DNA DAMAGE RECOGNITION PROTEINS AND THEIR INVOLVEMENT IN CISPLATIN RESISTANCE

Karen M^{C}Laughlin B.Sc.

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Dept. Medical Oncology
CRC Beatson Laboratories
Glasgow
G61 1BD

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To

Colin for being there whenever.....

and

Mum and Dad for their constant support and encouragement throughout my education

"The problem of cancer remains a Gargantuan challenge to the science of biology. Some day it may yield, but until it does, biologists have their work cut out for them."

William S. Beck (Modern Science and the Nature of Life, 1957)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

^0C	Degrees centigrade
3bpMM	3 base pair mismatch
7bpMM	7 base pair mismatch
A,T,C,G	Adenine, Thymine, Cytosine, Guanine
APS	Ammonium per sulphate
ATP	Adenine triphosphate
CAT	Chloramphenicol acetyl transferase
CDDP	cisDiammine-dichloroplatinum (II)
cm	Centimetre
cpm	Counts per minute
cps	Counts per second
DDRP	CDDP DNA damage recognition proteins
DNA	Deoxyribonucleic acid
DRP	DNA damage recognition proteins
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-Acetic acid
HPLC	High performance liquid chromatography
KD	Kilodalton
M	Molar
mA	Milliamps
µg	microgram
mg	milligram
$MgCl_2$	Magnesium Chloride
µl	microlitre
ml	millilitres

mm	Millimetre
MMC	Mitomycin C
$MnCl_2$	Manganese Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	nanogram
NH_3	Ammonia
NH_4HCO_3	Ammonium bicarbonate
PBS	Phosphate buffered saline
SB	Salt buffer
SDS	Sodium Dodecyl Sulphate
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	N, N, N', N'- tetramethylethylenediammine
UDS	Unscheduled DNA synthesis
UV	Ultra-violet
XP	Xeroderma Pigmentosum
ICPMS	Inductively coupled plasma mass spectroscopy

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ABSTRACT

cis-Diamminedichloroplatinum(II) (CDDP) is a chemotherapeutic agent widely used in the treatment of various types of cancer. Its mechanism of cytotoxicity is unclear although it is believed that DNA is the critical target. CDDP binds to DNA forming a variety of adducts including intrastrand adducts, interstrand adducts, monofunctional adducts and DNA-protein crosslinks. This thesis presents evidence that there are protein(s) present in mammalian cells which recognise CDDP-damaged DNA.

To assay for these DNA damage recognition proteins (DDRPs) conditions for two very separate assays were developed. The gel mobility shift assay, which detects protein complexes under non-denaturing conditions, identified two retardation complexes which bound to CDDP damaged DNA in human, murine and feline tumour cell extracts. Binding of these complexes is shown to CDDP treated oligonucleotide of 54 base pairs but not to a CDDP treated oligonucleotide of 27 base pairs, therefore suggesting binding is dependent on having normal DNA duplex.

The other system used in the detection of the DDRPs is the South-Western assay. This allowed the detection of proteins of sizes 25, 50, 100KD binding to CDDP treated DNA. The proteins in the South-Western system are run under denaturing conditions. It is not entirely clear as to whether the proteins detected in both systems are the same or whether they represent entirely different species.

CDDP has been reported to bind to DNA and cause areas of single-strandedness around the adducts. The results presented in this thesis demonstrate that the 50KD and 100KD DDRP which bind to CDDP treated double-stranded DNA may also have an affinity for single-stranded DNA. The 25KD DDRP, however, only recognises double-stranded DNA treated with CDDP suggesting that it is recognising the CDDP adducts and not the areas of single-strandedness generated around the adducts.

Resistance to CDDP proves a major problem area in treatment regimes. Many cell lines resistant to CDDP have been derived *in vitro* by multiple exposures to the drug. Many mechanisms of resistance to CDDP have been suggested from these lines. If a role of the DDRPs was to process damage in the DNA then cell lines resistant to CDDP may show an increase in expression of the DDRPs. This thesis presents evidence that an ovarian tumour cell line resistant to CDDP in comparison to its parental line shows an increase in the binding to the 50KD and 100KD DDRPs.

Work in chapter 5 presents the isolation of CDDP resistant cell lines, by acute exposure to the drug, with an increase of up to seven fold resistance levels. Evidence is presented for the resistant clones being of a mutational origin. Resistant variants occur at a frequency of 3.2×10^{-6} per viable cell. This frequency can be increased to 3.4×10^{-5} by treatment of the cells with the chemical mutagen ethyl methane sulphonate, EMS. The CDDP resistant phenotype is maintained after six months growth in drug free medium. This single step selection may provide clones which are more clinically relevant than the lines isolated by multiple exposures to CDDP. They may therefore provide a superior model for the study of drug resistance mechanisms to CDDP. However examination of the DDRPs showed no detectable difference in the resistant clones derived from the A2780 human ovarian tumour cell line.

The thesis therefore presents evidence of the existence of DDRPs in mammalian cells. The role of these damage recognition proteins will be discussed.

CHAPTER 1

GENERAL INTRODUCTION

1.1 DISCOVERY OF CISPLATIN

Chemotherapy has proved curative for some types of advanced cancers and useful in compliment with surgery and radiotherapy in others. Unfortunately, many cancers still do not respond to chemotherapy, and other cancers that initially do respond later become resistant. The question of how tumours become resistant to these drugs is obviously of major importance. One of the major sets of drugs in the clinic to-day, is the platinum coordination complexes which have proved successful in the treatment of ovarian, testicular, bladder, head and neck cancers (Loehrer and Einhorn, 1984). However cellular resistance to these drugs has been suggested as a reason for platinum based treatment failure.

The antitumour activity of the platinum complexes was discovered as a result of a fortuitous observation by Rosenberg during the study of effects of electric current on growing bacteria (Rosenberg et al, 1965). When alternating current was delivered through platinum electrodes to a growing culture of *Eschericia coli*, the *E.coli* cells stopped dividing and grew into long filaments. It was found that a compound was formed from the platinum electrodes during electrolysis which inhibited cell division - cis$Pt(II)(NH_3)_2Cl_2$. This product is now more commonly known as cis-diamminedichloroplatinum(II) or cisplatin (CDDP). The stereochemistry of these compounds was found to be important for inhibiting cell division since Rosenberg deduced that the corresponding trans isomers of those compounds did not inhibit cell division but acted only as a bacteriocide (Rosenberg, 1965).

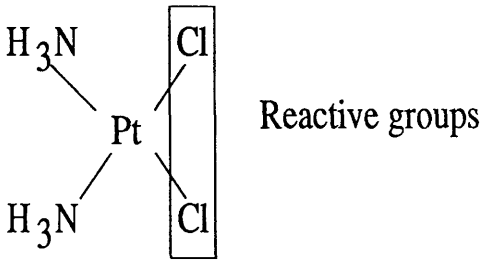
1.2 HISTORY OF CISDIAMMINEDICHLOROPLATINUM (II) (Cisplatin) AS AN ANTITUMOUR AGENT

After the initial discovery by Rosenberg in 1965, of the inhibition of bacterial cell division by the platinum compounds, interest was stimulated concerning whether these compounds can inhibit cell division in other mammalian cells. Two years later, Rosenberg demonstrated that platinum coordination compounds cis-diamminedichloroplatinum(II) and cis-diamminedichloroplatinum (IV), have potent antitumour activity against Sarcoma 180 and leukemia L1210, whereas the trans geometrical isomer of these compounds are ineffective (Rosenberg et al, 1969, 1970). The importance of steric conformation is therefore highlighted by the fact that the trans isomer has virtually no antitumour activity. Other groups showed that these compounds were also effective against a virus-induced reticulum cell sarcoma in mice (Talley, 1970), the Dunning ascitic leukemia, Walker 256 carcinoma (Kociba, 1970) and a dimethyl benzanthracene induced mammary carcinoma (Welsh, 1971). In 1972, the CDDP compound was released for clinical trials and generally referred to as the antitumour drug cisplatin, abbreviated throughout this thesis as CDDP. Seven years later, FDA approval of the drug was passed and to date it has proved to be one of the most successful drugs in the treatment of testicular, ovarian, bladder and head and neck carcinomas (Loehrer and Einhorn, 1984).

1.3 CHEMICAL STRUCTURE OF CDDP

CDDP, is a neutral square planar coordination complex (**Figure 1.1**).

Figure 1.1 Chemical structure of CDDP



It has two reactive chloride groups and two relatively inert ammine groups. At extracellular chloride concentrations, the chloride groups are stable. However the intracellular concentration of chloride is lower and once CDDP enters the cell, the two chloride groups are exchanged for hydroxyl groups. This exchange leads to a bifunctional charged electrophile which can react with nucleophilic sites in the cell e.g. DNA, RNA, proteins etc.

1.4 DNA AS THE CRITICAL TARGET

It is not entirely clear as to how CDDP is cytotoxic in cells, although there is extensive evidence in the literature implicating DNA as the critical target for cytotoxicity (Pinto and Lippard, 1985), although it should be borne in mind that much of the evidence in mammalian cells is circumstantial. The initial observation, of CDDP induced filamentous growth, was the first indication of interaction with DNA (Rosenberg, 1965). Further evidence came from the induction of growth of phage from lysogenic strains of *E.coli* (Reslova 1971). Normally, release of phage DNA to synthesise new DNA is a rare event. However upon treatment with low concentrations of CDDP, induction of the lytic cycle was found. There was found to be a correlation between the antitumour activity of the platinum compounds and their ability to induce lysogenic *E.coli* to enter the lytic

cycle. Most of the proposed mechanisms of CDDP induced cytotoxicity involve DNA and the following section will introduce each of these.

1.5 MECHANISMS OF CYTOTOXICITY

Various different hypotheses have been suggested as to how CDDP kills the cell, these include :

(1) inhibition of DNA synthesis

(2) apoptosis

(3) inability to repair specific adducts

(4) mutagenesis

1.5.1 Inhibition of DNA synthesis

There have been many studies since the discovery of CDDP to suggest inhibition of DNA synthesis as the pathway for its cytotoxicity. Studies in human AV_3 cells in culture (Harder & Rosenberg, 1970) and Ehlrich cells *in vivo* (Howle & Gale, 1970) have demonstrated that CDDP inhibits DNA replication. It has generally been shown that concentrations at which DNA synthesis is inhibited does not affect either RNA or protein synthesis (Roberts & Thomson, 1979). It has however been reported that superlethal concentrations of CDDP were required before inhibition of DNA synthesis was observed (Salles et al, 1983). In this report however, DNA synthesis was only measured during 90 minutes after incubation. Reports have previously demonstrated progressive slowing of DNA synthesis over several days (Sorenson and Eastman, 1988). It has been suggested that DNA synthesis may be inhibited for two main reasons :

(1) direct inhibition of the polymerases - DNA polymerases have been shown to be stopped *in vitro* by CDDP intrastrand cross-links (Pinto and Lippard, 1985).

(2) synchronization in a phase of the cell cycle in which DNA synthesis would not be anticipated - cells have been reported to arrest in G_2 upon treatment with CDDP, (Sorenson and Eastman, 1988).

1.5.2 Apoptosis

Since the discovery of the G_2 arrest, Eastman has presented several hypotheses to try to explain this. It was first suggested that the G_2 arrest allowed the cells time for post replication repair. However gaps in newly synthesised daughter strands were unable to be detected (Sorenson and Eastman, 1988). It was then suggested that G_2 arrest may be due to the inability to produce transcripts needed for mitosis. This was then disproved by studying cell lines deficient and proficient in DNA repair, the repair proficient cells being expected to circumvent this arrest. They demonstrated that in both cell lines the G_2 arrest occurred, however in the repair proficient cells a higher concentration of CDDP was required before arrest was observed. It is therefore presumably the number of adducts present in the DNA that is important.

In the yeast system it has been demonstrated that G_2 arrest caused by radiation is controlled by RAD9 (Weinert and Hartwell, 1988). Cells deficient in RAD9 fail to arrest in G_2; they continue to cycle and then die. Therefore there may be a similar system in mammalian systems that prevent the passage of cells into mitosis. The events occurring in the G_2 arrest are similar to those which occur in programmed cell death - apoptosis.

Apoptosis (first defined by Kerr et al 1972) is a phenomenon which is morphologically defined by cell shrinkage and especially in epithelial cells, loss of cell to

cell contact. Double strand breaks are a characteristic of the cell about to undergo apoptosis. The cells show a specific pattern of chromatin condensation, giving a dense mass close to the nuclear margin (Arrends, 1990). The next stage is the development of apoptotic bodies which express new cell surface markers. These are recognised by phagocytes and engulfed so that the cell dies without inflicting damage on its nearest neighbours. Protein synthesis is required for apoptosis. Apoptosis occurs during embryonic development, metamorphosis, differentiation and general cell turnover.

Many of the anticancer agents including CDDP appear to activate apoptosis (for review of biochemical and morphological evidence see Eastman, 1990). Many unanswered questions on apoptosis may lead to the understanding of toxicity in anticancer drugs

(1) what causes G_2 arrest in mammalian cells, and is there a mammalian homolog to RAD9?

(2) how do cells overcome G_2 arrest either to survive or die?

(3) what factors discriminate normal from abnormal mitosis?

(4) activation of endonuclease, how and where?

(5) what is the role of endonuclease in normal cell regulation and death?

(6) how do so many agents activate the same pathway?

1.5.3 CDDP-DNA adducts and the cells ability to repair them

Approximately 1% of the total cellular platinum binds to DNA, inducing various types of inter and intra-strand crosslinks (Eastman, 1988). Over the past decade the interaction of CDDP with DNA has been studied by a variety of techniques :

(1) Alkaline elution (Kohn and Grimek-Ewig, 1973)

(2) DNA renaturation (Eastman, 1982)

(3) DNA sedimentation (Roberts and Pascoe, 1972)

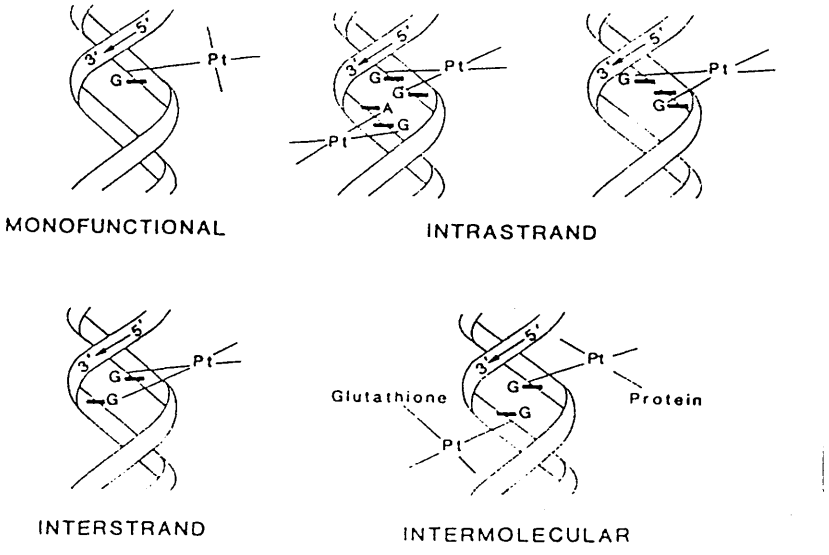
(4) Digestion of platinated DNA to nucleotides and analysis by HPLC fractionation (Fichtinger-Schepman, 1982)

(5) Antibodies to specific adducts (Fichtinger-Schepman et al, 1987; Poirer et al, 1982)

The first three methods however depend upon the capacity of CDDP to form either DNA interstrand cross-links or DNA protein cross-links - which have been shown to represent less than 1% of platinated DNA (Eastman 1982). All the above techniques have subsequently shown that CDDP can form a variety of adducts **(Figure 1.2)**

- Monofunctional
- Intrastrand cross-links including 1,2 d(GpG), 1,2 d(ApG), 1,3 d(GpG)
- Interstrand cross-links
- DNA-protein cross-links

Figure 1.2



This figure is adapted from Eastman, (1990)

These major adducts have been reported in treatment of DNA *in vitro* (Eastman, 1986), and in DNA of CDDP exposed bacteria (Fichtinger-Schepman et al, 1986), CHO cells (Plooy et al, 1985[a]) and human white blood cells (Fichtinger-Schepman et al, 1987).

The majority of cross-links formed have been found to be DNA-intrastrand crosslinks with 65% of these cross-links between two neighbouring guanine bases at the N7 atom 1,2 d(GpG), 25% cross-linking neighbouring adenine and guanine bases 1,2 d(ApG) and the remaining cross-linking two guanine bases separated by one or more bases 1,3 d(GpG). The formation of cross-links at the guanine bases suggests this is a site of favoured reaction on the DNA for the CDDP. A small percentage of cross-links (less than 1%) are present in the interstrand formation and also as DNA-protein cross-links.

In the initial reports on the measurement of adducts, DNA interstrand crosslinks were suggested to be the cytotoxic lesion of CDDP. This stems from work when only DNA interstrand cross-links were the measurable adducts and so were found to correlate with cytotoxicity. There was the general hypothesis that cytotoxicity could be due to the persistence of a specific cross-link which was not repaired by the cell. Some reports have shown the persistence of the interstrand cross-links (Erickson et al, 1981; Plooy et al, 1985[b]). Conflicting reports in other cell lines have shown that a defective repair of inter-strand crosslinks is not due to the cytotoxicity of CDDP (Rawlings & Roberts, 1986). Even if a correlation did exist between the persistence of interstrand cross-links and CDDP, it must be kept in mind that less than 1% of the cross-links are of the interstrand formation *in vivo* (Plooy et al, 1984). Thus although the number of interstrand cross-links might be proportional to the decrease in cell survival, large numbers of other types of adducts are being ignored which may also exhibit the same relationship and ultimately be the cause of platinum induced cytotoxicity.

It is now believed that the major intrastrand adduct, the 1,2 d(GG) adduct is the cytotoxic lesion of CDDP. Studies have demonstrated that testicular cell lines which were five fold more sensitive to CDDP in comparison to bladder lines were deficient in the repair of 1,2 d(AG) and 1,2 d(GG) intrastrand cross-links (Bedford et al, 1988). A study by Bohr and co-workers demonstrated that inter-strand crosslinks were removed more efficiently in specific gene regions than intra-strand adducts (Jones et al, 1991). This therefore suggests that the intra-strand adducts, which have shown to be the major CDDP adduct, may be the persistent lesion which causes cytotoxicity.

To measure adduct levels *in vivo*, antibodies have been raised to the CDDP adducts by two different groups (Fichtinger-Schepman et al, 1987; Poirer et al, 1982). This has allowed the measurement of adduct levels in white blood cells of patients who have been treated with CDDP. There is however great debate going on between the two groups due to discrepancies occurring when both their assays are directly compared

(Fichtinger-Schepman et al, 1989; Reed et al, 1990). To measure the adducts Poirer and co-workers have used a polyclonal rabbit antiserum raised against calf thymus DNA modified with CDDP (Poirer et al, 1982) whilst Fichtinger-Schepman has used polyclonal antibodies raised against CDDP containing dinucleotides, as haptens coupled to proteins (Fichtinger-Schepman et al, 1985). The antiserum raised by Poirer has been reported to show a high specificity for poly(dG).poly(dC) therefore it recognises the major adducts (Lippard et al, 1983) formed at the guanine nucleotides. The antibodies raised by Fichtinger-Schepman have been shown to be specific for platinum attached to guanine nucleotides. Both sets of antibodies therefore recognise the major CDDP adducts. Comparing both groups of antibodies, Fichtinger-Schepman detect 14-300 fold higher adduct levels in identical human blood samples to Poirer (Fichtinger-Schepman, 1989). It has been suggested that the antibodies recognise different epitopes. Whatever the reasoning for the disagreement over the detection of adducts by the antibodies they will prove of major importance in the detection of the *in vivo* adduct forming the important lesion.

It is necessary at this point to make a distinction between two not necessarily related phenomena: cytotoxicity and antitumour activity. Cytotoxicity is a non-selective process of killing whereas when discussing antitumour effects of drugs, the idea of selectivity to tumour cells is introduced. Unlike the cis isomer CDDP, the trans isomer transplatin (TDDP) has been demonstrated to exhibit cytotoxicity but no antitumour activity. Both CDDP and TDDP bind to DNA therefore the differences between the CDDP exhibiting antitumour activity and the TDDP, may be related to the differences between the two isomers' reactions with the DNA. The following explanations can be put forward for the differences in both cytotoxicity and antitumour activity between the two:

(1) TDDP has been shown to form a majority of DNA-protein cross-links unlike CDDP, where DNA protein cross-links are present in a minority of the population. The comparison between the two, may argue against the DNA-protein cross-links being the important antitumour lesion. This evidence is by no means definitive as both CDDP and TDDP may cross-link to different proteins and this could be related to both the cytotoxicity and antitumour activity.

(2) CDDP but not TDDP forms intrastrand cross-links between neighbouring bases on a single strand. TDDP being in the trans configuration can only form cross-links between guanine bases separated by one or more bases 1,3 d(GpG). Therefore the cytotoxicity and the antitumour activity may be related to the major 1,2 d(GpG) adduct observed.

(3) It has been reported that adducts formed by CDDP accumulate in the cell over a period of 48 hours whereas those formed by TDDP accumulate for the first six hours and then decreased presumably by repair mechanisms. It has therefore been suggested that a specific adduct namely the 1,2 d(GpG) adduct formed only by CDDP are not efficiently recognised or repaired and hence remain to kill the cell (Ciccarelli et al, 1985).

The above lists three possibilities on the basis of differences between CDDP and TDDP for the cytotoxicity and antitumour activity of CDDP. It is however, at this stage not definite, as to which, if any, of the DNA adducts are either the cytotoxic lesion, antitumour lesion or both, although the general opinion is that the 1,2 d(GpG) adduct is the important cytotoxic lesion.

1.5.4 Mutagenesis

CDDP has been reported to be mutagenic in *E.coli* (Brouwer et al, 1983), and *Salmonella typhimurium* (Leopold et al, 1981) and carcinogenic in mouse and rat (Leopold et al, 1979; Leopold et al, 1981). It may therefore induce mutations that lead to lethal effects in the cell.

In bacteria it has been shown that chemical mutagenesis requires the involvement of an inducible cellular system leading to increased mutation rates. This type of system has been described as SOS processing or error prone repair (review, Little and Mount 1982; Walker, 1984). SOS repair generally involves the activation of a protease, Rec A, which cleaves a repressor Lex A which normally acts to inhibit the function of different genes. On the proteolytic cleavage of Lex A, the products of the SOS target genes are expressed at much higher levels and the secondary functions of the SOS pathway are expressed, e.g

- Prophage induction
- Enhanced DNA repair capacity, both excision repair and post-replication repair
- Filamentous growth
- Enhanced mutagenesis

A signal must activate the Rec A before the SOS processing can occur: signals include DNA damage and inhibition of replication.

The contribution of the different CDDP adducts to the mutagenic potency is unknown. Studies in *E.coli* have demonstrated 5'Gs in GAG and GCG sequences to be hotspots for base substitution by CDDP (Brouwer et al, 1981). Base substitution has been suggested to be SOS-dependent. Mutants in Rec A (Rec A^-) do not show base

substitutions (Brouwer et al, 1981). Studies in *E.coli* also demonstrated the requirement for the SOS pathway for the full mutagenic effects of CDDP (Burnouf et al, 1987). Evidence is emerging from the mammalian system to suggest that inducible pathways are involved in how the cell responds to DNA damage. Studies in cells exposed to carcinogens or tumour promoters suggest an inducible pathway is present in mammalian cells (Elespuru, 1987).

