the cytotoxic consequences of cisplatin. However, the effects of tsHMG depend on the drug treatment conditions and the extent of cell death. The results may be relevant to the clinical requirement of achieving a balance between the antineoplastic and toxic properties of cisplatin. A model proposed to explain the observations will provide a basis for future studies of cellular factors that can affect cisplatin sensitivity, and for the design of more comprehensive antineoplastic therapies.

Thesis Supervisor: Stephen J. Lippard
Title: Arthur Amos Noyes Professor of Chemistry

This thesis is dedicated to my family.

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Abbreviations

1,2-d(GpG)	*cis*-[$Pt(NH_3)_2${d(GpG)-N7(1)-N7(2)}]
1,2-d(ApG)	*cis*-[$Pt(NH_3)_2${d(ApG)-N7(1)-N7(2)}]
1,3-d(GpTpG)	*cis*-[$Pt(NH_3)_2${d(GpTpG)-N7(1)-N7(3)}]
bp	base pair
BSA	bovine serum albumin
CFE	cell free extract
chx	cycloheximide
cisplatin	*cis*-diamminedichloroplatinum(II)
CMV	cytomegalovirus
cpm	counts per minute
DAPI	4',6-diamidino-2-phenylindole
DEPC	pyrocarbonic acid diethyl ester
DME	Dulbecco's modified Eagle medium
DMF	N',N'-dimethylformamide
DMS	dimethyl sulfate
DNA	deoxyribonucleic acid
dNTP	deoxynucleobase triphosphate
Dox	doxycycline
DTT	ethylenedinitrilo-tetracetic acid
E. Coli	*Eschericia coli*
EGFP	enhanced green fluorescent protein
EGTA	ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetracetic acid
excinuclease	active protein complex of the nucleotide excision repair pathway
FA	Fanconi's anemia
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G418	geneticin
Hepes	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HMG	high mobility group
HPLC	high pressure liquid chromatography
ICL	interstrand cross-link
K_d	apparent dissociation constant
NER	nucleotide excision repair
NLS	nuclear localization sequence
nt	nucleotides
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RHO	rhodamine
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
Staph A	*Staphylococcus aureus*
trans-DDP	*trans*-diamminedichloroplatinum(II)
TRE	tetracycline-responsive element
Tris	tris(hydroxymethyl)aminomethane
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
XP	Xeroderma Pigmentosum
XPAC	XP group A complementation protein

Chapter One

Introduction: The Responses of Cellular Proteins to Cisplatin-Damaged DNA

Introduction (1, 2)

The chemotherapeutic agent *cis*-diamminedichloroplatinum(II), or cisplatin (Figure 1.1A), can form covalent adducts with many cellular macromolecules, but there is convincing evidence that its cytotoxic properties are a consequence of bifunctional-DNA adduct formation (3, 4). Platinum binds to the N(7) position of purine nucleotides (Figure 1.1B), resulting predominantly in 1,2-d(GpG) and 1,2-d(ApG) intrastrand, but also in 1,3-d(GpNpG) intrastrand, interstrand and protein-DNA cross-links (5, 6). The 1,2-intrastrand cross-links, which comprise 90% of the DNA adducts, are not formed by the clinically inactive *trans*-DDP because of geometric constraints, and attention has therefore focused on these adducts as the active lesions in the anticancer activity of the drug.

Numerous studies, carried out to determine how ligation of platinum affects the conformation and stability of normal B-DNA, revealed significant destabilization of the double helix (reviewed in 7; see also 8). More detailed information has been provided by recent structural analyses of the 1,2-d(GpG) intrastrand cross-link in duplex DNA (Figure 1.2), performed both by X-ray crystallography (9, 10) and NMR spectroscopy (11-13). Although these structures differ in some details, they all reveal that the platinum induces a roll of 26°-50° between the guanine bases involved in the cross-link, displacement of platinum from the planes of the guanine rings, and a global bend of the helix axis towards the major groove. In addition, hydrogen-bonding within the 5'-coordinated GC base pair is severely perturbed, resulting in enhanced solvent accessibility. The cisplatin cross-link also unwinds the duplex and induces a widening and flattening of the minor groove in the vicinity of the adduct. These structural features strikingly resemble those observed in some DNA-protein complexes (14), the consequences of which will be discussed below.

NMR solution structures of two cisplatin-DNA decamers containing interstrand cross-links demonstrated that this minor adduct also has characteristic structural features (15, 16). In particular, the platinum atom lies in the minor groove, the complementary cytosines are extrahelical, and there is a switch in the double helix to a left-handed form localized at the site of the adduct. These distortions bend the helix axis towards the minor groove by approximately 20°-40° and unwind the duplex by 76°-80°. More detailed descriptions of Pt-DNA complexes may be found in other chapters in this volume.

The unusual conformations at the sites of cisplatin-DNA adducts suggest that they might serve as recognition elements for proteins that bind to and process damaged DNA. Studies with cell-free extracts, designed to test this hypothesis, demonstrated the presence of factors that bind DNA modified with cisplatin but not *trans*-DDP (17-19). Concomitant gel mobility shift assays revealed the binding activity of one of these proteins to be specific for the 1,2-intrastrand cross-links (17). The possibility that these cisplatin-damage recognition proteins play a role in the cytotoxic mechanism of the drug was supported by studies revealing that some proteins in this class are overexpressed in cell lines having acquired cisplatin resistance (19-22). In addition, several damage recognition proteins could be induced in cultured cells exposed to cisplatin (21, 23).

The purpose of this chapter is to review the types of proteins that interact with or respond to cisplatin-damaged DNA and to discuss how these proteins can modulate cellular sensitivity to the drug through their participation in various biochemical pathways (Figure 1.3). Included are proteins dedicated to dealing with genotoxic stress, such as components of the DNA repair and p53-regulated pathways, but also proteins affected by the DNA structural distortions induced by cisplatin adduct formation, such as transcription factors and architectural proteins. The manner by which such proteins affect the processing of cisplatin-DNA

adducts can determine whether a cell attempts to repair the damage or activates an irreversible cell death program. These proteins most likely contribute to the anticancer activity of this drug. Moreover, differential protein expression and activity may explain why certain types of tumors, such as testicular, ovarian, and head and neck, are successfully treated by cisplatin whereas others are resistant (24, 25). Because many factors are involved in the mechanisms of action and resistance, we make no attempt to cover this field comprehensively. Topics beyond the scope of this review include oncogene activation, replication bypass and mutagenesis, reactions with sulfur-containing molecules, and mechanisms of drug uptake and export (26-28).

DNA Repair Pathways

Nucleotide Excision Repair

There are numerous sources of DNA damage in the environment, including both naturally and artificially introduced substances. To limit genetic mutations and prevent the ensuing malignant transformation that might arise from exposure to such agents, there are a variety of cellular defense mechanisms that remove lesions from DNA and correct any unwanted changes. One of the most versatile systems is nucleotide excision repair (NER). In this pathway, many types of DNA lesions are excised as a small, single-stranded oligonucleotide fragment, and new DNA is synthesized to fill the resulting gap (Figure 1.4; for reviews see 29, 30). The study of NER has been facilitated by several genetic diseases associated with DNA repair deficiencies including xeroderma pigmentosum (XP), an inherited disorder characterized by unusual sensitivity to sunlight and a predisposition to neurological abnormalities and cancer (31). Most of the essential mammalian excision repair factors have been cloned by complementation studies with such human XP (XPA-XPG) or rodent (ERCC1-

ERCC11) mutant cell lines. Experiments with purified proteins demonstrated that 14-16 polypeptides are necessary to reconstitute fully the dual incision activity if the excinuclease (Table 1.1) (32-34).

Recognition of DNA Damage by NER Proteins

If cisplatin-DNA adducts are substrates for NER, they should be recognized specifically by proteins involved in the first, and possibly rate-limiting, damage recognition step (29). The zinc finger protein XPA plays a central role in sensing genetic damage and recruiting the excinuclease to the site of the lesion (35). Gel mobility shift and nitrocellulose binding assays demonstrated that XPA binds to cisplatin-modified DNA, although the affinity for the damaged over undamaged DNA was fairly modest, only a factor of 5 or less (36-38). Another essential component of the excision nuclease, the human single-stranded binding protein RPA, was detected in a complex with cisplatin-modified DNA isolated from cell extracts (39, 40). XPA binds tightly to RPA in vitro (29), so it is likely that together they recognize DNA damage with an increased specificity of binding. Recent work with purified repair factors, however, did not detect specific binding of the two proteins to a damaged substrate (41). Rather, it was suggested that XPA/RPA may bind DNA lesions weakly, and that recruitment of TFIIH and XPC, as well as ATP-dependent DNA-unwinding, are all required to form the first stable complex in the NER pathway (41, 42).

Another DNA-damage binding activity was found to be deficient in some XPE cells (43). This activity is expressed at higher levels in some human tumor cells selected for cisplatin resistance, accompanied by enhanced levels of repair (20). In another study, the small subunit of the putative XPE protein was induced by cisplatin and its induction was greater in resistant cell lines. The diminished cisplatin sensitivity was attributed to replicative bypass, however, not to excision

repair (44, 45). Further investigation of the XPE phenotype, which presents with only mild XP symptoms and partial reduction in repair capacity, demonstrated that the damaged-DNA binding activity is absent in cells from only a few XPE patients (reviewed in 29). Moreover, the purified protein does not complement the repair activity of XPE extracts and it is not required to reconstitute the excinuclease completely (32, 33). It is therefore impossible at present to delineate what role this protein might play in excision repair or the cytotoxic activity of cisplatin.

Repair of Cisplatin-DNA Adducts

It is now certain that cisplatin-DNA cross-links are removed by the excision repair pathway (4). In fact, one of the original pieces of evidence indicating that cisplatin-damaged DNA is cytotoxic was the enhanced sensitivity of bacterial mutants deficient in components of the UvrABC excision nuclease (46, 47). Parallel studies in mammalian cells revealed the abnormal cisplatin sensitivity of human XPA and XPF cell lines (48-50) and of a Chinese hamster ovary cell line lacking ERCC1 (51). The sensitivity of these cells was attributed to defective adduct removal from genomic DNA. This conclusion was supported by work with an in vitro repair synthesis assay that measures the formation of new DNA patches following removal of the platinum adducts by the excinuclease. The signal was diminished in extracts from XPA cells when compared to extracts from normal lymphoid cells (52, 53).

Experiments were also performed with an alternative assay that makes use of a linear DNA substrate containing a site-specific lesion positioned close to a radioactive phosphodiester group. Incubation with cell-free extracts and resolution of the DNA by denaturing gel electrophoresis afforded small oligonucleotides containing the adduct and the label (54). By using this excision

assay, it was revealed that both cisplatin 1,2-d(GpG) and 1,3-d(ApG) intrastrand cross-links are substrates for the mammalian excinuclease (55, 56). The minor 1,3-d(GpTpG) cross-link is removed much more efficiently, however (Table 1.2). The relative rates of repair of the various cisplatin-DNA adducts by the mammalian excinuclease (GTG >> GG) agree with prokaryotic excinuclease recognition of DNA adducts formed by a diaminocyclohexane platinum compound (57, see however 58) and reflects the degree of unwinding caused by the different adducts (59). The correlation between helix-destabilization and relative rates of repair is not surprising given that components of the damage-recognition complex (XPA and RPA) preferentially bind single-stranded over double-stranded DNA (35).

Although cisplatin interstrand cross-links also significantly distort DNA (see above), it is unclear whether they are repaired by NER. Study of a psoralen interstrand cross-link suggested that the excinuclease recognizes this type of damage, but with an unusual outcome (60). Two cleavage sites were identified, both 5' to the damage, such that the adduct was not removed from the DNA. It was suggested that the ensuing gap might serve as a recombinogenic signal to activate subsequent removal of the cross-link through a different repair pathway. When a cisplatin interstrand adduct was incubated with mammalian cell-free extracts, however, no such reaction products were detected (56). Cells from Fanconi's anemia patients (FA) are very sensitive to cross-linking agents but proficient in NER (61). The unusual sensitivity of this cell type to cisplatin is associated with an inability to remove interstrand adducts from the DNA (49, 50), indicating that the adducts are repaired by an alternative pathway. There is some evidence to suggest that mammalian repair of interstrand cross-links requires a few proteins that are also components of excision repair (62). The repair synthesis signal arising from a cisplatin-modified plasmid enriched for the interstrand cross-link was larger than that detected for randomly-modified DNA (63), and this

activity was absent in extracts made from XPA cells. Although these experiments provide some information about the repair of cisplatin interstrand cross-links, the detailed mechanism still remains to be elucidated.

Cisplatin is also a carcinogen (3), at least in part because mutations across from its DNA adducts are generated during replicative bypass (6). Studies of a cisplatin 1,2-d(GpG)-containing substrate revealed that a thymine across from the adduct enhanced the excinuclease activity when compared with the correct complementary bases (64, 65). This effect probably reflects the superimposition of structural distortions. The action of an excinuclease on the Pt-damaged strand of such a compound lesion would permanently fix the mutation in the genome. A compound lesion would also be recognized by the mismatch repair proteins, the consequences of which are discussed below.

The Role of NER in the Anticancer Activity of Cisplatin

Over the years, it has become apparent that the antitumor activity of cisplatin is a consequence of several different cellular phenomena (26, 28). DNA repair has been investigated as one factor that could influence cisplatin sensitivity, since failure to remove DNA lesions would allow them to persist and interfere with essential cellular systems. Several early studies provided evidence that *trans*-DDP was ineffective because its DNA adducts were repaired more efficiently than those of the cis isomer (6). This result might be a consequence of differential recognition of the platinum adducts by the repair enzymes or by some other factor that modulates repair. In addition, in contrast to previous reports (reviewed in 5), recent evidence suggests the *trans*-DDP forms predominantly monofunctional and interstrand DNA cross-links (66), which might be processed in a different fashion than intrastrand adducts.

Sublines with reduced sensitivity to cisplatin have been produced in vitro by first exposing cells to low levels of the drug and then increasing the concentration in a stepwise fashion as the cells adapt to its presence. Enhanced DNA repair has been implicated as a mechanism of drug tolerance in a variety of model systems, including murine leukemia (67-69), rat colon adenocarcinoma (70), human ovarian carcinoma (71), and human testicular tumor cell lines (72, 73). In some cases, it was evident that other factors were involved since the increase in DNA repair was not proportional to the level of resistance. In addition, the resistance phenotype may be caused by mutations that reverse a DNA repair deficiency in the parental cells. For example, the murine leukemia L1210/0 cell line has been used in many studies of acquired resistance to cisplatin. A recent investigation revealed the parental cell line to be deficient in nucleotide excision repair, due to an XPG genetic defect. In the course of selection for drug resistance, the XPG function was restored, probably by a platinum-induced mutation (74). Whatever the explanation, the possibility that increased DNA repair could confer resistance to cisplatin led to studies of agents that inhibit repair (7, 27). Although the results seem to depend on cell type, many of the compounds identified in this manner produced a synergistic response with cisplatin in vitro, and provided promising leads for clinical evaluation.

In *E. coli*, RNA polymerases stalled at sites of DNA damage are recognized by the coupling factor TCRF, which recruits repair factors (29). In mammalian cells, the coupling factor has not been clearly identified, but mutations in the two genes responsible for Cockayne's syndrome, another repair-related disease, result in defective transcription-coupled repair. Various types of DNA damage including cisplatin adducts are removed more rapidly from actively transcribed genes (75), and intrastrand adducts in particular are preferentially removed from the transcribed strand (76). Transcription-coupled repair of the major cisplatin-

DNA adducts has not been linked to drug sensitivity. In contrast, enhanced gene-specific repair of the cisplatin interstrand cross-link was detected in several cell lines with acquired cisplatin resistance (77, 78), but its repair mechanism has not been delineated.

A reduced capacity to repair cisplatin-DNA adducts may be responsible for the clinical effectiveness of the drug in the treatment of certain types of cancer. Cell lines derived from human testicular tumors are hypersensitive to a variety of DNA-damaging agents, including cisplatin (79). In comparison to relatively resistant bladder cancer cell lines, testis tumor cells have lower levels of platinum-adduct removal from the whole genome (80). This result was reproduced when the repair of both the actively transcribed N-ras gene and the inactive CD3∂ gene was analyzed. In particular, immunochemical analysis of the different cisplatin-DNA adducts demonstrated a deficiency in the repair of the 1,2-intrastrand cross-links (81, 82). The biological basis for this effect has not yet been established. Possible explanations include a defect in the nucleotide excision repair pathway, lower expression levels of the repair proteins, the absence of some excinuclease-activating signal, or the presence of a repair-inhibiting factor. Further work is required to ascertain whether DNA repair is the major determinant of cisplatin sensitivity in testicular cancer.

In vitro experiments with cultured cell lines have provided a wealth of information about the biological mechanism of cisplatin. Although there is some correlation between the sensitivity of these cells and related tumors (83), it is likely that they provide only a limited representation. An extreme case is a murine mammary tumor that acquired cisplatin resistance in vivo but seemed to lose drug tolerance when cultured in a monolayer in vitro (84). Reimplantation in vivo restored cisplatin resistance, demonstrating the importance of cellular

context when examining drug sensitivity. For this reason, the mechanism of action of cisplatin should be examined in human tissue when possible.

By using leukocytes from chemotherapy patients with squamous cell carcinoma of the head and neck region, it was demonstrated that damage removal from DNA was related to cisplatin resistance (85). This type of study assumes the profile of adduct formation and repair to be the same in peripheral and tumor tissue. The hypothesis was supported by several early studies that employed either atomic absorption spectroscopy or immunochemical techniques to demonstrate a relationship between DNA adduct formation in blood cells and disease response (86-91). Subsequent work revealed, however, that cisplatin-DNA adduct levels do not always correlate with survival (92) and can vary substantially between individuals (93).

DNA repair components have been analyzed in a few tumor samples. Elevated levels of RNA for ERCC1 and XPA were detected in the tumors of ovarian cancer patients who were clinically resistant to cisplatin chemotherapy when compared to the responders (94, 95). In these studies, the tissue was harvested before drug treatment, the higher levels of expression of the NER components providing a biochemical basis for *inherent* cisplatin resistance in some of the patients. The mechanism of *acquired* resistance was investigated in a malignant oligodendroglioma by examining tissue both before and after failed cisplatin therapy (96). The level of repair of cisplatin-DNA adducts, as determined by a host cell reactivation assay, was higher in the tumor procured after therapy and associated with increases in DNA polymerase β and ligase activity.

Clearly, the NER proteins play a major role in the biological processing of cisplatin-DNA adducts. As a consequence, it is likely that they can modulate cellular sensitivity to cisplatin. So far, however, there is only limited information about the relative repair capacities of malignant and normal tissue. To determine

whether DNA repair contributes to tumor responses during cisplatin chemotherapy, it is necessary to quantitate directly the DNA repair activity from the tissue. Recent studies of human tissue demonstrated a significant variability in the repair capacity of tumors from different patients (97). When the levels of repair in extracts from different organs were compared, no correlation with cisplatin toxicity was observed (98). Such experiments are difficult to evaluate, however, since current methods involve the preparation of crude extracts, which must be normalized to one another, and the use of in vitro repair assays which are complicated by inherent variability (99). Improved technical protocols, along with a better understanding of the intricacies of neoplastic transformation, should make it easier to study the excinuclease activity in the context of the complex cellular environment.

Mismatch Repair

Normal DNA processing activities are inherently error-prone, and the existence of several mechanisms to remove genetic misinformation indicates how disastrous the propagation of mutations can be. The consequences of such negligence was made evident by the discovery that almost all cases of hereditary nonpolyposis colon cancer as well as a variety of sporadic tumors arise from defects in the mismatch repair pathway (100). In addition to the maintenance of genetic integrity (for reviews on mismatch repair, see 100-102), however, it is becoming apparent that mismatch repair may also mediate the cytotoxicity of a variety of clinically effective drugs, including cisplatin (103, 104). A connection between cisplatin sensitivity and mismatch repair was made with the discovery that repair-deficient cell lines were resistant to cisplatin and carboplatin, but not to all DNA-damaging agents (105, 106). Furthermore, some ovarian cancer cell lines selected for cisplatin resistance lose the ability to express hHML1 (107-109), one of

the essential components of the mismatch repair machinery (101, 102). In a model proposed to explain drug tolerance associated with defects in mismatch repair, it is assumed that replication bypass of cisplatin-modified bases produces mutations (Figure 1.5A). During mismatch repair, the strand to be corrected is nicked, an oligonucleotide containing the mismatch is excised, and new DNA is synthesized. The mismatch repair proteins always replace the incorrect sequence in the daughter strand through some as yet undetermined signal, which would leave the exogenous platinum damage unrepaired. This activity initiates a futile cycle. During DNA synthesis to replace the excised oligonucleotide, the polymerases would again incorporate mutations, which would be followed by attempts to fix them. The repeated breaks in DNA formed at each ineffective cycle of repair could trigger a cell death response.

A more direct association was made by experiments showing that cisplatin-modified DNA is recognized by mismatch repair proteins. The MutSα heterodimer, a putative mismatch recognition factor, binds to a 32-bp oligonucleotide containing one 1,2-d(GpG) cisplatin intrastrand cross-link but only poorly to 1,2-d(ApG) and 1,3-d(GpTpG) adducts and not at all to a *trans*-DDP 1,3-intrastrand cross-link (110). Further work demonstrated that hMSH2, a component of MutSα, binds specifically to DNA globally modified with cisplatin but not with other, clinically ineffective platinum compounds (111). This protein also displays a weak selectivity for a single 1,2-d(GpG) adduct in 100-bp probe compared to the corresponding unmodified DNA.

The binding of mismatch repair proteins to cisplatin-modified DNA in the absence of mutations could activate cell death indirectly, by initiating the same futile cycle described above. Alternatively, recognition of DNA adducts by the repair apparatus could trigger cell cycle arrest and/or apoptosis by some mechanism (Figure 1.5B), such as activation of the JNK or c-Abl signaling

pathways (112). The absence of a functional complex to detect damage would increase cellular tolerance for cisplatin-DNA adducts and explain the drug resistance associated with diminished expression of these proteins. This hypothesis is interesting in light of the recent connection between mismatch repair and PCNA, a replication factor that has been implicated in a cell cycle checkpoint (113, 114), and the observation that hMLH1-deficient cells do not exhibit the prominent G_2-arrest usually observed following cisplatin exposure (109).

Cellular sensitivity to different platinum compounds and the recognition of the platinum DNA adducts by mismatch repair protein complexes appear to be linked (105). It may also be significant that hMSH2 is expressed to higher levels in testicular and ovarian tissue than in other organs such as heart, liver and colon (111). Whether or not mismatch repair plays a general role in the anticancer activity of cisplatin still remains debatable, however. Mismatch repair proteins bind to cisplatin-DNA adducts in vitro with weak specificity (111, 115). Although specificity is enhanced when a platinum lesion is combined with a mutation (115), it is still less than the affinity of these proteins for the unplatinated mutation (65, 110).

Mismatch repair mutants are more likely to afford a mechanism for acquired cisplatin resistance (107-109). Exposure to cisplatin can select for mismatch repair deficiency both in vitro (116, 117), and in vivo (109), and deleting MSH2 produces a small but significant level of cisplatin resistance in xenografts (116). The fact that several ovarian carcinoma cultured cell lines develop a defect in this pathway during the acquisition of resistance to cisplatin is intriguing, because the growth of resistant tumors is a significant problem in the treatment of ovarian cancer (118). The importance of overcoming resistance in cancer chemotherapy should further stimulate investigation of this area.

HMG-Domain Proteins and Cisplatin

As mentioned in the introduction, preliminary studies demonstrated the existence of cellular proteins that specifically recognize and bind to cisplatin-modified DNA. To determine what role these proteins might play in the cytotoxic mechanism of cisplatin, it was necessary to learn their identity and study them individually. To achieve this goal, a cDNA encoding one of these SSRPs (structure-specific recognition proteins) was isolated by expression library screening (119, 120), and a data base search with the predicted amino acid sequence revealed homology with the non-histone chromosomal protein HMG1 (120). The region of highest identity (47%) was an 80-amino acid DNA-binding motif known as the HMG domain, which occurs in a variety of proteins. In a complementary study, HMG1 and HMG2 were detected among a small group of nuclear proteins isolated from HeLa cell extracts by fractionation on cisplatin-modified DNA cellulose (121). Gel mobility shift assays demonstrated that HMG1 binds specifically to DNA treated with cisplatin, but not to DNA modified with *trans*-DDP (122), and the use of site-specifically modified probes revealed that it is the 1,2-intrastrand adducts that induce binding. These results stimulated a much more extensive study of the family of HMG-domain DNA-binding proteins.

The canonical HMG domain is a positively charged, predominantly α-helical 71 amino acid polypeptide (123-125). This L-shaped peptide occurs in a wide variety of minor groove DNA-binding proteins having little or no homology outside of the HMG domain. Members of this protein family lie on a continuum between two modes of DNA recognition. At one end are proteins that bind to a defined, AT-rich, consensus sequence. These proteins have only a single domain, usually function as transcription factors and regulators of cellular differentiation, and include the lymphoid enhancer binding factor LEF-1, the testis-determining factor SRY, and the Sox family of proteins. At the other

extreme are proteins such as HMG1 and 2, which contain more than one HMG-domain and display no DNA sequence specificity (for a review on HMG1/HMG2 see 125). The exact cellular function of these proteins is unknown, but because they bend DNA and recognize pre-bent DNA structures like other HMG-domain proteins, it has been proposed that they act as architectural factors during nucleic acid processing. In this role, the HMG-domain protein would facilitate the assembly of multi-protein complexes responsible for activities that include the maintenance of chromatin structure, DNA recombination, replication, transcription and repair.

The sequence homology between HMG-domains is quite low, identity typically being on the order of 25%, but recent modeling experiments indicate that the tertiary structure is highly conserved (126). This result may explain why HMG-domain proteins are all able to bind specifically to distorted DNA structures such as four-way junctions. Recent solution structures of the SRY and LEF-1 HMG-domains in complexes with their target sequences revealed a pronounced bend in the DNA accompanied by unwinding of the helix and widening of the minor groove (127, 128). Similar features have been observed in structures of DNA duplexes containing the cisplatin 1,2-d(GpG) intrastrand cross-link (10-13). Accordingly, cisplatin-DNA adducts might provide an optimal framework for specific recognition by this DNA-binding motif.

Binding to Cisplatin-DNA Adducts

Most of the HMG-domain proteins examined have the capacity to bind specifically to DNA cross-linked with cisplatin; Table 1.3 summarizes studies with site-specifically modified DNA (see also 121, 129-133). The binding affinity of these proteins to a site-specific 1,2-d(GpG) cisplatin intrastrand cross-link was determined by using gel mobility shift and other assays. The differences in

experimental conditions employed, the method for calculating K_d, components of the reaction solutions, and the DNA probes themselves, prohibit any quantitative comparison of the results. Several points of interest have emerged from these studies, however. First, there is no doubt that these polypeptides bind to a single 1,2-intrastrand cross-link with higher affinity than to the corresponding unplatinated DNA; the specificity ratio (ρ) ranges from 3-235. The higher specificity occurs with smaller DNA substrates, which is not surprising given that longer stretches of undamaged DNA provides more opportunity to form non-specific complexes with any DNA-binding protein.

The second observation is that isolated HMG-domains recognize the cisplatin lesion, establishing that it is this motif which confers such an unusual activity on the family of proteins (but see 134). That the domains do not bind to the cisplatin-DNA adducts with the same affinity and specificity as the whole proteins supports the fact that regions outside the domain influence the DNA-binding activity ((135) and references within).

Finally, the sequence context of the Pt-DNA adduct can have a significant impact on the strength of the protein-DNA interaction (136). In a study of a series of 15 bp platinated probes, changing the identity of the two nucleotides flanking a 1,2-d(GpG) cisplatin-DNA adduct modulated the binding affinity by more than 2 orders of magnitude. In particular, a striking effect was apparent for the base-pair 3' to the lesion, a position which has unusual minor groove accessibility and considerable hydrogen bond disruption (Figure 1.2) (10, 131, 137). It has not been determined whether this modulation of binding is due to differences in the structures of the cisplatin-modified duplexes, changes in the stability and flexibility of the duplex DNA, base-specific protein-DNA contacts or some combination thereof.

A recent study demonstrated that HMG1 can also apparently bind to DNA containing cisplatin interstrand cross-links (130). As described above, the duplex-DNA distortions induced by the interstrand cross-link are quite different from those resulting from a 1,2-intrastrand adduct, suggesting that there is no correlation between the bending or unwinding angles and HMG recognition. The authors propose that the proteins recognize fixed distortions in DNA, but that if the DNA is too denatured or flexible, as is the case following *trans*-DDP modifications, binding is inhibited. To date, this is the only reported example of such an interaction, and to appreciate its importance, a comparison of substrates containing the different types of cisplatin adducts will be required.

In addition to binding structural distortions in DNA, the ability to bend linear DNA is a common feature of this protein family (123). Thus HMG-domain proteins could effect the mechanism of cisplatin by further distorting DNA at the site of the lesions (138). Circular permutation assays demonstrated that HMG-domain proteins amplify the bend in a 1,2-d(GpG) site-specifically platinated probe (139). In addition to determining the bend angles, which ranged from 50° to 90°, these experiments also revealed that the platinum was near the center of the bend locus. The enhanced bending of the cisplatin-modified DNA suggests one reason why the interaction with HMG-domain proteins is favored. As described above, the roll between guanine bases is such that the platinum is under considerable strain, which could be released if the bend angle were increased to ≈ 90° through the formation of a protein-DNA complex (139).

The Role of HMG-Domain Proteins in Modulating the Cisplatin Sensitivity of Cells.

The binding of cisplatin-modified DNA by HMG-domain proteins, a consequence of features in common with the natural DNA targets, may

fortuitously play a role in the drug cytotoxicity. It is clear that HMG-domain proteins do respond to cisplatin, since both HMG1/2 and hUBF relocalize in cells exposed to the drug (140, 141). Interruption of the gene for Ixr1, a yeast HMG-domain protein, resulted in a 2-6 fold desensitization of the cells to the drug (142, 143), which correlated with a decrease in the number of platinum-DNA adducts (142). In addition, a recent report suggests that overexpression of HMG2 can sensitize cells to cisplatin (144).

If HMG-domain proteins function as a determinant of cisplatin cytotoxicity, the levels of these proteins would be expected to reflect cellular drug sensitivity. This issue is difficult to address, however, because it is not known whether only one member of this family, and if so, which one, or all HMG-domain proteins should be examined. Nevertheless, several studies have searched for a connection between the quantity of expressed HMG-domain protein and cellular response to cisplatin. No correlation was detected between hSSRP1 expression and the cisplatin sensitivity of several cell lines and tissue types (120). In contrast, elevated levels of HMG1/2 bound to cisplatin-modified DNA cellulose were detected in nuclear extracts from HeLa cells with acquired cisplatin resistance (145). HMG1 was also expressed in higher levels in human hepatocellular carcinomas than in the healthy tissue (146). Clearly, further research is needed to determine the importance of HMG-domain proteins sensitizing tumors to cisplatin.

Several models have been proposed to explain what specific role HMG-domain proteins could play in the cisplatin mechanism of action. When the ability of this family to recognize cisplatin-modified DNA was first detected, it was suggested that HMG-domain proteins might be factors that communicate the presence of the genetic damage to the repair pathways (119), but no evidence to date supports such a hypothesis.

In another potential scenario, the formation of tens to hundreds of thousands of genomic platinum lesions following a chemotherapeutic dose of cisplatin (88, 92) could titrate HMG-domain proteins away from a much smaller number of natural binding sites (Figure 1.6A). Both hUBF and SRY bind with similar affinity to the 1,2-d(GpG) intrastrand adduct and to their target sequences (Table 1.3) (147, 148). Furthermore, the addition of cisplatin-modified DNA to an in vitro ribosomal RNA transcription assay inhibited the hUBF-based activity (149). If gene regulation by an HMG-domain protein were essential for cell viability, then such a diversion could result in cell death. This hypothesis was tested in yeast by taking advantage of the function of Ixr1 (150), also known as Ord1 (151), which represses the transcription of Cox5b, an isoform of subunit V of cytochrome c oxidase. If cisplatin-DNA adducts can titrate Ixr1 away from its recognition sequence, the transcription of genes regulated by Ixr1 should increase specifically in the wild-type cells exposed to cisplatin when compared with *ixr1* mutants. No cisplatin-induced increase was observed either in the mRNA levels of the Cox5b gene or in the activity of a reporter gene placed downstream of the Ixr1 promoter. Thus, in this system at least, titration of Ixr1 cannot be invoked to explain the cisplatin resistance of cells with a mutant HMG-domain protein. It is possible, however, that transcriptional upregulation of Ixr1-controlled genes was not detected due to cisplatin-DNA adducts which blocked RNA polymerase activity, since a decrease in Cox5b mRNA was detected at the higher concentrations of cisplatin employed.

There is more experimental evidence to support an alternative, possibly synergistic but not exclusive, model (Figure 1.6B), which proposes that HMG-domain proteins bind tightly to cisplatin-DNA adducts and block repair complexes from removing the damage. Such repair shielding would enhance the cytotoxicity of the drug by allowing the lesions to persist in the cell. Gel mobility

shift assays demonstrated that the binding of HMG1 to cisplatin-modified DNA occurs slightly faster than binding by the repair factor RPA, and that a preformed HMG1-DNA complex is not disturbed by RPA (40). Moreover, addition of HMG-domain proteins to the in vitro repair assay specifically inhibited excision of the 1,2-d(GpG) but not the 1,3-d(GpTpG) intrastrand cisplatin-DNA cross-links (55, 56, 148). In vivo evidence for this model was provided by yeast double mutants. In this system the differential cisplatin sensitivity caused by inactivation of Ixr1 was directly related to damage recognition and formation of the excision repair complex (143).

The endogenous HMG-domain proteins in HeLa cell-free extracts do not seem to affect the relative rates of repair of cisplatin-DNA adducts (56, 64). Nevertheless, the hypothesis that HMG-domain proteins can enhance cellular sensitivity to cisplatin by blocking repair of the DNA adducts is still viable. Several HMG-domain proteins are specifically expressed in the testes ((148) and references cited therein), two of which, tsHMG and hSRY, inhibit the in vitro excision of cisplatin-DNA adducts at lower protein concentrations than any of the other HMG-domain proteins tested (56, 148). Selective expression of these or other such proteins in testicular tumors would provide an explanation for the unusual cisplatin sensitivity of this tumor type and the reduced repair of cisplatin-DNA adducts observed in testicular cell lines (discussed above).

Platinum Inhibition of Transcription

The formation of cross-links on DNA has the potential to affect directly two essential cellular processes, replication and transcription. Early work demonstrated that cisplatin could inhibit replication under conditions that did not block transcription or translation (3). Adducts formed by *trans*-DDP can also inhibit DNA polymerases (6), however, and it has become evident that cisplatin

lesions are not absolute blocks for replication (see the review by Villiani, this book). Furthermore, cisplatin commonly causes an arrest in the G_2 phase of the cell cycle (152), suggesting that inhibition of gene expression, and not replication, determines whether the cell will live and divide, or undergo apoptosis.

Accordingly, some effort has been devoted to studying the effects of cisplatin on transcription. In vitro experiments with RNA polymerases demonstrated that productive elongation activity was prematurely terminated by the whole spectrum of cisplatin-DNA adducts, but not by the *trans*-DDP 1,3-intrastrand adducts (153-155). Selective bypass of *trans*-DDP adducts was also demonstrated in XPA cells, suggesting that repair of the DNA lesions did not contribute to differential transcription inhibition by the platinum compounds (156). In vivo, hormone-induced chromatin remodeling and subsequent transcription from the MMTV promoter was specifically inhibited by cisplatin (157). In this case, platinum adducts seemed to cause a decrease in the DNA binding of one of the transcription factors, NF1. Several chromatin-associated proteins, such as the linker histone protein H1 or HMG1/2, could have produced through their known affinity for cisplatin-DNA adducts (158), however this possibility was not thoroughly investigated and it was suggested that NF1 is inhibited from binding by the cisplatin-induced distortions. This mechanism of cisplatin cytotoxicity could be countered by mutations that eliminate hot-spots for platinum binding, as observed in SV40 viruses selected for drug resistance (159).

It is possible that cisplatin cytotoxicity arises from more subtle effects than just general inhibition of transcription. The expression of some genes with a strong promoter is inhibited more in cultured cells by cisplatin than bulk RNA synthesis, and this differential sensitivity was not observed for *trans*-DDP (160, 161). On the other hand, the induction of several weak promoters by cisplatin is reminiscent of the bacterial SOS response (161, 162). Since the inhibition or

activation of RNA polymerase II-mediated transcription by cisplatin is gene-specific and modulated by the promoter region, the end result would not be to shut down the cellular machinery so much as to create an imbalance. The selective inhibition, or induction, of gene expression by cisplatin is particularly relevant with respect to the expression of oncogenes, several of which have been implicated in cisplatin resistance (26).

As mentioned above, one consequence of stalled RNA polymerase II at a DNA adduct is activation of transcription-coupled repair (29). This effect may depend on the type of polymerase, however, since the removal of some types of DNA damage is slower from RNA-polymerase I transcribed ribosomal DNA than from a nuclear gene (163). The lower level of repair in the nucleolus could also reflect the influence of other transcription factors, such as the HMG-domain protein UBF, which binds to cisplatin-modified DNA (147). When HeLa cells were exposed to cisplatin at concentrations that did not seem to affect nuclear transcription, inhibition of rDNA gene expression was associated with the redistribution of UBF, along with other factors responsible for rRNA transcription (141). These observations indicate how cisplatin might exert a combination of effects. Transcription is stopped due to titration of essential factors by the platinum-DNA adducts, and the same proteins could shield the lesions from the repair activity.

p53 and the Cellular Response to Cisplatin

To avoid the propagation of mutations leading to malignant transformation the cell has two options. It can arrest the cell cycle and attempt to repair the lesions or, if the damage is too extensive, it can initiate a suicide program. One of the major factors required for maintenance of genomic stability is the product of the p53 tumor suppressor gene, a nuclear protein that exerts its

effects through transcriptional regulation (for reviews see 164-166). Upon exposure to genotoxic compounds, p53 is induced by several post-transcriptional mechanisms, resulting in the activation or repression of a variety of downstream genes. The loss of p53 activity removes a crucial barrier to unrestrained neoplastic growth. More than 50% of solid human tumors have mutations in this gene, usually in highly conserved regions such as exons encoding the DNA-binding domain. In addition, the status of the p53 gene in a tumor can be an important prognostic indicator (167, 168). A non-functional p53 could confer resistance to chemotherapy by protecting tumor cells from drug-induced apoptosis, or it could sensitize the cells owing to the absence of p53-dependent growth arrest and repair.

p53 and Cisplatin Sensitivity

Given the importance of p53 in cancer, it is not surprising that many researchers have tried to establish a connection between p53 and cisplatin sensitivity, but unfortunately a consensus has not been attainable. Introduction of a wild-type p53 gene by viral transfer into both a human non-small cell lung cancer line and an ovarian cancer cell line selected for cisplatin resistance resulted in sensitization of the cells to cisplatin-induced apoptosis (169, 170). Similarly, ovarian carcinoma cell lines selected for cisplatin resistance had non-functional p53, associated with lower levels of apoptosis (171-173). In contrast, a comparison of nine human ovarian cell lines did not demonstrate any correlation between p53 and cisplatin-mediated growth inhibition, suggesting that p53-independent pathways were responsible for cisplatin cytotoxicity (174). In addition, in human breast cancer or foreskin fibroblast cells, which do not favor an apoptotic mechanism of cell death, inactivation of p53 sensitized the cells to cisplatin (175, 176). Finally, in a recent comparison of the drug sensitivity of 57 different human cell lines (177), on average p53-mutant cells were slightly less sensitive to

cisplatin-induced growth inhibition, but there was a large range of responses, depending on the cell line. These results confirm the importance of cell type and cellular context in studying p53-mediated responses to cisplatin (168).

Testicular cancer is particularly interesting with respect to cisplatin because the addition of this drug to the chemotherapeutic regimen has had such a dramatic effect on patient survival (178, 179). In addition, testicular tumors are unusual because they rarely have mutations in the p53 gene (180-185). High expression levels of the p53 protein, usually a characteristic of a genetic mutation, are also observed in testicular tumors (184-187), although a recent study of mouse testicular teratocarcinoma cells suggested that the protein is not transcriptionally active until the cells are exposed to a DNA-damaging agent (188). It is possible that the extreme sensitivity of testicular cancer cells to cisplatin is due to the induction of p53-regulated responses, such as apoptosis, but several in vitro studies failed to support this hypothesis. In an investigation of 4 testicular germ cell tumor cell lines, the p53 status was not a determinant of cisplatin sensitivity and p53-independent apoptosis was observed (189, 190). Similarly, although cisplatin-induced apoptosis observed in several murine testicular teratocarcinoma cell lines was dependent on a functional p53, the activation of a p53-independent cell cycle arrest resulted in comparable long term survival for p53-normal and p53-mutant cells (191).

As discussed above, it is important to confirm in vitro results with in vivo studies. In an examination of several germ cell tumors from male patients who failed cisplatin-based therapy, either because of inherent or acquired resistance, mutations in the p53 gene were found in a subset of these tumors (192). Defects in p53 could potentially cause some of the clinical resistance, due to the lack of an apoptotic response, but the majority of the resistance observed was not explained by p53 mutations. Together with the cell culture results, the clinical data suggest

that p53 is not a key determinant of cisplatin cytotoxicity in testicular cancer. Ovarian cancer is another type of malignancy that is managed with cisplatin chemotherapy, but patient survival is significantly limited by the development of resistance (118). For this type of tumor, p53 expression, either mutant or wild type, seems to be a marker for poor prognosis, which should be considered when planning the therapy regimen (for example, see (193-195) and references therein).

p53-Mediated Responses to Cisplatin

There are multiple pathways for p53 induction (166), but the specific mechanism for the activation of p53-mediated responses by cisplatin is still obscure. Details about the DNA-damage signal transduction pathway could be important for the mechanism of cisplatin resistance and must be provided by future research. In contrast, quite a lot is known about the downstream effects of p53. Several of these p53 activities have been implicated in the modulation of cellular sensitivity to cisplatin (Figure 1.7).

Apoptosis

One of the consequences of p53 induction is apoptosis, a cellular response that is activated by many DNA-damaging agents including cisplatin (152). Members of the Bcl-2 protein family, some of which are controlled by p53, can either accelerate or inhibit the programmed cell death pathway (196). For example, the p53-regulated bax protein usually promotes cell death, but its effects can be neutralized by Bcl-2. In several cases, cisplatin sensitivity was associated with the functional status of p53 and levels of the Bcl-2 proteins. In two different ovarian carcinoma cell lines, cisplatin resistance was acquired in vitro together with loss of p53-mediated transactivation of bax and initiation of apoptosis (172, 197). The introduction of a wild-type p53 gene resulted in an increase in bax

expression and cisplatin-induced apoptosis (170), whereas exogenous Bcl-2 delayed the activation of programmed cell death (198). A comparison of human testicular and bladder tumor cell lines, the latter of which are more resistant to cisplatin, revealed higher levels of apoptosis induced by the DNA-damaging agent etoposide in the testicular cancer cell lines (199). Programmed cell death was associated with functional p53 and high levels of Bax, whereas in most of the bladder cancer cells p53 was non-functional and the Bax:Bcl-2 ratio was much smaller.

DNA Repair

A connection between p53 and DNA repair was observed in p53-deficient cells that exhibited less global DNA repair (200-202) but see (203), as well as a reduced capacity to reactivate cisplatin- and UV-damaged reporter plasmids (175, 204, 205). Furthermore, pretreatment with low levels of UV activated a protective response in which the levels of repair activity were elevated, an effect not observed in p53-deficient cells (204, 206). It is possible that the p53 protein is directly involved in removing DNA damage since the protein recognizes both irradiated DNA and mismatches (164). There is also evidence that p53 can interact with several components of the excinuclease, including RPA and the TFIIH-associated factors XPB and XPD (207, 208). So far, however, there is no evidence to demonstrate an integral role for p53 in the excision repair pathway.

It is more likely that p53 influences repair in a regulatory capacity. One link between p53 and NER was made with the observation that the p53-regulated Gadd45 binds to PCNA, a component of both replication and repair (209). Overexpression of Gadd45 provided a small level of protection from cisplatin (210), whereas Gadd45 antisense DNA sensitized human colon carcinoma cells to cisplatin, an effect that was associated with a decrease in repair (205). It was

hypothesized that the Gadd45 protein could interact directly with the repair proteins because it stimulated repair synthesis in nuclear extracts (209); however, this result could not be reproduced under a variety of experimental conditions (211, 212). Thus, it seems likely that there are still some as yet unidentified factors which link p53 and the excision repair pathway.

The cyclin-dependent kinase inhibitor p21 is another downstream effector of p53 (164-166). There is evidence for p53-independent induction of p21 (164), and under these conditions the protein may be responsible for cisplatin-induced apoptosis (213, 214). The p21 protein usually plays a protective role in response to cisplatin (215, 216), however, an effect that correlated with enhanced repair of a damaged reporter plasmid (215-217). These observations are consistent with the hypothesis that DNA-damage induced p53 activates a G_1 cell cycle arrest through p21, affording the cell time to repair the lesions and precluding the genetic instability produced by replication of damaged DNA. In accord with this model, the addition of p21 to cell-free extracts blocked DNA replication but not excision repair (218), although, since p21 does interact with PCNA, it has the potential to block the repair activity of PCNA in addition to its activity in replication (219).

In *E. coli*, DNA-damaging agents produce what is known as the SOS response, a coordinated up-regulation of genes which contribute to cell survival at least in part by repairing the DNA (29). The p53 protein could provide a signal in a eukaryotic version of this type of general defense mechanism. The binding and activation of p53 by small oligonucleotides such as those generated by NER (164) suggests the existence of a feedback loop connecting DNA repair and p53-regulated gene expression. A p53-dependent increase in repair capacity has been reported for a variety of cell lines exposed to low levels of genotoxic agents (220). Similarly, in a recent study a dinucleotide, mimicking a signal produced by UV-DNA

damage, elicited a protective response to UV irradiation involving activation of the p53 pathway and enhanced repair (221).

Thus there is substantial evidence to suggest that p53 plays a central role in the cellular response to DNA-damage. It is also clear that p53 can control the processing of Pt adducts. Additional experiments are needed to clarify exactly which function p53 fulfills in the management of the DNA repair pathway.

