THE ROLE OF DAMAGED DNA RECOGNITION PROTEINS IN THE GENOTOXICITIES OF ULTRAVIOLET LIGHT AND THE ANTICANCER DRUG CISPLATIN

by

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B.A., Middlebury College, 1987

SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
September 1993

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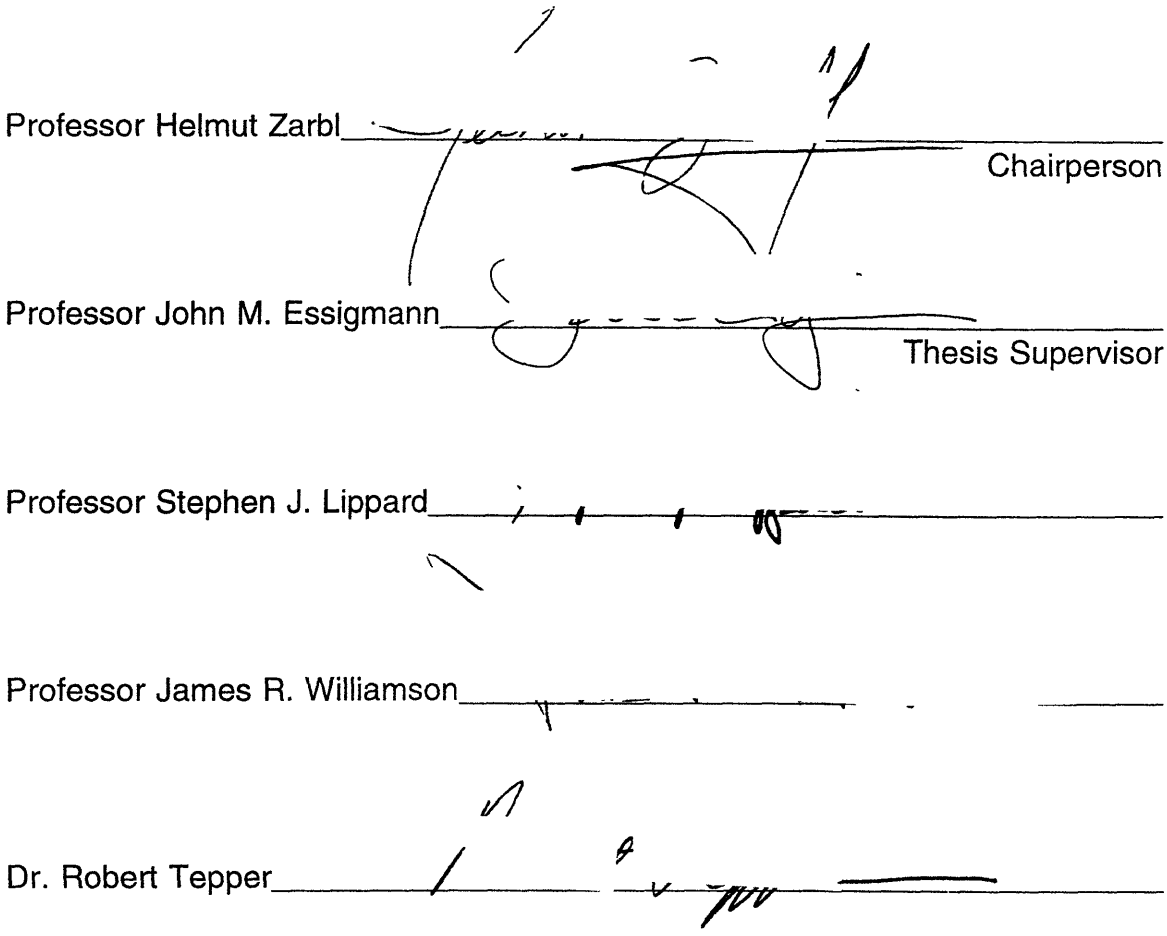

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ABSTRACT

DNA damaging agents can be mutagenic, carcinogenic, and effective as anticancer drugs. The cellular processes that contribute to these varied effects are poorly understood, but it is reasonable to speculate that an early event involves the recognition of DNA damage by cellular proteins. The results presented in this dissertation suggest that damage recognition proteins can contribute to cell survival and, interestingly, to cell death. A human ultraviolet light-damaged DNA recognition protein (UV-DRP) is absent in some xeroderma pigmentosum complementation group E cells suggesting a role in DNA repair. The binding specificity of the UV-DRP has been characterized as a first step toward elucidating a possible role in DNA repair. It is shown that the UV-DRP binds selectively to a minor UV-induced lesion, the pyrimidine (6-4) pyrimidone photoproduct (6-4 photoproduct). The major UV-induced photoproducts, cyclobutane pyrimidine dimers (CPDs), are not recognized by the UV-DRP. These findings suggest mechanisms to explain the preferential repair of 6-4 photoproducts in human cells. In other studies, it was demonstrated that the nucleolar transcription factor human upstream binding factor (hUBF), a critical regulator of rRNA synthesis, binds with striking affinity ($K_{d(app)} \sim 60$ pM) to DNA modified by the anticancer drug cisplatin. This value is comparable to that of hUBF for the rRNA promoter ($K_{d(app)} \sim 18$ pM). Furthermore, I observe that the hUBF-promoter interaction is cooperative and, consequently, highly sensitive to the antagonistic effects of cisplatin adducts. These results suggest that cisplatin could disrupt regulation of rRNA synthesis by a transcription factor hijacking mechanism that may contribute to the anticancer efficacy of this drug.

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ACKNOWLEDGEMENTS

Throughout the course of my studies in the Essigmann lab I've been lucky to have worked with many bright, generous, thoughtful people that made the entire process of obtaining this degree much less painful. My advisor, John Essigmann, always listened to my ideas, supported my research decisions, and showed confidence in me. He also understood what type of a person I was and didn't complain (too much) when I skipped out to play golf or to watch a baseball game. Most of all, I thank John for creating an environment that was pleasant - rarely did I dread coming to work. Our close collaborator, Steve Lippard, has also been a valuable source of ideas and encouragement. Steve has helped me to realize the importance of pursuing fields and techniques that are unfamiliar and often quite intimidating! I'd also like to thank my thesis committee, Helmut Zarbl, Jamie Williamson, and Bob Tepper, for being helpful and for letting me defend my thesis on short notice. I also acknowledge those who trained me when I was a new student. Lisa Naser Bradley and Mike Wood spent a lot of time and effort getting me up to speed in the lab and they showed great patience, and others, such as Susan Verghis, Ashis Basu and Ishrat Chaudhuri were always willing to help me out (and to point out when I'd committed a blunder). My less formal training in the lab came from arguing about science (and a host of other topics) with the likes of Brian Donahue and Zhenghuan Chen. These guys should be on a professional debating team. Mr. Chen was also a great coworker and I thank him for all of his technical and intellectual assistance. I also worked closely with Xiaoquan Zhai. X.Q. was the best coworker I could imagine - always enthusiastic to learn, to teach, to discuss ideas, and to unwind at the Muddy or over some Chinese food. I also acknowledge Pieter Pil and Mike Jantzen for sharing their hard earned reagents and Marjie Solomon for proofreading the dissertation. Other members of the platinum group, including Elizabeth Trimmer, Jill Mello, Tim Connors, Kevin Yarema, and Wendy Heiger-Bernays, were also valuable resources and a pleasure to work with. I thank KD&L for helping me with ordering and manuscript/thesis preparation and also my two UROP students, Greg Lubinieki and Priscilla Songsanand, for doing high quality work - I wish you guys stayed longer! Being surrounded by a diverse group of people that enjoys doing just about anything was the best part of graduate school. Each of the following people is a true friend that I will never forget: BAD, Susi, SOH&Regine, Kim&Mike, JAM, Woody&Marcia, Marjie, Tim, Kevin, Jill&Al, Z, and XQ. I'm sure the "youngsters" (also known as the Fantastic Four) will continue this fine tradition. I especially acknowledge my family and my girlfriend, Lisa. My parents, Tom and Betsy, and my sister, Sarah, have

supported everything I've ever done. This was not easy. There were times when I had nearly given up and they encouraged me to continue and to look on the bright side. I can never thank them enough. To acknowledge their lifelong support, I dedicate this dissertation to my family. Lisa is a coworker in the lab as well as my best friend and girlfriend. She has supported me in every possible way - always finding the energy to encourage me, even when her own studies were seemingly at an impasse. I thank her for her love, sacrifice, and devotion throughout our time together. I only hope I can properly repay her when she reaches this stage of her career.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AP	apurinic/apyrimidinic
bp	base pair
CPD	cyclobutane pyrimidine dimer
CMV	cytomegalovirus
DDP	diamminedichloroplatinum(II)
DRP	damage recognition protein
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EBV	Epstein-Barr virus
Endo V	T4 endonuclease V
FCS	fetal calf serum
hUBF	human upstream binding factor
kDa	kilodaltons
MEM	minimal essential media
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
r_b	bound drug/nucleotide ratio
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet
XP	xeroderma pigmentosum

I. INTRODUCTION

Several studies have revealed the existence of proteins in eukaryotic cells that bind specifically to DNA damaged by chemical or physical agents. DNA damage recognition proteins (DRPs) have been reported for a growing list of DNA modifications, including platinum crosslinks (Donahue *et al.*,1990; Pil and Lippard, 1992; Brown et al.1993), 1,N^6-ethenoadenine (Rydberg *et al.*,1991), G:T mismatches (Jiricny *et al.*,1988), apurinic/apyrimidinic (AP) sites (Lenz *et al.*,1990), *N*-acetyl-2-aminofluorene-guanine adducts (Moranelli and Lieberman, 1980), and ultraviolet light (UV)-induced photoproducts (Feldberg and Grossman, 1976; Chu and Chang, 1988; Hirschfeld *et al.*,1990). The cellular function of many DRPs is unknown, but it is reasonable to speculate that some of these proteins may play roles in DNA repair (Chu and Chang, 1988; Hirschfeld *et al.*,1990; Rydberg *et al.*,1991), or in other biological activities related to the genotoxicity of the agent under investigation (Donahue *et al.*,1990; Pil and Lippard, 1992; Brown *et al.*,1993).

The goal of my thesis research was to elucidate the role of DRPs in the genotoxicities of UV light and the anticancer drug cisplatin. A body of indirect evidence suggests that a UV-damaged DNA recognition protein (UV-DRP) may be a component of a versatile mammalian excision repair complex. The UV-DRP is overly expressed in human cells that display resistance to the anticancer drug cisplatin; these cells also exhibit an enhanced capacity to reactivate cisplatin-damaged plasmids (Chu and Chang, 1990). Furthermore, the UV-DRP is induced 2-4 fold above constitutive levels in primate cells pretreated with UV (Protic *et al.*,1989). These cells also reactivate UV-damaged plasmids with increased efficiency. The most compelling result linking the UV-DRP to DNA repair is the apparent absence of this activity in cells from two related persons afflicted with the human DNA repair disorder

xeroderma pigmentosum (XP) (Chu and Chang, 1988). More specifically, these individuals belonged to genetic complementation group E. The generality of this finding has recently been challenged because UV-DRP activity is detected in a number of XP-E cell lines derived from Japanese patients (Kataoka and Fujiwara, 1991; Keeney *et al.*,1992). It is possible, however, that these recently tested cell lines contain a form of the UV-DRP that is defective in a function other than DNA binding.

In my work the substrate specificity of the UV-DRP was examined as an initial step in elucidating a possible role for it in DNA repair. In the case of the UV-DRP, the true physiological substrate was not known before my study because UV induces many forms of DNA damage, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 photoproducts). Each of these photoproducts causes distortions in DNA architecture (Husain *et al.*,1988; Franklin *et al.*,1985; Rycyna and Alderfer, 1985; Kemmink *et al.*,1987a) and is believed to be both cytotoxic and mutagenic (LeClerc *et al.*,1991; Protic-Sabljic *et al.*,1986; Brash *et al.*,1987a; Glickman *et al.*,1986; Brash and Haseltine, 1982). The relative repair rates of these lesions in mammalian cells (Mitchell *et al.*,1985; Mitchell *et al.*,1990; Thomas *et al.*,1989) and cellular extracts (Wood, 1989) have been shown to be biased by a factor of 4-12 in favor of repair of the 6-4 photoproducts. The results of my studies show that the UV-DRP binds selectively to 6-4 photoproducts and may thereby contribute to the efficient repair of these lesions.

The second major topic of study described in this dissertation is an evaluation of the mode of toxicity of the anticancer drug cisplatin. Cisplatin is a highly effective antitumor drug used to treat a variety of cancers. The

mechanism of action of this drug is unclear, but it is likely that DNA is the critical intracellular target (Fraval *et al.*,1978). It is of importance to understand the mechanism of action of cisplatin in order to design rationally chemotherapeutic drugs of increased potency. We and others have reasoned that the recognition of cisplatin DNA adducts by cellular proteins may be an early event that modulates the genotoxicity of this drug. Several cisplatin damaged DNA recognition proteins (*cis*-DDP DRPs) have been discovered recently (Donahue *et al.*,1990; Pil and Lippard, 1992; Bruhn *et al.*,1992; Brown *et al.*,1993; Clugston *et al.*,1992), but their role in the genotoxicity of cisplatin remains elusive. Our studies have shown that a family of proteins homologous to the non-histone chromosomal protein HMG1 binds selectively to DNA modified by cisplatin (Bruhn *et al.*,1992; Pil and Lippard, 1992; Brown *et al.*,1993), and there is no evidence to suggest that these proteins are part of a DNA repair complex. This observation has led us to propose novel, non-mutually exclusive, mechanisms that contribute to the efficacy of cisplatin. One model suggests that *cis*-DDP DRPs fortuitously recognize the DNA structure induced by cisplatin lesions and thereby shield adducts from DNA repair enzymes (Donahue *et al.*,1990). As a consequence, adducts persist in the genome to mediate a genotoxic effect. Recent genetic studies in yeast are consistent with this model and established for the first time that an HMG1-related protein can modulate the toxicity of cisplatin (Brown *et al.*,1993). A second model proposes that *cis*-DDP DRPs function normally as critical regulatory proteins, such as transcription factors. In this model, cisplatin adducts act as molecular decoys to titrate a regulatory protein away from its natural site of action. Presumably, such a process would disrupt cellular homeostasis and contribute to genotoxicity. I report in this dissertation that the nucleolar transcription protein human upstream binding factor (hUBF), a critical

regulator of ribosomal RNA (rRNA) production (Bell *et al.*,1988), binds to cisplatin adducts and rRNA promoter sequences with similar affinities. These data are consistent with the possibility that cisplatin adducts inhibit the function of hUBF by acting as molecular decoys. This mechanism, if operative, could contribute to antitumor efficacy.

II. LITERATURE SURVEY

A. DNA LESIONS FORMED BY ULTRAVIOLET LIGHT AND THE ANTICANCER DRUG CISPLATIN

1. Ultraviolet light (UV)-induced DNA photoproducts

Irradiation of DNA with 254 nm light results in the formation of dimeric photoproducts occuring between adjacent pyrimidines (Py) in the same DNA strand (Figure 1). The most common photoproduct is the *cis-syn* cyclobutane pyrimidine dimer (CPD) in which the 5,6 double bonds have been saturated to form a cyclobutyl ring. CPDs form at all dipyrimidine sequences, but the efficiency of dimer formation at any particular dipyrimidine sequence is largely influenced by the flanking sequence context. In general, dimer formation is favored in runs of pyrimidines with the favored site of formation of CPDs at adjacent thymines (Brash and Haseltine, 1982). The next most common photoproduct is the pyrimidine (6-4) pyrimidone photoproduct (6-4 photoproduct), which occurs at roughly one-third the frequency of CPDs (Friedberg, 1985). The primary site of 6-4 photoproduct formation is at Py-cytosine (PyC) and CC sequences positioned 3′ to runs of pyrimidines. 6-4 photoproducts form rarely at other dipyrimidine sites, including TT sequences (Lippke *et al.*,1981). The structure of a TC 6-4 photoproduct has been determined by nuclear magnetic resonance (NMR) (Franklin *et al.*,1985). As shown in Figure 1, the 6-4 photoproduct has an unusual structure in which the 6 position of T is convalently linked to what was the 4 position of the 3′ neighboring C. The 5,6 double bond of T, but not C, is saturated, and the exocyclic amino group from the 4 position of C has been transferred to the 5 position of T.

CPDs are believed to cause distortions in DNA architecture. The

construction of oligonucleotides containing site-specific *cis-syn* TT CPDs has facilitated structural studies. NMR studies with short duplexes containing a centrally located *cis-syn* CPD indicate that, although weakened, base pairing between dimeric Ts and complementary A's is intact (Taylor *et al.*,1990; Kemmink *et al.*,1987b). Weakened base pairing is evidenced by a change in the helix - coil equilibrium. The ability of B-form DNA to accomodate *cis-syn* CPDs may reflect the observation that the Ts of a CPD retain the anti glycosyl conformation. Indeed, the rare trans-syn CPD, in which the 5' T is in a syn glycosyl conformation, is more helix destabilizing (Taylor *et al.*,1990). Model building and energy minimization studies suggest that the *cis-syn* CPD bends the helix by 27° toward the major groove and also causes a local helix unwinding of 20° (Pearlman *et al.*,1985). This prediction was supported by ligation experiments with oligonucleotides containing site-specific CPDs (Husain *et al.*,1988). In these experiments, 32-mer oliogonucleotides containing a site-specific CPD were multimerized by using DNA ligase, and the formation of DNA circles was monitored. The bending and unwinding angles were calculated by determining the multimer length that was optimal for circle formation. In a more recent study, however, the bending angle induced by a site-specific CPD was found to be much less severe, ~7° (Wang and Taylor, 1991). In these later studies the bend angle was measured by multimerizing CPD-containing duplexes and monitoring the electrophoretic mobility of the ligation products as a function of length. In this approach, the phased CPDs yield DNA bends that are additive, and the bend angle is calculated by comparing the electrophoretic mobility of the multimers containing CPDs to the mobility of control, unmodified multimers. It thus appears that CPDs do induce DNA bending, but the bend angle is still a matter of dispute. (Husain *et al.*,1988; Franklin *et al.*,1985; Rycyna and Alderfer, 1985; Kemmink *et al.*,1987a)

The degree of helix distortion induced by 6-4 photoproducts is unclear because oligonucleotides containing these lesions were synthesized only recently (Smith and Taylor, 1993). The NMR structure of the TC 6-4 photoproduct suggests that these lesions are likely to induce significant helix distortions as evidenced by the observation that the pyrimidine and pyrimidone rings occupy perpendicular planes. Furthermore, unlike the case with CPDs, a moiety important for base pairing (the exocyclic amino group of C) is disrupted in 6-4 photoproducts (Franklin *et al.*,1985).

2. Cisplatin-DNA adducts

cis-Diamminedichloroplatinum(II) (cis-DDP or cisplatin) reacts with DNA to form a variety of DNA adducts (Figure 2). In aqueous solution the labile chloride ligands of cisplatin are displaced by water molecules, and this process occurs efficiently only if the ambient chloride concentration is low. The aquated platinum species reacts with the N7 position of purines to form DNA adducts. The chemistry of cisplatin-DNA interactions has been reviewed extensively (Sherman and Lippard, 1987). The spectrum of DNA adducts formed by cisplatin, and their relative abundance, have been determined through the use of chromatographic and NMR spectroscopic procedures (Fichtinger-Schepman *et al.*,1985). The most abundant DNA adduct formed by cisplatin (~65% of the total adducts) is a 1,2 intrastrand crosslink formed at adjacent guanine residues (G^G). Intrastrand crosslinks at AG sequences are also abundant (~25%), and 1,3 intrastrand crosslinks at GNG sequences represent only ~5% of the total adducts formed (N = any nucleotide). Intrastrand crosslinks are also noted but constitute only ~1% of the adduct population. The geometric isomer of cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP) cannot, for steric reasons, form the

1,2 intrastrand crosslinks that constitute the majority of the cisplatin adducts. The GNG adduct is the most predominant lesion formed by *trans*-DDP. Interestingly, *trans*-DDP is much less toxic than cisplatin and is not an effective anticancer drug. This observation has lead to the hypothesis that the 1,2 intrastrand crosslinks at GG and AG sites are responsible for the therapeutic activity of cisplatin (Pinto and Lippard, 1985).

It is clear that cisplatin DNA adducts are helix distorting lesions. The X-ray crystal structure of a dinucleotide GG sequence containing a cisplatin crosslink reveals that the planes of the bases are completely destacked to accomodate the square planar platinum coordination complex; the angle between the planes of the bases is 76-87° (Sherman *et al.*,1985). Although the structure of duplex DNA containing a cisplatin adduct has not been solved, electrophoresis studies with site-specifically modified DNA fragments have revealed important features of the induced distortion (Bellon *et al.*,1991; Bellon and Lippard, 1990). Cisplatin G^G and A^G adducts bend and unwind the helix by 34° and 13°, respectively. The GNG adduct bends the helix to a similar extent, but the unwinding angle is larger (23°). The 1,3 *trans*-DDP adduct at a GNG sequence also bends the DNA, but the bend appears to be flexible making it impossible to measure the angle by the electrophoresis mobility approach. Although these structures appear to be quite similar, it is noteworthy that the subtle differences in DNA bending and unwinding may be of consequence to the processing of these lesions by DNA repair enzymes and to their recognition by HMG box proteins (*vide infra*).

B. CYTOTOXICITY AND MUTAGENICITY OF UV PHOTOPRODUCTS AND CISPLATIN ADDUCTS

1. Cytotoxicity and mutagenicity of UV photoproducts

CPDs and 6-4 photoproducts are believed to be cytotoxic and mutagenic in E. coli and mammalian cells (LeClerc *et al.*,1991; Protic-Sabljic *et al.*,1986; Brash *et al.*,1987a; Glickman *et al.*,1986; Brash and Haseltine, 1982), although the relative potency of these photoproducts as premutagenic lesions is a matter of debate. The major UV-induced mutation is a C to T transition occuring at CC and TC sites, and mutations at TT sequences are rare. In E. coli, mutational hotspots occur at photoproduct formation hotspots, and the frequency of mutations at particular sites correlates better to the frequency of 6-4 photoproducts than to the frequency of CPDs (Brash and Haseltine, 1982). Irradiated phage lamda DNA rendered devoid of CPDs by photoreactivation induce the same frequency of mutations as the non-photoreactivated control DNA. Photoreactivation did, however, increase the viability of the phage DNA (Wood, 1985). These results have been emphasized by those proposing that 6-4 photoproducts are the primary premutagenic lesions induced by UV. The studies also suggest that both types of photoproducts can reduce viability.

Many workers believe that CPDs are the primary premutagenic lesions in E. coli and mammalian cells, in spite of the evidence cited above. UV irradiated shuttle vectors that are passaged through mammalian cells have provided insight into the nature of the major premutagenic UV photoproduct. In monkey cells it was determined that photoreactivated shuttle vectors

induced 80% fewer mutations than the analagous non-photoreactivated plasmid (Protic-Sabljic *et al.*,1986). In agreement with these studies, photoreactivation reduced the mutation frequency of irradiated shuttle vectors by 90% in human cells (Brash *et al.*,1987b). Interestingly, in the human cell study it was shown that mutation hotspots did not correlate with the photoproduct formation hotspots. This contrasts with the results reported in E. coli discussed above and may suggest differences in mechanisms of lesion bypass in the two species. Recently, Taylor has argued that deamination and tautomer bypass of TC and CC CPDs lead to the major UV-induced mutations, C to T transitions (Jiang and Taylor, 1993). The evidence provided in support of these mechanisms was that TU pyrimidine dimers code as TT, and if U were derived from the deamination of cytosine, the result would be a T to C mutation. Although these results are consistent with the proposed mechanism, it is clear that additional studies are required to provide more conclusive evidence.

2. Cytotoxicity and mutagenicity of cisplatin DNA adducts

The critical intracellular target of cisplatin is generally accepted to be DNA. An early and profound effect of cisplatin treatment is the inhibition of DNA synthesis (Harder and Rosenberg, 1970). RNA and protein syntheses are also inhibited, but DNA synthesis is preferentially reduced. Cisplatin adducts inhibit the replication and transcription of DNA templates in a variety of prokaryotic and eukaryotic in vitro systems (Corda *et al.*,1991; Heiger-Bernays *et al.*,1990; Pinto and Lippard, 1985). Although these observations suggested the importance of DNA as a critical target for cisplatin, the most convincing evidence supporting this hypothesis is that DNA repair deficient cell lines are hypersensitve to cisplatin (Fraval *et*

al.,1978). In addition, some cisplatin resistant cell lines exhibit an increased capacity to repair cisplatin adducts and to reactivate cisplatin modified plasmids (Eastman and Schulte, 1988; Chu and Chang, 1990).

It is not immediately clear how the inhibition of DNA replication can cause cell death. In fact, recent evidence suggests that the inhibition of DNA synthesis does not correlate to the level of cell death after a single cisplatin treatment (Sorenson *et al.*,1990). In addition these same workers noted that cisplatin-treated cells become arrested in the G2 phase of the cell cycle where they remain for several days. Some cells eventually recover and begin to cycle, but others die. The dying cells have the hallmark qualities of cells undergoing apoptotic (or programmed) cell death. More specifically, the DNA of dying cells is degraded in a specific manner that is manifested as a nucleosome ladder in agarose gels. These results suggest that an event in G2 triggers cell death by apoptosis, but the signal transduction pathway leading to this type of cell death is unclear. It is likely that DNA damage and/or strand breaks are signals that trigger cell cycle arrest. Strand breaks apparently signal the arrest of gamma irradiated cells in the G1 phase of the cell cycle, and P53 is involved in the signal transduction pathway (Kastan *et al.*,1992). It is likely that a similar cell cycle checkpoint system exists in the G2 phase that contributes to apoptotic cell death induced by cisplatin. Taken together, these observations suggests that cycling cells, which must pass through G2, should be more sensitive to cisplatin than stationary cells that rest in the G0 phase of the cell cycle. This is indeed the case; stationary cells are less sensitive to cisplatin than are exponentially growing cells (Fraval and Roberts, 1979).

Cisplatin adducts are premutagenic lesions in E. coli and mammalian

cells (Brouwer *et al.*,1981; de Boer and Glickman, 1989). Recent studies in E. coli with site-specifically modified phage genomes have shown that the A^G, G^G, and GNG[1] adducts are all SOS-dependent premutagenic lesions that cause a preponderance of A/G to T tranversions at the 5′ base of the crosslink (Bradley *et al.*,1993). Interestingly, the A^G adduct is a more potent premutagenic lesion than the G^G adduct by a factor of 5-10 suggesting the desirability of platinum based drugs that form fewer A^G crosslinks. Recent studies in E. coli with an orally active platinum-based drug that forms A^G lesions with lower efficiency than cisplatin are consistent with the hypothesis that G^G adducts are less mutagenic than A^G adducts (K. Yarema, personal communication). Hopefully these studies will lead to the design of new platinum anticancer drugs that cause fewer genotoxic side effects.

[1]The mutagenicity of this adduct has been characterized recently by K. Yarema in our laboratory (manuscript in preparation).

C. THE REPAIR OF UV PHOTOPRODUCTS AND CISPLATIN ADDUCTS IN E. COLI AND MAMMALIAN CELLS

1. E. coli

Both types of pyrimidine dimers and the three major platinum adducts are repaired by the E. coli UvrABC excision nuclease (Page *et al.*,1990; Sancar and Rupp, 1983). CPDs can also be repaired by a photoreactivating enzyme, photolyase, that does not act on 6-4 photoproducts (Brash *et al.*,1985). T4 endonuclease V (T4 Endo V) is both a CPD-specific glycosylase and an apurinic site (AP site) endonuclease that acts upon CPDs to generate a strand break that can then be repaired by other bacterial enzymes (Nickell *et al.*,1992). By contrast, it appears that the major pathway for the repair of cisplatin adducts is excision repair mediated by the UvrABC excision nuclease.

The UvrABC repair nuclease binds to sites of DNA damage and then incises the DNA helix in the region flanking the adduct. More specifically, the eighth phosphodiester bond 5′ to the lesion and the fourth or fifth phosphodiester bond 3′ to the lesion are incised (Sancar and Rupp, 1983). Other gene products displace the damaged oligonucleotide, synthesize DNA to fill in the gap, and ligate the 3′ end to seal the repair patch. Many studies have aimed to determine the mechanism of the UvrABC repair nuclease. A leading model proposes that UvrA dimers act catalytically to deliver a monomer of UvrB to the site of damage. In doing so, the damage recognition subunit, UvrA, binds transiently to DNA in a complex with UvrB. UvrA then dissociates from the complex, and a stable UvrB - DNA complex is formed. Subsequently, UvrC recognizes the UvrB-DNA complex and

performs the incision reaction (Orren and Sancar, 1989). Several observations support this model. First, the protein DNA complex incised by UvrC contains only UvrB. Second, UvrB - DNA complexes form efficiently in the presence of catalytic amounts of UvrA. Since UvrB does not bind to damaged DNA in the absence of UvrA, this result suggested a catalytic, as opposed to a stoichiometric, role for UvrA.