The suggestion of the GCG and GAG being hot-spots for mutations occurring by CDDP treatment of *E.coli* has been questioned by Burnouf and co-workers, 1987. Using an assay which is not limited to the detection of nonsense mutations they have shown most mutagenic lesions in *E.coli* occurs at the ApG sites, giving predominantly A.T - T.A transversions. They also demonstrated that the GpG site is also prone to mutagenesis (Burnouf et al, 1987). Using this forward mutation assay, it is however not possible to determine the effects of individual adducts in the mutagenesis events. This is now beginning to be overcome by the transfection of vectors containing synthetic oligodeoxyribonucleotides with defined adducts into cells. Burnouf and co-workers have used such a system to examine the mutagenic effects of the d(ApG) site (Burnouf et al, 1990). They have described these sites as exhibiting a high mutation specificity. These sites have previously been reported to be potential sites for CDDP adducts (Eastman, 1986). Studies in *Saccharomyces Cerevisiae* have shown the 5'GG and 5'GA sequences to be hot-spots for mutation (Mis and Kunz, 1990) and other studies in the aprt gene of CHO cells have demonstrated mutations to occur at or near to the 5'AGG and 5'GAG sequences (Boer and Glickman, 1989). Evidence in the literature has therefore implicated the major intra-strand crosslink as a pre-mutagenic lesion.

The introduction so far has discussed how CDDP was first discovered and the way in which it is thought to kill cells. The following section of the introduction will focus on how tumour cells become resistant to CDDP. The following chapters in the

thesis will attempt to investigate how tumour cells may become resistant to CDDP *in vitro*. It is therefore apt at this point to introduce the phenomenon of drug resistance and the studies to date of reported mechanisms of resistance to CDDP.

1.6 RESISTANCE OF TUMOURS TO CHEMOTHERAPEUTIC DRUGS

Although chemotherapy has proved successful in the treatment of various forms of cancer, resistance of the tumours to the drug is still a main cause of treatment failure. The phenomenon of resistance was encountered in the first documented case of cancer chemotherapy (Gilman, 1963).

Resistance has been viewed as either temporary or permanent. Temporary resistance can be explained by a variety of factors. If a tumour mass is large, only a certain fraction will be killed leaving the remaining tumour cells behind to divide and grow again. It may also show resistance due to cell kinetics. If cells in the tumour are slow growing then it may not respond as well to drugs acting on dividing cells as will a fast growing tumour. Permanent resistance however, will occur even before a tumour cell population is subjected to chemotherapeutic drugs. Goldie and Coldman hypothesised that the best explanation for treatment failure in the clinic is that drug resistant mutants exist or are induced in the tumour cell population (Goldie and Coldman, 1984). This model is based upon the principles of the Luria-Delbruck fluctuation analysis (Luria and Delbruck, 1943), refer to chapter 6, section 6.1 for further introduction. Resistance is therefore the topic of study for many groups for two main reasons :

(1) in an attempt to prevent resistant cells occurring

(2) if resistance occurs, the understanding of the mechanisms leading to this resistance may allow the development of treatments to circumvent these.

Goldie and Coldman proposed that at the time of detection of a large tumour mass, there is a high probability of resistant cells being present. In terms of treatment of the tumour, this leads to the question of how to circumvent resistance in these cells. Successful circumvention of resistance has been reported experimentally in cells which are resistant to multiple drugs. These cells are referred to as exhibiting pleiotropic drug resistance or expressing the MDR phenotype and are found to contain an increase in a protein known as P-glycoprotein. This protein has been reported to be a membrane protein which actively pumps drug out of the cell (Kartner and Ling, 1989). The resistance phenotype described here can be overcome by the calcium channel blocker verapamil (Tsuro et al, 1981). For CDDP however, the mechanisms of resistance are only beginning to unfold and rational approaches to circumventing resistance are still in their early stages, although it has been demonstrated that resistance to CDDP is not mediated by increased P-glycoprotein expression.

1.7 MODELS FOR STUDY OF RESISTANCE MECHANISMS

Cell culture model systems have been useful in allowing an understanding of the mechanisms of resistance to various drugs, although there limitations must be bore in mind. Cell culture model systems, unlike *in vivo* studies are maniputable and factors within them can be controlled. Many studies on mechanisms of resistance to various drugs have been carried out in this type of cell model system. Cells can be made resistant to a drug by exposing them to increasing concentrations of drug over a period of months, even years. After each exposure, the cells exhibiting a resistance to the drug are selected for. Upon each increasing concentration of drug, cells with an increased resistant phenotype will be selected for. Many resistant cell lines have been isolated in this way (e.g Behrens et al, 1987). The disadvantage of studying mechanisms of resistance in cell

lines isolated in this way, is that cells present are bound to show different mechanisms of resistance because they have been isolated in multiple steps.

Cell lines resistant to drugs have also been isolated by a single exposure of the cell line to a predetermined concentration of drug which has been shown to give only one or two surviving mutants. Cell lines isolated in this way are more likely to exhibit a single mechanism of resistance and are less likely to show as great a resistance as those cell lines isolated by multiple exposures. These cell lines should make the unravelling of mechanisms of resistance to certain drugs much easier (for review see Thomson & Baker, 1973), (refer to chapter 6, section 6.1 for further information).

The key question is, which of the mechanisms of resistance characterised in these *in vitro* models contribute to the drug resistance that emerges in patients during the treatment of tumours in patients.

1.8 SUGGESTED MECHANISM OF RESISTANCE TO CDDP

The mechanism(s) by which a tumour becomes resistant to the treatment of CDDP is a vigorously ongoing area of research. Most of the following suggested mechanisms have been characterised in cell lines derived from tumours which have been selected for resistance to CDDP by stepwise increasing exposure to the drug. The following list presents suggested mechanisms of resistance to CDDP reported in the literature from *in vitro* studies.

(1) Accumulation Decreased CDDP accumulation has been reported in several cell lines resistant to CDDP (Andrews and Howell, 1990). It is however not entirely clear whether this is due to a decreased influx or increased efflux. Increased expression in a membrane protein of size 200KD has been observed in a murine lymphoma cell line resistant to CDDP, (Kawai et al, 1990). This is different

from the multi-drug resistance associated P-glycoprotein with a molecular weight of approximately 170KD.

(2) Glutathione The reactive electrophilic CDDP species will readily react with nucleophilic sites, such as GSH. Suggested mechanisms of resistance to CDDP include inactivation of the drug by glutathione (GSH). Many studies have demonstrated increased levels of GSH in CDDP resistant cell lines, although little correlation has been found between GSH levels and CDDP cytotoxicity (for review, Andrews and Howell, 1990). Elevated levels of GSH have been observed as part of the resistant phenotype, only when cells were selected for CDDP resistance by continuous exposure to CDDP *in vitro*. There is no clear evidence as to whether the increased levels of GSH are involved in CDDP resistance or whether they are part of a general stress response to CDDP.

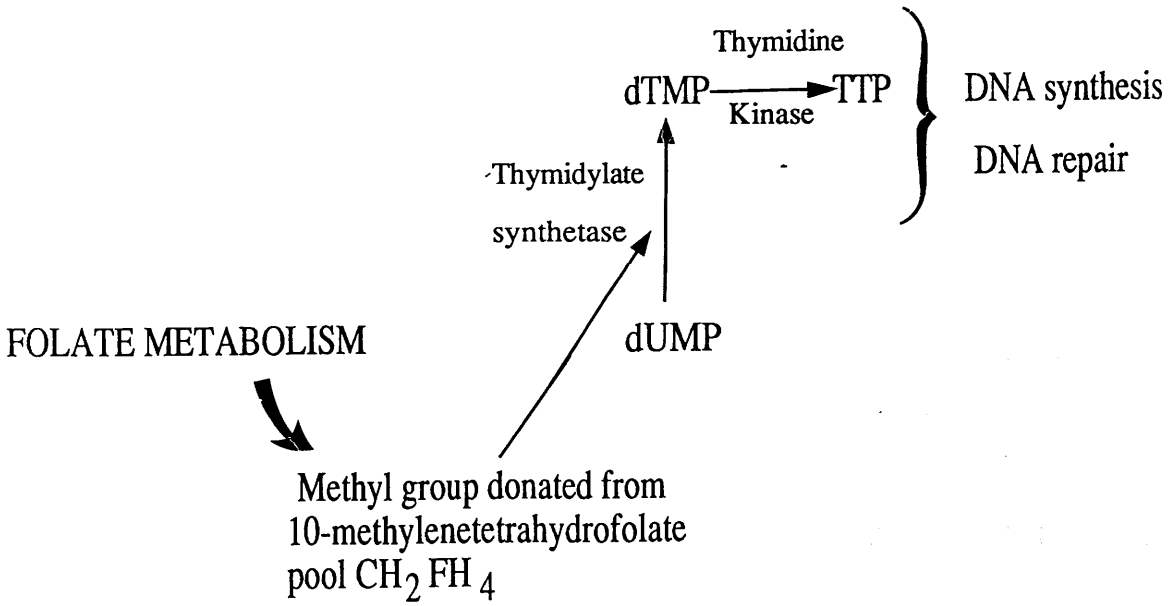
(3) Metallothioneins Metallothioneins, like the GSHs, may act as a nucleophile towards CDDP. The metallothioneins comprise of a family of proteins involved in Zn^{++} homeostasis and are also involved in the detoxification of other heavy metals. Metallothioneins have been shown to act as a sink and soak up any incoming CDDP. Studies have shown elevation of metallothioneins in some CDDP resistant cell lines (Kelley et al, 1988). These cell lines have been made resistant to CDDP *in vitro* by exposing to high concentrations of CDDP for long periods of time. It is not entirely clear whether metallothioneins are important in clinical specimens. The involvement of metallothioneins at this point in time is controversial.

(4) Signal transduction pathways Patient studies and cultured cell line studies have suggested amplification of oncogenes such as c-fos and c-Ha-ras occurs in CDDP resistance (for review, Scanlon et al, 1989[a]). There is however

considerable genetic variability among patients and cultured cell lines therefore it may be argued that increased expression of a gene may not be responsible for the CDDP resistance phenotype. Transfection studies where transfection of a single oncogene e.g ras, in cell lines have demonstrated that the ras oncogenes may be important in the CDDP resistant phenotype. Transfection of NIH3T3 with H-ras, activated by a missense mutation are 4-8 fold more resistant to CDDP than the control NIH3T3 lines (Sklar, 1988). More detailed studies are required to define the role, if any, of the oncogenes in CDDP resistance.

(5) Folate metabolism Rapidly dividing cells require an abundant supply of deoxythymidylate for the synthesis of DNA, (refer to **Figure 1.3**). The enzymes producing this have therefore become a choice target in cancer chemotherapy by drugs such as methotrexate, 5-flourouracil.

Figure 1.3 Synthesis of deoxythymidylate



dUMP = deoxyuridine monophosphate

dTMP = deoxythymidine monophosphate

TTP = thymidine triphosphate

Reports have suggested that the initial response of cells to CDDP is to enhance the 10-methylenetetrahydrofolate pool (Scanlon et al, 1986). CDDP resistant A2780 cells have shown a 2.5 fold increase in thymidine kinase, thymidylate synthase and dihydrofolate reductase activities (Scanlon et al, 1989[b]). Other reports have reported increases in other cell lines. The stimulated folate metabolism may be one of the first changes to occur during development of resistance, since it is detected in cells with low level CDDP resistance.

(6) **DNA repair** DNA has been reported to be the critical target in the mechanism of cytotoxicity of CDDP. On reaction of CDDP with DNA, both *in vitro* and *in vivo*, various adducts have been reported. Indirect evidence has shown that CDDP resistance in human cell cultures is associated with enhanced DNA repair (Ozols et al, 1988; Lai et al, 1988; Eastman, 1988). Evidence in the literature for enhanced DNA repair in the CDDP resistance phenotype will be presented in greater detail in the next sections of this introduction as it may play an important role in the results of this thesis.

The list presented above is by no means exhaustive, with evidence for new mechanisms being added continually. It has not been presented in any order of importance. As to date there is no evidence to support any of the above as the main mechanism of resistance to CDDP.

The next section of the introduction will present evidence for DNA repair being important in CDDP resistance and an introduction to the DNA repair pathway(s) which may be involved.

1.9 INVOLVEMENT OF DNA REPAIR IN CDDP RESISTANCE

A role of increased DNA repair capacity as a mechanism of CDDP resistance has been reported throughout the literature (for review, Andrews and Howell, 1990). In these studies DNA repair has been measured by different systems ;

(1) **unscheduled DNA synthesis** - this is a measure of the stimulation of DNA repair *in vivo* after exposure of cells to chemicals or drugs such as CDDP (Bianchi et al, 1982).

(2) **repair synthesis** - this *in vitro* assay system measures the incorporation of radio-labelled nucleotides into damaged DNA (Wood et al, 1988).

(3) **CAT reactivation** - involves transfection of a CDDP damaged plasmid containing the CAT gene into cells. The expression of CAT enzymatic activity in transfected cells is then used as a measure of repair of the CDDP adducts in the gene (Chu and Chang, 1991).

(4) **measure of DNA repair enzymes** - levels of mRNA enzymes thought to be important in DNA repair and DNA replicative synthesis are measured (Scanlon et al, 1989).

(5) **adduct removal** - use of systems to measure adducts investigates the removal rates of adducts in different cell lines (Plooy et al, 1985[a]; Dijt et al, 1988).

With these various methods listed above there are advantages and disadvantages to each. In summary the *in vivo* assays tend to be less manipulatable than the *in vitro* assays. They do however, represent the cell as a whole and therefore unlike the *in vitro* assays, will have all constituents necessary for repair processes which may be rate limiting in the *in vitro* systems.

In the *in vivo* assays, the analysis of DNA repair by directly exposing cells to CDDP may be affected by not only damaging the DNA, but also damaging the cellular repair machinery itself. The *in vitro* systems, such as the repair synthesis and CAT reactivation assays, will allow the measure of DNA repair without effects from other damaged systems. DNA *in vivo* is normally in the form of chromatin, however in the *in vitro* assay systems the DNA is introduced as a "naked" form. This may affect the cellular repair machinery if the chromatin structure proves to be important. It has been reported that the initial formation and repair processes are heterogeneous in different areas of the genome, (Bohr et al, 1987). Studies on the formation and repair of CDDP adducts have measured adducts only over the entire genome. Bohr has reported, however that the DNA intrastrand is preferentially repaired in transcribed regions of the gene (Jones et al, 1991). Among these assays there are various advantages and disadvantages to each and therefore it will probably take the uniting of results from the various assays to draw conclusions on the importance of DNA repair in CDDP resistance.

The table shown below, **Table 1.1**, provides a summary of evidence in different cell lines for enhanced DNA repair as a mechanism of resistance.

Table 1.1 Summary of evidence for DNA repair as a mechanism of CDDP resistance

CELL LINE	TUMOUR TYPE	FOLD RES	EVIDENCE FOR ENHANCED DNA REPAIR	REF
A2780	human ovarian carcinoma	20	UDS, repair synthesis	(1,2)
PEO4	human ovarian carcinoma	3	repair synthesis	(2)
L1210/ DDP5	mouse leukemia	50	CAT reactivation, adduct removal	(3,4)
P388	mouse leukemia	47	UDS, DNA polβ	(5,6)
HCT8	human colon carcinoma	4.3	DNA polα and β mRNA activity	(7)
ROT68 /C1	rat ovarian carcinoma	20	UDS	(8)
Hela	human	15-20	CAT reactivation	(9)

UDS = unscheduled DNA synthesis

(1) Ozols et al, 1988

(2) Lai et al, 1988

(3) Eastman and Schulte, 1988

(4) Sheibani et al, 1989

(5) Kraker and Moore, 1987

(6) Kraker and Moore, 1988

(7) Scanlon et al, 1989[b]

(8) Scanlon et al, 1989[a]

(9) Chao et al, 1990

Evidence in **Table 1.1** suggests that levels of enhanced DNA repair may be an important factor in the CDDP resistant phenotype in the human ovarian carcinoma cell line A2780 (Lai et al, 1988, Ozols et al, 1988). Studies on the L1210 mouse leukemia cells and human Hela cells have shown enhanced DNA repair in the CDDP resistant phenotype by reactivation of a damaged plasmid DNA more readily in the CDDP resistant subline in comparison to the sensitive parental cell line (Sheibani et al, 1989, Chao et al, 1991[b]). There has also been evidence presented from this cell line that the GG intrastrand cross-link, which is the predominant lesion, is removed more rapidly in resistant cells than in the sensitive parental cell lines (Eastman and Schulte , 1988).

Several polymerases have been shown to be involved in the gap filling step in the excision repair pathway of E.coli (review, Van Houten, 1990). If mammalian systems follow a similar excision pathway, mammalian polymerases may also be involved. There at least five mammalian DNA polymerases, (review, Linn, 1991). Pol α and δ are mainly involved in DNA replication, but may also play a role in DNA repair. Pol γ is specific for mitochondria and is involved in mitochondrial DNA replication. Pol β has been implicated to be involved in DNA repair only. Evidence from the P388 leukemia cell line resistant to CDDP have shown polymerase α and β activities to be increased in comparison to the sensitive parental cell line. This line has also shown an increase in unscheduled DNA synthesis (Kraker and Moore, 1987, Kraker and Moore, 1988). Scanlon and co-workers observed an overexpression in mRNA levels of DNA polymerase α and polymerase β in a human colon carcinoma cell line HCT8 and A2780 cells (Scanlon et al, 1989[b]). Many workers have tried to modulate the DNA repair pathways in an attempt to modulate resistance to CDDP. Many studies have focused on the inhibition of polymerases by aphidicolin . Aphidicolin is an inhibitor of DNA polymerase α (for further discussion on aphidicolin refer to chapter 6). Treatment of A2780 cells resistant to CDDP has shown to increase the cytotoxicity of this cell line to

CDDP (Masuda et al, 1988). As another means of modulation Scanlon and co-workers have targeted the biochemical events which they thought may occur in their CDDP resistant cell lines. The CDDP resistant cell lines they used were shown to have an increase in mRNA levels of dihydrofolate reductase, thymidine kinase, thymidine 5'-monophosphate which are believed to be involved in DNA repair and DNA replicative synthesis. Deoxyribonucleotides which are essential for DNA repair were therefore targeted in an attempt to increase the cytotoxicity of CDDP in these CDDP resistant cell lines, (Scanlon et al, 1989[b]).

Evidence therefore exists in the literature suggesting DNA repair as a mechanism of resistance to CDDP. Work is progressing in the modulation of CDDP resistance and pathways of DNA repair are being targeted. DNA repair can be classified into three major classes. The following section will introduce each, followed by evidence suggesting that CDDP adducts are repaired by the different pathways.

1.10 CLASSES OF DNA REPAIR PATHWAYS

DNA repair falls classically into three major classes :

(1) Direct repair

(2) Recombinational repair

(3) Excision repair

1.10.1 Direct Repair

Direct repair involves reversal of covalent modifications- it does not involve either removal or replacement of nucleotides. Examples of direct repair include O^6-methylguanine DNA methyl transferase and photoreactivation mediated by DNA

photolyase. Alkylating agents, of which methylating agents appear to be widespread environmental mutagens, act through covalent modification of the cellular genome to generate miscoding base derivatives and lesions that block DNA replication. These covalent modifications can be reversed by enzymes such as the O^6-methylguanine DNA methyl transferase. This enzyme corrects O^6-methylguanine, which can produce a miscoding of bases, by direct reversal of damage. This is accomplished by transfer of the methyl group to a cysteine residue in the repair enzyme itself, with an unmodified guanine simultaneously regenerated in the DNA sequence (for review, Lindahl and Sedgwick, 1988). The photoreactivation system, another example of direct DNA repair, has been extensively characterised in *E.coli* and two different strains of yeast, *Saccharomyces cerevisiae* and *Streptomyces gresius.* DNA photolyases repair pyrimidine dimers in DNA by breaking the cyclobutane ring joining the pyrimidines (for review, Sancar & Sancar, 1988).

1.10.2 Recombinational repair

Polymerases are involved in the elongation step of replication. If the polymerases when working their way along the DNA come in contact with certain nucleotide adducts e.g pyrimidine dimers, they will stop replicating and initiate again about 1000 base pairs beyond the adduct, thus generating a single-stranded gap that contains a modified nucleotide on the opposing strand. In *E.coli* this discontinuity is filled in by a process involving recombination controlled by the Rec genes (Rupp et al, 1971). Rec A protein fills in the gap by transfer of the complementary strand from the sister duplex (Cox and Lehman, 1987).

1.10.3 Excision repair

The best characterised systems of DNA repair involve pathways of excision repair. It can be classified into two types - the excision of incorrect nucleotides from DNA by hydrolysis of the N-glycosylic bond between the deoxyribose and the base by a DNA glycosylase (Lindahl, 1974), **Figure 1.4**, or the removal of damaged bases as a short oligonucleotide sequence with the resulting gap being patched by repair synthesis, **Figure 1.5**, for further detail refer to section 1.12.