HMG1

Finally, a surprising finding that might connect cisplatin and p53 was made with the discovery that HMG1 enhances both p53 binding to DNA and its transactivation activity (222). In particular, HMG1 was suggested to play an architectural role promoting the cooperative formation of protein complexes containing p53 at the site of transcriptional regulation. Although no direct interaction between p53 and cisplatin-modified DNA has yet been reported, the protein recognizes other types of DNA lesions and has been detected in association with some repair factors (164, 207, 208). It is therefore feasible that HMG1 and p53 could encounter each other at the sites of cisplatin-DNA adducts in response to cellular exposure to the drug.

Another intriguing scenario is that cisplatin-DNA adducts could modulate p53 activity by some manifestation of the titration hypothesis (Figure 1.6A). For example, if p53 and HMG1 were to interact in vivo as they do in vitro, HMG1 could mediate the specific binding of this complex to cisplatin-modified DNA, keeping the p53 protein away from its natural targets. At least some of the p53-regulated activities are enhanced by treatment with cisplatin, however, providing evidence against this theory. In an inverse situation, cisplatin-induced p53 might attract HMG1 away from the platinum adducts, preventing it from shielding the lesions from repair. This hypothesis provides another possible link between p53

and repair. More detailed information about such an interaction in vivo is required before any conclusions can be drawn about how it may contribute to the cisplatin mechanism of action.

Other Nuclear Proteins and Cisplatin-Modified DNA

DNA-PK

Several other proteins can bind cisplatin-modified DNA and hence might influence cellular processing of the damage. One example is the DNA-dependent protein kinase, DNA-PK. This protein plays a role in the repair of DNA double strand breaks, a product of ionizing radiation as well as V(D)J recombination (223, 224). There are two components of this complex. The first is the Ku heterodimer, a DNA-binding protein that specifically recognizes the ends of duplex DNA as well as single strand-to-double strand transitions, nicks, and hairpins. The second factor is the catalytic subunit, DNA-PK_{CS}, which functions as a kinase only when associated with DNA-bound Ku. Multiple phosphorylation substrates have been identified in vitro, including itself, p53, RPA, c-Jun, HMG1, and a variety of other transcription factors. DNA-PK might modulate cisplatin activity indirectly, since cells with a DNA-PK defect were sensitive not only to double-strand break inducing agents, but also to cisplatin and UV radiation (225). These cells exhibited lower levels of nucleotide excision repair, which was restored by transfection with the wild-type DNA-PK gene. In an in vitro assay, however, the levels of repair synthesis were unaffected by addition of purified DNA-PK, or by clearing the extracts by immunoprecipitation. No complementation was observed when two extracts with deficiencies in different components of DNA-PK were mixed together. It was suggested that DNA-PK must play a regulatory role in the excision repair pathway, possibly activating the cell to the presence of DNA-damage by phosphorylating transcription factors.

DNA-PK can directly bind to cisplatin-modified DNA, but the interaction does not enhance its phosphorylation activity (226). In fact the presence of the platinum adducts on the DNA substrates significantly decreased the phosphorylation activity when compared with undamaged DNA (226, 227). It is not immediately apparent how these results can be reconciled with the hypothesis discussed above, but only a few substrates were investigated, so it is possible that the full range of effects of cisplatin-DNA adducts on activity are still not known. In addition, since the affinity for damaged DNA was slightly less than for undamaged DNA, the significance of this interaction in the cisplatin mechanism of action may be minimal.

Histone H1

The histone H1 protein has higher affinity for cisplatin-modified DNA than for DNA damaged by *trans*-DDP or undamaged DNA (228). This interaction is similar to that of other abundant chromosomal proteins, HMG1 and HMG2, and may reflect common functions involving the organization and maintenance of chromatin structure. Like HMG1/2, H1 preferentially binds to altered DNA structures, including supercoiled DNA and four-way junctions (229). This lysine-rich protein is associated with the linker DNA of chromatin, and may bind to the DNA where it crosses itself as it enters and exits the core particles. H1 interacts with HMG1 in vitro, and it has been suggested that HMG1 functionally replaces H1 during the remodeling of chromatin that occurs during replication, transcription or repair (229, 230). It is feasible that this protein could effect cisplatin cytotoxicity in the same manner as the HMG-domain proteins. This hypothesis is made more attractive by the existence of tissue specific histone proteins, including testis-specific variants of H1 (229).

TBP

Another protein that binds to cisplatin-modified DNA is the TATA box-binding protein TBP (231, 232). The addition of either UV- or cisplatin-damaged DNA inhibited transcription in an in vitro assay, and activity was restored by the addition of the basal transcription factor TBP both in a reconstituted system and in whole cells. TBP activity might be titrated away by the presence of cisplatin-DNA lesions, as postulated for HMG-domain protein (Figure 1.6A). Structural similarities were noted between the TBP-bound DNA target and the crystal structure of the 1,2-d(GpG) cisplatin intrastrand adduct, including the DNA bend as well as an opening and flattening of the minor groove. This correspondence may promote the formation of similar protein-DNA complexes, since TBP bound to the two types of DNA generated comparable DNase 1 footprints (232), and the transcription factor was not associated with DNA modified with other compounds, such as *trans*-DDP. These experiments suggest that TBP has the potential to play a major role in the mechanism of action of cisplatin.

Telomeres/Telomerase

The ends of chromosomes are protected from fusion, degradation or rearrangements by a repetitive DNA sequence known as the telomere (233). In humans, telomeres comprise 500 to > 2000 tandem repeats of the 6 bp sequence TTAGGG. Such a G-rich strand provides an attractive target for cisplatin. A recent study of cisplatin-treated HeLa cells suggested that a low dose of the drug causes telomere shortening due to incomplete replication of the chromosome ends, followed by induction of the programmed cell death pathway (234). Whether such DNA adducts are important in the cytotoxic mechanism of cisplatin remains to be determined, but the unique telomere sequence does present a novel target for designing new platinum-based antitumor agents.

Telomeres also protect the cell from losing important genetic information due to the shortening of the chromosome ends at each replicative cycle. Since the telomeric sequence is finite, however, after a certain number of cellular divisions a critical length is reached and the cells enter senescence and die (235). To avoid such a crisis, some cells maintain the telomere structure through the activity of the ribonucleoprotein telomerase, which synthesizes new repeats at the ends of the DNA. Telomerase activity has been detected in the majority of human cancers, but not in most normal tissue, implicating this enzyme in cellular immortality and suggesting novel approaches to cancer therapy (236, 237). Inhibition of telomerase activity by an antisense vector increased the sensitivity of a malignant glioblastoma cell lines to cisplatin-induced apoptosis (238). A recent study investigated whether telomerase activity can be blocked by cisplatin. Exposure of testicular cancer cell lines to the drug inhibited telomerase activity, an effect not observed with bleomycin, doxorubicin or *trans*-DDP (239). The authors suggest that cisplatin-DNA adducts can specifically inhibit the transcription of the G-rich gene for the RNA component of the enzyme.

Concluding Remarks

Cells exposed to cisplatin can respond either by attempting to fix the damage or, if the injury is too extensive, committing suicide. It is unlikely that any one factor in the cell controls this crucial decision and thereby the clinical efficacy of the drug. Much is known about proteins that interact with cisplatin-DNA adducts. We are beginning to understand how cisplatin-induced DNA distortions provide a structural basis for protein recognition, and the identity and cellular function of many factors which mediate cisplatin cytotoxicity are now being defined. However, these proteins are involved in a complex, interwoven set of pathways. Many of the details of these systems, particularly the methods of

communication between critical cellular mechanisms, are still obscure and must be delineated in order to understand how the processing of the DNA lesions differs in sensitive and resistant tissue. The information on these diverse cellular responses should provide the basis for new therapeutic protocols and rational drug design.

A recent experiment with radiolabeled cisplatin has afforded new insight into its pharmacology. When radioactive [^{191}Pt]cisplatin was administered to cancer patients, gamma camera imaging revealed selective accumulation of platinum in tumor tissue as well as in several organs such as the liver, kidney, bladder, gastrointestinal tract, uro-genital region and the neck (Figure 1.8) (240). What distinctive features of the tissue produce this platinum localization? It is likely that differential delivery to the tissue as well as efficient uptake by the cells could have a significant role. It is also possible, however, that some of the proteins discussed above prevent rapid removal of the Pt-DNA adducts, allowing detectable levels of platinum to accumulate in the tissue. At this time, the relative importance of these two possibilities, uptake into the tissue versus intracellular processing, is unclear, but these preliminary studies suggest that this issue is worthy of further pursuit.

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Table 1.1. Components of the excinuclease[a].

Mammalian Protein	Yeast Homolog[b]	Role in Repair
XPA	Rad14	Damage recognition
RPA/HSSB	Rpa	Damage recognition, also involved in repair synthesis
TFIIH		Contains helicases and DNA-dependent ATPase activity Required for formation of preincision complex and transcription-repair coupling
includes: XPB/ERCC3	Rad25/Ssl2	
XPD/ERCC2	Rad3	
p62	Tfb1	
p52/Cdk7	Tfb2	
p44	Ssl1	
p34	Tfb4	
p38/CycH	Ccl1	
XPC	Rad4	Stabilization and protection of preincision complex, not required for repair of some lesions
hHR23B	Rad23	Binds XPC
XPG/ERCC5	Rad2	3' endonuclease
XPF/ERCC4	Rad1	Subunit of 5' endonuclease
ERCC1	Rad10	Subunit of 5' endonuclease

[a]Proteins required for repair synthesis include RFC, PCNA, RPA, DNA polymerase ε or δ and DNA ligase. [b]*S. cerevisae*

Table 1.2. Site-specific cisplatin-DNA adducts repaired by mammalian NER

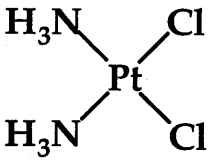
Adduct	Repair detected	Reference
GG	+	(55)
AG	+	(56)
GTG	+++	(55, 241)
GC ICL	-	(56)
GG mismatch	++	(64, 65)

Table 1.3. HMG-domain protein binding constants for cisplatin-DNA adducts.

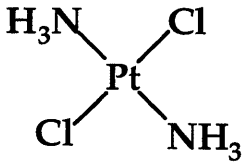
Protein[a]	probe[b]	k_d (M)	ρ[c]	Reference
HMG1	TGGT-100mer	$3.7 \pm 2.0 \times 10^{-7}$	≈ 100	(122)
HMG1 domain B	TGGT-100mer	3×10^{-7}	≈ 3-4	(242)
	AGGC-92mer	4×10^{-7}	≈ 2.5	(242)
	TGGT-20mer	5×10^{-7}	> 4	(242)
	AGGT-15mer	$4.8 \pm 0.9 \times 10^{-8}$	nd	(136)
	CGGC-15mer	$1.3 \pm 0.2 \times 10^{-6}$	nd	(136)
HMG domain A[d]	AGGA-15mer	$6.8 \pm 0.8 \times 10^{-9}$	≈ 235	(136)
	CGGC-15mer	$5.2 \pm 0.6 \times 10^{-7}$	≈ 3	(136)
Ixr1	AGGC-92mer	$2.5 \pm 0.1 \times 10^{-7}$	≈ 8	(150)
tsHMG[d]	TGGT-20mer	$3.0 \pm 0.5 \times 10^{-8}$	≈ 230	(243)
tsHMG domain A[d]	TGGT-20mer	$5.9 \pm 3.4 \times 10^{-7}$	≈ 20	(243)
hSRY[d]	TGGT-20mer	$1.2 \pm 0.2 \times 10^{-7}$	≈ 20	(148)
	SRY-20mer	$5.0 \pm 1.0 \times 10^{-8}$	≈ 40	(148)
hSRY domain	TGGT-100mer	$4 \pm 1 \times 10^{-9}$	≈ 5	(148)
	SRY-100mer	$3 \pm 0.7 \times 10^{-9}$	nd	(148)
	TGGT-20mer	$4 \pm 0.7 \times 10^{-9}$	≈ 5	(148)
	SRY-20mer	$3 \pm 0.4 \times 10^{-9}$	≈ 10	(148)
mSRY domain	AGGC-92mer	$\approx 10^{-6}$	nd	(139)
LEF-1 domain	AGGC-92mer	$\approx 10^{-7}$	nd	(139)
mtTFA	AGGC-92mer	$\approx 10^{-7}$	nd	(139)
hUBF[e]	TGGT-100mer	6×10^{-11}	nd	(147)
hUBF[e]	rRNA promoter	1.8×10^{-11}	nd	(147)

[a]The HMG1 peptides were from rat, tsHMG and mSRY peptides were from mouse, hSRY, Lef-1, mtTFA and hUBF peptides were from human. [b]The cisplatin-modified (underlined) and the flanking nucleotides are listed as well as the probe length. [c]Specificity of binding ratio for cisplatin-modified DNA compared with unmodified probe. [d]Determined by competition assays. [e] Determined by quantitative DNaseI protection assays. nd: not determined

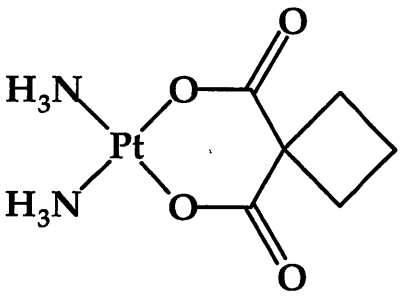
A



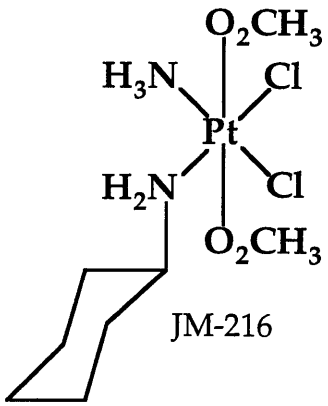
Cisplatin
cis-diamminedichloroplatinum(II)



trans-diamminedichlororoplatinum(II)

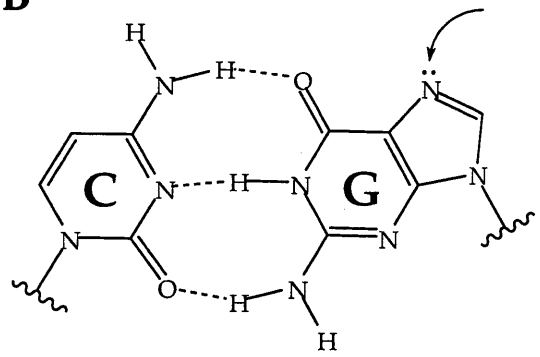


Carboplatin



JM-216

B



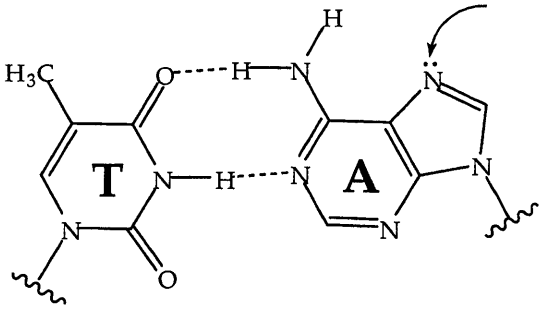


Figure 1.1. Relevant structures. (A) Therapeutically active platinum compounds, cisplatin, carboplatin and JM-216, and the inactive isomer *trans*-diamminedichloroplatinum(II). (B) Watson-Crick DNA base-pairs. Arrows indicate the N7 position of the purine bases, in the major groove, where the covalent Pt adducts form.

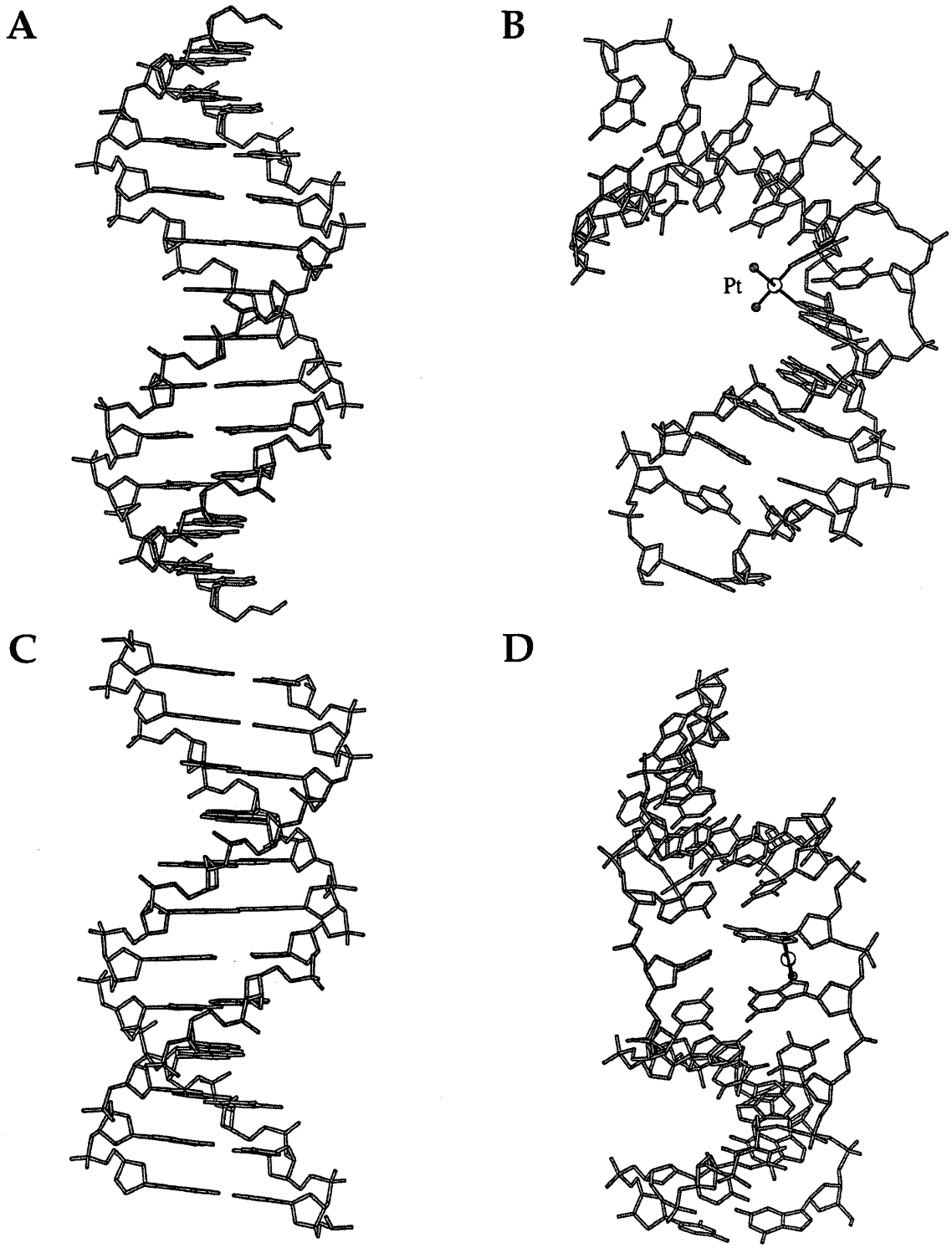


Figure 1.2. DNA distortions caused by a 1,2-d(GpG) cisplatin-intrastrand adduct in a double-stranded deoxyoligonucleotide with the sequence d(CCTCTG*G*TCTCC). (A) Major groove of normal B-DNA. (B) DNA bend caused by the cisplatin-DNA cross-link. (C) Minor groove of normal B-DNA. (D) Widening of the minor groove by the cisplatin-DNA cross-link. The cisplatin-DNA figures are from an NMR solution structure (12).

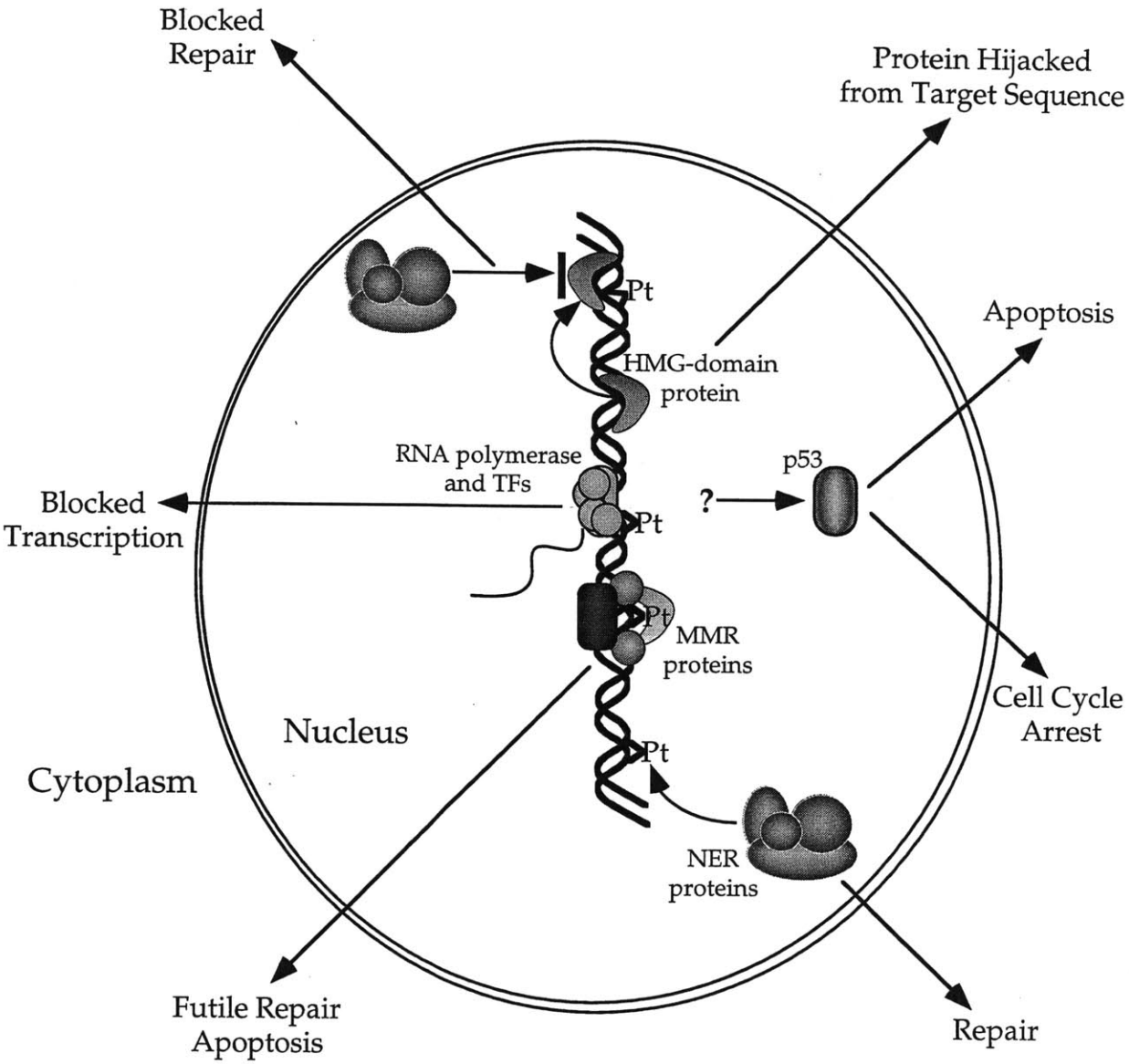


Figure 1.3. Effects of cisplatin-DNA adducts on some of the proteins in the nucleus that interact with the lesions.

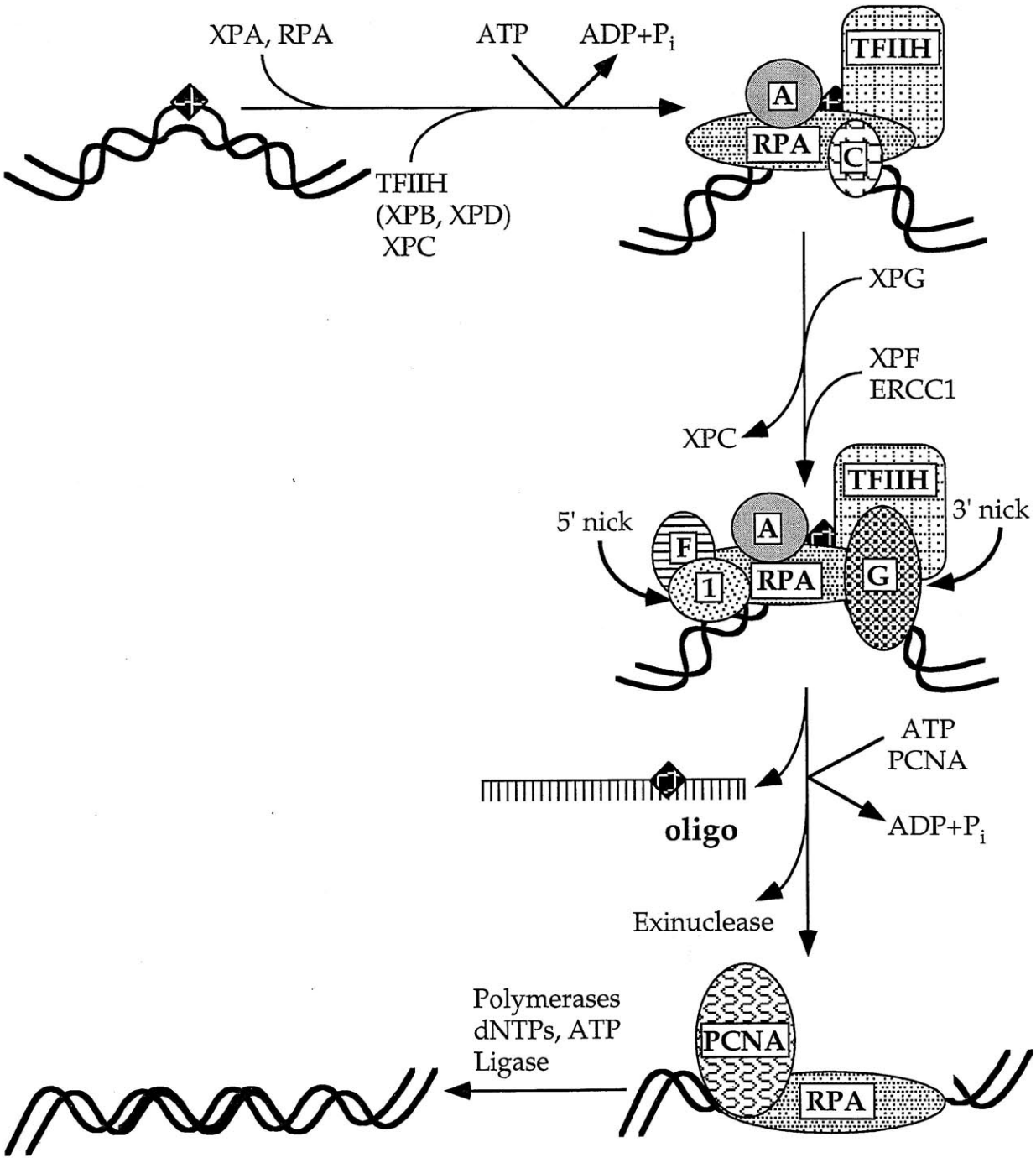


Figure 1.4. Nucleotide excision repair pathway. XPA and RPA are putative damage recognition factors, but the first stable complex is formed after ATP hydrolysis and the recruitment of TFIIH and XPC. This complex unwinds the DNA helix surrounding the lesion. The addition of XPG and the departure of XPC results in the next detectable preincision complex, and dual incision occurs after the final addition of XPF/ERCC1. The damaged oligonucleotide is excised and then most of the excinuclease dissociates and is replaced by PCNA in an ATP-dependent process, followed by repair synthesis by DNA polymerase δ or ε and DNA ligases.

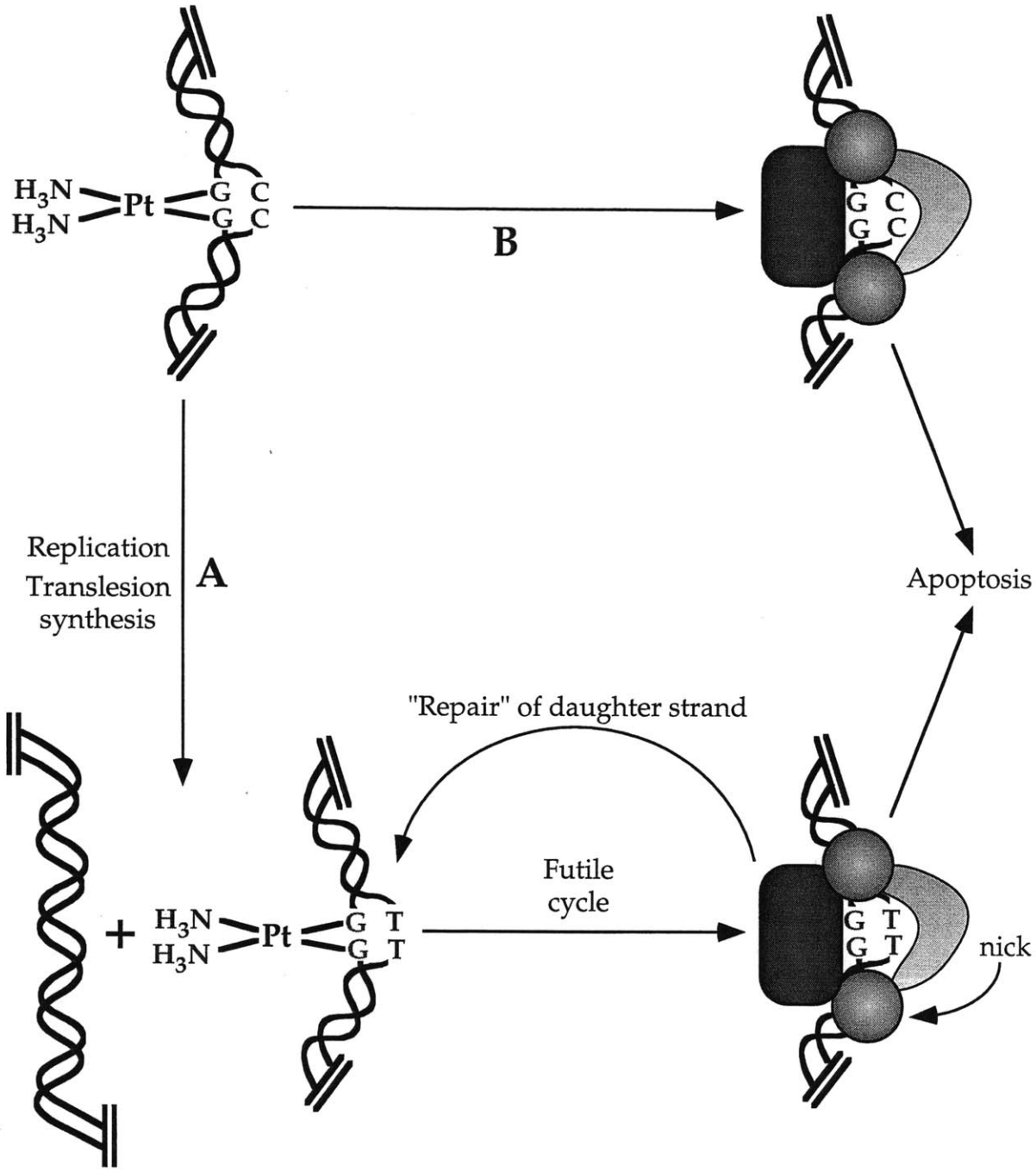


Figure 1.5. Possible roles for mismatch repair in the cytotoxicity of cisplatin. A) During replicative bypass, a mismatch is incorporated across from the cisplatin-DNA adduct. This compound lesion is recognized by the mismatch repair proteins, which cut the DNA on the strand opposite to the platinum. Repair synthesis reproduces the same mismatch, resulting in a futile cycle and possible the accumulation of DNA strand breaks which would activate apoptosis. B) Alternatively, the mismatch repair complex binds to the cisplatin-DNA adduct alone and generates a signal that triggers apoptosis.

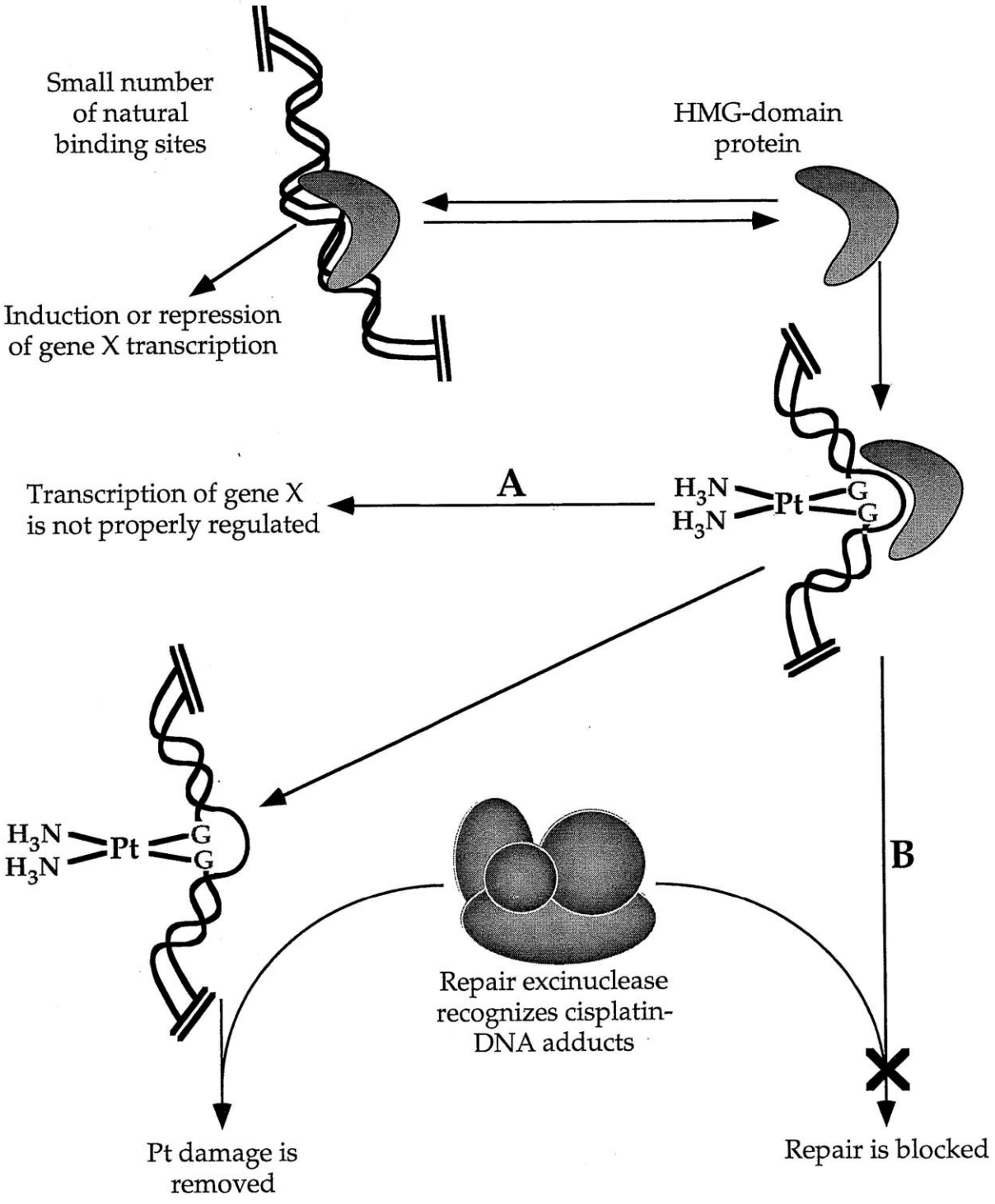


Figure 1.6. Models for HMG-involvement in the cisplatin mechanism of action. A) When a cell is exposed to a lethal dose of cisplatin, 10^4-10^5 DNA adducts are formed on the genome. If HMG-domain proteins bind with similar affinity to these lesions and to their natural target sites, the protein would be titrated away from their transcriptional regulatory function. B) The HMG-domain proteins block access of the excision repair complex and shield the adducts from repair.

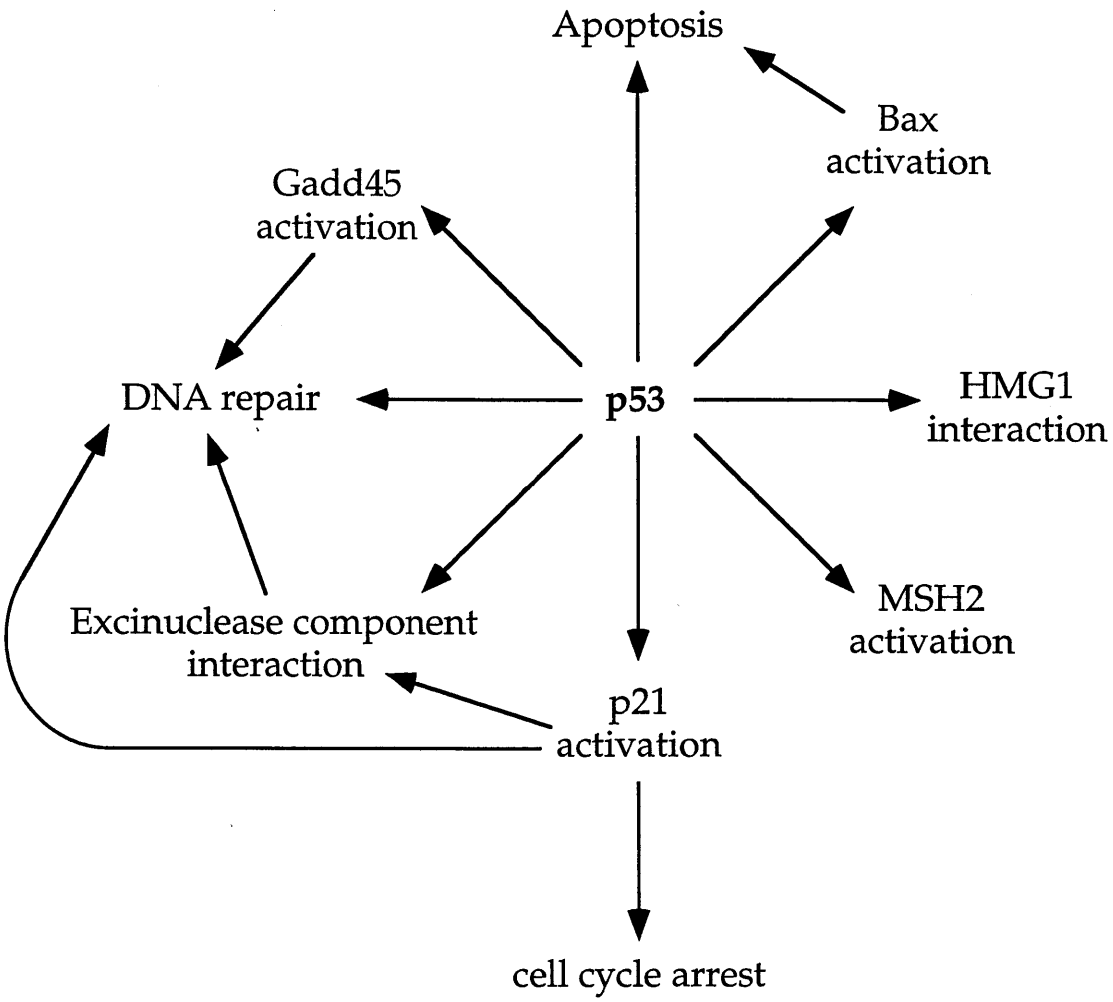


Figure 1.7. Downstream effects of cisplatin-induced p53

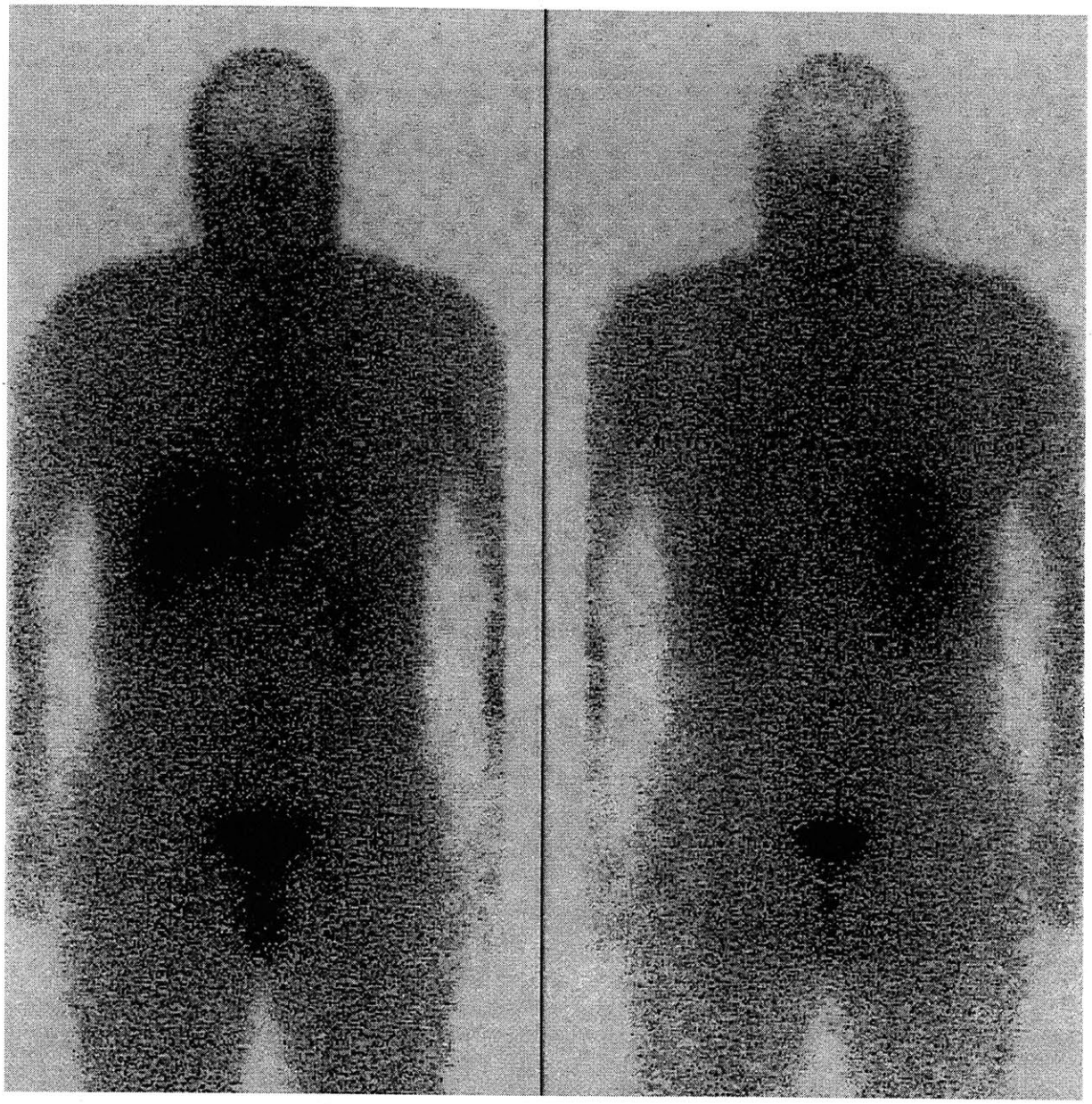


Figure 1.8. Whole body gamma camera image (anterior to the left, posterior to the right) of a testis cancer patient 1 h after the end of a [^{191}Pt]cisplatin infusion. The platinum was still clearly detectable after 65 h but only weakly visible 7 days after the infusion. Chemotherapy was performed following surgery, so no macroscopic tumor tissue remained in the testis. In a different patient, however, platinum was detected in a metastatic tumor of the neck.

Chapter Two

The Repair of Cisplatin-DNA Adducts by the Mammalian Excinuclease

Introduction (1-3)

Covalent adducts on genomic DNA are generated by cellular exposure to a variety of agents, including external sources such as sunlight and cigarette smoke, as well as internal sources such as oxidative metabolism. The presence of DNA lesions can promote mutagenesis, resulting in permanent genetic changes and neoplastic transformation (4). Alternatively, genotoxic stress can cause arrest in the cell cycle, inhibit essential cellular processes, and activate cell death pathways such as apoptosis (5). This second consequence of DNA damage is not always disadvantageous, and can be exploited to benefit an organism by eliminating malignant cells. A case in point is the current application of the toxic DNA-cross-linking agent *cis*-diamminedichloroplatinum(II) (cisplatin) in the clinical setting. This drug is used to treat a variety of human cancers, and is particularly effective against metastatic testicular and ovarian tumors (6, 7).

Cisplatin binds to many of the macromolecules found in the cell, but it is generally accepted that the drug exerts its effects through the formation of DNA adducts (8). The 1,2-intrastrand d(GpG) and d(ApG) cross-links account for 90% of all of the lesions formed, minor adducts include 1,3-d(GpNpG) intrastrand and DNA-interstrand cross-links (9, 10). It is not clear if any one type of cross-link is the active lesions in the anticancer activity of cisplatin, although the prevalence of the 1,2-intrastrand adducts, as well as the fact that they are not formed by the inactive isomer *trans*-DDP due to geometric constraints, makes them attractive candidates for this role. Early gel electrophoresis experiments (11, 12), as well as more recent high resolution structural analysis (13-18), established that all of the adducts induce significant and distinctive distortions in the DNA double helix. Thus, it is likely that they can be distinguished by cellular proteins that respond to and process the DNA damage generated by cisplatin.

Due to the disastrous consequences of genotoxic stress, the cell contains many defense mechanisms to remove flaws from its DNA (19). One of the most versatile pathways is nucleotide excision repair (NER), in which a multi-protein complex cleaves the DNA on either side of a lesion. Excision of the damage in a small, single-stranded, oligonucleotide fragment is followed by new DNA synthesis to fill the ensuing gap (19, 20). The substrate range of NER is very broad, extending from large, DNA-distorting adducts to mismatches (19), but the catalytic efficiency varies depending on the lesion. There is substantial evidence to suggest that cisplatin-DNA adducts are repaired by the NER pathway. Both mammalian and bacterial cells deficient in the excinuclease activity are unusually sensitive to the drug (21-26), an effect that was attributed to poor repair of the cisplatin-DNA cross-links. Furthermore, the repair synthesis assay, which monitors the ATP-dependent incorporation of a radioactive nucleotide into the repair patches of damaged DNA, was used to demonstrate that a plasmid globally modified with cisplatin was repaired in extracts from normal human lymphoid cell lines but not NER-deficient cell lines (27). Attempts to use this assay in the study of the repair of the 1,2-d(GpG) intrastrand adduct, however, were unsuccessful (28).