2. Mammalian cells

The mammalian excision nuclease also repairs both types of UV photoproducts and the three most abundant types of cisplatin DNA adducts. The process of nucleotide excision repair is considerably more complex in mammalian cells than in E. coli. Whereas the incision step of DNA repair in E. coli requires three gene products, at least seven gene products are required to complete this step in mammalian cells. The mammalian homologs of E. coli *uvr* a, b, and c mutants are the seven xeroderma pigmentosum complementation groups, A-G. Xeroderma pigmentosum is a human disorder characterized by extreme UV sensitivity and an overwhelming predisposition to skin cancer. The molecular basis of this disease is a defect in DNA repair. Since each XP complementation groups has a defect in the incision step of excision repair, it appears that at least seven gene products are required to complete this step. Although the genes for many of these complementation groups have apparently been isolated (all except groups E and F), the function of the respective gene products is still unknown, although some are putative helicases (XP-B, XP-D) (Weeda *et al.*,1990; Bootsma and Hoeijmakers, 1993), and another, which contains a putative zinc finger domain, binds selectively to UV-damaged DNA (XP-A) (Robins *et al.*,1991). An intriguing recent finding demonstrated that the

putative XP-B gene product is identical to a basic transcription factor, BTF2, which is required for transcription by RNA polymerase II (Schaeffer *et al.*,1993). This finding may help to establish the molecular basis for the preferential repair of DNA lesions in the coding strand of actively transcribed genes (Mellon *et al.*,1987). The cause of another DNA repair disorder, Cockaynes syndrome, is believed to be a defect in transcription-coupled repair. Further evidence supporting a possible role for BTF2 in transcription coupled repair is the observation that XP-B patients also suffer from Cockaynes syndrome.

Although the genes encoding many of the proteins involved in mammalian excision repair have been isolated, the biochemistry of excision repair remains poorly understood. In the last several years an in vitro DNA repair assay has been developed that employs crude human cellular extracts (Robins *et al.*,1991). This assay is valuable in isolating DNA repair proteins because biochemical complementation between extracts from different XP groups can be achieved in this system. In addition, the assay is useful for examining the repair of specific types of DNA lesions, including UV photoproducts and cisplatin adducts. Results from the in vitro repair assay have shown that 6-4 photoproducts are more efficiently repaired than CPDs. More specifically, photoreactivation of the UV-irradiated DNA that was used as the substrate for repair replication did not significantly reduce the repair signal (Wood, 1989). This result is consistent with in vivo studies which measured the repair of CPDs and 6-4 photoproducts by using antibodies raised against UV-damaged DNA (Mitchell *et al.*,1985). The repair protein that is responsible for the biased repair of 6-4 photoproducts is currently unknown, but, as shown in this dissertation, a damaged DNA binding protein that is absent in XP-E cells may be involved. The protein encoded by the

XP-A gene may also contribute to the selective repair of 6-4 photoproducts because it, too, has affinity for UV-damaged DNA (Robins *et al.*,1991).

The in vitro DNA repair assay has also been used to examine the repair of cisplatin DNA adducts. Studies with globally modified platinated DNA revealed that most of the repair synthesis was due to adducts other than the abundant G^G and A^G intrastrand crosslinks (Calsou *et al.*,1992). In agreement with this finding, other workers have shown that the cisplatin G^G adduct is refractory to repair by cellular extracts (Szymkowski *et al.*,1992). In vivo studies generally support the hypothesis that G^G adducts are poorly repaired. Most in vivo studies show that G^G adducts are removed rapidly in the time period immediately following the cisplatin treatment; however, repair activity becomes diminished at later time points, and a significant fraction of the adducts persist for several days (Terheggen *et al.*,1987). The inefficient repair of cisplatin adducts may contribute to antitumor activity. This hypothesis seems reasonable in light of the results cited above showing that cisplatin is more toxic to proliferating cells than to resting cells. Presumably, resting cells can survive, even if DNA repair is inefficient, because they are not committed to cell division and thus do not proceed through the stage of the cell cycle (most likely G2) in which the process of cell death is triggered. By contrast, inefficient repair of cisplatin would be expected to be deleterious to cycling cells that are committed to passage through all phases of the cell cycle. Furthermore, the efficient repair of DNA adducts of a cisplatin isomer, *trans*-DDP, has been proposed to explain the ineffectiveness of this drug to combat cancer (Ciccarelli *et al.*,1985; Heiger-Bernays *et al.*,1990).

D. THE ROLE OF DAMAGED DNA RECOGNITION PROTEINS IN THE GENOTOXICITIES OF UV LIGHT AND CISPLATIN

Damaged DNA recognition proteins (DRPs) are a class of proteins that binds selectively to damaged DNAs. DRPs have been reported for a growing list of DNA modifications, including platinum crosslinks (Donahue *et al.*,1990; Pil and Lippard, 1992; Brown *et al.*,1993), 1,N^6-ethenoadenine (Rydberg *et al.*,1991), G:T mismatches (Jiricny *et al.*,1988), apurinic/apyrimidinic (AP) sites (Lenz *et al.*,1990), *N*-acetyl-2-aminofluorene-guanine adducts (Moranelli and Lieberman, 1980), and ultraviolet light (UV)-induced photoproducts (Feldberg and Grossman, 1976; Chu and Chang, 1988; Hirschfeld *et al.*,1990). Studies leading to the identification of DRPs generally have the goal of isolating DNA repair enzymes since the initial step in repair is most likely at the level of damage recognition. This presumption is accepted as being valid since the best studied repair complex, UvrABC, has a damage recognition component, the UvrA protein. In the case of some DRPs there is a clear connection to DNA repair (Rydberg *et al.*,1991; Chu and Chang, 1988; Clugston *et al.*,1992); however, other DRPs are probably not involved in DNA repair and may actually contribute to the genotoxicity of the DNA damaging agent being examined (Donahue *et al.*,1990).

We have proposed three models to explain the role of DRPs in the genotoxicities of DNA damaging agents (Figures 3-5). Model 1 (Figure 3) proposes that DRPs are DNA repair proteins that act, like UvrA, as the lesion recognition subunit of a repair complex. In this case, the DRP would serve to enhance the survival of damaged cells. A body of indirect evidence suggests that a UV-damaged DNA recognition protein (UV-DRP) may be a

component of a versatile mammalian excision repair complex. The UV-DRP is overly expressed in human cells that display resistance to the anticancer drug cisplatin; these cells also exhibit an enhanced capacity to reactivate cisplatin-damaged plasmids (Chu and Chang, 1990). Furthermore, the UV-DRP is induced 2-4 fold above constitutive levels in primate cells pretreated with UV (Protic *et al.*,1989). These cells also reactivate UV-damaged plasmids with increased efficiency. The most compelling result linking the UV-DRP to DNA repair is the apparent absence of this activity in cells from two related XP patients (Chu and Chang, 1988). More specifically, these individuals belonged to genetic complementation group E. The generality of this finding has recently been challenged because UV-DRP activity is detected in a number of XP-E cell lines derived from Japanese patients (Kataoka and Fujiwara, 1991; Keeney *et al.*,1992). It is possible, however, that these recently tested cell lines contain a form of the UV-DRP that is defective in a function other than DNA binding.

The possible role of the UV-DRP in DNA repair has not been established but, based on genetic evidence in yeast, the protein has been hypothesized to be the human homolog of photolyase (Patterson and Chu, 1989), an enzyme that catalyzes the chemical reversal of CPDs back to normal dipyrimidine sequences in DNA. Although the UV-DRP lacks the ability to photoreactivate DNA *in vitro*, it may help to recruit repair complexes to sites of DNA damage (Patterson and Chu, 1989). In support of this model, the binding of E. coli photolyase to CPDs has been shown to stimulate the excision of these lesions by the UvrABC repair complex in vitro (Sancar *et al.*,1984). Another model suggests that the UV-DRP may perform a damage recognition function analogous to that of UvrA (Hirschfeld *et al.*,1990).

The second model (Figure 4) proposes that DRPs are anti-repair proteins. In this model, the DRP functions naturally in a process unrelated to DNA repair and fortuitously recognizes the adduct induced DNA structure. In this context, a consequence of DRP binding is the shielding of DNA adducts from repair enzymes. The DRP may hinder the accessability of adducts to repair enzymes or, alternatively, could alter DNA structure in such a manner that the adduct is no longer recognized by repair proteins. Below I present evidence to support the feasibility of this model as it applies to the toxicity of cisplatin.

We and others have reported a class of eukaryotic proteins that bind specifically to DNA damaged by the anticancer drug cisplatin (Chu and Chang, 1988; Donahue *et al.*,1990; Toney *et al.*,1989; Bruhn *et al.*,1992; Clugston *et al.*,1992; Pil and Lippard, 1992; Brown *et al.*,1993; Hughes *et al.*,1992). In only one of these reports is there evidence to suggest that the *cis*-DDP DRP is involved in DNA repair. More specifically, human single stranded DNA binding protein (hSSBP), which is known to have a role in DNA repair (Coverley *et al.*,1991), has been shown to be a *cis*-DDP DRP (Clugston *et al.*,1992). All of the remaining cis-DDP DRPs that have been cloned, or otherwise identified, are homologous to the abundant chromosomal protein HMG1. Indeed, HMG1 itself binds selectively to cisplatin modified DNA (Pil and Lippard, 1992). The homologous region in this family of proteins is a recently identified, novel DNA binding domain referred to as the HMG box (Jantzen *et al.*,1990). The HMG box is present in several putative transcription factors including human upstream binding factor (hUBF), the testis determining factor (SRY), and the lymphoid enhancing factor (LEF-1) (Jantzen *et al.*,1990; Giese *et al.*,1991; Harley *et al.*,1992). It is important to note that the normal cellular function of the

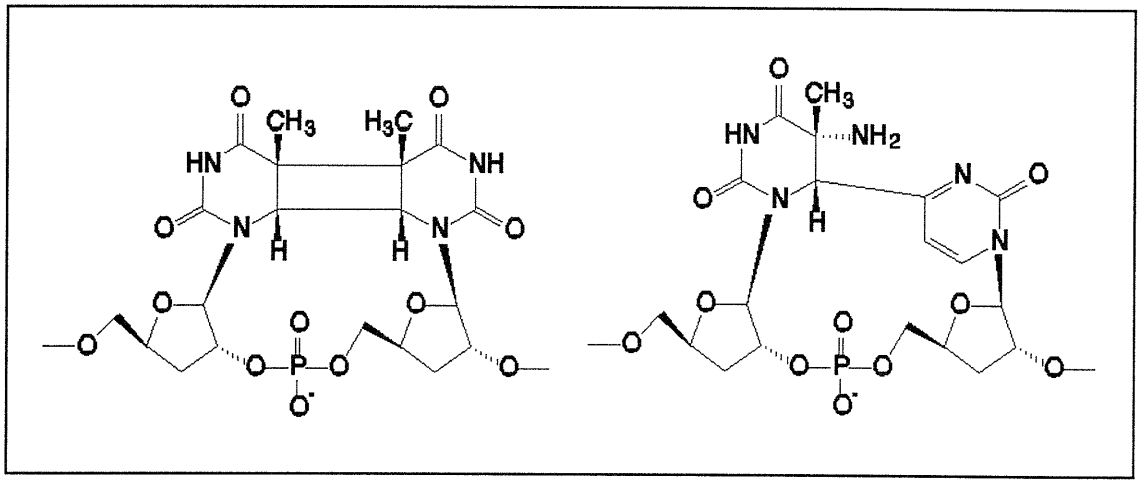
HMG1-related *cis*-DDP DRPs, which include SSRP1, HMG1, and IXR1 (Bruhn *et al.*,1992; Brown *et al.*,1993), remains a mystery; although there is evidence to suggest that HMG1 has a general role in transcription and in maintaining chromatin structure (Einck and Bustin, 1985). To date there are no known DNA repair proteins that are homologous to HMG1 suggesting that the HMG class of *cis*-DDP DRPs have a role in cisplatin toxicity that is unrelated to DNA repair.

We have proposed that HMG proteins can act as anti-repair proteins (Bruhn *et al.*,1992). Recent genetic studies in yeast are consistent with an anti-repair role of HMG box proteins. A yeast HMG box protein, IXR1, that binds selectively to cisplatin-modified DNA confers sensitivity to cisplatin. In mutants lacking this protein, lower levels of cisplatin adducts are observed and the sensitivity to cisplatin is decreased by a factor of two (Brown *et al.*,1993). Further support of the anti-repair model stems from the observation that the poorly repaired cisplatin adducts are high affinity binding sites for *cis*-DDP DRPs, whereas the efficiently repaired lesions are ignored by *cis*-DDP DRPs (Szymkowski *et al.*,1992; Donahue *et al.*,1990; Pil and Lippard, 1992).

The third model (Figure 5) proposes that *cis*-DDP DRPs function normally as critical regulatory proteins that are titrated away from their natural sites of action by cisplatin adducts. Presumably, if the function of the titrated protein were more critical to the viability of tumor cells than to normal cells, then the proposed "decoy effect" could contribute to antitumor efficacy. This model, although unproven experimentally, seems feasible since many HMG box proteins are indeed transcriptional regulators. In this dissertation, I provide evidence to suggest that the nucleolar transcription

factor hUBF may become titrated away from its natural sequence element by cisplatin adducts.

Figure 1. Structure and properties of the major UV-induced Photoproducts

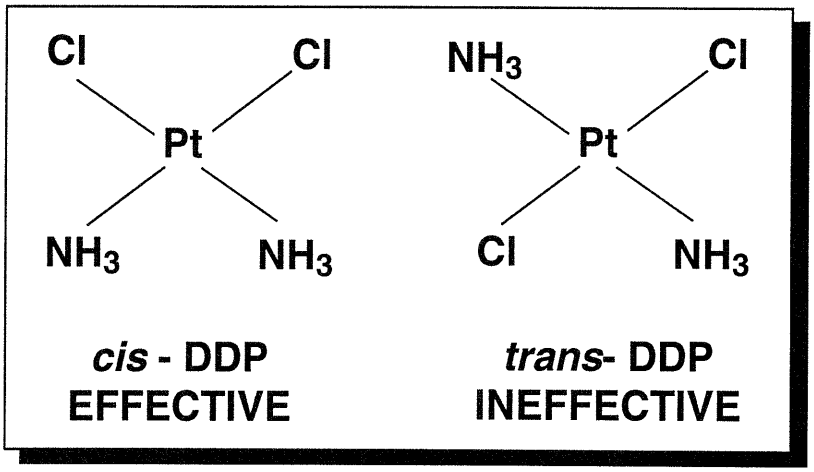


TT *cis-syn* Cyclobutane Pyrimidine Dimer

TC Pyrimidine (6-4) Pyrimidone Photoproduct (6-4 Photoproduct)

Cytotoxicity and Mutagenicity	+	+
Abundance	+ + +	+
Repair in Human Cells	+	+ + +
Repair by E. coli Photolyase	+	-
Alkali Lability	-	+
T4 Endonuclease V Sensitivity	+	-

Figure 2. Cisplatin DNA adducts



Adduct Profiles		
DNA-Protein	yes	yes
DNA-DNA		
Interstrand	yes	yes
Intrastrand		
GXG	yes	yes
GG	65%	no
AG	25%	no
Monofunctional	yes	yes

Figure 3. Model 1: DRPs are part of a repair complex.

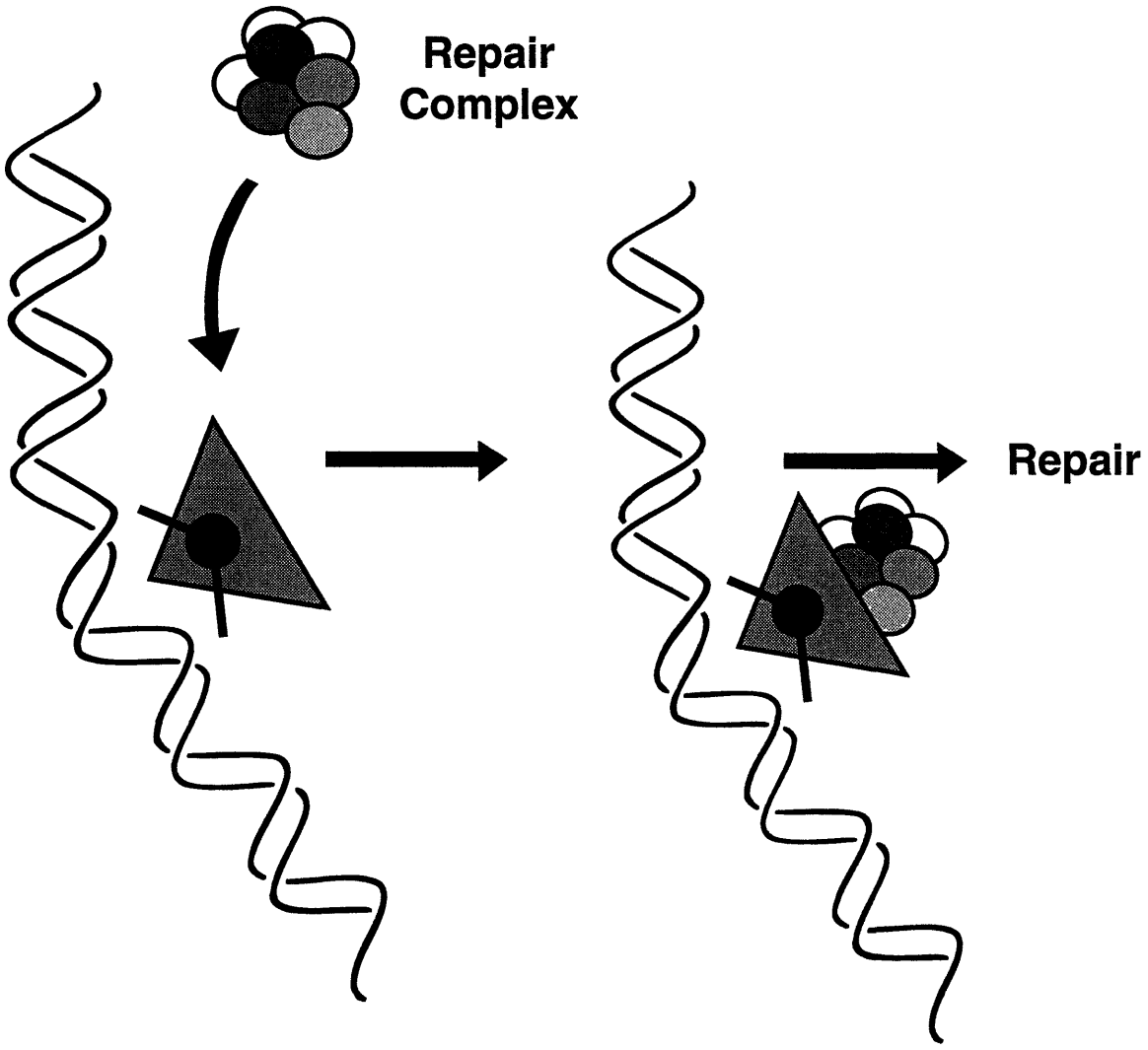


Figure 4. Model 2: DRPs are anti-repair proteins.

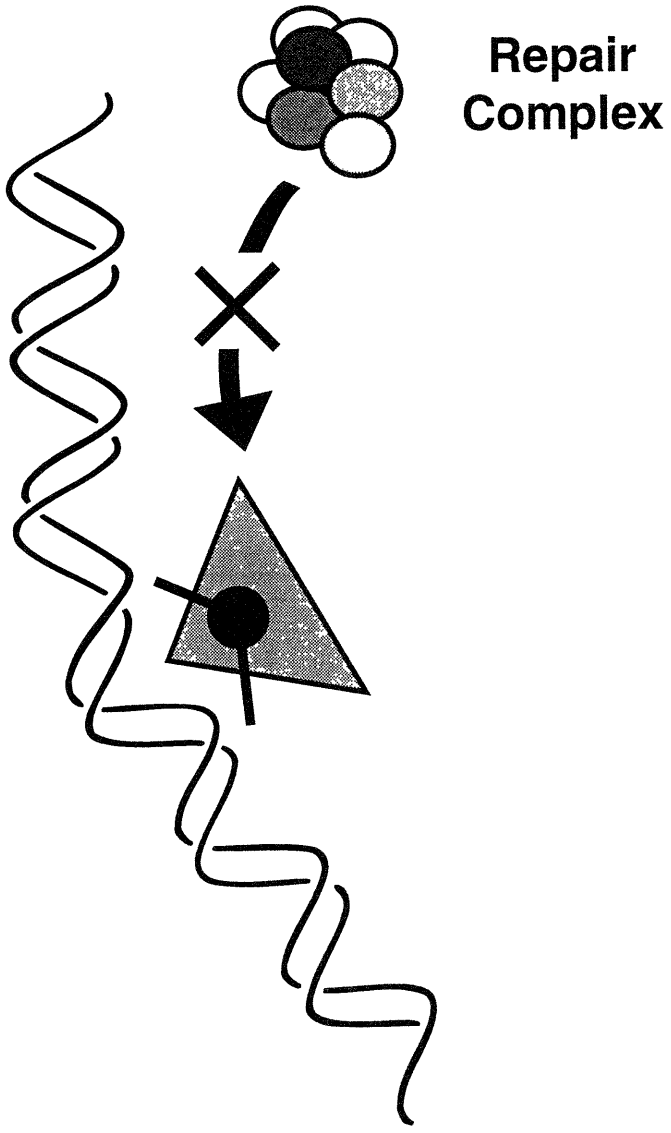
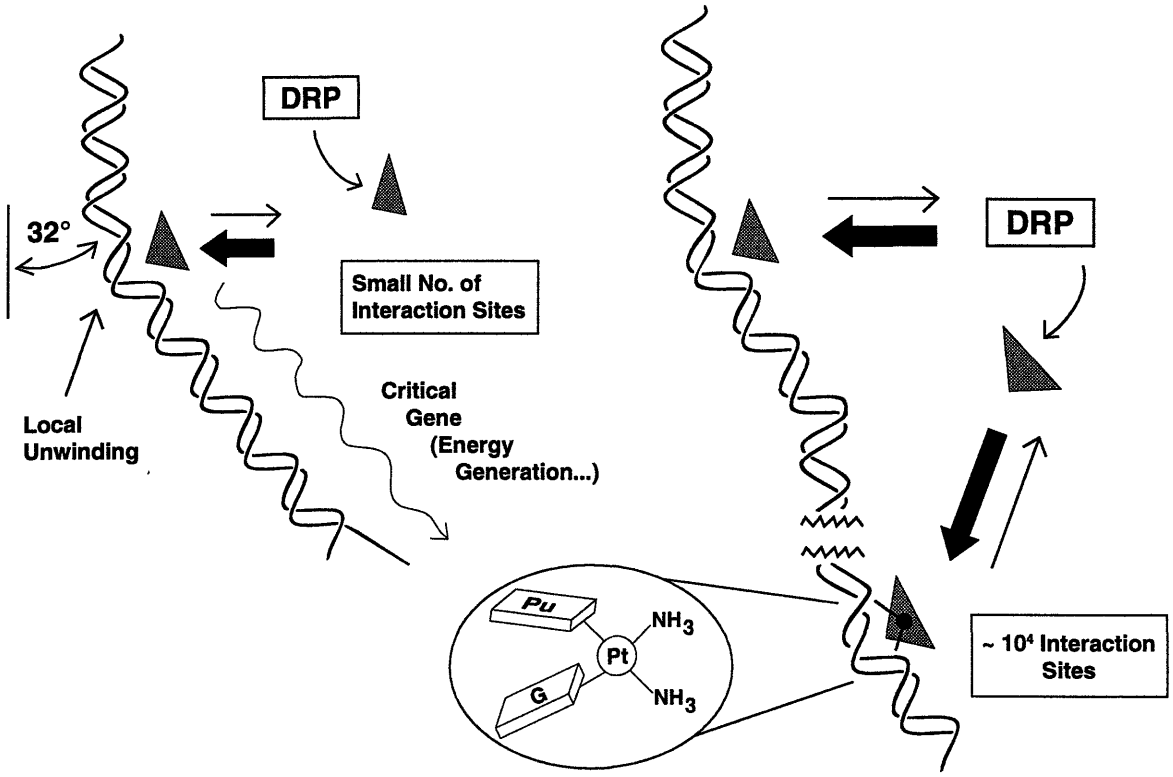


Figure 5. Model 3: DRPs are regulatory proteins that are diverted by cisplatin adducts.



III. MATERIALS AND METHODS

A. Materials

1. Enzymes and chemicals

Restriction endonucleases, DNA ligase and polynucleotide kinase were purchased from New England Biolabs or Pharmacia. The Klenow fragment of E. coli DNA polymerase I was obtained from Boehringer Mannheim Biochemicals or New England Biolabs and poly(dI-dC) · poly(dI-dC) from Pharmacia. DNase I (DPFF grade) was from Worthington and Hygromycin B from Sigma. Taq polymerase was from Perkin Elmer Cetus. T4 endonuclease V (T4 endo V) and E. coli photolyase were kindly provided by R.S. Lloyd (Vanderbilt University) and C. Walsh (Harvard University), respectively. Media, nutrient supplements, and fetal calf serum (FCS) for human cell culture were obtained from GIBCO and Sigma.

2. Cell Lines

Human lymphoid lines [XP-A (GM02250D), XP-D (GM02253D), and XP-E (GM02450D)] and a fibroblast cell line [XP-E (GM2415B)] were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository. HeLa cells were obtained from M. Chow and P. Sharp (M.I.T.) and the human hepatoma cell line Hep G2 from R. Rothstein (The Children's Hospital, Boston, MA). The Raji lymphoma cell line was obtained from the American Type Tissue Collection.

3. Plasmids

pSBr208 containing human rRNA promoter sequences was provided

by M. Jantzen and R. Tjian (University of California, Berkeley). pCMVβ-galactosidase was a gift of R. Tepper (Massachusetts General Hospital) and the Epstein-Barr virus (EBV)-based vector EBV-B108 was from B. Sugden (University of Wisconsin). pT7-RNHMG1 was from M. Bianchi (University of Milan), pcDNA1 was purchased from Invitrogen, and HEK1001 "bottom" was kindly provided by S. Bruhn (M.I.T.).

4. Proteins and Antisera

Affinity-purified HeLa human upstream binding factor (hUBF) was provided by M. Jantzen and R. Tjian (University of California, Berkeley). Rabbit antisera raised against rat HMG1 or human SSRP1 were generous gifts of P. Pil and S.J. Lippard (M.I.T.). Goat anti rabbit IgG antibodies conjugated to alkaline phosphatase, and the appropriate colorometric or chemiluminescent substrates were purchased from Promega and BioRad, respectively.

5. Site-specifically platinated 100 bp DNA constructs

100 bp DNA fragments, either unmodified, or containing a single, centrally located cisplatin 1,2 intrastrand crosslink at a d(GpG) sequence were generously provided by P. Pil and S. J. Lippard (M.I.T.) or by A. Barrasso (M.I.T.) The sequence of the adducted strand is: 5'-CAG ATC GAT GGA CTA GCC AGC TGC CTT GAT ATC ACG TCA GTC TCC TTC TG$\hat{}$G TCT CTT CTC AGT CGA TGA TAT CGC TCC AGC TGT TGA CTA CCC GGG TAC T-3'. G$\hat{}$G indicates the position of the cisplatin crosslink.

B. Methods

1. Cell Culture

Lymphoid cell lines were grown in suspension in RPMI 1640 medium containing 15% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine. Fibroblast cell lines were grown in minimal essential medium (MEM) supplemented with 20% FCS, 1x vitamins, 1x essential amino acids, and 1x non-essential amino acids. HeLa cells were grown in suspension in S-MEM supplemented with 5% FCS and 2 mM L-glutamine. Hep G2 cells were grown in D-MEM containing 10% FCS. Attached HeLa cells were grown in D-MEM containing 10% FCS and 1x non-essential amino acids. The Raji lymphoma line was grown in RPMI 1640 medium containing 15% FCS and 2 mM L-glutamine. All cultures contained a 1x penicillin/streptomycin mixture. Raji cells were maintained at 0.5-2x10^6 cells/ml. Suspension HeLa cells were maintained at 0.5-4x10^5 cells /ml.