1.11 EVIDENCE FOR REMOVAL OF CDDP ADDUCTS BY DIFFERENT PATHWAYS OF REPAIR

Beck and co-workers demonstrated that the recombination-repair deficient *E.coli* mutant rec A13 and the double mutant uvrA6 lex1 (mutation in excision repair pathway as well as recombination pathway) are very sensitive to CDDP (Beck and Brubaker, 1973). This suggests recombinational repair may be involved in the removal of CDDP adducts. The Rec A protein has been reported to be inducible by CDDP (Salles et al, 1982). This therefore suggests that an SOS type of response may be increasing cell survival by inducing a recombinational type of repair of the CDDP adducts. Interruption of replication or damage to the DNA generates an induction signal, which activates the constitutive level of rec A which in turn promotes cleavage of lex A and the generation of the secondary SOS responses. As a consequence the products of the SOS target genes are expressed at much higher levels. A number of the target genes are involved in either

Figure 1.4 Removal of incorrect bases

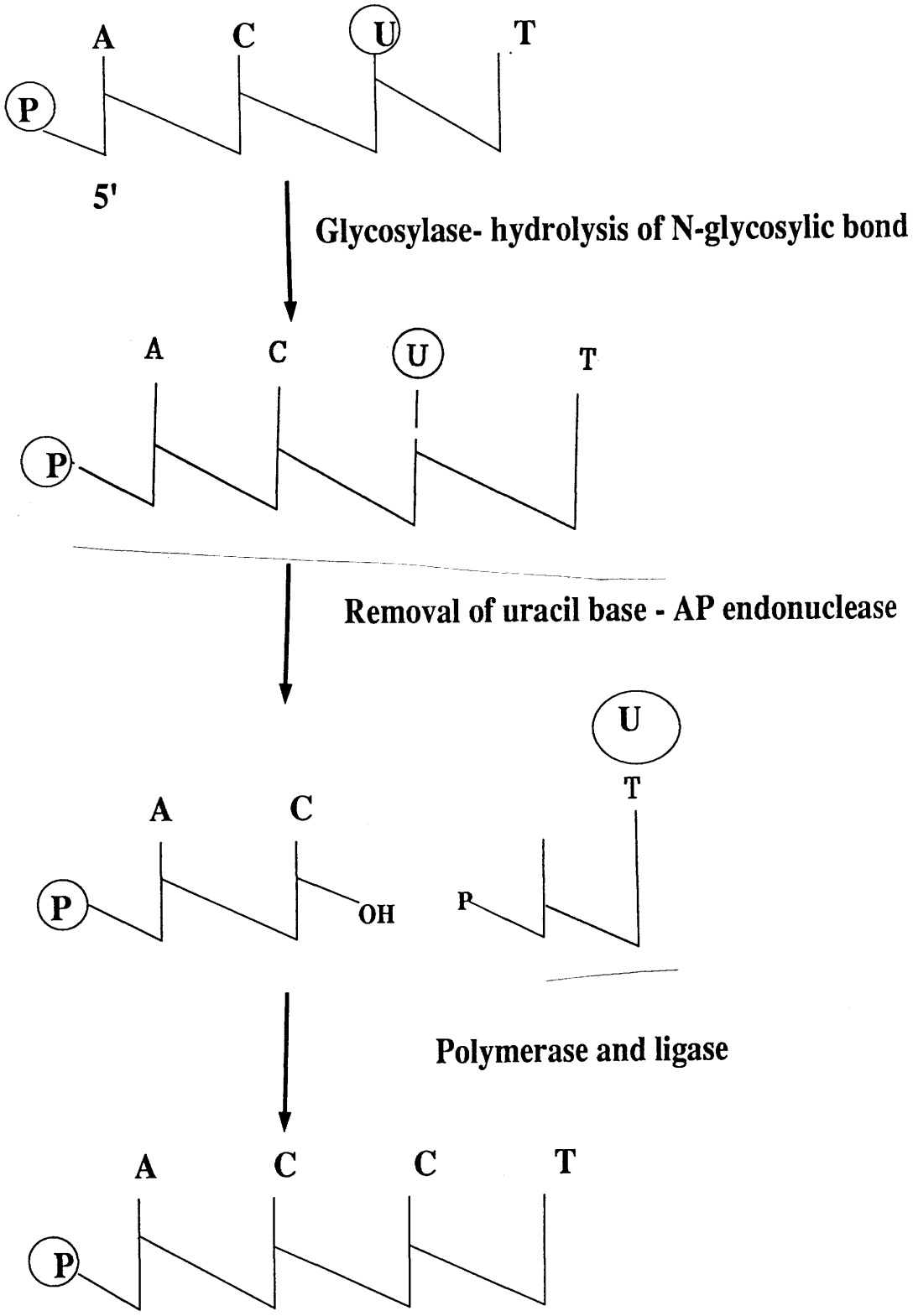
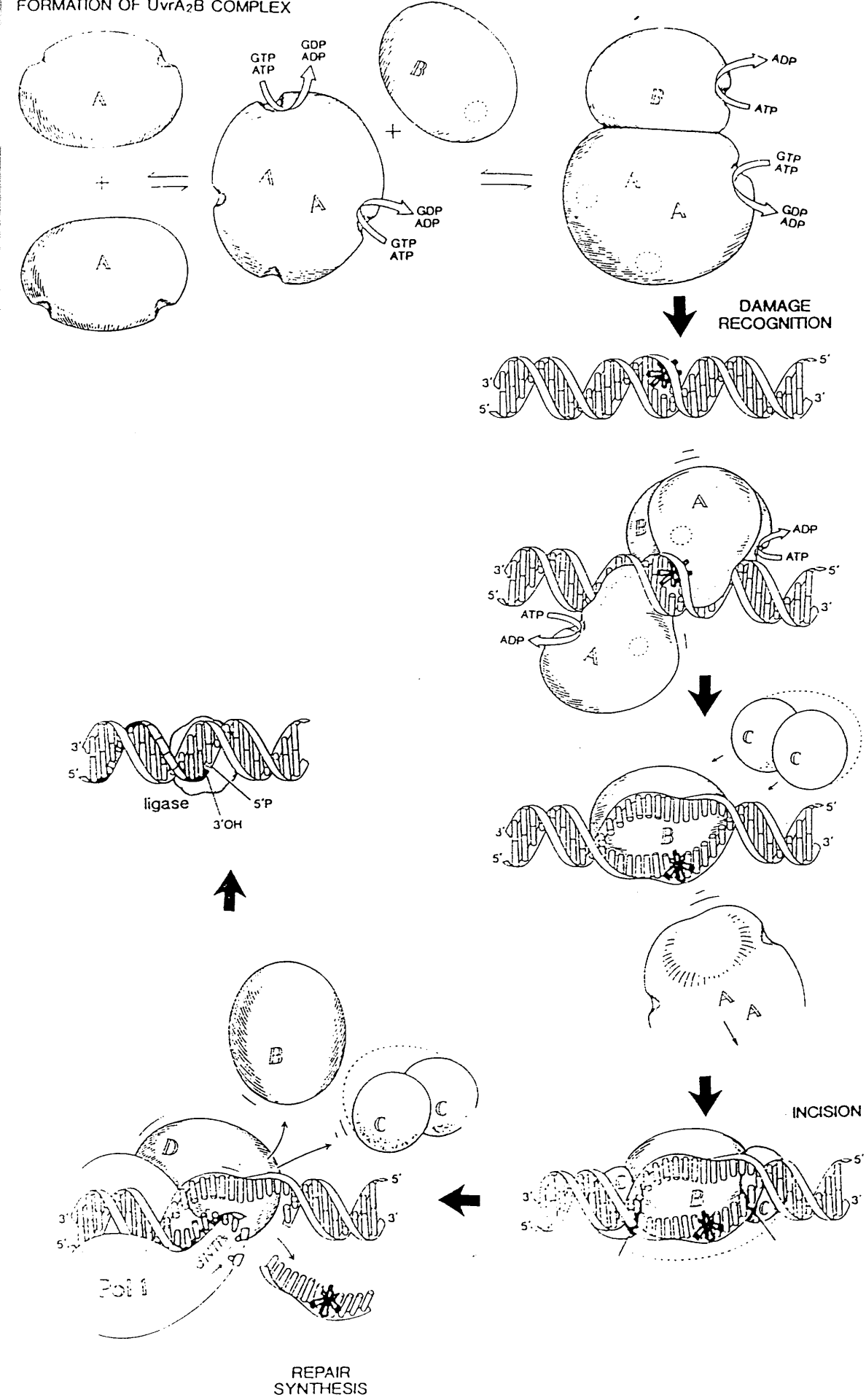


Figure 1.5 A hypothetical model of nucleotide excision repair. The main steps are depicted : formation of $uvrA_2B$ complex, damage recognition, incision, repair synthesis. Hydrolysis of ATP is shown where it is thought to occur. The diagram is adapted from a figure from Van Houten, 1990.


FORMATION OF UvrA2B COMPLEX
A
B
GTP
ATP
GDP
ADP
DAMAGE
RECOGNITION
INCISION
C
D
ligase
5'P
3'OH
Pol I
REPAIR
SYNTHESIS

excision or recombinational repair. The SOS response is therefore the *E.coli*'s way to deal with conditions that damage or inhibit DNA replication.

Another repair pathway, which occurs at the replication fork is known as mismatch repair. Evidence exists to suggest that mutants in *E.coli* which are defective in mismatch repair show a 2-15 fold increase in sensitivity to CDDP compared to wild-type (Fram et al, 1985). This demonstrates that mismatch repair is important in CDDP induced DNA damage.

Nucleotide excision repair is one of the best characterised repair pathways in *E.coli*. Many studies on CDDP cytotoxicity have focused on this particular pathway. The following section presented will therefore report on evidence in the literature implicating nucleotide excision repair to be involved in CDDP cytotoxicity.

1.11.2 EVIDENCE FOR EXCISION REPAIR IN REMOVAL OF CDDP ADDUCTS

Evidence exists to suggest that CDDP adducts are removed by the nucleotide excision repair pathway in *E.coli*. Mutants of *E.coli* which are blocked in excision repair are extremely sensitive to CDDP. This same study also reported that plasmid DNA damaged with CDDP but not TDDP is a substrate for the uvrABC excinuclease (Beck et al, 1985). Popoff has reported that upon transformation of various DNA repair proficient and deficient strains of *E.coli* with CDDP and TDDP damaged DNA, that excision repair plays an important role in the removal of CDDP adducts (Popoff et al, 1987).

Evidence is now beginning to emerge that nucleotide excision repair is important in the repair of CDDP adducts in mammalian systems. A system which was first defined by Wood and Lindahl in 1988 to measure the ability of excision repair by human cell extracts, was used by Hansson to measure the removal of CDDP adducts, (Hansson and Wood, 1989). It was shown that nucleotide patches were introduced into

plasmid DNA damaged with CDDP when incubated with soluble human cell extracts, in an ATP dependent reaction. This suggests that CDDP adducts in mammalian cells are removed by a pathway similar to that in *E.coli.* Further evidence from mammalian systems comes from studies on cell lines derived from XP patients and from CHO mutants. Cell lines derived from XP patients are normally deficient in the incision step of the excision repair pathway. They are extremely sensitive to UV and also show hypersensitivity to CDDP therefore suggesting that both types of adducts may be repaired by the same pathway.

It is therefore of interest to study the pathway of excision repair in the removal of CDDP adducts, as this may be involved in the cytotoxicity of CDDP. The excision repair pathway has been well characterised in the *E.coli* system therefore the following section will give an introduction to this system as a lead into excision repair in mammalian cells.

1.12 NUCLEOTIDE EXCISION REPAIR IN *E.COLI*

Nucleotide excision repair involves the removal of a damaged DNA adduct in a short oligonucleotide sequence and the resynthesis and religation of this patch within the duplex DNA. Nucleotide excision repair has been well studied in *E.coli* therefore a review of steps involved will be presented.

Studies in *E.coli* have isolated at least six proteins involved in this pathway; uvr A, uvr B, uvr C, uvr D, polymerase I and DNA ligase. Seeberg and co-workers in 1976, initiated the research into characterisation and partial purification of the proteins by complementation assays with cell extracts from uvr mutants (Seeberg, 1976). By 1981 the gene products of *uvr*A, *uvr*B and *uvr*C were partially purified and shown to interact to

produce strand cleavage at pyrimidine dimers, base adducts and interstrand cross-links in DNA (Seeberg, 1978, Seeberg, 1981).

Since these observations and the partial purification of the different subunits, several groups have gone on to clone each of the uvr genes. To date the uvr genes have now been cloned and sequenced, and detailed studies of the properties of the uvr proteins are described in the literature (for review, Van Houten, 1990). Due to the cloning of the uvr proteins in vectors designed for overexpression, large amounts can be purified to homogeneity therefore leading to a hypothetical description of the pathway for the removal of damage by nucleotide excision repair (**Figure 1.5**). There are five major steps described;

- Damage recognition
- Formation of the preincision complex
- Incision of the phosphate backbone
- Excision, repair synthesis and release of the post-incision complex
- DNA ligation

1.12.1 Damage recognition, formation of preincision complex & incision

In the presence of ATP two molecules of uvrA form a dimer, A_2 (refer to **Figure 1.5**). This dimer then interacts with uvrB forming a complex, uvr A_2B. The uvr A_2B complex binds to DNA non-specifically and since it has been demonstrated to have

an ATP-dependent helicase activity, is believed to move along the DNA scanning for any changes in conformation of the DNA (Grossman and Yeung, 1990). If the uvr A_2B complex encounters a damaged site, the helicase activity is inhibited and the uvr A protein dissociates from the complex, leaving uvr B behind at the site of damage. The uvr A protein is therefore in this model acting as a shuttle for uvr B to find DNA. Once the uvr A_2B is bound, the uvr A dissociates and therefore is likely to become available to participate in another loading of uvr B to a damaged site. The uvr B at the site of damage has been proposed to allow complexing of uvr C and this complex is then suggested to yield an active endonuclease that introduces two cuts in the damaged strand (Orren & Sancar, 1989).

1.12.2 Excision, repair synthesis and release of postincision complex

uvr D (helicase II) and polymerase I then becomes involved, presumably by allowing the post-incision complex to convert back to the proteins required for recognising new damage and refilling in the sequence of nucleotides removed around the DNA damage (Caron et al, 1985). It has normally been found that, in the majority of repair patches, DNA polymerase appears to fill the excised region with nucleotides. Although other polymerases have been shown to perform the gap-filling step and allow turn-over of the complex, only pol I will add sufficient nucleotides that the patch is repairable with DNA ligase.

1.12.3 Steps at which ATP are required

In **Figure 1.5**, it can be seen that the model shows, that in various steps, hydrolysis of ATP is required. At which points it is required in the pathways has not been fully established. Using nucleotide analogues it has been demonstrated that it is

absolutely necessary for incision to occur (Caron and Grossman, 1988[a]). ATP has been shown to cause an increase in the binding affinity of the uvr A protein to damaged DNA (Oh et al, 1989). Formation of the uvr A_2B complex results in a decrease in the uvr A ATPase and a stimulation of the uvrB complex which will only hydrolyse ATP. ATP is required for the unwinding activity of the uvr A_2B complex of DNA (Oh et al, 1989). Maintenance of the DNA in the unwound state does not seem to rely on the presence of ATP (Caron & Grossman, 1988[b]).

1.12.4 Damage recognised by the uvrABC excinuclease

The uvrABC excinuclease works on a large variety of DNA damage. It can recognise

(1) DNA containing large chemical modifications

(2) Linked bases e.g CDDP guanine-guanine adducts (Popoff, 1987)

(3) Minor modifications e.g O^6 alkylguanine (Voigt et al, 1989)

It has been shown however not to recognise mismatches (Thomas et al, 1986). The ability to recognise the above modifications described and not mismatches was suggested to be due to the substrates having a kinked DNA structure i.e a distortion in the DNA duplex. Many types of damage that are recognised by uvrABC excinuclease do cause a kink in the structure e.g CDDP has been reported to distort the DNA double helix 40^0 towards the major groove (Rice et al, 1988). However some damage which does not cause kinks are recognised substrates of the uvrABC excinuclease e.g thymine glycols, apurinic sites and O^6- methylguanine, (review, Van Houten, 1990).

The distortions that could be recognised by uvrABC can be split into at least six separate categories:

(1) covalent modifications

(2) bulky adducts

(3) localized unwinding of the two DNA strands

(4) site specific bend or kink

(5) charge distribution around the site of damage

(6) any changes in structural dynamics of the DNA helix

In summary the nucleotide excision repair in *E.coli* is a well characterised pathway in which a large spectrum of damage is recognised and repaired by a complex of proteins. These proteins have been purified and cloned and the properties of these proteins is an intensive research area.

1.13 NUCLEOTIDE EXCISION REPAIR IN EUKARYOTIC MODEL SYSTEMS

1.13.1 Studies in *Saccharomyces Cerevisiae*

The yeast system is an attractive model for the study of eukaryotic excision repair for three main reasons:

(1) low genome complexity

(2) can genetically manipulate the system with ease

(3) large numbers of repair mutants available for analysis

Many studies in mutant strains, abnormally sensitive to ultra-violet radiation and ionising radiation, have identified large numbers of genes involved in the repair of

these lesions (for review, Friedberg, 1988). A systematic nomenclature for radiation sensitive mutants was established at the International Conference on Yeast Genetics held in Chalk River, Ontario in 1970. It was decided that mutants abnormally sensitive to killing by radiation should be designated as rad, with identifying locus and allele numbers. Locus numbers 1-49 refer to genes which primarily affect sensitivity to ultra-violet radiation or to both UV and ionising radiation. Locus numbers greater than 50 designate genes which primarily affect sensitivity to ionising radiation.

Studies on the relative sensitivity of single and double mutants to killing by DNA damaging agents have separated the yeast mutants into separate epistasis groups (Cox and Game, 1974). In cases where a double mutant is no more sensitive than a single mutant, the two genes in question are considered to be epistatic. On the other hand, genes are placed in different epistasis groups if double mutants show synergistic sensitivity compared to single mutants (Haynes and Kunz, 1981).

Approximately 30 mutant loci have been analysed for mutant effects and have been placed into three separate epistasis groups referred to as :

	INVOLVEMENT
RAD 3	nucleotide excision repair
RAD 52	recombination repair
RAD 6	mutagenesis and post replication repair

The RAD 3 epistasis group therefore identifies the genes in *Saccharomyces Cerevisiae* involved in excision repair. The model for removal of damage has been suggested to be similar to that hypothesized in *E.coli* with proteins cloned which have

homologous function to those in *E.coli*. **Table 1.2** summarises the cloned genes of the RAD 3 epistasis group.

A high proportion of the predicted gene products harbour acidic regions that are thought to confer ability to bind chromatin, mediated by electrostatic interaction with basically charged histones. For a review of the genes involved in excision repair for *Saccharomyces Cerevisiae* refer to Friedberg, (1988).

1.13.2 Excision repair in mammals

In comparison to the *E.coli* system relatively little is known about nucleotide excision repair in eukaryotes. The previous section introduced the initiation of studies in the simplified eukaryotic system. Within mammals the study of the excision repair pathway has been carried out using two classes of excision repair defective cell lines :

(1) cell lines from patients suffering from Xeroderma Pigmentosum.

(2) Laboratory induced UV-sensitive rodent lines (mainly Chinese hamster ovary cell lines, CHO).

Xeroderma Pigmentosum is an autosomal recessive human repair disorder. This rare, cancer prone syndrome is clinically characterised by the extreme sensitivity of the skin to sunlight (UV) exposure, pigmentation abnormalities, predisposition to skin cancer, and frequently neurological degeneration (Cleaver and Kramer, 1989). The molecular defect in most XP patients occurs in the early steps of the excision-repair pathway - mainly defective in the incision step (Setlow et al, 1969, Hansson et al, 1990).

Table 1.2 Summary of cloned genes of RAD3 epistasis group

GENE	HOMOLOGIES TO HUMAN GENES	REMARKS
RAD 1	None	Acidic carboxy terminus. May be involved in recombination.
RAD 2	None	Transcription inducible by UV light.
RAD 3	ERCC-2	Nucleotide and DNA binding. Contains a region which resembles an ATPase. 5'-3' helicase activity. Acidic carboxyl terminus.
RAD 4	Unknown	Acidic carboxyl terminus. From amino acid sequence shows homology to known functional domains in DNA thought to be involved in DNA binding.
RAD 7	Unknown	Acidic stretches, membrane association? Partial excision defect.
RAD10	ERCC-1	DNA binding? Involved in recombination.
ERCC-3^{sc}	ERCC-3	Nucleotide DNA binding, DNA helicase? Acidic stretches, essential function.

Cell fusion studies have demonstrated so far eight different complementation groups ranging from XP-A through to XP-G and XP-V. A few years prior XP-H was designated to be a complementation group, however this has now been reassigned to XP-D (Johnson et al, 1989).

Cellular studies have demonstrated XP-A and XP-F to be hypersensitive to CDDP induced damage (Dijt et al, 1988; Plooy et al, 1985). Evidence is accumulating for deficient repair of CDDP damage by XP cells. XP-A cells are deficient in reactivating SV40 DNA damaged by CDDP (Poll et al, 1984) and cells from XP-A, XP-C, XP-F are less efficient at restoring expression of a marker gene after transfection of CDDP damaged plasmids (Chu and Berg, 1987). Dijt and co-workers reported that cells from XP-A remove CDDP adducts at a slower rate than normal repair proficient cells (Dijt et al, 1988). Recently *in vitro* studies have demonstrated that XP-A, XP-C, XP-D and XP-G are defective in the repair of plasmid DNA damaged with either CDDP or TDDP. This defective repair has been shown to be overcome by the addition of uvrABC into the system (Hansson et al, 1990).

The UV sensitive rodent lines possess a similar phenotype to the XP cell lines in being defective in the incision step of excision repair (Thompson et al, 1982). They are also characterised by their reduced ability for UDS (Thompson et al, 1980). These mutants can be classified into at least five complementation groups. There is however a UV mutant which shows a normal capacity for UDS (Busch et al, 1989). This has been placed into a complementation group of its own, Group 6. It is likely that this mutant does excise UV lesions successfully (for review of CHO mutants, Hickson and Harris, 1988).

Mutants from complementation group 1, but not group 2 have been shown to be highly sensitive to the cytotoxic effects of CDDP but not TDDP. They do however show some effects to TDDP, but at much higher concentrations (Hoy et al, 1985). This

suggests that group 1 mutants are defective in the repair of the cytotoxic lesion formed by CDDP.

The repair-deficient mutants have proven a valuable tool for the isolation of human DNA repair genes. Initial attempts to isolate human DNA repair genes from XP cell lines has proved unsuccessful in that human cells are poor recipients of DNA. The Chinese hamster ovary mutants have therefore been used to identify human nucleotide excision repair genes because they have a high uptake of exogenous DNA compared to the human cells. One of the strategies used in their isolation, is the transfection of genomic DNA into these DNA repair deficient mutants, selection of repair-proficient transformants and retrieval of the correcting repair gene. This technique has allowed the isolation of genes responsible for DNA repair termed Excision Repair Complementation Chinese hamster repair deficiency (ERCC) genes and these are presented in **Table 1.3**

(review, Bootsma et al, 1988; Tanaka et al, 1989; Tanaka et al, 1990)

Tanaka, K., Satokata, I., Ogita, Z.,Uchida, T., Okada, Y. (1989) Molecular cloning of a mouse DNA repair gene that complements the defect of group-A xeroderma pigmentosum. P.N.A.S. (USA) 86, 5512-5516

Tanaka, K., Miura, N., Satokata, I., Miyamoto, I., Yoshida, M.C., Satoh, Y., Kondo, S., Yasui, A, Okayama, H., Okada, Y. (1990) Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc-finger domain. Nature 348, 73-76

Table 1.3 Summary of cloned genes of human ERCC genes

GENE	CORRECT	REFERENCE
ERCC-1	Excision repair defect in CHO mutants of complementation group 1	Westerveld et al, 1984
ERCC-2	Defect in the incision step of excision repair of group 2 hamster UV sensitive mutants	Weber et al, 1988
ERCC-3	Restores repair synthesis to XP-B complementation group	Weeda et al, 1990[a]
	Corrects defect in CHO UV sensitive mutants in complementation group 3	Weeda et al, 1990[b]
	Strong possibility ERCC-3 is a DNA helicase	Weeda et al, 1990[b]
ERCC-5		MacInnes et al, 1990
ERCC-6	Corrects the cyclobutane dimer repair defect in the moderately UV sensitive complementation group 6 hamster mutants	Troelstra et al, 1990

Analysis of the ERCC gene products have revealed that there is some amino acid sequence homology to other nucleotide excision repair counterparts in other systems. **Table 1.4** briefly outlines these.

Table 1.4 Sequence homology of ERCC genes with other repair genes

GENE	HOMOLOGY	REFERENCE
ERCC-1	RAD 10 *uvr*A *uvr*C	vanDuin et al, 1986 Hoeijmakers et al, 1986 Doolittle et al, 1986
ERCC-2	RAD 3	Weber et al, 1990
ERCC-3	None so far. Suggestion of ERCC-3^{sc} although evidence exists to suggest not homologous to excision repair genes.	Weeda et al$^{(b)}$, 1990
ERCC-5		
ERCC-6	Nucleotide sequence yet to be defined.	

Initial studies have suggested that the ERCC1 gene may be important in CDDP resistance. When the ERCC1 gene was transfected into CHO cells, the transfectant was demonstrated to be four fold more resistant to CDDP than the wild-type cell. In the same study A2780CP cells which were 13 fold more resistant to CDDP demonstrated higher levels of ERCC1 expression, but these levels were variable (Reed et al, 1989). This study suggests that ERCC1 may be involved in CDDP resistance in human ovarian cancer.