Unfortunately, as with other chemotherapeutic agents, the clinical success of cisplatin is impeded by its inherently poor activity against some types of cancer as well as the development of resistance after only a short term remission (29). DNA repair has been implicated as a mechanism for both acquired and inherent cisplatin resistance in several model systems (30). In particular, testicular tumor cell lines have a reduced capacity to repair cisplatin-DNA adducts when compared to relatively resistant bladder cancer cell lines (31-33). At this time, there is limited information on the correlation between cisplatin sensitivity and DNA repair in human tissue. An examination of the blood leukocytes of cancer patients undergoing cisplatin chemotherapy revealed an inverse relation between adduct removal and clinical

response (34). Clinical cisplatin resistance has been observed in tumors expressing elevated levels of the mRNA for several of the excinuclease components (35, 36), or exhibiting enhanced repair capacity as determined by a host cell reactivation assay (37). Reports of DNA repair activity in extracts made from mammalian tissue demonstrated significant variability in the repair capacity of the extracts but no correlation with the clinical profile of the drug (38-40).

None of these previous studies have directly examined the mammalian excision repair activity on the individual cisplatin-DNA adducts. Elucidation of the differential processing of the cisplatin-DNA cross-links, which may be crucial to the biological mechanism of the drug, is essential in order to design new therapies which overcome the limitations of cisplatin. In the present study, the in vitro nucleotide excision repair of several different kinds of cisplatin-DNA adducts was observed and the activity of the excinuclease on the cross-links was compared. Furthermore, the influence of extraneous cellular proteins on the excision of the cisplatin-DNA lesions was determined by using purified repair factors. Finally, repair activity was examined in extracts prepared from several cell types as well as human tissue.

Materials and Methods

Materials. Cisplatin was obtained as a gift from Johnson-Matthey. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim. Glycogen, type XI from oysters, and piperidine were purchased from Sigma. DMS and hydrazine were purchased from Aldrich. Yeast tRNA, calf thymus DNA, and pBR322 plasmid DNA were purchased from Gibco BRL. Protease inhibitors and proteinase K were purchased from Boehringer Mannheim. The [γ-^{32}P]ATP was purchased from Amersham or Dupont, NEN. The pET-15b/XPAC plasmid containing the gene for XPAC with a 6-histidine tail was a gift from R. Wood (ICRF, UK) (41). The purified repair components were

prepared by D. Mu (University of North Carolina) as described (42). The cisplatin-modified 123 bp fragment was a gift from J. P. Whitehead (M.I.T.).

Cell-Free Extracts (CFEs). The HeLa S3 cell line was from the Lineberger Comprehensive Cancer Center (University of North Carolina) or was supplied by P. A. Sharp (M.I.T.). The wild-type rodent cell line (AA8), the xeroderma pigmentosum complementation group F (XPF) rodent cell line (CHO-UV41), the XPG rodent cell line (CHO-UV135), the F9 cell line, and the Nulli-SCC1 cell line were obtained from the American Type Culture Collection Repository (Rockville, Maryland). Whole cell-free extracts (CFEs) from all cell lines were prepared by the method of Manley *et al.*, stored at -80° and used through only two freeze/thaw cycles (43). The AA8, XPF and XPF CFEs were prepared by J. T. Reardon (University of North Carolina).

Purification of $XPAC_{his6}$. The pET-15b/XPAC plasmid was transformed into *E. coli* and the cells were grown and induced as previously described (41), except that 75 μg/ml of ampicillan was used. All subsequent steps were performed at 4°C. Thawed cells were resuspended in lysis buffer (20 mM Tris, pH 8, 200 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 8 μg/ml aprotinin, 0.5 mg/ml pefabloc) and lysed by two passages through a French press at 1500 psi. Following centrifugation at 40 000 rpm for 45 min in an ultracentrifuge, the supernatant was filtered through a 0.45 μm syringe filter, and then loaded onto a His-bind column (Novagen). $XPAC_{his6}$ was eluted according to the manufacturer's specifications. The protein started coming off the column at about 70 mM imidazole. $XPAC_{his6}$ was detected on Coomassie stained 12% SDS-polyacrylamide gels. The fractions containing $XPAC_{his6}$ were pooled, 1 mM DTT and 8 μg/ml aprotinin were added, and the resulting solutions were loaded onto a DNA cellulose column (Pharmacia) equilibrated in buffer A (25 mM Hepes, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol) containing 100 mM KCl. The protein was eluted with a linear gradient of 100 mM to 1 M KCl in buffer A. It eluted in a broad peak at

around 600 mM KCl. The fractions containing protein were concentrated in Centriprep-10 concentrators (Amicon), dialyzed against storage buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 16.5% glycerol) and stored at -20°C. The purity of the protein was estimated to be >95% by Coomassie staining of SDS-polyacrylamide gels.

Mobility Shift Assays with XPAC. A radioactively labeled 123-bp DNA fragment globally modified with cisplatin (44), or the site-specifically modified repair substrates, were incubated with the indicated amounts of protein and chicken erythrocyte DNA in binding buffer (4% glycerol, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 1 mM spermidine, 0.05% NP-40, 200 μg/ml BSA, 10 mM Hepes, pH 7.9) for 20-30 min on ice. The mixtures (10 μl) were combined with 0.6 μl of gel-loading solution (30% glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol) and loaded onto pre-run, pre-equilibrated (4°C) 6 or 8% native polyacrylamide gels (29:1 acrylamide:N,N'-diallyltartardiamide). The gel was run in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) at 4°C, 300 V for 2-3 h and visualized by autoradiography with Kodak X-Omat film at room temperature.

Platinated Oligonucleotides. Oligonucleotides were synthesized on a 1 μmol scale on a Cruachem PS250 DNA synthesizer and deprotected according to standard methods. All of the oligonucleotides to be platinated were converted to their sodium salts on a Dowex cation exchange resin (Aldrich) prior to platination.

Site-Specific Intrastrand Adducts. The synthesis of oligonucleotides containing the 1,2-d(GpG), the 1,2-d(ApG) and the 1,3-d(GpTpG) cisplatin intrastrand cross-links was performed as described (12); the 1,2-d(GpG) adduct was a gift from C. S. Chow or U.-M. Ohndorf (M.I.T.). Briefly, cisplatin was allowed to react in aqueous solution with 1.98 equiv of $AgNO_3$ for 14-16 h at room temperature in the dark. The precipitated AgCl was removed by centrifugation and the resulting aqueous solution of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ was used to treat the DNA at 37°C. Platination was carried

out using 100-400 μM DNA at a formal platinum:DNA strand ratio of 1.1-1.5. The reactions were monitored by using reverse phase HPLC, Vydac C4 or C18 analytical columns, linear gradients of 0-50% CH_3CN in 0.1 M NH_4OAc, pH 6.0. The platinated oligonucleotides were purified on preparative scale reverse phase HPLC columns. The purity of the platinated DNA was assessed by HPLC. The amount of DNA present was estimated by using the calculated extinction coefficient at 260 nm (45), and the amount of platinum bound per strand was determined by flameless atomic absorption spectroscopy. The nature of the platinum adducts was confirmed by enzymatic digestion of the oligonucleotides before and after platination (46). DNase 1, P1 nuclease and alkaline phosphatase were used to digest the oligonucleotides into their component nucleosides, which were separated on reverse phase HPLC. Authentic standards *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$, *cis*-$[Pt(NH_3)_2\{d(ApG)\}]^+$ and *cis*-$[Pt(NH_3)_2\{d(G)_2\}]^{2+}$ were employed to assign the products of digestion by HPLC (11).

Site-Specific Interstrand Adduct. The synthesis of the interstrand cross-link was performed in a manner analogous to that previously described (47, 48). Cisplatin was treated with 0.98 equiv of $AgNO_3$ for 14-16 h in DMF at room temperature in the dark, and the AgCl formed was removed by centrifugation. Platination was carried out with 0.4 mM DNA and a four-fold molar excess of platinum-to-oligonucleotide strand in 10 mM $NaClO_4$ at 37°C. The reaction was stopped after 10 min by making it 0.4 M in NaCl. The monofunctional adduct (20 nt) was purified by ion exchange FPLC on a Mono Q HR5/5 column (Pharmacia) with linear gradients of 0.25-0.4 M NaCl in 20 mM Tris, pH 8.0, then concentrated and washed with 0.4 M NaCl at 4°C in Centriplus-3 concentrators (Amicon). The site of platination was verified by Maxam-Gilbert sequencing analysis (48, 49). The platinated strand (20 nt) was allowed to anneal with 0.75 equivalents of its complement (32 nt) in 0.45 M NaCl, 2 mM $MgCl_2$, 20 mM Tris pH 7.4 for 2 h at room temperature and then for 2.5 h at 4°C. The DNA was washed extensively with 100 mM $NaClO_4$, 1 mM $Mg(NO_3)_2$ in Microcon-3

concentrators (Amicon) at 4°C and then incubated at 37°C for 40 h. The interstrand cross-link was purified on a Mono Q HR5/5 column with linear gradients of 0.4-0.6 M NaCl in 10 mM NaOH. The DNA was concentrated in Centricon-3 concentrators (Amicon) at 4°C, precipitated with ethanol, and stored in 5 mM Tris pH 7.5 containing 50 mM $NaClO_4$. The presence of the interstrand cross-link was verified by 5'-end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP followed by analysis on 15% denaturing polyacrylamide gels. The decreased mobility of the platinated duplex oligonucleotide, which could be reversed by NaCN treatment (0.2 M, pH 10-11, 45°C overnight), confirmed the presence of the cross-link. In addition, its location was determined by 5'-end labeling the monofunctionally platinated 20-mer or its complementary 32-mer prior to annealing the two strands. The interstrand cross-link was then isolated on 15% denaturing polyacrylamide gels and analyzed by Maxam-Gilbert sequencing (see below).

Maxam-Gilbert Sequencing Reactions. The DNA was dried, resuspended in water, and the radioactivity was counted. Up to 500,000 cpm were used for the sequencing reactions.

G specific reaction. The DNA (9 µl) was incubated on ice for 10 min with 190 µl of dimethyl sulfate (DMS) buffer (50 mM NaCacodylate, pH 7.0, 1 mM EDTA pH 8.0) and 4 µl of 1 mg/ml calf thymus DNA (Gibco BRL). DMS was added (5 µl of 10% DMS in ethanol) and the reaction was incubated for 10 min at room temperature before adding 50 µl of DMS stop solution (1.5 M NaOAc, pH 7.0, 1 M 2-mercaptoethanol, 250 µg/ml yeast tRNA), 750 µl of ethanol, and 20 µg of oyster glycogen.

C+T specific reaction. The DNA (20 µl) was incubated on ice for 10 min with 5 µl of 1 mg/ml calf thymus DNA. Hydrazine (30 µl) was added to the DNA and the reactions were incubated for 5 min at 37°C before adding 200 µl of hydrazine stop

solution (0.3 M NaOAc, pH 7.0, 0.1 mM EDTA, 100 μg/ml yeast tRNA), 20 μg of oyster glycogen, and 750 μl of ethanol.

All reactions were incubated at -80°C for at least 2 h and then the DNA was precipitated by spinning in a microcentrifuge for 30 min at 4°C. The supernatant was removed, the DNA was washed with 1 ml of cold 70% ethanol, and the DNA was precipitated again by adding 270 μl of water, 30 μl of 3 M NaOAc, 750 μl of ethanol, and incubating at -80°C for at least 2 h. Following centrifugation at 4°C, the DNA was washed with 100% cold ethanol and then dried. The reaction samples were incubated overnight in 50 μl of 0.2 M NaCN (pH > 10) at 45°C. The DNA was precipitated with 15 μg of oyster glycogen, 5 μl of 3 M NaOAc, and 130 μl of ethanol, washed with cold 95% ethanol, then with cold 70% ethanol, and dried. The DNA was then treated with 1.0 M piperidine for 30 min at 90°C. The reactions were lyophilized and washed several times with water before counting and loading onto a 20% denaturing sequencing gel.

Intrastrand Cross-Linked Substrate. Linear 156-bp duplexes were assembled from three sets of complementary oligonucleotides with overhangs 6 nt long following 5' end-labeling of the damaged 12-mer with [γ-^{32}P]ATP and T4 polynucleotide kinase (2). This procedure introduces a radioactive label at the fifth phosphodiester bond to the 5' side of the site of damage in the 1,2-d(GpG) and 1,2-d(ApG) substrates, and at the fourth phosphodiester bond to the 5' side of the damage in the 1,3-d(GpTpG) substrate. For some of the experiments with CFEs prepared from tumors or testicular teratocarcinoma cell lines, a 158 bp substrate was prepared with 3 phosphorothioate linkages and 1 nt overhangs at both of the 3' ends.

Cholesterol Substrate. The cholesterol substrate was analogous to the cisplatin intrastrand cross-linked substrates. It was a 140 bp duplex with a cholesterol moiety attached to a propanediol backbone at position 70 of the damaged strand (Operon Biotechnologies, Almeda, CA). This cholesterol-containing duplex is an efficient

substrate for the mammalian excinuclease and was prepared as previously described (50).

Interstrand Cross-Linked Substrate. The interstrand cross-linked substrate was also assembled from three sets of oligonucleotides except that both strands of the platinated duplex were 5'-end labeled with [γ-^{32}P]ATP before ligation to the annealed pairs of arms at 4°C. The ligation products were purified by electrophoresis on 5% native polyacrylamide gels. The presence of the interstrand cross-link was verified by comparing the mobility of the substrate before and after NaCN treatment (0.2 M, pH 10-11, 45°C overnight) on a 6% denaturing polyacrylamide gel. An impurity was detected, so the substrate was further purified on a 6% denaturing polyacrylamide gel. The product was not reannealed following this purification step, but restriction enzyme digests confirmed that the substrate contained double-stranded DNA (data not shown).

Excision Assay with CFEs. Whole cell extracts were used to measure excision of the damaged DNA according to a previously described method (2, 50). The reaction conditions varied slightly as improvements were made. Briefly, the standard reactions (25 µl) contained 4-5 µl of reaction buffer (300 mM Hepes pH 7.9, 400 mM KCl, 32 mM $MgCl_2$, 1 mM EDTA, 20-50 mM ATP, 2 mM DTT), 5 µl of p21 buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol), 20 µM of each dNTP, 0.2 mg/ml bovine serum albumin (BSA), 50-120 ng of pBR322 as non-specific competitor DNA, 50-250 pM substrate, and 3-5 µl of CFE in storage buffer (25 mM Hepes pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT and 12.5-16.5% glycerol). Several different types of assay conditions were used, but all reaction mixtures contained 50-100 µg of CFE and were incubated at 30°C for 45 min unless otherwise indicated. For the complementation assay, a 50 µl reaction volume was used containing 20 µl of CFEs from XP complementation groups F or G, a mixture of the two, or HeLa CFE. For the kinetic studies, 125 µl reaction volumes

were used containing 25 µl of CFE in storage buffer, and aliquots were removed from the reaction at each time point. The reactions were stopped by making them 0.5% in SDS and 0.4 mg/ml in proteinase K (Gibco BRL) and heating at 55°C for 15 min. After two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and an ether extraction, the DNA was precipitated with 30 µg of oyster glycogen and ethanol, washed with 70% ethanol, dissolved in formamide loading buffer (80% formamide (v/v), 10 mM EDTA, 1 mg/ml each xylene cyanol FF and bromophenol blue), and resolved on a 10% denaturing polyacrylamide gel. When the interstrand cross-linked substrate was used, reaction products were treated overnight with 0.2 M NaCN, pH 10-11, at 45°C, to remove the DNA-bound platinum, and precipitated with ethanol prior to resolution on the gel. The gels were vacuum dried on Whatman 3 mm chromatography paper and exposed to X-ray film (Kodak, X-OMAT AR) at -80°C with an intensifier screen. The level of excision was quantitated by an AMBIS Systems scanner, by using a PhosphorImager with ImageQuant software (Molecular Dynamics), or on a BioRad GS-525 Molecular Imager System.

Reconstituted Excision Assay. The excision assay with highly purified repair components was performed as previously outlined (42). The reaction buffer described above was used, except that the pBR322 and dNTPs were omitted and 4 mM ATP (Boehringer Mannheim) was added separately. The purified repair factors were in 8 µl of storage buffer for a 25 µl reaction, and consisted of 20 ng of XPA, 1-2 ng of TFIIH, 7 ng of XPC, 1-2 ng of XPG, 1-2 ng of XPF/ERCC1 and 250 ng of RPA. For the kinetic studies 25-50 µl reactions were incubated at 30°C and aliquots were removed at the times indicated.

Cell-Free Extracts from Tumor Tissue. Tumor tissue was obtained from the Massachusetts General Hospital Pathology Dept. under the direction of Dr. R. Colvin, or from the Cooperative Human Tissue Network, Western Division. Tumors were either used fresh (4°C) or snap-frozen in $N_{2(l)}$ following surgery, shipped on dry ice

and stored in $N_{2(l)}$. One of the three following methods for preparing cellular extracts was used. In some trials, protease inhibitors were added to the homogenization buffers (1 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mg/ml pefabloc, 10 μg/ml pepstatin and 1 mM benzamidine) and the dialysis buffer (1 μg/ml aprotinin, 1 μM leupeptin, 100 μM pefabloc, 0.5 μM aprotinin), but this step did not affect the repair activity of the extracts. The protein concentration of the extracts was determined by Bradford assay (BioRad or Pierce), with bovine serum albumin used as a standard.

Manley method. Several methods of breaking up the tissue were tested. Some tissue samples were pulverized in a mortar and pestle (pre-cooled at -80°C) under $N_{2(l)}$. Other tissue samples were minced with a razor blade, followed by suspension in hypotonic solution (10 mM Tris, pH 8, 1 mM EDTA, 5 mM DTT) and homogenization with 10 s pulses of a polytron (Brinkman Instruments, Kinematica, PCU-2) or with a Potter-Elvehjem Teflon-glass electric dounce homogenizer (Kontes). Some tissue samples were pushed through a metal tissue sieve with glass pestle (Thomas Scientific). Finally, one sample was digested with collagenase according to a previously described method (51). All work with the tissue was done at 4°C or on ice. All subsequent steps in the CFE preparation were performed as described (43). Some extracts were further concentrated either in microcon-3s (Amicon) or in Biomax-5K Ultrafree centrifugal concentrators (Millipore). Aliquots of the extracts were frozen in $N_{2(l)}$ and stored at -80°C.

Husain method. This method is slightly modified from a previously described procedure for the isolation of active topoisomerase 1 from human tumors (52). The tumor sample was ground in a mortar and pestle (precooled at -80°C) under $N_{2(l)}$, suspended in 4 ml of buffer A (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.25% Triton X-100) and homogenized with a Potter-Elvehjem Teflon-glass electric dounce homogenizer. After incubation on ice for 30 min, 4 ml of

buffer B (buffer A with 2 M NaCl) was added slowly and the mixture was stirred for 30 min at 4°C. The mixture was cleared by centrifugation at 10 000 rpm for 2 h in an SS-34 rotor. Polyethylene glycol 8000 was added slowly to the supernatant to a final concentration of 5% (w/v). The mixture was stirred for 30 min at 4°C and then cleared in a microcentrifuge. The supernatant was dialyzed (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol) for 2 h and concentrated in microcon-3 concentrators. Aliquots were frozen in $N_{2(l)}$ and stored at -80°C.

Nuclear extract method. This method is a modification of a previously described procedure for the isolation of transcriptionally active nuclear extracts from tissue (53, 54). The tissue was minced with a razor blade, suspended in 6 ml of homogenization buffer (10 mM Hepes pH 7.9, 25 mM KCl, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM benzamidine) and homogenized by using 10 sec pulses of the polytron. The homogenate was layered over 1/3 vol homogenization buffer in a centrifuge tube and centrifuged for 45 min at 26 000 rpm in a SW Ti41 ultracentrifuge rotor. The pellet was resuspended in 3.5 ml of lysis buffer (10 mM Hepes pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT) by using a dounce homogenizer (pestle A) and 1/10 vol of 4 M ammonium sulfate was added. The solution was stirred for 30 min on ice and the precipitate was removed by centrifugation for 1 h at 32 000 rpm in a Ti50.2 rotor. Solid ammonium sulfate was added to a final concentration of 0.3 g/ml; the solution was stirred for 30 min on ice and cleared by centrifugation for 30 min at 32 000 rpm. The protein pellet was resuspended in dialysis buffer (25 mM Hepes, pH 7.9, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT), dialyzed for 2 h, and concentrated in microcon-3 concentrators; aliquots were frozen in $N_{2(l)}$ and stored at -80°C.

Results and Discussion

The Binding of XPAC to Cisplatin-Modified DNA. The nucleotide excision repair pathway can be divided into three steps, damage recognition, dual incision and repair synthesis (19). The isolation and characterization of the individual excinuclease components were facilitated by cells from patients harboring the human disease xeroderma pigmentosum (XP), which is a condition arising from defective nucleotide excision repair (55). This disease is categorized into seven different complementation groups, each arising from a defect in one of the repair factors. Group A XP patients present with some of the more severe characteristics of this disorder (55), and the protein that complements the repair deficiency of XPA cells (XPAC) contains a zinc-finger DNA-binding domain (56). It has been suggested that XPAC plays a key role in sensing genetic damage, so if cisplatin-DNA cross-links are removed by NER, the adducts should be recognized by this repair factor. Previous studies revealed that XPAC does bind different types of DNA lesions, including those found in UV-, osmium tetroxide- and cisplatin-modified DNA (41, 57, 58). The mobility shift assay presented in Figure 2.1A confirms that XPAC (expressed with an N-terminal 6-histidine tail, $XPAC_{his6}$) does specifically bind to DNA globally modified with cisplatin. In this experiment, a protein known to specifically recognize cisplatin-DNA adducts, HMG1 (59), serves as a positive control.

Cellular proteins that specifically recognize cisplatin-modified DNA could interfere with the excinuclease and modulate the cytotoxicity of the lesions. Thus it would be informative to study the competition between such proteins and XPAC by using DNA-binding assays. For this type of experiment, the use of a single, site-specific, cisplatin-DNA adduct is preferred because some proteins can distinguish between the types of cross-links. Furthermore, a single adduct should avoid the complications arising from multiple binding sites. Accordingly, mobility shift assays were employed to compare the binding of XPAC and HMG1 to a 1,2-d(GpG)

cisplatin-DNA intrastrand adduct. Specific binding of HMG1 to the substrate produced a complete shift under conditions in which none of the unmodified DNA was bound (Figure 2.1B). However, that level of specificity was not achieved with $XPAC_{his6}$, even though many different conditions were used (Figure 2.1B and data not shown). This result is in agreement with previous reports that the affinity of XPAC for cisplatin-damaged over undamaged DNA is fairly modest (41, 57, 58). It is possible that XPAC is not the exclusive damage-recognition component of the excinuclease since the human single-stranded DNA binding protein RPA also specifically binds to cisplatin-modified DNA (60, 61). Furthermore, the first stable complex on the excision reaction pathway that can be isolated on substrate DNA contained XPAC, RPA, as well as several other factors (62). It is likely that XPAC acts as a DNA damage sensor with only weak specificity, and that tight binding is realized only upon the recruitment of more excinuclease components to the site of the lesion. This theory suggests that an in vitro investigation into the effects of cellular proteins such as HMG1 on excision repair of cisplatin-DNA adducts will require the use of multi-protein repair complexes.

Repair of the Cisplatin-DNA Intrastrand Adducts by the Mammalian Excinuclease. To study the repair of cisplatin-DNA adducts in vitro, the excision assay described in Figure 2.2A was used. A single, site-specific cisplatin-DNA adduct was prepared in a small oligonucleotide, which was then 5'-end labeled and annealed to a complementary strand of DNA. Ligation to two pairs of oligonucleotides yields a 156-bp substrate with a radiolabel several bases to the 5' side of the platinum lesion. When the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand adducts were incubated with HeLa CFE, and analyzed on a denaturing polyacrylamide gel, a pattern of small DNA fragments, 25-31 nt in length, was observed, indicating excision near the damaged site (Figure 2.3, lanes 1 and 5). Unlike the prokaryotic excinuclease, the mammalian counterpart does not have a very strict cutting pattern, with most of the variability

occurring primarily at the 5' incision, resulting in the observed ladder of bands (63, 64). This signal was not detected following incubation with CFEs from cell strains of XP patients (Figure 2.3, lanes 2, 3, 6 and 7). When extracts from two different complementation groups, in this case XPF and XPG, were incubated together with the substrate, repair activity was restored and excision of the damaged DNA was apparent (lanes 4 and 8). This behavior is characteristic of the specific action of the mammalian excinuclease on damaged DNA (65). Similarly, the second most abundant cisplatin-DNA lesion, the 1,2-d(ApG) intrastrand adduct, is also repaired by the mammalian excinuclease, resulting in excision of oligonucleotides 25-31 nt long (Figure 2.4). This ladder of bands was not observed upon incubation of an unmodified repair substrate with HeLa CFE (data not shown). The lower mobility bands near the top of the gel were present at comparable levels following incubation of both modified and unmodified probes in CFE (data not shown), suggesting that they are a product of non-specific nucleases.

The observation that the cisplatin-DNA intrastrand adducts are excised by the mammalian excinuclease expands our understanding of the biological mechanism of action of cisplatin. A previous report suggested that the 1,2-d(GpG) cross-link was not removed by nucleotide excision repair (28), but the experiments were performed with the repair synthesis assay. The assay employed here may be more sensitive, because it measures excision of the radiolabel as opposed to incorporation of radioactive nucleotides into a damaged substrate, which is difficult to detect above background. Further work with the repair synthesis assay, with perhaps more favorable reaction conditions, subsequently confirmed that the 1,2-intrastrand adducts are repaired by the mammalian excinuclease (66).

Many types of lesions that distort the DNA double helix are repaired by the mammalian excinuclease, but the efficiency of repair can vary by more than an order of magnitude (19, 67). Furthermore, there is evidence to suggest that the cisplatin-

intrastrand adducts are structurally distinct (12, 68), which may affect recognition and repair by the excinuclease and thus the cytotoxicity of the cross-links. The variations in conformation between the 1,2- and 1,3-intrastrand cross-links are manifest in the excinuclease activity on the cisplatin-modified substrates. As shown in Figure 2.3 (and data not shown), the repair of the 1,3-d(GpTpG) intrastrand adduct in HeLa CFE is significantly more efficient than that of the 1,2-d(GpG) intrastrand cross-link, a result that was recently confirmed by experiments with the alternative repair synthesis assay (66).

The relative rates of excision of the two 1,2-intrastrand cross-links were determined by incubating the site-specifically platinated probes in HeLa CFE for increasing amounts of time (Figure 2.5). As the incubation time lengthened, the 25-29 nt excision products were digested by non-specific nucleases in the whole cell extracts, resulting in the detection of additional bands corresponding to material smaller than 25 nt. The experiment shown in Figure 2.5A was repeated three times, but differences among the preparations of CFEs caused some variability in the levels of excision. For this reason, data from each experiment were normalized to the highest level of repair (consistently the 90 min time point for the AG substrate). The resulting plot reveals that both of the 1,2-intrastrand adducts are repaired in a similar fashion (Figure 2.5B). In all of the experiments, however, the 1,2-d(ApG) adduct was a better substrate for the excinuclease, with the level of excision about 30% higher than that detected for the 1,2-d(GpG) adduct after 90 min of incubation in HeLa CFE.

The relative rates of repair of the cisplatin-DNA intrastrand cross-links (GTG>AG>GG) observed in the HeLa CFE agree with studies of prokaryotic repair (69), but see (70), although that work was performed with a 1,2-diaminocyclohexane ligand bound to platinum in place of the two ammine groups of cisplatin. Both the d(GpG) and the (ApG) 1,2-intrastrand adducts bend and unwind the DNA to a similar extent (11, 12), so the small but reproducible difference in the relative rates of

repair is not a result of global changes in the DNA structure. These two intrastrand adducts produce distinct responses in several other biological pathways, however. For example, the d(ApG) adduct is more mutagenic than the d(GpG) adduct (10, 71), and it is more readily bypassed by a variety of bacterial DNA polymerases (72). This disparity must be due to local structural variations in the platinated bases, providing a slightly altered recognition element for the excinuclease or other cellular factors.

Repair of the Interstrand Cross-Linked Substrate. In addition to intrastrand adducts, cisplatin also covalently cross-links the guanosine nucleosides on opposing strands in 5'-d(GpC)-3' sequences (49). Biochemical methods, as well as high resolution NMR structures, demonstrated that the interstrand cross-links produce dramatic conformational changes in DNA (13, 16, 73). The DNA distortions include a bending and unwinding of the duplex, a local reversal to a left-handed helix, and extrahelical extrusion of the complementary cytosines. Although interstrand cross-links comprise fewer than 5% of the lesions formed when cisplatin binds to DNA (10, 74), they have been implicated as the cytotoxic lesion (75). Furthermore, removal of the interstrand cross-links from genomic DNA has been associated with cisplatin resistance of certain cultured cell lines (31, 76, 77). Thus, it is possible that interstrand cross-links contribute to the mechanism of action of cisplatin.

To test this hypothesis, a site-specific interstrand cross-link was constructed between two complementary oligonucleotide fragments 20 and 32 nt in length. Maxam-Gilbert sequencing was used to demonstrate that the platinum was covalently bound to the guanine bases of the d(GpC) sequence (Figure 2.6); the bond between the platinum and the N7 of the cross-linked guanines blocks the G-specific reaction with DMS (48, 49). A radioactive label was introduced on both strands of the duplex in order to facilitate detection of cleavage sites on either side, and this complex was ligated with two pairs of oligonucleotides to prepare a 164 bp interstrand cross-linked substrate (Figure 2.2B). The probe was incubated under

excision assay conditions in the absence of cell extracts, followed by analysis on a denaturing polyacrylamide gel (Figure 2.7, lane 5). To detect the low levels of excision repair in the other lanes, the whole gel was overloaded, resulting in the visualization of very small amounts of impurities surrounding the full length probes. Nevertheless, it is clear from the reduced mobility of the major band, in comparison to the intrastrand adduct, that most of the material contains the interstrand cross-link.

Extracts prepared from CHO AA8 cells were employed in the assay for repair of the interstrand adduct because they provide a high level of excinuclease activity, but HeLa CFE afforded the same results (data not shown). To detect products arising from incision on only one strand of the duplex, the platinum adduct was released after incubation with CFE by treating the DNA with NaCN (49). The 1,2-d(GpG) intrastrand adduct was run as a positive control to ensure that the extract was active in nucleotide excision repair (Figure 2.7, lane 3). After NaCN treatment, the pattern of bands resulting from excision of the control substrate increased in mobility by one nucleotide (compare lanes 3 and 4), as expected for removal of the positively charged platinum complex from the excised oligonucleotide fragments. NaCN treatment of the interstrand cross-linked substrate released two oligonucleotides having slower mobility than that of the 30 nt marker (lane 6), even after extensive native and denaturing polyacrylamide gel purification steps. The impurity might arise from some interstrand cross-linked oligonucleotide ligated to the arms of the probe DNA only at the ends of the 20-mer; incomplete NaCN reversal would result in the release of two products, an unmodified 32 nt fragment and a platinated 32 nt fragment. Two solution structures of cisplatin-modified DNA containing an interstrand cross-link revealed that the adduct can cause a marked disruption of the B-DNA duplex (13, 16), which might account for the poor ligation efficiency observed in the present study. The two new bands were not observed when the substrate was incubated with CFE prior to cyanide treatment (lane 8), presumably because of nuclease degradation of

the impurity. Of greater consequence, however, is that following incubation in cell extracts no excision products were detected, either before or after NaCN treatment. Varying the incubation times and the amount of substrate used did not alter this result (data not shown). Although a faint pattern of bands was observed in the range between the 30 and 24 nt markers, these features were no more prominent than in other regions of the autoradiograph and are ascribed to non-specific nuclease activity.

In vitro removal of the cisplatin interstrand cross-link by the repair synthesis assay was previously reported (78). This assay measured the amount of new DNA synthesized after damage removal in whole cell extracts, however, so the signal could have been a result of repair other than nucleotide excision. Studies in cell strains derived from patients with Fanconi's anemia (FA) suggest that NER may not be the primary pathway for removing the cisplatin-DNA interstrand cross-links (23, 25). Although NER is active in FA cells, they have a deficiency in the repair of interstrand cross-links, a fact that explains their extreme sensitivity to various DNA cross-linking agents including cisplatin (79).

A recent investigation of a psoralen interstrand cross-link suggested that the mammalian excinuclease attempts to repair this type of lesion, but in a different fashion than for any other type of damage (80). The excinuclease hydrolyzed two phosphodiester bonds about 22-28 nt apart, both 5' to the cross-link and on the same strand, producing a gap several nucleotides 5' to the cross-link. The cisplatin interstrand cross-linked substrate used in the study described here contained a radioactive label at the 10^{th} phosphodiester bond to the 5' side of the damage on one side of the duplex and 16 nt to the 5' side of the damage on the other strand. Thus, if nucleotide excision occurred by the standard mechanism, with cleavage sites about 5 nt 3' and 20-24 nt 5' from the adduct, or in the same manner as the psoralen cross-link, it would be detected in this assay. The fact that we were unable to detect any nucleotide excision products of the cisplatin-interstrand cross-linked substrate might

be the result of impurities in our probe that obscured its repair. We consider this possibility to be unlikely, however, and conclude that the cisplatin interstrand cross-link is not removed by the mammalian NER pathway in the same manner as the intrastrand adducts, unless the efficiency is so low that it is undetectable in this assay. This conclusion is in agreement with the recent demonstration that the *trans*-DDP interstrand adduct is also refractory to repair by NER (S. S. Marla, personal communication).

Repair of the Cisplatin Intrastrand Adducts in a Reconstituted System. There are many cellular proteins that bind to cisplatin-DNA adducts and might, in this manner, affect their processing (30). Some proteins, such as HMG1 (59), specifically recognize the 1,2-intrastrand adducts and could selectively block access of the excinuclease to these lesions. In contrast, the 1,3-intrastrand cross-links are poor targets for recognition by HMG1 and its analogues, providing a possible explanation for the more efficient repair of the 1,3-intrastrand adduct observed in the HeLa CFE. To study the nucleotide excision repair of cisplatin-DNA adducts in the absence of any potentially interfering factors, the relative rates of repair of the site-specifically platinated substrates were compared in a reconstituted system of highly purified repair components (64). The repair signal of all three intrastrand adducts increased with time over a 3 h period (Figure 2.8). This result differs from the plateau observed in the whole cell extracts after 40-50 min (Figure 2.5B). The amount of repair detected was fairly small (0.5-2%) (42).

In the reconstituted system, the most rapidly excised cisplatin-DNA intrastrand adduct is the 1,3-d(GpTpG) cross-link (Figure 2.8B). The fact that more efficient repair of the 1,3-intrastrand cross-link compared with the 1,2-intrastrand adduct was observed both here and in the HeLa CFEs reflects the degree of unwinding of the platinated DNA (12). Although the difference is more pronounced in the cell extracts, these results suggest that extraneous cellular proteins in the HeLa

CFE can only influence the repair of the cisplatin-DNA adducts to a small degree. Similarly, a comparison of the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand adducts in the repair synthesis assay revealed a 15-20 fold difference in repair efficiency, and fractionation of the HeLa cell extracts did not isolate any factors which changed the relative levels of repair (66). An examination of the rate of incision of cisplatin-DNA adducts by the bacterial UvrABC endonuclease demonstrated the 1,3-d(GpCpG) intrastrand adduct to be incised less efficiently than the 1,2-d(GpG) intrastrand cross-link (70). The rate of UvrC binding to the 1,3-d(GpCpG) preincision complex was faster than to the 1,2-d(GpG) preincision complex, however. Thus both mammalian and prokaryotic excision repair systems can distinguish between the two types of cisplatin-intrastrand cross-links, even if some divergence between the two pathways is reflected in the relative rates of repair. It should be noted that each adduct was studied in the context of only a single sequence, which can influence the recognition by cellular proteins (81).

The relative rates of excision of the two 1,2-intrastrand adducts in the reconstituted system were indistinguishable over a period of several hours (Figure 2.8), in contrast to the HeLa CFE (Figure 2.5). This result demonstrates that the slightly faster rate of excision of the 1,2-d(ApG) adduct observed in the whole cell extracts, compared to the 1,2-d(GpG) adduct, is not caused by differential recognition of the two adducts by the excinuclease. Instead, it is possible that cellular factors that can distinguish between the 1,2-intrastrand adducts (discussed above) affect the relative rates of repair. A recent study of the levels of bifunctional DNA adducts formed in CHO cells exposed to cisplatin or carboplatin revealed a positive correlation between cytotoxicity and the 1,2-d(ApG) adduct (82). This result might be explained by differential processing of the adducts, but since the 1,2-d(ApG) cross-link was repaired at least as efficiently as the 1,2-d(GpG) intrastrand adduct (Figures

2.5 and 2.8), this difference in cytotoxicity between the 1,2-intrastrand adducts cannot be ascribed to excision repair.

Repair of Cisplatin-DNA Intrastrand Adducts in Murine Testicular Teratocarcinoma CFEs. The introduction of cisplatin as a chemotherapeutic option has had the largest impact in the treatment of testicular cancer, which is now one of the most curable types of solid tumors (83, 84). A significant amount of work on cisplatin has focused on testicular tumors, in the hope that information about the efficacy of the drug will lead to better therapies for other types of cancer. Many studies of cisplatin employed cultured cell lines that retain the relative clinical chemosensitivities of their tumors of origin. One hypothesis to explain the cisplatin hypersensitivity of testicular tumor cell lines (85, 86) involves a deficiency in repair of the DNA adducts (33). In particular, several studies which examined the removal of specific cisplatin-DNA adducts by immunochemical analysis could not detect any repair of the 1,2-intrastrand cross-links in some cell lines (31, 32).

To investigate the excision repair of the different site-specific cisplatin-DNA adducts in testicular tumor cells, CFEs were made from the F9 mouse testicular teratocarcinoma cell line. Following incubation of the cisplatin-DNA intrastrand adducts with HeLa or F9 CFEs, the repair products were analyzed on a denaturing polyacrylamide gel. Figure 2.9 demonstrates that the 1,3-d(GpTpG) cross-link is excised in a similar manner in both the F9 and HeLa CFEs (compare lanes 7 and 11). When the 1,2-d(GpG) intrastrand adduct was incubated with F9 CFEs, however, the signal was different then that generated by HeLa CFEs (compare lanes 1 and 5). The typical pattern of bands 25-29 nt was barely detectable above the background of non-specific degradation. Instead, smaller DNA fragments were observed, which are due to an alternative repair pathway or some other DNA nuclease activity. Similarly, deficient excision of the 1,2-d(GpG) intrastrand adduct was observed in extracts

made from the Nulli-SCC1 mouse testicular teratocarcinoma cell lines (data not shown).

The levels of repair of the 1,2-d(GpG) intrastrand adduct in the F9 extracts were so low that it was difficult to isolate this signal from the background of non-specific degradation. To eliminate this problem, each reaction was performed in duplicate, with or without ATP. In the absence of ATP, the percentage of radioactivity detected in the region of the excision products was designated as background and this value was subtracted from the amount of repair detected in the presence of ATP. This calculation is based on the fact that the excinuclease activity is ATP-dependent (19), and the assumption that non-specific nucleases are ATP-independent (87).

Table 2.2 is a summary of the repair of the cisplatin-intrastrand adducts following incubation with HeLa or F9 CFEs for 30 or 60 min. It is inappropriate to compare the levels of DNA repair between two different extract preparations because there are no internal controls to normalize the data. Accordingly, the amount of the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand cross-links repaired was compared in each extract preparation. The percent repair was calculated by two different methods. The data listed in Table 2.2.1 were calculated by assuming that all of the DNA fragments smaller than about 32 nt were generated by nucleotide excision and degradation of the excision products. In Table 2.2.2, the data were calculated by assuming that the excision repair products were only about 22-32 nt long (19), and that smaller DNA fragments were due to non-specific DNA degradation of the repair substrate. By using either method, the ratio of repair of the 1,3-d(GpTpG) and 1,2-d(GpG) adducts is much larger in the F9 CFEs than in HeLa, reflecting the poor repair of the 1,2-intrastrand adduct observed in the mouse testicular teratocarcinoma cell extract (Figure 2.9).

A recent report demonstrated that protein recognition of the 1,2-d(GpG) cisplatin-DNA adduct is influenced by the nucleotides flanking the cross-link (81). For this reason, a site-specifically cisplatin-modified repair substrate was prepared with the adduct in a different sequence context than the G*G* substrate used in the experiments described above (CG*G*C, Table 2.1, the asterisks indicate the sites of platination). The ratio of repair levels of the CG*G*C substrate in comparison with the 1,3-d(GpTpG) adduct is smaller in the HeLa CFE than that of the G*G* substrate. This result is due to a larger level of repair of the CG*G*C substrate in the HeLa CFE (data not shown). This experiment demonstrates that the sequence context of a cisplatin-DNA adduct can affect the excinuclease activity. In a similar manner as the G*G* substrate, however, the CG*G*C substrate was also poorly repaired in the F9 CFEs (data not shown), resulting in a larger G*TG*/CG*G*C ratio of repair in the F9 CFEs than in the HeLa CFEs (Table 2.2). Thus the deficient repair in the F9 extracts is not limited to the 1,2-d(GpG) intrastrand adduct in a single sequence.

Although these data support the hypothesis that testicular tumor cells are sensitive to cisplatin due to deficient repair of the major DNA adduct, there are several caveats such a conclusion. First, the comparison is being made between a mouse testicular teratocarcinoma cell line and a human cervical carcinoma cell line. The HeLa CFEs were used because they are known to be proficient in excision repair of cisplatin-DNA adducts. Future work should focus on human testicular teratocarcinoma cell lines, and the relative levels of repair in extracts from multiple cell types should be determined and correlated with cisplatin sensitivity. Second, there is an inherent variability in the in vitro repair assay (88), most of which probably arises during extract preparation. As shown in Figure 2.9, the levels of repair can vary significantly between extract preparations from the same cell type. The ratio of repair of the 1,2- and 1,3-intrastrand adducts in two HeLa CFE preparations differs by a factor of 2.5 (compare lanes 7 and 9). It might be possible to

use some form of internal control to normalize the repair activity of CFEs, however nothing appropriate has yet been described. Hence, the in vitro repair assay may not be suited for this type of experiment, and repair of the cisplatin-DNA adducts in different models may be best monitored in vivo.

Repair of Cisplatin-DNA Adducts in Tumor Tissue CFEs. Although it is clear that the biological activity of cisplatin involves multiple cellular pathways (89, 90), it has been suggested that cisplatin resistance, either inherent or acquired, is a consequence of enhanced repair activity (8, 89). Many studies have explored this connection in cell culture, but only one group examined the repair activity of cisplatin-DNA adducts in extracts made from mammalian tissue (39, 40). The repair capacity of ovarian tumors from different individuals varied by as much as 10-fold (39), and a comparison of different rat tissue did not reveal any correlation between the levels of repair and the clinical profile of cisplatin sensitivity (40). In these experiments, DNA randomly modified by cisplatin was used as a substrate in the repair synthesis assay. Cellular sensitivity to cisplatin may depend on differences in repair of the cisplatin-DNA adducts, which would not have been detected. Furthermore, since some DNA synthesis is observed even with undamaged DNA (27), the origin of this activity is unclear. For this reason, attempts were made to study the repair of the site-specific cisplatin-DNA adducts in extracts made from human tissue.

Cell-free extracts were prepared from samples of human tissue, either tumor or normal. Several different methods were used to make the extracts (see Materials and Methods). The Manley method is a procedure that was originally designed for the isolation of cellular proteins from cultured cells (43). The nuclear extract and Husain methods were successfully applied in the isolation of active transcription factors and topoisomerase 1, respectively, from human tissue (52, 53). Several features of these protocols were varied in order to optimize the isolation of excision repair-active proteins. The most consequential factor is the method of breaking up

the tissue to a cellular level. The methods that proved to be successful were either grinding the frozen tissue in $N_2(l)$ or homogenization with a polytron. Ineffective methods include an enzymatic collagenase digestion (51), pushing the tissue through a metal sieve with a glass pestle, and an electric dounce homogenizor.

All of the extracts were tested for repair activity by using site-specific cisplatin-DNA adducts and the excision assay. An example of such an experiment is shown in Figure 2.10. Out of 63 tissue samples, only 12 extracts were active in repair, and in most of the extracts the repair capacity was weak such that only the 1,3-d(GpTpG) cross-link produced a detectable signal. Active extracts were prepared from kidney, brain, endothelial, and testis tumors, but none from normal tissue. Both active and inactive extracts were prepared from the same type of tissue. This result has two possible explanations. One interpretation is that tumor cells from different individuals have a large variability in the DNA repair capacity. Whereas this hypothesis may be true, given the success rate in preparing active extracts it is more likely that the variability in repair activity is due to problems in extract preparations, as discussed above. Furthermore, the tissue samples were not screened for homogeneity. Since different cell types in the same organ exhibit variable levels of repair (91), inconsistencies between the tumors would affect the repair capacity of the extracts. Attempts were made to correlate repair activity and the presence of proteins that recognize cisplatin-DNA adducts, by using both Southwestern and Western assays, but no relationship was detected (data not shown).

Conclusions

These experiments provide some valuable insights into the cellular processing of genetic damage caused by cisplatin. The three types of major cisplatin-DNA adducts, the 1,2-d(GpG), the 1,2-d(ApG), and the 1,3-intrastrand cross-links, are all repaired by the mammalian excinuclease. In a reconstituted system of highly

purified proteins the 1,3-intrastrand adduct is the most efficiently excised, and the relative repair rates of the two 1,2-intrastrand adducts are very similar. In whole cell extracts, the 1,2-d(ApG) cisplatin-DNA adduct is excised at a slightly faster rate than the 1,2-d(GpG) cross-link. Thus the distinct structures of the cross-links significantly influence the excinuclease activity, but it is also possible that there are cellular proteins in the extracts that can affect the relative rates of repair of the cisplatin-DNA adducts. In vitro repair of the 1,2-d(GpG) intrastrand adduct is deficient in murine testicular teratocarcinoma cell lines, a result that needs to be confirmed in human cell lines. Finally, it is possible to detect excision repair in extracts made from human tissue, but difficulties arise because of the low success rate in preparing active extracts and in normalizing the repair activity between samples.