2. Cellular extract preparation

Nuclear extracts were prepared according to a published procedure (Dignam *et al.*,1983). Protein concentrations were determined by the BioRad Bradford assay (Bradford, 1976). Whole cell extracts on small numbers of cells were prepared by a sonication procedure (Samson *et al.*,1986). 2x10^6 cells were harvested, washed with ice-cold phosphate buffered saline, resuspended in 500 μl of sonication buffer (50 mM Hepes (7.8), 0.5 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.5 mM phenylmethylsulfonyl flouride), and sonicated for 10 sec at the lowest power setting with a Branson probe sonicator. Lysates were centrifuged for 10 min at 12,000 x G, and the supernatant was concentrated to 50 μl in an Amicon centicon-10 filtration unit. Extracts were stored at -80° C.

3. Preparation of UV-treated p422 probes

M13mp18 replicative form DNA was digested with AvaI, and the resulting fragments were labeled with [α-^{32}P]dCTP (5000 Ci/mmole, New England Nuclear) by using the Klenow fragment of DNA polymerase I. Both strands of each restriction fragment are radiolabeled by this procedure. The labeled fragments were separated by using native polyacrylamide gel electrophoresis (PAGE), and the 422 base pair (bp) fragment, designated p422, was electroeluted with an Amicon Centrilutor. Labeled probes were irradiated on a chilled surface with a 15-W germicidal lamp (maximum output at 254 nm) at a fluence of 5 J/m^2·sec until the desired dose was achieved (fluence measured with a UVX-25 sensor).

4. Preparation of UV-treated synthetic oligonucleotide probes

Sixty nine-mer oligonucleotides T_5, T_4C, and Δ (*vide infra*) were synthesized on an Applied Biosystems 381-A DNA synthesizer. Oligomers were purified on 10% denaturing (7 M urea) polyacrylamide gels and electroeluted as above. Urea was removed from oligomers by several distilled water washes in Amicon Centricon 10 microconcentrators. T_5, T_4C, and Δ were end labeled with [γ-^{32}P]ATP (3000 Ci/mmole, New England Nuclear) by using polynucleotide kinase. Unincorporated label was removed by centrifugation through G-25 spun columns. Labeled strands were annealed to unlabeled complementary strands, and the resulting duplexes were purified by native PAGE to remove contaminating single stranded DNA. UV-irradiation was as described above.

5. Analytical electrophoretic mobility shift assays

DNA-protein complexes were detected by using a published procedure (Carthew *et al.*,1985). End labeled probes either unmodified, UV-treated, or cisplatin modified (10^4 cpm p422 or 7 X 10^4 cpm 69-mer synthetic probes T_5, T_4C, and Δ) were incubated in the presence of crude nuclear extracts (10 μg protein) and poly(dI-dC) · poly (dI-dC) (6 μg protein) for 15 min at 37°C in binding buffer (2 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 10 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 1% glycerol, and 0.2 mM dithiothreitol) in a final volume of 15-25 μl. Protein-DNA complexes were then resolved on 4% (p422) or 6% (69-mer probes) polyacrylamide gels [29:1 acrylamide/*N,N'*-methylenebis(acrylamide)]. Gels were electrophoresed in Tris-glycine buffer (50 mM Tris-HCl (pH 8.5), 380 mM glycine, 2 mM EDTA) for 4 hr at 30 mA. Gels were dried and autoradiographed overnight at -80°C with an intensifying screen. In some cases gels were imaged and bands quantified by using a Molecular Dynamics Phosphorimager. The level of binding was calculated under the assumption that the DNA in the B2 protein/DNA complex was occupied by twice the number of UV-DRP molecules as the DNA in the B1 complex (*vide infra*).

6. Preparative electrophoretic mobility shift assays

Purification of UV-DRP-bound T_4C probes was achieved by scaling up the protein-DNA binding reaction by one order of magnitude. Electrophoresis was performed for 14 hr to separate the UV-DRP-specific band from a non-damage-specific band. The UV-DRP-specific band and the non-protein bound band were excised from the gel and electroeluted. Gel-purified bound and free probes were deproteinized by a phenol:chloroform:isoamyl alcohol (29:29:1) extraction. Deproteinized material was extracted with chloroform,

ethanol precipitated, and resuspended in buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE).

7. Photolyase treatment of UV-irradiated DNA

End-labeled p422 bp probe DNA (25 μl at 5,000 cpm/μl) treated with a UV dose of 1.5 kJ/m^2 was incubated in the presence of E. coli photolyase (170 ng) in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 100 - μg/ml gamma globulin, and 10 mM 2-mercaptoethanol in a final volume of 50 μl. Samples were incubated under yellow light (>400 nm) for 30 min to allow enzyme binding and then photoreactivated for 1 hr under a 15-W General Electric F15T8.BLB Black Light (peak emission at 366 nm) at a fluence of 6.5 J/m^2·sec (fluence measured with a UVX-36 sensor). Photoreactivated DNA was then phenol:chloroform extracted, chloroform extracted, ethanol precipitated, and resuspended in TE buffer.

8. 6-4 photoproduct and cyclobutane pyrimidine dimer assays

6-4 photoproducts were detected as alkali-labile sites in irradiated DNA (Mitchell *et al.*,1990; Franklin *et al.*,1982). Piperidine (Fisher) was diluted freshly in distilled water before use and added to end labeled DNA samples to a final concentration of 1 M. Samples were heated at 90°C for 0.5 hr. In some cases it was necessary to extend the incubation time to several hr. Piperidine was removed by vacuum centrifugation and samples were washed by resuspension in distilled water followed by vacuum centrifugation. Washed samples were resuspended in TE buffer. Piperidine treated samples were subjected to denaturing PAGE to quantify and to map 6-4 photoproducts. Chemical sequencing reactions (Banaszuk *et al.*,1983)

were used as calibration standards. CPDs were detected as T4 endo V-sensitive sites in DNA. End labeled DNA fragments were treated with T4 endo V (1 μl of a 0.02 mg/ml solution of enzyme in TE and 100 μg/ml BSA) in the presence of 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 50 mM NaCl, and 1 mg/ml BSA at 37°C for 1 hr. Digestion products were analyzed by denaturing PAGE. The T4 endo V reaction was shown to proceed to completion by 1 hr. The average number of photoproducts in irradiated p422 was calculated by quantitating the fraction of strands refractory to T4 endo V or piperidine and applying the Poisson distribution equation, $P_o = e^{-x}$, where x is the average number of photoproducts per strand and P_o is the fraction of strands lacking a photoproduct.

9. Radiolabeling DNase I Footprinting Probes

The EcoR1-BstEII fragment of pSBr208 containing the -208 to +78 region of the human rRNA gene was labeled with ^{32}P for footprinting studies. The noncoding strand was 3' end-labeled by using the Klenow fragment of DNA polymerase I. pSBr208 was with digested with BstEII, extracted with phenol:chloroform, chloroform, and then precipitated with ethanol. Linearized pSBr208 (0.24 pmole) was treated with 5 units of New England Biolabs Klenow enzyme in the presence of 75 μM dTTP and 100 μ Ci of each α^{32}P-dATP, α^{32}P-dCTP, and α^{32}P-dGTP. The specific activity of the labeled dNTPs was >6,000 Ci/mmole. The labeling procedure was most efficient if the labeled dNTPs were used on the day of manufacture. The reaction (35 μl total volume) was performed in 1x Klenow buffer, supplied by New England Biolabs. The reaction proceeded for 30 min at 25° C at which time a chase of unlabeled dNTPs was added. The final concentration of each dNTP was 150 μM. The cold chase was incubated for 30 min at

25° C, and the reaction was terminated by heating for 15 min at 75° C. After cooling the reaction to room temperature, NaCl was added to a final concentration of 100 mM as were 20 units of EcoR1. Digestion proceeded for 2 hr at 37° C. The labeled footprinting probes were gel purified by PAGE (5%), and the specific acitivity was determined by scintillation counting.

The 5′ end of the noncoding strand of the rRNA promoter was labeled by using polynucleotide kinase. pSBr208 was digested with EcoR1, and the 5′ phosphate was removed with calf intestinal phosphatase. The DNA was phenol:chloroform extracted and precipitated with ethanol. Digested pSBr208 (0.96 pmole) was 5′ end-labeled by using polynucleotide kinase in the presence of 300 μ Ci of γ^{32}P-ATP (>6,000 Ci/mmole). The kinase was inactivated for 15 min at 65°, and NaCl was added to a final concentration of 100 mM. The DNA was digested for 2 hr at 60° C with BstEII to generate the footprinting probes. The probes were then gel purified by PAGE (5%).

The adduct containing strand of GˆG-100 and the analogous strand of Un-100 were 5′ end labeled by using polynucleotide kinase. Thirty ng of each fragment was treated with kinase in the presence of 50 μ Ci γ^{32}P-ATP (>6,000 Ci/mmole) for 1 hr at 37° C. Kinase was inactivated for 15 min at 64°C, and the 5′ end of the unadducted strand was removed by digestion with Ava1 for 2 hr at 37°. Unincorporated label and the 10 bp Ava1 fragment were removed by using G-25 spun columns (Boehringer Mannheim).

10. DNase I footprinting assays

Purified HeLa hUBF at a concentration of 4.0 nM was stored in 20 μl aliquots at -80° C in TM^+0.1 M KCl (50 mM Tris-HCl (7.9), 17.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20% glycerol, 100 mM KCl, and 0.1% Nonidet-P40). DNase I (Worthington DPFF grade) was dissolved at 2.5 mg/ml in water and stored in 10 μl aliquots at -80° C. hUBF and DNase I were thawed in an ice water bath immediately before use. hUBF was diluted, if necessary, in binding buffer (25 mM Tris-HCl (7.9), 14 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol, 50 mM KCl, 0.05% Nonidet-P40, 2.5 mM $CaCl_2$). DNase I was diluted 1/500 in ice-cold water immediately before use. Footprinting reactions (50-60 μl) were performed in 1x binding buffer and contained 10^3-10^4 cpm of the appropriate labeled probe (final DNA concentration 0.7-50 pM, depending on the experiment). The reactions were started on ice by the addition of hUBF followed by gentle mixing (e.g., flicking the tube). All steps involving hUBF were performed using siliconized test tubes tubes and pipette tips (USA Scientific. The mixture was incubated for 10 min at 30° C and allowed to cool to room temperature for 5 min. DNase I (generally 2 μl of a 1/500 dilution of the 2.5 mg/ml stock) was added, the reaction was mixed gently and incubated for 1 min at room temperature. Staggering the samples by 15 sec facilitated this procedure. The DNase I reaction was terminated after 1 min by adding 100 μl of DNase I stop buffer (20 mM EDTA, 1% SDS, 0.2 M NaCl, and 50 μg/ml yeast total RNA) and vortexing. Samples were extracted with phenol:chloroform, ethanol precipitated, and resuspended in formamide containing xylene cyanol and bromophenol blue. Resuspended samples were transferred to new tubes and subjected to scintillation counting. The samples were denatured by heating to 90° C for 3 min and loaded on wedged (0.4-1.0 mm) sequencing gels (6% or 12% for promoter footprints and GˆG-100 footprints, respectively) in 0.8 cm wells. Gels were prerun for 30 min at 70

W, loaded, and run at 70 W (constant power) until the xylene cyanol was three quarters of the way to the bottom (promoter footprint) or until the bromophenol blue was to the bottom (GˆG-100). Gels were fixed for 20 min in 20% methanol/20% acetic acid, dried, and exposed with an intensifying screen to preflashed Kodak XAR film at -80° C. Gels were also analyzed by using a PhosphorImager.

11. Plasmid constructions

CMV-rHMG1 was constructed by subcloning the ~700 bp Nde1/HindIII fragment of pT7-RNHMG1 into the EcoRV site of pcDNA1. Both the sense (S) and antisense (AS) orientations were obtained. CMV-hHMG1 was constructed by inserting a polymerase chain reaction (PCR) fragment of the human HMG1 cDNA (-1 to +179) into the BamH1 site of pcDNA1. Again, both orientations were obtained. EBV-SPA was constructed by subcloning the HindIII/Fok1 fragment of pcDNA1 encompassing the splice and polyadenylation sequences into a HindIII/Sal1 digest of EBV-B108. EBV-rHMG1 was prepared by subcloning the BamH1 fragment of CMV-rHMG1 into the BamH1 site of EBV-SPA and both orientations were obtained. EBV-SSRP1-5′ was constructed by subcloning the ~600 bp EcoR1 fragment of HEK 1001 "bottom" into the HindIII site of EBV-SPA and both orientations were obtained.

Restriction mapping was used to verify each construct and to determine the orientation of the subcloned fragment. All mini plasmid preparations were made by using the Promega Magic Miniprep system. Large scale plasmid preparations were made by using Qiagen Maxi Prep columns. pcDNA1-based and EBV-SPA-based plasmids were maintained in

E. coli strains MC1061/P3 and DH5, respectively.

12. Western blot analysis

Whole cell extracts (10 μg) prepared by a sonication procedure were boiled for 2 min in SDS-PAGE sample buffer and loaded on 8% (SSRP1) or 12% (HMG1) discontinuous gels. The Biorad minigel/minitransfer unit was used in these studies. Minigels were run at 200 V for 1 hr, and the proteins were transferred to nitrocellulose according to the protocol provided with the transfer unit. The blocking, antibody probing, and alkaline phosphatase-based colorometric/chemilumiscent detection steps were performed by using commerically available systems from Promega (colorometric) and Biorad (chemiluminescent). Human HMG1 was detected by probing the protein blots for 30 min with a 1/1000 dilution of rabbit serum raised against rat HMG1. Human SSRP1 was detected by probing the protein blots for 30 min with a 1/500 dilution of rabbit serum raised against human SSRP1. All other procedures were provided by the commercial supplier.

13. Transient transfection of HeLa cells.

A suspension culture of exponentially growing HeLa cells was harvested and resuspended in room temperature D-MEM containing 10% FCS and 1x non-essential amino acids to a concentration of $1.25x10^7$ cells/ml. The cell suspension (0.4 ml, $5x10^6$ cells) was mixed with 35 μg of the test vector (antisense, sense, parental), 5 μg of the reporter construct, CMV-β-galactosidase, and added to a 0.2 cm electroporation cuvette (Biorad). Electroporation was performed by using a BTX electroporator. The electroporator settings were: Voltage = 246 volts, Timing Resistance = 13

ohms, Capacitance = 1150 μfarads. These settings combined with the conductivity of the culture media resulted in a voltage decay time constant of ~7 ms. After pulsing, the samples were allowed to recover for 10 min at room temperature in the cuvette. Subsequently, the cell suspension was diluted into 10 ml of media and plated on 100 mm dishes. Fresh media was added after 12 hr, and the cells were harvested after 35 hr.

14. Stable transfection of Raji cells.

A suspension culture of exponentially growing Raji cells was harvested and rsuspended in room tempertature RPMI 1640 containing 15 % FCS and 2 mM L-glutamine to a concentration of $1.25x10^7$ cells/ml. The cell suspension (0.4 ml, $5x10^6$ cells) was mixed with 10 μg of the appropriate EBV plasmid (*vide infra*) and added to a 0.2 cm electroporation cuvette. The electroporator settings were: Voltage = 200 V, Timing resistance = 13 ohms, Capacitance = 1,200 μfarad. These settings combined with the conductivity of the culture media resulted in a voltage decay time constant of ~7 ms. After pulsing, the samples were allowed to recover for 10 min at room temperature in the cuvette. Subsequently, the cell suspension was diluted in 10 ml of media an transferred to an upright T-25 flask. After 48 hr, the cells were diluted into fresh media containing hygromycin B (200 μg/ml) and plated in 96 well dishes at 10, 100, and 1000 cells/well, or transferred to a new flask to initiate a culture of pooled clones. Hygromycin B-resistant clones were scored after 2-4 weeks, and several clones were isolated and expanded into cell lines. All manipulations of hygromycin B-resistant clones were performed in the presence of this selective agent.

15. *β*-galactosidase assays

Solution-based and *in situ* *β*-galactosidase assays were performed as described (Ausubel *et al.*,1993).

IV. AN ULTRAVIOLET LIGHT DAMAGED DNA RECOGNITION PROTEIN ABSENT IN XERODERMA PIGMENTOSUM GROUP E CELLS BINDS SELECTIVELY TO PYRIMIDINE (6-4) PYRIMIDONE PHOTOPRODUCTS

A. RESULTS

1. Identification of a human UV-damaged DNA recognition protein (UV-DRP)

A protein was present in human nuclear extracts that retarded the electrophoretic mobility of UV-damaged DNA fragments. This protein, the UV-DRP, has been observed by others by gel mobility shift assays (Chu and Chang, 1988; Hirschfeld *et al.*,1990) and is possibly a protein observed earlier by other techniques (Feldberg and Grossman, 1976). The proteinaceous nature of the UV-DRP was established by demonstration that the activity was abolished by pronase but not by RNase A. Binding of the UV-DRP to an irradiated (1.5 kJ/m^2) 422 bp fragment (p422) excised from M13mp18 DNA resulted in two low mobility complexes, B1 and B2, that were not observed if extracts were probed with undamaged DNA. The relative mobility of B1 and B2 is believed to reflect the number of UV-DRP molecules bound per molecule of probe (i.e., two molecules of protein per probe in B2 and one molecule per probe in B1). The binding of a non-damage specific protein to p422 was precluded by performing the binding reactions in low salt and buffer concentrations (2 mM Tris·HCl, 10 mM NaCl), conditions that enabled binding of the UV-specific protein but were not favorable for binding of the non-specific DNA binding protein. Furthermore, the level of UV-DRP binding was increased by 4-5 fold if a high concentration of EDTA (50 mM) were included in the binding reactions. This finding has been observed by others and may be attributable to inhibition of UV-DRP activity by the presence of trace amounts of zinc in the binding buffer (Gilbert Chu, personal communication).

The initial focus of these studies was to determine if the UV-DRP activity was caused by the same protein(s) that were responsible for the reduced mobility of cisplatin-modified DNA fragments in gel shift assays performed in our laboratory by Brian Donahue and in the laboratory of Dr. Gilbert Chu (Chu and Chang, 1988; Donahue *et al.*,1990). To address this issue, competitive gel mobility shift assays were performed (Figure 6) in which binding to UV-damaged DNA was competed with either unlabeled, UV-damaged DNA or unlabeled, cisplatin-modified DNA (lanes 9-16). The converse experiment was also performed in which the cisplatin-damaged DNA recognition protein (*cis*-DDP DRP) activity was competed with both competitor DNAs (lanes 1-8). Lanes 1 and 9 show the mobilities of the cisplatin-modified and UV-treated probes, respectively, in the absence of nuclear extract. It is noteworthy that the electrophoretic mobility of the platinated probe is greatly reduced, even in the absence of extract, relative to the UV-damaged probe. This slower mobility is attributed to the positive charge of cisplatin adducts, to the DNA bending induced by bifuntional cisplatin DNA crosslinks, or both effects. The *cis*-DDP DRP activity is shown in lane 2 in the absence of any competitor DNA, and the UV-DRP activity is likewise shown in lane 10. It should be pointed out that each of these reactions contain a significant excess of the nonspecific competitor DNA poly(dI-dC)*poly(dI-dC) in order to mask the effect of nonspecific DNA binding proteins present in the crude nuclear extracts. As shown in lanes 3-8, the *cis*-DDP DRP activity is efficiently competed by platinated DNA, but not by UV-treated DNA. In lanes 11-16 it is clear that the UV-DRP activity is competed strongly by UV-treated DNA, whereas cisplatin-modified DNA is a poor competitor. If the protein(s) responsible for the UV-DRP and *cis*-DDP DRP activities were identical, then it would not be possible to obtain this pattern of competition; therefore, it was concluded that the two activities

were due to distinct proteins.

We suspected that the UV-DRP activity detected in Figure 6 was identical to that reported to be lacking in two cell lines from XP patients belonging to complementation group E (Chu and Chang, 1988). A survey of several human cell lines, both repair proficient (Hep G2 and HeLa) and deficient (XP-A, XP-D, and XP-E), confirmed this notion because the binding activity was present in all extracts except those from XP-E cells (Figure 7). This result establishes that the factor characterized is indeed the one lacking or defective in certain XP-E lines. By contrast, the *cis*-DDP DRP activity was found in XPE and further supports the conclusions of Figure 6.

2. Substrate specificity of the UV-DRP

Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 photoproducts) are the major lesions formed in UV-treated DNA (see Figure 1). We hypothesized that the UV-DRP recognizes structural distortions in DNA caused by one or both of these photoproducts. An initial goal was to estimate the relative contribution of each lesion to UV-DRP binding. A UV dose-response relation was generated that compares UV-DRP binding to the relative frequency of CPDs and 6-4 photoproducts in UV-irradiated probes (Figure 8). It is well established that 6-4 photoproducts are alkali-labile, and in the presence of hot piperidine will degrade to form a single strand break (Franklin *et al.*,1982). By contrast, CPDs are stable to hot piperidine, but are sensitive to T4 endonuclease V (T4 endo V). T4 endo V possesses both glycosylase and apurinic (AP) site endonuclease activity and acts on CPDs to form single strand breaks (Nickell *et al.*,1992). Hence, piperidine lability and T4 endo V sensitivity can be used in DNA nicking

assays to measure the levels of 6-4 photoproducts and CPDs, respectively. In such assays, the relative number of adducts is determined by measuring the fraction of the DNA that remains unnicked and, hence, contains zero adducts. Once the "zero fraction" is known, the Poisson relation can be used to calculate the average number of photoproducts per DNA fragment.

In Figure 8, nicking assays and gel mobility shift assays were performed on labeled DNA probes that were UV-irradiated to a range of final doses. UV-DRP binding was dose dependent and did not show a plateau in the range of doses tested. This response did not parallel the formation of CPDs, which formed as a logarithmic function of dose that reached a plateau at 5.0 kJ/m^2. Conversely, 6-4 photoproducts accumulated as a linear function of dose in a manner that more closely mirrored the binding curve. The most striking feature of this result is the steady increase in UV-DRP binding at doses above the CPD equilibration point - an effect that can best be explained by the existence of non-CPD binding sites. These data are consistent with the formal possibility that 6-4 photoproducts are recognized by the protein.

E. coli photolyase in the presence of near UV light repairs CPDs, but not 6-4 photoproducts, with high efficiency (Brash *et al.*,1985). We used enzymatic photoreactivation to prepare a p422 substrate (1.5 kJ/m^2) that lacked CPDs. Photolyase was added to DNA samples and the reaction mixture was exposed to yellow light for 30 min (enzyme binding step) and then to near UV light for 1 hr (photoreactivation step). Samples were deproteinized and desalted before being used in subsequent experiments. Photoreactivated DNA was refractory to T4 endo V digestion (Figure 9A), thereby showing that the photolyase reaction had proceeded to completion and that the probes were devoid of CPDs. Conversely, p422 not treated with photolyase was cleaved by T4 endo V yielding a characteristic distribution of truncated products. The UV-DRP recognized CPD-free probes and non-photoreactivated probes equally well (Figure 9B). Binding to CPD-free probes was not due to residual photolyase as photoreactivated samples incubated in the absence of nuclear extract showed no low mobility bands

(lane 5B-1). UV-DRP binding was a linear function of UV dose between 0.1 and 5.0 kJ/m^2 (see Figure 8); hence, one would expect that

removing a putative major recognition site from DNA treated with 1.5 kJ/m^2 would reduce binding significantly. Since photoreactivation had no such effect, the most straightforward conclusion is that CPDs are not major binding cues of the UV-DRP.

The next goal in this work was to assess the importance of 6-4 photoproducts in UV-DRP binding. The following experiments were done in collaboration with Mr. Zhenghuan Chen. In the absence of an enzyme that can repair 6-4 photoproducts specifically (such an enzyme has apparently been disovered very recently; refer to discussion), it was deemed useful to employ three nearly identical 69-mer oligonucleotides: T_5, T_4C, and Δ. Sequences were chosen such that adjacent pyrimidines are found exclusively in five bp "UV boxes" that reside near the center of the oligonucleotides. GC clamps on both ends of the oligomers increased the annealing efficiency but did not alter the outcome of experiments performed with these sequences. These oligonucleotides permitted an evaluation of the relative importance of CPDs, 6-4 photoproducts, and non-dimer damage in UV-DRP binding. The oligonucleotide Δ contains no adjacent pyrimidines and therefore cannot form pyrimidine dimers. The T_5 probe has a run of Ts, making it an excellent target site for CPD formation; 6-4 photoproducts form rarely at TT sequences (Mitchell *et al.*,1990; Lippke *et al.*,1981) and, hence, irradiation of this probe should yield a product that contains mainly CPDs. The T_4C sequence differs from T_5 by a single T to C transition, which provides a TC sequence following a run of pyrimidines. Such sequences are known hotspots for the formation of 6-4 photoproducts (Brash and Haseltine, 1982; Lippke *et al.*,1981). As a result, T_4C should have a much higher frequency of 6-4 photoproducts than either Δ or T_5..

Photoproduct analysis of irradiated T_5 T_4C and Δ revealed that these sequences formed the predicted distribution of photoproducts (Figure 10). T4 endo V analysis of T_5 showed that this sequence has a high frequency of CPDs mapping primarily to the 3′ TT sites in the UV box (lane 7). Conversely, 6-4 photoproducts were not detected in the T_5 oligomer as UV specific piperidine cleavage products were not observed (compare lanes 9 and 10). The T_4C sequence formed both CPDs and 6-4 photoproducts (lanes 13 and 15, respectively). CPDs were most abundant in the central TT sites, but were also evident in the flanking TT and TC dipyrimidines. The exclusive hotspot for the formation of 6-4 photoproducts was the TC site as evidenced by a UV specific piperidine cleavage product (lane 15) migrating 1 nucleotide above the corresponding C in the chemical sequencing lane (lane 17). This banding pattern was not surprising because piperidine cleavage of TC 6-4 photoproducts present on a 5′ end-labeled fragment has been shown to yield a product that has a reduced mobility relative to sequencing standards (Brash *et al.*,1985). As expected, the Δ oligomer contained neither CPDs nor 6-4 photoproducts (lanes 1-4). When irradiated UV box oligonucleotides were incubated with HeLa nuclear extracts, two low mobility protein/DNA complexes formed (Figure 11). The higher mobility complex (NS) is due to a non-damage dependent DNA binding protein, because it was observed with unirradiated probes (lanes 1 and 3). The slower migrating of the two bands (B1) is due to the UV-DRP; it was UV-specific (lane 2), and it was not formed when XP-E cells were the source of nuclear extracts (lane 4). A UV dose-response experiment was performed to compare binding of the UV-DRP to T_5, T_4C, and Δ. Results from this experiment are in Figure 12 and illustrate that T_4C was a superior substrate for the UV-DRP by a five to seven fold margin at all doses tested. In agreement with the photoreactivation studies, T_5 and Δ were equally poor

substrates. On the basis of these findings it was speculated that the major UV-DRP binding site is the 6-4 photoproduct while a cryptic non-pyrimidine dimer photoproduct(s) represents a rare or low affinity class of binding sites. Once again, there was no evidence to suggest that CPDs are recognized. If 6-4 photoproducts represent the major class of UV-DRP binding sites, then it follows that protein-bound DNA should contain these lesions. The aim of the next experiment was to determine whether 6-4 photoproducts were disproportionately represented in the material retarded in gel shift assays (UV-DRP-bound T_4C). Since a small fraction (2-5%) of the total T_4C used in a gel shift assay becomes incorporated into a protein-DNA complex, it was necessary to scale up the assays by an order of magnitude in order to generate sufficient bound DNA for analysis. Preparative gel-shift reactions were electrophoresed longer than normal to maximize separation of the damage dependent and damage independent complexes. The UV-DRP-bound DNA was purified and subjected to T4 endo V or piperidine treatment. A similar protocol was followed for unbound DNA.

A prolonged exposure of these DNAs to piperidine revealed that UV-DRP-associated material was enriched for 6-4 photoproducts mapping to the UV box sequence of T_4C (Figure 13 Top). The enrichment was substantial in that 90% of bound T_4C contained a 6-4 photoproduct (detected as an alkali-labile site) at this position relative to 20% for the unbound material (the mode of data analysis is explained in the Figure 13 legend). The major site of 6-4 photoproduct formation was at the TC sequence as evidenced by the electrophoretic mobility of the piperidine cleavage product relative to standards (not shown, refer to Figure 10). A minor cleavage product, $\sim$1 nucleotide smaller than the more abundant fragment, was also enriched in the bound population..

This fragment is possibly the consequence of a rare 6-4 photoproduct occurring between the third and fourth Ts. These findings demonstrate clearly that the UV-DRP can distinguish between DNA possessing or lacking 6-4 photoproducts. Photoproduct frequencies in bound T_5 and Δ oligonucleotides were not determined because these sequences are recognized by the UV-DRP so weakly that it was not possible to obtain sufficient material for analysis.