Evidence is therefore accumulating from XP cell lines and CHO mutants which are sensitive to CDDP that nucleotide excision repair may be an important pathway for removal of CDDP adducts in humans. Certain key features suggest it is reasonable to consider mechanisms of nucleotide excision repair in humans as having similarities to the that in *E.coli*.

(1) Human DNA repair enzymes which correct DNA base damage in humans such as O^6-methylguanine-DNA methyl transferase, DNA glycosylase have similar activities to those enzymes found in *E.coli* (Lindahl, 1982).

(2) Damage specific incision of DNA in UV-irradiated permeabilised cells requires ATP (Dresler and Lieberman, 1983) as it does in *E.coli*.

(3) Pyrimidine dimers in human cells are excised within short oligonucleotides (Weinfeld et al, 1986) again as in *E.coli*.

(4) The DNA repair syndrome, Xeroderma Pigmentosum has various different complementation groups. This therefore suggests that a multi-subunit repair nuclease exists which is analogous to the uvr ABC in the *E.coli* system.

1.14 Advantages of *in vitro* assays

A large amount of information on DNA excision repair in mammalian cells has been gained by the analysis of transfecting damaged DNA into cells. Popoff and co-workers have demonstrated the *uvr*B gene to be essential for the repair of CDDP induced DNA damage, by introduction of CDDP damaged plasmid into UV mutants of *E.coli* (Popoff et al, 1987). White and Sedgwick have also assigned a role of post-incision UV

repair to the products of RAD 1, 4, 7 and 14 gene products by transfection experiments into yeast (White and Sedgwick, 1987). Problems in interpretation of results of *in vivo* assays occur. Difficulty in ensuring equivalent DNA uptake in cells therefore making comparisons between cell lines very hard. It is also difficult to determine the involvement of cellular metabolic processes in the observed outcome. In the analysis of repair of a damaged substrate, complications can arise if the damage itself can affect the uptake or integration of DNA.

Many groups have begun to unravel the pathway of excision repair in mammalian cells by use of *in vitro* assays. *In vitro* studies have a number of advantages over transfection systems. These systems are free of complications arising from the interaction of cellular metabolism on the system under investigation. They also provide a means for purification of proteins involved in specific reactions either by assaying protein fractions for a defined activity in a model system (Eisen et al, 1988) or by restoration of the relevant activity (Wood et al, 1988; Hansson and Wood, 1989). The *in vitro* systems are also maniputable, in that factors such as enzyme inhibitors, antibodies, etc. can be added to test different hypothetical pathways.

1.15 Dissection of mammalian excision repair pathway using *in vitro* assays

In vitro assays have been used to either study individual steps of the excision repair pathway in human cells or a succession of steps leading to removal of the damage. The excision repair pathway can be separated into four major areas

recognition

removal

resynthesis

religation

In vitro assays for these steps are discussed in the next two sections of this chapter.

1.15.1 Identification of DNA damage recognition proteins (DRPs)

If CDDP adducts are repaired by an excision repair pathway in mammalian cells it is reasonable to suggest that prior to incision the adducts must be recognised by a protein similar to that of the uvrABC complex in the *E.coli* nucleotide excision repair pathway.

Studies were stimulated in this area of research when Chu and Chang identified a factor which recognised adducts formed by ultra-violet radiation (Chu and Chang, 1988). They provided evidence that this factor was absent in an extract of cell line derived from complementation group E (XPE). The importance of the absence of this factor in XPE was later questioned by Kataoka and Fujiwara who verified the absence of the factor in the same XPE cell line but found it present in four other XPE fibroblast strains (Kataoka and Fujiwara, 1991). Chu and Chang identified another factor which recognised DNA damaged with CDDP (Chu and Chang, 1988), which was independent of the factor recognising UV damage.

In a separate study a factor which recognises the 1, 2 d(GpG) and d(ApG) only, has been identified (Donahue et al, 1990). These lesions have been reported to be the major lesions formed *in vitro* (Eastman, 1986). For a more extensive introduction to DNA damage recognition proteins refer to chapter 3, section 3.1.

Some studies have reported an increase in a damage recognition protein in cell lines resistant to CDDP. Chu and Chang reported that the factor they found to be absent in an XPE cell line was overexpressed in a Hela cell and fibrosarcoma cell line made resistant to CDDP *in vitro* (Chu and Chang, 1990). An independent study also reported an increase in a damage recognition protein recognising UV damage in a Hela

cell line selected for resistance to CDDP (Chao et al, 1991[a]). Chao and co-workers had previously provided evidence for their CDDP resistant cell line to have an increased DNA repair capacity by plasmid reactivation (Chao et al, 1991[b]). A study which identified damage recognition proteins in A2780 cells recognising CDDP damage reports not to show an increase in these proteins in CDDP resistant cell lines (Andrews and Jones, 1991).

Evidence exists suggesting that the DRPs may be involved in a DNA repair pathway. Studies have shown an absence of DRPs in an XPE strain (Chu and Chang, 1988) and also an increased expression of DDRPs in cell lines with an enhanced DNA repair activity (Chao et al, 1991[a, b]). This however only provides circumstantial evidence for the involvement of the DRPs in an excision repair pathway and steps are being made in the direction of isolation of the protein recognising CDDP damage and further cloning of the gene before direct evidence can be presented as to whether this protein is a DNA repair protein (Toney et al, 1989).

1.15.2 Repair synthesis assay

Recently an *in vitro* assay for investigations of nucleotide excision repair by extracts from human cells has been developed (Wood and Lindahl, 1988). This assay basically monitors the formation of repair patches in exogenously added damaged plasmid DNA. This cell free system has demonstrated the repair of pyrimidine dimers, psoralen and CDDP adducts (Woods and Lindahl, 1988, Ullah et al, 1989). The cell free system has also been used in the investigation of different cell extracts. Cell extracts of XP complementation groups A, C, D and G were shown to be deficient in DNA repair synthesis in plasmid DNA treated with UV, CDDP, TDDP (Hansson et al, 1990). The repair synthesis assay is therefore of value in the determination of types of adducts

repaired in an excision repair pathway in mammalian cells similar to that described for *E.coli.*

1.15.3 Study of the ligation step

The final step in the excision repair process is the formation of a phosphodiester bond after the removal of the damage from the DNA. The eukaryotic DNA ligase I has been demonstrated to play a role in both replication and repair (for review, Lasko et al, 1990). An altered DNA ligase I has tentatively been associated with the defective DNA repair syndrome known as Bloom's syndrome. DNA ligase II is another ligase which has been identified in eukaryotic cells although, as of yet, its' physiological function is unknown. *In vitro* assays exist which allow the measurement of both enzymes (Lasko et al, 1990). It would be of interest to assay the enzyme activities in CDDP resistant cell lines to determine if the final ligation step in the excision repair process is altered.

1.16 Importance of study of damage recognition step

The damage recognition step is the first step required prior to removal, resynthesis, religation. It is therefore an important step in that the three later stages all follow sequentially. If damage recognition does not occur the latter steps will not occur. If a protein was found which acts as a damage recognition protein, then it may prove of importance if its overexpression in cell lines hypersensitive to DNA damaging agents, allowed their return to normal DNA repair synthesis. At the opposite end of the spectrum, modulation of this protein may prove important in overcoming resistance to damage induced by agents recognised by this protein. The study of damage recognition proteins may therefore prove clinically relevant if they prove to be important either in

being repair enzymes or being enzymes which recruit repair enzymes onto the site of damage.

1.17 Substrates for damage recognition

The DNA damage recognition proteins like the uvrABC complex in *E.coli* are more likely to recognise a range of damage rather than one specific type of damage. It is more economical for a cell to contain proteins which recognise classes of damage rather than damage induced by specific agents. Through evolutionary terms it is more likely proteins will not have evolved to recognise specific damage but will have evolved to recognise structural alterations.

CDDP is thought to bind to DNA and cause local denaturation around the adduct (Sundquist et al, 1986). A single-stranded binding protein, gene 32 protein, from bacteriophage T4 has been reported to bind more efficiently to DNA modified with CDDP (Toulme et al, 1983). Toulme and co-workers have suggested a role for single-stranded binding proteins in an early step of recognition of damage due to conformational changes prior to recruiting other DNA repair enzymes onto the site of damage.

Recently a role for the human single-stranded binding protein (hSSB) involved in DNA replication has been reported in DNA excision repair (Coverley et al, 1991) - see chapter 4, section 4.1. It was shown that monoclonal antibodies against hSSB caused extensive inhibition of DNA repair in a system measuring excision repair (Wood and Lindahl, 1988). It was also reported that upon reintroduction of purified SSB into the system, DNA excision repair was stimulated. It therefore may be possible that the identified DNA damage recognition proteins binding to CDDP adducts reported in the literature, may be recognising regions of single-strandedness generated by the CDDP adducts.

Reports by Donahue and co-workers have identified DNA damage recognition proteins recognising only the 1,2 d(GpG). They have suggested that either this protein is involved in repair or it may impede repair by binding to this specific cross-link and shielding it from DNA repair enzymes.

1.18 AIMS OF THE THESIS

Evidence from the literature has been presented for the involvement of a nucleotide excision repair pathway similar to that found in *E.coli*, for the removal of CDDP adducts in mammalian cells. The main aim of this thesis is to attempt to identify a protein complex in mammalian cells which recognises CDDP damaged DNA. If the DDRPs are found to be involved in handling damage to the DNA then they may be important in the generation of a CDDP resistant phenotype. A role for DDRPs in CDDP resistance will therefore be investigated in cell lines resistant to CDDP (their resistance derived *in vitro*). Previous reports have shown that cell lines resistant to CDDP exhibit increased levels of DNA damage recognition proteins recognising UV damage (Chu and Chang, 1990; Chao et al, 1991[a]). These CDDP resistant cell lines were demonstrated to have increased levels of DNA repair synthesis therefore the author made the tenuous link of the DRPs being involved in DNA repair.

In the *E.coli* system the uvrABC complex has been reported to recognise a large variety of damage. CDDP has been reported to cause areas of single-strandedness. Recently human single-stranded binding protein (hSSB) normally involved in DNA replication, has been suggested to have a role in DNA excision repair (Coverley et al, 1991). The study of the recognition of single-stranded DNA by DDRPs will be presented with some evidence suggesting that the DDRPs recognising CDDP adducts may also be recognising regions of single-strandedness generated around the adducts.

In summary the following results chapters of this thesis will present:

Chapter 3 The identification of damage recognition proteins in mammalian cell extracts recognising CDDP adducts by gel mobilbity shift assay. The characterisation of the CDDP treatment of the substrate used in the identification of the DDRPs will be presented fully.

Chapter 4 The identification of DDRPs in mammalian cell extracts by South-Western analyses and a study of their involvement in CDDP resistance in two ovarian tumour cell lines resistant to CDDP (resistance derived *in vitro* by multiple exposures to CDDP). Binding of the DDRPs to single-stranded oligonucleotides is also investigated. The DDRPs in this chapter are also examined for induction by pretreatment with CDDP.

Chapter 5 The isolation of CDDP resistant clones of the A2780 ovarian tumour cell line by a single step selection with CDDP. Evidence is presented in this chapter to suggest that the clones are derived from a mutational origin. The DDRPs from the resistant clones are then analysed on a South-Western blot to determine if differences in binding can be detected.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Chemicals : Sigma, Biorad, Aldrich

Enzymes & Buffers : Pharmacia

Cisplatin : Sigma

Radiochemicals : Amersham International

Oligonucleotides	αG7	Oswell DNA Service
	2αG7	Oswell DNA Service
	3bpMM	Oswell DNA Service
	7bpMM	Oswell DNA Service

Protein molecular weight markers : BRL

Transfer membrane : Alderman & Co.LTD

Acrilamide:bis (30:0.8) : Severn Biotech Ltd

Filters : Nalgene

Spinex Tubes : Costar

Dialysis tubing and Collodion bags : Sartorius

Autoradiography film : Kodak

Plastics : Falcon

Tissue Culture Media : NBL

Supplements for media : Gibco

Tissue Culture Plastics : Becton Dickinson

96 Microwell Plates : Nunclon

2.2 OLIGONUCLEOTIDES

Oligonucleotides were synthesised on an automatic DNA synthesiser by Oswell DNA service and HPLC purified.

2.2.1 General Buffers

TEN : 100mM NaCl, 10mM Tris pH7.5, 10mM EDTA

TE : 10mM Tris pH7.5, 1mM EDTA

TBE : 89mM Tris borate, 89mM Boric acid, 2.5mM EDTA

2.2.2 Reannealing

Complementary strands of oligonucleotides were reannealed at a concentration of 0.05μg/μl by heating a mixture of both strands at 80^0C for ten minutes in a waterbath and then allowing the waterbath to reach room temperature overnight. For reannealing of both strands to occur, the temperature must be lowered gradually. The double stranded oligonucleotides shown in table 2.1 were reannealed in this way.

2.2.3 Cisplatin Treatment of Oligonucleotides

Both double stranded and single stranded oligonucleotides at 15ng/μl were incubated with various concentrations of CDDP (as described for each particular set of experiments) in a final reaction mixture with TEN buffer at 37^0C for one hour. A final concentration of 0.1M NH_4HCO_3 was then added and the reaction was left at 4^0C for 16 hours. This procedure allows any remaining CDDP to be inactivated. Reaction with CDDP initially forms monofunctionally bound platinum and then this reaction progresses

Table 2.1 Sequences of oligonucleotides

αG7	GATCCGGGCAACTGATAGGGATTCCCA GCCCGTTGACTATCCCTAAGGGTTCGA
2αG7	GATCCGGGCAACTGATAGGGATTCCCAGATCCGGGCAACTGATAGGGATTCCCA GCCCGTTGACTATCCCTAAGGGTCTAGGCCCGTTGACTATCCCTAAGGGTCTAG
3bp MM	GATCCGGGCAACTGATA**TTT**ATTCCCAGATCCGGGCAACTGATAGGGATTCCCA GCCCGTTGACTATCCCTAAGGGTCTAGGCCCGTTGACTATCCCTAAGGGTCTAG
7bp MM	GATCCGGGCAACTGA**ACTTTCA**TCCCAGATCCGGGCAACTGATAGGGATTCCCA GCCCGTTGACTATTCCCTAAGGGTCAGGCCCGTTGACTATCCCTAAGGGTCTAG

towards the formation of bifunctional adducts. Addition of NH_4HCO_3 will generate NH_3 which will bind to the remaining coordination sites of monofunctionally bound CDDP. Free CDDP was then removed by precipitating the oligonucleotide with 2 $^1/_2$ volumes of ice cold ethanol and $^1/_{10}$ th volume of sodium acetate at 0^0C for at least two hours. The pellet is then washed with 75% ethanol and then resuspended in TE buffer to a stock concentration of 0.025 μg/ul.

2.2.4 KINASE LABELLING OF OLIGONUCLEOTIDES

Buffer : One-Phor-All Buffer Plus (Pharmacia)

(stock concentration of buffer is ten times concentrated at which the working concentration is used.)

100mM Tris-acetate

100mM Magnesium acetate

500mM Potassium acetate

Ten picomoles of oligonucleotide were incubated in One-Phor-All Buffer Plus with $^{32}P\gamma$ ATP (specific activity 10mCi/ml), 12 units of T4 polynucleotide kinase and the reaction made up to 25μl with sterile distilled water. The labelling of the 5' terminii was carried out at 37^0C for 45-60 minutes.

2.2.4.1 Purification of Labelled Oligonucleotide

After the 45-60 minute incubation the labelling reaction was loaded on a 1xTBE 8% polyacrylamide non-denaturing gel and electrophoresed for 90 minutes at a constant voltage of 150 Volts. After electrophoresis the gel was exposed to Kodak XOMAT-AR autoradiography film and this was then sequentially used as a template for

cutting out the 5' end labelled oligonucleotide. The fragment was placed in TE and eluted overnight at 37^0C.

The following day the fragment was placed in a Spinex tube, and the TE from the overnight elution was used to wash out any remaining oligonucleotide from the fragment of acrylamide. The labelled oligonucleotide was stored in a lead pot at -20^0C.

2.2.5 LIGATION OF OLIGONUCLEOTIDES

5' end labelled CDDP treated and non-CDDP treated αG7 was ligated to an excess of cold non-phosphorylated αG7 with 2 units of T4 Ligase in manufacturer's supplied ligation buffer (50mM tris-HCl, pH7.8, 10mM $MgCl_2$, 1mM DTT, 1mM ATP, 100μg/ml BSA) at 4^0C for 16 hours. An excess of cold unphosphorylated αG7 was used in the reaction to favour the ligation of the phosphorylated αG7, both with and without CDDP, to the untreated αG7. After ligation the ligated products were purified on an 8% polyacrylamide gel (see section 2.2.4) and eluted overnight at 37^0C in TE. The ligation products were checked on a denaturing gel (refer to section 2.2.6.2).

2.2.6 ANALYSIS OF CDDP TREATMENT

2.2.6.1 Polyacrylamide gels - Basic Concepts

Polyacrylamide gel results from the polymerisation of acrylamide monomer into long chains and the cross-linking of these by bifunctional compounds such as N,N'-methylene bisacrylamide reacting with free functional groups at chain terminii. Polymerisation of acrylamide is initiated by the addition of ammonium persulphate and addition of N,N,N',N'-tetramethylethylenediamine (TEMED) is added as a catalyst of the process.

In the ammonium persulphate - Temed system, TEMED catalyses the formation of free radicals from persulphate and these in turn initiate polymerisation. Oxygen inhibits polymerisation and so gel mixtures are usually degassed prior to use.

Polyacrylamide gels have been used throughout this work (section 2.2.6.2, 2.2.6.4, 2.4.2).

2.2.6.2 Denaturing Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gels were cast by mixing 45ml 30:0.8, w/w acrylamide:bisacrylamide with 7.5ml 10xTBE , 36g urea (7M urea). This acrylamide solution is then degassed. Polymerisation is aided by the addition of ammonium persulphate with TEMED (see section 2.2.6.1). The gel was finally cast by pouring this mixture between two glass plates of 55cm long separated by 0.4mm spacers and a comb inserted on top to allow wells to form. Once solidified the gel was placed on a vertical apparatus with each end submerged in a reservoir of 1xTBE buffer. Gels were run at 25mA for 3-4 hours at 55^0C after which they were transferred to Whatman 3MM filter paper and exposed to XOMAT autoradiography film overnight.

2.2.6.3 Exonuclease III Treatment

Both CDDP and non-CDDP treated end-labelled oligonucleotides were incubated with six units of exonuclease III in 50mM Tris, pH 8.0, 5mM $MgCl_2$, 10mM B- mercaptoethanol at 37^0C for 30 minutes. After the incubation period with the enzyme, treated oligonucleotides of 200 cps were loaded on a 25% polyacrylamide denaturing gel (refer to section 2.2.6.1).

2.2.6.4 Non-Denaturing Polyacrylamide Gel Electrophoresis

A 1xTBE 8% polyacrylamide was cast in a 2mm notched glass plate and oligonucleotides of 200cps were loaded and run at 20mA for 90 minutes in 1xTBE buffer. The gel was then transferred to Whatmann 3MM filter paper and dried down under vacuum for 60 minutes at 80^0C. The dried gel was then exposed to XOMAT film overnight with the use of an intensifying screen.

2.3 EXTRACTION OF PROTEIN FROM CELLS

2.3.1 Buffers

TMS
10mM Tris, pH 7.5
5mM $MgCl_2$
0.25M sucrose

E_{50}
50mM $(NH_4)_2SO_4$
20mM Hepes, pH7.9
5mM $MgCl_2$
0.1mM EDTA
0.1% Brij 35
1mM DTT (add fresh each time)
20% glycerol

SB
50mM NaCl
20mM Hepes, pH7.9
5mM $MgCl_2$
0.1mM EDTA

20% glycerol

1mM DTT (add fresh each time)

2.3.2 Protease Inhibitors

Leupeptin	0.1mg/ml
Chymostatin	0.1mg/ml
Benzamidine	50mM
Aprotinin	0.1mg/ml
Pepstatin	0.1mg/ml
PMSF	50mM

2.3.3 Extraction Protocol

2.3.3.1 Nuclear Extract Preparation

At least $1x10^8$ cells from desired lines were harvested during exponential phase of growth. All the following manipulations were carried out at 4^0C. The cells were washed with ice cold PBS and osmotically swollen with a TMS wash and lysed in 0.25% Triton X-100. The resulting pellet of nuclei was washed three times and the DNA content estimated by measuring the optical density at 260nm of a small sample after sonication. The pellet was resuspended in TMS to a concentration of 7.5 mg/ml DNA, NaCl added to a final concentration of 0.3M and nuclear proteins eluted by incubation on ice for ten minutes. Debris was removed by a 15K spin and the supernatant clarified further by a 36K ultracentrifuge spin. Proteins were pelleted by incubation on ice with ammonium sulphate at 0.35 g/ml followed by a 10K spin. The pellet was then dissolved in 0.6ml E_{50} buffer and dialysed overnight at 4^0C against one litre of storage buffer

(SB). The following morning the solution was centrifuged in an IEC/B60 ultracentrifuge using an SB-405 rotor at 35K for 60 minutes at 4^0C and the resultant supernatant aliquoted into eppendorf tubes and stored in liquid nitrogen. This protocol generated protein extracts which were used in gel mobility shift assays (refer to section 2.5.1), however it was adapted to a simplified version to give a much cruder extract which was used in South-Western blotting. **N.B. The 10K and 15K spins were carried out using a fixed angle Sorvall GS-3 rotor, each for 30 minutes. The 36K spin was in a IEC/B60 ultracentrifuge using an SB-405 rotor for 60 minutes.**

2.3.3.2 Cell free salt extract

10^6 -10^7 cells were harvested during exponential growth of the cells. The cells were washed with ice cold PBS and again osmotically swollen with a TMS wash and lysed in 0.5ml TMS-0.25% TX-100. After lysis NaCl was added to give a final concentration of 0.3M NaCl. This is then spun in a microfuge at 4^0C for 15 minutes. The supernatant is then removed and added to it 20% glycerol, 0.1% Brij 35and 1mM DTT (final concentration). The extract is then transferred to mini colloidan bags and dialysed against SB overnight at 4^0C. The following morning the solution is microfuged at 4^0C for 15 minutes and then aliquotted into eppendorf tubes and stored in liquid nitrogen.

2.4 QUANTIFYING PROTEIN CONCENTRATIONS IN EXTRACTS

2.4.1 Biorad Assay

Protein concentrations were estimated using the Bio-rad protein assay. The assay is based on the method by Bradford 1976, and is a dye binding assay based on the differential colour change of a Coomassie blue dye in acidic conditions to various concentration of protein (for detailed protocols see manufacturer's instruction manual).

Each protein concentration was estimated in triplicate at varying amounts of the protein sample.

2.4.2 Discontinuous-SDS Polyacrylamide Gels

Buffers

Gel Buffer	Tris 1.5M pH 8.9
	SDS 10%
Tank Buffer	Tris 0.05M
	Glycine 0.05M
	SDS 1%
Stack Buffer	Tris 1.0M (pH6.8)
	SDS 10%
Loading Buffer	0.2M Tris, pH6.8
	SDS 8%
	EDTA 8mM
	Glycerol 40%

Loading buffer is four times more concentrated than that of the final.