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1. Some of this work was previously published in a slightly different form. See references 2 and 3.
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Table 2.1. Sequences of the oligonucleotides used to prepare the site-specific cisplatin adducts. Only one strand of the interstrand cross-link (ICL) is shown. The platinated nucleosides are in bold.

Abbreviation	Sequence (5' to 3')
G*G*	TCTA**GG**CCTTCT
A*G*	TCTT**AG**TTCTCT
G*TG*	TCT**GTG**CACTCT
ICL (GC20)	CTCCTCCTT**GC**TCTCCTCTC
CG*G*C	CCTCTCC**GG**CTCTTC

Table 2.2.1. Summary of experiments comparing the excision of cisplatin-DNA intrastrand adducts in extracts made from HeLa or mouse testicular teratocarcinoma (F9) cells. The reactions were incubated at 30°C for the times indicated prior to resolution on 10% denaturing polyacrylamide gels. The data was analysed on a BioRad phosphorimager. The averages of the ratios of percent repair of the different adducts are listed ± 1 esd.

	30 min incubation		60 min incubation	
CFE	GTG/GG[a]	GTG/CGGC[b]	GTG/GG	GTG/CGGC
HeLa	4.8 ± 0.55	2.7 ± 0.21	3.5 ± 0.58	2.5
F9	13 ± 4.8	4.9 ± 0.64	13 ± 1.3	7.8
n[c]	3	2	4	1

[a]The percent repair was calculated by dividing the signal from all of the products smaller than appoximately 33 nt by the radioactivity in the whole lane, then the percent repair of the same reaction performed in the absence of ATP was subtracted. The GTG/GG ratio was calculated by dividing the percent repair of the 1,3-d(GpTpG) intrastrand adduct by the percent repair of the 1,2-d(GpG) intrastrand adduct. [b]The CG*G*C substrate contains a 1,2-d(GpG) intrastrand adduct in a different sequence context than the G*G* substrate (see Table 2.1). [c]Number of individual experiments.

Table 2.2.2. Summary of experiments as in Table 2.2.1 except that the percent repair was calculated in a slightly different fashion (see below).

	30 min incubation		60 min incubation	
CFE	GTG/GG[a]	GTG/CGGC[b]	GTG/GG	GTG/CGGC
HeLa	5.2 ± 0.43	2.5 ± 0.05	4.1 ± 0.59	2.4
F9	22 ± 7.7	6.3 ± 0.65	31 ± 2.7	11
n[c]	3	2	4	1

[a]The percent repair was calculated by dividing the signal of the products approximately 32-22 nt long by the signal in the whole lane, and the percent repair of the same reaction performed in the absence of ATP was subtracted. [b]The CG*G*C substrate contains a 1,2-d(GpG) intrastrand adduct in a different sequence context than the G*G* substrate (see Table 2.1). [c]Number of individual experiments.

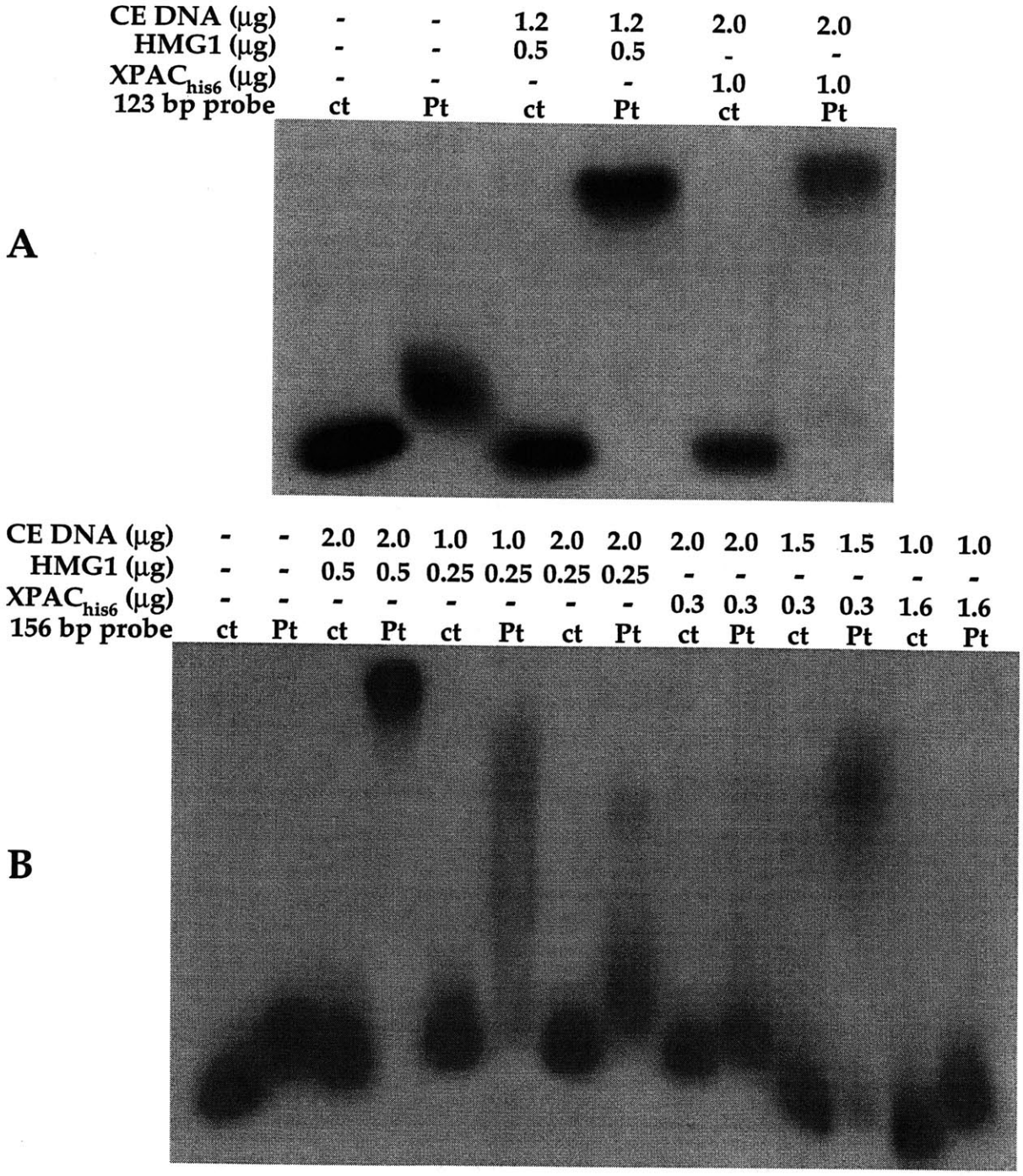


Figure 2.1. XPAC$_{his6}$ binds specifically to cisplatin-modified DNA. (A) The indicated amounts of HMG1 or XPAC$_{his6}$ were incubated in a 10 μl reaction with CE DNA and either 0.2 ng of unmodified or 0.7 ng of cisplatin-modified 123 bp DNA (20 000 cpm) for 30 min on ice. The reactions were resolved on an 8% native polyacrylamide gel. (B) HMG1 or XPAC$_{his6}$ was incubated for 30 min on ice with the indicated amounts of CE DNA and 10 000 cpm of the 156 bp site-specific 1,2-d(GpG) intrastrand adduct or the unmodified control. The reactions were resolved on a 6% native polyacrylamide gel. The gel was slightly distorted during separation from the plates.

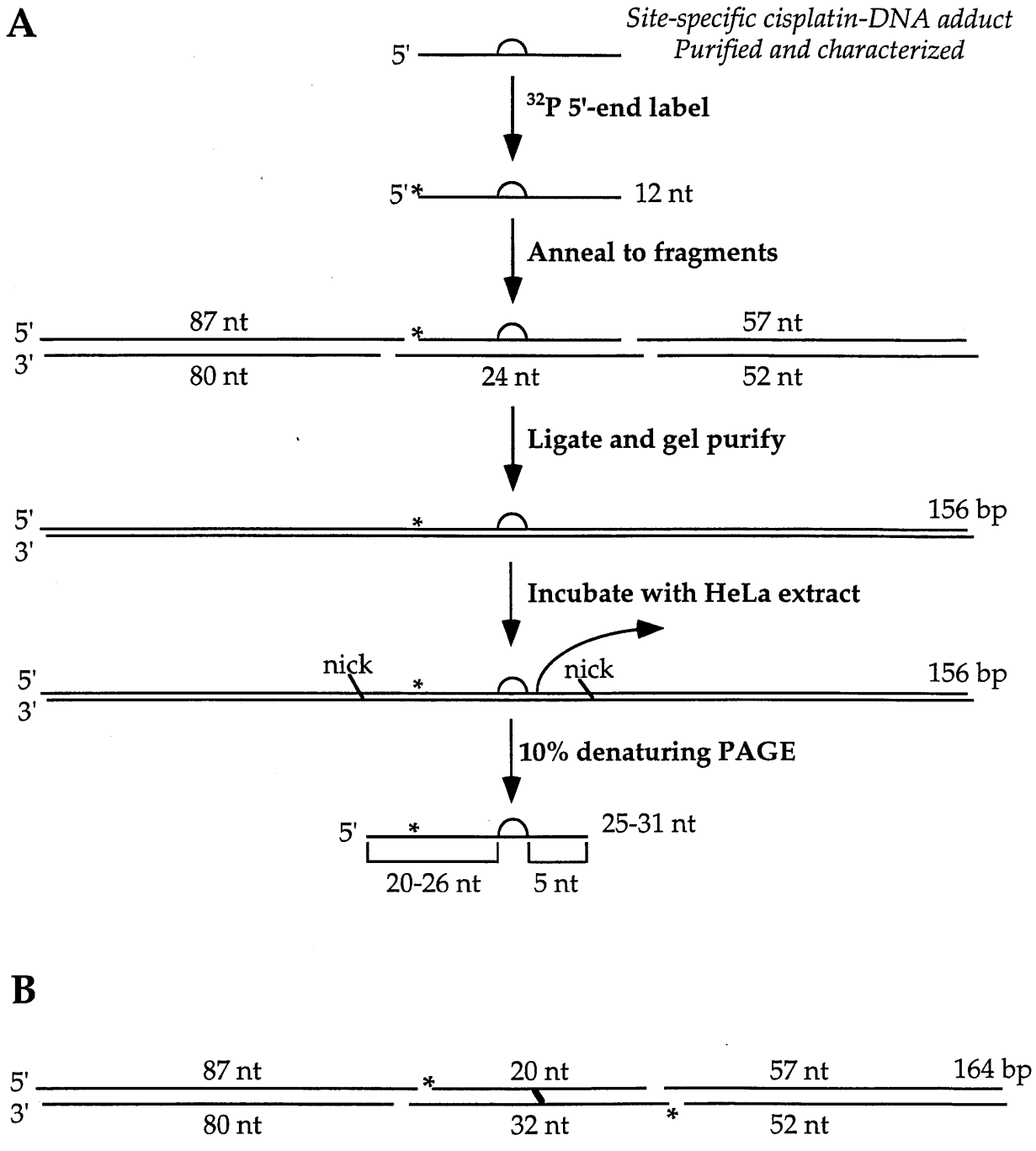


Figure 2.2. Schematic representation of the in vitro excision repair assay. (A) A cisplatin-DNA intrastrand adduct is indicated by the half-sphere. (B) The cisplatin-interstrand cross-linked substrate constructed for the repair assay. The DNA fragments are not drawn to scale. The asterix indicates the presence of a ^{32}P radioactive label in the phosphodiester bond.

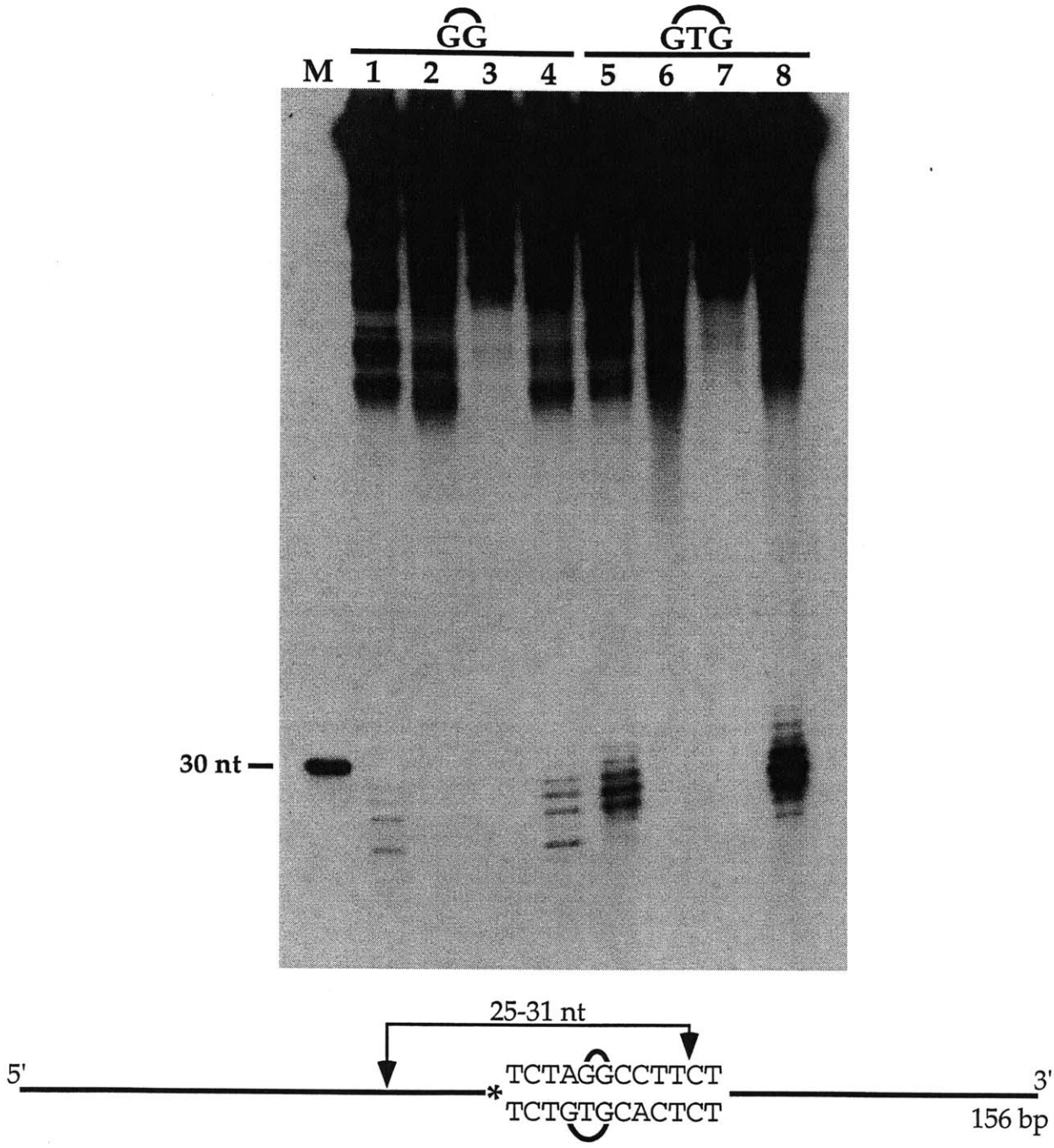


Figure 2.3. Excision of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin-DNA intrastrand adducts by the mammalian excinuclease. The substrate indicated was incubated with HeLa CFE (lanes 1 and 5), or with extracts from XP complementation groups F (lanes 2 and 6), or G (lanes 3 and 7), or a mixture of the two (lanes 4 and 8), for 75 min at 30°C. The reaction products were resolved on a 10% denaturing polyacrylamide gel. The bands corresponding to 25-31 nt located just below the marker are the excision products, as indicated on the scheme below. Only the top strand of the double stranded substrate is shown, the asterix indicates a ^{32}P radioactive label in the phosphodiester bond. Lane M, 30 nt marker.

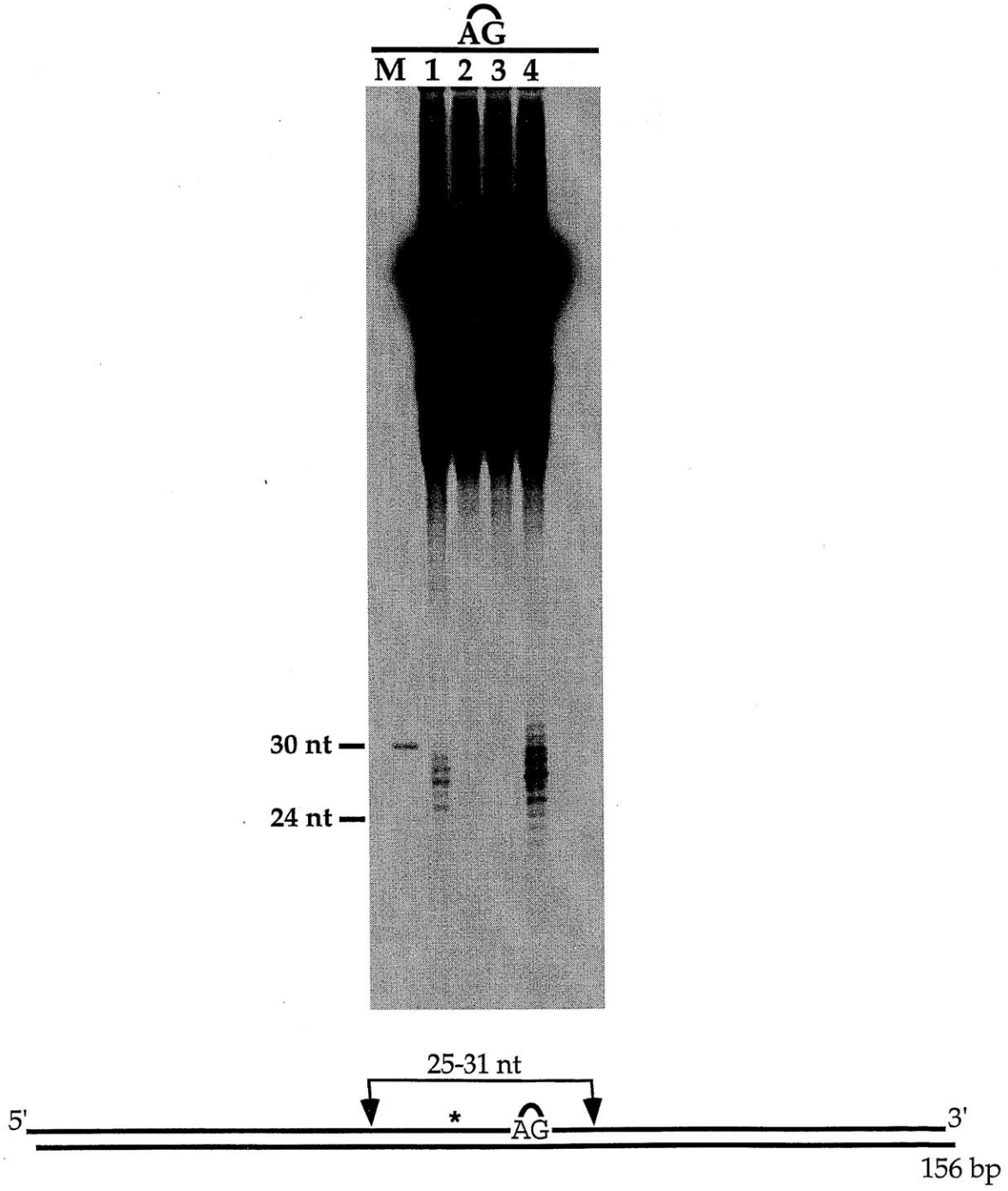


Figure 2.4. Excision of the 1,2-d(ApG) cisplatin-DNA intrastrand adduct by the mammalian excinuclease. The substrate was incubated with Hela CFE (lane 1), or with extracts from XP complementation groups F (lane 2), or G (lane 3), or a mixture of the two (lane 4), for 45 min at 30°C. The reaction products were resolved on a 10% denaturing polyacrylamide gel. The bands corresponding to 25-31 nt located between the two markers are the excision products, as indicated on the scheme below. The asterix indicates a ^{32}P radioactive label in the phophosdiester bond. Lane M, 24 and 30 nt markers.

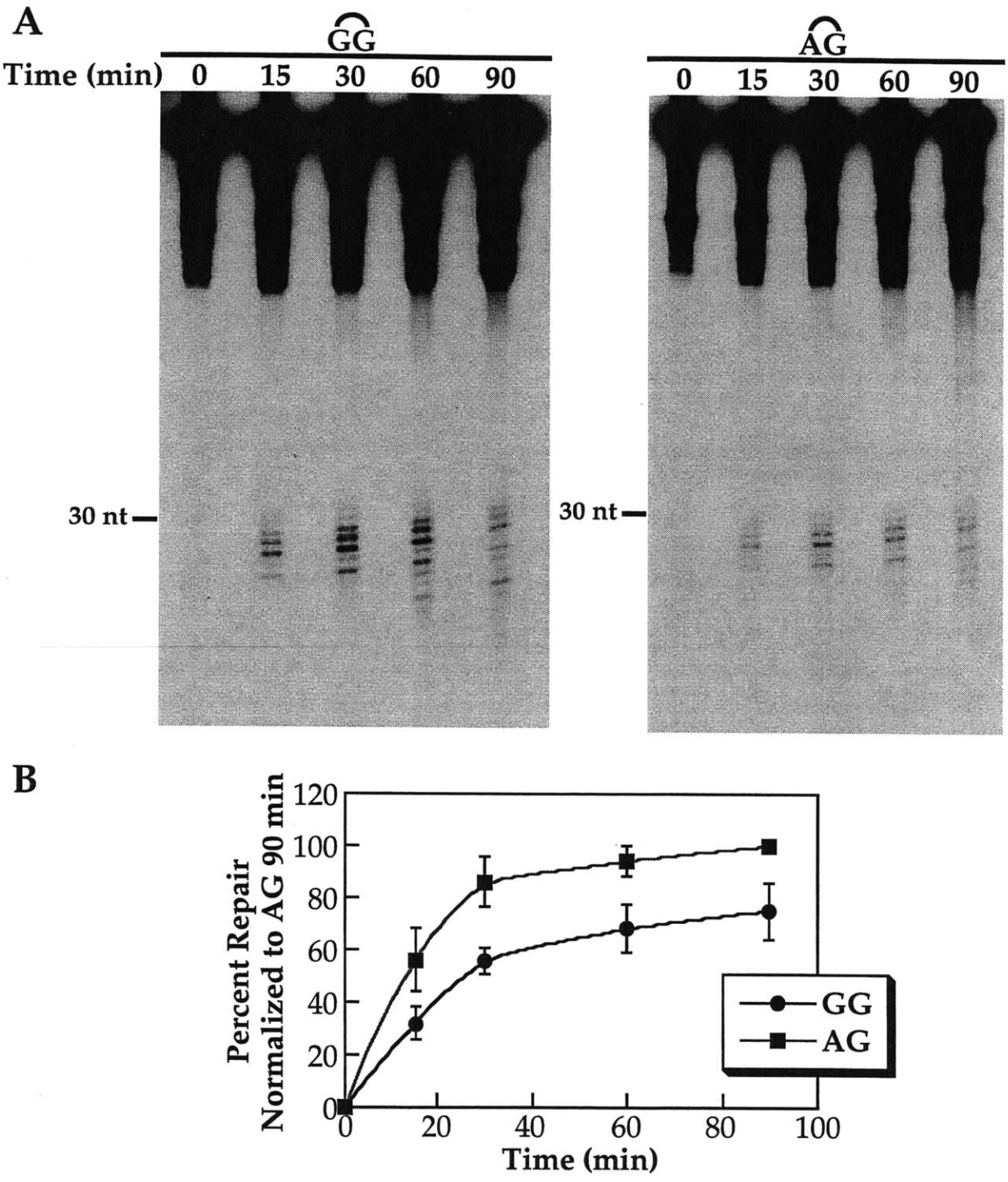


Figure 2.5. Kinetic studies of excision of the 1,2-d(GpG) and 1,2-d(ApG) cisplatin-DNA intrastrand adducts in HeLa CFE. (A) Increasing amounts of excision products with time are revealed on denaturing 10% polyacrylamide gels. Substrates were incubated with HeLa CFE for the times indicated. (B) Plot of three excision experiments such as that shown in panel A. The data for each experiment were normalized to the highest level of repair. Error bars represent ± 1 esd.

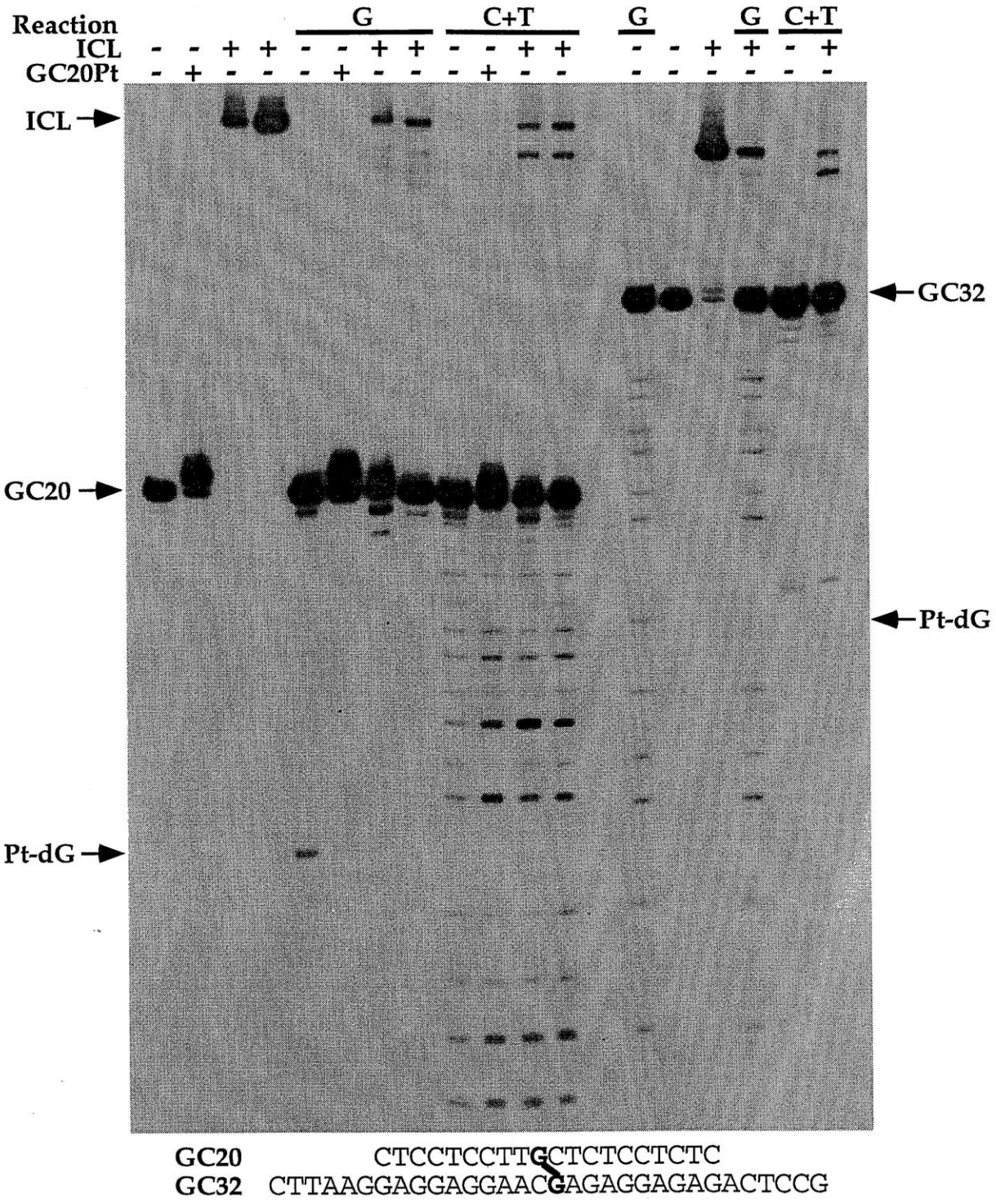


Figure 2.6. Maxam-Gilbert sequencing of the cisplatin-DNA interstrand cross-link. The radioactive label was put on 5' end of GC20 (lanes 1-12) or GC32 (lanes 14-19) before the interstrand cross-link was made, as described in Materials and Methods. All of the sequencing reactions were treated with NaCN prior to analysis on a 20% denaturing polyacrylamide gel. The modified guanine nucleotides are indicated (Pt-dG). GC20Pt is a monofunctional cisplatin adduct on the guanine of the GC20 oligonucleotide.

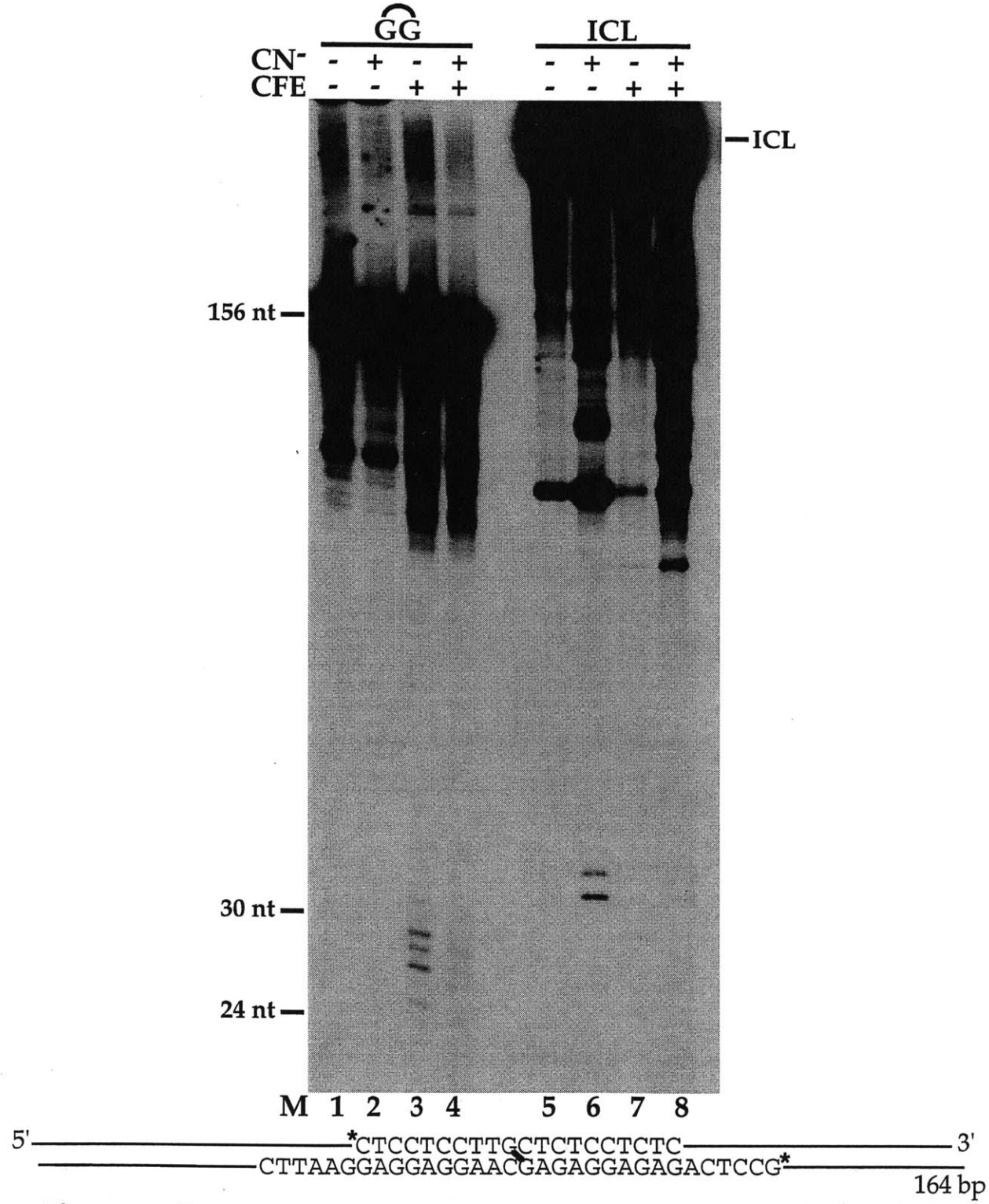


Figure 2.7. Excision of the 1,2-d(GpG) intrastrand and interstrand cisplatin-DNA cross-links by the mammalian excinuclease. The substrates were incubated with CHO AA8 CFE for one hour at 30°C (lanes 3, 4, 7, and 8) and subsequently treated overnight with NaCN (lanes 2, 4, 6, and 8) prior to analysis on a 10% denaturing polyacrylamide gel. The asterixes on the scheme below indicate a ^{32}P radioactive label in the phophodiester bonds. Lane M, 24 and 30 nt markers.

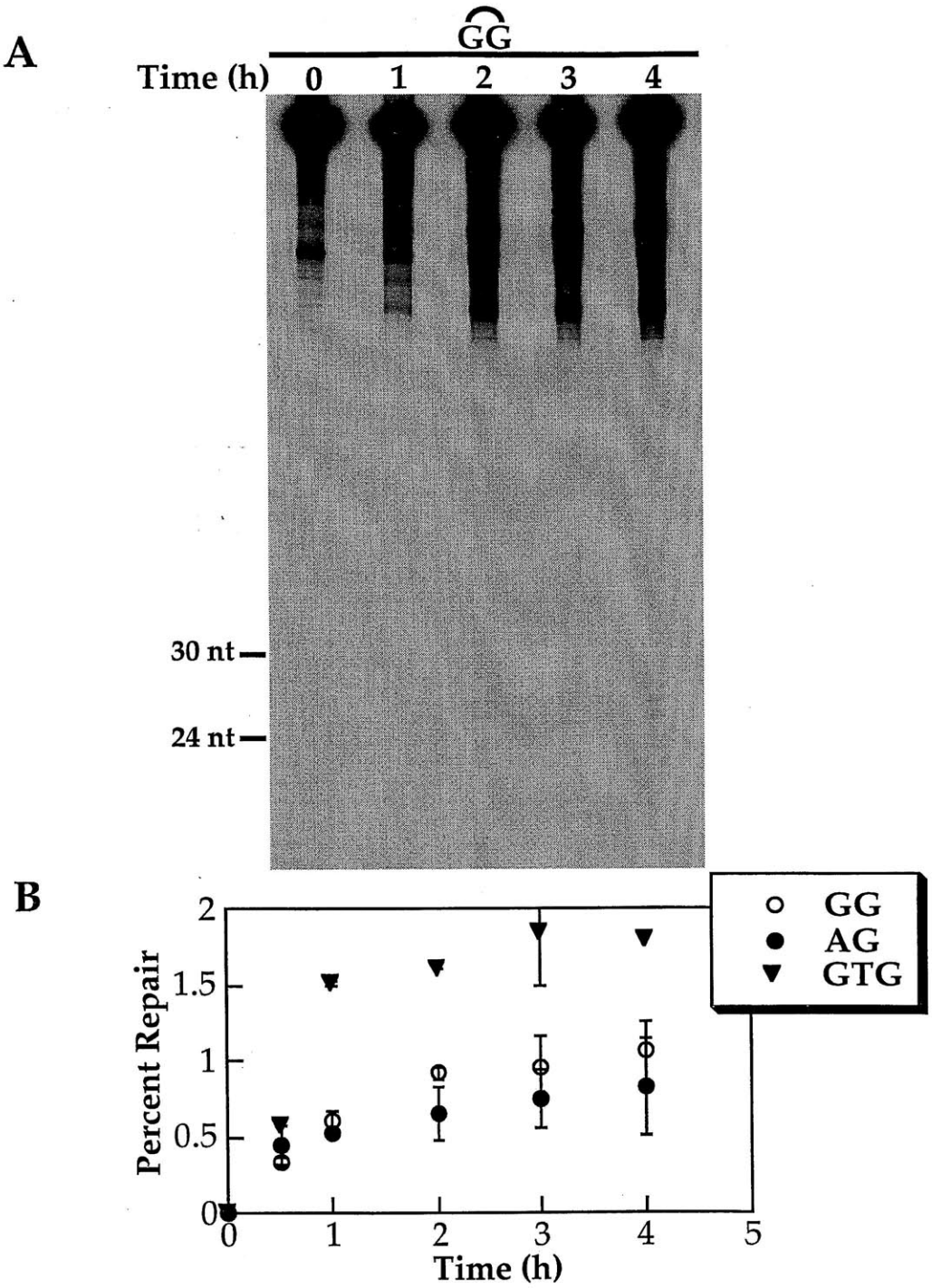


Figure 2.8. Kinetics of excision of the cisplatin-DNA intrastrand adducts by the reconstituted excinuclease. (A) The 1,2-d(GpG) intrastrand cross-linked substrate was incubated with the purified repair factors at 30°C for the times indicated. Increasing amounts of excision products with time are revealed on a 10% denaturing polyacrylamide gel. The 24 and 30 nt markers are indicated. (B) Plot of excision experiments of the 1,2-d(GpG) (open circles), 1,2-d(ApG) (closed circles), and 1,3-d(GpTpG) (triangles) cross-linked substrates, such as that shown in panel A. Each data point is an average of two or three experiments, the error bars represent ± 1 esd.

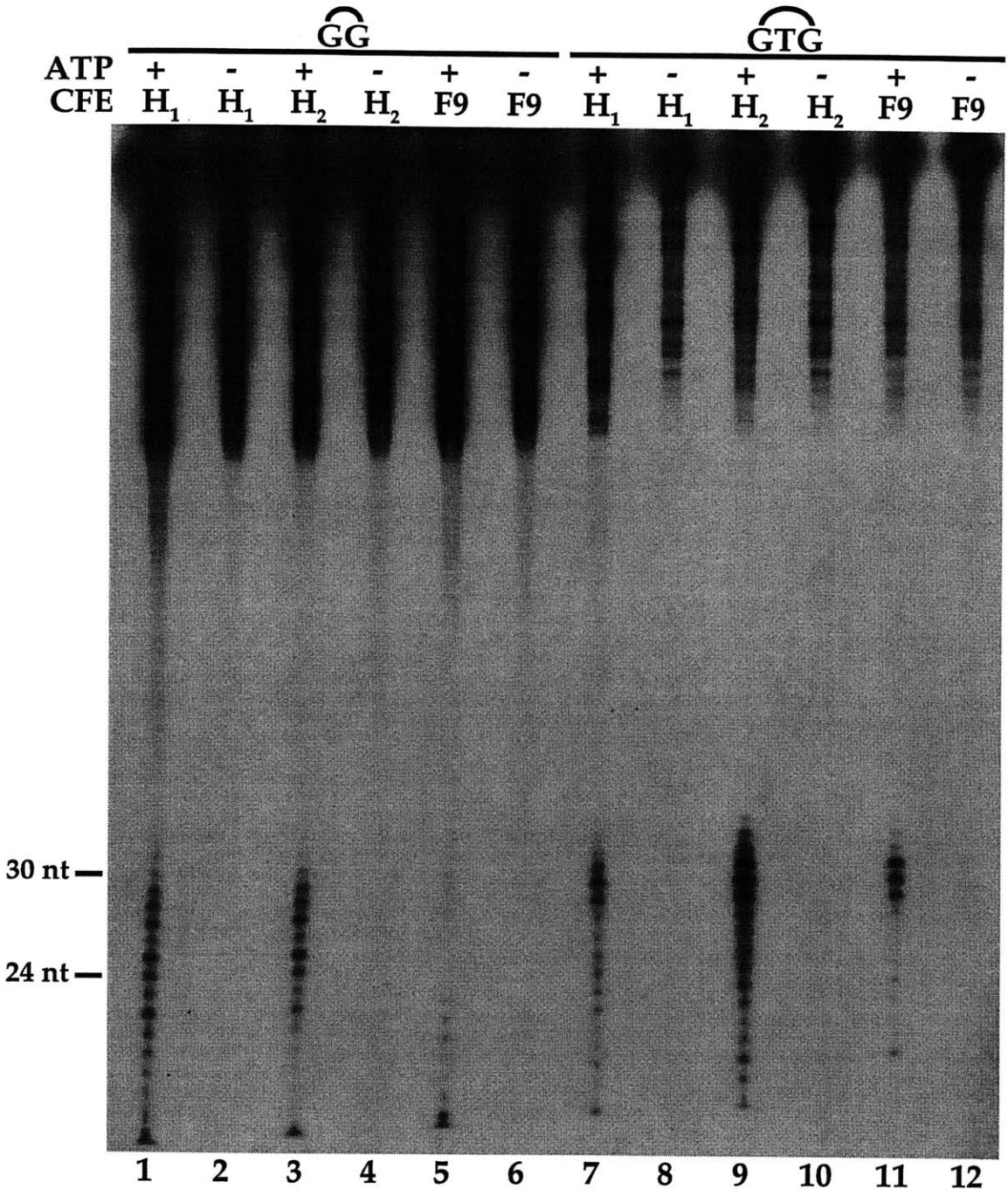


Figure 2.9. Excision of cisplatin-DNA intrastrand cross-links in HeLa and F9 CFEs. The substrates were incubated with two different preparations of HeLa extracts (H_1: lanes 1, 2, 7, and 8, H_2: lanes 3, 4, 9, and 10) or with a CFE from the F9 mouse testicular teratocarinoma cells (F9: lanes 5, 6, 11, and 12) for one hour at 30°C. Half of the reactions were performed in the presence of ATP (lanes 1, 3, 5, 7, 9, and 11). The products were resolved on a 10% denaturing polyacrylamide gel. Levels of excision in terms of percent repair (calculated as on Table 2.2.1) were 0.9%, 0.8%, 0.3%, 3.1%, 6.6% and 4.3%, lanes 1, 3, 5, 7, 9, and 11, respectively. The ratios of excision of the 1,3-d(GpTpG) and 1,2-d(GpG) intrastrand adducts (GTG/GG) were 3.4 (H_1), 8.2 (H_2) and 14.3 (F9). The 24 nt and 30 nt markers are indicated.

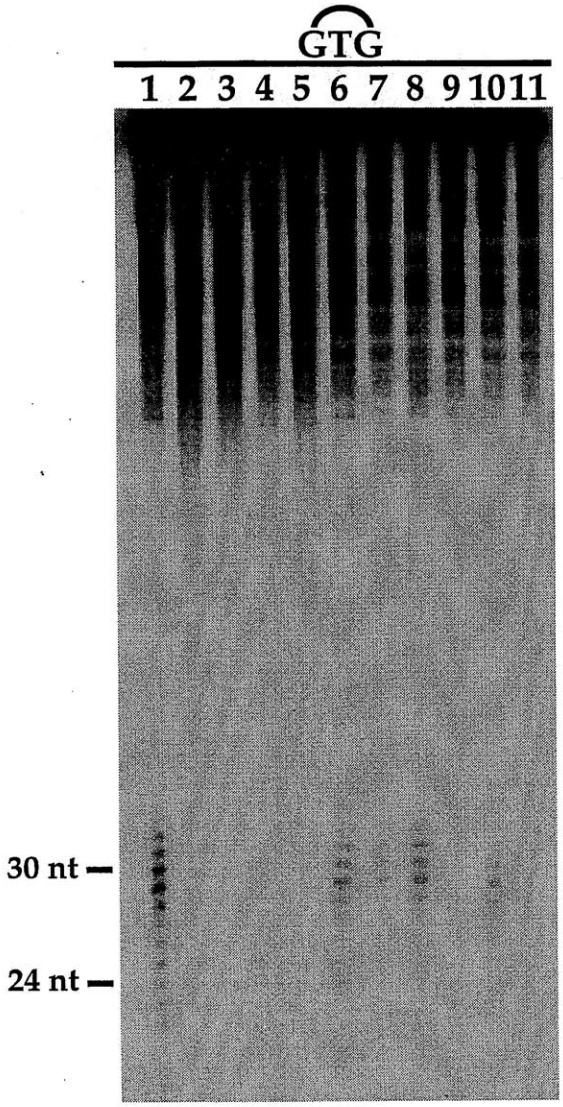


Figure 2.10. Excision repair of the 1,3-d(GpTpG) cisplatin-DNA intrastrand adduct in extracts prepared from human tissue. The substrate was incubated with the extracts at 30°C for one hour prior to analysis of the excision products on a 10% denaturing polyacrylamide gel. The CFEs used were from HeLa (lane 1), lung tumor (lanes 2, 3), normal lung tissue (lanes 4, 5), kidney tumor (lanes 6, 7, 10), endothelial tumor (lanes 8, 9), and normal kidney tissue (lane 11). The reactions in lanes 6-11 were all performed with the same amount of protein from extracts prepared from different tissue samples. The 24 nt and 30 nt markers are indicated.

Chapter Three

The Shielding of Cisplatin-DNA Adducts from Excision Repair in Vitro

Introduction (1-4)

The platinum-DNA cross-links through which the anticancer drug cisplatin exerts its cytotoxic effects significantly destabilize the double helix (5-8). The distinct conformational changes in the DNA generated by the most prevalent lesion, the 1,2-d(GpG) intrastrand cross-link, were examined in several recent NMR and X-Ray crystallography studies (9-13). Some of the global disturbances common to the solid state and solution structures include bending of the helix axis towards the major groove, unwinding of the duplex, and widening and flattening of the minor groove. These properties are very similar to those observed in the DNA of some protein-DNA complexes (14), and may serve as recognition elements for cellular factors that process the cisplatin-DNA damage. One of the details revealed by the high-resolution structural analyses is that the roll between the guanine bases involved in the cross-link fell within 26-50°. This value is significantly less than the dihedral angle of 90° required for a stable, square planar complex, and it suggests a putative driving force behind the formation of protein-DNA complexes at cisplatin-DNA adducts. Since many DNA-binding proteins induce bends in their target sites (14), binding at the platinum locus could increase the helical roll and release the considerable strain at the platinum.

One family of proteins that specifically recognize cisplatin-modified DNA are those which contain the high mobility group (HMG) DNA-binding motif (15-17). The HMG domain is an 80 amino acid polypeptide found in a wide variety of minor groove-binding proteins. These include transcription factors as well as architectural components essential for the formation of multi-protein complexes involved in DNA structure, maintenance, and manipulation. Although the homology between all of these proteins is quite low, they possess common structural features which allow them to bind to distorted DNA such as that found in four-way junctions, as well as cisplatin-modified DNA (18). In particular, HMG-domain proteins specifically bind

to, and bend, the 1,2-intrastrand adducts of cisplatin, but not the minor 1,3-intrastrand cross-links or DNA modified with clinically ineffective platinum compounds (19-21).