T4 endo V analysis of T_4C complexed, and uncomplexed, with the UV-DRP showed that free and, to a lesser extent, bound T_4C contained CPDs mapping to the UV box region (Figure 13 Bottom). As predicted from Figure 10, the internal TT sequences in the UV box were hotspots for CPD formation, and CPDs formed to a lesser extent at the flanking TT and TC sites. Interestingly, bound T_4C was impoverished by two fold for the CPD photoproducts relative to free T_4C (as evidenced by 11% degradation of T_4C by T4 endo V in the bound fraction vs. 22% degradation in the free fraction). In light of the concomitant enrichment for 6-4 photoproducts, this result may be attributable to two non-mutually exclusive phenomena. First, the UV-DRP may not bind with high affinity to a UV box containing both a CPD and a 6-4 photoproduct. Second, the presence of a 6-4 photoproduct in the UV box may significantly lower the propensity of upstream Ts to form CPDs. The UV-DRP did not appear to promote the formation of strand breaks nor AP sites in bound DNA. The UV-DRP-bound DNA exhibited the same mobility in denaturing gels as full length control DNA. The lack of AP sites in this DNA is evidenced by its resistance to the AP endonuclease activity of T4 endo V.

Figure 6. A human UV-DRP is distinct from a protein that binds to DNA modified by the anticancer drug cisplatin. End-labeled 422-bp fragment modified with *cis*-DDP at $r_b = 0.038$ was incubated in the presence of 10 μg of HeLa nuclear extract and 0.1-10 ng of unlabeled M13mp18 modified with either *cis*-DDP at $r_b = 0.041$ (lanes 3-5) or UV at 1,500 J/m^2 (lanes 6-8). Binding in the absence of competitor is shown in lane 2, and DNA incubated in the absence of extract is shown in lane 1. End-labeled 422-bp fragment treated with UV at 1,500 J/m^2 was used in lanes 9-16. Unlabeled UV-modified M13mp18 modified with *cis*-DDP was added to the reactions in lanes 14-16. Binding of the UV-modified DNA binding factor is shown in lane 10, and the labeled UV-modified 422-bp fragment incubated in the absence of extract is shown in lane 9. Binding reactions in this experiment were performed in 50 mM Na_2EDTA, which is optimal for the UV-modified DNA binding factor.

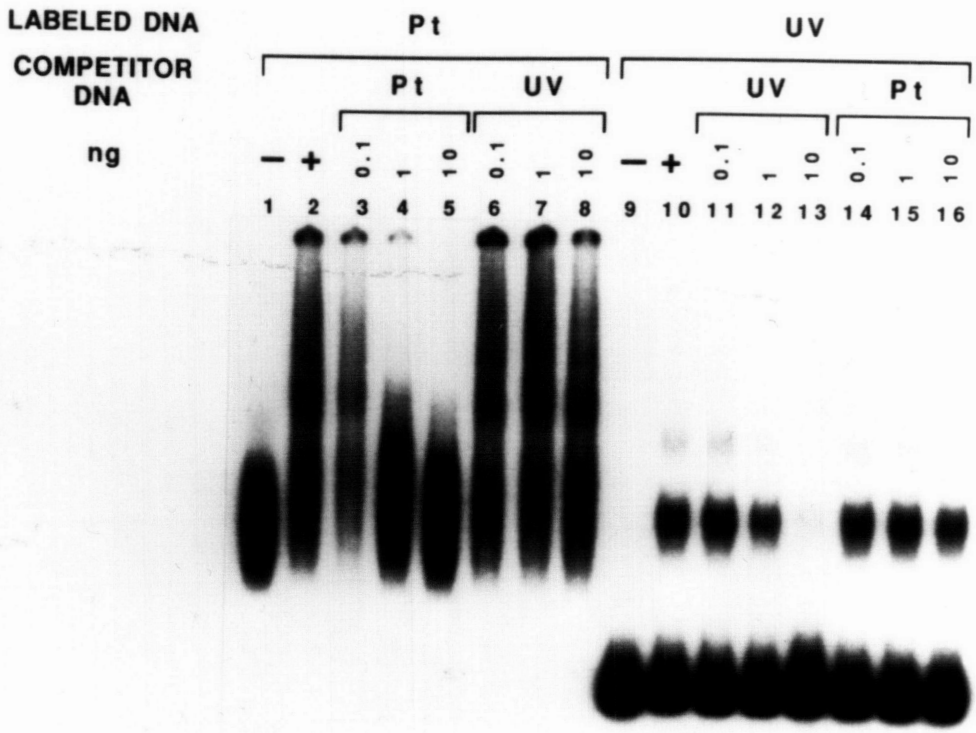

LABELED DNA
Pt
UV
COMPETITOR DNA
Pt
UV
UV
Pt
ng
– + 0.1 1 10 0.1 1 10 – + 0.1 1 10 0.1 1 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 7. UV-DRP activity in DNA repair proficient and deficient cell lines. p422 irradiated to a final UV dose of 1.5 kJ/m^2 was incubated in the presence of 10 μg of nuclear extract from various human cell lines. A: XP-A lymphoblasts (GM02250D), D: XP-D lymphoblasts (GM02253D), E: XP-E fibroblasts (GM2415B), 2: Hep G2, H: HeLa. The mixture was subjected to native PAGE and autoradiographed. Binding of the UV-DRP to irradiated probes is evidenced by the formation of two low mobility bands, B1 and B2. Note the absence of UV-DRP activity in XP-E extracts (lane E)

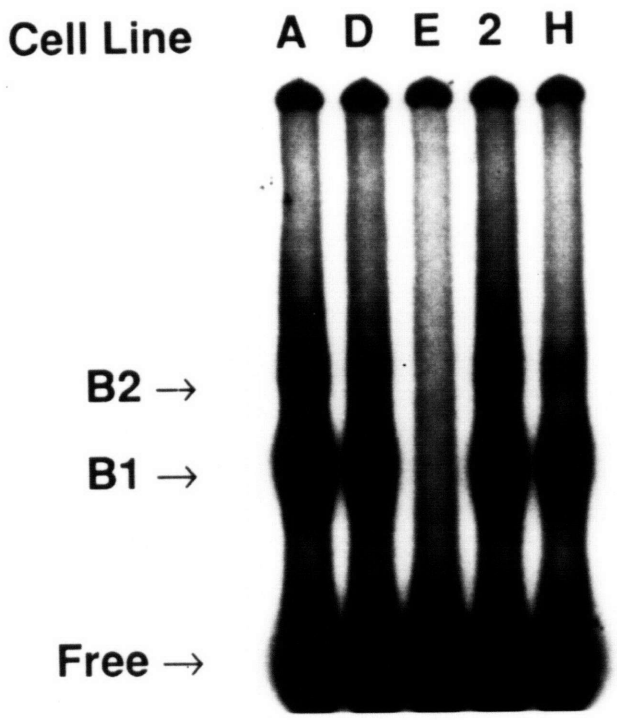

Cell Line
A
D
E
2
H
B2 →
B1 →
Free →

Figure 8. Correlation of UV-DRP binding to the frequency of CPDs and 6-4 photoproducts in irradiated p422. p422 was UV-irradiated to various degrees and subjected to either photoproduct analysis or electrophoretic mobility shift assays. CPDs were detected as T4 endo V-labile sites, whereas 6-4 photoproducts were detected as alkali-labile sites apparent after a 0.5 hr treatment with 1 M piperidine at 90°C. Quantitation of strand breaks was as described in **Material and Methods**. UV-DRP binding was measured by using analytical electrophoretic mobility shift assays. UV-DRP binding is reported in arbitrary phosphorimager units (PIU). For each curve, the individual points are the average of duplicate trials.

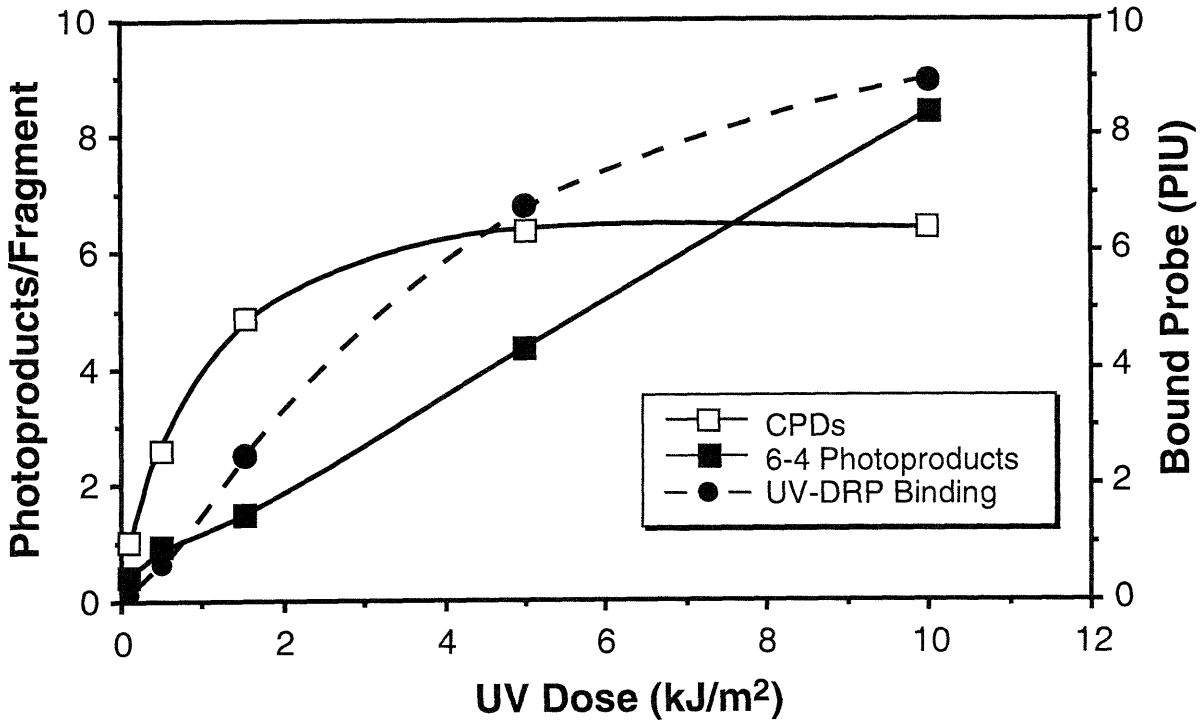

Photoproducts/Fragment
Bound Probe (PIU)
UV Dose (kJ/m2)
CPDs
6-4 Photoproducts
UV-DRP Binding
0
2
4
6
8
10
12

Figure 9. UV-DRP binding to photoreactivated p422. (A) Photoreactivation of p422. p422 irradiated to 1.5 kJ/m^2 was digested with T4 endonuclease V (Endo V) following photoreactivation by photolyase (lane A-4) or a mock treatment (lane A-2). The reaction products were subjected to denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiographed. The nested set of T4 endo V digestion products in lane A-2 is indicative of the distribution of CPDs in irradiated p422. The sensitivity of p422 to T4 endo V was precluded by a prior photolyase treatment (lane A-4). In lanes A-1 and A-3, unirradiated p422 shows the lack of nonspecific digestion by T4 endo V (the doublet that appears under the 422N band, best seen in lane A-1, is probably double stranded probe that had reannealed). These data also show that the photolyase pretreatment did not produce CPDs. (B) Binding of the UV-DRP to photoreactivated p422. Irradiated p422 from (A) that had been photoreactivated (lane B-2; this fragment was enriched for 6-4 photoproducts) or mock treated (lane B-3; this fragment contained both CPDs and 6-4 lesions) was incubated with 10 μg of HeLa nuclear extract and subjected to native PAGE. Nuclear extract was omitted in lane B-1 to show the absence of binding by residual photolyase.

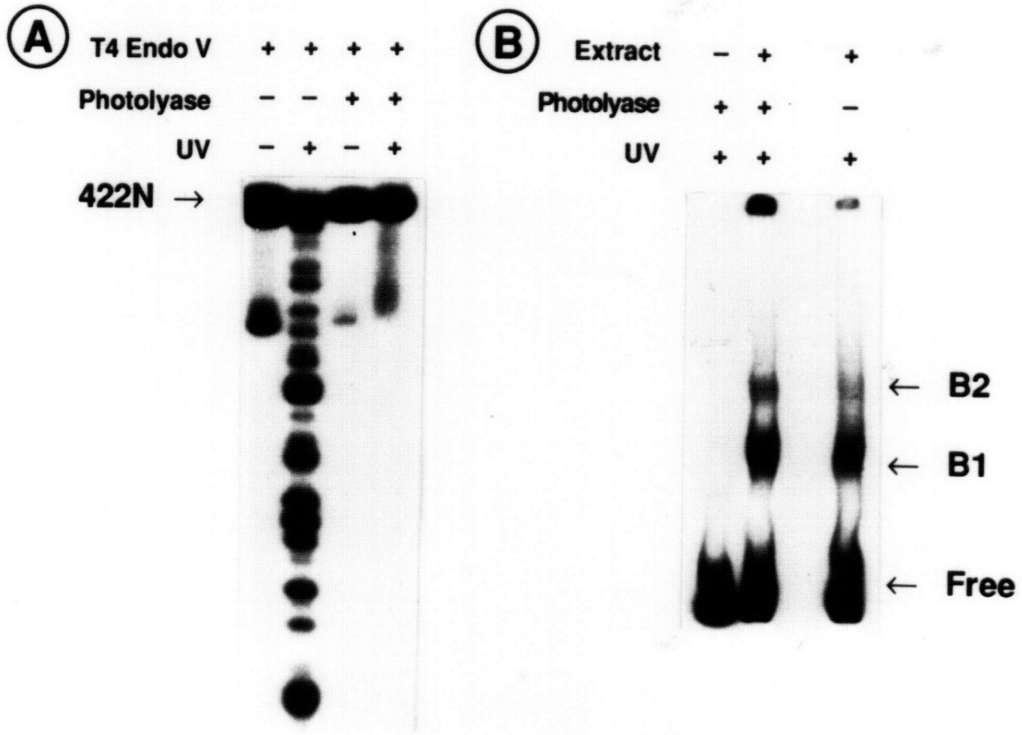

A
T4 Endo V + + + +
Photolyase − − + +
UV − + − +
422N →
B
Extract − + +
Photolyase + + −
UV + + +
← B2
← B1
← Free

Figure 10. Characterization of UV box oligonucleotides. The relevant portions of the UV box oligonucleotide sequences are shown to the right. Sixty nine-mer oligonucleotides T_4C, T_5, and Δ were designed such that adjacent pyrimidines are absent in both strands except in the "UV boxes" shown in large type near the center of each sequence. Δ contains no adjacent pyrimidines as the UV box was replaced with a purine/pyrimidine stretch. Ten bp GC clamps are present on the ends of each oligonucleotide, and the intervening DNA sequences between the GC clamps and the UV box were ATATGCGTACATGTGCG for the region upstream of (5′ to) the UV box and ACGCACGCACGTACATGTGCACGTGTAT for the region downstream. The autoradiogram to the left shows the photoproduct distribution in irradiated UV box oligonucleotides. Control and irradiated (10^4 J/m^2) UV box oligonucleotides were treated with either T4 endo V (Endo V) for 1 hr at 37°C or with 1M piperidine (PIP) for 0.5 hr at 90°C to determine the distribution of CPDs and 6-4 photoproducts, respectively. The resulting cleavage products were resolved on a DNA sequencing gel adjacent to the appropriate chemical sequencing markers. Lanes 1-6, Δ, lanes 7-12, T_5, and lanes 13-18, T_4C. T4 endo V analysis of irradiated oligonucleotides is shown in lanes 1 (Δ), 7 (T_5), and 13 (T_4C) and a similar analysis of unirradiated oligonucleotides is shown in lanes 2 (Δ), 8 (T_5), and 14 (T_4C). Analysis of piperidine treated irradiated oligonucleotides is shown in lanes 3 (Δ), 9 (T_5), and 15 (T_4C) and in lanes 4 (Δ), 10 (T_5), and 16 (T_4C) a similar analysis of unirradiated oligonucleotides is shown. C + T chemical sequencing reactions are shown in lanes 5 (Δ), 11 (T_5),and 17 (T_4C), and A + G reactions are shown in lanes 6 (Δ), 12 (T_5), and 18 (T_4C).

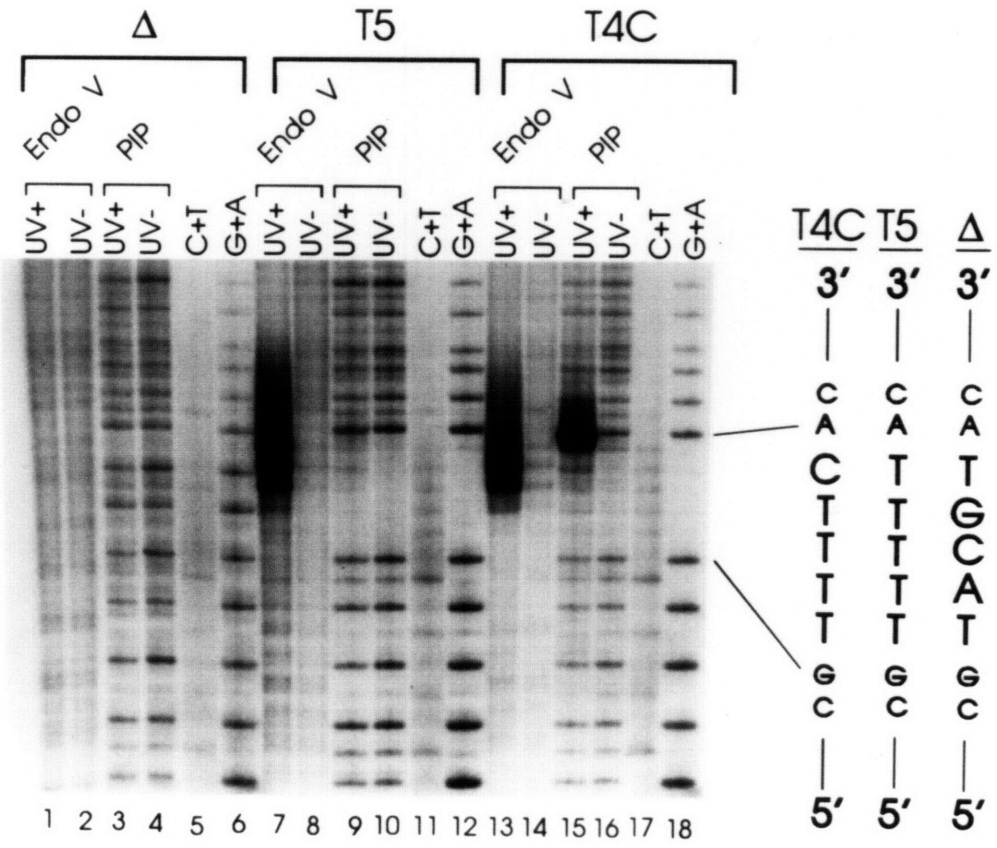

Δ
T5
T4C
Endo V
PIP
UV+
UV-
C+T
G+A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
T4C
T5
Δ
3′
5′
CACTTTTGC
CATTTTTGC
CATGCATGC

Figure 11. Binding of the UV-DRP to irradiated T_4C. T_4C, either untreated (lanes 1 and 3) or irradiated (1 kJ/m^2, lanes 2 and 4), was incubated with HeLa (lanes 1 and 2) or XP-E (GM02450D) (lanes 3 and 4) nuclear extracts and subjected to native PAGE. A damage specific low mobility band (B1) is present in the HeLa sample (lane 2) but not in the XP-E sample (lane 4). A non-damage specific low mobility band (NS) is evident in both HeLa and XP-E reactions (lanes 1 and 3). Some other minor non-damage specific bands are apparent in all four lanes; we have not pursued these activities as they are unrelated to DNA damage.

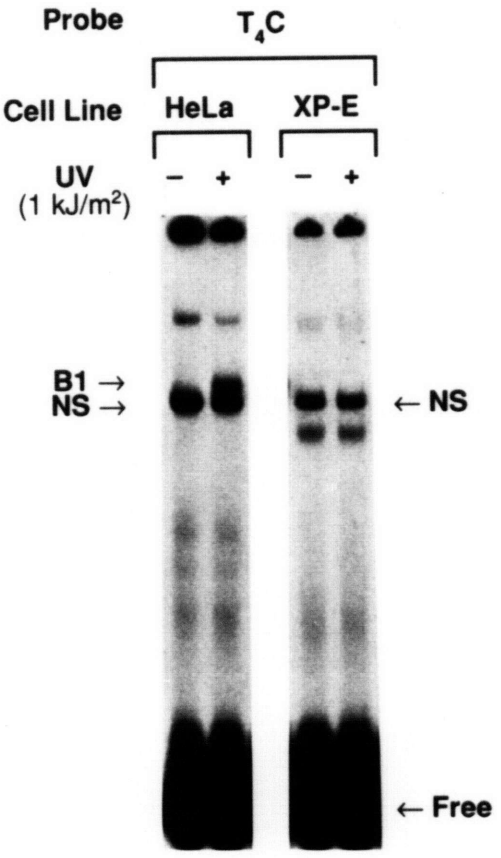

Probe
T4C
Cell Line
HeLa
XP-E
UV
(1 kJ/m2)
–
+
–
+
B1 →
NS →
← NS
← Free

Figure 12. UV dose-response analysis of UV-DRP binding to UV box oligonucleotides. UV box oligonucleotides T_4C, T_5 and Δ were irradiated to doses of 0.1-20 kJ/m^2 and incubated with 10 μg of HeLa nuclear extract. The mixtures were then analyzed by native PAGE. The amount of probe incorporated into the B1 complex (Figure 11) was quantified and is reported in arbitrary phosphorimager units (PIU).

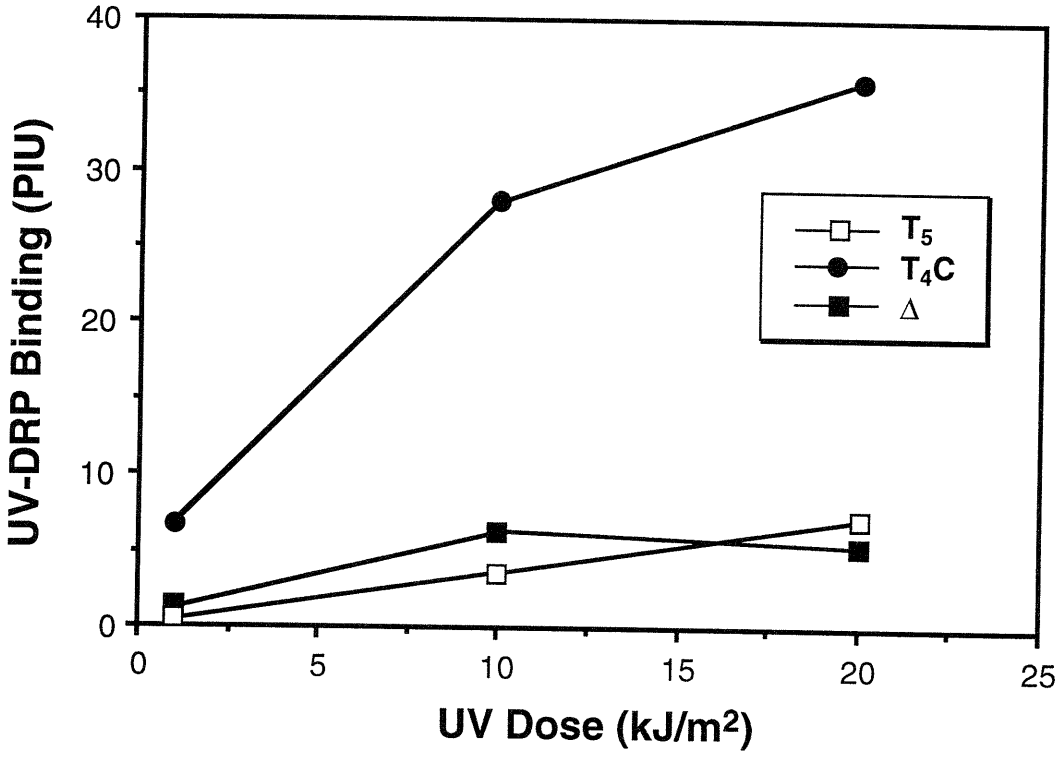
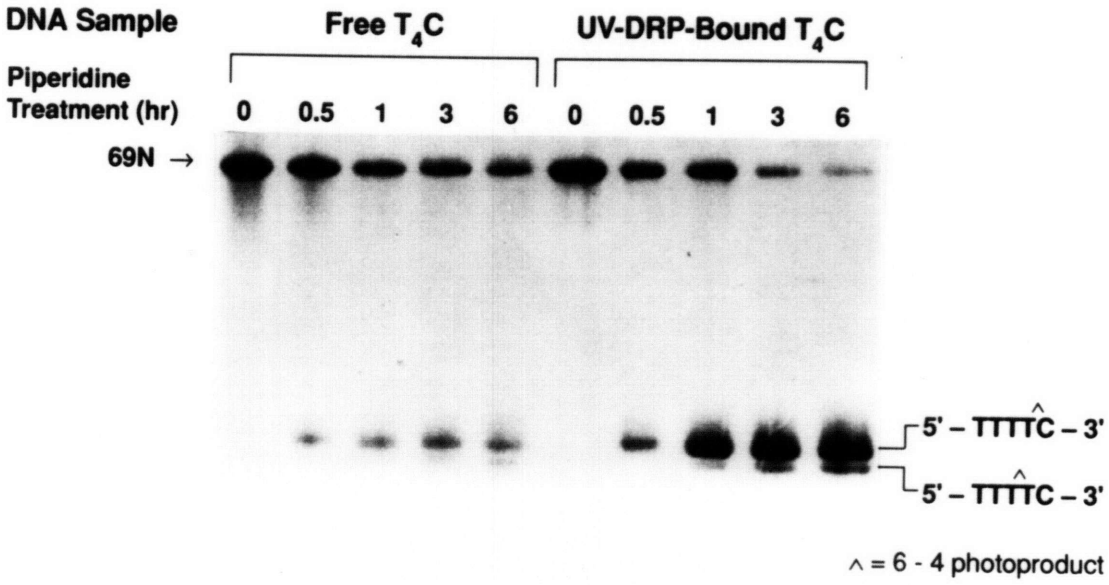
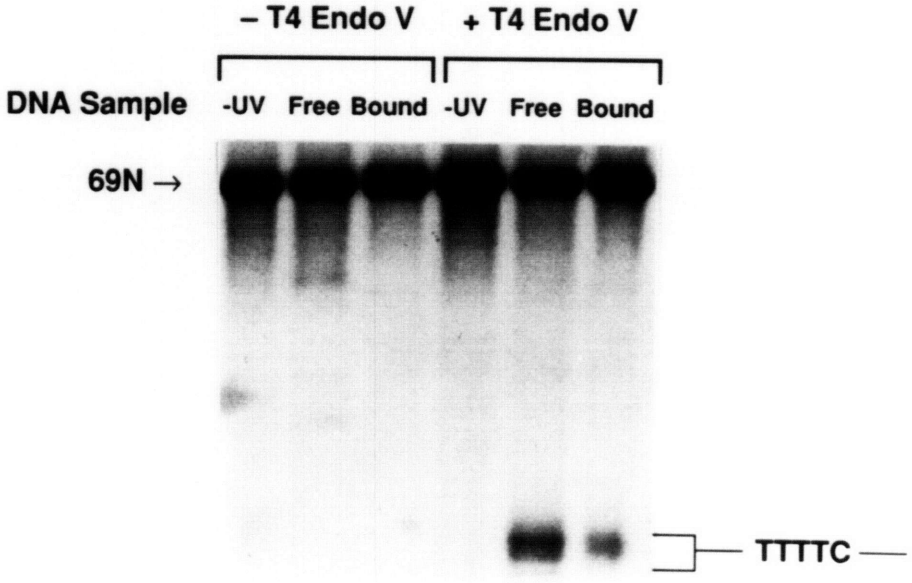

40
30
20
10
0
UV-DRP Binding (PIU)
0
5
10
15
20
25
UV Dose (kJ/m2)
T5
T4C
Δ

Figure 13. Photoproduct analysis of UV-DRP-bound T_4C. **Top.** 6-4 photoproduct analysis. T_4C, either non-protein bound (Free, lanes 1-5) or associated with the UV-DRP (Bound, lanes 6-10), was purified from a preparative electrophoretic mobility shift assay and subjected to 1 M piperidine at 90°C for 0.5-6.0 hr. Equal amounts of each reaction mixture were analyzed by denaturing PAGE. The two major degradation products map to the UV box as determined by comparison to standards (not shown, refer to Figure 10). The percentage of T_4C containing a 6-4 photoproduct in the UV box was determined by quantifying the radioactivity of the two major cleavage products after 6 hr and dividing by the amount of full length DNA (69 nucleotides; 69N) in the 0 hr lane. Nonspecific degradation of the full length probes by piperidine necessitated this mode of data analysis as it was not feasible to measure the photoproduct frequency accurately by monitoring the disappearance of full length probes. **Bottom.** CPD analysis of UV-DRP-bound T_4C. Bound and Free T_4C were purified as in A. These samples were digested by T4 endo V and subjected to denaturing PAGE (lanes 5 and 6). Equal amounts of DNA were loaded in each lane. The four major cleavage products mapped to the UV box as determined by comparison to standards (not shown, refer to Figure 10). The percentage of T_4C containing a CPD in the UV box was determined by quantifying the amount of full length DNA (69N) remaining after digestion and dividing by the amount of full length DNA in the T4 endo V-treated, unirradiated control (lane 4). In lanes 1-3, the DNAs were not digested with T4 endo V to show that the cleavage products in lanes 5 and 6 were caused by neither UV light nor UV-DRP binding.