Gradient Gel

	5%	15%
Running Buffer	8ml	8ml
Acrylamide	2.67ml	8ml
Polyacrylamide	1.6ml	1.6ml
Water	2.4ml	7.73ml
Sucrose	/	2.25g
10% APS	30µl	90µl
TEMED	6.5µl	7.5µl

A Tris - SDS based resolving gel was first prepared using a concentration gradient of acrylamide varying between 15% at the bottom of the gel through to 5% at the top hence the gradient gel having a decreasing pore size. During electrophoresis in gradient gels, proteins migrate until the decreasing pore size inhibits further progress. Once the pore limit is approached the protein banding pattern does not change appreciably with time although migration does not stop completely. This thus ensures tighter bands of proteins through the gel.

To ensure easier handling of the gel 1% polyacrylamide solution was added to the mixture. Sucrose is also added to the 15% polyacrylamide solution to give a stabilising density gradient. Polymerisation was initiated by the addition of 10% APS and TEMED (refer to section 2.2.6.1). The gel is poured between two glass plates separated by 1mm spacer to about 5cm from the top and the gel overlayed with isobutanol. When polymerisation was complete, approximately 1 hour, the isobutanol is poured off. A stacking gel was then added of 5% acrylamide in a stack buffer (again

being polymerised by the APS -TEMED system). Polymerisation of the stack occurs within minutes. Protein samples were loaded in the SDS loading buffer onto the gel and samples were run through the stack in a Tris-glycine SDS buffer at 60mA. Once the samples reach the resolving gel the current is turned down to 30mA and run for 2-3 hours at room temperature in a BIO-RAD PROTEAN II tank system. To check protein loading the gel is stained in 0.1% Coomassie blue stain for one hour and then destained overnight in destain solution, 5% methanol, 7% acetic acid.

2.5 ANALYSIS OF CISPLATIN-DNA PROTEIN INTERACTIONS

2.5.1 Gel Mobility Shift Assay

This assay is a modified version of that by Garner and Revzin, 1981. ^{32}P end labelled oligonucleotides (1.5ng) were incubated on ice for 35 minutes in the presence of 2μg poly(dI.dC):(dI.dC) with 1-2 μg nuclear extract of interest (if different amounts used then this is stated in the figure legend). This reaction is then incubated in a final volume of 20μl with storage buffer (SB) (refer to section 2.3.2).

After an incubation period the reactions were electrophoresed through a 8% 0.5x TBE non-denaturing polyacrylamide gel at 4^0C. Following electrophoresis the gel was dried down under vacuum at 80^0C and autoradiographed on Kodak XOMAT film.

2.5.2 South-Western Blotting

2.5.2.1 Buffers

BLOTTO 5% Carnation nonfat milk powder
50mM Tris pH7.5

50mM Na Cl

1mM EDTA

1mM DTT (add fresh each time)

TNE-50 10mM Tris pH7.5

50mM NaCl

1mM EDTA

1mM DTT

Binding Buffer 30mM Hepes-NaOH, pH7.5

10mM $MgCl_2$

2mM $MnCl_2$

0.25% Carnation dried milk

Wash Buffer 30mM Hepes-NaOH, pH7.5

0.25% Carnation dried milk

2.5.2.2 Protein Blotting

The gel for proteins to be analysed by a South-Western blot is run in exactly the same way, under identical conditions, as that described in section 2.4.2. Once the samples have electrophoresed through the gel, it is then taken and proteins are transferred from it by the semi-dry electroblotting procedure (Kyhse-Andersen, 1984) onto a nitrocellulose membrane (BA852, 0.45μm; Schleicher and Schuell). After blotting the filters were stained with Ponceau-S stain for ten minutes and then destained in 5% acetic acid for a further ten minutes. This allows to check for even transfer of the proteins from the gel to the nitrocellulose filter where further manipulations will be carried out. The

filters were immersed in BLOTTO for at least 1 hour at room temperature with gentle shaking. They were then washed twice with TNE-50 for 5 minutes for each wash. The filters were then taken and incubated for 60 minutes at room temperature in binding buffer with labelled oligonucleotide at $2x10^4$ cpm/ml. Included in this reaction mixture is poly(dI.dC):(dI.dC), 3µg/ml, to act as a competitor for non-specific binding of proteins. Unbound DNA was then removed by washing at 4^0C with wash buffer and filters left to air dry. Protein-DNA complexes were detected by autoradiography. By running protein molecular weight standards, estimation of the size of the detected proteins can be made:

Protein Standards (BRL)

	Daltons
Lysozyme	15 300
B-Lactoglobulin	17 950
Carbonic anhydrase	28 500
Ovalbumin	43 750
Bovine serum albumin	70 600
Phosphorylase B	110 900
Myosin (H-chain)	206 450

2.6 TISSUE CULTURE TECHNIQUES

2.6.1 General Cell Culture Methods

2.6.1.1 Maintenance of Cells

Cell lines were maintained as monolayers in RPMI (Rosswell Park Memorial Institute) medium supplemented with 10% foetal calf serum, 2mM L-Glutamine, 1mM sodium pyruvate and sodium bicarbonate added in equilibrium with 5% CO_2. All medium used had 0.05 units/ml of penicillin present. Cells were grown in a 37^0C, 5% CO_2 dry incubator. All lines were free of mycoplasma contamination. **Table 2.2** shows cell lines used and seeding density at which these lines were maintained at.

2.6.1.2 Storage of Cells

To reduce chances of genetic drift, all cell lines were replaced from frozen stocks every 3 months. Frozen stocks were maintained in liquid nitrogen in culture medium at a concentration of at least $1x10^6$/ml and 10% DMSO added. Cells were frozen with a cooling rate of approximately 1^0C/min to -70^0C and retained at -70^0C overnight before being transferred to liquid nitrogen. **Table 2.3** shows cell lines used and descriptions.

2.6.1.3 Aseptic Technique

All aseptic manipulations were carried out in a class II microbiological safety cabinet with vertical air flow. All glassware was dry heat sterilised at 160^0C for a minimum of 1 hour.

Table 2.2 Growth conditions of cell lines

CELL LINE	SEEDING DENSITY	GROWTH MEDIUM
A2780	$1x10^5$ per $75cm^2$flask	RPMI medium
A2780CP	$1x10^5$ per $75cm^2$flask	RPMI medium
Ov1	$1x10^5$ per $25cm^2$flask	RPMI medium
Ov1 DDP	$2x10^5$ per $25cm^2$flask	RPMI medium supplemented with CDDP at a final concentration of 1mg/ml
GM02145	$1x10^5$ per $25cm^2$flask	Special Liquid medium
Hela	$1x10^5$ per $75cm^2$flask	RPMI medium

Table 2.3 Origins of cell lines

CELL LINE	SOURCE	CELL TYPE	PATHOLOGY
A2780	R.F. Ozols, T.C.Hamilton Fox Chase Cancer Centre, Philadelphia	Epithelial	Adenocarcinoma
A2780CP	CDDP resistant counterpart to A2780 (as above)	Epithelial	Adenocarcinoma
Ov1	J.Benard, Intitut Gustave Roussy, France	Epithelial	Adenocarcinoma
Ov1DDP	CDDP resistant conterpart(as above)	Epithelial	Adenocarcinoma
GM02145	Human genetic mutant cell repository(Camden,NJ)	Fibroblast	XP group E

2.6.2 Clonogenic Assay

A2780 cells were plated at $2x10^6$ per $F75cm^2$ flask for 5-30 μM CDDP concentrations. After 24 hours the cells were treated with the various concentrations of CDDP for a period of 24 hours, washed twice with phosphate buffered saline, incubated in drug free RPMI medium for two weeks in a 37^0C incubator, 5% CO_2 and surviving colonies of more than 60 cells were counted. The A2780 cells had a plating efficiency of approximately 20% using these conditions.

2.6.2.1 Isolation of CDDP resistant colonies by single step selection

Clonogenic assays gave a survival curve and from this a concentration of CDDP was chosen at which to select colonies. A concentration of CDDP at which 0-3 colonies survived was chosen at the selecting concentration. From the surviving colonies a concentration of CDDP of 15μM was used for selection of resistant colonies.

Cells were plated out as in section 2.6.2 at $2x10^6$ per $F75cm^2$ flask and resistant colonies selected at the predetermined concentration of CDDP as described above. After two weeks incubation the surviving colonies were picked as separate clones and placed in a $F25cm^2$ flask and grown in RPMI medium.

The frequency of resistant colonies were either calculated as surviving colony number per viable cell or using fluctuation analysis (Luria,S.E. & Delbruck,M.) based on the number of cultures containing no surviving colonies. This latter analysis avoids any uncertainties due to the daughter colony formation.

2.6.2.2 Mutagen Exposure Prior to CDDP Selection

A2780 cells were seeded at $5x10^6$ per F175cm^2 flask. The cells were allowed to settle for a period of 24 hours and then treated for 2 hours with 2.5mg/ml of ethyl methane sulphonate (EMS) in serum free medium.

Prior to choosing this concentration of CDDP a dose response of A2780 to various concentrations of EMS was carried out as in section 2.6.2 (2.5mg/ml EMS was chosen because at this concentration the survival fraction of A2780 was 22%. At various times after exposure to EMS the cells were selected at $2x10^6$ per F75cm^2 flask as described in section 2.6.2.1.

2.6.2.3 Pretreatment with BSO and Aphidocolin

Aphidicolin (2.5 μg/ml and 5 μg/ml) was added 1 hour prior to CDDP and maintained during the CDDP selection as described in section 2.6.2.1. These are conditions previously shown to inhibit removal of DNA bound platinum in A2780 cells treated with CDDP (Masuda, et al 1990).

Buthionine sulfoximin (BSO) was added 24 hours prior to CDDP selection and maintained during the CDDP selection (selection is again as in section 2.6.2.1). Concentrations of BSO were used (25μM and 50μM) previously shown to deplete glutathione levels in A2780 cells and increase the sensitivity of A2780CP cells to CDDP in short term cell viability assays (Hamilton et al, 1985).

2.6.2.4 MTT Assay

As a rapid method of analysing the selected clones for sensitivity to CDDP the MTT assay was employed.

This assay is based upon a method described by Mossman (1983) and the modified version by Plumb was used in this thesis presentation (Plumb et al, 1989).

The assay is dependent on the reduction of a tetrazolium dye, MTT (3-(4, 5-dimethylthiozoyl-2-yl, 5 diphenyl-tetrazolium bromide) to a purple formazan product by live but not dead cells. Cells are plated onto 96 well plates and allowed to attach and grow. Cells are then exposed to cytotoxic drug for a fixed period of time. Drug is removed and cells are allowed to recover and divide. Surviving cells are then indicated following incubation with MTT. Formazan production is thus an endpoint to this assay.

Cells in exponential growth were plated out at $1x10^3$/well in medium and left for two cell doubling times before drug was added. The cells were exposed to CDDP for 24 hours and then subsequently incubated for 2-3 cell doubling times in drug free medium. Metabolically active cells were measured using the reduction of MTT as a measure of cell viability as described above.

CHAPTER 3

GEL MOBILITY SHIFT ANALYSIS OF DNA DAMAGE RECOGNITION PROTEINS (DDRPs) RECOGNISING DNA DAMAGED WITH CDDP

3.1 INTRODUCTION

In the search for a mammalian equivalent to the *E.coli* uvrABC complex, which recognises and excises damage from DNA, (refer to section 1.12), the gel shift assay has been used to identify proteins from mammalian cells binding to damaged DNA. The gel shift system has previously been used to identify proteins which bind to specific sequences involved in control of gene transcription - otherwise termed transcription factors (Mitchell & Tijan, 1989).

The technique is based on the observation that the movement of a DNA molecule through a non-denaturing polyacrylamide gel is hindered when a protein molecule is bound to it. When a reaction containing nucleic acid protein complexes and unbound DNA molecules is subjected to gel electrophoresis, initially the free DNA will quickly enter the gel leaving the DNA-protein complexes behind. The highly negatively charged DNA will eventually pull bound proteins into the gel, but the DNA protein complexes are retarded in moving through the gel. The free DNA will always run much faster than the DNA bound protein complex, therefore allowing the two to be separated. With the DNA being ^{32}P labelled, an autoradiograph can be taken to visualise the complexes. In the study of transcription factors, binding of the complexes can be shown to be specific for a certain sequence by saturating the system with an excess of the specific sequence of DNA or a different sequence. Only sequences able to bind the transcription factor will compete in the gel shift, leading to disappearance of the retardation complex.

By use of the gel shift system various groups have reported factors in mammalian cells recognising DNA damage (Chu and Chang, 1988; Toney et al, 1989; Hirschfeld et al, 1990; Chao et al, 1991[a]; Donahue et al, 1990, Andrews and Jones,1991). Prior to the use of gel shift systems, proteins recognising DNA damage in mammalian cells were identified and partially purified using a DNA cellulose column, in which the

substrate bound to the column was damaged by UV-irradiation (Lehmann and Kirk-Bell, 1978; Feldberg and Grossman, 1976; Huang et al, 1975) or AAAF (Moranelli and Lieberman, 1980; Tsang and Kuhnlein, 1982). The proteins identified proved to be different from each other. The DNA binding protein characterised by Tsang binds efficiently to supercoiled DNA treated with UV light (Tsang and Kuhnlein, 1982) whereas the protein isolated by Moranelli does not recognise UV induced damage (Moranelli and Lieberman,1980). The protein characterised by Feldberg does recognise UV induced damage but it was found to elute off columns at a different salt concentration compared to Tsang's protein (Feldberg and Grossman, 1976). In 1988 Chu and Chang identified at least one nuclear factor in Hela cell extracts that binds to DNA damaged with ultra-violet radiation by use of the gel shift system. They reported that the factor was absent in an extract of a cell strain of XPE (refer to section 1.13.2 for introduction on XP), (Chu and Chang, 1988) and therefore called it the XPE binding factor. They therefore suggested that the XPE binding factor may be important in the recognition of damage prior to removal by DNA repair enzymes. The absence of the XPE binding factor in XPE cell strains was later questioned by Kataoka and Fujiwara. They verified the absence of the binding factor in the same XPE cell strains as Chu and Chang demonstrated, but found it present in other XPE cell strains (Kataoka and Fujiwara, 1991). The uvrABC complex recognises a broad range of damage (review, vanHouten, 1990). It has therefore been hypothesized that a DNA damage recognition protein complex present in mammalian cells is likely also to recognise a broad range of damage. In the gel shift system, as previously described, binding of the protein to a particular adduct can be competed for when excess of the substrate is added to the system. Cross-competition assays demonstrated that the binding to the XPE factor could be competed with an excess of CDDP damaged DNA. It was however shown that the XPE binding factor did not directly recognise CDDP damaged DNA (Chu and Chang, 1988). This was

suggested to be due to the abundance of the damage recognition protein recognising CDDP damage, masking the binding of the XPE binding factor.

In an attempt to characterise the XPE binding factor in mammalian cells Chu and co-workers searched for an equivalent factor in the yeast system. It was thought that this approach would prove fruitful, since many proteins involved in cellular metabolism are conserved between yeast and humans. A factor was found in yeast which binds to UV damaged DNA and shares many characteristics with the XPE binding factor (Patterson and Chu, 1989). This factor is the photoreactivating enzyme photolyase. Photolyase is an enzyme which will bind to the site of a pyrimidine dimer, absorb energy from visible light through chromophores and reduce the cyclobutane ring *in situ* to restore DNA to its native state (review, Sancar and Sancar, 1988). In *E.coli* the addition of purified photolyase causes a two-fold stimulation in the repair of pyrimidine dimers by uvrA, uvrB, uvrC proteins *in vitro* (Sancar et al, 1984). Furthermore, genetic experiments in yeast suggest that expression of the PHR1 gene, the gene coding for photolyase, enhances excision repair by the RAD 3 epistasis group (Sancar and Smith, 1988). These lines of evidence suggest that photolyase may be involved in excision repair.

Mentioned previously, the *E.coli* uvrABC recognises various types of DNA damage. CDDP is known to form intrastrand adducts that are unique to the cis configuration. TDDP which does not demonstrate antitumorigenic activity cannot form these adducts. The intrastrand adducts have therefore been reported to be the important lesion in the killing of tumour cells (Eastman, 1986). A factor has been identified in mammalian cell extracts that recognises these unique 1,2 d(GpG) and d(ApG) intrastrand cross-links generated by CDDP, (Donahue et al, 1990).

This CDDP recognition protein could either recognise the CDDP adduct itself, or more likely, recognise a change in structural conformation within the DNA. CDDP has been reported to bind to DNA and cause a local denaturation around the

adduct itself, (Sundquist et al, 1986). In the bacterial system it has been found that gene 32 from bacteriophage T4 which exhibits a high specificity for single-stranded nucleic acids, binds more efficiently to DNA treated with CDDP than to native DNA, (Toulme et al, 1983). It has been suggested that with various forms of damage which have been reported to cause a local melting around the adduct, (Van Houten, 1990), single stranded binding proteins may be important in the recognition step prior to incision of the damaged DNA. Recent evidence has found a role for human single stranded binding proteins (hSSB) in DNA excision repair. Human SSB is normally involved in replication of DNA by acting with T antigen and topoisomerases to unwind DNA, allowing the access of other DNA replication proteins, (Wold and Kelly, 1988). By making use of a cell free system that can carry out nucleotide excision repair *in vitro* , (Wood and Lindahl, 1988), it was found that monoclonal antibodies against hSSB caused extensive inhibition of DNA repair in plasmid molecules damaged by UV light or acetylaminofluorene. On addition of purified hSSB the repair was stimulated, (Coverley et al, 1991). This suggests a role for hSSB in excision repair.

In this present chapter the identification of damage recognition proteins by gel shift assays using CDDP modified oligonucleotides is described (these will be abbreviated to DDRPs). The CDDP treatment of the oligonucleotide is examined by denaturing gel electrophoresis and exonuclease III treatment. Exonuclease III is a 3'-5' nuclease which has previously been used to determine sites of CDDP modification, (Royer-Pokora et al, 1981). The adducts present in the oligonucleotide are analysed by ICPM spectroscopy, (Tothill et al, 1990). The involvement of single-stranded binding proteins are discussed in the context of DDRPs and excision repair.

RESULTS

3.2 Choice of Oligonucleotide

Many of the groups who have identified damage recognition proteins (DRPs) have used DNA derived from plasmids as their substrate in the gel shift assay. Oligonucleotides however, can be generated with a defined sequence. They are also maniputable in that the sequence can be altered if need be. In the systems described in the next two chapters an oligonucleotide was chosen as the substrate in the detection of damage recognition proteins recognising CDDP adducts (DDRPs).

The choice of oligonucleotide sequence and its preparation for use in the gel shift assay was found to be a crucial factor in the detection of the DDRPs. It has been reported that CDDP binds preferentially to a sequence of guanines in DNA, (Fichtinger-Schepman et al, 1982). The oligonucleotide chosen had therefore to be rich in guanine nucleotides. A DNA sequence was chosen which had two areas of three consecutive guanines and had previously shown little non-specific binding to nuclear proteins, (Plumb et al, 1988). Preliminary experiments with oligonucleotides of random sequence showed high levels of non-specific protein binding. The sequence chosen, (αG7) (**Table 2.1**), is a mutated form of a sequence which is important in binding an erythroid specific nuclear factor. Normally the erythroid-specific nuclear factor will bind to GATTAG motifs which are found to be conserved in the promoter regions and 3' enhancer regions of globin and non-globin genes, (Plumb et al, 1989). It is important at this point to emphasise that αG7 is known not to bind proteins in most cell types. HPLC purification after synthesis, and gel purification after radio-labelling the oligonucleotides, has shown to be necessary in the use of the oligonucleotides in the gel shift system in clearly allowing the identification of the binding factors.

3.3 CDDP Treatment of Oligonucleotides

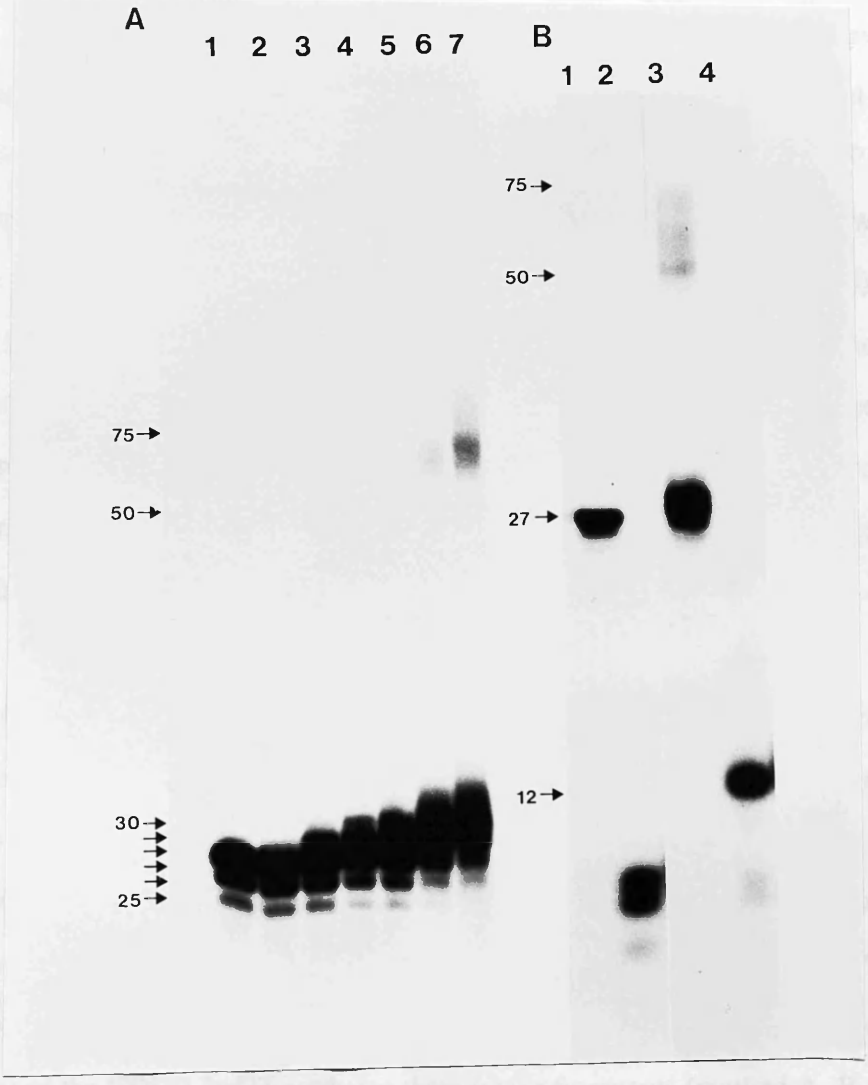
During the synthesis of the oligonucleotide, the crude reaction mixture collected at the end of each cycle contains different lengths of oligonucleotide sequence, depending on the efficiency of coupling of each nucleotide to the next. The major species is the desired product. There are also small quantities of premature termination sequences that range in size from one nucleotide to 26 nucleotides i.e one nucleotide shorter than the desired product. Upon examination of **Figure 3.1a**, lane 1, one sees products also running at 25 and 26 nucleotides in size as well as the 27 base pair αG7. The smaller forms represent oligonucleotides which have not coupled completely in the synthesis process.