Evidence to suggest that HMG-domain proteins play a role in the biological mechanism of cisplatin was provided by experiments in yeast. Interruption of the gene encoding the HMG-domain protein Ixr1 produced elevated resistance to cisplatin but not to other DNA-damaging agents (22), an effect which was linked to an intact excision repair pathway (23). When Ixr1 expression was reintroduced to the knockout cells, the cisplatin resistance decreased (24). Attempts to overexpress the protein above endogenous levels and enhance sensitivity to the drug were unsuccessful, however (24). Several non-exclusive models have been formulated to explain how HMG-domain proteins might affect the cytotoxicity of cisplatin-DNA adducts (25). The first hypothesis suggests that the protein-DNA complex could serve as a signal to stimulate repair, but there is no experimental evidence to support this theory. The second model is based on the fact that some of these proteins have essential functions, so titration away from their natural binding sites by the cisplatin-DNA adducts would activate cell death. This potential consequence of cisplatin-modified DNA was modeled by its ability to disrupt reconstituted ribosomal DNA transcription, which requires the HMG-containing human upstream binding factor hUBF for promoter activation (26, 27). Finally, the shielding hypothesis, which is most consistent with the yeast work, proposes that HMG-domain proteins bind to the cisplatin-DNA adducts and block cellular defense mechanisms such as repair, allowing the damage to persist, obstruct vital DNA transactions, and activate cell death.

In addition to HMG-domain proteins, it is becoming apparent that other cellular factors specifically recognize the structural distortions of cisplatin-modified DNA and could affect the processing of the lesions (28). Included are XPAC and RPA

(29-33), the putative damage recognition components of the nucleotide excision repair pathway that efficiently removes the cisplatin-DNA lesions (2, 3). Although it is less certain how any other proteins such as the transcription factor TBP (34, 35), the histone H1 protein (36), and the mismatch repair protein hMSH2 and its active complex, MutSα (37, 38) might affect the cytotoxicity of cisplatin, the models proposed for HMG-domain proteins could also be applied.

In the present study, we explored the shielding hypothesis by using site-specific cisplatin-DNA adducts and an in vitro excision repair assay. These experiments focus on the effects of the HMG-domain family of proteins on DNA repair, and particular attention was paid to proteins that may be expressed in testicular tumors. The results provide insights into the cellular processing of cisplatin-DNA lesions and may be relevant to the unique sensitivity of testicular cancer to this anticancer drug (39, 40). In addition, the putative role of mismatch repair proteins in the cisplatin mechanism of action was examined in the context of excision repair, and no connection was observed. Furthermore, our data reveal that a mismatch repair deficiency is not required for the development of cisplatin resistance.

Materials and Methods

Materials. Cisplatin was obtained as a gift from Johnson-Matthey. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim. The [γ-^{32}P]ATP was purchased from Dupont, NEN. The cisplatin-modified 123-bp fragment was a gift from J. P. Whitehead (M.I.T.). The cisplatin-resistant HeLa and V79 cell lines were grown in the appropriate concentrations of cisplatin as described (41), and the mismatch repair capacity of these cell lines was tested in collaboration with P. Modrich (Duke, NC). The HeLa S3 cell line was from the stock of Lineberger Comprehensive Cancer Center (University of North Carolina) or was supplied by P. A. Sharp (M.I.T.). Whole cell-

free extracts (CFEs) were prepared by the method of Manley et al. and stored at -80°C (42).

HMG-domain and Mismatch Repair Proteins. Recombinant rat HMG1 (20), HMG1 domain B (residues K86-K165) (43), and mouse testis-specific HMG (44), were expressed and purified as described by K. E. Sandman (M.I.T.), S. A. Kane (M.I.T.), and U.-M. Ohndorf (M.I.T.), respectively. These proteins were stored in buffer A (50 mM Tris, pH 7.3, 50 mM NaCl, 5 mM 2-mercaptoethanol) at -20°C, and diluted into p21 buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) immediately prior to use. Recombinant human SRY and HMG-domain (residues D58-K136) were expressed and purified by E. E. Trimmer (M.I.T.) as described (4). The hSRY was stored in 20 mM Hepes pH 7.9, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol. The hSRY HMG-domain polypeptide was provided in the same buffer as the whole protein except that it contained only 10 mM NaCl. The proteins were diluted in storage buffer immediately prior to use. Human MutSα, kindly provided by Dr. Modrich (Duke University, NC), was purified as described (45). MutSα was stored at -80°C in 50 mM Hepes, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10% sucrose, 1 mg/ml BSA, and diluted in the same buffer. Once an aliquot was thawed, it was used up because the protein is not stable through multiple freeze/thaw cycles. Functional assays for Mutsα activity were not performed in this laboratory.

Oligonucleotides. The 156-bp oligonucleotides site-specifically modified with cisplatin were synthesized as previously described (3). The 123-bp DNA fragment was obtained by digestion of a commercially available 123-bp DNA ladder (Gibco BRL) with the restriction enzyme *AvaI;* the resulting DNA fragment was purified by extraction from an agarose gel. The 123-bp fragment was globally modified with cisplatin as previously described (25), and the platinum/nucleotide ratio was determined to be 0.02 by using atomic absorption spectroscopy. The DNA was 3'-end

labeled in a fill-in reaction with the Klenow fragment of DNA polymerase I, [α-^{32}P]dCTP (Dupont/NEN) and the four deoxynucleotide triphosphates, and purified on a Sephadex G-50 Quick spin column (Boehringer Mannheim).

Mobility Shift Assays with tsHMG. The cisplatin intrastrand cross-linked probes (5000 cpm) were incubated in a 20 µl volume containing 2 µl of the excision assay reaction buffer, 108 ng of pBR322 plasmid DNA, 0.25 mg/ml of BSA, and 5 µl of tsHMG in dilution buffer, for 30 min on ice. Gel loading solution (30% glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF) was added (0.6 µl) and the reactions were resolved by electrophoresis on pre-run, pre-equilibrated (4°C), 0.5X TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3), 5% native polyacrylamide gels (29:1 acrylamide: N,N'-methylene-bisacrylamide, 0.08% ammonium persulfate). Gels were run at 300 V, 4°C, vacuum dried and exposed to film (Kodak, X-OMAT AR) at -80°C with an intensifier screen.

Mobility Shift Assays with MutSα. The band shift assays were performed as previously reported (38). Briefly, the protein was incubated with either 0.3 fmol of the site-specifically modified oligonucleotides or 1.6 fmol of the randomly platinated 123-bp oligonucleotide. The 20 µl reactions contained 2 µl of MutSα in storage buffer, 10 mM Hepes, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.05 mg/ml BSA, and 16 fmol or 32 fmol of cold 123-bp unmodified DNA included with the site-specific or globally platinated probes respectively. After incubation on ice for 20 min, 2 µl of loading solution was added (10 mM Hepes, pH 7.5, and 50% sucrose) and the reactions were loaded onto pre-run, pre-equilibrated, 6% native polyacrylamide gels. The gels were run in 0.5 × TBE at 4°C, 300 V, vacuum dried, and exposed to film at -80°C with an intensifier screen.

Inhibition of Excision Repair by HMG-domain and MutSα Polypeptides. The repair inhibition experiments were performed as previously described with a few modifications (3). The polypeptides were incubated with the site-specific cisplatin-

DNA repair probes for 10 min at 30°C prior to the addition of HeLa CFE. The volume of polypeptide added in dilution buffer was kept constant within each experiment. The reaction conditions varied slightly as improvements were made. Briefly, the standard reactions (25 µl) contained 1.4-5 µl of protein in dilution buffer, 4-5 µl of reaction buffer (300 mM Hepes pH 7.9, 400 mM KCl, 32 mM $MgCl_2$, 1 mM EDTA, 20-40 mM ATP, 2 mM DTT), 5 µl of p21 buffer except when the protein was diluted into this buffer, 20 µM of each dNTP, 0.2 mg/ml BSA, 50-120 ng of pBR322 plasmid as non-specific competitor DNA, 50-250 pM substrate, and 3-5 µl of CFE in storage buffer (25 mM Hepes pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT and 12.5-16.5% glycerol). The reactions were incubated at 30°C for the indicated length of time and then stopped by adding 10 µg of proteinase K, making the reaction 0.5% in SDS and incubating at 55°C for 20 min. The reactions were extracted, precipitated and resolved on 10% denaturing polyacrylamide gels as previously described (3). The data were quantitated by using an AMBIS systems scanner or by Phosphorimager analysis (Molecular dynamics).

Results

Inhibition of Excision Repair by HMG-Domain Polypeptides. Almost all of the HMG-domain proteins studied to date specifically bind to cisplatin-modified DNA (46). A recently reported testis-specific HMG-domain protein (tsHMG) was of particular interest (47), however, since cisplatin is particularly effective in the treatment of testicular cancer (39, 40). The binding specificity of this protein for cisplatin-DNA adducts was tested in an electrophoretic mobility shift assay (Figure 3.1). Like HMG1 (20), tsHMG specifically binds to the 1,2-d(GpG) intrastrand adduct, but not to the 1,3-d(GpTpG) cross-link. One possible consequence of this specific recognition is that tsHMG could prevent the excinuclease from reaching and repairing the major cisplatin-DNA adducts. To test this hypothesis, the ability of

tsHMG to influence the excision repair of cisplatin-modified DNA was examined by adding the HMG-domain protein to the in vitro excision repair assay. In a reflection of the specific binding capacity (Figure 3.1), 25-100 nM of tsHMG inhibits repair of the 1,2-d(GpG) intrastrand adduct, whereas excision of the 1,3-d(GpTpG) intrastrand adduct was not significantly blocked and a modest stimulation was observed (Figures 3.2 and 3.3A). At these protein concentrations, only the 1,2-intrastrand adduct is specifically bound (Figure 3.1), but at higher concentrations non-specific binding observed on both the 1,3-intrastrand substrate and unmodified DNA (data not shown) inhibits repair of both of the cross-links.

Another testis-specific HMG-domain protein, the human sex-determining factor hSRY, similarly blocks repair of the major cisplatin-DNA adduct, although at slightly higher concentrations (Table 3.1). Various HMG-domain proteins are capable of this specific repair inhibition, but none are as effective as the testis-specific proteins (Table 3.1 and data not shown). For example, 25 nM of tsHMG inhibited the excision of the 1,2-d(GpG) adduct by 35% (Figure 3.3A), whereas 1-2 μM of HMG1 was necessary to achieve a similar level of inhibition (data not shown). HMG-domain proteins exhibit very little homology outside of the DNA-binding domain (48), so it is unlikely that the repair inhibition is due to direct interactions with the excinuclease components. In support of this hypothesis, the isolated HMG-domain of SRY could inhibit the excision of the 1,2-d(GpG) cisplatin-DNA adduct at concentrations that did not block repair of the 1,3-d(GpTpG) cross-link in HeLa CFE (Figure 3.3B).

The other major cisplatin-DNA adduct, the 1,2-d(ApG) intrastrand cross-link, is also recognized by HMG1 (20) and is processed in a similar fashion as the 1,2-d(GpG) intrastrand adduct by the mammalian excinuclease (3). To determine whether HMG-domain proteins also shield the 1,2-d(ApG) adduct from excision repair, HMG1 was added to the in vitro assay (Figure 3.4). Increasing amounts of HMG1 caused a decrease in the excision of the 1,2-d(ApG) cross-linked substrate,

inhibition was observed at 0.5 μM HMG1, and 2 μM resulted in a 60% drop in the level of excision. Similarly, excision of the 1,2-d(GpG) intrastrand adduct, but not the 1,3-d(GpTpG) cross-link, is blocked by comparable concentrations of HMG1 (Table 3.1). Although the exact expression levels of HMG-domain proteins have not been determined, the isolation of nuclear chromatin yields about 0.8-2.4 million molecules of HMG1 per cell (49, 50), suggesting that micromolar concentrations of the proteins are biologically relevant. The phosphorylation status of the HMG-domain polypeptides employed in these experiments was not determined, and this type of modification has been shown to influence the DNA-binding properties of members of the HMG family (51, 52). However, it is not clear how much these proteins are phosphorylated in their native state, and there is evidence to suggest that the recombinant proteins are appropriately modified. For example, recombinant tsHMG exhibits similar DNA-binding and topological activities as protein that was dephosphorylated and then incubated with extracts from mouse testes supplemented with protein kinase cofactors (52).

Inhibition of Excision Repair by tsHMG in a Reconstituted System. It is possible that the repair inhibition by HMG-domain proteins is influenced by interactions with other factors in the whole cell extracts. To address this issue, the effect on repair was also tested in a reconstituted system of purified repair factors (53). As in the HeLa cell extracts, a concentration of tsHMG as low as 25 nM inhibited the excision of the 1,2-d(GpG) intrastrand adduct whereas the repair of the 1,3-d(GpTpG) intrastrand adduct was slightly stimulated (Figure 3.5).

Binding of Cisplatin-DNA Adducts by MutSα. It was recently demonstrated that hMSH2, a human homologue of the prokaryotic mismatch recognition repair protein, specifically binds DNA modified by cisplatin (37). MutSα, the active form of the same protein in a complex with another 160 kDa polypeptide (45), also binds specifically to the 1,2-d(GpG) cisplatin intrastrand adduct (38). To examine this

behavior, mobility shift assays were performed with MutSα and either random or site-specific cisplatin-modified DNA. Specific binding of 4-8 nM MutSα to the cisplatin-DNA adducts is evident with the globally platinated 123-bp DNA fragment (Figure 3.6A). At higher protein concentrations (>16 nM) several bands due to complexes with slower mobility were observed for both the modified and unmodified substrates, presumably due to non-specific binding.

In contrast, although several conditions were tested, only a small variability was observed in the binding affinity of MutSα for the 1,2-d(GpG), 1,3-d(GpTpG), and unmodified substrates (Figure 3.6B and data not shown). The mobility of the only band specific for the 1,2-d(GpG) adduct was faster than the other bands, and it was only detected at the lower protein concentrations used (4-8 μM). Additional bands of slower mobility were produced with all of the substrates and might be made up of a combination of specific and non-specific binding, since more DNA was shifted by the 1,2-d(GpG) intrastrand adduct. In a previous report, a similar binding pattern of site-specific lesions was described (54), but the authors designated all of the shifted material as specific binding products, including the lower mobility bands observed in reactions with the unmodified DNA.

Inhibition of Excision Repair by MutSα. The binding of MutSα to cisplatin-modified DNA could be relevant to the cisplatin resistance of some cultured cell lines that is associated with loss of mismatch repair (55-57). In order to determine whether this interaction could affect the cytotoxicity of the adducts through modulation of the excinuclease, MutSα was added to the in vitro repair assay. Figure 3.7A demonstrates that repair of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin-DNA adducts was not influenced by 9.6 nM of MutSα. ATP inhibits MutSα DNA binding activity (45), so in additional experiments the ATP required for the excinuclease was added to the reaction only after incubation of MutSα with the substrate and addition of the

HeLa CFE. Concentrations of MutSα up to 66 nM were employed under these conditions, and no inhibition of repair was observed (Figure 3.7B).

Discussion

Excision Repair Shielding by HMG-Domain Proteins. The binding of HMG-domain proteins to the major cisplatin-DNA cross-links is presumably due to a structural correspondence with the natural target sites. This fortuitous resemblance has the potential to play a role in the anticancer activity of the drug. Of the multiple models proposed to explain how HMG-domain proteins could mediate the cytotoxicity of cisplatin (25), one is refuted and another is supported by the data presented here. It was originally postulated that HMG-domain proteins could assist in the excision of platinum damage by binding to the adducts and serving as recognition elements for repair. The fact that HMG-domain proteins do not stimulate the in vitro excision of the 1,2-intrastrand adducts is incompatible with this theory, however. A slight increase in the excision of the 1,3-d(GpTpG) intrastrand cross-link was detected, but appears to be caused by non-specific binding. Although the HMG-domain proteins do not activate excision repair, the possibility that the specific binding of these proteins to the cisplatin lesions could stimulate other cellular pathways that respond to the DNA damage, such as programmed cell death, cannot be ruled out.

The fact that all of the HMG-domain proteins tested specifically inhibit excision of the major cisplatin-DNA adducts supports the alternative repair shielding hypothesis. This effect is unlikely to be due to any contacts between the HMG-domain proteins and the excinuclease, since an isolated HMG domain can also block repair. In addition, the same range of tsHMG concentrations inhibits repair in both the whole cell extracts and the reconstituted system. This result suggests indirectly that the inhibition of repair is not regulated by interactions between the HMG-

domain proteins and any other cellular constituents. The ability of cisplatin-DNA bound HMG-domain proteins to block the excinuclease raises the possibility that this complex may also inhibit other DNA-processing pathways. This proposal is supported by several recent reports demonstrating that HMG1 can inhibit both DNA replication and helicase activity at the site of a cisplatin-DNA adduct (58, 59).

The ubiquitously expressed HMG-domain proteins, such as HMG1, are present in the HeLa cell extracts used in this assay (20, 60), but the relative repair rates of the 1,2- and 1,3-intrastrand adducts were similar to those observed in the reconstituted system of purified repair proteins (3). Furthermore, fractionation of HeLa cell extracts did not affect the relative repair of the cross-links measured with the repair synthesis assay (61). It should be noted that sequence context can significantly influence the strength of the interaction between HMG-domain proteins and cisplatin-DNA adducts (62), but only one sequence context was employed in these studies (3, 61). Nevertheless, these results suggest that the levels of HMG-domain proteins in the extracts were not sufficient to block the repair of the 1,2-intrastrand adducts. It is likely that, if HMG-domain proteins do play a role in the cytotoxicity of cisplatin, this property is not an attribute of the whole family of proteins, but rather of certain proteins that have tissue restricted expression (15). Thus the question of which HMG-domain to examine in an investigation of the cisplatin mechanism of action can be crucial.

Both of the testis-specific HMG-domain proteins investigated here inhibit repair of the 1,2-d(GpG) intrastrand cisplatin-DNA adduct at concentrations lower than any other member of this family. The murine tsHMG protein, a nuclear isoform of mtTFA (63), is expressed in the nuclei of elongating spermatids (47) and may play a role during chromatin structure transitions (52). Testis-specific isoforms of mtTFA are abundant in human male germ cells, but no nuclear protein has yet been detected in human tissue (64). In contrast, hSRY, the human sex-determining factor (65), is

expressed in testicular and prostatic tumor tissue (66). Thus it is possible that SRY, or other HMG-domain proteins highly expressed in testis tissue (65, 67-70), could provide the basis for the cisplatin sensitivity of testicular tumors by enhancing the toxicity of the DNA lesions through repression of repair. This hypothesis is in accord with several reports that have suggested that the sensitivity of cultured testicular tumor cell lines is due to poor repair of the major cisplatin-DNA adducts (71-73). Malignant tissue frequently develops with genetic amplification and changes in protein expression (74), so the proteins present in tumors cells may be different from the tissue of origin. It would be of interest to examine the expression levels of HMG-domain proteins in tumor cells and to determine whether there is any correlation with cisplatin responsiveness.

The Effects of MutSα on Cisplatin-Modified DNA. In addition to the excinuclease, there are a variety of cellular mechanisms to correct genetic defects. Errors in DNA originating from replication, recombination, or deamination, are removed from the genome by the mismatch repair pathway (75, 76). A predisposition to hereditary non-polyposis colon cancer, as well as a wide variety of sporadic cancers, have been associated with mutations in the mismatch repair genes (75, 77). Another consequence of defects in this pathway is cellular resistance to a variety of chemotherapeutic drugs (78, 79). Several different mismatch repair-deficient cell lines exhibit diminished cisplatin sensitivity in comparison with the repair-proficient controls (80, 81). Similarly, expression of hMLH1, an essential mismatch repair factor (76), is lacking in multiple ovarian cell lines with acquired cisplatin resistance (55-57). The connection between cisplatin and mismatch repair was reinforced by the observation that components of the putative human mismatch recognition complex, hMSH2 and MutSα (76), also recognize cisplatin-modified DNA (37, 38). This result is of particular relevance to the cisplatin mechanism of action because hMSH2 is expressed at elevated levels in testis tissue (37).

In analogy with HMG-domain proteins, experiments were performed to examine whether the mismatch repair proteins could shield the cisplatin-DNA adducts from excision repair. MutSα did not inhibit the excinuclease activity on the 1,2-d(GpG) or 1,3-d(GpTpG) cross-linked substrates. This result was surprising, since the concentrations of protein added to the in vitro assay (up to 66 nM) should have been sufficient to achieve specific binding of the 1,2-d(GpG) intrastrand adduct (38). Accordingly, the affinity of MutSα for cisplatin-DNA adducts was re-examined. Experiments that employed randomly modified oligonucleotides confirmed that MutSα recognizes DNA damaged by cisplatin. The specific affinity for the 1,2-d(GpG) cisplatin-DNA adduct was fairly weak, however, in comparison with the 1,3-d(GpTpG) cross-linked and unmodified substrates, as previously reported (37, 54). Although the MutSα protein was provided by an outside source and not independently tested for mismatch repair capabilities, there was no indication that the protein had lost any DNA-binding activity. The fact that this complex did not block the excinuclease suggests that it is not strong enough to compete with cellular factors involved in processing the DNA damage, and that the mismatch repair pathway must alter cisplatin sensitivity independent of excision repair. MutSα functions in the context of multi-protein complexes, so it is possible that the native affinity for cisplatin-DNA damage was not achieved. The in vitro excision assay experiments were performed in extracts prepared from human HeLa cells, however, which are proficient in mismatch repair (J. Drummond, P. Modrich, personal communication), and should have provided the relevant cellular components for the formation of the active complexes. Furthermore, a recent study of excision repair in extracts prepared from both mismatch repair proficient and deficient cell lines supports the conclusion that there is no functional overlap between the two pathways (54).

The discovery that cultured cell lines selected for cisplatin resistance have deficient mismatch repair could have potential clinical relevance. This relationship has been observed in several ovarian cell lines (55-57) and could explain the severe problem of acquired cisplatin resistance in many ovarian cancer patients (82). In addition to the shielding hypothesis, several other models have been postulated to explain the putative connection between cisplatin-sensitivity and the mismatch repair pathway (37). One proposal suggests that the mismatch repair proteins bound to the cisplatin-DNA adducts may be a signal for cell death. Another possibility is that the enzymes enter a futile cycle of attempted repair and replication bypass, which would produce endless DNA nicking and activate apoptosis. The latter hypothesis is supported by the fact that the affinity of the mismatch repair proteins for a cisplatin-DNA cross-link is strengthened when the adduct is incorporated across from a mismatch (54, 61, 83). It is important to note, however, that a mismatch repair defect is not required for the evolution of cisplatin resistance. Two cell lines selected for cisplatin resistance, a hamster V79 cell line and a human HeLa cell line (41), were tested for mismatch repair activity in collaboration with the laboratory of P. Modrich. Both of these cell lines, as well as the parental cells, were proficient in mismatch repair (data not shown). Furthermore, western analysis was used to examine the levels of the hMSH2 and hMLH1 proteins, which are frequently deficient in association with cisplatin resistance (55-57), but equal quantities of both proteins were expressed in the resistant HeLa clone and the parental cells.

Conclusions

The experiments presented here provide information about how cellular proteins that recognize cisplatin-DNA adducts could affect the cytotoxicity of the lesions. HMG-domain proteins specifically block repair of the 1,2-d(GpG) cisplatin-DNA intrastrand cross-link in an in vitro excision repair assay. This effect was

observed in whole cell extracts, a reconstituted repair system, and with an isolated HMG-domain. The testis-specific HMG-domain proteins are most effective at inhibiting repair, which may be relevant to the unique sensitivity of testicular tumors to cisplatin chemotherapy. The mismatch repair protein, MutSα, exhibits weak selectivity for the major 1,2-d(GpG) cisplatin-DNA adduct and does not inhibit excision repair of the intrastrand cross-links. Although the mismatch repair pathway may be a key to the development of drug resistance in certain cell types, it is not an absolute requirement.

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1. Some of this work was published in a slightly different form. See references 2, 3 and 4.
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Table 3.1. Inhibition of excision of the cisplatin-DNA intrastrand adducts by several HMG-domain polypeptides. Specific inhibition indicates the concentration of HMG-domain protein which blocked excision of the 1,2-d(GpG) but not the 1,3-d(GpTpG) cisplatin intrastrand adducts.

Protein	Specific Inhibition (μM)	Expression	Function
HMG1[a]	1-4	ubiquitous	(?)architectural factor/transcription regulation
HMG1 Domain B[b]	0.5-1		
hSRY	0.1-0.4	embryonal genital ridge	sex-determining transcription factor
hSRY Domain	0.1-0.3	adult testis tissue	
tsHMG	0.025-0.1	elongating spermatids	(?)topological factor mtTFA homolog

[a]The specificity of inhibition of HMG1 was determined in collaboration with A. Sancar (U. of N. Carolina) (2). [b]The specificity of binding of HMG1 domain B was determined by comparing the inhibition of a 1,2-d(GpG) intrastrand cisplatin-DNA adduct with the cholesterol repair substrate.

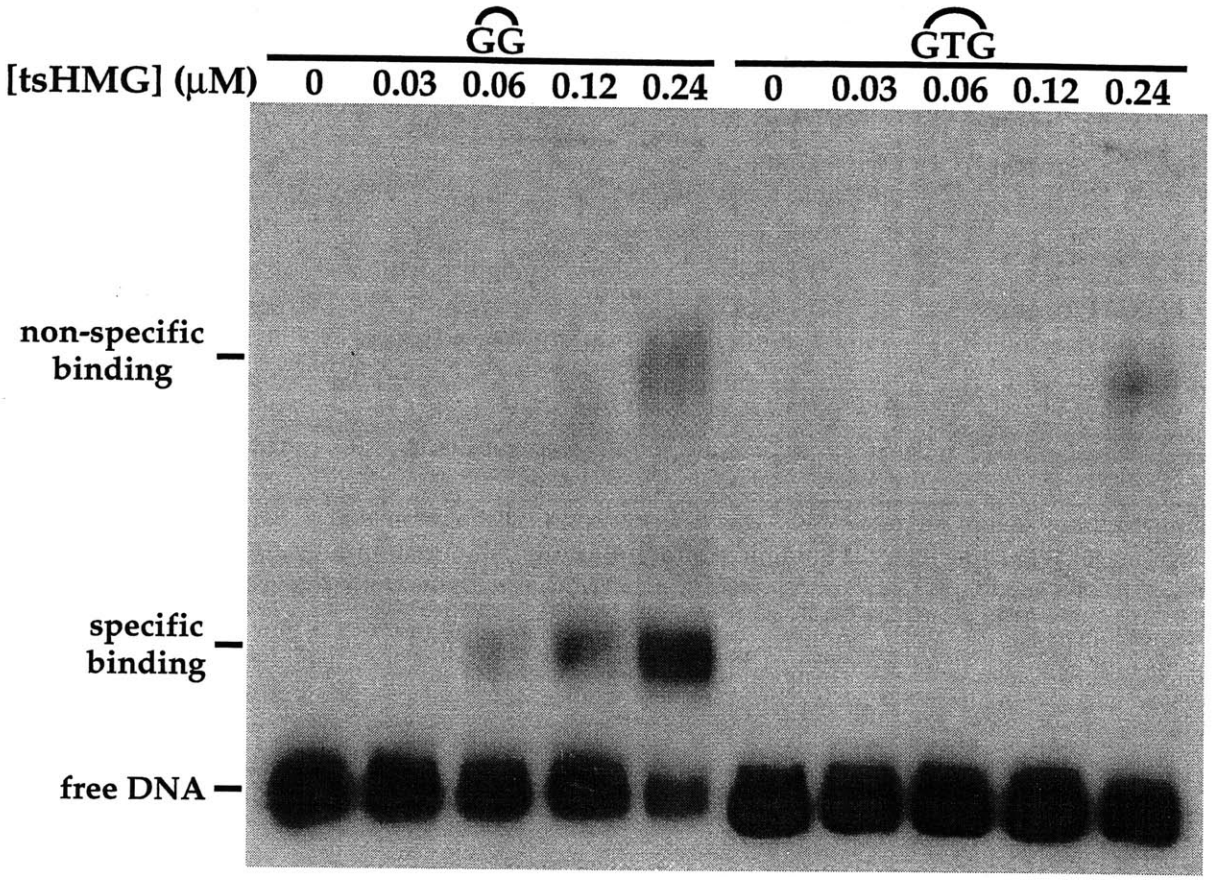


Figure 3.1. tsHMG specifically binds to the 1,2-d(GpG) cisplatin-DNA intrastrand adduct. The 1,2-d(GpG) and 1,3-d(GpTpG) site-specific adducts (156 bp) were incubated with the indicated concentrations of tsHMG for 30 min on ice under excision assay conditions prior to analysis on a 5% native polyacrylamide gel.

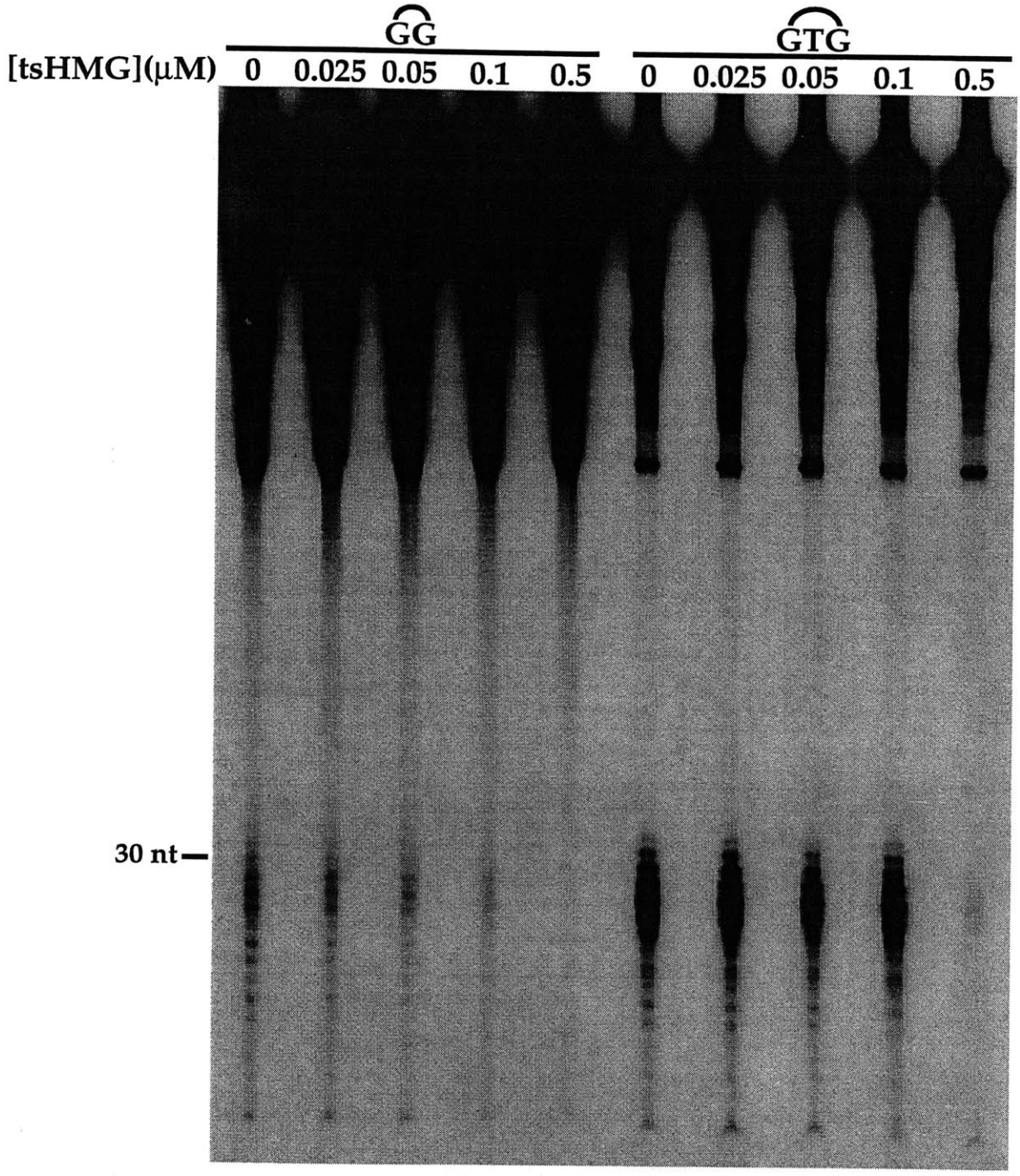


Figure 3.2. Effect of tsHMG on excision of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin-DNA adducts by the human excinuclease. The 1,2-d(GpG) and 1,3-d(GpTpG) site-specific cross-linked substrates were incubated with tsHMG for 10 min at 30°C followed by incubation for an additional 45 min with HeLa CFE. The reaction products were analyzed on a 10% denaturing polyacrylamide gel. The 30 nt marker is indicated.

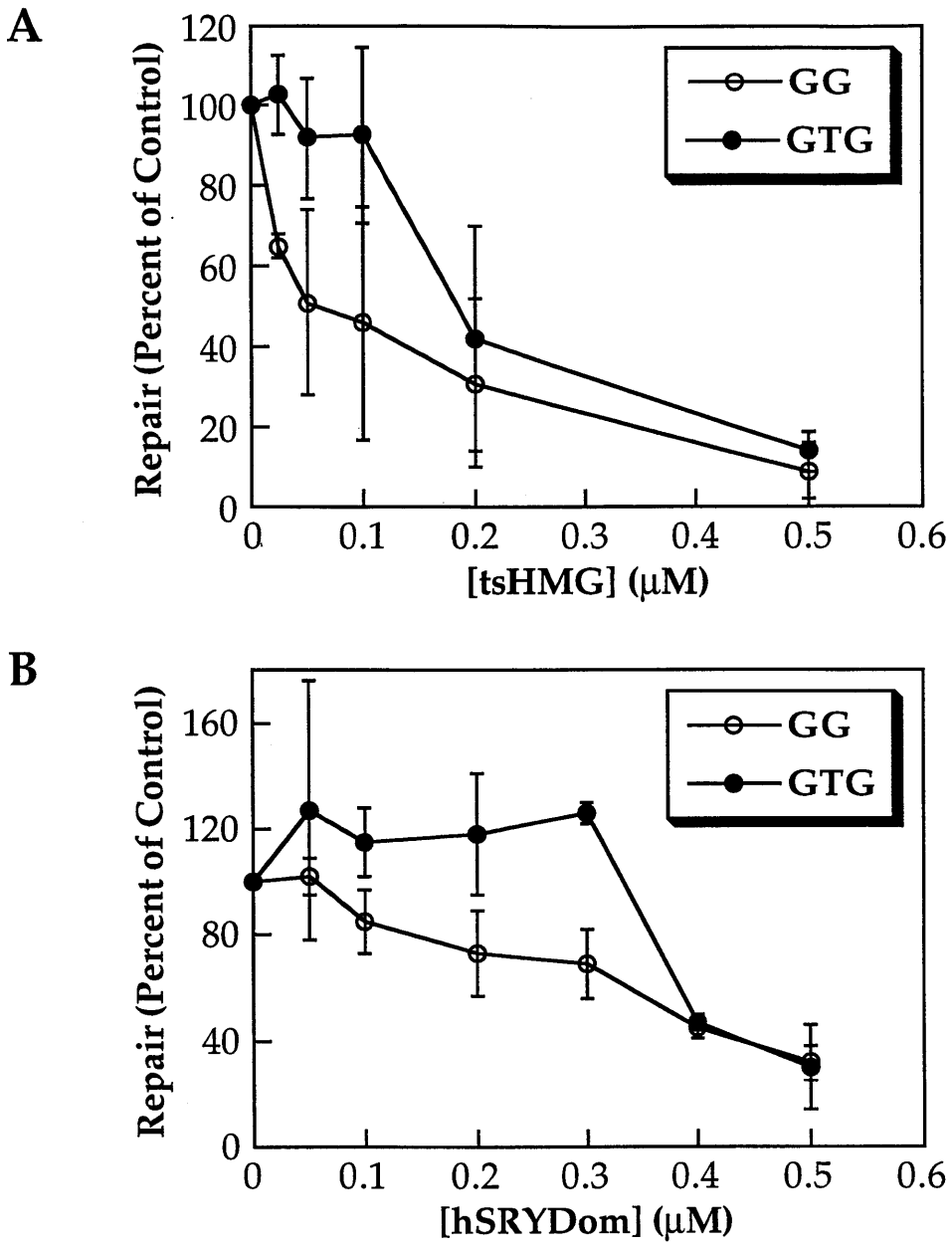


Figure 3.3. Inhibition of excision repair of the cisplatin-DNA intrastrand adducts by tsHMG and the HMG domain of SRY. (A) The 1,2-d(GpG) (open circles) and 1,3-d(GpTpG) (closed circles) cross-linked substrates were incubated with tsHMG for 10 min at 30°C, followed by an additional 45 min incubation with HeLa CFE. The reactions were analyzed on 10% denaturing polyacrylamide gels. Several different preparations of HeLa CFE and tsHMG were used to compile the data, and data points are averages of four or five experiments. (B) The repair substrates were incubated with the SRY HMG domain for 10 min at 30°C before the addition of HeLa CFE and further incubation for 50 min. Data points are averages of two or three experiments. The level of repair in the absence of HMG domain polypeptide for each experiment is designated 100%. Error bars represent ± 1 esd.

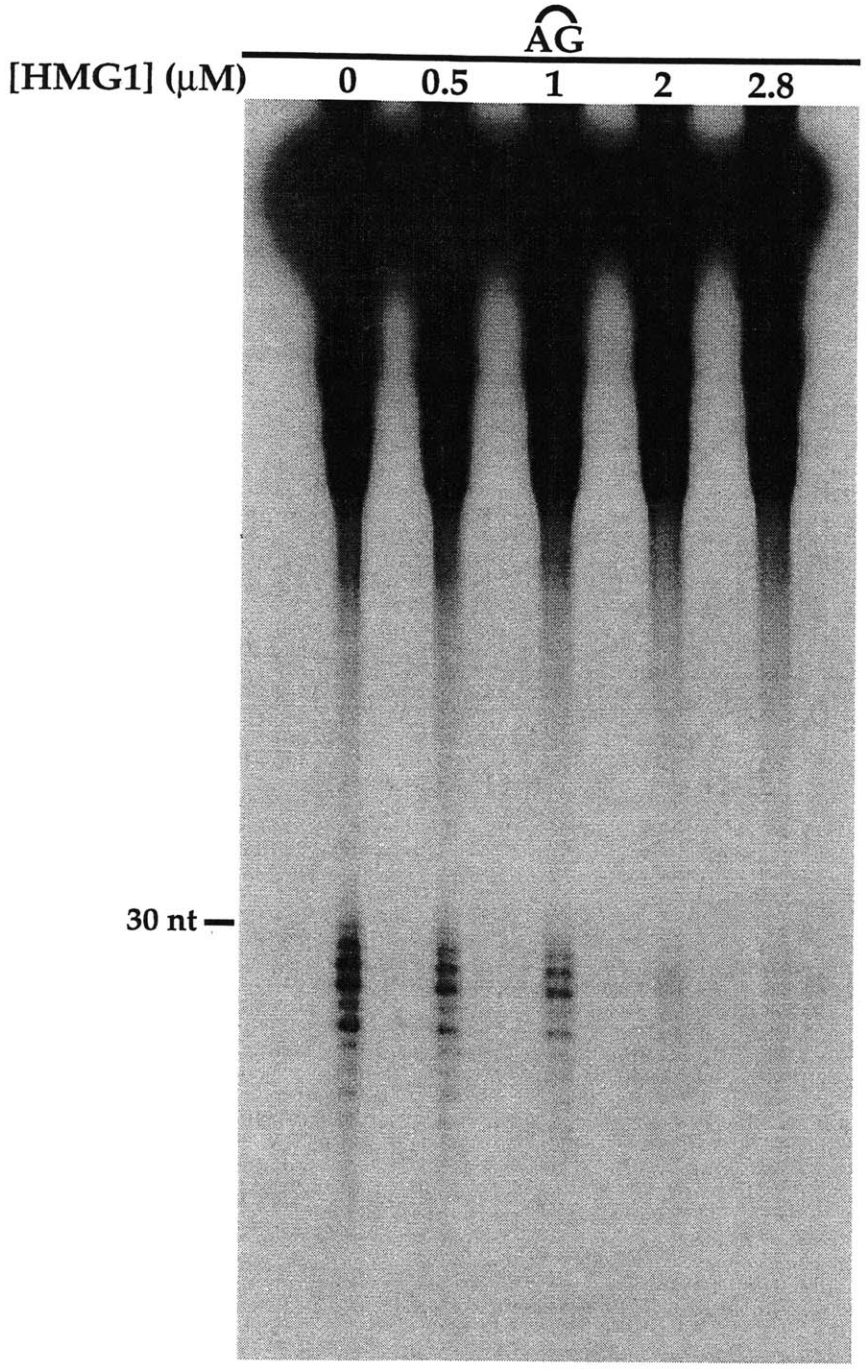


Figure 3.4. HMG1 blocks excision of the 1,2-d(ApG) cisplatin-DNA cross-link. The substrate was incubated with HMG1 at the concentrations indicated for 10 min at 30°C followed by incubation for an additional 45 min with HeLa CFE. The reaction products were analyzed on a 10% denaturing polyacrylamide gel. The 30 nt marker is indicated.

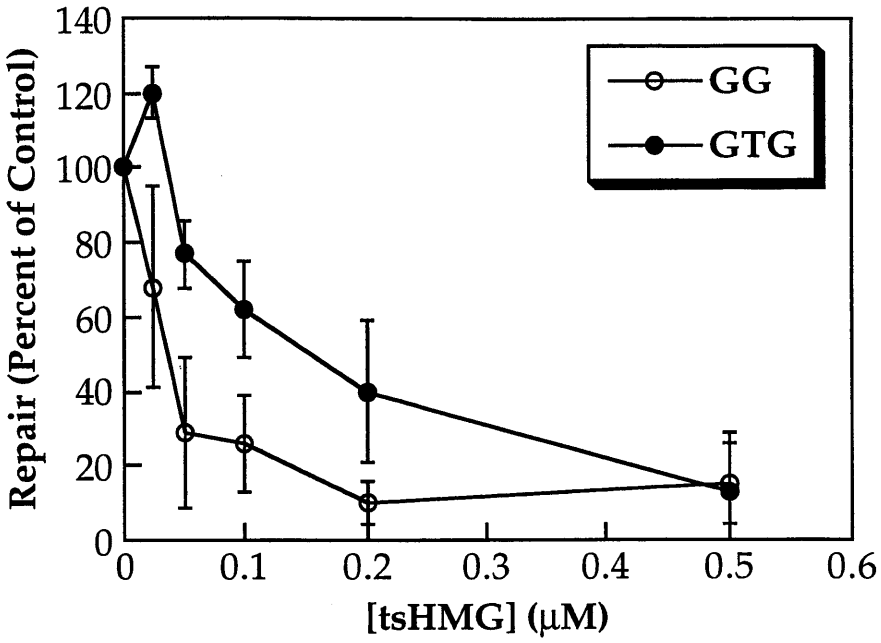


Figure 3.5. tsHMG inhibits reconstituted excision repair of the cisplatin-DNA intrastrand adducts. The 1,2-d(GpG) (open circles) or 1,3-d(GpTpG) (closed circles) cross-linked substrates were incubated with tsHMG for 10 min at 30°C followed by the addition of the purified repair proteins and further incubation for 110 min. Each data point is an average of three experiments. The error bars represent ± 1 esd.

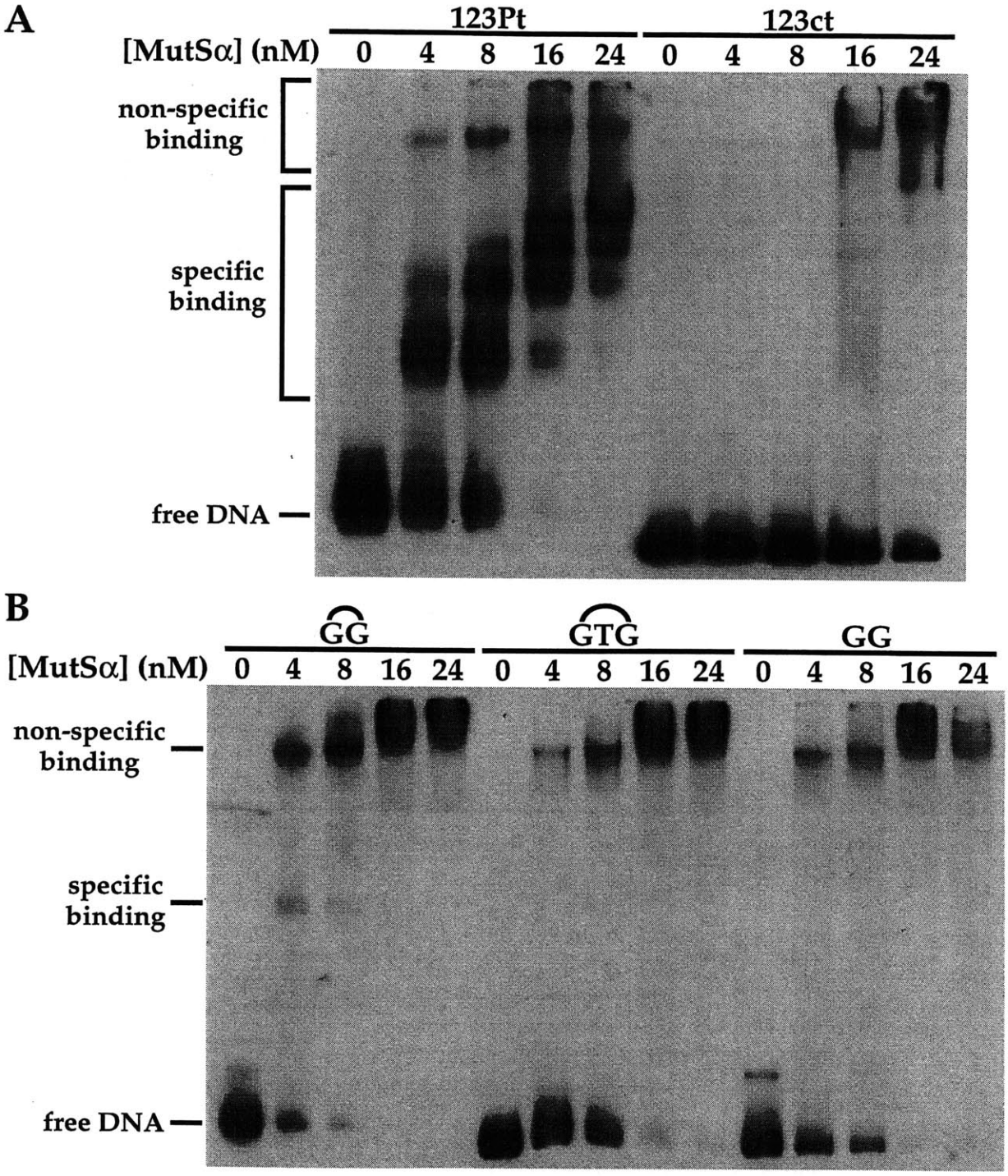


Figure 3.6. Mobility shift assays of cisplatin-modified DNA with MutSα. (A) The indicated concentrations of protein were incubated with either globally platinated 123 bp DNA (123Pt) or unmodified DNA (123ct) for 20 min on ice followed by analysis on a 6% native polyacrylamide gel. (B) The 1,2-d(GpG) or 1,3-d(GpTpG) cisplatin cross-linked or unmodifed 156 bp excision repair substrates were incubated with the indicated concentrations of protein for 20 min on ice prior to analysis on a 6% native polyacrylamide gel.