DNA Sample
Free T_4C
UV-DRP-Bound T_4C
Piperidine Treatment (hr)
0 0.5 1 3 6 0 0.5 1 3 6
69N →
5' – TTTT^C – 3'
5' – TTT^TC – 3'
^ = 6 - 4 photoproduct


– T4 Endo V
+ T4 Endo V
DNA Sample
-UV Free Bound -UV Free Bound
69N →
TTTTC

B. DISCUSSION

1. Identification of a human UV-DRP

Several groups have reported an activity in human cell extracts that is characterized by tight binding to UV-damaged DNA. The first such study was performed fifteen years ago by Feldberg and Grossman (Feldberg *et al.*,1982). The activity was purified, but lacked enzymatic activity, had an elusive substrate specificity, and was present in each of the xeroderma pigmentosum complementation groups that were examined; however, only a subset of the XP groups was tested. For the above mentioned reasons, these investigators were at a loss to explain the significance of the UV-DRP activity. More recently Chu and coworkers apparently rediscovered the UV-DRP activity and, importantly, obtained evidence that suggested a possible role in DNA repair processes. More specifically, Chu and Chang (Chu and Chang, 1988) discovered that the UV-DRP activity was absent in two cell lines from XP group E (XPE) patients. The UV-DRP activity identified in my thesis research is similarly lacking in XPE cells (Figure 7) and is most likely identical to the protein studied by Chu and coworkers. My work focused on the identification of the substrate specificity of this protein.

A cisplatin-damaged DNA recognition activity (*cis*-DDP DRP) has been reported by Dr. Chu's group and our laboratories. This factor, which was shown to be proteinaceous, reduces the electrophoretic mobility of platinated DNA probes (Chu and Chang, 1988; Donahue *et al.*,1990). Competition experiments done by me showed that the UV-DRP and *cis*-DDP DRP activities are due to different proteins (Figure 6). Moreover, the *cis*-DDP DRP activity was present in XPE cells further establishing that it is

independent of the UV-DRP activity. It is likely that the *cis*-DDP DRP activity is multifactorial in nature, whereas the UV-DRP is most likely a single polypeptide. To date, at least seven eukaryotic proteins have been identified that bind to cisplatin-modified DNA. These proteins include HMG1 and HMG2 (Pil and Lippard, 1992; Hughes *et al.*,1992), SSRP1 (Bruhn *et al.*,1992), IXR1 (Brown *et al.*,1993), hUBF (this work, chapter V), SRY (E. Trimmer, personal communication), and human single strand binding protein (hSSBP) (Clugston *et al.*,1992). With the exception of hSSBP, all of these proteins contain at least one region of homology to the abundant chromosomal protein HMG1. This region, the HMG box, is known to be a novel DNA binding domain (Jantzen *et al.*,1990). Some HMG proteins, including hUBF (Jantzen *et al.*,1990) and SRY (Harley *et al.*,1992), are most likely transcriptional regulators and others, such as HMG1 and HMG2 (Einck and Bustin, 1985), have a more elusive function that is likely to be related to maintaining chromosomal structure; however, there is no evidence to suggest that any protein with an HMG1 homology is involved in DNA repair. By contrast, hSSBP is known to be involved in human nucleotide excision repair (Coverley *et al.*,1991). It is not certain which, if any, of these proteins is(are) responsible for the *cis*-DDP DRP activity, but more than one may be involved. It thus appears that the electrophoretic mobility shift assays with cisplatin-modified DNA can detect both repair proteins and proteins that fortuitously recognize kinked DNA structures. The role of these proteins in cisplatin toxicity is explored further in chapter V.

As alluded to earlier, the UV-DRP activity is likely to be the consequence of a single polypeptide. Two groups have reported the purification of the UV-DRP from human and monkey sources (Hwang and Chu, 1993; Abramic *et al.*,1991). The proteins appear to be identical based

on both molecular weight (125 kDa) and their ability to recognize UV-damaged DNA. However, some differences are noteworthy. The monkey protein is apparently a homodimer in solution and exhibits only a 17-fold preference for UV-treated double stranded DNA over unmodified DNA. The human protein behaves as a monomer in glycerol gradients and has a striking (10,000-fold) preference for UV-damaged DNA. These incongruities may, in part, reflect differences in the assay conditions and the experimental designs in the two studies. These proteins are also most likely identical to a UV-DRP purified from human placenta by Feldberg several years ago (Feldberg *et al.*,1982).

The UV-DRP is presumably a component of a versatile DNA repair complex. The results reported herein concur with those of others that the UV-DRP is lacking in some XPE cell lines (Figure 7). Since each XP group is deficient in the incision step of DNA excision repair, the UV-DRP may be part of the preincision complex, possibly at the level of damage recognition, a role analogous to that of the E. coli UvrA protein (Orren and Sancar, 1989). The UV-DRP is induced 2-4 fold above constitutive levels in primate cells pretreated with UV or other agents that block DNA polymerases or cause DNA damage (Protic *et al.*,1989). These cells also reactivate UV-damaged plasmids with increased efficiency. The UV-DRP is overly expressed in human cells that display resistance to the anticancer drug cisplatin; these cells also exhibit an enhanced capacity to reactivate cisplatin-damaged plasmids (Chu and Chang, 1990). This result seemed at first to be paradoxical in light of the competition experiment of Figure 6 which showed that the UV-DRP activity did not recognize cisplatin-modified DNA with high affinity. Cisplatin adducts are apparently UV-DRP substrates, although the relative affinity for UV-damaged DNA is much higher (Chu and

Chang, 1988).

The UV-DRP is most likely the defective/missing protein in XPE cells. A caveat exists in that the UV-DRP activity is found in some XPE cell lines derived from Japanese patients (Kataoka and Fujiwara, 1991; Keeney *et al.*,1992). It is possible, however, that these recently tested cell lines contain a form of the UV-DRP that is defective in a function other than DNA binding. Clearly, caution must be exercised until more definitive genetic evidence is obtained. A cDNA clone has recently been isolated that apparently encodes the monkey UV-DRP (Takao *et al.*,1993). The predicted amino acid sequence shows homology to a slime mold protein of unknown function, but no homology was found to any proteins, including DNA repair proteins, of known function. Interestingly, northern analysis showed that the XPE cell lines defective in UV-DRP activity expressed a cognate mRNA of the correct length. DNA sequence analysis of UV-DRP alleles in the various XPE cell lines should definitively establish if the UV-DRP is the XPE protein.

2. Binding specificity of the UV-DRP

The major goal of the work presented in this chapter was to determine which, if either, of two UV-induced photoproducts is a binding site for the human UV-DRP. We have found that this DRP binds almost exclusively to 6-4 photoproducts and has no apparent affinity for CPDs. Several lines of evidence support this conclusion. UV-irradiated DNA rendered devoid of CPDs by enzymatic photoreactivation showed no loss of affinity for the UV-DRP (Figure 9). Furthermore, experiments with UV box oligonucleotides

showed that T_4C sites, but not T_5 sequences, formed high affinity UV-DRP binding sites upon irradiation (Figure 12). Subsequent photoproduct analysis revealed that the UV-DRP-bound T_4C oligonucleotides were enriched by 4-5 fold for 6-4 photoproducts as compared to the unbound fraction (Figure 13). Consistent with these findings was the observation that the level of UV-DRP binding to a random sequence probe correlated with the frequency of 6-4 photoproducts, but not CPDs (Figure 8).

These findings both support and extend what has been reported in the literature with regard to the binding specificity of the UV-DRP. The first UV-DRP studies in the late 1970s were unable to establish the nature of the high affinity UV-DRP binding site although photoreactivation studies similar to those presented here also suggested a minimal role for CPDs. 6-4 photoproducts were essentially unknown entities at that time and hence were not considered as potential binding sites. Studies with the primate UV-DRP similarly ruled out CPDs and also showed that a synthetic oligonucleotide containing a single CPD at a TT sequence was not bound (Hirschfeld *et al.*,1990). The authors of this work speculated on the basis of this indirect evidence that the 6-4 lesions were possibley high binding sites for the UV-DRP. The results reported herein establish directly that the 6-4 lesion is indeed the primary UV-DRP binding site. Recent studies with the human UV-DRP also concluded that CPDs at TT sequences are not UV-DRP binding sites; however, photoreactivation studies on UV-damaged probes that had been irradiated to a range of final doses suggested that minor CPD species at dipyrimidine sequences other than TT may be substrates (Hwang and Chu, 1993). The results are not at odds with the results presented here since the affinity of the UV-DRP for non-TT CPDs was not rigorously tested.

Both CPDs and 6-4 photoproducts are believed to distort the DNA helix and hence the UV-DRP does not simply seem to be recognizing general DNA distortion with high affinity. It is unclear at present how the UV-DRP differentiates between these lesions, but chemical and structural data suggest that the two types of photoproducts induce dissimilar helix irregularities. Hydrogen bonding moieties critical to base pairing are chemically disrupted in 6-4 photoproducts but not in CPDs (Franklin *et al.*,1985), and NMR studies with dinucleotides suggest that 6-4 photoproducts, in which the pyrimidine and pyrimidone rings occupy perpendicular planes, cause a perturbation in DNA structure more severe than that of CPDs (Franklin *et al.*,1985; Rycyna and Alderfer, 1985; Kemmink *et al.*,1987a). Whether the grossly distorted structure observed for the 6-4 photoproduct dinucleotide is also manifested as a similar alteration in double stranded DNA is unknown. Circle formation studies with duplex DNA containing CPDs shows that these lesions bend ($\sim$30°) and probably unwind (14-20°) the helix (Husain *et al.*,1988). However, more recent evidence in which the CPD-induced bend was estimated by the reduced electrophoretic mobility of DNA fragments containing phased CPDs suggests that the bend angle is much lower, $\sim$7° (Wang and Taylor, 1991). It is possible that subtle differences in the degree of helix bending, unwinding, and melting induced by the two classes of photoproducts mediate differential recognition by the UV-DRP. Such differences have been hypothesized to explain the differential recognition of various structurally similar cisplatin-DNA crosslinks by a human cisplatin-DRP (Donahue *et al.*,1990) and the chromosomal protein HMG-1 (Pil and Lippard, 1992). It is also noteworthy that competition experiments have shown that cisplatin modified DNA competes for UV-DRP binding, but only 10-fold more efficiently than unmodified DNA (Chu and Chang, 1988), and that some

CPDs may be recognized weakly (Hwang and Chu, 1993). The UV-DRP most likely recognizes a variety of DNA distortions, but it is clear that 6-4 photoproducts are recognized with the highest affinity of any lesion yet tested.

In general it is not understood how DNA excision repair enzymes recognize a diverse array DNA damage. This observation stems mainly from studies with the versatile E. coli uvrABC excision repair complex. This repair system recognizes and repairs a wide spectrum of DNA modifications. Recognized lesions include those likely causing major perturbations of the helix, such as 6-4 photoproducts and benzo[α]pyrene diol epoxide N-2-guanine adducts, as well as damages producing more subtle effects, such as O^6-methylguanine. Indeed, damage recognition by UvrABC can not be ascribed to any one class of DNA distortion. All kinked DNA structures, for example, are not recognized, nor are mismatched base pairs. Lesions that disrupt base pairing however, such as O^6- methylguanine, are recognized With this precedent in mind, it is possible that the specificity of the UV-DRP in damage recognition will not correlate with the severity of a single type of DNA distortion alone. It is more likely that the efficiency of damage recognition is dictated by the number of structural determinants that are disrupted by a particular lesion. Lesions that both kink DNA and disrupt base pairing, for example, may be better substrates than those that only disrupt base pairing. More subtle changes, such as effects on DNA dynamics are also likely to be involved. This subject has been extensively reviewed by Van Houten (Van Houten, 1990).

The UV-DRP also has detectable affinity for non-pyrimidine dimer UV damage as evidenced by the observation that irradiated oligonucleotides

lacking adjacent pyrimidines showed a weak, but significant, level of binding to the UV-DRP (Figure 8). The modest binding of the UV-DRP to these probes suggests that the non-dimer photoproduct(s) is either rare or recognized with low affinity. The chemical nature of the photoproduct(s) responsible for this activity is unknown, but interstrand crosslinks are not likely candidates because they were not detected by denaturing PAGE. Pyrimidine glycols and pyrimidine hydrates may be UV-DRP binding sites, but we have yet to address this issue. The relative contribution of this unidentified photoproduct(s) to overall UV-DRP binding in random DNA sequences is likely to be considerably lower than in the case of the UV box oligonucleotides in which sequences that permit 6-4 photoproduct formation are underrepresented.

3. The UV-DRP may contribute to the efficient repair of 6-4 photoproducts

The binding specificity of the UV-DRP is of possible importance to the mechanism by which mammalian DNA repair complexes remove 6-4 photoproducts more efficiently than CPDs. The relative repair rates of these lesions in mammalian cells (Mitchell *et al.*,1985; Mitchell *et al.*,1990; Thomas *et al.*,1989) and cellular extracts (Wood, 1989) have been shown to be biased by a factor of 4-12 in favor of repair of the 6-4 photoproducts. The protein(s) responsible for this differential repair are currently unknown. The UV-DRP could play an active role in the physical delivery of repair complexes to sites in DNA containing 6-4 photoproducts. Acting in this manner the UV-DRP would function analogously to the E. coli uvrA protein. This model, however, is inconsistent with the XP-E phenotype. XP-E cells exhibit a reduced, but still significant, level of DNA repair and UV resistance when compared to normal cells (Lehmann *et al.*,1977; Andrews *et*

al.,1978), suggesting that a non-vital component of the repair machinery is defective in this complementation group. Other XP groups have a more severe repair deficiency and are likely to be lacking, or have defects in, proteins as essential to DNA repair in mammalian cells as uvrA is to the *E. coli* excision nuclease.

Alternatively, the UV-DRP may, as hypothesized previously (Patterson and Chu, 1989), increase the efficiency of excision repair by the mode proposed for the *E. coli* photolyase, which enhances the rate of the UvrABC catalyzed excision of CPDs *in vitro* (Sancar *et al.*,1984). By this model, the UV-DRP would occupy DNA regions containing 6-4 photoproducts and act as an antenna to recruit repair complexes. The antenna function of the UV-DRP could be mediated by protein-protein interactions, or by inducing structural changes in the DNA (e.g., bending and/or unwinding) that increase accessibility to repair proteins. This model is consistent with the XPE phenotype because it predicts a repair-enhancing role that may increase the overall rate of repair but is not necessary for the repair complex to function with near normal efficiency. The observation that CPDs are not bound by the UV-DRP does, however, contradict the hypothesis that this protein is an evolutionary descendent of yeast photolyase, an enzyme that photoreactivates CPDs (Patterson and Chu, 1989).

Interestingly, it has been reported recently that two UV-DRP activities are present in *Drosophila melanogaster* (Todo *et al.*,1993). One of these proteins recognizes only CPDs and does, indeed, have the ability to photoreactivate these lesions. The second UV-DRP does not bind to CPDs and, in the presence of fluorescent light, apparently catalyzes the conversion of 6-4 photoproducts to another structure that no longer has the hallmark qualities (e.g., alkali lability) of 6-4 lesions. It remains unclear if this

photoreactivating activity converts 6-4 photoproducts to normal bases or to another type of lesion. The human UV-DRP which, as shown here, also binds selectively to 6-4 photoproducts apparently lacks photoreactivation capabilities (Todo *et al.*,1993). It is tempting to speculate that the human and Drosophila 6-4 photoproduct binding proteins are evolutionarily related, but the human protein has lost its ability to photoreactivate and serves a non-vital role in enhancing the repair of 6-4 lesions by the excision nuclease.

If the model based on the photolyase analogy presented above is valid, then it also follows that XP-E cells should lack the repair bias in favor of 6-4 photoproducts that exists in normal cells. The literature contains data showing that the repair of 6-4 photoproducts is deficient in XP-E cells (Mitchell, 1988). A deficient repair of CPDs is also evident in these cells, but the extent of the deficiency is unclear as conflicting data have been reported (Mitchell, 1988; Zelle and Lohman, 1979). Further repair studies are clearly required in order to test the proposed model conclusively.

The data cited above suggest that XP-E cells may be somewhat deficient in CPD repair. The UV-DRP may be involved indirectly in CPD repair by facilitating the release of repair complexes from 6-4 photoproducts subsequent to the DNA incision event. This process would effectively increase the turnover number for the repair complex and lead to the enhanced repair of all photoproducts. A similar model was proposed to explain the stimulation of UvrABC activity by photolyase (Sancar *et al.*,1984). This model is not necessarily consistent with the UV-DRP having a role in the biased repair of 6-4 photoproducts and may necessitate the existence of other protein(s) that mediate this phenomenon. Recent results with the putative XPA correcting factor (XPAC) reveal that this protein binds

selectively to UV-damage DNA (Robins *et al.*,1991); however XPAC, unlike the UV-DRP, does not demonstrate a dramatic preference for UV-damaged versus undamaged DNA. It is possible that XPAC also binds selectively to 6-4 photoproducts and contributes to the selective repair of these lesions. Since the XPA phenotype is quite severe relative to that of XPE, the XPA protein may have a function homologous to UvrA in damage recognition.

In summary, the results show that a human UV-DRP has 6-4 photoproducts as a primary high affinity binding site in irradiated DNA. We showed that the factor does not demonstrate any significant binding to CPDs, although an as yet unknown form of non-pyrimidine dimer damage seems to be a rare or low affinity binding cue. It is possible that this protein mediates the efficient repair of 6-4 photoproducts in UV-damaged DNA. Alternatively, the UV-DRP could be of more general importance in the repair of UV damage by increasing the rate at which repair complexes dissociate from 6-4 photoproducts subsequent to DNA incision. The methodologies we have employed could be of broad use for determining the binding specificities of other DRPs.

C. CONCLUSIONS

The binding specificity was defined of a human ultraviolet light-damaged DNA recognition protein (UV-DRP), the activity of which is absent in some xeroderma pigmentosum complementation group E cells. UV-DRP binding to irradiated DNA was better correlated to the level of alkali-labile pyrimidine (6-4) pyrimidone photoproducts (6-4 photoproducts) than to the level of cyclobutane pyrimidine dimers (CPDs). Furthermore, UV-damaged DNA rendered devoid of CPDs by enzymatic photoreactivation showed no loss of affinity for the UV-DRP suggesting that these lesions are not high affinity binding sites; these data are consistent with other reports on this protein. A major role for 6-4 photoproducts in UV-DRP binding was suggested in studies showing that irradiated oligonucleotides containing a T_4C UV box sequence, which efficiently forms a TC 6-4 photoproduct, was a superior substrate for the UV-DRP when compared to a similar irradiated oligonucleotide having a T_5 sequence. The latter sequence forms CPDs at a much higher frequency than 6-4 photoproducts. T_4C-containing oligonucleotides complexed with the UV-DRP were separated from the unbound oligonucleotide fraction and the frequencies of 6-4 photoproducts in the two DNA populations were compared. The UV-DRP-bound fraction was highly enriched for the 6-4 lesion over the unbound fraction supporting the conclusion that 6-4 photoproducts are the principal binding cues for the UV-DRP. The binding specificity of the UV-DRP may be of importance to the mechanism by which mammalian repair complexes selectively excise 6-4 photoproducts.

D. FUTURE EXPERIMENTS

1. Role of the UV-DRP in the repair of 6-4 photoproducts

The repair of CPDs and 6-4 photoproducts in XPE cells needs to be tested rigorously. In normal cells, 6-4 photoproducts are repaired much more efficiently than CPDs, and it is of interest to see if the same is true in XPE cells that lack the UV-DRP. The repair bias in favor of 6-4 photoproducts may not exist in XPE cells that lack the UV-DRP activity. In addition, repair studies should be performed with XPE cells that possess the UV-DRP activity to determine if the presence or absence of UV-DRP binding activity alters the repair spectrum. Presumably, XPE cells that exhibit UV-DRP binding activity have a form of the UV-DRP that is altered in a domain unrelated to damage recognition. Binding of the UV-DRP may be unproductive since a downstream function, such as interactions with other repair proteins, is lacking. If this is the case, then UV-DRP binding may actually impede repair complexes from excising 6-4 photoproducts.

2. Structural studies

Structural features of the [UV-DRP - 6-4 photoproduct] complex should be elucidated by footprinting studies. We have attempted these studies but the lack of pure protein was prohibitive. Many other interesting studies are apparent but also require either the UV-DRP gene or purified UV-DRP protein. Until one or both of these reagents is obtained further studies are rather limited.

V. DNA-PLATINUM COMPLEXES ARE MOLECULAR DECOYS FOR THE NUCLEOLAR TRANSCRIPTION FACTOR hUBF

A. RESULTS

1. Identification of human upstream binding factor as a damage recognition protein for cisplatin modified DNA.

Several eukaryotic proteins homologous to the high mobility group chromosomal protein HMG1 bind to DNA modified by clinically effective platinum anticancer drugs, including *cis*-diamminedichloroplatinum(II) (cisplatin) (Hughes *et al.*,1992; Bruhn *et al.*,1992; Pil and Lippard, 1992; Brown *et al.*,1993). The existence of this family of proteins has suggested novel mechanisms by which the efficacy of platinum drugs could be mediated (Donahue *et al.*,1990). The current models include (1) that protein-bound adducts would be shielded from DNA repair enzymes and (2) that the sequestered proteins would be unable to perform their natural functions.

Evidence in support of the first model was suggested by genetic studies in yeast (Brown *et al.*,1993). More specifically, a yeast cDNA encoding an HMG box protein that binds specifically to cisplatin modified DNA was isolated, and a strain was constructed in which both copies of this gene were disrupted. The gene is referred to as *IXR1* (intrastrand crosslink recognition). Although the in vivo function of the IXR1 protein has not been reported, it appears that it is not essential as the knockout strain (*ixr1*) was viable. Toxicity assays showed that the *ixr1* was two-fold resistant to cisplatin relative to the wild type strain. However, the sensitivity of *ixr1* to *trans*-diamminedichloroplatinum(II) (*trans*-DDP) and ultraviolet irradiation was unchanged. Furthermore, *ixr1* accumulated three-fold fewer cisplatin adducts than the wild type strain after a single dose of the drug. These

results are consistent with a mechanism by which the IXR1 protein shields cisplatin adducts from repair enzymes. The model is by no means established, however, since it is also possible that the uptake of cisplatin is reduced in *ixr1*.

The second model for the role of HMG box proteins in the mechanism of cisplatin proposes that essential HMG box proteins are sequestered by cisplatin adducts. The adduct-bound proteins are unable to perform a critical cellular function. Early southwestern blot and gel mobility shift studies performed by Drs. Jeffrey Toney and Brian Donahue showed that a human protein of ~97 kDa binds selectively to cisplatin modified DNA (Toney *et al.*,1989; Donahue *et al.*,1990). Ms. Xiaoquan Zhai repeated the southwestern experiments using high resolution gradient gel electrophoresis and found that the 97 kDa species resolved into a doublet of 97 kDa and 94 kDa. I proposed that the protein responsible for this doublet was the nucleolar transciption factor human upstream binding factor (hUBF). hUBF contains 4-6 regions of homology to HMG1 (Jantzen *et al.*,1990) and exists as both 97 kDa and 94 kDa species owing to alternative splicing events (Chan *et al.*,1991). hUBF is a critical positive regulator of rRNA synthesis (Bell *et al.*,1988). To confirm that hUBF binds selectively to cisplatin modified DNA, Ms. Zhai performed southwestern analysis on in vitro translated hUBF. The results showed that hUBF binds to cisplatin modified DNA but not to unmodified DNA or DNA treated with the clinically ineffective compound *trans*-DDP. We proposed that cisplatin adducts may act as molecular decoys for hUBF and thereby disrupt the synthesis of rRNA. Since rRNA synthesis is critical for proliferating cells (Tata, 1968), this mechanism, which I have termed "transcription factor hijacking", could possibly contribute to the antitumor efficacy of cisplatin.

2. Binding of hUBF to rRNA promoter sequences

In order to test the proposed transcription factor hijacking mechanism, it was first necessary to compare the affinity of hUBF for the rRNA promoter and a cisplatin adduct. The affinity constant (also referred to as the dissociation constant (K_d)) for the hUBF - rRNA promoter interaction had yet to be determined by groups studying RNA polymerase I transcription factors. A collaboration was established with Dr. Hans-Michael Jantzen of Dr. Robert Tjian's laboratory. These coworkers provided me with purified hUBF and with the gene encoding this transcription factor. hUBF binds to a large, GC-rich region upstream of the start site for rRNA transcription. The region contains two genetically-defined elements (the CORE and upstream control element (UCE)) that are critical for RNA polymerase I initiation (Haltiner *et al.*,1986). hUBF - promoter interactions are not efficiently detected by gel mobility shift assays. Binding is, however, detected by DNase I footprinting analysis. Upon the binding of hUBF to DNA fragments containing the rRNA promoter, a region in the UCE (positions -75 to -115) is protected from DNase I. In addition to the protected region, position -95, which is centered within the protected region, becomes hypersensitive to the nuclease as does position -21 in the CORE element (Bell *et al.*,1988); thus the DNase I footprinting assay provides a useful quantitative tool for measuring binding constants.

The K_d is defined as the **equilibrium** concentration of uncomplexed protein when 50% of the DNA binding sites are occupied.

K_d = [DNA][PROTEIN]/[DNA-PROTEIN] (Eq.1)

In many cases of protein-DNA interactions, one assumes that the concentration of free protein at equilibrium [PROTEIN] is equal to the total amount of protein added to the binding reaction. Since the concentration of DNA-protein complexes at equilibrium [DNA-PROTEIN] is generally much lower than the total concentration of protein added, this assumption is often valid. The validity of this assumption is determined by two parameters: (1) the K_d and (2) the concentration of the DNA probe in the reactions. Point (2) is limited by the specific activity of the probe being used. If the DNA probe concentration is well below the K_d, then the assumption that total protein = free protein is a good one. If the probe concentration exceeds the K_d then a significant fraction of the total protein added is bound and the K_d must be estimated by subtracting the concentration of bound protein from the total protein concentration. This method, however, that the stoichiometry of binding is known and, furthermore, that the protein concentration can be measured with high accuracy. The latter is often difficult to achieve since the concentrations of purified transcription factors are often low and are determined by techniques, such as silver staining, that are only semi-quantitative.

Initial attempts at determining the K_d of the hUBF-promoter interaction revealed that the affinity was sufficiently high to make it impossible t make the simplifying assumption that total protein = free protein at the probe concentration being used. The probe concentration in these studies was 50 pM, and the hUBF concentration required to give half-maximal binding was 100 pM. Hence, it was clear that protein excess was not being achieved. The stoichiometry of binding is not known, and the concentration of hUBF is estimated roughly by silver staining. For these reasons, it did not seem feasible to use the experimentally simpler "subtraction" method to determine

the K_d. It was necessary to reduce the concentration of DNA in the binding reactions so that protein excess could be achieved.

Several parameters of the binding reaction were altered to achieve protein excess. The goal of these experimental manipulations was to lower the concentration of the probes below 1 pM, while maintaining at least 1,000 cpm of ^{32}P in each reaction; this value is the lower limit of radioactivity that can be detected in a footprinting experiment. The major change from the conventional method of binding analysis was to 3' end label the footprinting probes by using the Klenow fragment of DNA polymerase I in the presence of three α-^{32}P dNTPs (dATP, dCTP, and dGTP). The polymerase step filled in, at least in part, the underhang on the probes, which had four base 5' overhanging ends. This procedure can generate a probe that contains as many as four molecules of ^{32}P. It should be noted that this procedure proceeded to 50-80% completion, and required that radiolabeled nucleotides be used on the day they were manufactured. A specific activity of at least 12,000 ci/mMole was obtained routinely. By contrast, the standard labeling method, 5' end labeling with polynucleotide kinase, incorporates only one ^{32}P per probe molecule. The second experimental parameter modified for this experiment was to increase the total reaction volume was increased, and the total number of cpm used was decreased. Taken together, these changes allowed the probe concentration in footprinting reactions to be reduced from 20 pM to 0.7 pM.