CDDP has a molecular weight of 300, this is similar to the molecular weight of a nucleotide which averages at 300. Therefore one CDDP adduct present on the 27 base pair oligonucleotide was likely to increase the weight of the oligonucleotide to that of a 28 base pair oligonucloetide and could be observed on a denaturing gel as a shift to a form running at the 28 base pair size assuming there was no alteration in charge.

Treating double-stranded 27 base pair αG7 with increasing concentrations of CDDP produces an increase in slower migrating forms of the oligonucleotide observed on denaturing gels, (**Figure 3.1a**). Two of these forms migrate to a position consistent with intrastrand CDDP crosslinks or monofunctional adducts. These make up more than 90% of the forms at the higher CDDP concentration. At the higher concentrations of CDDP treatment there is gradual disappearance of the 27 base pair oligonucleotide to slower migrating forms, so suggesting at these concentrations most of the oligonucleotide present contains CDDP adducts. The estimated molecular weight from labelled size markers of the majority of the forms would suggest they are oligonucleotides containing one and two CDDP adducts.

Figure 3.1

Denaturing gel analysis of oligonucleotide treated with CDDP



Panel A : Double stranded 27 base pair αG7 was treated with increasing concentrations of CDDP for 1hr. Lanes 1-7 are αG7 treated with 0, 1, 10, 50, 75, 100, 200 μg/ml CDDP. The CDDP treated oligonucleotides were then end labelled with γ^{32}P ATP, separated on a 20% denaturing polyacrylamide gel and autoradiographed. Size of the bands in bases were estimated from size markers.

Panel B : Exonuclease III digestion of oligonucleotide. Lanes 1 and 2 untreated αG7, lanes 3 and 4 αG7 treated with 200μg/ml CDDP for 1 hr. Lanes 2 and 4, oligonucleotides incubated with exonuclease III prior to denaturing polyacrylamide gel electrophoresis.

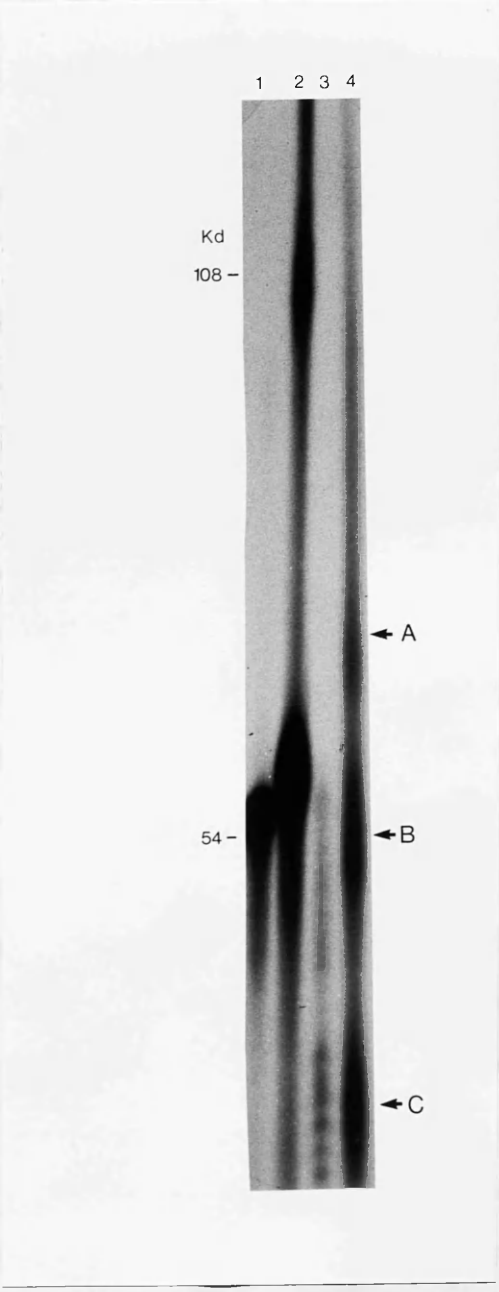
A smaller amount of the oligonucleotide migrates at a position consistent with interstrand CDDP cross-links. (**Figure 3.1a**, lanes 6 and 7) observed on the gel migrating between the 50 and 75 base pair size marker.

Exonuclease III, a double strand specific 3'-5' nuclease has previously been used to define the site(s) of CDDP modification, (Royer-Pokora et al, 1981). Exonuclease III digestion of the oligonucleotide αG7 with or without CDDP treatment is shown in **Figure 3.1b**. Digestion of the CDDP treated oligonucleotide shows one major and one minor DNA fragment which are resistant to Exonuclease III (lane 4) and are not present in the Exonuclease III digested unplatinated αG7 (lane 2). This would suggest that the CDDP adducts are not randomly distributed in the oligonucleotide sequence, but rather are localised at one main site. The size of the minor nuclease resistant fragment is also consistent with nuclease digestion stopping at one main site in oligonucleotides containing interstrand crosslinks.

The larger 2αG7 oligonucleotide (54 base pairs) was analysed in the same way as above, by denaturing gel electrophoresis (**Figure 3.2**) . However with the 2αG7 being larger than the αG7, the resolution in the denaturing gel system is not as sharp and also the gel is overloaded. It can be seen however, that treatment of the 2αG7 with 400μg/ml CDDP causes a shift to a slower migrating form of the oligonucleotide. Again one sees that a smaller amount of the oligonucleotide (approximately 10-20%) migrates to a position consistent with interstrand crosslinks, (**Figure 3.2**). The exonuclease III treatment of the CDDP-2αG7 produces three major fragments (marked A, B, C on figure 3.2), two of which may represent the site of major adduct formation of intrastrand crosslinks (sites B, C) and one which may represent nuclease digestion stopping at one main site in the oligonucleotide containing interstrand crosslinks (site A) as previously discussed.

Figure 3.2

Denaturing gel analysis of 2αG7

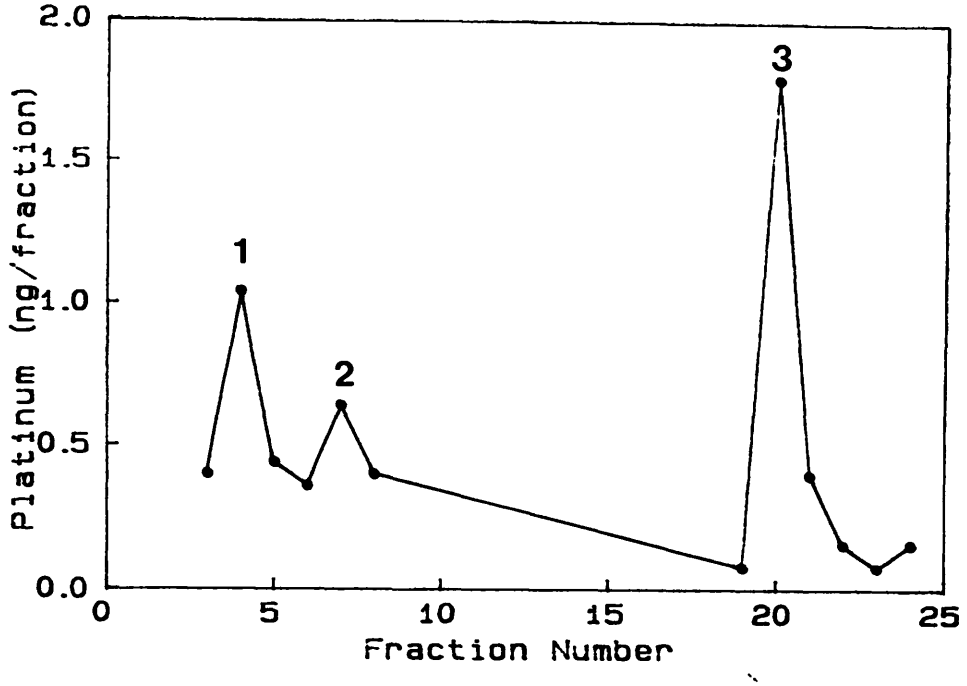


lanes 1 and 3, 2αG7. Lanes 2 and 4, 2αG7 treated with 400μg/ml CDDP for 1 hour. Prior to denaturing gel electrophoresis oligonucleotides were end labelled with γ^{32}P ATP. Lanes 3 and 4 treated with exonuclease III.

The larger platinated 2αG7 was also analysed by ICPM spectrometery, (Tothill et al, 1990), **(Figure 3.3)**, in collaboration with Dr.D.Bissett, Dept. Medical Oncology, University of Glasgow and the Scottish Universities Reactor Centre, East Kilbride. Nuclease digested and HPLC separated nucleotides of the CDDP treated oligonucleotides showed a platinum to nucleotide ratio of 8.96×10^{-4}. (Platinum levels measured by ICPM spectrometry). From the trace **(Figure 3.3)** the major peak, peak 3 represents PtG-G adducts, (the peaks are compared to known standards of CDDP adducts run at the same time). The method cannot differentiate, however, between PtG-G intrastrand or interstrand adducts. However upon comparison of the denaturing gel electrophoresis, **(Figure 3.2)**, 90% of the adducts prove to be migrating at a size consistent with intrastrand and monofunctional adducts, therefore suggesting that the major peak of the trace may represent PtG-G intrastrand adducts. Directly after the major peak PtGxG (x is any nucleotide) adducts come off. It is therefore difficult to distinguish if the major peak also has a population of PtGxG adducts within it. It has been reported that PtGxG adducts form less than 1% of the total adducts, therefore suggesting that a small proportion, if any, of the major peak would represent PtGxG adducts. In the trace **(Figure 3.3)** a peak representing PtA-G cross-links is shown, (peak 2), however it was again not possible to distinguish between intrastrand or interstrand cross-links. As demonstrated in **Figure 3.3** approximately 25% of the adducts are in the monofunctional adduct form, (peak 1). Less than 1% of adducts formed are normally of the monofunctional form (Eastman,1986). It has generally been accepted that in the reaction of CDDP with DNA monofunctional adducts are formed which will gradually react to form the bifunctional cross-links. The time of CDDP treatments, prior to quenching of the monofunctional adducts with NH_4HCO_3, may cause these differences in the amount of monofunctional adducts. Normal protocols for CDDP treatment of DNA follows a 18-24 hour treatment with drug whereas the protocol used here follows a 1 hour treatment of DNA with CDDP.

Figure 3.3

Platinum detected in HPLC fractions of a 2αG7 digest



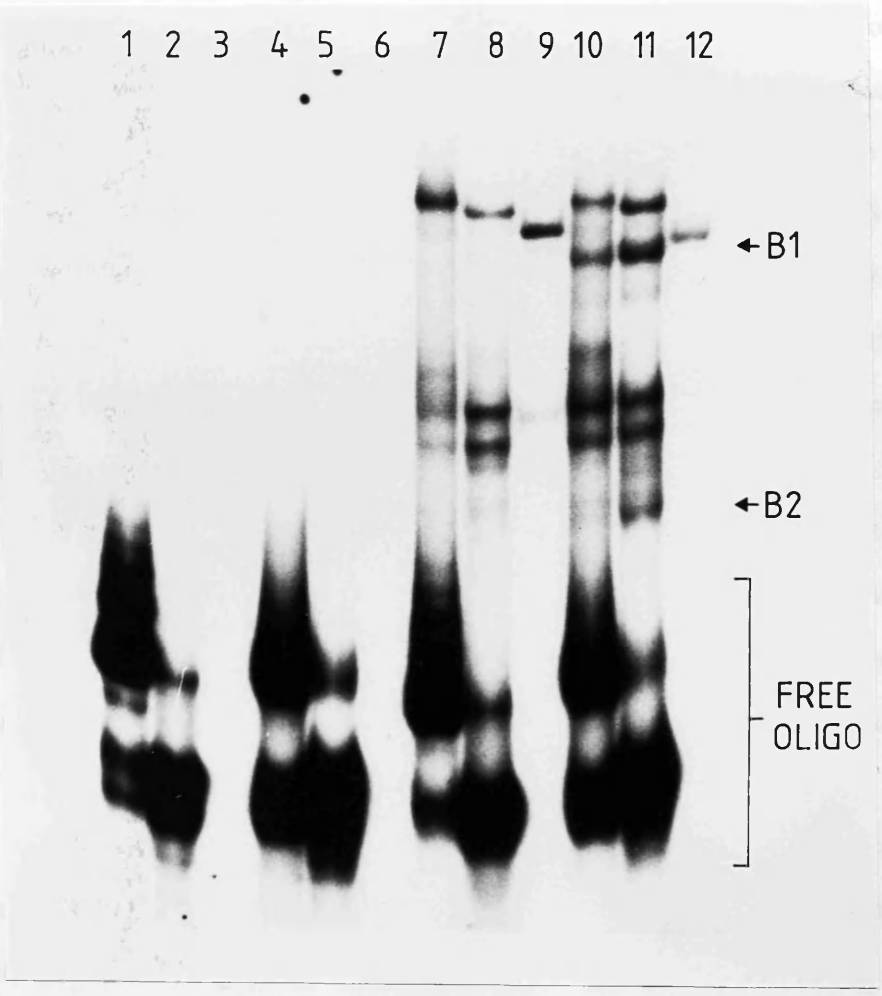
2αG7 treated with 400μg/ml CDDP, digested to the nucleotide level by treatment with DNAseI and Nuclease P1, HPLC purified and platinum detected within the fractions by ICPMS. Peaks 1, 2, 3 represent peaks at which major adducts are formed based on standards which have been characterised by NMR studies and obtained from Fichtinger-Schepman (Fichtinger-Schepman et al, 1982). Peak 1 = monofunctional adducts, Peak 2 = PtA-G adducts, Peak 3 = PtG-G adducts. This work was carried out in collaboration with Dr.D.Bissett, Medical Oncology Dept., Glasgow and Scottish Universities reactor centre, East Kilbride.

3.4 Identification of DDRPs by gel mobility shift assay

Use of the 27 base pair oligonucleotide, after CDDP treatment, in the gel shift system did not show a different pattern of binding compared to the unplatinated sequence (**Figure 3.4**, lane 9 cf. lane 12). It can be seen that retarded bands do appear in the unplatinated αG7, lane 9. The bands however bind both to the the platinated and unplatinated forms of the sequence. They therefore represent non-specific factors which bind to the sequence but not to CDDP adducts. It was decided to use a longer piece of oligonucleotide to investigate if there was a DNA length requirement for binding of DDRPs. The platinated αG7 after kinase labelling was ligated to a cold excess of untreated, unphosphorylated αG7. This was in the hope to favour the reaction of the platinated αG7 ligating to the unplatinated αG7, so forming a longer section of DNA, with one section containing the CDDP adducts. This should therefore form a more stable DNA duplex with CDDP present nearer the centre of the DNA. Before a ligation process will occur at least one end of the oligonucleotide must be phosphorylated, therefore ligation of the unphosphorylated, unplatinated αG7 should be an unlikely event. With the unplatinated form of the αG7 being in excess in the reaction, it is likely that the reaction will be between platinated αG7 with unplatinated αG7. After ligation monomer, dimer and trimer forms were separated by gel electrophoresis and isolated. To verify the forms isolated represented monomer, dimer and trimers, equal amounts of each fraction were analysed on a denaturing gel (data not shown).

Figure 3.4

Gel mobility shift assay of monomer, dimer and trimer forms of αG7



^{32}P labelled non-CDDP treated αG7 and CDDP treated αG7 were ligated to an excess of untreated αG7. After ligation monomer, dimer and trimer forms were gel purified. Lanes 1-6 represent oligonucleotides incubated without extract and lanes 7-12 oligonucleotides incubated with 1μg Hela nuclear extract. Oligonucleotides in lanes 4-6 and 10-12 were treated with 200μg CDDP prior to ligation and gel purification. The assay conditions are as those described in section 2.5.1. Lanes 1, 4, 7, 10 represent trimer forms of αG7, lanes 2, 5, 8, 11 dimer forms of αG7, lanes 3, 6, 9, 12 monomer forms of αG7. After the ligation process the purified forms were analysed on a denaturing gel to check the sizes, data shown in figure 3.5. The position of unbound oligonucleotide (free oligo) is shown, although in the case of monomer αG7 this was electrophoresed off the gel.
Bands B1 and B2 represent retarded complexes.

Figure 3.4 shows a gel mobility shift assay using the ligated substrates with nuclear extract from Hela cells. Two new bands are observed to bind to the CDDP-dimer containing fraction upon incubation with Hela extract, bands B1 and B2 (lane 11). In the CDDP-trimer containing fraction (lane 10), band B1 is observed much stronger than band B2. There is a very faint appearance of band B2 in the CDDP-trimer fraction (lane 10) which is totally absent in the unplatinated ligated form of the αG7 (lane 7). Bands B1 and B2 are only observed when the αG7 has been CDDP treated prior to ligation (lanes 10-12). (The binding to Bands B1 and B2 is proteinase K sensitive and RNAse resistant). Binding of complexes to the untreated oligonucleotides are also observed, but these are not affected by the CDDP treatment of the oligonucleotides and represent non-specific binding complexes. No difference in binding of complexes to the monomer before or after CDDP treatment is observed. It is therefore found that DDRPs can be detected using a platinated oligonucleotide of 54 bases in length but not of 27 bases in length. It can therefore be postulated that the critical length of the platinated oligonucleotide used in the detection of proteins recognising CDDP adducts must be between the limits of 27 and 54 bases in length.

To investigate the altered binding to the CDDP treated dimer and trimer forms of the αG7, an oligonucleotide of 54 base pairs in length containing two direct repeats of the αG7 sequence was synthesised. This oligonucleotide is therefore 54 base pairs in length. The following work described is carried out using this longer oligonucleotide 2αG7 unless otherwise stated.

To investigate if the protein(s) recognising the damage to the oligonucleotide, by CDDP treatment, are binding at saturation level in the extract, dilutions of Hela extract incubated with both non-CDDP and CDDP treated 2αG7 oligonucleotide were carried out.

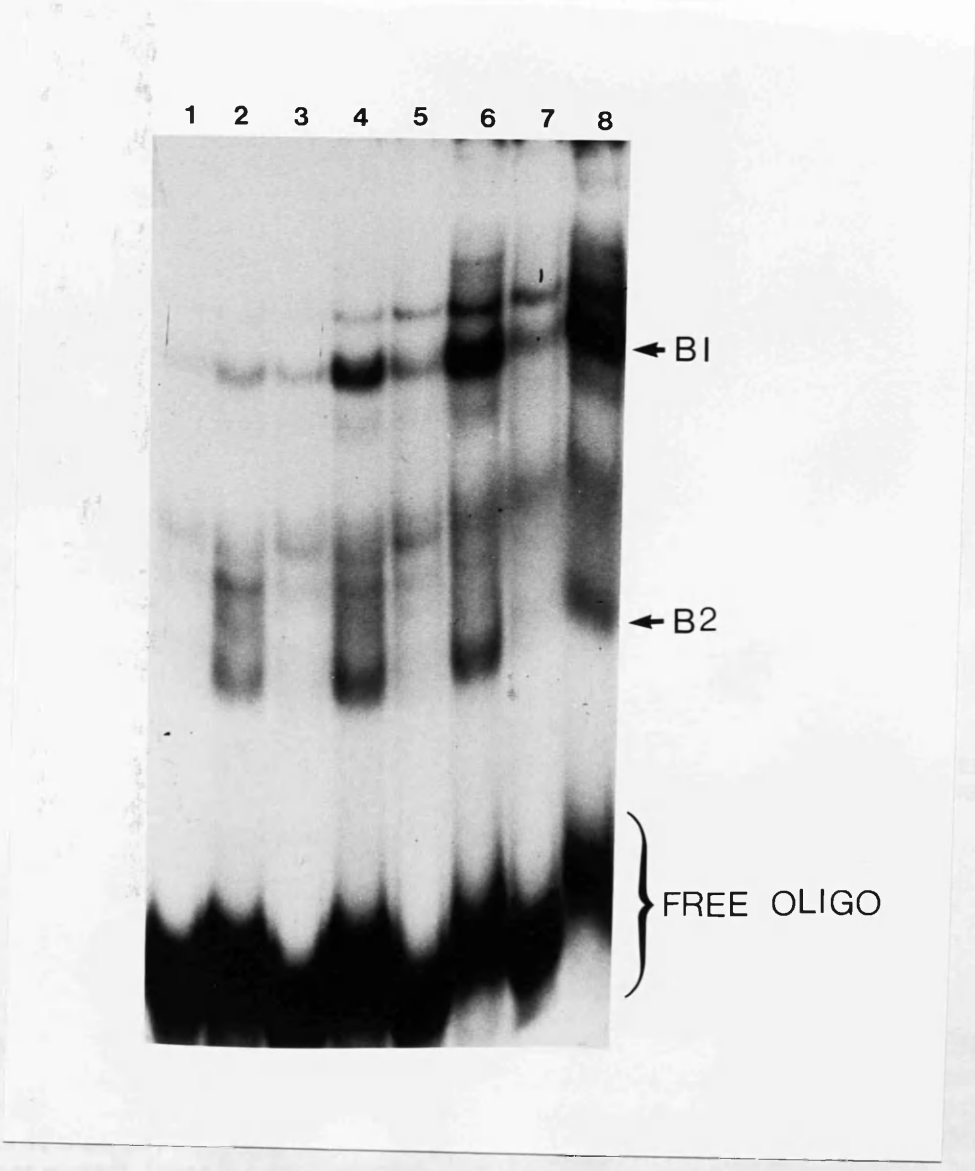
Figure 3.5 shows that with increasing concentrations of Hela extract an increase in the binding of protein complex B1 to the platinated 2αG7 occurs (lanes 2, 4, 6, 8), however only at the two lowest concentration is a difference seen with band B2 (lanes 2, 4). If increasing concentrations of extract are incubated with unplatinated 2αG7 (lanes 1, 3, 5, 7) there is little difference in band B1, although a slight increase may be seen but almost negligible in comparison to that seen with the platinated 2αG7. This therefore suggests that the protein represented in band B1 is limiting within the extract whereas the protein complex represented by band B2 is in excess at concentrations greater than 0.5μg of extract. The concentration of Hela extract used in the previous set of experiments (**Figure 3.4**), 1μg, therefore allowed one to detect actual changes occurring in the binding of proteins to the platinated oligonucleotide. This knowledge also allowed the following competition experiments described in the next section to be analysed.

3.5 Competition experiments of the DDRPs in the gel shift system.

The increased binding of complexes from Hela cells to platinated 2αG7 compared to unplatinated 2αG7 is shown in **Figure 3.6** (lanes 3 and 4). Coincubation of calf thymus DNA with CDDP-2αG7 and Hela extract has no effect on the binding of the complexes, **Figure 3.6** (lanes 5-7). However coincubation with CDDP treated calf thymus DNA shows a dose dependant decrease in the complex represented by band 1. Band 2 completely disappears upon incubation with even the lowest concentration of CDDP treated calf thymus DNA (Figure 3.6, lanes 8-10). ICPM spectrometery of the CDDP adducts in calf thymus DNA, **Figure 3.7**, showed it to have a platinum to nucleotide ratio of 1.5×10^{-3} and for more than 80% of the population to consist of dinucleotide platinum crosslinks with the d(GpG) to be the main lesion.