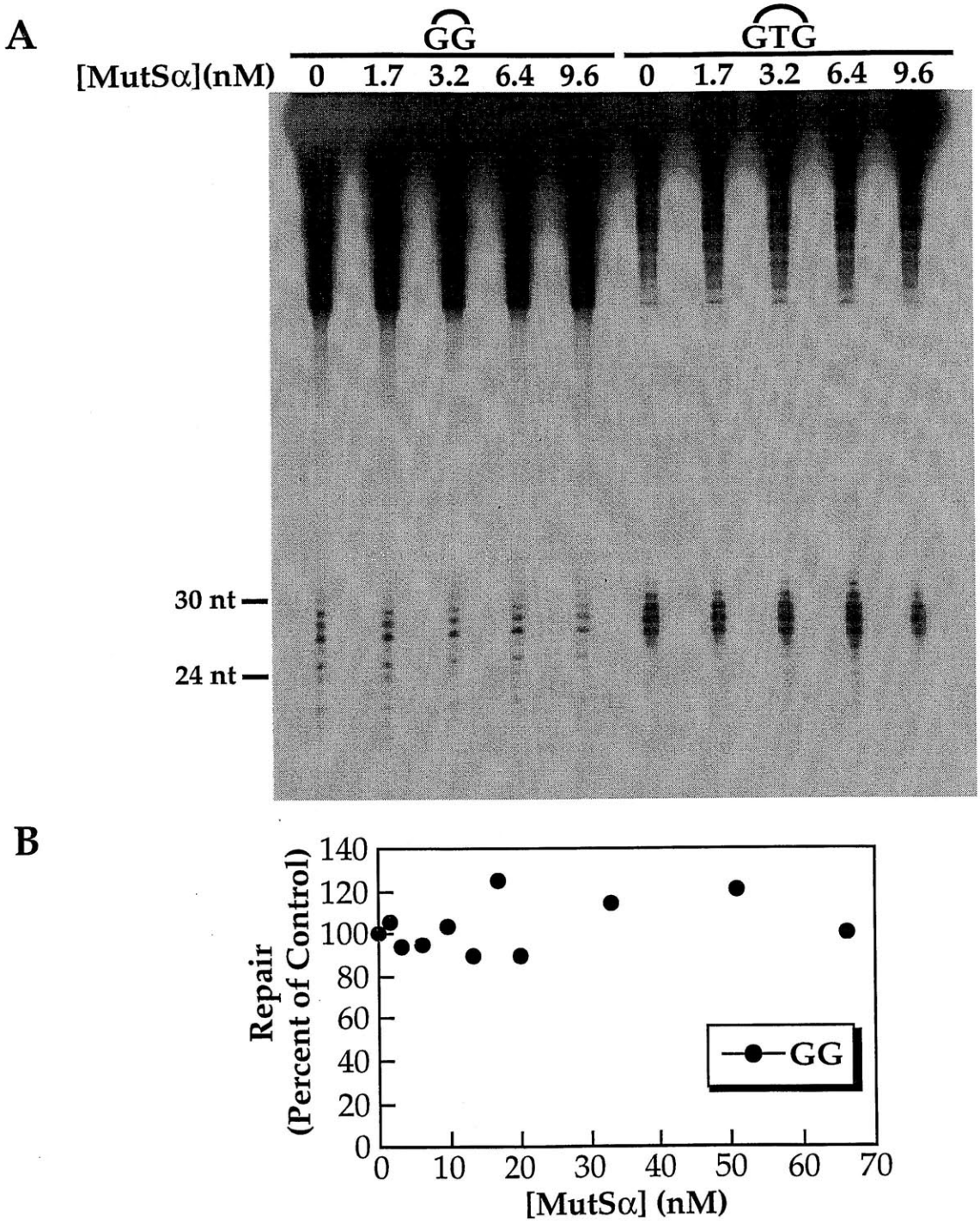


Figure 3.7. The effects of MutSα on excision of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin-DNA adducts by the human excinuclease. (A) The cisplatin-modified substrates were incubated with MutSα for 10 min on ice followed by the addition of HeLa CFE and an additional 50 min incubation. The reactions were resolved on a 10% denaturing polyacrylamide gel. (B) Inhibition of excision repair of the 1,2-d(GpG) cisplatin-DNA adduct by MutSα. Data from several experiments such as that shown in panel A are plotted here.

Chapter Four

p53-Dependent and -Independent Responses to Cisplatin in Mouse Testicular Teratocarcinoma Cells

Introduction (1, 2)

Chemotherapeutic agents and radiation are often used in the treatment of cancer to augment surgical procedures, particularly to address metastatic complications. In most cases, however, chemotherapy has limited success in conferring long-term patient survival. One notable exception is in the management of testicular cancer, which is the most common malignancy found in men under 35 years of age (3, 4). Several recent changes in the treatment of this disease have caused the patient mortality rate to diminish to less than 10 percent, making it one of the most curable solid neoplasms (3). In particular, the largest effect in the disease response was afforded by the addition of the platinum compound *cis*-diamminedichloroplatinum(II) (cisplatin) to the chemotherapeutic regimen (3, 4).

Cisplatin is used to treat a wide variety of cancers in addition to testicular tumors (5, 6), although its effectiveness is often limited by its inherently poor activity against many tumor types and by the development of resistance (7). The anticancer mechanism of cisplatin is not clearly understood, but it is generally accepted that it acts through the formation of DNA adducts (8). The fact that some cells are killed by the DNA damage while others are resistant is most likely a consequence of several cellular pathways (9, 10) which must be unraveled in order to provide more comprehensive treatment strategies.

One response to genotoxic stress involves the p53 tumor suppressor gene product (11, 12). This nuclear phosphoprotein accumulates following DNA damage and controls cellular proliferation predominantly through its activity as a transcription factor. The expression of downstream genes contributes to tumor suppression either by activating cell cycle arrest, possibly to give the cell time to repair the damage and avoid genetic instability, or by initiating apoptosis in the injured cell. The fact that the loss of p53 activity promotes malignant

transformation is confirmed by the high incidence of mutations in this gene in a wide spectrum of human cancers (13, 14). Moreover, a non-functional p53 might provide the ensuing tumors with resistance to chemotherapy because of the lack of drug-induced apoptosis. In this regard, the status of the p53 gene in a tumor can be an indicator of the clinical prognosis of a cancer patient (15).

Testicular tumors are atypical because the p53 gene is usually not mutated in this type of cancer (16-18). In addition, testicular tumors express high levels of the p53 protein, a characteristic that is usually a manifestation of a genetic mutation (19, 20). Recently, experiments with murine testicular teratocarcinoma cell lines demonstrated that the elevated levels of p53 protein did not result in increased transcriptional activity of p53-regulated genes (18), suggesting that the protein was predominantly inactive. Upon exposure of the cells to the DNA-damaging agent etoposide, however, their p53-mediated functions were restored, including activation of the programmed cell death pathway.

Although p53-dependent apoptosis suggests how DNA-damaging agents such as cisplatin kill neoplastic cells, the relationship between cisplatin sensitivity and p53 status remains unclear. In support of a role for p53 in the cytotoxic mechanism of cisplatin, several studies of ovarian carcinoma cell lines demonstrated that disruption of p53 function afforded drug resistance (21-23), although recent work has indicated that p53 is not a determinant of cisplatin cytotoxicity in ovarian cancer cells (24). In addition, non-small cell lung cancer cells were sensitized to cisplatin-induced apoptosis after the introduction of a wild-type p53 gene (25). In contrast, cell lines which do not exhibit a predominant apoptotic response to chemotherapy, such as breast cancer or human foreskin fibroblast cell lines, were sensitized to cisplatin by the inactivation of p53 (26, 27). Furthermore, although a screen of human cancer cell lines revealed a trend in which the p53-mutants were more resistant to cisplatin, there was significant

variability in drug response (28). Finally, while the present manuscript was in preparation, a report appeared indicating that the sensitivity of human testicular tumor cell lines to cisplatin-induced apoptosis was independent of p53 status (29).

These investigations reveal that the success of cisplatin-based chemotherapy may be due in part to apoptosis controlled by wild-type p53, but there is only limited information about the role of this protein in mediating cisplatin cytotoxicity in testicular cancer cells, the most responsive tumor type. Moreover, previous work demonstrates the importance of cellular context and cell type in examining the effect of p53 on the cytotoxicity of cisplatin. In the present study, several mouse testicular teratocarcinoma cell lines, having both wild-type and mutant p53, were used in order to examine the response of p53-controlled pathways to cisplatin in a relevant cellular background. Experiments were also performed to determine whether or not p53 is essential for the cytotoxic activity of this drug. Our results clearly show that, whereas p53 is an important factor in the short-term response to drug treatment, wild-type p53 function is not an absolute requirement for cisplatin-induced cytotoxicity.

Materials and Methods

Materials. Cisplatin was a gift from Johnson-Matthey. Fresh aqueous stock solutions were prepared for each experiment. The [α-^{32}P]dCTP, [γ-^{32}P]ATP, [^{35}S]amino acids, and [^{125}I]Protein A were purchased from Dupont, NEN. Protease inhibitors and RNase were purchased from Boehringer Mannheim. Igepal CA-630, DAPI, and cycloheximide were purchased from Sigma. Fixed *Staphylococcus aureus* (*Staph A*) was supplied by H. Ploegh (M.I.T.). The anti-p53 monoclonal antibody mAb421 was obtained from A. J. Levine (Princeton, NJ).

Cells and Cell Culture. F9 (ATCC CRL 1720) and Nulli-SCC1 (ATCC CRL 1566) cells were obtained from the American Type Culture Collection, Rockville,

Maryland. The p53 mutant teratocarcinoma cell line (EB16) was a gift from A. J. Levine, Princeton (18), and was derived from a testicular tumor of a p53 knockout mouse. The teratocarcinoma cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine and antibiotic antimycotics (Sigma) in addition to 10% fetal bovine serum (EB16), 15% fetal bovine serum (F9) or 10% calf serum (Nulli-SCC1). All plates were coated with a 0.1% (w/v) gelatin solution (Sigma) for 30 min and washed with PBS before use. The cells were grown at 37°C in a 5% $CO_{2(g)}$ atmosphere. Unless otherwise indicated, cisplatin was administered to cells growing in logarithmic phase on 10 cm plates in fresh media; continuous exposure means that the drug-treated media was not replaced.

p53 Protein Analysis. At each time point, the cells were washed with cold PBS and then lysed by incubation for 30 min on ice in lysis buffer (50 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.5% Igepal CA-630, 1 mM DTT, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, and 0.5 mg/ml pefabloc). The cell lysate was centrifuged, and the supernatant was stored on ice until use. All subsequent steps were performed at 4°C with pre-chilled solutions.

Fixed *Staph A* was washed three times in NET buffer (50 mM TrisHCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Igepal CA-630). Samples containing equal amounts of protein in the same volume (1-1.5 ml) were pre-cleared with 100 µl of *Staph A* and 6 µl each of normal mouse and rabbit sera (Sigma) for 1 h. After centrifugation the supernatant was incubated with 200 µl of mAb421 for 12-16 h. *Staph A* (80 µl) was incubated for 45 min in the sample, followed by centrifugation. The pellet was washed three times in NET buffer, boiled for 5 min in SDS loading buffer (50 mM TrisHCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue), and loaded onto an 8% SDS-polyacrylamide gel.

For western analysis, the proteins were electroblotted onto Immobilon-P membrane (Millipore) at 450 mA for 100 min, at 4°C, in 20 mM Tris, 200 mM

glycine, 20% methanol. The blot was blocked with 5% BSA in TPBS (0.05% Tween 20 in PBS), probed with mAb421 diluted 20-fold in TPBS containing 1% BSA. The blot was then washed, probed with 10 μCi [^{125}I]Protein A in the same buffer, and exposed to film at -80°C with an intensifier screen. The data were quantitated by Phosphorimager analysis (Molecular dynamics).

For metabolic labeling, either untreated or cisplatin-treated (24 h) cells were exposed to methionine/cysteine free media for 30 min and then pulse-labeled for 80 min with 0.3 mCi of [^{35}S]methionine and [^{35}S]cysteine. The cells were then washed and fed fresh media. Immunoprecipitations were performed as described above. Following SDS-PAGE analysis, the gel was fixed for 30 min in 25% isopropanol, 10% acetic acid, then in Amplify (Amersham) for 30 min. The gel was vacuum dried on Whatman filter paper and exposed to film at -80°C with an intensifier screen. The data were quantitated as described above.

Northern Assay. Total cellular RNA was isolated by using a single-step acid guanidinium thiocyanate-phenol-chloroform extraction (Ultraspec II, Biotecx). Plasmids containing the cDNA needed for probing the p53, p21, mdm-2, cyclin G and GAPDH genes were provided by T. Jacks (M.I.T.) The DNA was grown in *E. Coli,* purified by Quiagen preparation, cut with the appropriate restriction enzymes, resolved on an agarose gel, and eluted. Total mRNA was resolved on agarose-formaldehyde gels. The RNA was heated to 65°C for 15 min in 1X MOPS (40 mM MOPS, pH 7, 10 mM NaAcetate, 1 mM EDTA), 6% formaldehyde and 50% deionized formamide. The samples were chilled on ice and loading buffer was added (50% glycerol, 1 mM EDTA, 0.2% bromophenol blue) prior to loading onto an agarose gel containing 6% formaldehyde (Fisher) and 1X MOPS buffer, which was run in 1X MOPS buffer at 70-90 V. The RNA was transferred to Hybond-N nylon membrane (Amersham) by using the Schleicher and Schuell turboblotter, cross-linked to the membrane by using a Stratalinker (Stratagene). The blot was

hybridized to randomly primed cDNA probes (Prime-It II, Stratagene) as previously described (30). Briefly, the blot was blocked for 1-4 h at 65°C (0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS), and hybridized for 14-18 h at 65°C to the radiolabeled cDNA probe in 30% formamide, 0.2 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS with 0.1 mg/ml herring sperm DNA (GibcoBRL) as a non-specific competitor. The blot was washed twice in 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS for 30 min at 65°, and exposed while still damp to film at -80°C with an intensifier screen. The data were quantitated by Phosphorimager analysis (Molecular dynamics). Blots were reprobed after being stripped twice by pouring boiling water onto the membrane and then incubating at 65°C for 15 min.

DNA Degradation Assay. The cells were treated with cisplatin for 24 h, harvested, and washed twice in cold TBS (100 mM Tris, pH 7.4, 154 mM NaCl). DNA from 7.2 x 10^5 cells was isolated as described previously (18). Briefly, the cells were resuspended in 200 µl of PK solution (10 mM Tris, pH 8, 100 mM NaCl, 10 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K) and incubated at 37°C for 3 h. The mixture was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then once with ether. The DNA was precipitated with two volumes of isopropanol and then resuspended in TE containing 50 µg/ml RNase and incubated for 2 h at 37°C. Loading buffer was added (10% glycerol, 20 mM Tris, pH 7.5, 0.02% SDS, 20 mM EDTA, 0.002% bromophenol blue and xylene cyanol) and the DNA was loaded onto a 1.1% agarose gel. The gel was run at 20 mA for 12 h and then stained in 0.5 µg/ml ethidium bromide for 30 min and destained in deionized water.

Apoptosis Assay. Cells were treated continuously with cisplatin in 24-well plates (3-5 x 10^4 cells/well). At each time point, both attached and detached cells were collected and resuspended in PBS. To check for membrane perforation, an

aliquot was stained in 0.2% trypan blue and counted on a hemacytometer. The remainder of the cells were fixed in cold methanol, stained with 1 μg/ml of DAPI in PBS, and mounted on a slide. Chromatin structure was observed under a fluorescent light microscope (Axioscope, Zeiss Germany).

FACS Analysis. At each time point, both attached and detached cells were collected, washed with PBS, fixed by dropwise addition of ethanol, and stored at 4°C. The cells were washed once with PBS followed by resuspension in PBS containing 50 μg/ml propidium iodide (Sigma) and 0.2 mg/ml RNase. The samples were incubated at 37°C for 30-60 min and then analyzed on a Becton-Dickinson FACScan. Quantitation of cell cycle distribution was performed with ModFitLT software.

Excision Assay with CFEs. Whole cell-free extracts (CFEs) were used to measure excision of the damaged DNA according to a previously described method (31, 32). Extracts were prepared by the method of Manley et al. and stored at -80° (33). The 1,3-d(GpTpG) cisplatin intrastrand cross-linked substrate containing a radiolabel four nucleotides to the 5' side of the site-specific adduct has been described previously (32, 34). The excision assay experiments were performed as described. Briefly, 125 μl reactions contained 20 μl of reaction buffer (300 mM Hepes pH 7.9, 400 mM KCl, 32 mM $MgCl_2$, 1 mM EDTA, 20-50 mM ATP, 2 mM DTT), 25 μl of p21 buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol), 20 μM of each dNTP, 0.2 mg/ml bovine serum albumin (BSA), 500 ng of pBR322 as non-specific competitor DNA, 50-250 pM substrate, and 17.5 μl of CFE in storage buffer (25 mM Hepes pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT and 12.5-16.5% glycerol). When comparing two different CFEs, the same amount of protein was used. The reaction mixtures were incubated at 30 °C and 25 μl aliquots were removed from the reaction at each time point. The reactions were stopped by making them 0.5% in SDS and 0.4 mg/ml in

proteinase K (Gibco BRL) and heating at 55 °C for 20 min. The reactions were extracted, precipitated, and resolved on 10% denaturing polyacrylamide gels as previously described (34). The data were quantitated by using a PhosphorImager with ImageQuant software (Molecular Dynamics).

Clonogenic Assays. Dilute cells were plated on 6-well plates (150-200 cells/well) the day before cisplatin treatment. Exposure to drug was either for 2 h, at which point the cells were washed with PBS and fed fresh media, or continuous. Colonies were allowed to grow for 5-7 d, stained with 1% methylene blue (Fluka) in 50% ethanol, and counted.

Results

Cisplatin Induction of the p53 Protein. Previous reports have demonstrated that DNA-damaging agents induce the p53 protein in testicular tumor cells (18, 35, 36). In the present study, immunoprecipitation followed by western blot analysis revealed that continuous treatment of the F9 teratocarcinoma cells with cisplatin resulted in a rapid (2 h) elevation (2-6 fold) in the steady-state levels of the p53 protein (Figure 4.1). The amount of cellular p53 protein increased over a 6 h period, reaching levels more than 10-fold higher than in untreated cells, followed by a decrease observed 4 h later (data not shown).

One possible explanation for the cellular accumulation of p53 protein is drug-induced protein stabilization (18, 37). To test whether such a mechanism is active in cisplatin-treated cells, the half-life of the p53 protein was determined by pulse-labeling the F9 cells prior to drug exposure (Figure 4.2). In untreated cells, the half-life of the p53 protein was 3-4 h, as previously reported (38), and the requirement for active protein synthesis in p53 degradation was demonstrated by stabilization of the protein after the addition of cycloheximide to the media (Figure 4.2A). The 10 μM dose of cisplatin that produced a rapid increase in the

steady-state levels of p53 did not significantly alter the half-life of the protein, although a higher dose (50 μM) did stabilize p53. When the cells were exposed to 10 μM cisplatin 24 h prior to the radioactive pulse, many of them died and detached from the plate, resulting in less total protein for immunoprecipitation (Figures 4.2B and C). It is clear, however, that a 24 h dose of 10 μM, but not 1 μM, cisplatin stabilized the p53 protein. The mechanism of cisplatin-mediated p53 protein induction in the F9 cells was further examined by northern blot analysis. No significant changes in the p53 mRNA levels were observed following exposure of the cells to the drug (Figure 4.3).

Cisplatin Activation of p53 Activity. To investigate whether the p53 protein in the cisplatin-treated F9 cells was active, a northern blot containing total RNA was probed for the expression of p21, a transcriptional target of p53 (39). The p21 message was elevated after a 2 h treatment (data not shown) and continued to increase over the 10-h period of exposure (Figure 4.3). In addition, the induction of two other p53-regulated genes, mdm-2 and cyclin G (40, 41), was observed. Similarly, the RNA of all three genes was induced by cisplatin in another p53-wild-type teratocarcinoma cell line, Nulli-SCC1. The blots were also analyzed for expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene to control for gel loading and to demonstrate that cisplatin damage did not enhance gene expression nonspecifically.

Induction of p53-Dependent Apoptosis in Teratocarcinoma Cells. One of the p53-regulated responses to genotoxic stress is initiation of the programmed cell death pathway (12), an effect that could be involved in the cytotoxic mechanism of cisplatin (42). To determine whether cisplatin kills the testicular teratocarcinoma cells through an apoptotic pathway, chromatin condensation, an early indicator of apoptosis (43), was evaluated in cells stained with DAPI (Figure 4.4). The nuclear change was observed in a significant number of the p53-normal

cells following a 24 h exposure to 10 μM cisplatin (Figure 4.5A). After treatment for 2 d, most of the cells (>80%) were apoptotic and had detached from the plate. The results of the DAPI assays correlate with the loss of viable cells as determined by a Trypan blue exclusion test (Figure 4.5B), suggesting that at these time points the teratocarcinoma cells were killed exclusively by apoptosis. The requirement for functional p53 in the apoptotic response was examined in the EB16 cell line derived from testicular tumors of mice with a defective p53 gene (18). After 48 h of drug treatment, the majority of these p53-mutant teratocarcinoma cells remained attached to the plate and exhibited very little of the altered chromatin structures (Figures 4.4 and 4.5).

Another feature of programmed cell death is endonuclease digestion of genomic DNA (43). Resolution of the DNA from cisplatin-treated F9 and Nulli-SCC1 cells by agarose gel electrophoresis revealed the characteristic ladder of oligomers (Figure 4.6). Such fragmented DNA was not present upon similar analysis of the p53-mutant EB16 cells.

Effect of Cisplatin on Cell Cycle. To investigate further the response of the teratocarcinoma cells to cisplatin, flow cytometry was used to analyze the cell cycle distribution during exposure to the drug (Figure 4.7 and Table 4.1). A 1 μM dose of cisplatin allowed the effects of the drug on the cell cycle to be examined without immediately killing the cells. Treatment of F9 cells with this low dose for 24 h resulted in an accumulation of cells in the G_2 phase. After 2 d of exposure, the cells were still arrested in G_2, although some evidence for cell death (cellular debris) was observed. Significant cell death was apparent after a 1 d treatment with a higher dose (10 μM) of cisplatin and was complete after 48 h (Figure 4.7), in accord with the experiments described above (Figure 4.5). The p53-wild-type Nulli-SCC1 cells exhibited similar cell cycle profiles, a low dose of cisplatin activated G_2 arrest and some cell death, whereas a higher dose rapidly killed the cells (Table 4.1).

Cisplatin-Induced DNA-Repair. It has been suggested that cisplatin-treated cells arrest in the cell cycle so that attempts can be made to repair the DNA damage (42). In addition, several lines of evidence suggest that p53 modulates the nucleotide excision repair pathway, either through direct interactions with the excinuclease components or through transcription-controlled expression of DNA repair factors (44, 45). Pretreatment of human fibroblast or colon carcinoma cells with UV irradation resulted in an increase in the levels of DNA repair, an effect not observed in p53-deficient cells (46). To determine whether cisplatin-induction of the p53 protein was accompanied by an increase in repair activity in the teratocarcinoma cells, cell-free extracts (CFEs) were prepared from F9 cells that were exposed to 10 μM cisplatin for 5 h. The repair capacity of the extracts prepared from the treated cells was slightly higher than the extracts from untreated cells (Figure 4.8). This protective response was also observed in cells which had been exposed to cisplatin for 12 h (data not shown), at which time the p53-protein levels have subsided, supporting the hypothesis that the p53 protein is not directly involved in DNA repair, but instead may have a regulatory role.

Cell Cycle Response of p53-Mutant Cells. The effect of cisplatin on the p53-mutant teratocarcinoma cells was also examined. A low dose of cisplatin caused accumulation of the EB16 cells in the G_2 phase of the cell cycle (Figure 4.9 and Table 4.1). In contrast, the EB16 cells treated with 10 μM cisplatin for 1 d accumulated in the G_1/early S phase of the cell cycle. The block in the cell cycle was sustained through 4 d of continuous drug treatment and only a small amount of cellular debris was observed. This result was surprising because one of the mediators of G_1 arrest is the p53-regulated p21 gene product (47-49). There is evidence, however, for a p53-independent pathway of p21 induction and cell cycle arrest (11, 50). In fact, in our system, northern blot analysis revealed that the p21 message was induced during cisplatin treatment of the EB16 testicular

teratocarcinoma cells, even in the absence of wild-type p53 (Figure 4.3), providing a possible explanation for the observed G_1 arrest. A similar p53-independent induction of two other p53-regulated genes, mdm-2 and cyclin G (40, 41), was not observed in the mutant cell line (Figure 4.3).

Cisplatin Cytotoxicity. Colony formation assays were used to determine the overall sensitivity of the p53-wild-type and p53-mutant mouse testicular teratocarcinoma cell lines. This type of experiment differs from the apoptosis assays described above in that it only assesses viable cell division and does not distinguish between various mechanisms of cell death and/or cell cycle arrest. Such a limitation was noted in a recent study of human colon cancer cells in which a p21 mutation sensitized xenografts to γ-irradation, an effect not observed by clonogenic assay (51). However, the response of cell lines tested by this type of in vitro assay does reflect the differential chemosensitivity of tumors treated in vivo (36). In the present study, the cells were either treated for 2 h or continuously exposed to cisplatin and grown until visible colonies had formed. Under both conditions, no significant difference was observed in the sensitivity of the different cell lines to cisplatin (Figure 4.10), regardless of p53 status. Although the plating efficiencies of the three cell lines varied, for example in the experiments shown they ranged from 46-74% (Figure 4.10A) and 13-49% (Figure 4.10B), the p53 mutant cells did not have a consistently lower plating efficiency when compared to the other cell lines.

Discussion

Previous work investigated the relationship between the status of the p53 gene and cellular sensitivity to cisplatin, with contradictory results (21-29). The present study addresses the issue of cell type by investigating the p53 response to cisplatin in murine testicular teratocarcinoma cells. These cell lines resemble

testicular cancer, for which cisplatin is a particularly effective treatment, in that the product of a wild-type p53 gene is overexpressed (38, 52).

Treatment of the $p53^{+/+}$ teratocarcinoma cells lines with etoposide both induces and activates the p53 protein (18). Similarly, we report here that the p53 protein was elevated and transcriptionally active in the testicular teratocarcinoma cells after a 2 h exposure to cisplatin. One mechanism of p53 cellular accumulation is inhibition of the protein degradation pathway (28), and in support of this hypothesis the half-life of the p53 protein was prolonged in cisplatin-treated cells. In contrast to the rapid protein induction observed at the same drug concentration, however, protein stabilization was not immediately apparent and was only manifest after a much longer exposure to the drug (24 h). This result, coupled with the observation that the DNA-damaging agent did not result in increased levels of p53 mRNA, suggests that the rapid induction of the p53 protein by cisplatin occurs at least in part through a translational mechanism. Experiments with human leukemic blast cells also provided evidence for translational control of p53 expression (53). Cells collected from different patients with acute myelogenous leukemia displayed variable levels of p53 protein expression, which did not correlate with the levels of mRNA. In addition, a comparison of leukemia cell lines demonstrated that the p53 mRNA was preferentially associated with large polysomes in the cell populations with larger ratios of p53 protein to mRNA. In fact, it is possible that p53 is involved in autoregulatory control of its expression, because the protein binds to, and inhibits the translation of, its own mRNA (54, 55).

In a similar fashion to other DNA-damaging agents, cisplatin activates the programmed cell death pathway in the mouse testicular teratocarcinoma cells. Immediate activation of apoptosis, however, was avoided by the use of a smaller dose of cisplatin, which permitted an investigation of the effects of the drug on

the cell cycle. Both the F9 and Nulli-SCC1 cells accumulated in the G_2 phase during drug treatment. A G_2 block is a characteristic effect of cisplatin treatment and is considered to be a prerequisite for apoptosis induced by cisplatin (42, 56, 57), or a transient response during which the genetic damage is repaired. In addition, there is evidence that cisplatin-induced apoptosis depends on the active cell cycle of proliferating cells, a requirement that was not observed for etoposide (58). Although there is support for p53 involvement in the G_2/M checkpoint (11), it is unlikely that the protein participates in the cisplatin-mediated block of the cell cycle observed here, because some G_2 accumulation was evident in the p53-mutant cells exposed to the low dose. The lack of a functional p53 may be more relevant to the fate of the cells after cell cycle arrest. Whereas the clonogenic experiments demonstrate that the cellular proliferation of all of the teratocarcinoma cells is inhibited by the low dose of cisplatin, preliminary studies suggest that, in contrast to the p53-wild type cells, the p53-mutant cells do not undergo programmed cell death (data not shown). Non-apoptotic death following G_2 arrest would be consistent with a report suggesting that cells can die by a mechanism involving failure to overcome the cell cycle block when treated with a dose of cisplatin that does not immediately activate apoptosis (59).

The rapid drug-induced apoptosis observed in the F9 and Nulli-SCC1 cells depends on functional p53, since the characteristic chromatin condensation and DNA fragmentation were not observed in the p53-mutant cells. Instead, cell cycle analysis revealed a prolonged block in G_1/early S phase in the mutant cells. This result was unexpected because an effector of the G_1 checkpoint is the cyclin-dependent kinase inhibitor, p21 (47, 49), which is transcriptionally regulated by p53 (39), and is specifically induced in p53-dependent cellular responses to DNA damage (48). Exposure to cisplatin caused an elevation in p21 expression in the p53-mutant teratocarcinoma cells, however, providing an explanation for the

observed arrest in the cell cycle. Several reports have proposed the existence of p53-independent G_1 arrest and p21 induction (11, 24, 50, 60). This pathway seems to be selectively activated by different DNA-damaging agents, depending on the cellular context. For example, UV light, but not ionizing radiation, induced p21 and G_1 arrest in p53-deficient fibroblasts from Li-Fraumeni syndrome patients (60), but G_1 accumulation was observed in irradiated $p53^{-/-}$ T lymphoma cells expressing Bcl-2 (50).

A p53-independent induction of p21 was not detected in human testicular tumor lines exposed to cisplatin (29). In that study, however, similar chemosensitivities of cells with both functional and non-functional p53 were observed and attributed to the activation of p53-independent apoptosis. Therefore, with respect to the lack of p53-involvement in ultimate sensitivity to drug treatment, the results from the human and mouse testicular tumor cell lines are consistent, but important differences in cellular mechanisms may exist. The p53-mutant mouse teratocarcinoma cells did not undergo cisplatin-induced apoptosis, although clonogenic assays revealed an overall sensitivity similar to that of the p53-wild-type cell lines. It is conceivable that the p53-mutant cells experience a permanent cell-cycle arrest mediated by p21, which has been linked with senescence (61, 62). Preliminary cell viability studies suggest that these cells eventually die (data not shown), however, making this possibility unlikely.

Whatever the reason for the loss of clonogenic capability, these experiments demonstrate that, despite the failure of cisplatin to induce programmed cell death in the p53-mutant cells, this deficiency does not confer resistance. In fact, it is possible that the p53-mutant cells are slightly more sensitive than the p53-normal cells in this assay (Figure 4.10). A similar decrease in resistance was observed in fibroblast cells treated with paclitaxel, an anticancer drug that stabilizes cellular microtubules, when the cells were depleted of

functional p53 (63). In contrast, preclinical trials demonstrated that paclitaxel was effective in the treatment of human tumor xenografts with and without p53 mutations (64). The inconsistency was explained by the observation that paclitaxel activates the release of tumor necrosis factor α (TNF-α) by murine macrophages. TNF-α, which would have been induced in the in vivo experiments but not in the in vitro cell culture experiments, activates p53-independent apoptosis (64). These experiments, and others (51), demonstrate that it is not always possible to extrapolate data from cell culture to whole animals. Although the data presented here suggest that the unusual sensitivity of testicular cancer to cisplatin is not due solely to a wild-type p53 gene, it is impossible to make such a conclusion without in vivo evidence. A mutation in the p53-gene is an indicator of poor prognosis in patients with well or moderately differentiated ovarian cancer (65); however, overexpression of both wild-type and mutated p53 was found in advanced ovarian carcinomas refractory to cisplatin-based chemotherapy (66). In light of the results presented, both here and elsewhere (29), it would be informative to investigate whether the few testicular cancer patients who fail cisplatin-based chemotherapy have developed p53 mutations. In a recent study of a small panel of male germ cell tumors from relapsed patients, a mutant p53 gene was found in only a subset (<20%) of the specimens (67), supporting the conclusions of the present study. A much larger screen, however, will be necessary to confirm these results.

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1. Most of this work has been published in a slightly different form. See reference 2.
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Table 4.1. Cell cycle analysis of teratocarcinoma cells treated with cisplatin. The p53 wild-type (F9 and Nulli-SCC1) and p53-mutant (EB16) murine teratocarcinoma cell lines were treated continuously with cisplatin. At the time points indicated, the cells were collected and analyzed by FACScan as described in Material and Methods.

Dose (μM)	Time (d)	F9				N.S.				EB16			
		G_1	S	G_2/M	debris	G_1	S	G_2/M	debris	G_1	S	G_2/M	debris
0	0	21	62	17	2	31	51	18	5	43	45	12	1
1	1	2	51	47	2	10	63	27	7	14	40	46	17
1	2	13	5	82	54	9	32	59	19	16	33	51	34
10	1	31	68	1	43	30	40	30	59	100[a]		0	13
10	2	49	38	13	95	48	29	23	75	100[a]		0	26
10	4									100[a]		0	23

[a]In analysis of the cell cycle of EB16 cells treated with 10 μM cisplatin, the fraction of cells in the G_1 or S phase of the cell cycle could not be confidently determined.

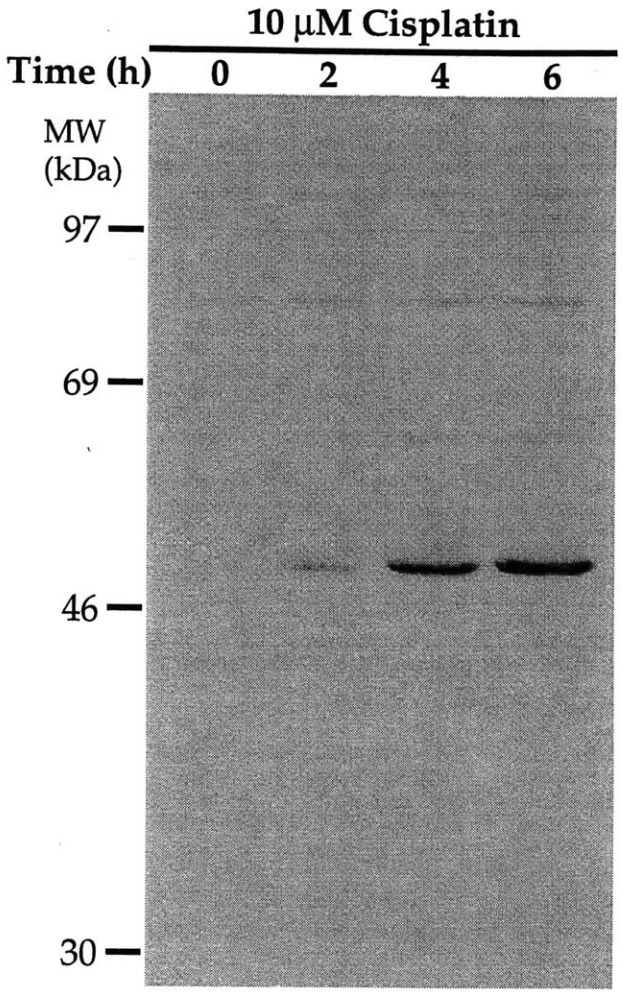


Figure 4.1. Cisplatin induction of p53 in mouse teratocarcinoma cells. F9 cells were treated with 10 μM cisplatin and whole protein extracts were prepared at the times indicated. The p53 protein was immunoprecipitated from equal amounts of total protein with an anti-p53 monoclonal antibody (421) and resolved on an 8% SDS-polyacrylamide gel. The proteins were transferred to Immobilon-P membrane, probed with the anti-p53 antibody and then with [^{125}I]protein A.

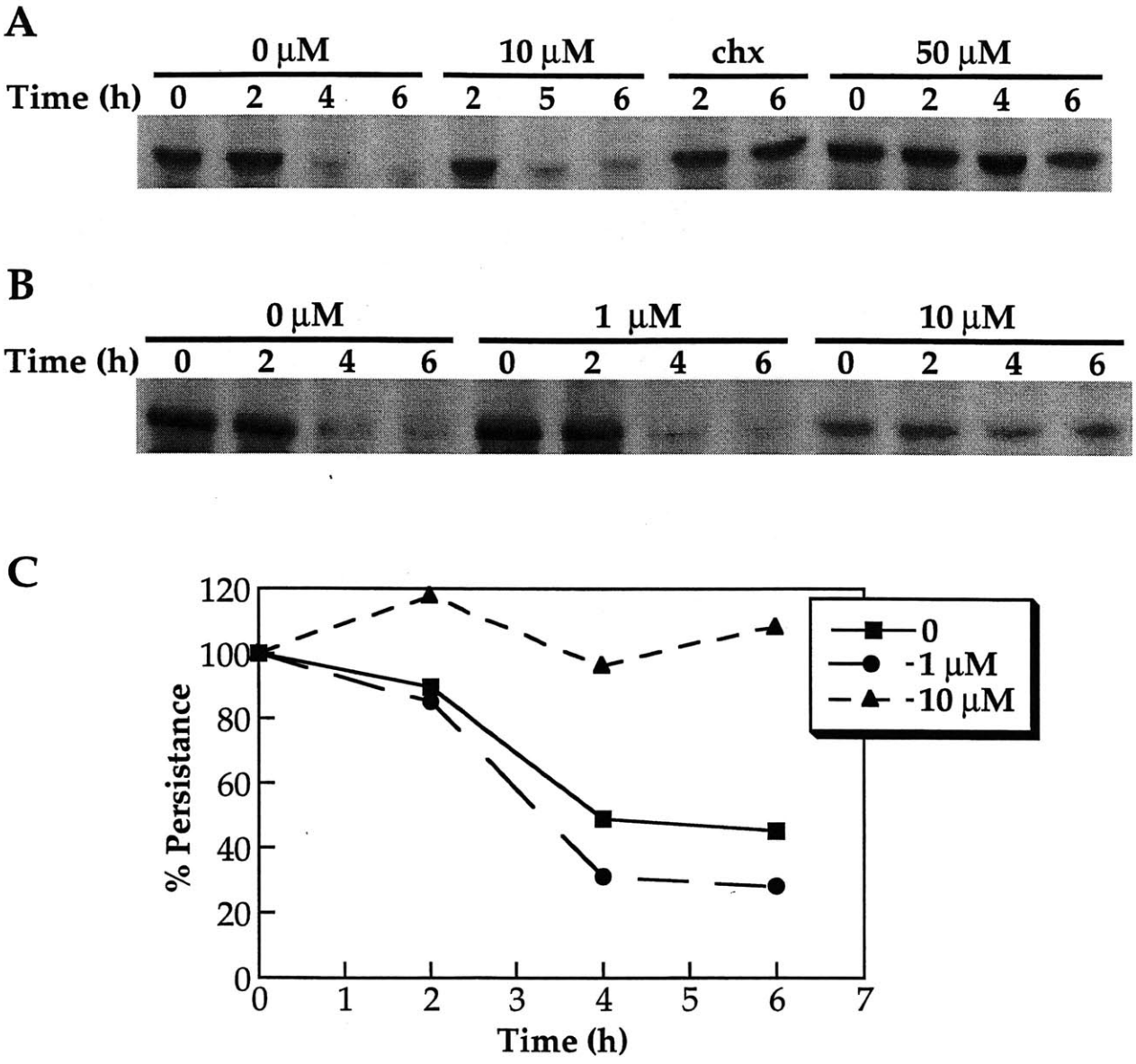


Figure 4.2. Cisplatin stabilization of p53 protein in mouse teratocarcinoma cells. The F9 cells were starved for 30 min in methionine/cysteine-free media, pulsed with [^{35}S]methionine and [^{35}S]cysteine for 80 min, and then washed and incubated in complete media. The cells were harvested at the indicated time points. Equal amounts of protein were immunoprecipitated with an anti-p53 monoclonal antibody (421), and analyzed on an 8% SDS-polyacrylamide gel. (A) The F9 cells were exposed to cisplatin or cycloheximide (chx, 50 μg/ml) immediately following the radioactive pulse. (B) The cells were exposed to cisplatin 24 h before the pulse. Less total protein was immunoprecipitated for the time course of the cells treated with 10 μM cisplatin. (C) Quantitation of the experiment shown in panel B by phosphorimager analysis. For each cisplatin concentration, data were normalized to the first time point (0 h).

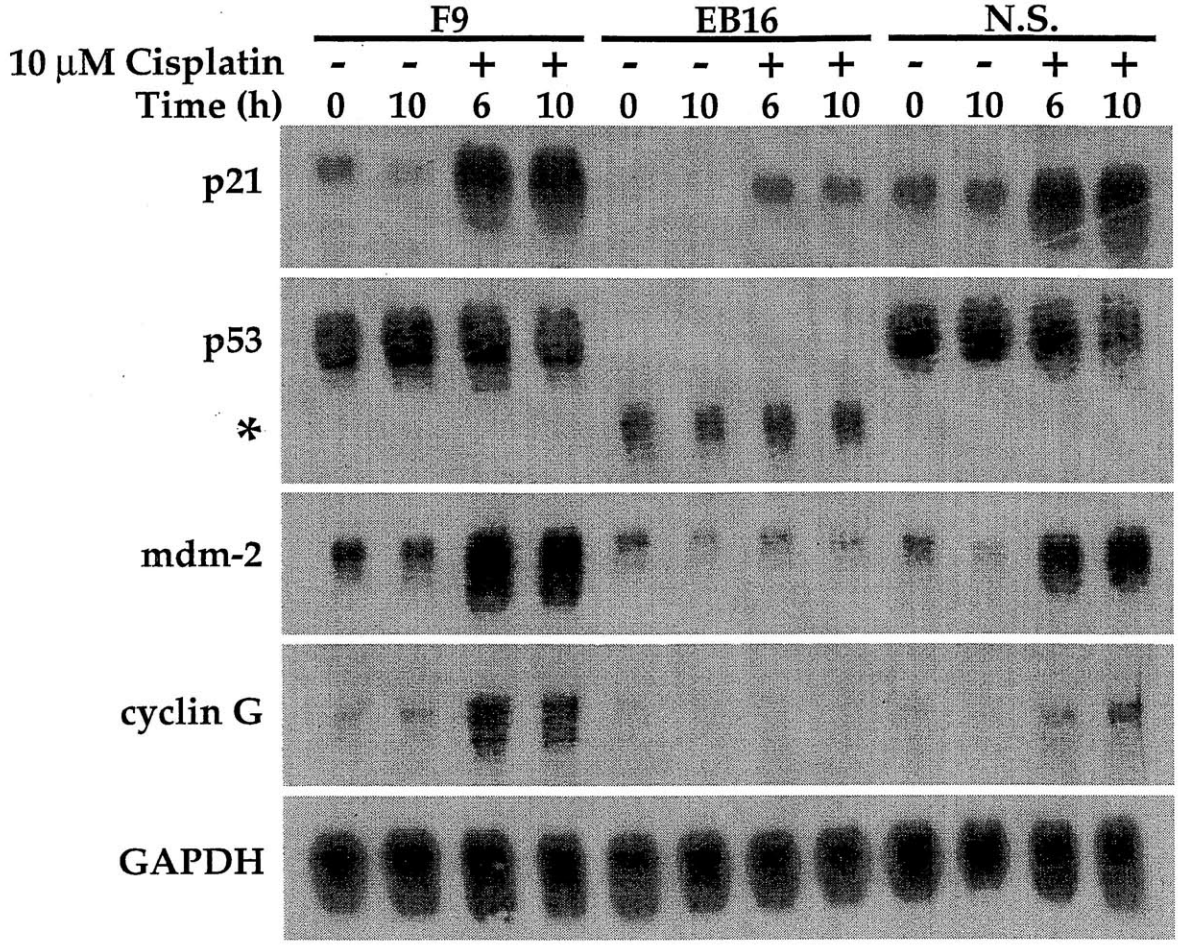


Figure 4.3. Cisplatin induction of p21, but not cyclin G or mdm-2, in a p53-independent manner. The p53-wild-type (F9, Nulli-SCC1), and p53-mutant (EB16) teratocarcinoma cells were treated with 10 μM cisplatin. Total RNA was isolated at the indicated time points; 12 μg of RNA was resolved on a 1.1% agarose-formaldehyde gel, transferred to nitrocellulose, and probed with randomly primed cDNA. Two different blots were prepared from the same samples of RNA, one blot was probed with the p21, p53, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes, the other with the cyclin G and mdm-2 genes. The asterisk indicates the truncated p53 gene from the p53-mutant mouse.