An hUBF - promoter binding isotherm was constructed under conditions of protein excess by using the high specific activity probes described above. Preliminary time course studies showed that the binding equilibrium was established under standard binding conditions. At a fixed

concentration of hUBF, no significant change in binding was observed between 5 and 100 min incubations. The standard 10 min incubation was deemed suitable for these studies. The binding isotherm is shown in Figure 14, and reveals that hUBF binding increases sharply over a narrow concentration range (7-78 pM hUBF)[2]. Since low amounts of radioactivity were used in this experiment, it is somewhat difficult to visualize the DNase I protection in the -115 to -75 region of the (UCE). What is quite clear, however, is the enhanced cleavage that occurs at position -95 as the result of hUBF binding. The intensity of the -95 band was quantified by PhosphorImager analysis to generate the binding curve. The enhanced cleavage at position -21 in the CORE element is not shown in this figure, but the appearance of this enhanced cleavage follows a similar protein dependency as the enhanced cleavage at -95. The high affinity nature of this interaction is evidenced by the low concentration of hUBF required to give half-maximal binding (18 pM hUBF).

The hUBF-promoter interaction also appears to be cooperative. A cooperative interaction between DNA and protein results in a steep binding isotherm. In a cooperative binding isotherm the fraction of DNA bound (Y) increases from 0.1 to 0.9 over a narrow range (within one order of magnitude) of protein concentrations. This is clearly the case for hUBF-rRNA promoter interactions. The data yield a Hill constant of 2.7, which also suggests cooperativity. Hill constants greater than 1 indicate positive cooperativity. It is also noteworthy that the data cannot be fit to equation

[2]It should be noted that at the higher hUBF concentrations, the binding appears to decrease. This is a common artefact of DNase I footprinting. At high protein concentrations, nonspecific binding globally inhibits DNase I and diminishes the specific footprinting pattern. In some experiments this problem was overcome by increasing the DNase I concentration at some hUBF concentrations.

(1), which does not take into account cooperativity. The data fit better to an equation that describes cooperative binding of two monomeric protein species to two DNA binding sites and assumes that the microscopic binding constants are similar; even this curve, however, is not as steep as the experimentally determined hUBF-promoter binding isotherm. This observation may reflect difficulties and assumptions associated with measuring binding by DNase I footprinting. Alternatively, the binding of hUBF to the promoter may be more complicated. hUBF dimers, for example, may be the ultimate DNA binding species. Consequently, the monomer⇆dimer equilibrium would be coupled to the DNA binding equilibrium. In this type of multiple-equilibria system, binding isotherms can be extremely steep as evidenced by the binding of phage P22 Arc repressor tetramers to a recognition sequence (Brown and Sauer, 1993).

The finding that hUBF apparently binds cooperatively to the rRNA promoter lends insight into the mechanisms by which rRNA transcription is regulated. Also, importantly, it suggests that small changes in hUBF concentrations within cells can greatly alter promoter occupancy. This effect is directly relevant to a mechanism by which cisplatin-DNA adducts act as molecular decoys to divert hUBF from rDNA sequence elements.

3. Characterization of hUBF-cisplatin adduct interactions

The southwestern analysis described above showed that hUBF, like several other HMG box proteins, binds selectively to cisplatin modified DNA. Southwestern analysis is a useful qualitative technique, but is not a very effective quanititative tool for measuring binding constants. Gel mobility shift assays with hUBF are ineffective at monitoring promoter binding, and it

seemed likely that they would also be ineffective at detecting platinated DNA binding. For these reasons, DNase I footprinting was employed to characterize hUBF-promoter complex formation. For such studies to be informative, it is essential to employ a DNA substrate that has a single, site-specific platinum lesion. Such a substrate was prepared by P. Pil and S. Lippard, and, more recently, by A. Barrasso (Pil and Lippard, 1992). This 100 bp DNA fragment contains a single-centrally located cisplatin (*cis*-$[Pt(NH_3)_2]^{2+}$) 1,2-intrastrand d(GpG) crosslink in which the N7 atoms of adjacent guanines are coordinated to the Pt atom. This DNA fragment is referred to as GˆG-100.[3] hUBF was added to GˆG-100 and the analogous unmodified sequence (Un-100), and DNase I analysis was performed on the adduct containing strand. The results were striking as a distinct protected region is observed in the 14 bp region flanking the cisplatin adduct (Figure 15, lanes 1&2). This result provides direct evidence that hUBF recognizes the bent (34°) (Bellon and Lippard, 1990) and underwound (13°) (Bellon *et al.*,1991) DNA structure induced by the lesion. No protection was afforded to the unmodified sequence (lanes 3&4). The cisplatin adduct is centered within the protected region, although the phosphodiester bond immediately 5′ to the lesion remains nuclease sensitive. This footprinting pattern resembles that observed in the UCE element upon hUBF binding in which a protected region symmetrically flanks a nuclease-sensitive site (Figure 14). The similar footprinting patterns may reflect conformational homologies between the [hUBF - UCE] and [hUBF - GˆG-100] complexes.

The formation of [hUBF - GˆG-100] complexes is exceptionally

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[3] The GˆG intrastrand crosslink was chosen for these studies because previous work has shown that 1,2 intrastrand crosslinks at both AG and GG sites, but not 1,3 intrastrand crosslinks at GXG sites, are recognized by cisplatin-damaged DNA recognition proteins.

favorable. DNase I protection assays estimate the apparent dissociation constant ($K_{d(app)}$)[4] to be 60 pM (Fig. 15). For comparison, the [HMG1 - GˆG-100] complex has a $K_{d(app)}$ of 370 nM (Pil and Lippard, 1992). The kinked DNA structure induced by GˆG may mimic a DNA conformation that occurs during the formation of a stable [hUBF - promoter] complex and thereby precludes a sequence requirement. Unlike the case for promoter binding, hUBF binding to the single cisplatin lesion does not appear to be cooperative as the data fit equation (1). It is striking that promoter sequences are only three-fold better substrates for hUBF than are cisplatin adducts. This similarity in affinity makes it reasonable to suggest that adducts can act as molecular decoys for hUBF in a cellular milieu. Since the cellular levels of hUBF and cisplatin lesions are similar ($\sim 5x10^4$/cell) (Reed *et al.*,1993; Bell *et al.*,1988), the decoy effect could result in a significant reduction in the quantity of hUBF available for promoter binding.

4. hUBF-promoter interactions are antagonized by cisplatin adducts.

The results presented above showed that hUBF interacts specifically with both the rRNA promoter and a single cisplatin adduct. Indeed, similar footprinting patterns were detected on both types of DNA probes, and the relative affinities of the interactions were similar. These observations suggested that cisplatin adducts could efficiently reduce promoter binding by a decoy mechanism. As shown in Figure 16, GˆG-100 does indeed efficiently antagonize hUBF-promoter interactions. The reduced intensity of bands at positions -21 and -95 in the CORE and UCE elements and the

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[4]The $K_{d(app)}$ is defined as the concentration of free protein at which 50% of the DNA is bound. This simplification was made since the actual binding equations for the interactions being studied have not been determined rigorously.

reappearance of bands in the -75 to -115 region illustrate this effect (lanes 7-12). At a saturating concentration of hUBF, the formation of promoter complexes is completely inhibited by a platinum adduct concentration of $5x10^{-9}$ M. It is noteworthy that this level is well below the adduct levels in cancer patient DNA (10^4-10^5/cell, or 10^{-7}-10^{-6} M) (Reed *et al.*,1993).

The results also indicate that hUBF can be competed away effectively with the unmodified DNA fragment, Un-100, albeit with less efficiency than with G^G-100. Although there is no evidence of a specific interaction of hUBF with unmodified DNA in the footprinting studies (Figure 15), Un-100 was a 10-30 fold weaker competitor of hUBF binding than G^G-100 (Figure 16). This result is not surprising since HMG box proteins often exhibit a significant affinity for random DNA sequences (Giese *et al.*,1992). Nonspecific binding was examined further by using unmodified, random-sequence DNAs of various lengths as competitors. On a mass basis, chicken erythrocyte (CE) DNA averaging 175 bp in length was a ~ 4-8 fold more potent competitor than Un-100. The length dependence suggests that hUBF requires a large tract of DNA for binding. This is consistent with studies on Xenopus UBF which showed that a minimal binding site is ~60-80 bp (Putnam and Pikaard, 1992). If 100 bp were the minimal hUBF binding site, then CE DNA would be ~75-fold more effective as a competitor than Un-100; a single molecule of CE DNA would represent 75 overlapping nonspecific binding sites. The 4-8 fold difference in efficiency between CE DNA and Un-100 suggests that a minimal hUBF binding site is 80-90 bp, which is in agreement with the Xenopus UBF results. Accordingly, Un-100 likely contains 10-20 overlapping nonspecific hUBF binding sites. By comparison, G^G-100 contains only 1 specific binding site. When these numbers are taken into consideration, it is evident that hUBF has a 100-600

fold preference for a platinated versus an unplatinated site.

Interestingly, calf thymus DNA averaging 1,000 bp was 5-10 fold more effective as a nonspecific competitor of hUBF activity than CE DNA. This finding cannot be explained solely by comparing the number of available nonspecific binding sites on the two types of fragments; such arguments could only explain a 2-fold difference since a minimal hUBF binding site is apparently 80-90 bp, or one-half the length of the CE DNA. It is possible that extremely long tracts of DNA allow multiple hUBF species to bind cooperatively and to assemble a stable nucleoprotein superstructure. Indeed, Xenopus UBF binds cooperatively to enhancer repeats, and the degree of cooperativity is positively correlated to the number of repeats. This finding suggests that multiple UBF molecules can interact cooperatively provided that DNA length is not limiting (Putnam and Pikaard, 1992). A similar phenomenon may occur with nonspecific binding.

Figure 14. *TOP,* The -115 to -75 region of the rRNA promoter becomes DNase I resistant upon hUBF binding. Enhanced nuclease sensitivity is observed, however, at positions -95 and -21 (Bell *et al.*,1988). Promoter binding at hUBF concentrations ranging from 7-78 pM is shown and is most easily visualized by the increased DNase I sensitivity of the -95 position in the upstream control element (UCE). *Bottom,* hUBF binding in the top panel was quantitated by measuring the intensity of the enhanced cleavage at -95, and relative binding is reported to the left in arbitrary PhosphorImager units (PIU). To the right the data are expressed as the apparent fractional saturation (Y). A Hill plot of these data yielded a best fit line ($r = 0.997$) with a Hill constant (n_H) of 2.7. A Hill constant > 1 indicates positive cooperativity.
METHODS. *Top*, DNase I footprint reactions were performed on the noncoding strand of the rRNA promoter in the presence of affinity-purified HeLa hUBF as described (Jantzen *et al.*,1990) with the following exceptions. The footprinting probe was 3' end-labeled by using the Klenow enzyme in the presence of three α-^{32}P dNTPs (dATP, dCTP, and dGTP). Since this labeling procedure often does not proceed to completion, the bands appear as doublets. The probe concentration in each reaction was 0.7 pM (10^3 cpm). *Bottom*, Quantitation was performed by PhosphorImager analysis.

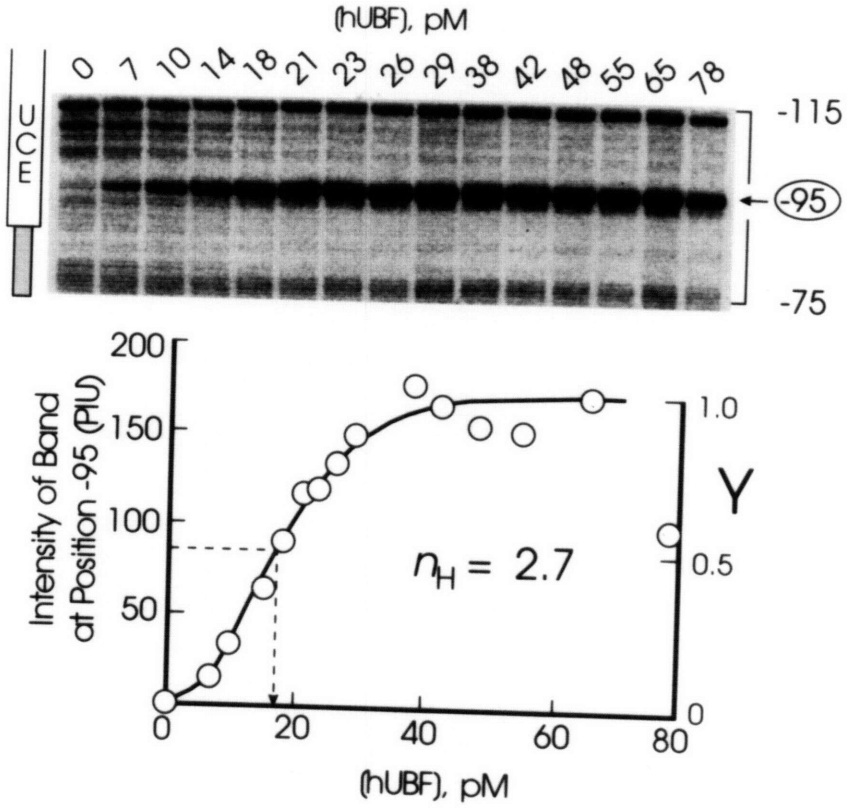

(hUBF), pM
0 7 10 14 18 21 23 26 29 38 42 48 55 65 78
EcoC
-115
-95
-75
Intensity of Band at Position -95 (PIU)
200
150
100
50
0
20
40
60
80
(hUBF), pM
1.0
0.5
0
Y
n_H = 2.7

Figure 15. *A*, A 100 bp DNA fragment containing a single, centrally located cisplatin (*cis*-$[Pt(NH_3)_2]^{2+}$) 1,2-intrastrand d(GpG) crosslink (GˆG-100), is protected from DNase I cleavage in the presence of hUBF (compare lanes 1&2). The relevant sequence is shown to the left, and the protected residues are displayed within the box. The broken line indicates a residue immediately 5′ to GˆG that remains DNase I-sensitive. The analogous unmodified 100-mer, (Un-100) gives the same DNase I cleavage pattern both in the presence and in the absence of hUBF (lanes 3&4). *B*, The DNase I protection assay described in panel *a* was used to characterize hUBF binding to GˆG-100. Y is the fractional saturation of GˆG-100. The data fit the equation K_d [hUBF][GˆG-100] / [hUBF-GˆG-100] when K_d 60 pM. The $K_d$is indicated by the broken line.
METHODS *A.* The adduct-containing strand of GˆG-100 and the analogous strand of Un-100 were 5′ end labeled by using polynucleotide kinase. The concentration of affinity purified HeLa hUBF (Bell *et al.*,1988) was estimated by silver staining. hUBF was added to the labeled DNA fragments to a final concentration of 400 pM in binding buffer (25 mM Tris-HCl (7.9), 14 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol, 50 mM KCl, 0.05% Nonidet-P40, 2.5 mM $CaCl_2$), and the mixture was incubated for 10 min at 30° C. The DNA fragments were at a final concentration of 90 pM (10^4 cpm). The subsequent DNase I and electrophoresis steps were essentially as described (Jantzen *et al.*,1990). *B*, hUBF was added to GˆG-100 to final concentrations of 6-400 pM. GˆG-100 was present at 20 pM (10^3 cpm). Quantitation was performed by using a Molecular Dynamics PhosphorImager. Y was estimated by monitoring the intensity of three bands in the protected region at each [hUBF]. The best fit line was generated by utilizing Microsoft Excel.

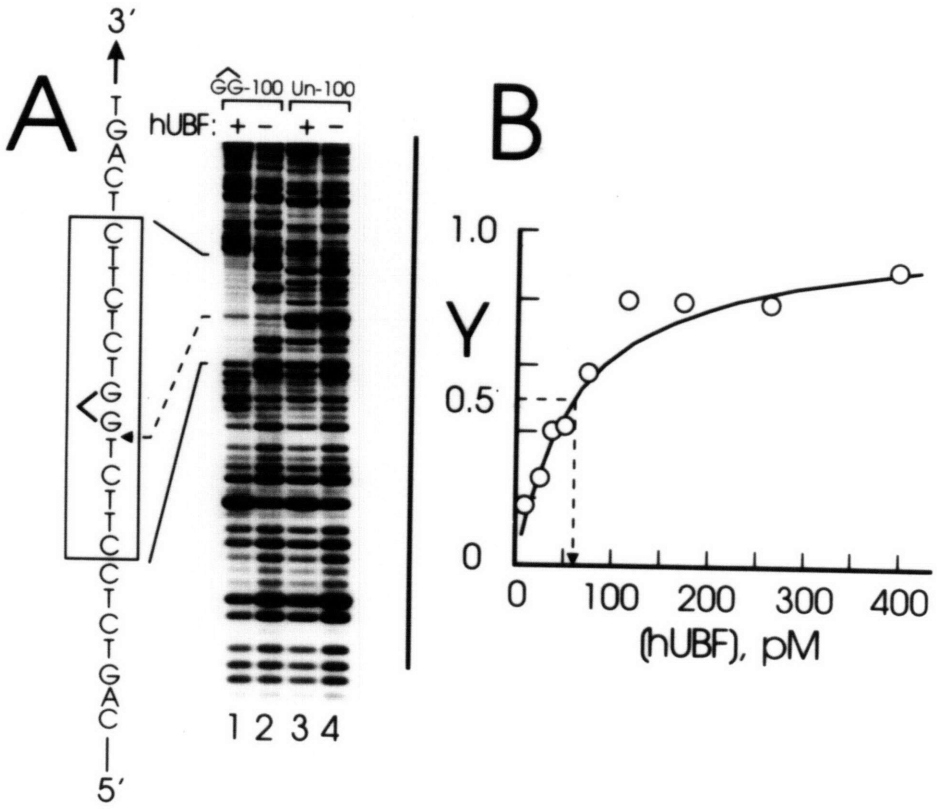
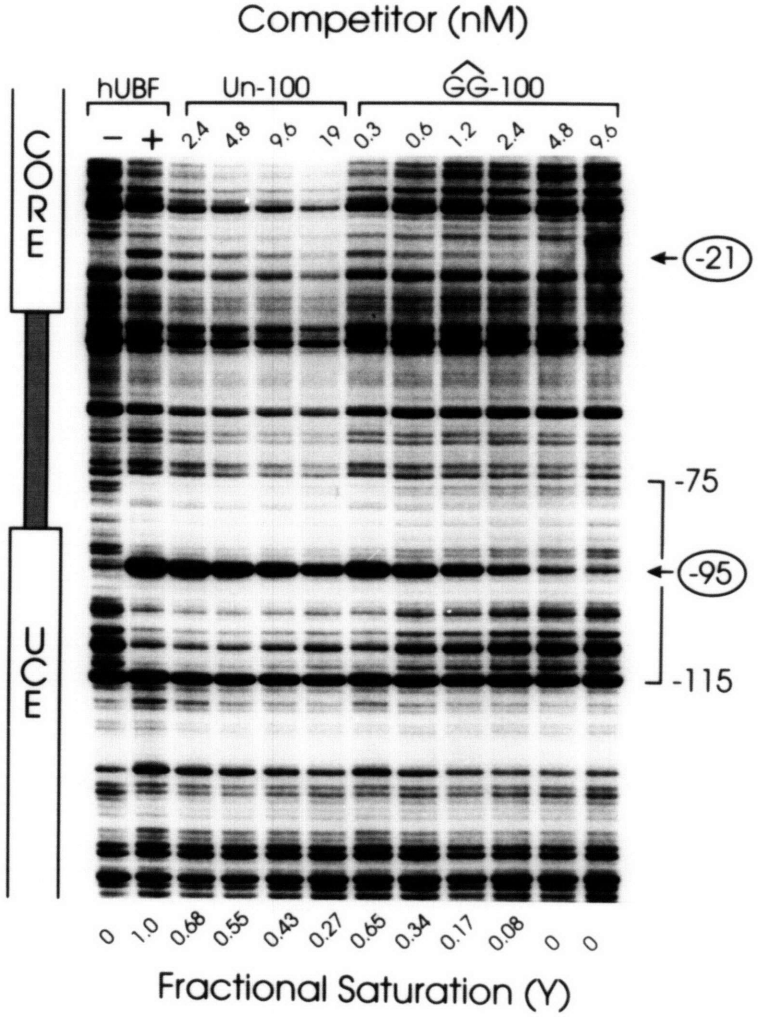

A
3′
TGACT
CTTCTCTGGTCTTTCCTCTGAC
5′
GG-100
Un-100
hUBF: + − + −
1 2 3 4
B
1.0
Y
0.5
0
0
100
200
300
400
(hUBF), pM

Figure 16. The ability of GˆG-100 to compete with rRNA sequences for hUBF binding has been evaluated. Promoter binding is visualized by DNase I footprinting as in Figure 14 except that the enhanced cleavage at -21 in the CORE is shown. hUBF was added to all samples, except the negative control (lane 1), to a final concentration of 168 pM. This level of hUBF is safely above that which results in an apparent fractional saturation (Y) of 1 in the positive control (lane 2). Un-100 (lanes 3-6) and GˆG-100 (lanes 7-12), were added as unlabeled competitors to the final concentrations listed. The competitive effect was estimated by measuring Y of the promoter probe. Y values are shown at the bottom. Lanes 1and 2 were used as standards to calculate Y in lanes 3-12.


Competitor (nM)
hUBF
Un-100
GG-100
– +
2.4 4.8 9.6 19
0.3 0.6 1.2 2.4 4.8 9.6
CORE
UCE
-21
-75
-95
-115
0 1.0 0.68 0.55 0.43 0.27 0.65 0.34 0.17 0.08 0 0
Fractional Saturation (Y)

B. DISCUSSION

1. Specific binding of hUBF to DNA containing a single cisplatin 1,2 intrastrand d(GpG) crosslink.

Specific binding of hUBF to cisplatin modified DNA has been demonstrated in three different assay systems: southwestern analysis, DNase I footprinting (Figure 15), and promoter binding competitions (Figure 16). The affinity of this specific interaction is striking (Kd = 60 pM) (Figure 15), and the critical DNA contacts occur in the 14 bp region flanking the G^G crosslink (Figure 15). Other HMG box proteins have been shown to bind selectively to cisplatin-modified DNA, including HMG1,2 (Hughes *et al.*,1992; Pil and Lippard, 1992), SSRP-1 (Toney *et al.*,1989; Bruhn *et al.*,1992), IXR-1 (Brown *et al.*,1993), and SRY (E. Trimmer, unpublished results). In all of these cases, however, specific binding was detected by techniques that do not specify the critical protein-DNA contacts. In addition, the specific binding constant has been determined only for HMG1 (Pil and Lippard, 1992), where the observed K_d (370 nM) was four orders of magnitude higher than for hUBF.

hUBF contains at least four (Jantzen *et al.*,1990), and possibly six (E. Chan, personal communication) HMG boxes. By contrast, HMG1 has two HMG boxes (Bianchi *et al.*,1992). The relative affinity of hUBF and HMG1 for cisplatin adducts is likely to reflect the disparity in HMG box content. Ms. Zhai in our laboratory has shown that the affinity of hUBF for cisplatin adducts is greatly reduced when HMG boxes are sequentially deleted, and at least two boxes are required for specific binding. Additional HMG boxes are likely to increase the binding affinity by altering the rates of both association

and dissociation. First, the presence of multiple boxes may increase the rate of association by increasing the local concentration of DNA binding domains. Second, the dissociation rate could be reduced because the adduct-specific binding would be stabilized by cooperative nonspecific interactions occuring in the flanking DNA region. Since the HMG box is a nonspecific DNA binding domain, it can be predicted from these arguments that hUBF would also have a higher nonspecific binding component than HMG1. This prediction is supported by the competition experiment (Figure 16) which showed that hUBF has a 100-600 fold preference for a platinated site over an unplatinated site. This corresponds to a nonspecific binding constant (K_{ns}) of 6-36 nM. The affinity of HMG1 for an unmodified 100 bp DNA fragment was much lower, K_{ns} = 10 μM (Pil and Lippard, 1992). It is noteworthy that the fold-preference of both HMG1 and hUBF for a platinated versus an unplatinated site is similar, ~ 100-fold.

The footprinting data show that hUBF makes specific DNA contacts in the 14 bp region flanking the G^G lesion. Furthermore, the adduct is approximately centered within the protected region suggesting that the DNA binding domain(s) is symmetrically placed relative to the adduct. Since the protected region spans ~1.5 helix turns, it appears that hUBF binding is not restricted to one face of the DNA. The interaction may also occur preferentially in the minor groove on the convex side of the DNA bend; other HMG proteins, including LEF-1, bind in the minor groove (Giese *et al.*,1992). Footprinting reagents such as hydroxyl radicals may ultimately prove useful in further resolving the positions of DNA-protein contacts. It is interesting that the phosphodiester bond immediately 5′ to the G^G is not efficiently protected from nuclease cleavage. The G^G lesion both bends (34° toward the major groove) (Bellon and Lippard, 1990) and unwinds (13°) (Bellon *et*

al.,1991) the helix. hUBF recognizes this structure and may induce further bending upon binding; however, the elbow of the bend does not appear to be an important contact point and remains exposed to the solvent. This footprinting pattern qualitatively resembles the pattern observed when hUBF binds to the UCE element of the rRNA promoter (Figures 14&15). [hUBF - UCE] complexes are characterized by a distinct DNase I-protected region that symmetrically flanks a nuclease hypersensitive site. It is likely that the nuclease hypersensitive site results from protein-induced DNA bending. Indeed, DNase I activity is sensitive to the width of the minor groove suggesting that DNA bends can alter nuclease susceptibililty (Brown and Sauer, 1993). Hence, two markedly different binding targets, a single GˆG, and the UCE, yield similar fooprinting patterns upon hUBF binding[5]. More specifically, a putative bend site is flanked symmetrically by regions of nuclease resistance. This result suggests a possible structural homology between the [hUBF - GˆG-100] and [hUBF - UCE] complexes. DNA bending and wrapping are likely to be common features of these complexes. Indeed, HMG box proteins are known to recognize specific DNA sequences, structures, or both, and to alter DNA conformation. HMG1 and SRY recognize DNA cruciforms, and mtTF1, LEF-1 and SRY bind to, and bend, specific sequences (Ferrari *et al.*,1992; Fisher *et al.*,1992; Giese *et al.*,1992; Harley *et al.*,1992). An NMR structure of an HMG box has recently been solved and shows that this domain has a unique "L" shape (Weir *et al.*,1993). Basic amino acids are located on the concave face of the L, and may be responsible for DNA binding. It is possible that bent DNA structures can fit snugly into the L-shaped binding pocket, but further studies are

[5]The sequence of GˆG-100 was compared to the UCE and CORE elements of the rRNA promoter by using a Pustell sequence analysis program. GˆG-100 shares no homology with either the UCE or CORE elements.

required to test this model.

In order to elucidate the mechanism by which hUBF recognizes cisplatin adducts, it is useful to review the current models proposed to explain DNA recognition by HMG box proteins. HMG box proteins have been categorized into three classes (Ferrari *et al.*,1992). The first class consists of HMG1 and 2 and related low molecular weight, high abundance proteins. HMG1 is a sequence-independent DNA binding protein that is known to interact specifically with DNA containing sharp angles, such as cruciform structures (Bianchi *et al.*,1989). Negatively supercoiled DNA is recognized preferentially by HMG1, and protein binding results in DNA unwinding which has been attributed to the acidic tail domain (Sheflin *et al.*,1993). The second class of HMG box proteins is characterized by sequence specific binding to promoter sequences. The testis determining factor SRY and the lymphoid enhancing factor LEF-1 fall into this class and are most likely to function as specific RNA polymerase II transcription factors (Harley *et al.*,1992; Giese *et al.*,1991). SRY can also bind to DNA cruciforms showing that structure and sequence-specific DNA recognition are not mutually exclusive (Ferrari *et al.*,1992). Structure-specific binding by LEF-1 has yet to be reported but is likely to occur. The third class of HMG box proteins includes general transcription factors for polymerase I and mitochondrial sequences, including hUBF and mtTF1 (Fisher *et al.*,1992). mtTF1 and hUBF bind selectively to regions upstream of polymerase I start sites, but a consensus binding sequence has yet to be identified in either case. As shown in this dissertation, hUBF also binds selectively to bent DNA structures. The same is likely to be true for mtTF1. This HMG class has an intermediate phenotype that is characterized by a relaxed sequence specificity - "long region-specificity" may be an apt description. Certain long

DNA regions may transiently form tertiary structures that are recognized by this HMG class. This "transient structure" mechanism could explain the lack of a consensus binding sequence for hUBF. It is clear from this discussion that structure, not sequence, binding specificity is intrinsic to HMG box proteins. Another common property of HMG box proteins is the ability to bend and/or wrap DNA. LEF-1 is an appropriate example for this discussion because it binds to a short, linear consensus sequence and induces a dramatic DNA bend of ~130°; SRY behaves similarly (Giese *et al.*,1992). Indeed, the bending theme is conserved throughout the HMG family because bent structures are recognized ubiquitously and linear DNA structures become bent upon binding.