Figure 3.5

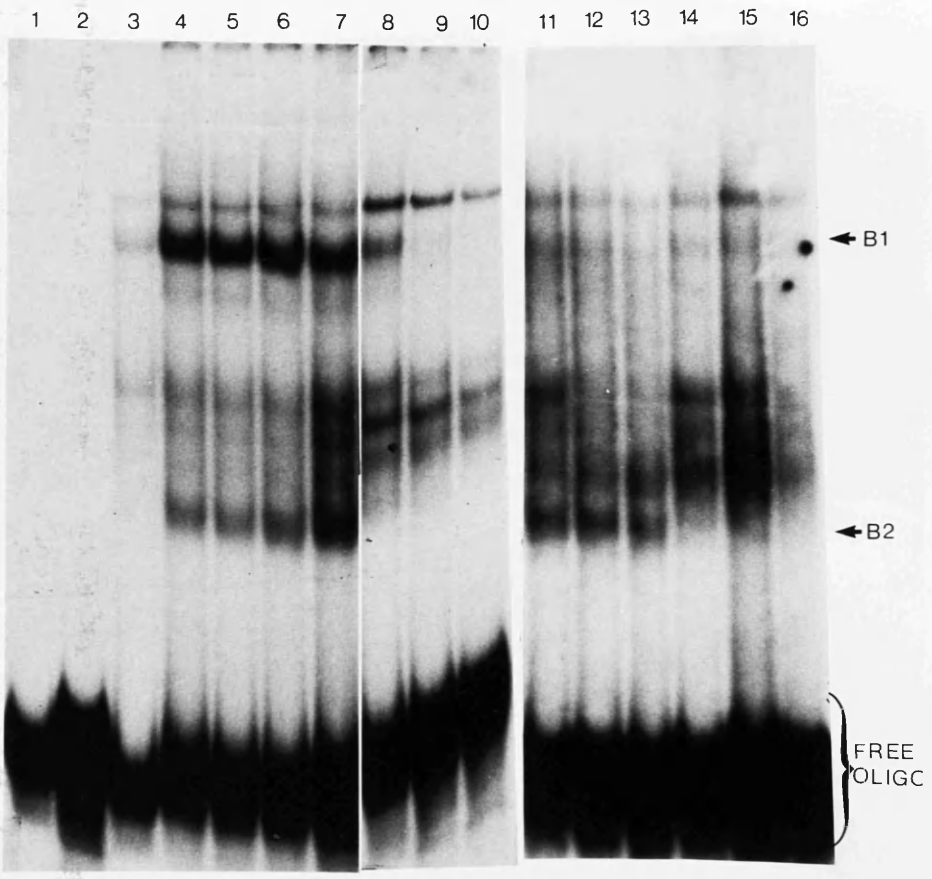
Gel mobility shift assay of 2αG7 with increasing concentrations of Hela nuclear extract



^{32}P labelled non-CDDP and CDDP treated 2αG7 incubated with increasing concentrations of Hela nuclear extract in binding conditions as section 2.5.1. Lanes 1, 3, 5, 7 non-CDDP 2αG7 incubated with 0.1, 0.5, 1, 2 μg Hela nuclear extract respectively. Lanes 2, 4, 6, 8 CDDP treated 2αG7 incubated with 0.1, 0.5, 1, 2 μg Hela nuclear extract. Nuclear extract prepared as by section 2.3.3.1) CDDP treatment of oligonucleotide at a concentration of 400μg/ml CDDP. Retardation complexes represented by bands B1 and B2.

Figure 3.6

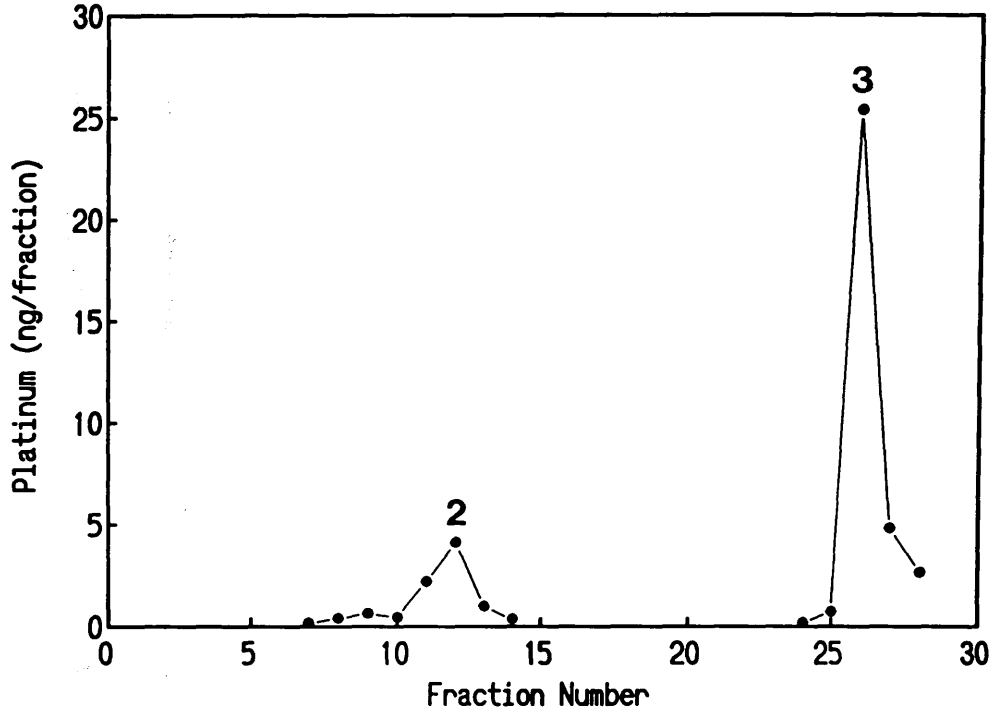
Competition of binding complexes detected in gel mobility shift assay



Coincubation of the complexes with calf thymus DNA ± CDDP. ^{32}P labelled CDDP-2αG7 and untreated 2αG7 were incubated with 1μg of Hela nuclear extract (binding conditions, nuclear extraction method and CDDP treatment as in previous figures). Lanes 1, 3 2αG7, lanes 2,4-16 CDDP 2αG7. Lanes 3-16 incubation with 1μg Hela nuclear extract, lanes 5-7 coincubation with 2.5, 7.5,12.5 μg calf thymus DNA, lanes 8-10 coincubation with 2.5, 7.5, 12.5 μg CDDP treated calf thymus DNA (representing an excess of 1600x, 4800x, 8000x CDDP adducts).
Lanes 11-13 coincubation with 0.05, 0.15, 0.25μg 2αG7 respectively, lanes 14-16 coincubation with 0.05, 0.15, 0.25μg CDDP-2αG7. (representing a 1300x, 3900x, 6500x Molar excess). Specific retardation complexes represented by bands B1 and B2.

Figure 3.7

Platinum detected in HPLC fractions of treated calf thymus DNA digest



Calf thymus DNA is treated at a drug : nucleotide ratio of 0.08 with CDDP. It is then digested to the nucleotide level by treatment with DNAse I and Nuclease P1, HPLC purified and platinum detected within the fractions by ICPMS. The standards used for the CDDP adducts were characterised by NMR and were obtained from Fichtinger-Schepman. This work was done in collaboration with Dr.D.Bissett, Dept.Medical Oncology.

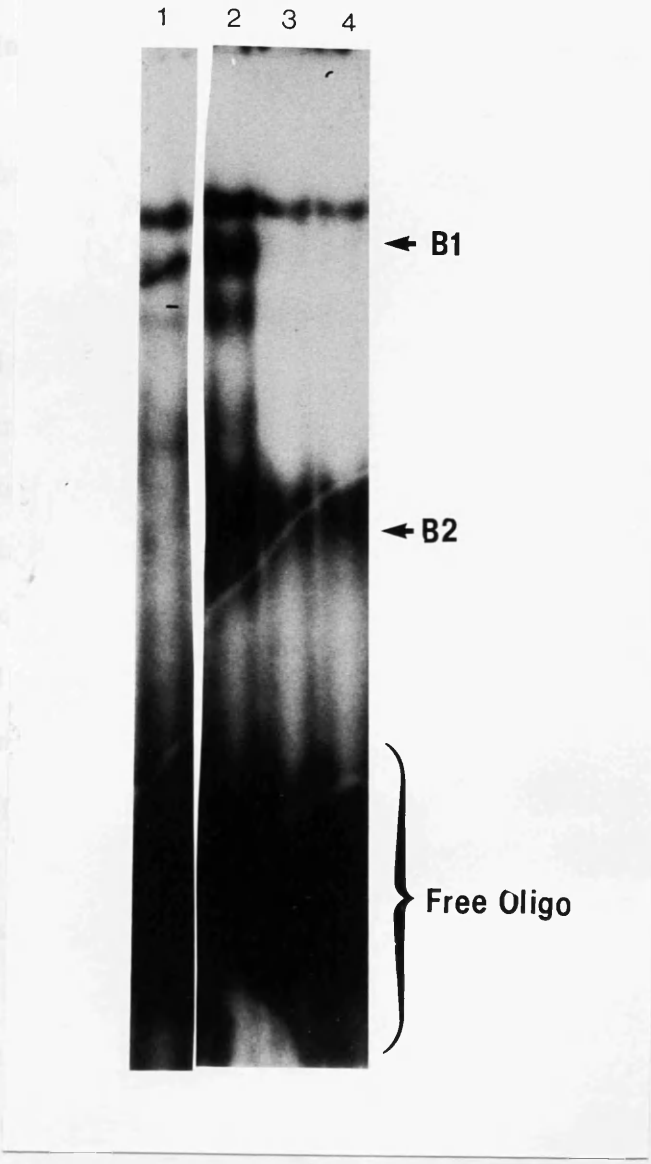
Therefore an excess of at least 3900 fold of CDDP adducts is required to compete for binding to band B1 and less than a 1600 fold excess of CDDP adducts to compete for band B2. At most a 1300 fold Molar excess of CDDP treated oligonucleotide is required for competition of Band B2 whilst at least a 1300 fold Molar excess of unplatinated oligonucleotide competes for binding to band B1. Thus binding of the DDRPs to the CDDP-2αG7 can be competed with DNA of unrelated sequence containing CDDP adducts, showing that the complex formation is independent of the DNA sequence used. Coincubation with an excess of 2αG7 competes for binding of the protein complex represented in band B1 but not of the protein complex represented in band B2 (lanes 11-13). However coincubation with a cold excess of CDDP-2αG7 competes for the binding in band B2 (lanes 14-16).It therefore can be concluded that binding in the complex B1 can be competed for with an excess of platinated calf thymus DNA, platinated oligonucleotide, unplatinated oligonucleotide but not with unplatinated calf thymus DNA. The binding in B2 can however only be competed for with platinated forms of calf thymus DNA or oligonucleotide.

3.6 DDRPs recognising single-stranded DNA

CDDP is known to bind to DNA and generate areas of local base pair disruption around the adduct. It therefore may be hypothesized that DDRPs could also recognise single-stranded DNA. To investigate this hypothesis, coincubation of single stranded M13 with CDDP-2αG7 and Hela was carried out, **(Figure 3.8)**. This shows that, upon coincubation with single stranded M13 within the reaction, there is competition of band B1, but not band B2. This therefore suggests that the protein complex is recognising single strands of oligonucleotide or single-stranded areas generated around the CDDP adduct.

Figure 3.8

Competition of binding complexes B1 and B2 with single stranded DNA



^{32}P labelled non-CDDP and CDDP 2αG7 coincubated with single-stranded DNA. Lane 1, non-CDDP 2αG7, lanes 2, 3, 4 CDDP 2αG7. All lanes incubated with 1μg A2780. Lanes 3, 4 coincubated with 0.2μg M13 and 0.4μg M13 respectively. Conditions as described in section 2.5.1.

The binding of non-specific retardation complexes observed in both the unplatinated and platinated oligonucleotides are not competed by the CDDP treated calf thymus DNA, nor by the platinated 2αG7. For a further investigation and discussion of single stranded binding proteins as DDRPs and their involvement in excision repair refer to chapter 4.

3.7 Binding complexes are conserved across mammalian species

The gel purified trimers which were analysed by denaturing gel analysis, were used in gel mobility shift assays with nuclear extracts from different species, **(Figure 3.9)**. CDDP-DNA binding complexes are observed after incubation with the CDDP treated trimer oligonucleotide with human (Hela and ovarian cell lines), murine (Mouse Erythroid Leukemia cells) and feline (T cell leukemia) nuclear extracts. The main complexes, bands B1 and B2 are seen with all extracts, however additional, less strongly binding complexes, are observed with some of the extracts. In the mouse erythroid leukemia cell extract a new complex is detected which binds to both the platinated and unplatinated forms of the oligonucleotide sequence. The oligonucleotide as described in section 3.1 is a mutated form of a sequence which is important in binding an erythroid specific nuclear factor. This sequence has been shown to weakly bind the factor (Plumb et al, 1989). The new complex observed could be a representation of the binding of this erythroid factor.

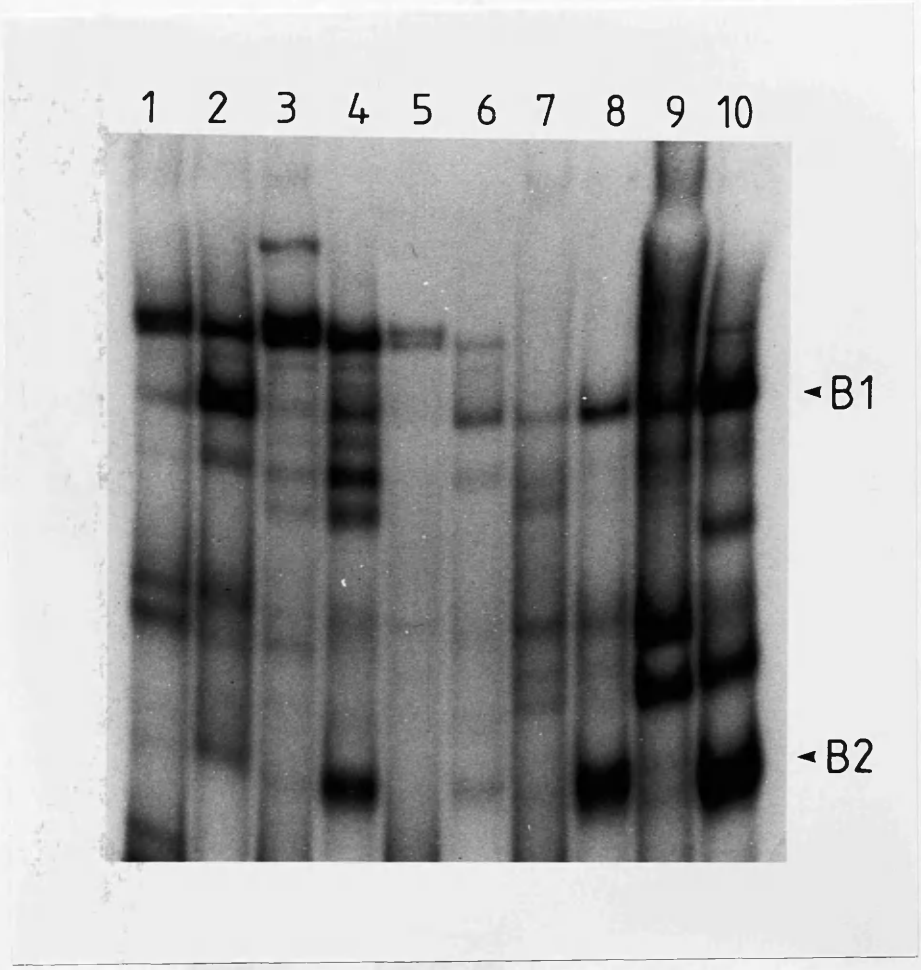
It therefore can be concluded that binding of complexes represented by bands B1 and B2 can be demonstrated in different species therefore suggesting that these protein complexes which recognise the CDDP damage may be important in a variety of different species.

3.8 Comparison of binding complexes in Hela and XPE

Chu and Chang (1988), identified two factors which represent binding to UV damaged DNA. Binding of both of these factors was shown to be competed for with CDDP damaged DNA therefore the question arises of the importance of these factors in recognition of CDDP damage. It was also reported by Chu and Chang that these two damage binding factors are both absent in Xeroderma complementation group E (XPE) cell extracts. To compare the damage recognition proteins detected by Chu and Chang with those identified in this chapter, experiments were performed using the gel mobility shift assay with CDDP-2αG7. **Figure 3.10** shows that there is no difference in the binding factors B1 and B2 in comparison of the Hela cell extract with the XPE cell extract. It therefore can be assumed that the proteins being detected in the gel shift assay by the platinated 2αG7 are different from those binding factors which recognise the UV damage, (Chu and Chang, 1988).

Figure 3.9

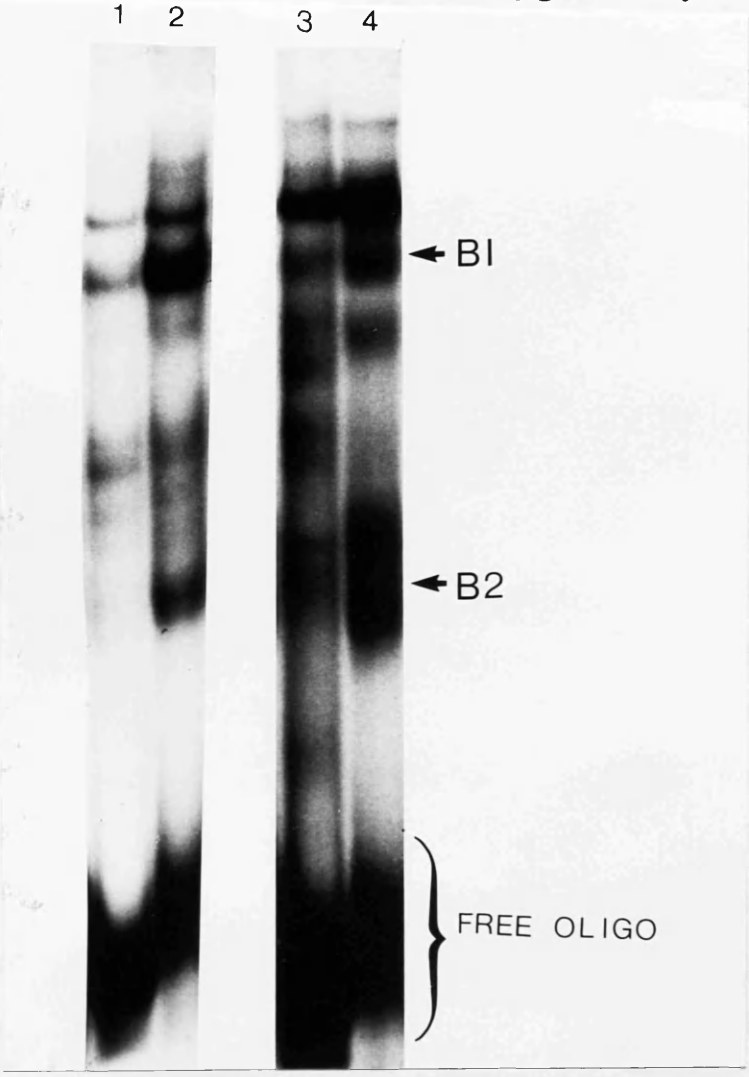
Detection of complexes B1 and B2 across different mammalian species



^{32}P labelled CDDP-2αG7 and 2αG7 incubated with different mammalian extracts in conditions described in 2.5.1. Lanes 1, 3, 5, 7, 9 2αG7, lanes 2, 4, 6, 8, 10 CDDP-2αG7. Lanes 1 and 2 incubated with 1μg Hela, lanes 3 and 4 1μg A2780, lanes 5 and 6 1μg Ov1, lanes 7 and 8 1μg Feline T cell leukemia, lanes 9 and 10 1μg mouse erythroid leukemia. All of the extracts used were prepared by the nuclear extract method as in section 2.3.3.1. Specific retardation complexes represented by bands B1 and B2.

Figure 3.10

Comparison of Hela with XPE nuclear extracts by gel mobility shift assay



^{32}P labelled 2αG7 and CDDP-2αG7 incubated with either Hela or XPE nuclear extract (conditions as described in section 2.5.1). Lanes 1 and 3 2αG7, lanes 2 and 4 CDDP 2αG7. Lanes 1 and 2 incubation with 1μg Hela nuclear extract, lanes 3 and 4 incubation with 1μg XPE nuclear extract. Specific retardation complexes represented by bands B1 and B2.

3.9 DISCUSSION

The work carried out in this chapter has attempted to illustrate the presence of mammalian protein(s) that binds specifically to DNA modified with the antitumour drug CDDP. The identification of DDRPs recognising CDDP damage in this chapter is by gel mobility shift assay. An oligonucleotide was chosen which has a sequence with two areas of three guanines. CDDP has been reported to bind to DNA forming adducts preferentially at N7 positions of guanine nucleotides, (Fichtinger and Schepman et al, 1985), therefore, the sequence of guanines within the oligonucleotide should provide an excellent target for CDDP adduct formation. The major DNA adducts of CDDP reported in the literature are d(GpG) and d(ApG) 1,2 intrastrand crosslinks, (Sherman and Lippard, 1987), representing 65% and 25% of adducts respectively. The remaining adducts are made up of 6% GNG intrastrand crosslinks (N representing any base) and the final 4% of DNA interstrand crosslinks, DNA protein crosslinks and monofunctional adducts, (Eastman, 1986). In the quantification of adducts by Eastman it is observed that as few as 4% of the total adducts contain DNA interstrand cross-links, DNA protein cross-links and monofunctional adducts. However, upon comparison of these results with the adducts formed in the 2αG7 oligonucleotide, ICPM spectroscopy shows approximately 25% of the adducts are in the monofunctional form. It has been suggested as a model, that for the formation of CDDP cross-links the reaction must go through a two step process where there is a fast formation of CDDP-DNA monoadduct followed by slow closure to a bidentate adduct over 6-12 hours (Eastman, 1986). In Eastman's Protocol, CDDP treatment is for a period of 16 hours at 37^0C. This time period should therefore give time for any monofunctional adducts being converted to bidentate adducts. The CDDP treatment followed in the experiments described in this chapter is a 1 hour CDDP treatment prior to quenching the formed adducts with NH_4HCO_3. This shorter period of CDDP treatment is therefore likely to result in a larger majority of adducts

being in the monofunctional form. This could therefore explain the discrepancies in the amount of monoadducts reported in the literature to that observed in this chapter. The cell may contain DDRPs which recognise monofunctional adducts. This may be advantageous in the prevention of the cross-linking process which is thought to generate the cytotoxic lesion.

The αG7 oligonucleotide is a mutated form of the sequence which weakly binds an erythroid specific nuclear protein factor. As this factor is specific for erythroid cells it should not cause any problem in detection of the DDRPs by masking their binding when analysed in different cell extracts. The αG7 oligonucleotide was chosen to be used in the identification of the CDDP DDRP entirely for the sequence which contained a run of guanines and also the unplatinated form of the oligonucleotide showed little non-specific binding in the gel shift assay. The choice of an oligonucleotide which binds an erythroid factor even weakly, may cause difficulties if the gel shift assay was to be extended into analysis of blood and tumour samples. The binding of the erythroid factor in these samples may mask differences in detection by the platinated oligonucleotide.