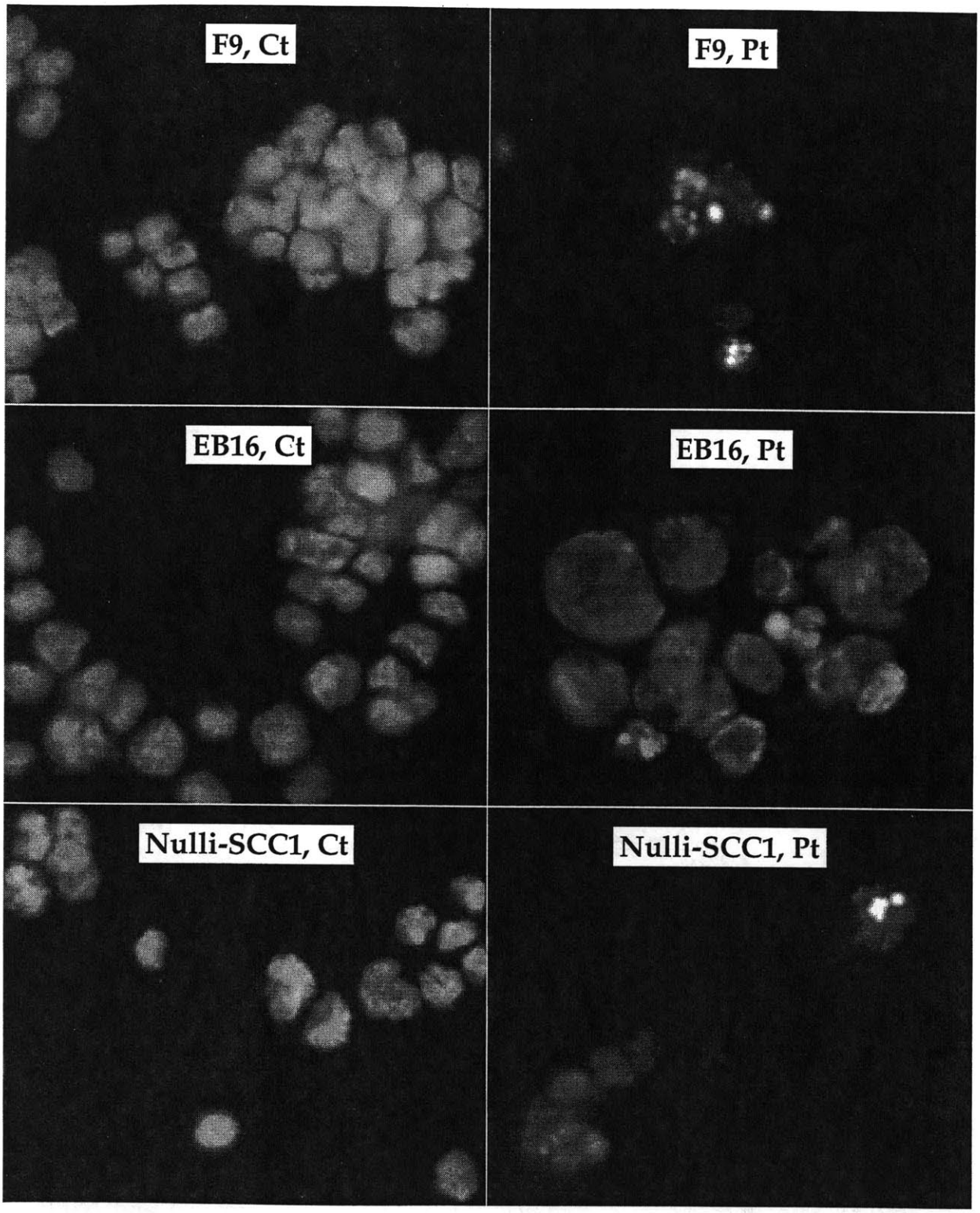


Figure 4.4. Chromatin condensation assay of mouse testicular teratocarcinoma cells exposed to cisplatin. The F9 (top), EB16 (middle), or Nulli-SCC1 (bottom) cells were either untreated (left) or exposed to 10 μM cisplatin (right) for 48 h. Following trypsinization, the cells were fixed in methanol and stained with DAPI.

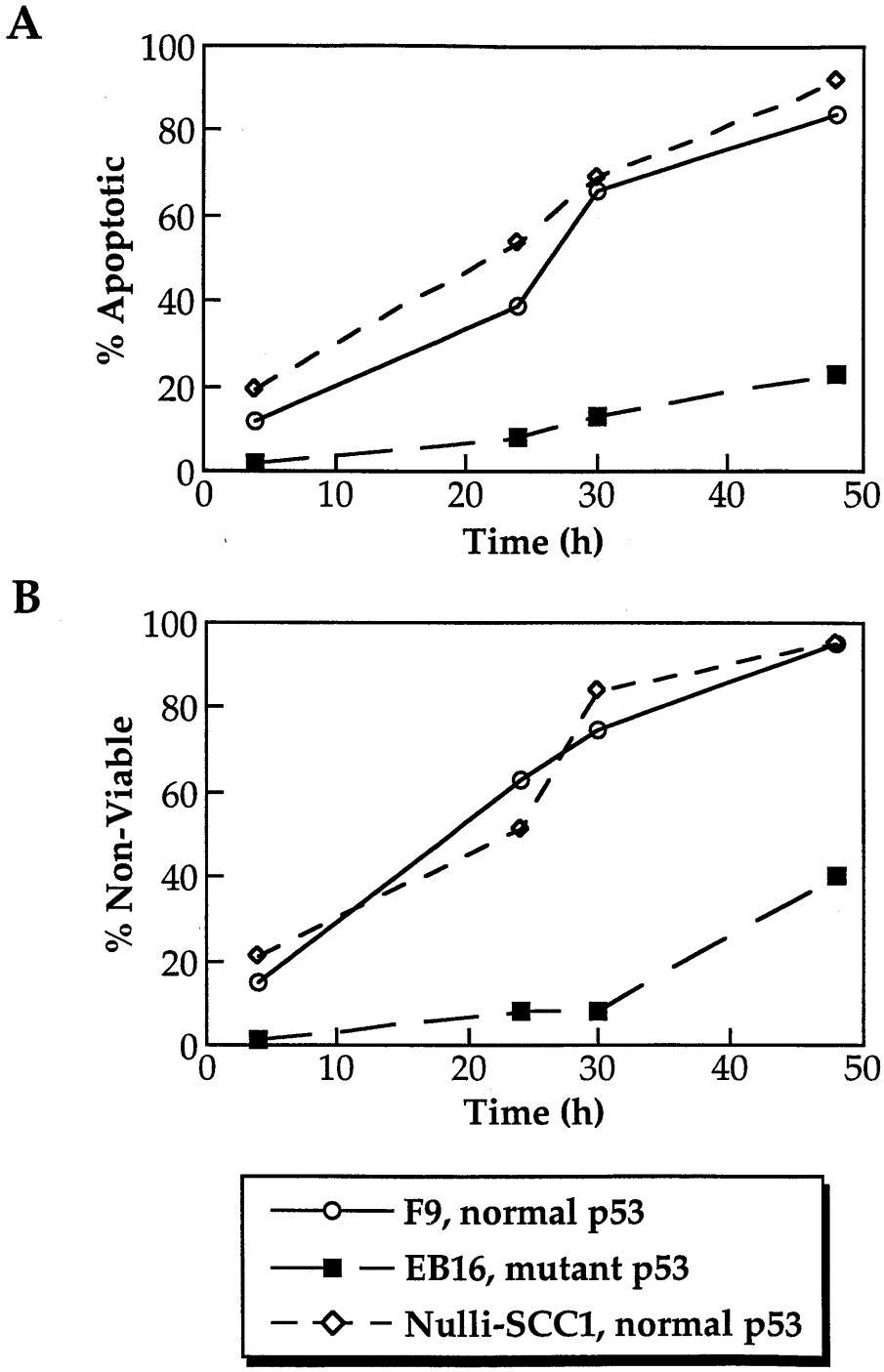


Figure 4.5. Induction of p53-dependent apoptosis by cisplatin. Three murine testicular teratocarcinoma cell lines, the p53-normal F9 (circles) and Nulli-SCC1 (diamonds) and the p53-mutant EB16 (squares), were treated continuously with 10 μM cisplatin. At the time points indicated, the cells were collected and stained with either (A) DAPI to quantitate cells with condensed chromatin or (B) trypan blue to quantitate cells with permeable membranes.

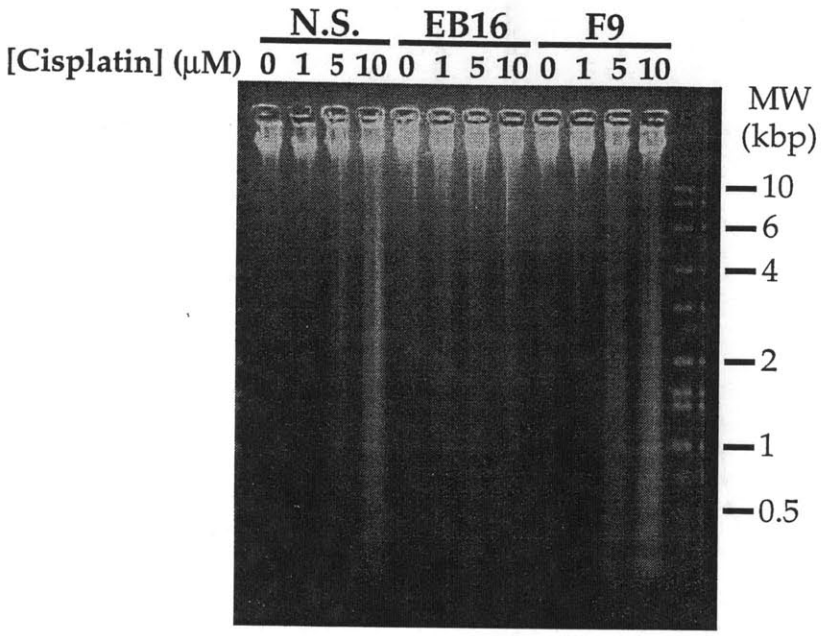


Figure 4.6. Cisplatin induction of p53-dependent DNA fragmentation. DNA was isolated from p53-wild-type (Nulli-SCC1 and F9) or p53-mutant (EB16) mouse teratocarcinoma cells after 24 h continuous exposure to the concentrations of cisplatin indicated and resolved on a 1.1% agarose gel.

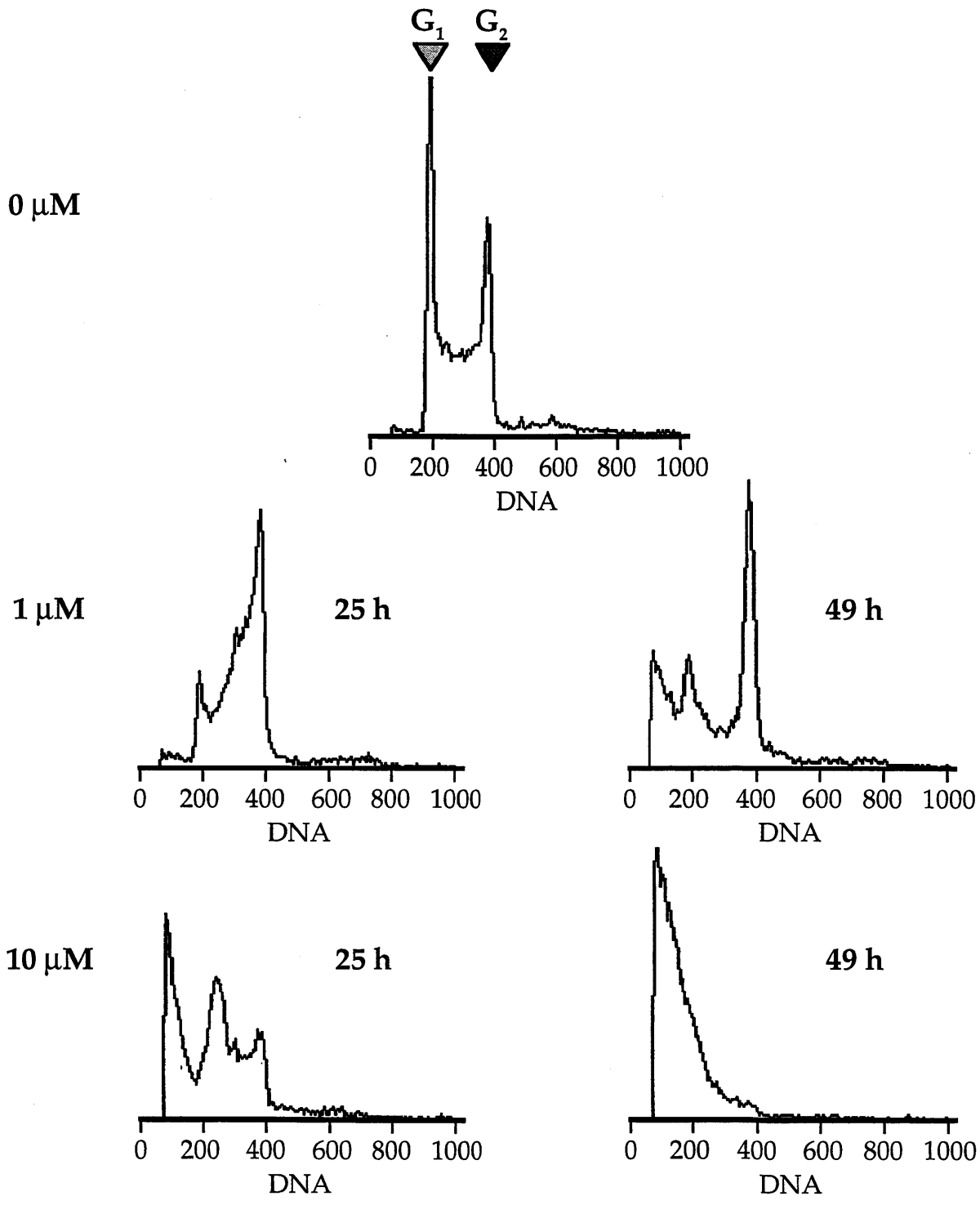


Figure 4.7. Cell cycle analysis of p53-normal mouse testicular teratocarcinoma cells treated with cisplatin. The p53-normal F9 cells were continuously exposed to 0, 1, or 10 μM cisplatin for the indicated times, fixed in ethanol, stained with propidium iodide, and sorted according to DNA content. The results of the flow cytometry are summarized in Table 4.1.

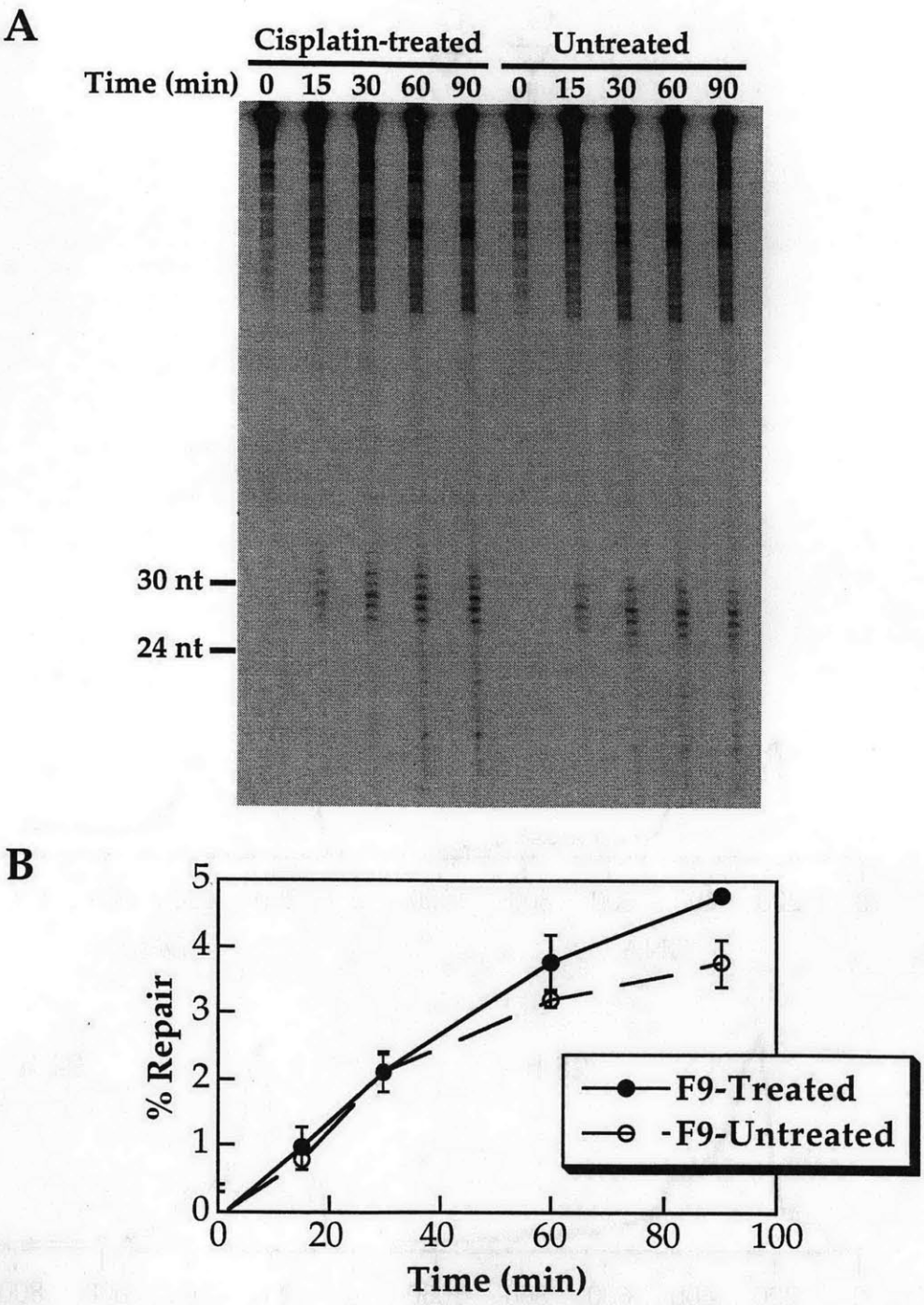


Figure 4.8. Excison of the 1,3-d(GpTpG) intrastrand cisplatin-DNA adduct in F9 extracts. (A) The substrate was incubated for the indicated amounts of time in whole cell extracts made either from teratocarcinoma cells exposed to 10 μM cisplatin for 5 h (F9-Treated) or from untreated cells (F9-Untreated). The products were resolved on a 10% denaturing polyacrylamide gel. The 24 and 30 nt markders are indicated. (B) Phosphorimager analysis of two experiments such as that shown in panel A. Error bars represent ± 1 esd.

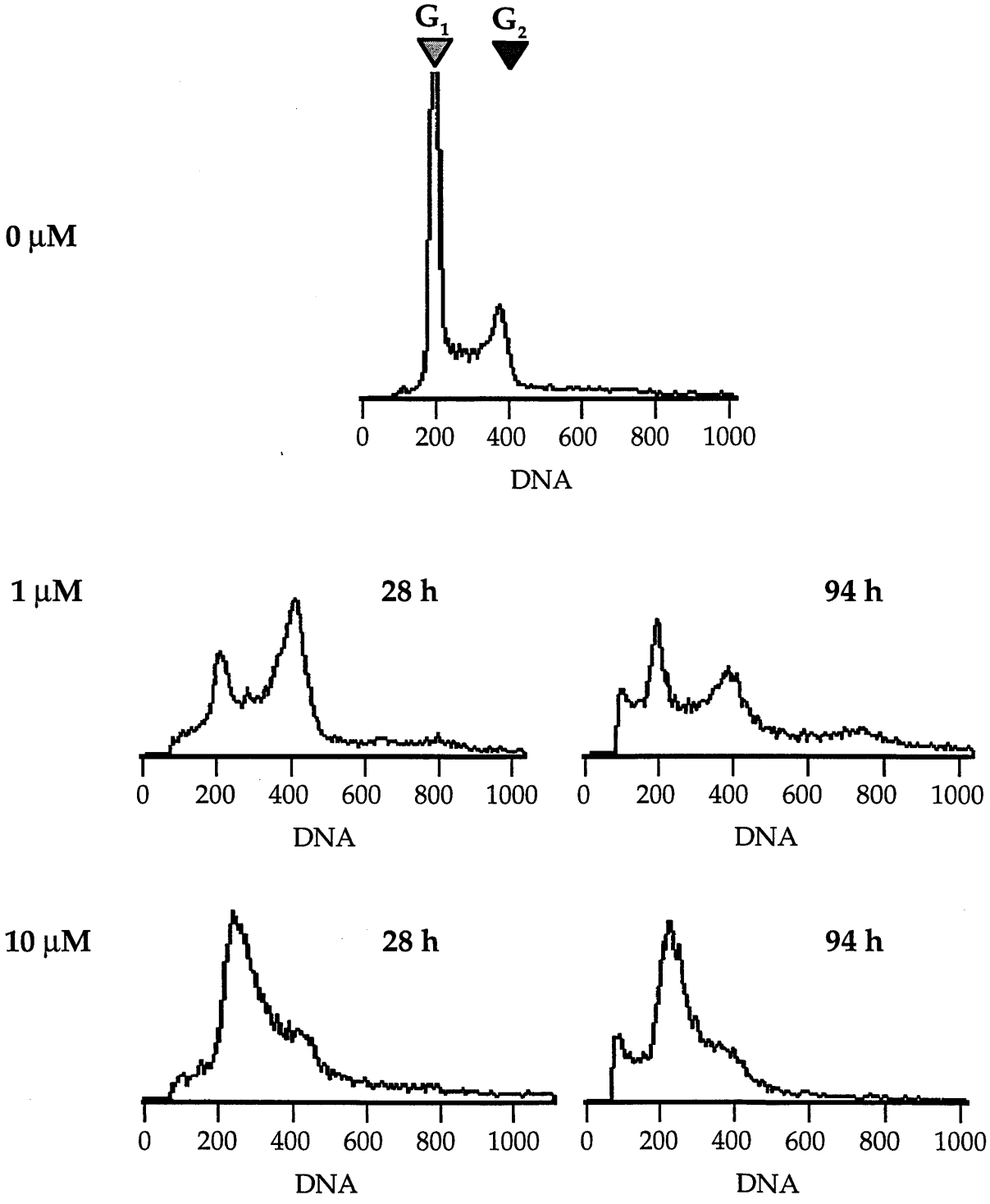


Figure 4.9. Cell cycle analysis of p53-mutant mouse testicular teratocarcinoma cells treated with cisplatin. The p53-mutant EB16 cells were continuously exposed to 0, 1, or 10 μM cisplatin for the indicated times, fixed in ethanol, stained with propidium iodide, and sorted according to DNA content. The results of the flow cytometry are summarized in Table 4.1.

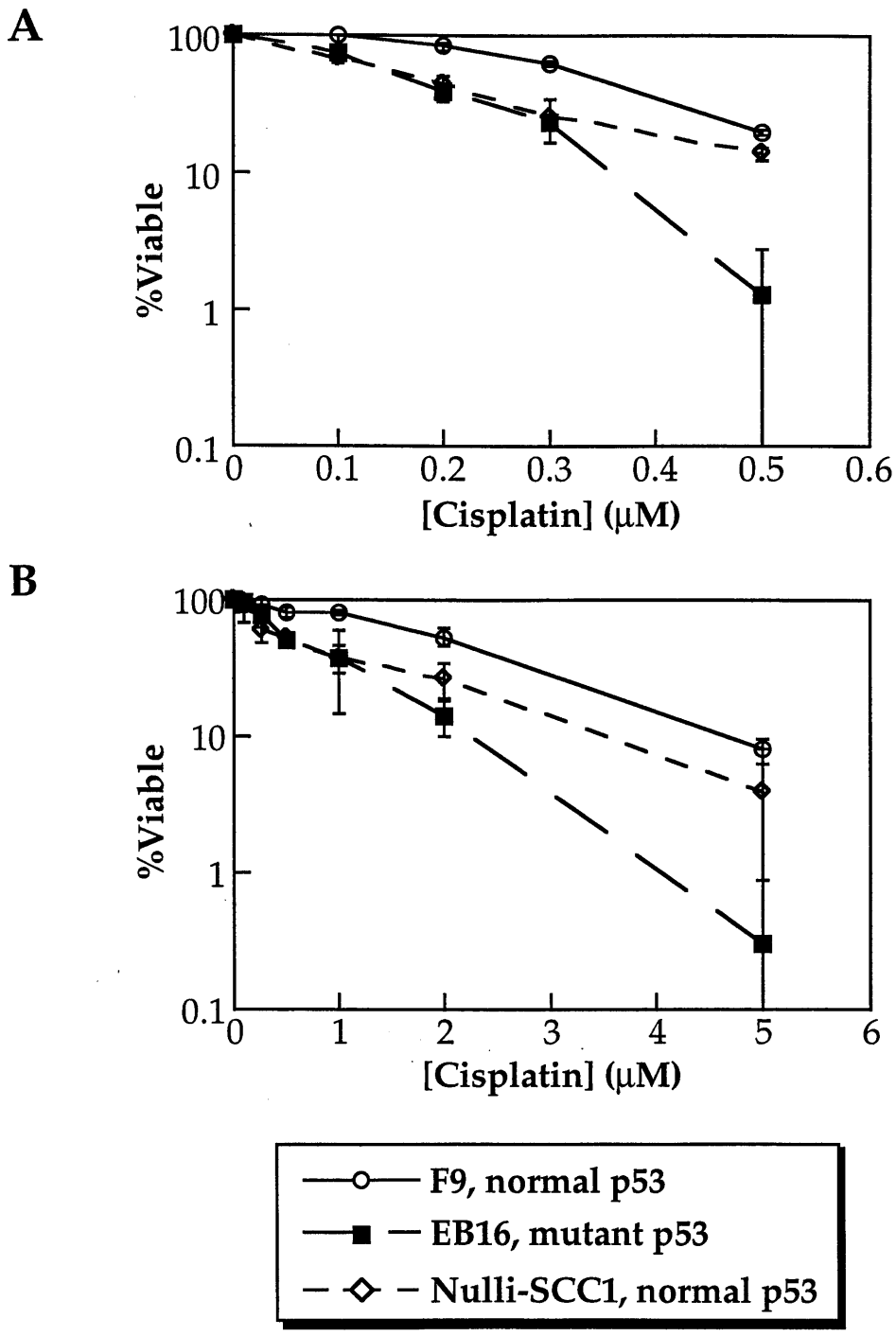


Figure 4.10. Colony formation assays. The p53-wild-type F9 (circles) and Nulli-SCC1 (diamonds) and p53-mutant EB16 (squares) teratocarcinoma cells were treated either (A) continuously or (B) for 2 h with cisplatin. After incubation for 5-7 days, visible colonies were stained with methylene blue and counted. For each cell line the numbers were normalized to the untreated data point. Error bars represent ± 1 esd.

Chapter Five

The Influence of the Testis-Specific HMG-Domain Protein on the Responses of HeLa Cells to Cisplatin

Introduction

Almost a third of the North American population will develop some type of cancer in their lifetime (1). Due to the high incidence of this complicated disease, considerable effort has been devoted to the generation of preventative and curative measures. One question which needs to be addressed is why the currently available therapies are often effective against only a subset of tumors. For example, *cis*-diamminedichloroplatinum(II) (cisplatin) is an essential component of the successful chemotherapeutic regimen used to manage testicular tumors (2-4), but it achieves an appreciable disease response in only a few other types of neoplasms (2, 5). Several reported properties of testicular cancers could contribute to the selective cytotoxicity of cisplatin. Included are chromosomal amplification, detected in both sensitive and resistant male germ cell tumors (4, 6), alternative gene splicing (7), the relative expression levels of particular proteins (8), or the production of serum tumor markers which correlate with clinical prognosis (4). Unfortunately, the expression of a specific cellular component has yet to be definitively associated with the mechanism of action of cisplatin in any human tumor.

It is generally accepted that the therapeutic effects of cisplatin originate from the formation of covalent-DNA adducts at the N7 position of purine nucleotides (9, 10). Structural analysis revealed that both the intrastrand and interstrand cross-links significantly distort B DNA from its normal conformation (11). The distinctive features of these cisplatin-DNA lesions, such as a local unwinding and bending of the double helix, are also observed in some protein-DNA complexes (12), so they might serve as recognition elements for factors that process the DNA damage. Recent studies have demonstrated that a variety of cellular proteins have the capacity to bind cisplatin-DNA adducts, although the consequences of this interaction are frequently unresolved (13). One group of proteins that specifically recognize cisplatin-modified

DNA are those which contain one or more copies of the high-mobility group (HMG) DNA-binding motif (14).

The HMG-domain family comprises DNA architectural components and transcription factors, some of which facilitate the formation of multi-protein complexes responsible for a variety of DNA transactions (15-17). Despite differences in sequence specificity, the domain confers certain properties on all of these minor-groove-binding proteins, including the ability to bend linear DNA and to bind distorted DNA structures. The affinity for cisplatin-modified DNA provides and explanation for why cisplatin treatment stimulated an intracellular redistribution of several HMG-domain proteins in human lung tumor and cervical carcinoma cells exposed to the drug (18, 19). This effect was not observed upon exposure to the clinically ineffective isomer, *trans*-DDP, the DNA adducts of which are not recognized by HMG-domain proteins (14). In vivo evidence supporting a role for HMG-domain proteins in the cytotoxic mechanism of cisplatin was provided by experiments performed in yeast. Interruption of the gene encoding the HMG-domain protein Ixr1 desensitized the cells to cisplatin but not to other DNA damaging agents (20). Reintroduction of protein expression in the knockout yeast caused a decrease in cisplatin resistance, although overexpression of the protein slightly above endogenous levels did not enhance drug sensitivity (21). Furthermore, a preliminary report suggested that there was a connection between elevated expression of the non-histone chromosomal protein HMG2 and the cisplatin sensitivity of a lung adenocarcinoma cell line (22).

Although many of the mammalian HMG-domain proteins are ubiquitously distributed in cells of all origins, the expression of some is restricted to certain tissue types (15). Of relevance to the organotropic response to cisplatin is the fact that multiple HMG-domain proteins are specifically expressed in testis tissue at various stages of differentiation (23-30). These proteins include the sex-determining factor

SRY, as well as several of the SRY-related (Sox) proteins that share considerable homology within their HMG-domains. The recently described murine testis-specific HMG-domain protein (tsHMG), a nuclear isoform of the mitochondrial transcription factor mtTFA, is expressed in the spermatocyte nuclei of seminiferous tubules and elongating and condensing spermatids (26, 30, 31). The function of tsHMG is currently unknown, although it may mediate chromatin restructuring during spermatogenesis (31) or sequence-specific regulation of transcription in analogy with mtTFA (32). Since this protein binds to cisplatin-modified DNA with unusually high affinity and specificity (33), it could potentially mediate the cisplatin sensitivity of tumors originating from testicular tissue.

To examine this hypothesis, tsHMG was exogenously expressed in HeLa cells, and its effect on several different cellular responses to cisplatin was examined. The experiments revealed that an HMG-domain protein can modulate the cytotoxic properties of cisplatin, depending on the intensity of drug exposure. These results suggest that testis-specific HMG-domain proteins such as tsHMG have the capacity to influence cisplatin cytotoxicity and contribute to the unique sensitivity of testicular tumors to the drug. Although the expression levels of HMG-domain proteins in testicular tumors have not yet been quantitated, this information could be used to develop of a wider range of anticancer strategies.

Materials and Methods

Materials. Cisplatin was a gift from Johnson-Matthey. Geneticin (G418) was purchased from Gibco BRL. Doxycycline, paraformaldehyde, and 4'-6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. Tet-system approved fetal bovine serum was purchased from Clontech. Hygromycin B and protease inhibitors were purchased from Boehringer Mannheim. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. Pfu polymerase was purchased from

Stratagene. The mouse mtTFA antiserum (30) was a generous gift from D. A. Clayton (Stanford, CA).

Construction of tsHMG Vectors. PCR primers were synthesized on an Applied Biosystems 392 DNA synthesizer, deprotected with NH_4OH, and purified on denaturing polyacrylamide gels according to standard procedures. Two different tsHMG-containing plasmids were constructed. To prepare the pTRE-tsHMG vector, the gene for tsHMG was PCR-amplified out of the pET-15b-tsHMG vector (supplied by G. Boissonneault, University of Sherbrooke, Quebec (31)). The 659-bp product was cut with *EcoRI* and *BamHI* and cloned into the corresponding sites of the pTRE expression vector (Clontech). The PCR primers used were 5'-TGTATTCCGAAGGAATTCTCCAGCATGGGTAGCTATCC-3' and 5'-TGGGCCT-AATCCGGATCCCAACTCCGTCTTCCATCTTA-3. The pTRE-tsHMG plasmid expresses the previously reported 199 amino acid tsHMG protein (26) under the control of an inducible tetracycline response element (TRE) and a minimal CMV promoter. To prepare the pcDNA3.1-tsHMG vector, the tsHMG gene was cut out of a pBluescript-tsHMG plasmid (supplied by N. Larsson, Karolinksa Institutet, Stockholm) with *EcoRI* and *KpnI* and cloned into the corresponding sites of the pcDNA 3.1/neo(-) plasmid (Invitrogen). This plasmid constitutively expresses a tsHMG protein (215 amino acids) with sixteen additional residues (MAGAWGLVCIPKCFSS) at the amino terminus of the originally reported sequence (30), under the control of a complete CMV enhancer promoter. The resulting plasmids were purified with a maxi-prep Qiagen kit. Restriction mapping and DNA sequencing (MIT Biopolymers Facility) confirmed the correct orientation and sequence of the genes.

Cell Culture and Transfections. The HeLa Tet-On cell line (Clontech) is a stable transfectant of a neomycin resistance gene and the "reverse" Tet repressor, which activates transcription in the presence of doxycycline. These cells were grown in

DME containing 10% fetal bovine serum, 2 mM glutamine, antibiotic antimycotics (Sigma), and 100-400 μg/ml G418, at 37°C in a 5% CO_2(g) atmosphere. Transfections were performed by using the calcium-phosphate precipitation method (34). The pTRE or pTRE-tsHMG plasmids were linearized with *Ssp1* and 15 μg of DNA was cotransfected with 0.75 μg of *HindIII*-digested pTK-Hyg plasmid (containing a hygromycin resistance gene, Clontech), to provide a selection marker. Following a 16 h incubation with the precipitate, the cells were washed and incubated for 48 h in fresh media. The cells were then replated at different dilutions and exposed to 200 μg/ml hygromycin. After 14 days selection the drug-resistant colonies were isolated, expanded, screened for tsHMG production by western analysis, and maintained in 100 μg/ml hygromycin. Unless otherwise indicated, cisplatin was administered to cells growing in logarithmic phase in fresh media; continuous exposure means that the drug-treated media was not replaced. Fresh aqueous stock solutions were prepared for each experiment, and the platinum concentration was confirmed by using a Varian atomic absorption spectrophotometer AA-1475, equipped with a graphite tube atomizer.

Western Analysis. Cells growing on 10-cm plates were induced for 24 h with the indicated concentrations of doxycycline in fresh media, washed with PBS, and lysed in 250 μl of cell lysis buffer (2% SDS in PBS, 10 μg/ml each of pepstatin, leupeptin, and aprotinin, 0.5 mg/ml pefabloc). The lysates were passed through a 25G5/8 syringe needle, boiled for 5 min, and the protein concentrations were determined by using the BioRad DC assay. The proteins (240 μg) were resolved on a 10% SDS-polyacrylamide gel and then electroblotted onto Immobilon-P membrane (Millipore). The blots were blocked for 1 h in TPBS (0.05% Tween-20 in PBS) containing 5% BSA, washed, and then incubated for 60-90 min with α-mtTFA antibody diluted in TPBS with 1% BSA. The membrane was then washed, and incubated with an α-rabbit IgG whole antibody conjugated to horseradish peroxidase

(Amersham) diluted 5000-fold in TPBS and 1% BSA. After washing, the blot was developed with an enhanced chemiluminescent (ECL) detection system. The membrane was incubated for 1 min in a freshly made solution containing 67 mM Tris, pH 8, 1.25 mM luminol (Fluka), 0.2 mM p-coumeric acid (Sigma) and 0.009% hydrogen peroxide, and then exposed to film.

Clonogenic Assays. The cells were exposed to 4.2 μM (2 μg/ml) doxycycline for 2 days, plated onto 6-well plates (400 cells per well) and incubated with doxycyline for an additional day prior to cisplatin treatment. Exposure to cisplatin was either for 2 h, at which point the cells were washed with PBS and fed fresh media containing doxycycline, or continuous. Colonies were allowed to grow for 6-7 days, stained with 1% methylene blue (Fluka) in 50% ethanol, and counted.

Immunofluorescence and Apoptosis Assays. In preparation for experiments involving continuous cisplatin exposure, 5-8 x 10^4 cells were plated into each well of 6-well plates and treated with 4.2 μM doxycyline for two days prior to drug treatment. For short drug exposure times, cells growing on 10-cm plates were treated with doxycycline for 2 days, then replated onto 6-well plates (5-8 x 10^4 cells per well). A day later the cells were exposed to cisplatin in fresh media for the indicated length of time, washed twice with PBS and then incubated in media containing doxycycline. At the appropriate time point, both the detached and attached cells were collected, centrifuged onto to Vectabond-treated glass slides (Vector Labs) by using a cytospin, and air dried. The cells were then fixed with 4% paraformaldehyde in PBS, rinsed with PBS, and permeabilized with 0.1% Triton-X 100 in 0.1% (w/v) sodium citrate for 2 min at 4°C. The cells were then washed with PBS, incubated for 60 min at 37°C with terminal deoxynucleotidyl transferase and fluorescein-conjugated dUTP (Boehringer Mannheim) according to the manufacturer's specifications, and washed extensively in PBS. The cells were blocked in PBS containing 0.1% Triton X-100 and 5% goat serum (Sigma) for 15 min at RT, incubated for 40 min at 37°C with mtTFA

antiserum diluted 500-fold in blocking buffer, and washed. After incubation for 40 min at 37°C with goat α-rabbit IgG conjugated to rhodamine (Cappel) diluted 1000-fold in blocking buffer, the cells were washed, stained with 1 μg/ml DAPI in PBS for 5 min, washed and mounted with Mowiol. The cells were then examined with a fluorescent light microscope (Axioscope, Ziess Germany). The fraction of tsHMG-expressing and -non-expressing cells that were apoptotic was determined, at least 400 cells were counted on each slide. The significance of the data from each experiment was determined by using the two-sample unpaired t-test (35). Each experiment was performed in triplicate, and the mean values and standard deviations (s, eq 1) for the

$$s = \sqrt{\frac{\sum\left(X_i - \overline{X}\right)^2}{N-1}} \qquad (1)$$

percentage of tsHMG-expressing and non-expressing apoptotic cell was determined. These values were then used to calculate the population variance (s_p^2) and t (eq 2 and 3), which was compared with values on a t-table. The means were considered as significantly different if the probability that the null hypothesis was true (p) was less than 5%.

$$s_p^2 = \frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2} \qquad (2)$$

$$t = \frac{\overline{X}_1 - \overline{X}_2}{s_p\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}} \qquad (3)$$

Transcription Inhibition Assay. This experiment was performed by K. E. Sandman (M.I.T.). HeLa cells expressing the enhanced green fluorescent protein (EGFP) under the control of the doxycycline-inducible promoter (36) were transiently transfected with pcDNA3.1 or pcDNA3.1-tsHMG by the calcium phosphate method, using 6 μg of circular plasmid per well of 6-well plates. The precipitate was washed

away after 16 h, and the cells were incubated in fresh media for 48 h. The cells were then co-treated for 13.5 h with cisplatin and doxycycline in fresh media, and the EGFP expression assay was performed as previously described (37).

Results

tsHMG Expression in HeLa Cells. To investigate whether exogenous HMG-domain proteins can influence the cytotoxic effects of cisplatin in a cell-based system, the gene for the mouse tsHMG was placed under the control of an inducible promoter, the tetracycline responsive element (TRE). The pTRE-tsHMG vector was cotransfected into HeLa cells with a hygromycin-resistance gene, and stable clones were selected, isolated, and expanded. The HeLa cell line employed expresses a mutant form of the Tet repressor fused to a VP16 transcriptional activation domain. This complex activates transcription of TRE-controlled genes in the presence of tetracycline or a derivative such as doxycycline (Figure 5.1). The HeLa cell line is derived from a human cervical carcinoma infected with a human papillomavirus (HPV) which modulates the activity of the wild-type p53 protein (38, 39). Although the HPV could afford some unusual drug response which is not relevant to the treatment of uninfected cell types, many of the p53-regulated responses to cisplatin, including p53 protein relocalization, p21 mRNA induction, and apoptosis, are observed in this cell type (40-42).

Following induction with doxycycline, clones that inducibly expressed tsHMG were identified by using a polyclonal antibody for mtTFA in western blot analysis of whole cell extracts. Murine tsHMG and mtTFA arise from alternative splice variants such that mtTFA only differs by an extra 44 residues at the amino terminus (30). The tsHMG protein was detected in multiple clones following a 24 h induction with 4.2 μM doxycycline (Figure 5.2 and data not shown). No significant difference in tsHMG expression was observed upon induction with a range of doxycycline concentrations

(2.1-21 μM). The levels of tsHMG expression increased as a function of induction time up to, but not beyond, 48 h (data not shown). For all subsequent experiments tsHMG protein expression in the HeLa cells was induced by exposure to 4.2 μM doxycycline for at least 48 h. Finally, tsHMG was still detectable in the HeLa cells 48 h after doxycycline had been removed from the media (data not shown), possibly because of a long protein half-life or persistent induction of the response element.

The Effect of tsHMG on Cisplatin Cytotoxicity. Colony formation assays were used to compare the cisplatin sensitivity of the tsHMG-expressing HeLa cells with uninduced cells (Figure 5.3). Two sets of conditions were employed, either a two hour exposure or continuous drug treatment. Some variability in sensitivity was observed between the different clones, particularly for the longer exposure times. No reproducible difference in the sensitivity of the induced cells was observed when compared with that of the uninduced cells, however, under either of the drug-treatment conditions. The plating efficiencies of the different cell lines varied. For example, in the experiments shown they ranged from 39-69% (Figure 5.3A) and from 16-38% (Figure 5.3B). When there was a difference between induced and uninduced cells, the doxycycline-treated cells had a slightly lower proliferative capacity after plating.

One possible explanation for the absence of any effect of tsHMG on the sensitivity of the HeLa cells to cisplatin is that the protein localized in the cytoplasm and did not have access to the drug-damaged DNA. Recent analysis of murine testis-specific cDNA suggested that tsHMG might be expressed with sixteen additional N-terminal residues (30), proposed to serve as a nuclear localization sequence (NLS). The exact location of the translational start site of tsHMG remains uncertain (26, 31), however, and mutational analysis of several HMG-domain proteins established that nuclear localization is determined by sequences contained within the HMG domain (43, 44). To resolve this issue, the subcellular localization of tsHMG in the HeLa cells

was evaluated with immunofluoresent staining. A high concentration of primary antibody was employed in these experiments, resulting in a low level of background fluorescence in all parts of the control pTRE cells, similar in both doxycycline-treated and -untreated samples (Figure 5.4). The same degree of cellular staining was observed in an uninduced stable transfectant of pTRE-tsHMG (Figure 5.5). After a 24 h exposure to doxycycline, however, strong immunoreactivity was observed in some of the nuclei, confirmed by parallel DAPI staining.

Although these experiments demonstrate that tsHMG localizes to the nucleus, they also suggest why there was no significant effect on cisplatin toxicity in the clonogenic assays. Examination of the doxycycline-induced HeLa revealed that only a fraction of the cells contained detectable levels of the HMG-domain protein (Figure 5.5), and the levels of expression of tsHMG varied from about 20% to 70% of the cells, depending on the clone.

Sensitization to Cisplatin-Induced Apoptosis. A common cellular response to cisplatin exposure is the activation of the programmed cell death pathway (45), which is accompanied by several characteristic transformations including chromatin condensation and endonuclease digestion of genomic DNA (46). To investigate how tsHMG affects cisplatin-induced apoptosis in individual HeLa cells, tsHMG expression was detected with a rhodamine-conjugated secondary antibody. The number of expressing or non-expressing cells undergoing apoptosis was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (47), which enzymatically incorporates a fluorescein label at the ends of DNA strand breaks. Low levels of apoptosis were observed in the HeLa cells 48 h after a 2 h exposure to cisplatin. A representative experiment is shown in Figure 5.6 and listed in Table 5.1. Under these conditions, a larger percentage of tsHMG-expressing cells exhibited the distinctive nuclear changes than did non-expressing cells. Although this difference was small, never more than two-fold, it was

statistically significant, and the effect was reproduced in several tsHMG-expressing clones, indicating that the HMG-domain protein sensitized the HeLa cells to cisplatin-induced apoptosis.

Enhanced Transcription Inhibition. A recent study of HeLa cells inducibly expressing the enhanced green fluorescent protein (EGFP) reporter gene demonstrated that platinum drugs block transcription to a degree that correlates with the cytotoxicity of the different compounds (37). In order to study the effect of an HMG-domain protein on this cisplatin-mediated transcription inhibition, the pcDNA3.1-tsHMG expression plasmid was transiently transfected into the EGFP-expressing cells. Induction with doxycycline, followed by fluorescence measurements and Western analysis, confirmed that tsHMG and EGFP could be simultaneously expressed (data not shown). Transiently transfected HeLa were then exposed simultaneously to doxycyline and cisplatin. In the absence of tsHMG, exposure to low concentrations of cisplatin (1-4 μM) resulted in a slight increase in the EGFP expression (Figure 5.7), but higher drug doses monotonically reduced the protein expression, as previously reported (37). This inhibitory effect of cisplatin on the EGFP expression was enhanced in the presence of tsHMG, such that only a decrease in fluorescence was observed upon exposure to the drug. Preliminary experiments revealed that, under the conditions producing the greatest effect of tsHMG on EGFP expression, significant apoptosis was not detectable (data not shown).

Protection from Cisplatin-Induced Apoptosis. In order to explore further the effects of tsHMG on cisplatin-induced apoptosis in HeLa cells, several different drug treatment conditions were used. When the cells were exposed to cisplatin for two hours and then incubated for 61 h, instead of 48 h as described above, slightly higher levels of apoptosis were achieved but tsHMG sensitization of the HeLa cells was no longer apparent (Table 5.1). Furthermore, when the drug-exposure time was

extended to 3 h, the increase in apoptosis was accompanied by the opposite effect, such that tsHMG seemed to be protecting the cells from cisplatin-induced programmed cell death (Table 5.1). Finally, upon continuous exposure to cisplatin, the tsHMG-expressing cells were more resistant to cisplatin-induced apoptosis than the uninduced cells (Figures 5.8, 5.9 and Table 5.1). Some of the signaling pathways activated during apoptosis involve a controlled activation of proteases (48), so it is possible that the number of tsHMG-expressing apoptotic cells was artificially low because the protein was degraded. This explanation is unlikely, however, since the overall levels of tsHMG expression were not significantly reduced in the cisplatin-treated samples of cells (Table 5.1).