2. hUBF recognizes specific DNA sequences and structures.

A set of models has been devised to reconcile sequence and structure specific binding by HMG box proteins (Ferrari *et al.*,1992) (Figure 17). The lock and key model may explain structure-specific recognition. DNA distortions match the shape of the HMG binding pocket and favorable DNA protein contacts can occur. It is possible that further bending of the DNA substrate occurs after binding with a concomitant rearrangement of protein structure. It is clear that similarly bent structures can be recognized with vastly different affinities. The 1,3 GTG cisplatin crosslink is not recognized by HMG1, although it produces a bend similar to G^G. The unwinding angles induced by these lesions are different, however, and suggest that a combination of bending and unwinding contributes to recognition (Bellon *et al.*,1991). It is peculiar that subtle differences in unwinding can dramatically alter recognition when it is considered that DNA cruciforms, which contain much sharper angles (60° and 120°) than G^G,

are also recognized. It is clearly of interest to determine if the bend angle induced by G^G is augmented upon complexation with HMG box proteins.

The induced fit model is intended to explain both sequence and structure recognition by HMG box transcription factors. In this model, the HMG protein binds to a linear sequence and induces a DNA bend. The free energy generated by favorable DNA-protein contacts and by allosteric changes in the HMG protein drives the complex formation. It is possible that "bendable" DNA sequences are involved in this type of recognition.

The "transient structure" model may be invoked to explain the recognition of rDNA sequences by hUBF. The long GC-rich regions upstream of the rRNA promoter may form transient bent, or otherwise distorted, structures that are "captured" by hUBF. A caveat is apparent regarding this particular example. A specific HMG box from hUBF is both necessary and sufficient for recognition of this sequence to occur (Jantzen *et al.*,1992), whereas any of the hUBF HMG boxes can participate in cisplatin adduct recognition (as shown by Ms. Zhai in our laboratory). It is possible that the specific box required for promoter binding has partial characteristics of the LEF-1 and SRY boxes and therefore can recognize sequence cues in addition to structural distortion.

The results of my work show that hUBF, like SRY, binds to both specific structures and to putative sequence cues. The models presented above suggest that the [DNA - HMG Box] complexes formed via both sequence-specific and structure-specific interactions will have similar conformational features. The findings that [hUBF - G^G-100] and [hUBF - UCE] complexes yield similar DNase I footprinting patterns and have similar

dissociation constants support this prediction. The high affinity interaction between hUBF and G^G-100 suggests that the specific DNA distortion induced by G^G mimics a DNA structure naturally occuring in rDNA, or a favorable DNA structure that is formed during the assembly of promoter complexes.

3. Binding of hUBF to rRNA promoter sequences

This work is the first study in which the thermodynamics of [hUBF - Promoter] complex formation have been described quantitatively. Although the appropriate reagents for such studies have been available for some time, the high affinity nature of the interaction and the relative difficulty associated with quantitating DNase I footprinting experiments have served as roadblocks. The problems associated with achieving "protein excess" in these studies resulted from the high affinity nature of the interaction. This obstacle was overcome by preparing probes with the high specific activity required to detect the low DNA concentrations present in the assay. Furthermore, quantitation of the binding was rendered trivial by the recent development of PhosphorImager technology.

The affinity of hUBF for the rRNA promoter is very high ($K_d = 18$ pM, see Figure 14) and, importantly, binding is cooperative. Several previous studies hinted that hUBF may bind cooperatively. First, Xenopus UBF, which is highly homologous to hUBF, binds cooperatively to repeats of an enhancer sequence that is located upstream of the Xenopus rRNA start site (Putnam and Pikaard, 1992). The $K_{d(app)}$ for this interaction is $\sim$ 10 nM. Since the organization of the human and Xenopus rRNA promoters is quite different, this result did not insure that hUBF would also bind cooperatively.

Second, the importance of protein-protein interactions in promoter binding was established; deletion of a putative dimerization domain from the N-terminus of hUBF abolished promoter binding in the footprinting assay (Jantzen *et al.*,1992). In addition, there is sequence homology between regions of the UCE and CORE elements suggesting that these regions may be functionally linked (Bell *et al.*,1988). It is tempting to speculate that binding of hUBF to one of these elements facilitates binding to the other element through protein-protein interactions.

Cooperative binding of hUBF to the rRNA promoter is likely to have several important consequences for the regulation of ribosome production. The in vivo consequences of cooperativity are not immediately clear, however, since most of the transciptional activation studies with hUBF have been performed in vitro. There are 400 rRNA genes in a diploid human cell, whereas the estimated number of hUBF molecules is 50,000 (Bell *et al.*,1988). Immunostaining studies have shown that hUBF is detectably localized only in the nucleolus (Jantzen *et al.*,1990) and is associated with rDNA at all stages of the cell cycle (Chan *et al.*,1991)[6]. These studies also show that hUBF levels are not reduced concomitantly with the transcriptional activity of rRNA genes. Taken together, these studies suggest that hUBF is not the limiting factor regulating rRNA synthesis. Many cooperative protein-DNA interactions contribute to the tight regulation of transcription. Since the formation of cooperative complexes is a steep function of ligand concentration, subtle changes in ligand concentration can

[6] It is unclear if these immunostaining studies are of sufficient sensitivity to detect hUBF that is diffused throughout the nucleous. Indeed, it is possible that hUBF is only detected in the nucleolus where it is concentrated on the rDNA. If this is the case, then the conclusion that hUBF is exclusively located in the nucleolus would require reevaluation.

dramatically alter activity. Hence, cooperativity confers exquisite sensitivity to many biological regulatory systems. With these comments in mind, it is not clear why a factor that is apparently in excess would exhibit cooperativity. It must be emphasized, however, that the data suggesting hUBF is in excess stem from crude estimations of abundance extrapolated from protein purification schemes. Furthermore, the high levels of nonspecific binding exhibited by hUBF (Figure 16) suggest that a significant fraction of intracellular hUBF may be bound nonspecifically. It thus remains possible that the concentration of hUBF available for promoter binding is in the range of the K_d where cooperativity would be an important effect.

The most probable limiting factor in rRNA transcription is the selectivity factor, SL-1; this factor is apparently present in catalytic amounts relative to hUBF (Bell *et al.*,1988). SL-1 alone does not bind to the rRNA promoter, but once hUBF has bound SL-1 binds sequentially to form a cooperative complex that is competent for transcription (Bell *et al.*,1988). Recent studies have shown that SL-1 is a multiprotein complex that includes the TATA binding protein, which was once thought to be exclusively a polymerase II transcription factor (Comai *et al.*,1992).

Cooperative binding of hUBF may be important for reasons unrelated to the tight regulation of rRNA synthesis. Cooperativity may (1) be important for the nucleolar targeting of hUBF or (2) displace transcriptional repressors from rDNA sequences. It is unclear how hUBF is transported to the nucleolus. It has been suggested that accumulation occurs due to the increased affinity of hUBF for rDNA sequences (Jantzen *et al.*,1990). This suggestion is inconsistent with hUBF's high nonspecific binding activity; however, specific, cooperative binding may contribute to affinity-driven

accumulation. Nonspecific binding appears to be favored by the availability of long tracts of naked DNA (see discussion above). Since DNA is coated with protein in vivo, long tracts of naked DNA will be rare, and nonspecific binding may be significantly reduced. Alternatively, the high nonspecific binding of hUBF may surprisingly be of value to nucleolar localization. Nonspecific binding may result in the facilitated linear diffusion (tracking) of hUBF to target sequences. The prokaryotic DNA repair enzyme T4 Endo V is more efficient at locating damaged substrates under assay conditions that favor nonspecific binding, and a tracking mechanism seems to be involved (Nickell *et al.*,1992). In this model, tracking would occur until the target site is reached, and the cooperative binding phenomenon would serve to "lock in" specific complex formation.

hUBF does not appear to contain a consensus nucleolar localization sequence. Studies with mouse UBF showed that the acidic tail domain was critical for nucleolar localization (Maeda *et al.*,1992). Another study showed that the acidic tail of hUBF was required for protein-protein interactions with SL-1 to occur (Jantzen *et al.*,1992). Perhaps cooperative interactions between hUBF and SL-1 enhance nucleolar targeting. A caveat to this model, however, is that SL-1 is apparently far less abundant than hUBF. Furthermore, the mouse UBF studies were performed on mutants that were also lacking the N-terminal dimerization domain; the acidic tail may only be required for nucleolar targeting in the absence of dimerization.

There is evidence to suggest that a "repressor displacement" process contributes to transcriptional activation by mouse UBF. In these studies, hUBF was shown to activate transcription efficiently only when crude RNA polymerase I fractions were used. The interpretation is that transcriptional

repressors are present in crude polymerase preparations that bind to DNA and are displaced by hUBF. The putative inhibitor protein has recently been identified as the Ku antigen - a protein of ambiguous function (Kuhn *et al.*,1993). Cooperative interactions occuring during hUBF binding may provide the free energy required for repressor displacement. Alternatively, cooperative interactions between hUBF molecules may facilitate the formation of a nucleoprotein superstructure that is required for productive interactions to occur with SL1, RNA polymerase 1, or both.

4. Cisplatin adducts may be molecular decoys for hUBF in vivo.

hUBF binds to rRNA promoter sequences and cisplatin adducts with comparable affinity. This result suggests that adduct binding can occur in the intracellular environment. One argument against adduct binding in vivo is the high level of nonspecific binding by hUBF. Since the number of nonspecific binding sites is orders of magntude higher than the numbers of both cisplatin lesions and rRNA promoters, nonspecific binding would be expected to largely inhibit specific binding. Promoter binding occurs in vivo, so there must be a mechanism (possibly tracking, *vide supra*) that allows nonspecific binding to be evaded. Presumably, specific binding to adducts could exploit similar mechanisms. The issue of nonspecific binding is common with HMG box proteins and it is often unclear how proteins with such high nonspecific binding affinities can find their specific genomic targets. LEF-1 exemplefies this paradox (Giese *et al.*,1991).

As discussed in the results section, the number of platinum adducts formed in cancer patient DNA is similar to the number of cellular hUBF molecules. It is thus possible that adduct binding could significantly reduce

promoter binding and disrupt nucleolar targeting. Since hUBF-promoter interactions are cooperative, reductions in available hUBF could have a profound effect on promoter occupancy (such an effect is evident in the competition experiment, Figure 16). The validity of these predictions ultimately depends on the normal intracellular concentration of unbound hUBF which, in turn, depends on the degree of nonspecific binding. If, for example, unbound hUBF is in vast excess in the nucleolus, then large decreases in hUBF concentration caused by adduct binding would be required to alter promoter binding significantly. If nonspecific binding is significant, then the concentration of unbound hUBF may be in the range of the K_d. In this case, promoter binding would be sensitive to even subtle changes in unbound hUBF concentrations.

Another relevant issue is the availabilty of cisplatin adducts for hUBF binding. hUBF is not the only HMG protein known to recognize adducts, and it is possible that other HMG proteins, such as HMG1, would shield adducts from hUBF. In the case of HMG1, this effect does not seem likely. HMG1 is $\sim$10-fold more abundant than hUBF, but has a binding affinity for adducts that is four orders of magnitude lower than that observed for hUBF. This suggests that HMG1 would not effectively compete for adduct binding sites with hUBF. The abundance and binding constants of other HMG box proteins that bind to cisplatin adducts is not known making it difficult to speculate further on this issue.

An abnormal nucleolar morphology has long been recognized as a hallmark of transformed cells, suggesting that nucleoli may be attractive targets for chemotherapy (Busch and Smetana, 1970; Egan and Crocker, 1991). We propose that cisplatin adducts target nucleoli by diverting hUBF.

If this hijacking mechanism is operative in cisplatin-treated cells, then a dramatic effect on rRNA production should be evident. Proliferating cells have higher demands for rRNA and would be particularly sensitive to inhibited rRNA production (Larson *et al.*,1991). The synthesis of ribosomal proteins and rRNA is coupled such that productive ribosome assembly can occur in the nucleolus. The diversion of hUBF may disrupt ribosome assembly by upseting the delicate balance of rRNA and ribosomal protein in the nucleolus. Since mature ribosomes have a long half life (up to 10 days) (Larson *et al.*,1991), reduced ribosome synthesis may not have an immediate impact on cellular welfare, but could inhibit proliferation. Unfortunately, the literature contain few studies monitoring the effect of cisplatin on rRNA production; however, it is clear, however, that total RNA and mRNA production are decreased dramatically in cisplatin-treated cells (Harder and Rosenberg, 1970; Sorenson *et al.*,1990). Since at least 50% of newly synthesized RNA in proliferating cells is ribosomal, the observation that total RNA production is reduced most likely reflects decreased rRNA synthesis. Nucleolar segregation, a morphological change associated with inhibited rRNA synthesis, is also observed in cisplatin treated cells (Jones *et al.*,1985). These results are consistent with the hijacking model, but hardly provide proof since many processes are inhibited in cisplatin-treated cells. DNA synthesis, protein synthesis, and mRNA synthesis are also inhibited (Harder and Rosenberg, 1970). In general, effects on a multitude of intracellular processes are observed and make it difficult to ascribe any one effect to a specific mechanism. Furthermore, cisplatin DNA adducts can inhibit RNA polymerases directly by blocking the extension of nascent RNA chains (Corda *et al.*,1991). Ms. Zhai is performing several experiments to test the proposed hijacking mechanism directly (refer to the "Future Experiments" section).

5. hUBF may shield cisplatin adducts from DNA repair enzymes.

Molecular hijacking of hUBF by cisplatin lesions would be expected to disrupt the regulation of rRNA synthesis. A concomitant effect would be the blocking of DNA repair enzymes from accessing sites of damage. It is clear from the DNase I cleavage pattern of [hUBF - G^G-100] complexes that the 14 bp region flanking the adduct is nuclease-resistant. This result suggests that the human excision nuclease may also be inefficient at recognizing and repairing hUBF-bound lesions. In support of this notion, the prokaryotic UvrAB repair complex binds to G^G with an affinity ($K_{d(app)}$) >1 nM) that would be insufficient to displace hUBF (Visse *et al.*,1991; Visse *et al.*,1992). In addition, G^G lesions are repaired poorly by human cell extracts (Szymkowski *et al.*,1992; Calsou *et al.*,1992) suggesting that repair replication on globally platinated DNA occurs primarily at less abundant 1,3 GTG intrastrand crosslinks. Perhaps the binding of HMG box proteins in the extracts shields G^G lesions from repair enzymes. HMG1 is not a likely candidate protein because it is not detected in repair extracts (P. Pil, personal communication). Furthermore, if repair extracts are supplemented with purified HMG1, the repair of globally platinated DNA is unaltered (D. Bissett, personal communication). hUBF levels in repair extracts have not been measured. A possible caveat to the shielding model is that the repair of G^G in crude extracts can be rescued by the addition of purified UvrABC repair proteins (Szymkowski *et al.*,1992). This result suggests that G^G is accessable to repair enzymes; however, it is possible that an excess of Uvr proteins was added and was thus able to mask lesions from HMG proteins. Careful protein titration and time course studies are required to interpret this result more conclusively.

Cisplatin G^G adducts are repaired in mammalian cells. The efficiency of repair, however, is a matter of dispute. Studies with cancer patient DNA and rodent organs show that efficient repair (75% removal) occurs during the first 21 hr, but the remaining lesions are persistent (Fichtinger-Schepman *et al.*,1987; Fichtinger-Schepman *et al.*,1990; Terheggen *et al.*,1987). These studies did not take into account, however, adduct dilution resulting from DNA replication. Adducts are removed less efficiently from the unreplicated DNA of chinese hamster ovary cells (Fraval and Roberts, 1979; Jones *et al.*,1991) and murine leukemia cells (Gibbons *et al.*,1990) (40% removal after 24 hr), although repair in an active hamster gene was 50% more efficient. In general the data are consistent with a shielding mechanism since inefficient repair is noted in cases where DNA replication has been considered. In the cases where repair is apparently efficient, a biphasic repair curve suggests that a fraction of the lesions is refractory to removal - a shielding mechanism could be invoked to explain this phenomenon as well. Alternatively, an intrinsic property of excision repair complexes may preclude the efficient repair of G^G lesions. Indeed, the UvrABC complex repairs the G^G lesion with the lowest efficiency of the platinum adducts tested (Page *et al.*,1990). Preferential repair of one class of UV-induced photoproducts is also evident in human cells (refer to the preceeding chapter of this document). A shielding model is not likely to explain this effect, however, since the poorly repaired photoproduct is not recognized by any known human damage recognition proteins.

Inefficient repair of DNA adducts may contribute to the therapeutic efficacy of cisplatin. DNA repair-deficient cells derived from xeroderma pigmentosum patients are hypersensitive to cisplatin proving that inefficient DNA repair increases toxicity (Fraval *et al.*,1978). Likewise, elevated DNA

repair is observed in some cisplatin-resistant cell lines (Eastman and Schulte, 1988). These results are significant because proliferating cells are more sensitive to cisplatin than stationary cells (Fraval and Roberts, 1979). More specifically, stationary cells treated with cisplatin, allowed to recover for 1-3 days, and plated for colony forming ability, recovered from the cisplatin insult during the incubation period in a time-dependent manner. Conversely, the cisplatin treated proliferating cells had a lower survival. These results suggest that cell cycling is required for death to occur. Consequently, inefficient repair would be selectively deleterious to cycling cells, such as those of tumors.

6. The role of hUBF hijacking in apoptotic cell death.

Concomitant negative effects on both DNA repair and rRNA transcription are predicted by the proposed hUBF hijacking mechanism. Cisplatin may kill cells by triggering apoptotic cell death (Sorenson *et al.*,1990). Since the signal transduction pathway leading to drug-induced apoptosis is unknown, it is difficult to speculate how reduced DNA repair or reduced rRNA synthesis would contribute to this phenomenon. Presumably an event occuring in the G2 phase of the cell cycle induces apoptosis because cisplatin treated cells arrest at G2 for several days and subsequently recover, or become apoptotic (Sorenson *et al.*,1990). It is reasonable that indicators of cellular welfare, such as rRNA levels, or the integrity of the genome contribute to the onset of apoptosis. The signal transduction pathway leading to G2 arrest and apoptosis is unclear. It is suspected, however, that p53 is a G1/S checkpoint factor (Kastan *et al.*,1992). Other toxic agents that are less effective anticancer drugs than cisplatin also induce apoptosis (Eastman, 1993). This result indicates that

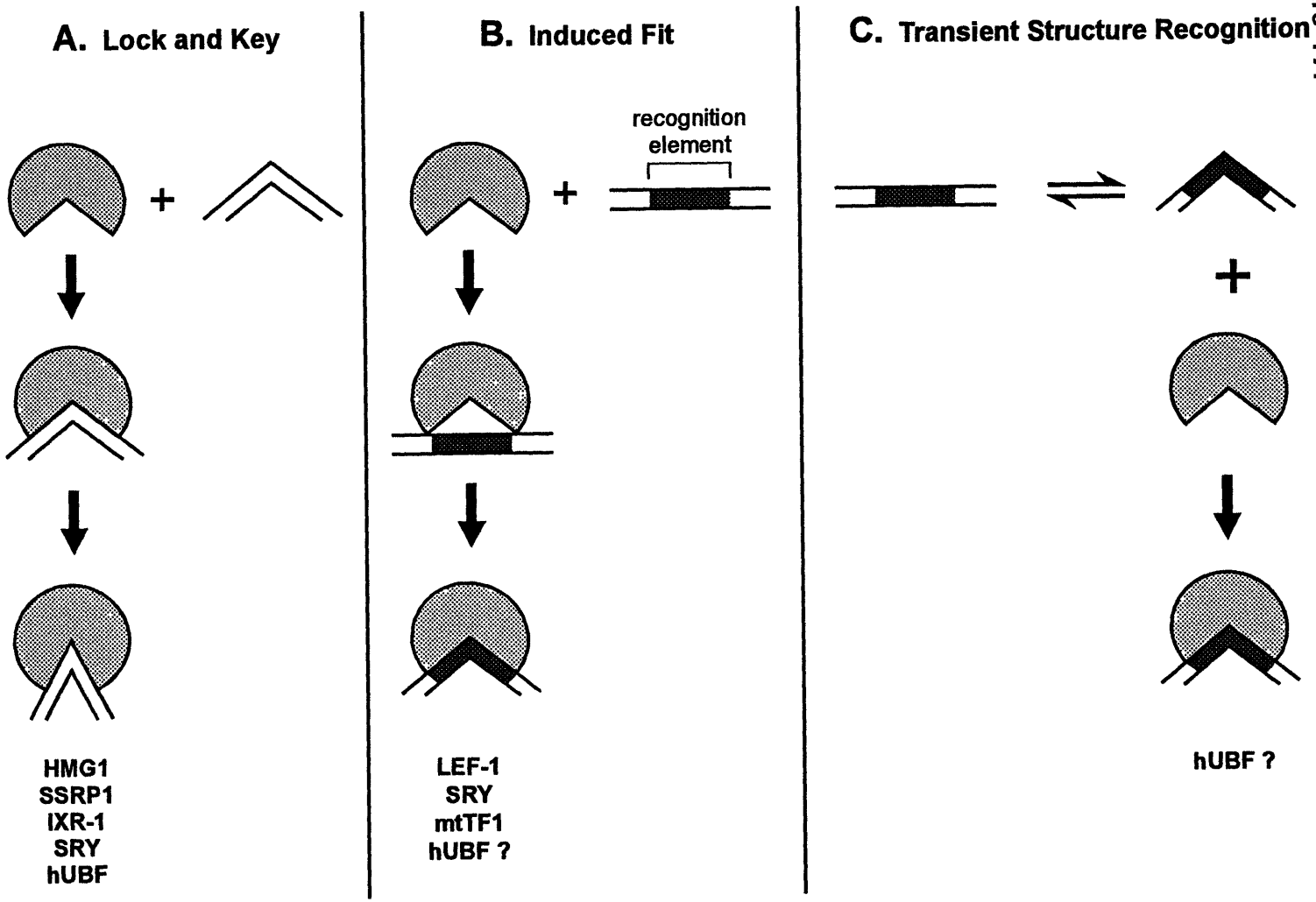
attributes other than the capacity to induce apoptosis may contribute to chemotherapeutic efficacy. The hUBF hijacking mechanism may be important to these currently unknown processes, perhaps by limiting availability of an essential protein or substrate. It is also noteworthy that a cell could not become resistant to the effects of hUBF hijacking by altering hUBF levels; increased levels of hUBF may rescue rRNA production but would also lead to further inhibition of DNA repair. Likewise, decreased levels of hUBF may partially restore DNA repair, but would have negative effects on rRNA synthesis.

7. Implications for novel drug design.

Molecular decoys that inhibit the function of disease-specific proteins are currently being developed as potential therapeutic drugs. One strategy is to construct double stranded oligonucleotides that contain a recognition site for a disease-specific DNA binding protein. Recent advances in this technology include the development of DNA dumbell structures that have increased potentcy owing to reduced nuclease sensitivity (Chu and Orgel, 1992). The finding that DNA with a specific cisplatin-induced distortion can act as a molecular decoy for a nucleolar transcription factor suggests that other DNA damaging agents may induce structures recognized by a variety of DNA binding proteins. It is reasonable that this will be true since several non-HMG transcription factors are known to induce DNA bending upon binding. Furthermore, the specificity of recognition is exquisite - the similar DNA structures induced by various cisplatin adducts are recognized with vastly diffferent affinities by HMG box proteins. This result suggests that drugs that induce similar, but nonidentical structural distortions may have different specificities as decoy agents and may confer programability to the

approach. In addition cells could be treated directly with the drug and the ultimate decoys would be created inside the cell and would thereby circumvent the drug delivery problems associated with therapeutic oliognucleotides.

Models for DNA Sequence and Structure Recognition by HMG Box Proteins



*Adapted from Ferrari et al., (1992) EMBO J., **11**, 4497-4506*

Figure 17.

C. CONCLUSIONS

The toxicity of DNA damaging agents is widely believed to result from the formation of lesions that block polymerases or disrupt the integrity of the genome. A mechanism heretofore not addressed is that specific forms of DNA damage may divert critical regulatory proteins from their natural sites of action. This work has shown that the nucleolar protein hUBF, a critical regulator of rRNA transcription, binds with striking affinity (Kd ~ 60 pM) to DNA modified by the potent anticancer drug cisplatin, and the specific interaction is characterized by a distinct DNase I footprint in the 14 bp region flanking the adduct. This footprinting pattern is similar to the one observed when hUBF binds to rRNA sequences and suggests that the two complexes have conformational homologies. Furthermore, we observe that the hUBF-promoter interaction is cooperative and, consequently, highly sensitive to subtle changes in hUBF concentration. This result, taken together with the cellular levels of both cisplatin adducts and hUBF, suggests that cisplatin could disrupt regulation of rRNA synthesis by a transcription factor hijacking mechanism. Diminished rRNA production would be selectively deleterious to proliferating cells. In addition, the binding of hUBF to cisplatin adducts would be expected to inhibit DNA repair enzymes and thereby contribute to the clinical efficacy of cisplatin. This study also suggests a novel strategy for the design of therapeutic molecular decoys. Transcription factor decoys generally consist of short duplexes containing recognition sites for sequence specific binding proteins. DNA-platinum complexes represent a new class of molecular decoys. Drugs that form DNA adducts recognized by disease-specific proteins may ultimately be of therapeutic value and may allow the drug delivery problems associated with synthetic oligonucleotides to be circumvented.

D. FUTURE EXPERIMENTS

1. In vivo testing of the transcription factor hijacking mechanism.

If the transcription factor hijacking mechanism is correct, then rRNA production should be depressed in cisplatin-treated cells. Unfortunately, cisplatin adducts can block transcription directly by inhibiting polymerases from progressing along the DNA template. For this reason it is not feasible to treat cells with cisplatin, monitor rRNA production, and to then draw conclusions about the validity of the hijacking model.

The construction of a plasmid that expresses proteins from a polymerase I promoter has been reported recently (Palmer *et al.*,1993). This was a significant breakthrough since earlier vectors harboring polymerase I promoters were transcribed, but the messages were not translated efficiently. This construct could be of significant value for testing the proposed model because it will allow one to separate the polymerase-blocking and transcription factor hijacking effects of cisplatin on transcription. In these studies, cells are treated with cisplatin, washed, and transfected with the polymerase I promoter construct. As an internal control, a polymerase II reporter gene construct would be cotransfected that does not require hUBF for expression. If the proposed hUBF hijacking method is operative, one would expect to see a selective dimunition of the signal from polymerase I promoter-driven reporter gene. A similar experiment could be performed using cells that were not treated with cisplatin. The two reporter plasmids would be contransfected with a vast excess of cisplatin-modified salmon sperm DNA. Presumably, the platinated salmon sperm DNA could hijack hUBF and prevent efficient expression of the

polymerase I promoter-driven reporter gene.

The nucleolar localization of hUBF can be exploited to test the hijacking mechanism. hUBF can be detected *in situ* by immunostaining and fluorescence microscopy. We have obtained the proper reagents to perform these studies. If cisplatin adducts are diverting hUBF, then nucleolar localization should be lost, and the staining should be evident throughout the nucleus where the cisplatin adducts are localized. Fibrillarin is a nuclear protein that most likely does not bind to platinated DNA and should retain nucleolar localization upon cisplatin treatment; fibrillarin will serve as a negative control in these studies. All of the aformentioned studies are being pursued by Xiaoquan Zhai in our laboratory.

2. Does hUBF block the activity of DNA repair enzymes?

The repair blocking model can be tested by using purified UvrABC proteins or mammalian cell extracts competent for DNA repair. In both cases, DNA substrates damaged with cisplatin can be preincubated with an excess of hUBF and then added to repair reactions. The UvrABC system is more amenable to these studies because it contains only purified components and is known to repair cisplatin G^G lesions. The repair of G^G in mammalian extracts is inefficient and the effects of hUBF binding could be below the limit of detection.