Discussion

The mouse tsHMG protein exhibits strong affinity and specificity for the most prevalent type of cisplatin-DNA adduct (33, 49), so it has the potential to affect cellular responses to the platinum-induced genotoxic stress. To explore this possibility, the influence of ectopic tsHMG expression on cisplatin cytotoxicity was examined in several stable transfectant clones. No reproducible effect on cisplatin inhibition of colony formation was observed when the HeLa cells were induced to express tsHMG. Although the clonogenic assay is frequently used to provide accurate information about the relative chemosensitivities of different cell types (50), it does not distinguish between two of the possible responses to cisplatin, cell cycle arrest and death (45). Since arrested cells can recover, the results from this assay do not always reflect the ultimate consequences of toxic stress (51). Moreover, a closer examination revealed that the nuclear tsHMG protein was expressed in only a fraction of the cells, even though all of the clones were each isolated from a single colony. The high background of non-expressing cells could preclude detection of the effects of tsHMG on cisplatin cytotoxicity.

Accordingly, to minimize the limitations of heterogeneous tsHMG expression, a technique designed for the study of individual cells was used to investigate cisplatin-induced apoptosis. Under drug treatment conditions that generated low levels of apoptosis, expression of tsHMG sensitized the HeLa cells to cisplatin. Furthermore transient transfection of tsHMG into HeLa cells enhanced the cisplatin-dependent inhibition of the reporter gene expression. These results are consistent with one of the models proposed to explain how HMG-domain proteins could modulate the cytotoxicity of the cisplatin-DNA damage (52). The theory is that HMG-domain proteins bind to the major cisplatin-DNA adducts and block repair or other DNA-processing pathways. Diminished DNA repair would allow the adducts to persist in the cell, block essential cellular pathways, such as transcription, and activate programmed cell death. In support of the shielding hypothesis, the differential cisplatin resistance of yeast cells lacking a native HMG-domain protein was dependent on an intact nucleotide excision repair pathway (53). Furthermore, HMG-domain proteins inhibit repair of the cisplatin-DNA cross-links (49, 54, 55), replication bypass (56), and helicase activity (57), when added to in vitro assays, and immunoprecipitation of HMG1/HMG2 from cell extracts enhanced repair synthesis activity on cisplatin-modified plasmid DNA (58). Since the ubiquitous HMG-domain proteins that are expressed in HeLa cells did not modulate the repair of cisplatin-modified DNA (49, 59), it is unlikely that they are responsible for the variability in disease response to cisplatin. However, many members of this protein family have a distribution pattern limited to specific tissue types, such as testicular (23-30). Moreover, the testis-specific proteins tested, tsHMG and hSRY, were the most effective at blocking the in vitro repair of cisplatin-DNA adducts (49, 55).

The tsHMG-sensitization of HeLa cells to cisplatin was observed at low levels of apoptosis. Although the difference between induced and uninduced cells was significant and reproducible, it was not very large. Lengthening the post-treatment

incubation time, in an effort to amplify this difference, actually diminished the effect. This result suggests that the cells were sensitized by tsHMG only at the early stages of apoptosis. It is therefore likely that the ability of the HMG-domain protein to block repair or replicative bypass of the major cisplatin-DNA adducts is only temporary. The tendency of DNA-binding proteins to dissociate from and reassociate to their target site within a period of seconds to hours (for example, see 60-62) would, over time, provide opportunities for the repair proteins to access and remove the lesions, allowing the cell to recover from the effects of the damage.

Longer exposure times to cisplatin were also investigated, and under these conditions tsHMG expression appeared to protect the HeLa cells from cisplatin-induced apoptosis. This observation supports an alternative model to the shielding hypothesis described above, which involves the variety of cellular proteins that specifically recognize cisplatin-modified DNA including transcription factors, architectural components, and elements of several repair pathways (13, 14). The genetic damage ensuing from cisplatin exposure may divert these essential proteins away from their natural target sites and prevent them from accomplishing their designated functions. For example, it has recently been demonstrated that both the ribosomal transcription factor, hUBF, and the basal transcription factor, TBP, are titrated away from in vitro transcription activity by cisplatin-modified DNA (63-66). Elevated expression of a protein such as tsHMG, which also binds to cisplatin-DNA adducts, could conceal the lesions and alleviate the lethal consequences, such as transcription inhibition, arising from the titration of critical cellular factors.

It is also feasible that tsHMG influences cellular responses to cisplatin through a mechanism that does not involve direct interaction with platinum-DNA adducts. The function of tsHMG has not yet been established, although the protein modulates DNA packaging and processing in a topoisomerase I-dependent fashion (31). HMG-domain proteins act in a variety fundamental pathways through their ability to

distort DNA conformation and facilitate the assembly of multi-protein complexes (15, 16). Members of this family can functionally interact with nuclear hormone receptors (67, 68), HOX proteins (69), the DNA-dependent protein kinase (70), TBP (71), octamer transcription factors (72), and a variety of other general transcription regulators (73-76). Moreover, several connections have been made between HMG-domain proteins and the programmed cell death pathway, including interactions with the tumor suppressor proteins p53 and Rb (77, 78), stimulation of an apoptotic nuclease activity (79), and modulation of the apoptotic response to cellular stress (80). Thus, flooding the nuclei of HeLa cells with an HMG-domain protein could intensify or diminish one of the intrinsic reactions to the cisplatin damage. It should be noted that these experiments involved ectopic expression of a mouse protein in human cells. The human homologue of the nuclear tsHMG has not yet been detected (81), so it is not clear whether the effects of specific protein-protein interactions fully apply to this system.

The tsHMG protein exerts two different effects on cisplatin-induced apoptosis in HeLa cells. At the early stages of a low but lethal drug dose, tsHMG enhances cell death, but the protein plays the opposite role if the drug exposure time is extended. The importance of drug dose severity on how tsHMG can influence the cellular response may reflect the clinical usage of cisplatin. As with many other chemotherapeutic agents, the effectiveness of cisplatin is undermined both by the development of resistance and by a variety of deleterious side effects (2, 82). The balance required between toxic and antineoplastic activities results in a fairly constrained window of acceptable cisplatin doses (83, 84). Small variations in cell responses within the tumor-specific range, such as that observed in the HeLa cells expressing tsHMG, could have significant consequences in the potency of the anticancer therapy. Beyond the tolerable dose, it is possible that other response

signals become dominant, and cisplatin kills the cells through a different, generally cytotoxic, mechanism.

The results presented here support the notion that an HMG-domain protein can modulate cellular responses to cisplatin, but further experiments are required to resolve the mechanisms underlying the divergent effects. As discussed above, many HMG-domain proteins are expressed in specific tissue types, and several members of this family, including the testis-specific SRY, have been detected in human tumors (85-88). Furthermore, since the process of tumorigenesis frequently involves gene amplification and changes in protein expression (89), particular HMG-domain proteins could be expressed in tumors at levels distinct from the tissue of origin. The experiments presented here and elsewhere indicate that it would be informative to examine the expression levels of various HMG-domain proteins in normal and malignant tissue to determine whether or not there is any correlation with cisplatin sensitivity.

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Table 5.1. Representative examples of cisplatin-induced apoptosis in tsHMG expressing cells. The HeLa cells were induced with doxycyline for 2-3 days prior to drug treatment. Immunofluorescence and TUNEL assays were performed as described in Materials and Methods. Each number is the average of at least 3 data points ± esd.

Exposure[a]	[Cisplatin] (μM)	Dox	% Apoptotic +tsHMG	% Apoptotic -tsHMG	p	% expressing
2 h + 48 h	0	+	1.9 ± 0.79	1.9 ± 0.69		32 ± 2.4
	10	-		10 ± 0.42		0.35 ± 0.26
	5	+	12 ± 1.8	8.9 ± 0.41	<0.05	32 ± 3.0
	10	+	14 ± 2.7	8.3 ± 0.83	<0.05	31 ± 1.9
2 h + 61 h	0	+				37 ± 8.8
	10	-		25 ± 3.3		2.7 ± 0.63
	10	+	22 ± 3.7	18 ± 1.6	<0.2	29 ± 0.55
3 h + 61 h	0	+	2.8 ± 0.42	3.0 ± 0.21		31 ± 1.6
	5	+	23 ± 0.90	23 ± 2.20		32 ± 4.0
	10	+	21 ± 2.06	27 ± 0.25	<0.01	31 ± 2.1
24 h	0	+	0.50 ± 0.47	0.67 ± 0.11		38 ± 2.3
	10	-		33 ± 0.62		3.2 ± 0.42
	10	+	10 ± 2.22	35 ± 4.4	<0.01	36 ± 1.6
37 h	0	+	1.9 ± 0.17	2.7 ± 0.89		32 ± 3.2
	5	-		19 ± 2.5		0.41 ± 0.71
	5	+	6.8 ± 2.07	23 ± 3.7	<0.01	37 ± 2.2
	10	+	27 ± 4.70	62 ± 3.0	<0.001	38 ± 2.8

[a]Exposure indicates the drug treatment conditions. In the first three sets of conditions, the cells were exposed to cisplatin for a short time, washed, and then incubated in fresh media. A single time means that the cells were treated continuously with cisplatin.

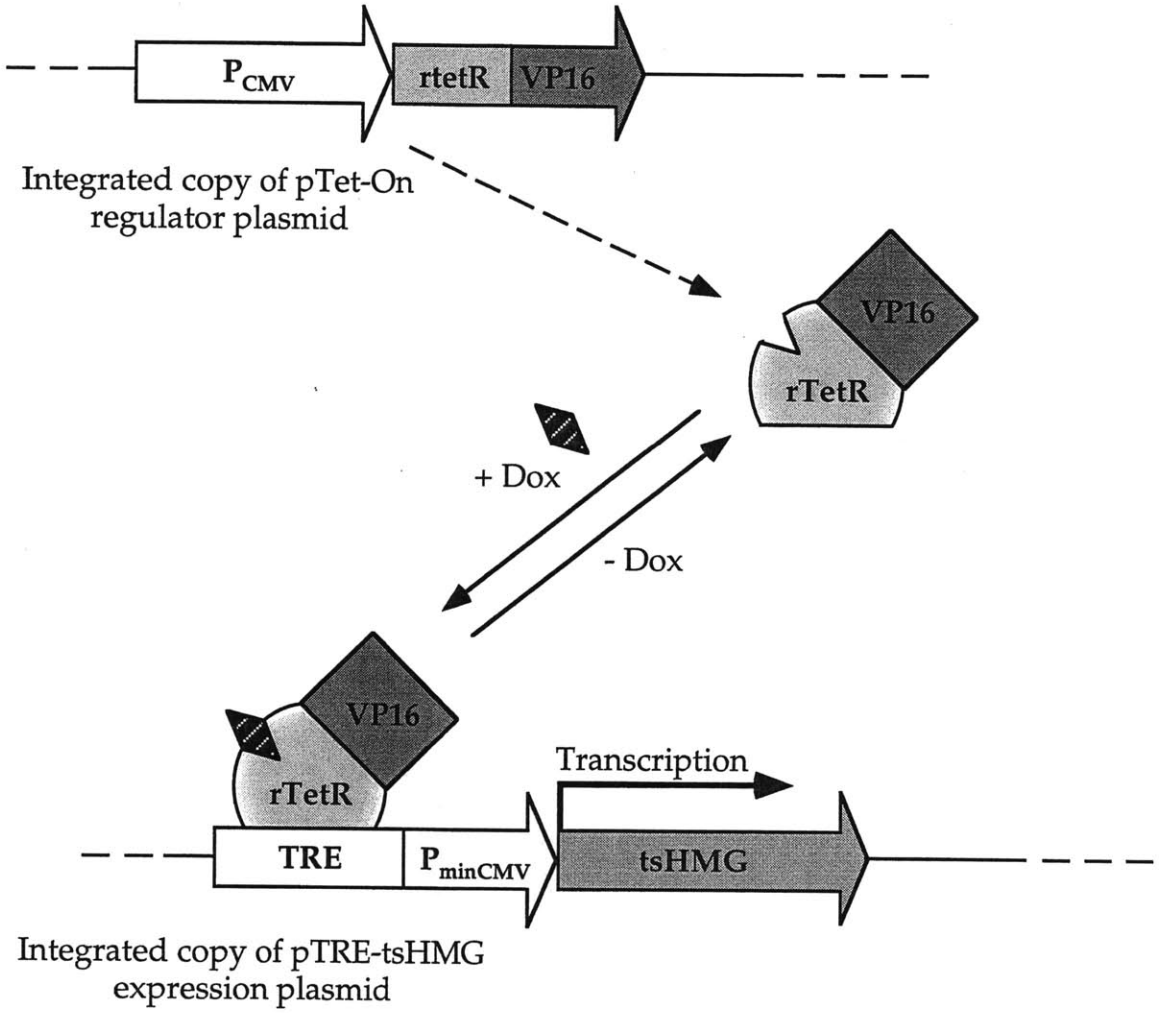


Figure 5.1. Schematic of gene regulation by the Tet-On system. The "reverse" Tet repressor (rTetR), fused to the VP16 acivation domain of herpes simplex virus, binds the tet-responsive element (TRE) in the presence of tetracycline or doxycycline (Dox). The minimal CMV promoter is silent in the absence of TRE activation, so tsHMG is only expressed in the presence of Dox.

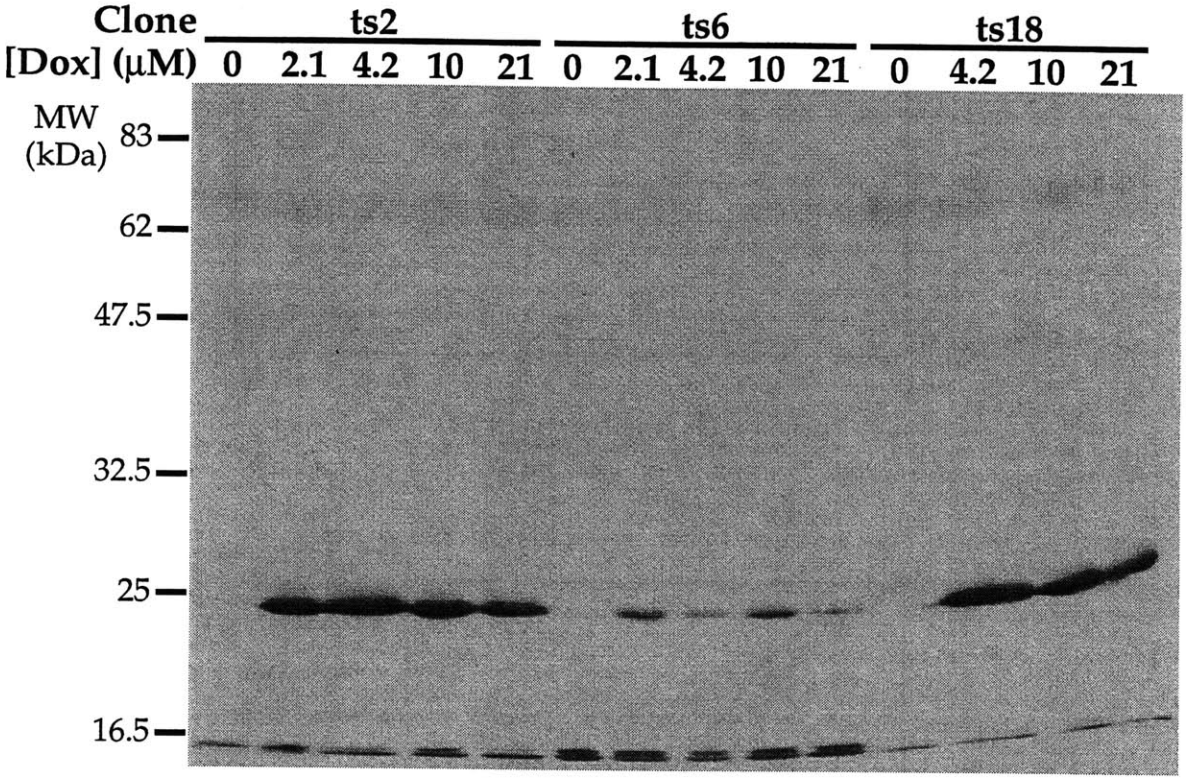


Figure 5.2. Inducible tsHMG expression in HeLa cells. Three different clones of HeLa cells transfected with pTRE-tsHMG were exposed for the indicated concentrations of doxycycline (Dox). After a 24 h induction, cell extracts were prepared, resolved on a 10% SDS-polyacrylamide gel, and transferred to Immobilon-P membrane. Western blot analysis was performed with the mtTFA antiserum as described in Materials and Methods.

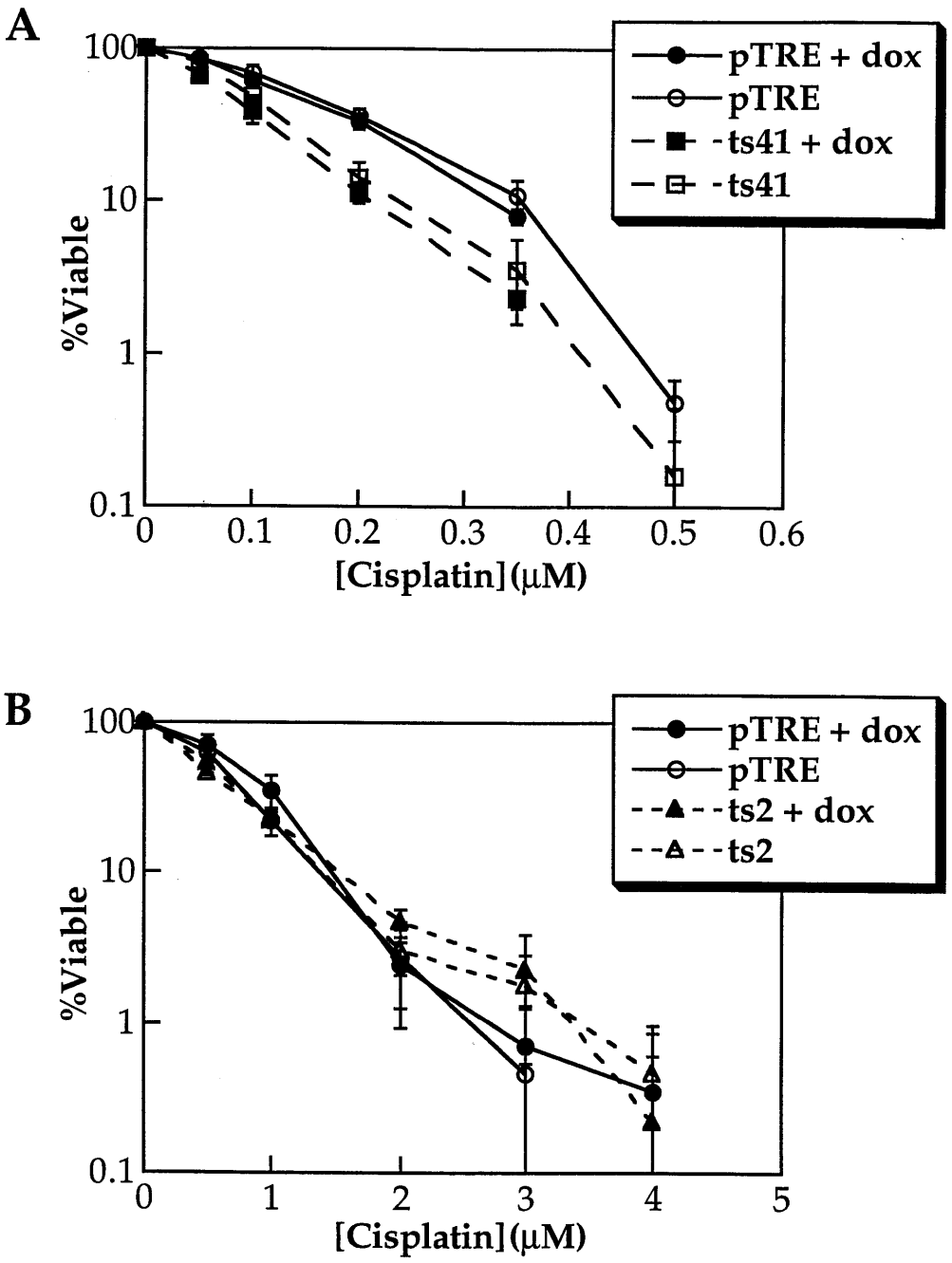


Figure 5.3. Colony formation assays. Two different clones of tsHMG-expressing HeLa cells, ts41 (squares) and ts2 (triangles), or HeLa cells transfected with the control vector, pTRE (circles), were induced with doxycycline (Dox, filled symbols) for 3 days before either (A) continuous or (B) a 2 h exposure to cisplatin. For each cell line (induced or uninduced), the numbers were normalized to the untreated data point. Each experiment was performed in triplicate, error bars represent ± 1 esd.

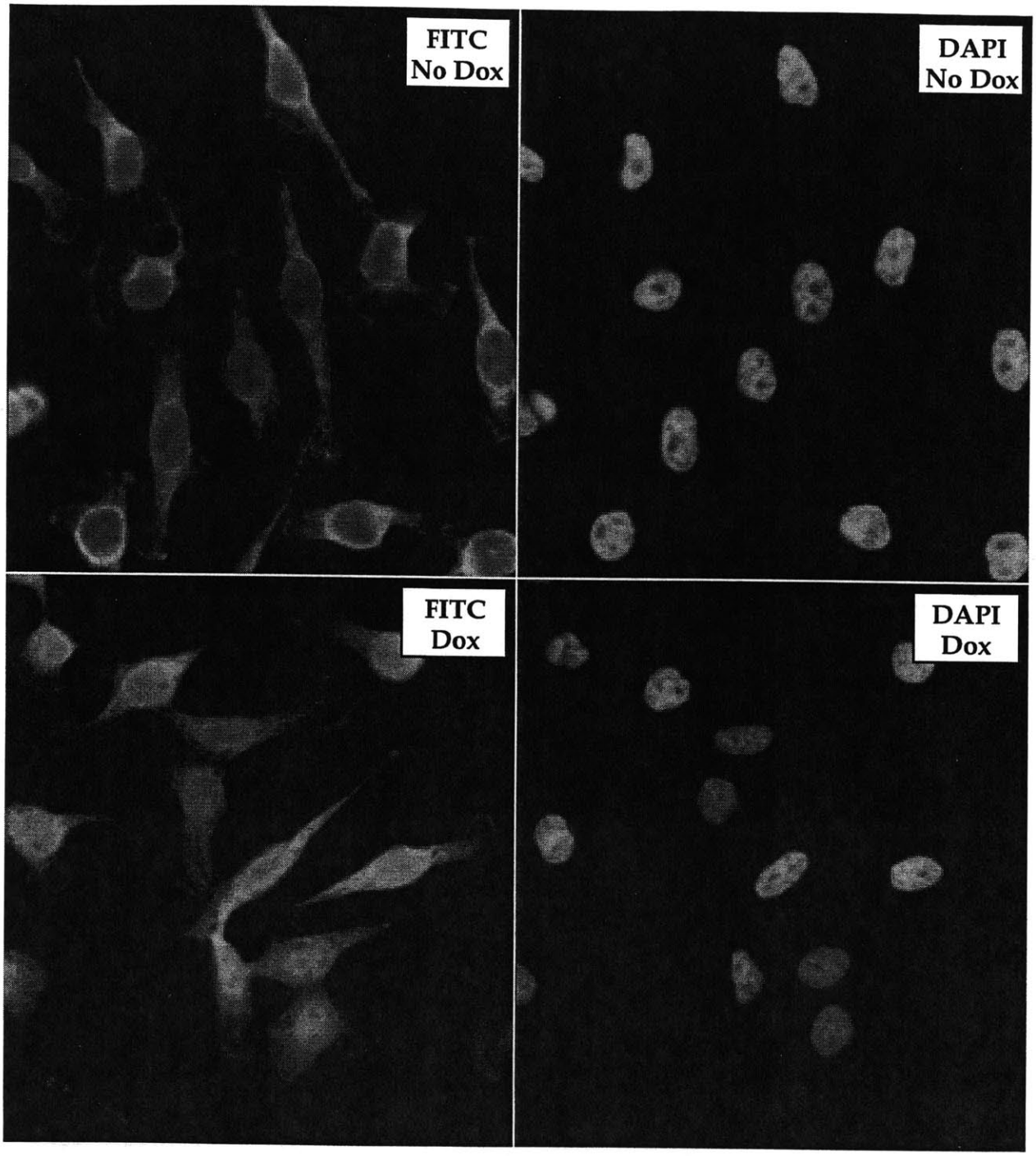


Figure 5.4. Immunofluorescence of HeLa cells transfected with the control pTRE vector. Transfected cells were either untreated (top) or exposed to doxycycline (Dox) for 2 days (bottom) while growing on coverslips. Immunofluorescence (left) was performed as described in Materials and Methods, except the primary antibody was used at a 50-fold dilution, and the secondary antibody was a fluorescein isothiocyanate (FITC) conjugate. The cells were also stained with DAPI (right).

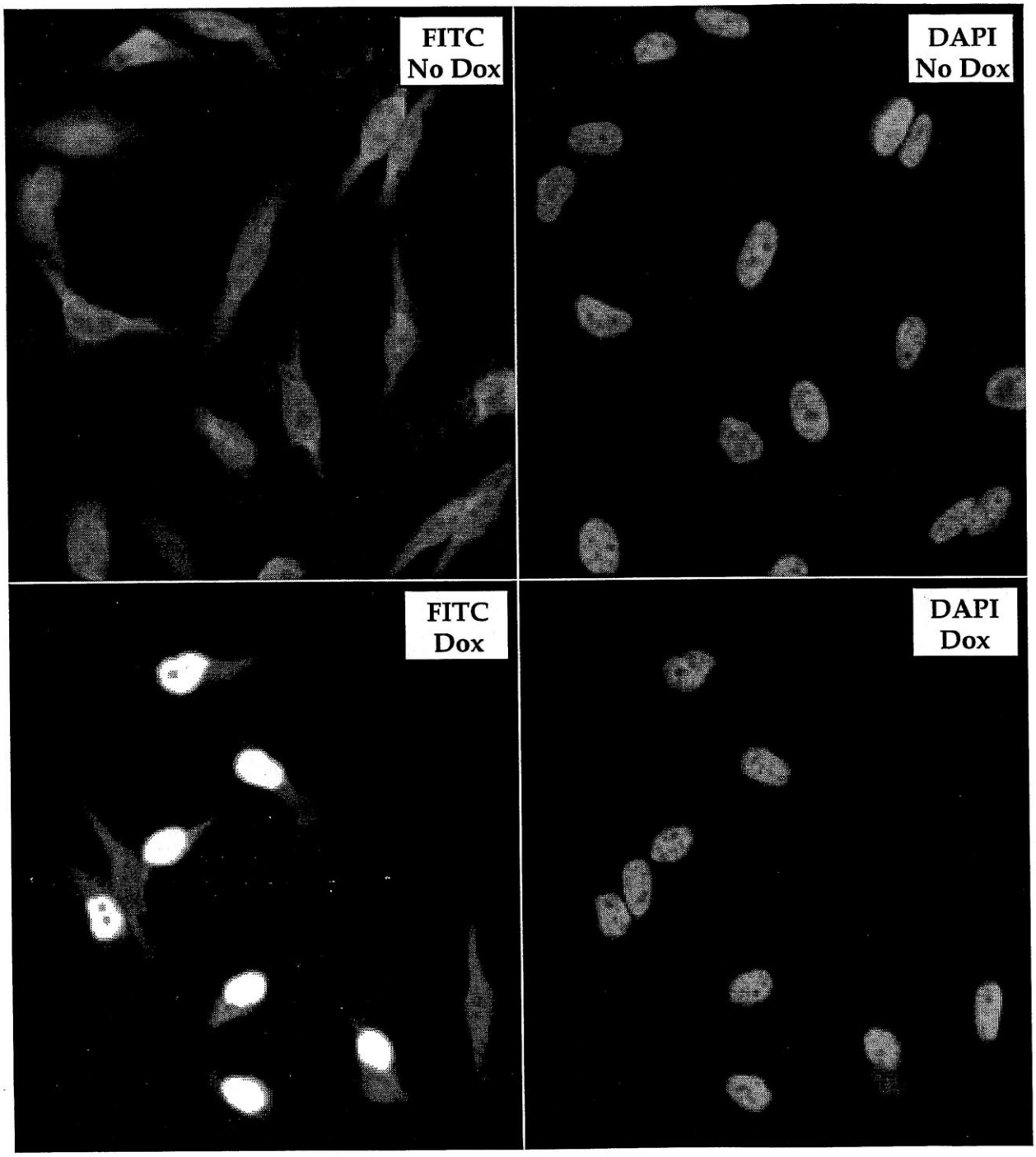


Figure 5.5. Immunofluorescence of HeLa cells transfected with the pTRE-tsHMG vector. Transfected cells were either untreated (top) or exposed to doxycycline (Dox) for 2 days (bottom) while growing on coverslips. Immunofluorescence (left) was performed as described in Materials and Methods, except the primary antibody was used at a 50-fold dilution, and the secondary antibody was a fluorescein isothiocyanate (FITC) conjugate. The cells were also stained with DAPI (right).

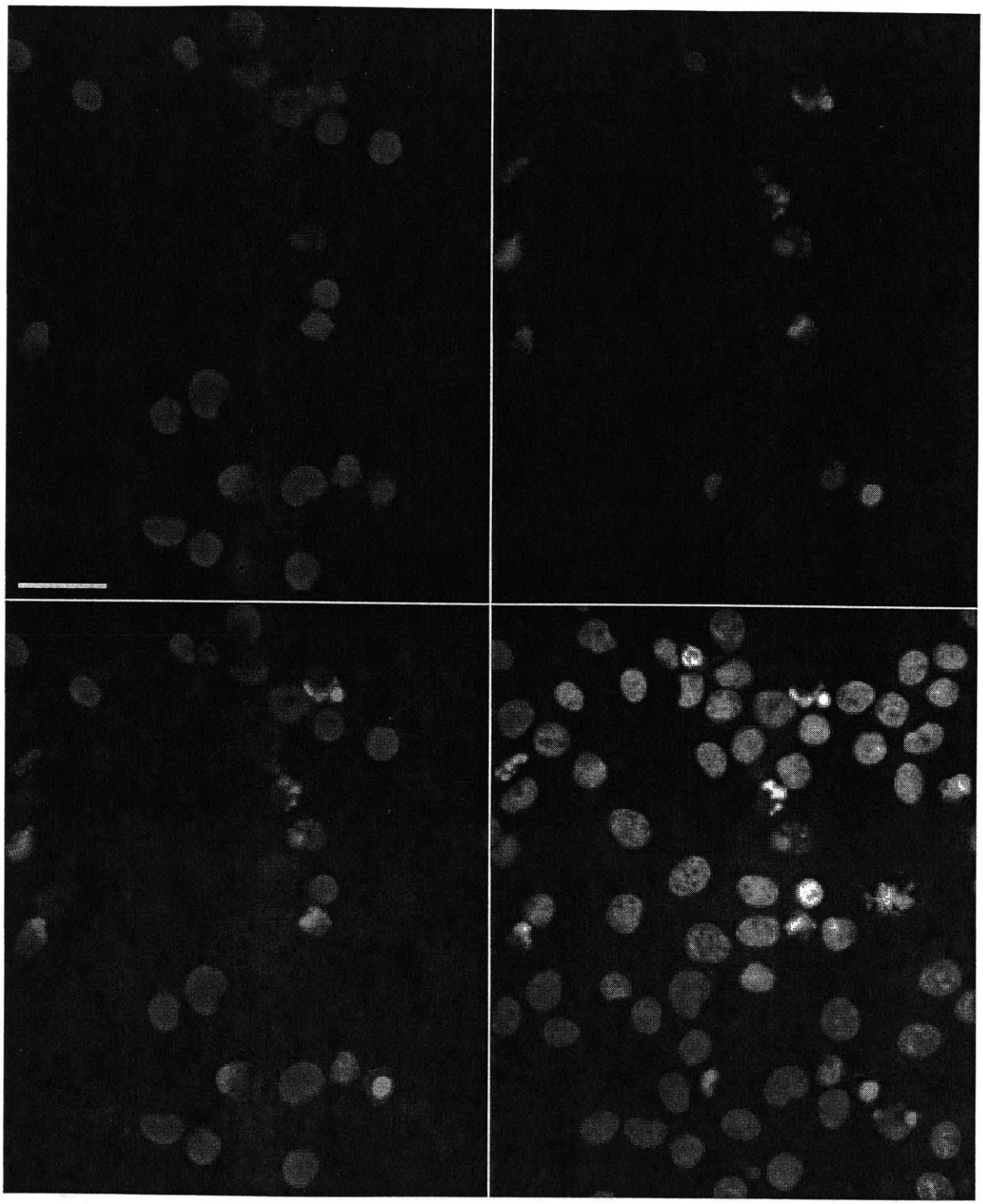


Figure 5.6. The tsHMG protein sensitizes HeLa cells to cisplatin-induced apoptosis. HeLa cells containing the tsHMG gene were induced with doxycycline for 48 h, plated onto 6-well dishes, and induced for an additional 24 h. The cells were then exposed to 10 μM cisplatin for 2 h, washed, and incubated for 48 h. The immunofluorescence (top left), TUNEL (top right), and DAPI staining (bottom right), were performed as described in Materials and Methods. The bottom left panel is an overlay of the rhodamine (immunofluorescence) and fluorescein (TUNEL) pictures. The bar represents 50 μm.

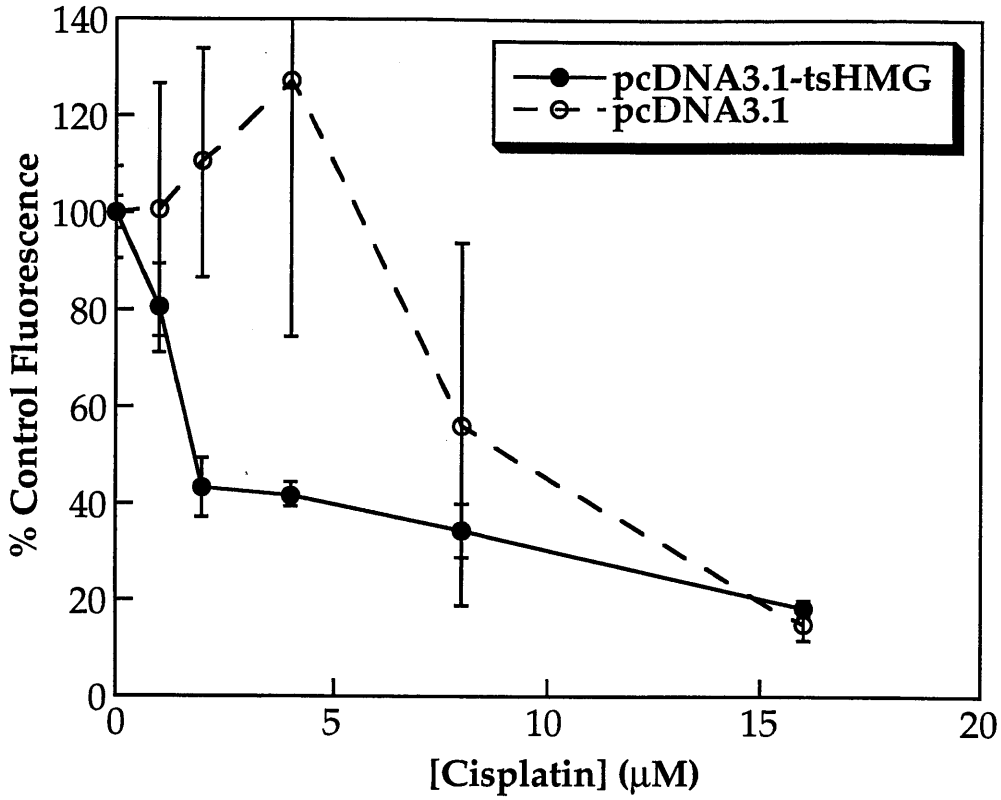


Figure 5.7. Effect of tsHMG on cisplatin inhibition of EGFP expression in HeLa. The cells were transiently transfected with 6 μg pcDNA3.1-tsHMG or pcDNA 3.1. Following a 13.5 h induction with doxycycline and concurrent exposure to the indicated concentrations of cisplatin, the cells were lysed and the EGFP fluorescence was determined and normalized to total protein content. The experiment was performed in triplicate, error bars represent ± 1 esd.

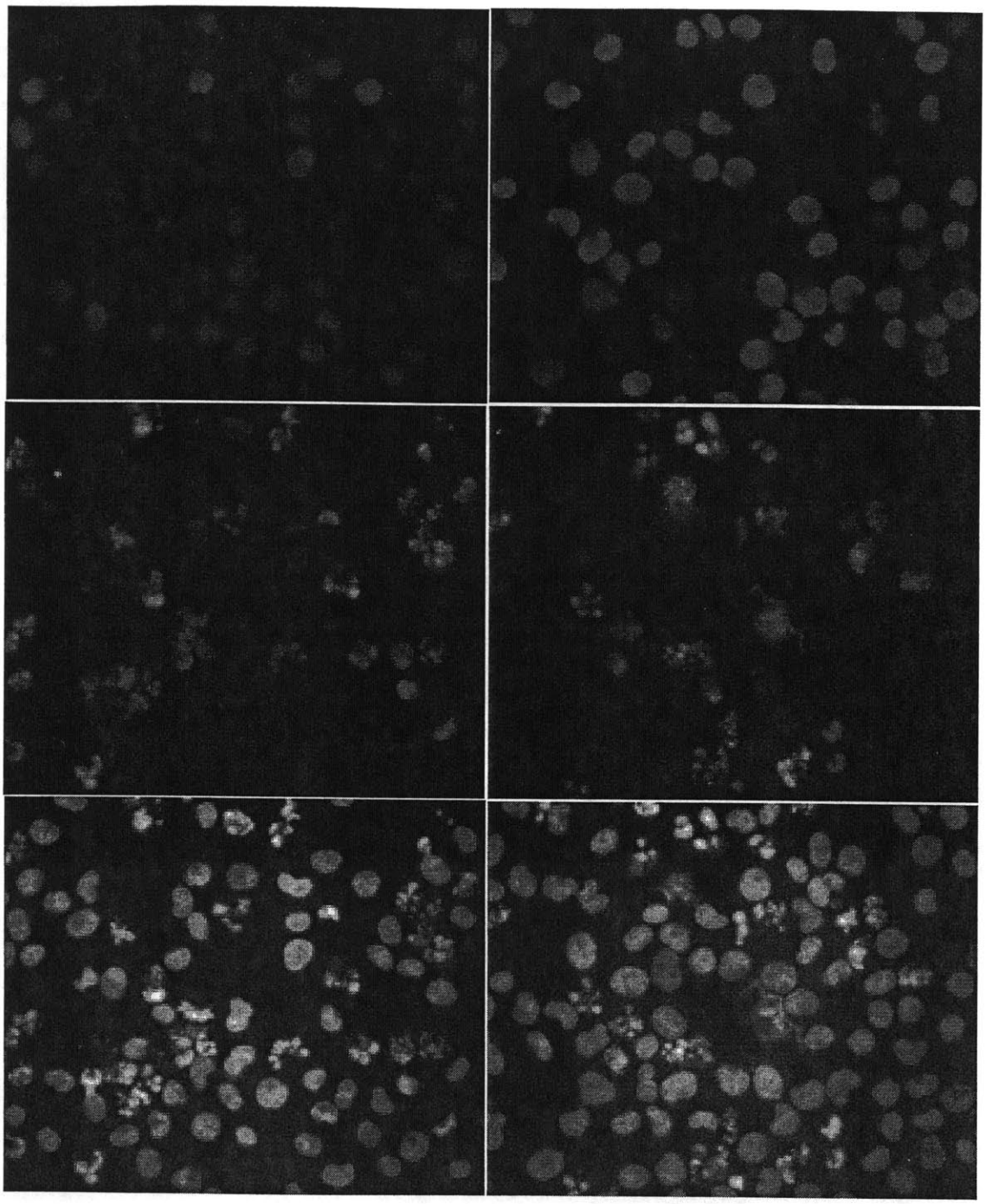


Figure 5.8. The tsHMG protein protects HeLa cells from cisplatin-induced apoptosis. HeLa cells containing the tsHMG gene were either uninduced (left) or induced with doxycycline (right) for 48 h prior to treatment with 10 μM cisplatin for 24 h. The immunofluorescence (top) was performed with a rhodamine-conjugated secondary antibody, TUNEL (middle) was performed with fluorescein labeled dUTP, and the cells were also stained with DAPI (bottom), as described in Materials and Methods.

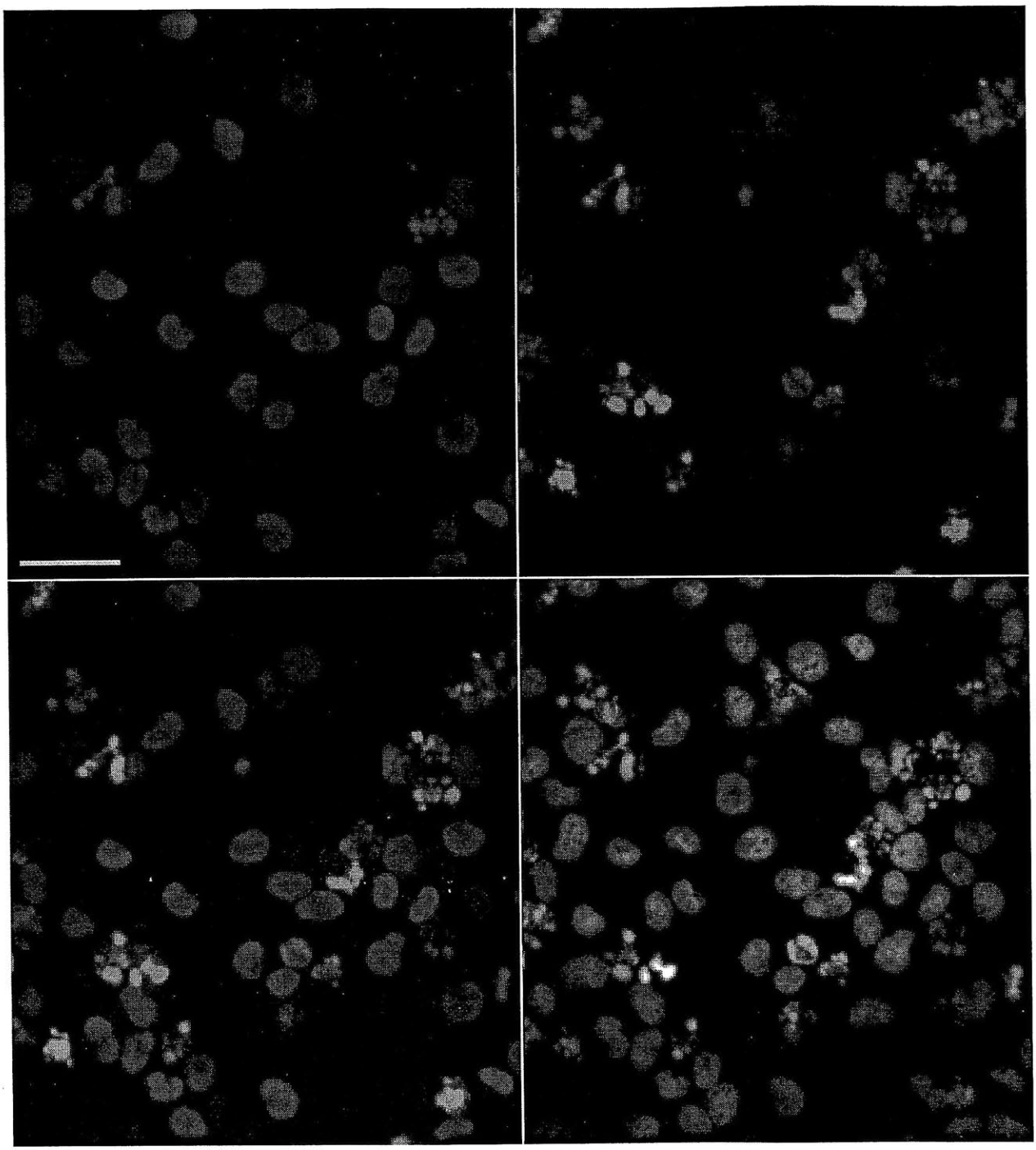


Figure 5.9. The tsHMG protein protects HeLa cells from cisplatin-induced apoptosis. HeLa cells containing the tsHMG gene were induced with doxycycline for 48 h prior to a 24 h exposure 10 μM cisplatin. The immunofluorescence(top left), TUNEL (top right), and DAPI staining (bottom right), were performed as described in Materials and Methods. The bottom left panel is an overlay of the rhodamine (immunofluorescence) and fluorescein (TUNEL) pictures. The bar represents 50 μm.

Biographical Note

The author was born Deborah Beth Zamble on October 5, 1971, to Edward and Marcia Zamble, in Kingston, Ontario, where she grew up with one sibling, Rebecca. She graduated from Kingston Collegiate and Vocational Institute in 1989 with an Ontario Secondary School Diploma and a Certificate of French Studies. She then moved to University College, at the University of Toronto, where she received a 1993 B.Sc. degree in Chemistry and Biochemistry, with a minor in Philosophy. For one summer during this time, she performed research in the laboratory of Prof. I. A. Manners, investigating the synthesis and characterization of small molecules containing silicon and ferrocene. Her 4th year thesis, supervised by Prof. B. Sarkar, at the Hospital for Sick Children, involved an investigation of protein-protein interactions between a family of zinc-finger DNA-binding proteins. In September 1993, the author began graduate studies in biological chemistry at M. I. T. As a Howard Hughes Predoctoral Fellow she joined the laboratory of Prof. S. J. Lippard where she studied the biological mechanism of action of the anticancer drug cisplatin. In addition to independent research, she mentored the laboratory studies of an undergraduate student, Christina Eng. Following graduation, she plans to do postdoctoral studies with Prof. C. T. Walsh at Harvard Medical School.