The host cell reactivation assay may also be of use in these studies. In this assay, damaged plasmids expressing a reporter gene are transfected into human cells, and the amount of repair can be assessed by measuring the activity of the reporter protein. The validity of this assay is underscored

by the inability of repair-deficient cell lines to express genes from damaged plasmids. In these experiments, hUBF would be preincubated with damaged plasmids prior to transfection, and the reactivation assay would be employed to monitor repair. Several control experiments are necessary to show that hUBF-bound plasmids are taken up by transfected cells.

3. Can cisplatin adducts substitute for rRNA promoter elements?

The results presented in this thesis show that hUBF binds to cisplatin adducts and the UCE element of the rRNA promoter with near equal affinities. In addition, the two footprinting patterns are similar suggesting a structural homology between the complexes. These observations raise the intriguing possibility that adducts can functionally substitute for sequence elements. I propose experiments in which critical sequence elements of the promoter are removed and replaced with cisplatin adducts. The various constructs can be tested for promoter activity using the polymerase I plasmids described above.

4. Structural studies

The DNase I footprinting pattern revealed some structural features of [hUBF - G^G-100] complexes. The footprinting pattern on the unadducted strand should also be determined, and other reagents, such as hydroxyl radicals, can be used to give more detailed footprinting patterns. Methylation protection assays may help elucidate if hUBF makes primarily major or minor groove contacts. It is also of interest to see if a single HMG box from hUBF can bind to the adduct and yield a DNase footprinting pattern. The minimal DNA sequence length in the region flanking the adduct

should also be determined. If the system is simplified by using only the minimal protein and DNA regions required for binding, then it may ultimately be possible to do detailed spectroscopic studies.

VI. ANTISENSE RNA APPROACHES TO STUDYING THE FUNCTION OF TWO CISPLATIN DAMAGED DNA RECOGNITION PROTEINS, HMG1 AND SSRP1

A. RESULTS

1. Transient expression of antisense HMG1 RNA in human cells

The role of human *cis*-DDP DRPs in mediating the genotoxicity of cisplatin is not known. Cell lines that lack a particular cis-DDP DRP would be valuable tools for addressing this issue. One method for reducing the expression of genes is through the use of antisense RNA/DNA. This subject has been discussed in a recent review paper (Stein and Cheng, 1993). Antisense RNA is complementary to the normal cellular mRNA and most likely disrupts protein expression by annealing to, and inactivating, the mRNA. In this chapter, antisense RNA has been employed to study the role of HMG1 in cisplatin-mediated toxicity.

Several transient expression vectors were designed to study the effect of antisense HMG1 RNA on human cells. In transient expression studies, the vectors producing the antisense RNA were transfected into human cells, and 24-72 hr later the cells were harvested and the protein of interest was quantified. The plasmids constructed for these studies are shown in Figure 18 and are derived from the parental vector pcDNA1. The salient features of pcDNA1 are as follows: (1) High level expression is driven by the cytomegalovirus immediate early promoter and (2) the expressed RNA is fused to a vector-encoded RNA sequence that contains an intron and a polyadenylation signal. Splicing and polyadenylation may increase the stability of the expressed RNA. CMV-rHMG1(AS) and CMV-rHMG1(S) contain a 5'-truncated version of the rat HMG1 gene in either the sense or antisense orientation, respectively. The 45 nucleotides at the 5' end of the rHMG1 cDNA (including the start codon) are absent in this construct. As a

result, CMV-rHMG1(S) does not express HMG1. This deletion was intentional since CMV-rHMG1(S) was designed to serve as a control for the antisense vector and not as an HMG1 expression vector. CMV-hHMG1(S) and CMV-hHMG1(AS) contain the -1 to +179 region (one-third) of the human HMG1 cDNA.

Human HeLa cells were transfected with the sense, antisense, and parental (pcDNA1) vectors by using electroporation[7]. After ~48 hr the cells were harvested, extracts were prepared, and HMG1 levels were measured by western blotting. The HMG1 levels in the antisense and control samples were indistinguishable. In addition, no truncated HMG1 species were noted in the sense sample that might have resulted from translational initiation at internal methionine codons. Although electroporation is an efficient transfection procedure, most of the cells that survive the procedure do not take up and express exogenous DNA. If only 10% of the cells surviving the transfection express antisense RNA, then HMG1 levels in the cell population as a whole would appear unchanged. The transfection efficiency was measured by electroporating HeLa cells with CMV-β-gal and staining the cells *in situ* for enzyme activity. The results of this experiment showed that ~15% of the cells were transfected. It was concluded that the transfection efficiency was too low to detect antisense RNA-mediated reductions in HMG1 levels.

An interesting effect was discovered serendipitously when CMV-β-gal was cotransfected with the antisense HMG1 vectors. The antisense vectors were transfected in a molar excess of CMV-β-gal to insure that all cells

[7]Prior to these experiments, the transfection procedure was optimized by using the plasmid CMV-β-gal which expresses a useful reporter gene, β-galactosidase.

expressing β-galactosidase were concomitantly expressing antisense HMG1. The results of this experiment are in Figure 19 and reveal that β-galactosidase expression was reduced by 40% in cells transfected with the antisense rat HMG1 construct. Expression of the sense RNA did not effect enzyme production. This effect was reproducible and was also apparent, albeit to a lesser degree, with the human HMG1 antisense construct. The human vector encodes a smaller portion of the HMG1 gene and may not inhibit HMG1 expression to the same extent.

2. Construction of Epstein-Barr virus based episomal vectors for the stable expression of antisense HMG1 and SSRP1 RNA.

The goal of these studies was to create cell lines that constitutively express antisense HMG1 or SSRP1 RNA. The aim was to obtain the highest possible expression of antisense RNA to lower significantly the cellular levels of HMG1 or SSRP1. Epstein-Barr virus (EBV) based episomal vectors are ideal for studies that require stable, high level RNA expression. Vectors that harbor an EBV replication origin maintain a high copy number (10-100 copies/cell) in cell lines that express the EBV nuclear antigen (Sugden *et al.*,1985). Dr. Bill Sudgen kindly provided an EBV-based vector that confers hygromycin B resistance to transfected cells. The appropriate cell line for these studies was the Raji Burkitt lymphoma line which expresses the EBV nuclear antigen and thereby maintains EBV vectors as high copy number episomes. In addition, this vector has a novel version of the CMV immediate early promoter that is ~10-fold stronger than commonly used CMV-based promoters (Bill Sugden, personal communication).

Several EBV-based vectors were constructed from the parental

plasmid (EBV-B108) that was provided by Dr. Sugden (Figure 18). EBV-SPA was constructed by subcloning the polylinker, splice, and polyadenylation sequences from pcDNA1 into EBV-B108. EBV-SPA served as the parental vector for all future constructions. The 5'-truncated rat HMG1 sequence described in the transient assays discussed above was subcloned in both orientations into the polylinker of EBV-SPA. These vectors are named EBV-rHMG1(S) and EBV-rHMG1(AS).

It was also of interest to lower the cellular levels of the human SSRP1 protein. SSRP1 is a *cis*-DDP DRP that was cloned and sequenced through the collaborative efforts of several individuals from our laboratory and that of Dr. Lippard (Toney *et al.*,1989; Bruhn *et al.*,1992). The normal cellular function is unknown, and the antisense approach was intended to elucidate the role of SSRP1 in the genotoxicity of cisplatin. The 5' EcoRI fragment of the SSRP1 cDNA (position - 174 to +447)[8] was subcloned into EBV-SPA in both orientations. These vectors are named EBV-SSRP1-5'(S) and EBV-SSRP1-5'(AS).

3. Stable expression of HMG1 antisense RNA

Raji cells were transfected with either EBV-rHMG1(AS), the cognate sense construct, or the parental vector EBV-SPA. Forty eight hr post transfection the cells were diluted into selective media containing 200 μg/ml hygromycin B. For each transfection, the cells were selected as pooled clones or plated at limiting dilution in 96 well dishes to facilitate the isolation

[8]This EcoRI fragment contains the 5' 20% of the SSRP1 coding sequence

of clonal cell populations. Twelve days after plating in selective media an unusual effect on survival was noted. The concentration of viable cells in the pooled clone population was much lower in the samples transfected with EBV-rHMG1(AS) and (S) than in the EBV-SPA sample. Surviving cells were not found in a control sample that was transfected with a vector lacking the hygromycin B resistance gene. This result indicated that expression of the rHMG1 cDNA in either orientation leads to significant toxicity. Clone formation in the 96 well dishes also reflected the putative toxic effect of HMG1 RNAs: the sense and antisense constructs yielded only 3-8% and 15-33% of the clones obtained with EBV-SPA, respectively (Table 1). This effect was reproduced in four transfections with different plasmid preparations, and the pattern of survival was consistent: EBV-SPA>>rHMG1(AS)>rHMG1(S).

Cell populations derived from pooled or individual clones were harvested and crude extracts and poly A^+ RNA were prepared. Western blotting revealed that the antisense and control cell lines expressed similar levels of HMG1. A total of 29 cell lines was examined, including 12 that harbored the antisense episome. The expression of truncated HMG1 species was not detected in cell lines harboring the sense HMG1 construct.

4. Stable expression of SSRP1 antisense RNA

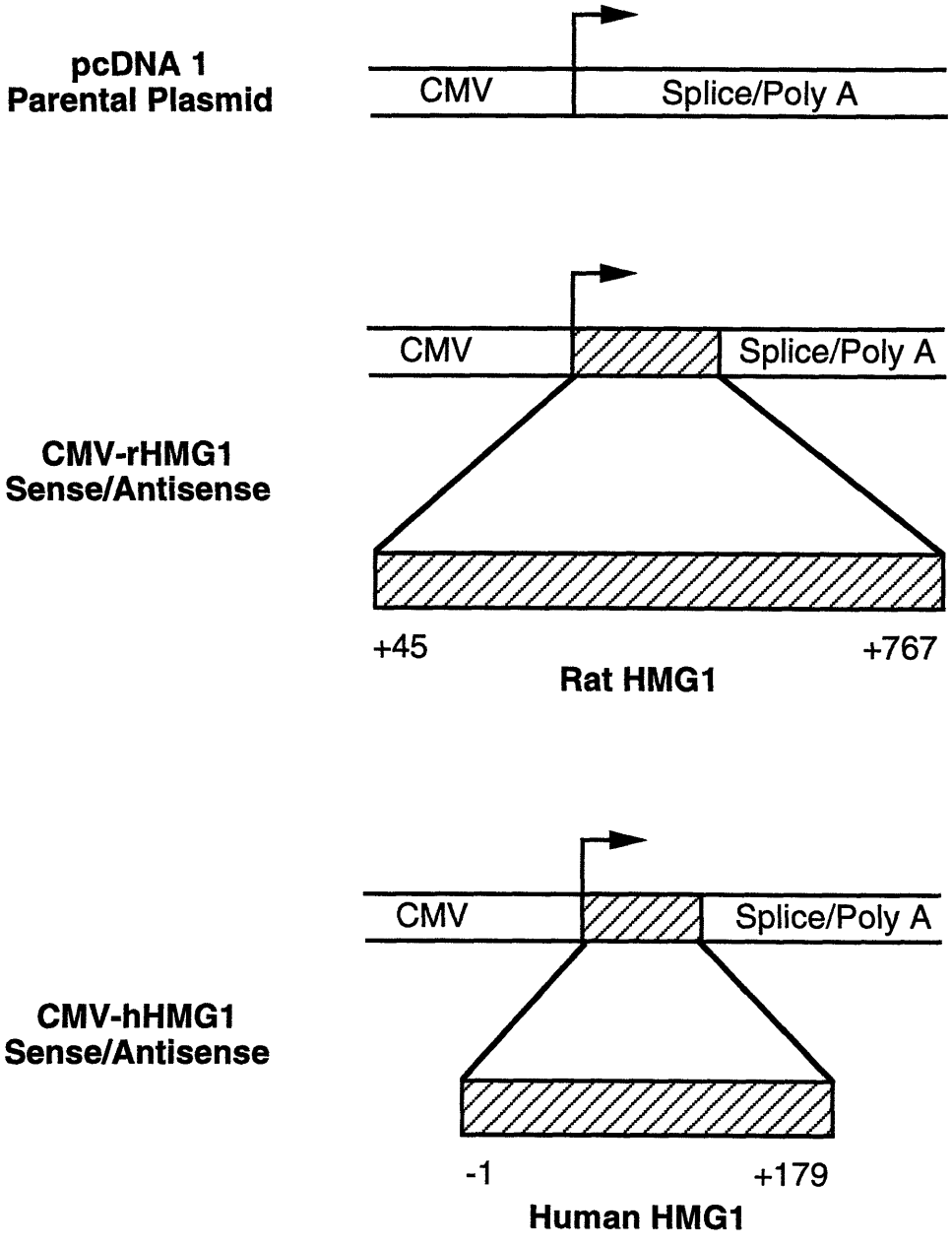
Raji cells were transfected with EBV-SSRP1-5'(AS) and the two control vectors, and selected in hygromycin B as above. As with the HMG1 constructs, a toxic effect of the SSRP1 constructs was noted, albeit less severe. The sense and antisense plasmids yielded 80% and 45% of the clones obtained with EBV-SPA (Table 2). This transfection was perfomed

only once, but there was good agreement between the duplicate transfections, and the survival values obtained for the pooled and isolated clone populations were in agreement. The pattern of relative survival can be summarized as EBV-SPA > SSRP1-5'(S) > > SSRP15'(AS).

The levels of SSRP1 in the cell lines harboring the antisense and control episomes was compared by western blotting. A total of six antisense SSRP1 cell lines were examined, four derived from isolated clones and two derived from pooled clones. The level of SSRP1 in these cell lines was indistinguishable from the level observed in control lines harboring the sense or parental episome.

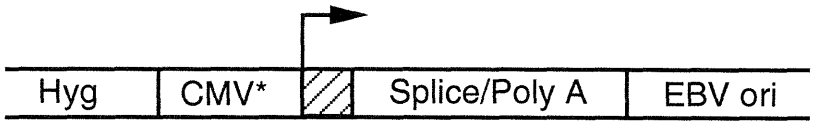
Figure 18. Antisense RNA plasmid constructs

Ⓐ Transient Expression

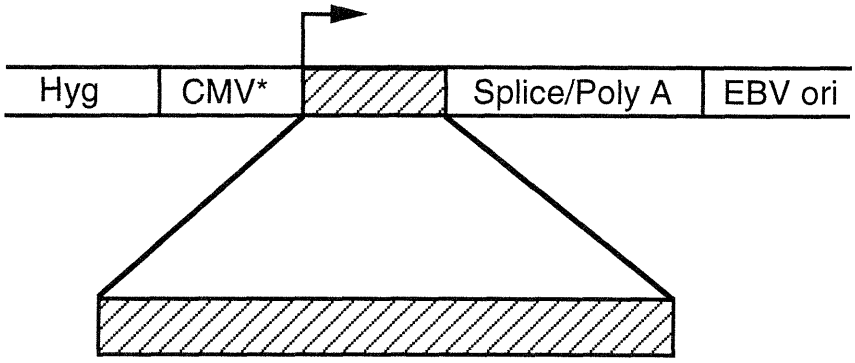


Ⓑ Stable Expression

EBV-SPA Parental Plasmid

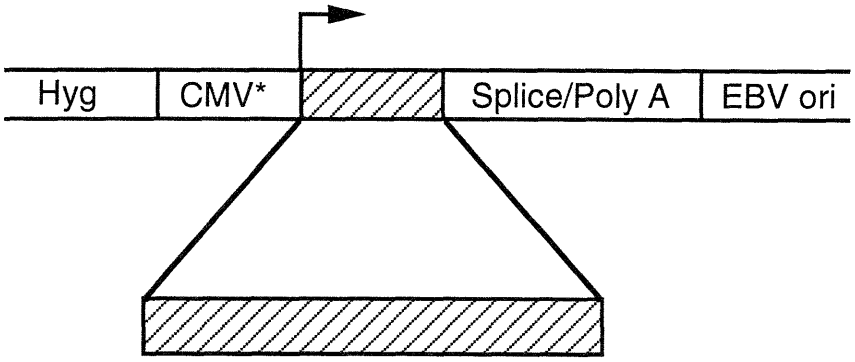


EBV-rHMG1 Sense/Antisense



Rat HMG1

EBV-SSRP1-5' Sense/Antisense



Human SSRP1

Figure 19. Antisense HMG1 RNA inhibits β-galactosidase activity. A reporter gene construct construct, pCMV-β-galactosidase, was cotransfected with vectors expressing rat HMG1 RNA and a control construct (pcDNA1). Enzyme activity was measured after 35 hr and is reported in arbitrary units. pcDNA1, parental control vector. CMV-rHMG1(S), sense rat HMG1 RNA. CMV-rHMG1(AS), antisense rat HMG1 RNA.

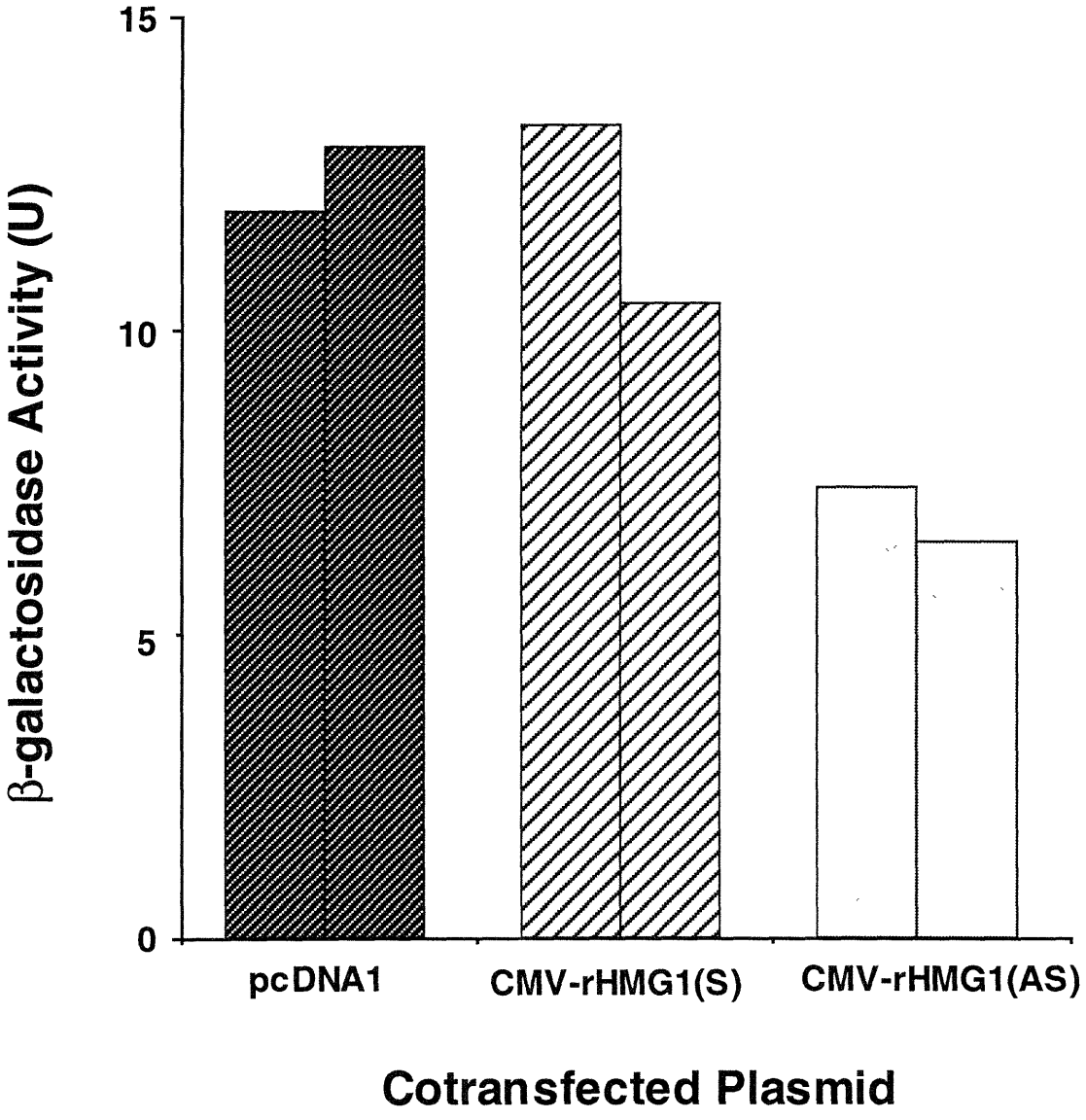

15
10
5
0
β-galactosidase Activity (U)
pcDNA1
CMV-rHMG1(S)
CMV-rHMG1(AS)
Cotransfected Plasmid

Table 1. Transfection efficiency of EBV constructs expressing rat HMG1 RNAs

PLASMID	Wells with Hyg^r clones (100 cells/well)	Wells with Hyg^r clones (1000 cells/well)	Relative transfection efficiency
EBV-SPA	14/96 17/96	96/96 96/96	100%
EBV-rHMG1(S)	0/96 1/96	42/96 76/96	<5%
EBV-rHMG1(AS)	3/96 2/96	96/96 96/96	16%

Table 2. Transfection efficiency of EBV constructs expressing human SSRP1 RNAs

PLASMID	Wells with Hyg^r clones (100 cells/well)	Wells with Hyg^r clones (1000 cells/well)	Relative transfection efficiency
EBV-SPA	78/96 85/96	96/96 96/96	100%
EBV-SSRP1-5'(S)	73/96 78/96	96/96 96/96	81%
EBV-SSRP1-5'(AS)	66/96 42/96	96/96 96/96	46%

B. DISCUSSION

1. Antisense HMG1 RNA inhibits the expression of a reporter gene

Transient transfections in HeLa cells showed that the expression of a reporter gene, β-galactosidase, was reduced when an antisense HMG1 construct was cotransfected (Figure 19). This phenomenon resulted from a specific antisense RNA-mediated event since control transfections with the cognate sense construct had no effect on β-galactosidase expression. Presumably, reduced HMG1 levels negatively affected the expression of the reporter gene. Unfortunately, the low transfection efficiency achieved in this experiment precluded this hypothesis from being tested directly.

Three models are proposed to explain the reduced-expression phenomenon. First, reduced HMG1 levels may be toxic to transfected cells, and dead or dying cells would not express a cotransfected reporter gene with high efficiency. This model is not consistent with the data, described above, of the stable transfection experiments that employed EBV vectors. In these studies the sense HMG1 construct was more toxic than the antisense construct. Hence, if toxicity were involved then the sense construct should have caused the reduced-expression effect as well. However, it may not be reasonable to compare the transient and stable transfections directly since the time scales and the DNA constructs differed in the two systems. A second model proposes that HMG1 facilitates the transfer of the reporter gene construct from the cytoplasm to the nucleus, where transcription occurs. Reduced HMG1 levels would negatively affect reporter gene expression because nuclear targeting would be diminished. There is evidence in the literature to support this model. To give one example,

liposome-mediated cell transfections are more efficient if HMG1 is mixed with the reporter gene construct (Kaneda *et al.*,1989). The authors speculated that HMG1 binds to the plasmid DNA and acts to facilitate its nuclear localization. A third model proposes that HMG1 is a general transcription factor. In this case reduced HMG1 levels would have a globally negative effect on transcription. There is indeed evidence to suggest that HMG1 has a general role in transcription as evidenced by the observation that antibodies against HMG1 reduce transcription in vitro (Singh and Dixon, 1990). Additionally, HMG proteins 14 and 17 interact preferentially with nucleosomes containing actively transcribed sequences (Einck and Bustin, 1985).

2. HMG1 RNAs are toxic to Raji cells

Vectors expressing sense or antisense HMG1 RNA were toxic to Raji cells. Interestingly, the sense RNA was significantly more toxic than the antisense RNA. Antisense-mediated toxicity was not surprising since HMG1 is likely to have a critical cellular function. In support of this hypothesis, the yeast homolog of HMG1 is essential for viability (Haggren and Kolodrubetz, 1988). The sense RNA has a 45 N deletion that encompasses the initiation codon; as a result the full length HMG1 cannot be expressed. It is possible that truncated HMG1 species were produced from translational initiation at internal methionines. HMG1 deletion mutants may have a dominant-negative effect on the function of normal cellular HMG1. In one possible scenario, HMG1 mutants would effectively compete with wild type HMG1 for DNA binding but would not be able to execute a critical subsequent function. This model seems feasible since an internal methionine codon is near the 5′ end of the truncated sense RNA. A caveat is that truncated

HMG1 species were not evident in cell lines harboring the sense construct. A similar lack of truncated products was noted in HeLa cells transiently transfected with sense HMG1 plasmids.

It is noteworthy that several hygromycin B-resistant clones were isolated that harbored the HMG1 sense or antisense constructs. These cell lines had normal HMG1 levels and did not appear to express truncated HMG1 species. Since expression levels can vary widely from clone to clone, it seems most likely that the "interesting" clones expressing high levels of antisense RNA or truncated HMG1 species were lost from the population. A similar effect has been reported previously with EBV vectors that express a toxic gene product (Hammerschmidt *et al.*,1989). These results suggest that HMG1 has an essential function. Antisense studies are still possible but will require new strategies and plasmid constructs (refer to the Future Experiments section).

3. SSRP1 may be an essential gene product

A 50% reduction in survival was noted with a vector expressing antisense SSRP1 RNA. By contrast, the sense RNA was minimally, if at all, toxic. The toxic effect of antisense SSRP1 RNA was evident in duplicate transfections within a single experiment, but the experiment should be repeated to substantiate this observation. Cell lines harboring the antisense episome expressed normal levels of SSRP1, suggesting that the SSRP1-deficient cell lines may have been lost from the population. It is also possible, however, that the antisense SSRP1 RNA may not reduce SSRP1 levels. The construct used in these studies expresses only 20% of the SSRP1 coding region. Perhaps a longer antisense RNA is required for

inhibition of SSRP1 expression.

The function of SSRP1 is unknown, but the results reported here suggest that whatever that function may be is critical. The mouse homolog of SSRP1 binds to V(D)J recombination sequences suggesting a possible role in gene rearrangement or recombination (Shirakata *et al.*,1991). It is unclear if V(D)J binding is specific or fortuitous. In my opionion the binding is fortuitous since most HMG proteins bind promiscuously to a variety of DNA sequences and structures. In addition, SSRP1 mRNA is ubiquitously expressed and does not have tissue specificity. This argues against a specific role in V(D)J recombination but does not rule out a more general function in recombination. A possible role of SSRP1 in transcriptional regulation has also been suggested. The chicken homolog of SSRP1 was shown to recognize an enhancer sequence in the collagen II gene (Wang *et al.*,1993). Although this is an interesting result, it does not demonstrate a role in transcriptional regulation.

C. CONCLUSIONS

It appears that HMG1 has an essential function in human cells. Expression of a truncated sense or antisense version of the HMG1 RNA resulted in significant toxicity. These experiments did not reveal how the sense RNA mediates toxicity, but a dominant negative effect has been proposed. In addition, a general role for HMG1 in transcription was suggested in transient transfection studies; antisense HMG1 RNA inhibited the expression of a cotransfected reporter gene. The role of HMG1 in cisplatin-mediated toxicity remains elusive.

The studies with SSRP1 antisense RNA were less complete but suggested that this gene may also be essential. The cellular function of SSRP1 as well as its role in cisplatin toxicity remain a mystery.

D. FUTURE EXPERIMENTS

The antisense studies should be performed with inducible promoters, which may allow the toxicity problem to be circumvented. One of the problems associated with the transient transfection studies is the low transfection efficiency in that only 15% of the cells surviving the transfection procedure take up and express a reporter gene construct. It would be of use to isolate the transfected cells. If the antisense plasmids are cotransfected with the β-galactosidase construct, then fluorescence activated cell sorting may facilitate the isolation of a homogeneous cell population consisting of transfected cells.

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BIOGRAPHY

The author was born December 22, 1965 in Putnam, Connecticut and spent most of his childhood in Willington, Connecticut. He attended Hall Memorial Elementary School and Windham High School where he played soccer, ice hockey, and baseball in addition to avidly pursuing the sciences. In 1983 he entered Middlebury College where he majored in biology. During the summers of his college years the author performed toxicology research at the University of Connecticut in the laboratory of Dr. Richard Dicapua. While at Middlebury College, he completed a senior research project under the supervision of Dr. Robert Cluss for which he received the Egbert C. Cole biology award. In 1987 he earned a B.A. degree *magna cum laude* and was elected to *Phi Beta Kappa*. To pursue further his interest in toxicology, the author entered the Department of Applied Biological Sciences at MIT and joined the laboratory of Dr. John Essigmann. His doctoral research was focused on the function of cellular proteins that respond to damaged DNA. Upon completion of his doctoral studies September 29, 1993, he became a postdoctoral associate in the laboratory of Dr. James Williamson in the MIT department of chemistry