Using denaturing polyacrylamide gel electrophoresis, it can be seen that with increasing concentration of CDDP treatment, the 27 base pair labelled oligonucleotide begins to shift to slower migrating forms. These forms migrate to positions consistent with intrastrand cross-links. It has been suggested that 1,2 intrastrand crosslinks formed by CDDP may be responsible for the chemotherapeutic effectiveness, since trans-DDP the ineffective chemotherapeutically trans isomer, does not form this type of cross-link, (Pinto and Lippard, 1985). The concentration of CDDP which causes complete disappearance of the 27 base pair form of the oligonucleotide on a denaturing gel was chosen for subsequent experiments of DDRPs. In the platinated oligonucleotide population 90% consists of intrastrand and monofunctional adducts, whilst the remaining 10% migrates at a position consitent with interstrand cross-links.

The type of cross-links contained within the population of platinated oligonucleotide have been determined in the 2αG7 oligonucleotide. ICPMS analysis of the platinated 54 base pair oligonucleotide 2αG7 shows the majority of adducts to be formed between guanine-guanine and adenine-guanine nucleotides. Previous reports have suggested that approximately 90% of the total platinum in the DNA is linked to N7 atoms of the nucleobases adenine and guanine in the form of intrastrand cross-links, (Fichtinger-Schepman et al, 1985). By comparing the analysis of the CDDP treatment by the denaturing gel analysis, approximately 90% of the forms are observed to migrate to a position consistent with intrastrand cross-links and monofunctional adducts. It can therefore be postulated that although ICPM spectrometry cannot differentiate between PtG-G intrastrand and interstrand cross-links, the major peak represents a population mainly of PtG-G intrastrand cross-links. Normally upon reaction of CDDP *in vitro* with DNA, 90% of the adducts prove to be intrastrand with a small percentage being monofunctional in character. However as can be seen by ICPMS the CDDP treatment protocol used in this work leaves a larger than normal proportion of the adducts in the monofunctional form. Comparing the ICPMS trace of the platinated 2αG7 with that of the ICPMS trace of the platinated calf thymus DNA used in competition experiments, there is an extensive difference in the amount of monofunctional adducts formed. The protocol used for CDDP treatment of the calf thymus DNA was treatment for 4 hours in comparison to the 1 hour treatment for the 2αG7 oligonucleotide. The protocol in the calf thymus DNA gave a longer time for bidentate adducts to form in comparison to the oligonucleotide treatment. To date there is no direct evidence to which particular adduct forms the important cytotoxic lesion, although at present the literature favours the 1,2 intrastrand crosslink.

The CDDP treated oligonucleotide (2αG7) was employed in a gel shift assay and differences in gel shift were seen comparing platinated 2αG7 to unplatinated 2αG7. At present the differences which were observed (bands B1 and B2) have not been shown

to be specific to any one type of crosslink. Recent work has shown a protein complex binding to oligonucleotides which have specific 1,2 intrastrand d(GpG) and d(ApG) crosslinks. The proteins do not bind to 1,3 intrastrand crosslinks nor to interstrand crosslinks (Donahue et al, 1990). This suggests that DDRPs are present in mammalian cells which recognise and bind to what is presumed to be the important cytotoxic lesion formed by CDDP. It would be of use to take the 2αG7 sequence and generate specific crosslinks and then use these to characterise which type of cross-link the protein complex is recognising. Introduced in chapter 1, antibodies have now become available to the different CDDP-DNA adducts (Fichtinger-Schepman et al, 1985; Reed et al, 1982). These may help to provide a method of understanding, if introduced into the gel shift system, which adducts the DDRPs are recognising. The platinated 2αG7 sequence has many more monofunctional adducts than normally reported for CDDP treated DNA. The formation of the complexes in the gel shift assay could be due not only to the binding to the intrastrand cross-links but also to the monofunctional adducts prior to the formation of the intrastrand cross-links. Further experiments to investigate the importance of the monofunctional adducts would be to compare a time course of CDDP treatment prior to quenching the adducts in their monofunctional form. The increase in time of treatment of the oligonucleotide will allow more of the monofunctional forms to progress to their cross-link forms and so each point of time could be examined in the gel shift assay. If the DDRPs are involved in excision repair, the gel shift assay may be looking at a step occurring in the cell prior to any incision steps, where the cell detects the damage of the CDDP in the monofunctional form before the adduct progresses towards cross-link formation. This early recognition step could be beneficial to the cell as a means of removing any damage to the DNA before it forms cross-links which may cause more damage to the cell as a whole.

Studies of the DDRPs with the platinated 27 base pair αG7 did not show any difference in the gel shift therefore suggesting that protein complexes were not binding to

the damaged DNA. If however the protein complexes being assayed were proteins of a scanning nature i.e binding to double-stranded DNA and moving its way along the DNA duplex until it reaches the site of damage, then it may be expected that the protein has a requirement for a stable duplex of DNA. CDDP being such a large bulky adduct, when reacted *in vitro* with the 27 base pair oligonucleotide will cause disruption of the duplex and hence if the protein present within cells is a scanning protein then it will not have a chance to bind to the DNA and hence recognition of the damage will not occur. This hypothesis was therefore tested by taking CDDP treated oligonucleotide, end label and ligating it to an excess of cold non-phosphorylated αG7. The different forms of the oligonucleotide were gel purified and used in a gel shift assay. A difference in gel shift patterns can only be seen in the 54 base pair and 81 base pair platinated forms of the oligonucleotide. No difference can be seen in binding, comparing the 27 bp unplatinated oligonucleotide to the 27bp platinated. This therefore suggests that the DDRPs do require a stable duplex of DNA for binding to occur. The size dependency of protein complexes binding to oligonucleotides has been reported previously, (Donahue et al, 1990). They found that binding of their identified damage recognition protein occurred only to oligonucleotides 88 or 110 bp in length but not to probes that were 44 or 66 bp in length. Other groups who have detected CDDP damage recognition proteins have used DNA sequences of greater than 100 base pairs,(Chu and Chang, 1988, Chao et al, 1991[a]).

Competition experiments with platinated DNA of unrelated sequence were carried out to show that the binding of the DDRPs were independent of the platinated DNA sequence. It is observed that coincubation of the extract and platinated oligonucleotide with calf thymus DNA does not affect the binding of bands B1 and B2, whereas coincubation with platinated calf thymus DNA competes out the binding of band B1 in a dose dependant manner and competes band B2 completely at the lowest concentration of the platinated calf thymus DNA. This therefore suggests that the binding of complexes B1 and B2 is independent of DNA sequence. Although bands B1

and B2 are competed with platinated calf thymus DNA and not unplatinated calf thymus DNA, it cannot be presumed that the DDRPs are specific for CDDP damage. It is more likely that a cell will have evolved factor(s) which recognise different types of damage. It would be expected for the cell to contain repair protein(s) which will recognise specific classes of damage, such as a distortion in the DNA. It is therefore necessary to carry out cross competition experiments with DNA damaged by different agents such as ultraviolet radiation, mitomycin C, before any specificity of the DDRPs can be concluded.

If however, competition experiments are carried out using the sequence of 2αG7, one sees that coincubation with unplatinated DNA competes for the binding in band B1, whereas coincubation with platinated DNA competes for the binding in both B1 and B2. CDDP treatment of DNA has been reported to cause base pair disruption around the adduct and hence generate single-stranded areas around the adducts, (Sundquist et al, 1986). Competition experiments using either single-stranded 2αG7 or single-stranded M13 as the competitor competes out band B1. Therefore the competition of band B1 by single-stranded oligonucleotides and also by double stranded 2αG7 suggests that the protein complex recognises single-stranded regions, as well as having an affinity for double-stranded DNA. The discrepancy however in the hypothesis is if band B1 has an affinity for double stranded DNA, why is it not competed out by calf thymus DNA. One explanation for this could be due to the number of ends present in calf thymus DNA compared to double stranded 2αG7. Calf thymus DNA has very large fragments of DNA present in its population compared to 2αG7, therefore the number of free ends within the calf thymus DNA will be much less than that within the 2αG7. If the protein complex has an affinity to bind to free ends then it is likely that it will be competed out much easier with 2αG7 than calf thymus DNA. It therefore can be suggested from this set of experiments that band B2 may represent a protein complex recognising a type of damage formed by CDDP, whilst band B1 may represent a single-stranded binding protein being detected. Evidence exists to suggest that single-stranded binding (SSB) proteins may be

involved in the recognition of damage caused by certain DNA damaging agents. Toulme has suggested a role for SSB proteins in the damage recognition step on the basis of binding of gene 32 protein from bacteriophage T4 to DNA that has been chemically modified with carcinogens or antitumour agents. They found that gene 32 protein shows a high specificity for single-stranded DNA and binds more efficiently to DNA damaged with agents which cause structural changes to the duplex. The efficient binding is related to the formation of locally unpaired regions which are strong binding sites for the SSBs, (Toulme et al, 1983). Recently evidence has been presented that a mammalian DNA replication protein, human SSB is also involved in repair. Coverley et al (1991), showed that by using a cell free system that can carry out nucleotide excision repair *in vitro*, (Wood et al, 1988), addition of human SSB stimulated repair synthesis. Human SSB is a multi-subunit protein containing polypeptides of 70, 34, and 14KD. Antibodies to the sub-units 70 and 34KD have been isolated, (Kenny et al, 1990). Preliminary experiments in the gel shift system described in this chapter, with the monoclonal antibodies to the various subunits of the SSBs, have demonstrated that by including in the incubation the antibodies to the single-stranded binding proteins the binding of the complex represented by band B1 is abolished but no change to band B2 occurs, (personal communication, Dr.C.Clugston, Department of Medical Oncology, University of Glasgow). This is therefore strong evidence to suggest that the complex identified in band B1 is binding to a single-stranded form of the oligonucleotide. A further discussion of single-stranded binding proteins and their involvement in excision repair will be included in chapter 4.

Similar work in the identification of DDRPs has been carried out by several groups to identify proteins which bind to ultra-violet (UV) damage in DNA, (Chao et al, 1991[a], Chu and Chang, 1988, Patterson and Chu, 1989). UV as well as other agents such as mitomycin C (MMC) and CDDP all cause bulky adduct damage. It would therefore seem more economical for a cell to evolve a protein complex to deal with various types

various types of damage. It would be of interest to test whether bands B1 and B2 which were detected by platinated 2αG7 are competed out with the various types of damage which cause bulky adduct formation. It is more likely that the damage recognition proteins within a cell recognises a structural conformation common to these various types of damage.

As discussed earlier, various groups are working on proteins recognising CDDP adducts. It is therefore important to compare results. Chu and Chang (1988), found a difference in a damage binding protein in Hela extracts in comparison to XPE extracts, they found their DRP to be absent in XPE cells. Cell lines derived from Xeroderma Pigmentosum (XP) patients are deficient in repair of DNA lesions induced by many types of DNA damaging agents including CDDP. XPE is simply one of, at least, eight complementation groups. Before any conclusions can be made about the XPE cell line being hypersensitive to the damaging agents because of the absence of the XPE binding factor, experiments in which the exogenous introduction of a single gene coding for this factor, corrects the DNA repair defect in intact cells, will have to be shown. The complexes described in these reports are detected by UV damaged probe and binding competed for with CDDP damaged DNA. It was therefore suggested that these proteins recognise both UV damage and CDDP damage. To provide evidence that retarded complexes represented as bands B1 and B2 in this chapter are different from Chu and Chang's binding factor, studies in comparing XPE cell extracts with Hela cell extracts are presented. It is shown that complexes B1 and B2 are present in both cell extracts, therefore suggesting that the proteins being detected by the CDDP treated 2αG7 are different from those detected by Chu and Chang.

The DDRPs if important in biological systems in the recognition of damage to DNA, would be expected to be present within different mammalian species. It can be seen in the extracts tested in this chapter that the DDRPs are present in mouse, cat and human extracts. However as well as bands B1 and B2 being detected other binding

complexes are shown to bind to both the platinated and unplatinated forms of the oligonucleotide. These complexes generally represent non-specific binding complexes present in the extract. Detected within the mouse erythroleukemia extract, with both platinated and unplatinated oligonucleotide, is the binding factor which recognises the GATAAG sequence present as a mutated form in the oligonucleotide sequence. The DDRPs are therefore present within all the extracts tested and suggests that they may be ubiquitous to all mammalian cells.

In summary the experiments described in this chapter have led to the characterisation of mammalian cellular proteins which recognise and bind to DNA containing CDDP adducts. The DDRPs are shown to bind independently of sequence. They have also been shown to bind to various mammalian extracts hence suggesting that they may be ubiquitous to all mammalian extracts. It has been suggested that one of the protein complexes identified may represent a single-stranded binding protein and this is discussed with its involvement in excision repair.

CHAPTER 4

INCREASED DAMAGE RECOGNITION PROTEINS IN AN OVARIAN CELL LINE RESISTANT TO CDDP - IDENTIFICATION BY SOUTH-WESTERN ANALYSIS

4.1 INTRODUCTION

As described in the previous chapter, proteins recognising DNA damaged by CDDP have been identified in different mammalian cell extracts. These proteins may be involved in the recognition of damage prior to recruitment of other DNA repair enzymes to remove the damage. They could also be involved in other aspects of DNA metabolism affecting toxicity of CDDP. If the DDRPs are involved in how the cell handles CDDP damage and since DNA repair has been implicated as a mechanism of CDDP resistance *in vitro*, it would be reasonable to examine if any changes occur in the DDRPs of CDDP resistant tumour cell lines.

During the progression of the work presented in this chapter, evidence was reported by two different groups to suggest that CDDP resistant cells express increased levels of a factor that recognises UV damaged DNA. By use of the gel mobility shift assay, it was reported that the factor which had been previously found to be deficient in XPE extracts, denoted as XPE binding factor, (Chu and Chang, 1988), was found to be increased in two independent cell lines resistant to CDDP, (Chu and Chang, 1990). In a separate study it was shown that a factor which recognises DNA damaged by ultra-violet radiation was increased in a CDDP resistant Hela cell line, (Chao et al, 1991[a]). Both the studies presented an increase in a protein present in CDDP resistant cell lines, which had previously shown enhanced levels of DNA repair. Both studies were carried out using gel shift assays. The gel shift assay has limitations in that the gel system used will detect protein(s) binding to a factor, but it will not differentiate between proteins binding as a complex to binding as a single protein species. The gel shift system allows one therefore to identify a factor which may represent various proteins acting within a complex to recognise the damaged DNA. The South-Western technique described below allows one to identify the specific proteins involved in the recognition of the damaged DNA.

The South-Western technique was first defined by Bowen et al, (1980), in the detection of DNA-binding proteins. The method involves three steps:

(1) separation of proteins by gel electrophoresis in a SDS polyacrylamide gel system.

(2) transfer of the separated proteins to a nitrocellulose filter.

(3) analysis of the adsorbed proteins by incubating the filter with a defined labelled probe. In the case of the work to follow in this chapter, γ^{32}P ATP CDDP treated 2αG7 was used as the probe in the detection of proteins recognising DNA damaged with CDDP.

The oligonucleotide 2αG7 was chosen as the sequence to be used in the detection of the DDRPs for similar reasons to those described in detail in chapter 3:

(1) sequence contains guanines to which evidence has shown that CDDP binds preferentially to.

(2) the sequence shows little non-specific binding.

CDDP is known to bind to DNA and cause local denaturation around the adducts (Sundquist et al, 1986). DDRPs recognising CDDP damage may therefore recognise these areas of single-strandedness. Evidence in the literature has suggested a role for single-stranded binding proteins, known to be involved in DNA replication, in excision repair (Coverley et al, 1991). Single stranded binding proteins have been extensively studied in replication of the adenovirus, SV40 system. SV40 has long been regarded as an excellent model for mammalian chromosomes since all the replication machinery for SV40 replication is given by the host cell i.e the mammalian cell. The only part of the machinery not derived from the host is the SV40 large T antigen protein.

Initiation of SV40 DNA replication requires interaction of T-antigen with the origin of DNA replication, (for general review on DNA replication refer to Campbell, 1986). Human single stranded DNA binding protein (SSB) is found to be absolutely required for DNA replication. SSB is also known in the literature as RF-A and RP-A (Wold and Kelly, 1988, Fairman and Stillman, 1988). SSB consists of three polypeptides 70, 34, and 14KD. The 70KD subunit is found to bind to single-stranded DNA, whereas the other subunits do not. Antibodies have been generated towards the 70KD and 34KD subunits leading to the result that DNA unwinding and polymerase α and δ stimulation are required functions of human SSB in SV40 DNA replication, (Kenny et al, 1990). Recently there has been a report on the isolation of a DNA helicase that requires hSSB for activity (Soo Seo et al, 1991). By use of these antibodies in a cell free system that can carry out nucleotide excision repair, (Wood et al, 1988)., extensive inhibition of DNA repair of plasmid molecules damaged with ultraviolet light or acetylaminofluorene was observed, so suggesting a role for the SSB DNA replication protein in excision repair, (Coverley et al, 1991).

In the following chapter the identification of DDRPs recognising DNA damaged with CDDP are detected by South-Western analysis. Two out of the three identified DDRPs are found to be increased in a CDDP resistant human ovarian tumour cell line. Upon incubation of the South-Western blot of ovarian cell extracts, with single-stranded oligonucleotide 2αG7, proteins binding to the single-stranded DNA are observed which are of similar size to those detected with ds-CDDP-2αG7. These proteins were found to be increased in the CDDP resistant ovarian cell line extract, Ov1DDP. It is therefore discussed as to whether the DDRPs increased in the CDDP resistant cell line extract have an affinity for both single-strand and double-strand DNA.

4.2 RESULTS

4.2.1 Cell lines Investigated

CDDP is known to be effective in the treatment of human ovarian cancers, however the majority of patients with bulky ovarian cancer are not cured with standard CDDP regimes. The selection of subpopulations of resistant cells in the tumour has been suggested to account partly for chemotherapy treatment failure, (Ozols and Young, 1984). As a way to study mechanisms of drug resistance, cell lines have been generated from different tumours and resistance to certain drugs have been developed *in vitro*. Although this is not an ideal situation, it allows one to study the mechanisms of resistance *in vitro*. Mechanisms of resistance to CDDP have been reported from *in vitro* studies, (see section 1.8).

To investigate if DDRPs are involved in the development of resistance to CDDP, two independent human ovarian cancer cell lines, both their sensitive and resistant sublines were analysed. A2780, (Behrens et al, 1987), Ov1 originally named IGROV1, (Benard et al, 1985) are two independent human cell lines which were derived from ovarian carcinomas of two different untreated patients. CDDP resistant cell lines have been developed from both of these lines, denoted in this chapter as A2780CP and Ov1DDP, by exposing them to increasing concentrations of CDDP, (Behrens et al, 1987), (Teyssier et al, 1989). The sensitivities to CDDP has been confirmed by MTT assay and **Table 4.1** presents ID_{50} values for each cell line.

Table 4.1 ID_{50} OF OVARIAN TUMOUR CELL LINES

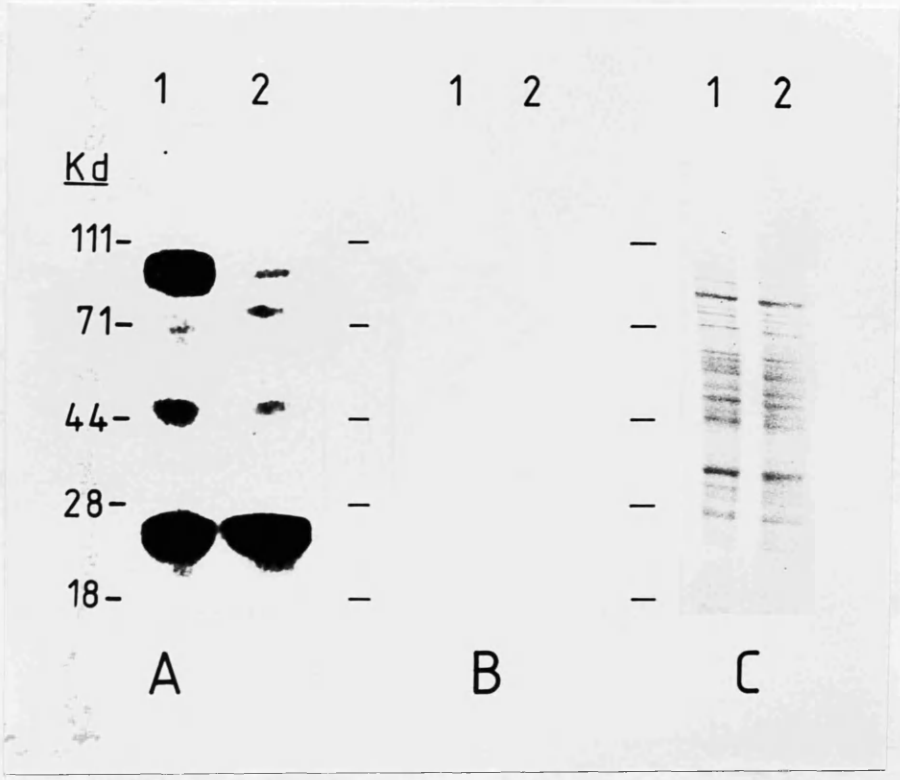
Cell line	ID_{50} (M)	Fold Resistance
A2780	2.4×10^{-7}	
A2780CP	5×10^{-6}	20
Ov1p	9.5×10^{-8}	
Ov1DDP	4×10^{-7}	4.2

4.2.2 Investigation of DDRPs in Ov1 and Ov1DDP by South-Western analysis

The probe used in the detection of the DDRPs is the 2αG7 oligonucleotide, CDDP treated, under identical conditions as that described in chapter 3. By incubating a South-Western blot of Ov1 and Ov1DDP probed with double stranded platinated 2αG7 (ds-CDDP-2αG7), three separate factors of approximate size 25, 50 and 100KD are identified (**Figure 4.1a**). The 25KD and 100KD factors have been observed each to separate into two species, (data not shown). To determine if the three factors are recognising the damaged DNA or if they are simply binding to the oligonucleotide, a South-Western blot of the extracts was probed with ds-2αG7, (**Figure 4.1b**) . It can be seen that binding does not occur to the labelled ds-2αG7 using different extract preparations of the cells. The binding of the three factors to the damaged DNA is destroyed by prior incubation of the extract with proteinase K suggesting that the ds-CDDP-2αG7 is recognised by three different proteins. The 100KD protein recognising the damaged DNA is increased to levels of almost 7 fold greater in the Ov1DDP resistant subline than in the Ov1p sensitive parental cell line (densitometry analysis).

Figure 4.1

South-Western analysis of CDDP resistant ovarian cell line extract for DDRPs recognising DNA damaged with CDDP



Cell free extracts (100μg total protein per lane) were separated on a 5-15% gradient SDS-polyacrylamide gel transferred to nitrocellulose and incubated with $\gamma^{32}P$ ATP labelled double-stranded oligonucleotide, 2αG7, both platinated and unplatinated in the presence of 3μg/ml poly (dI-dC): poly (dI-dC) and 0.25% non-fat milk proteins.

Lane1 : Ov1DDP, Lane 2, Ov1p

Panel A : blot incubated with CDDP treated double-stranded oligonucleotide 2αG7.
Panel B : blot incubated with unplatinated oligonucleotide 2αG7.
Panel C : Coomassie Blue stain of 40μg total protein on a SDS-PAGE gel.

Sizes in Kilodaltons estimated from molecular weight size markers.

The 50KD protein is also increased in the Ov1DDP cell line by values of 2 fold, (results by densitometry scan analysis).

Equivalent amounts of each protein extract from the different cell lines were analysed on the South-Western blot. Protein concentrations of the cell line extracts were estimated using the Biorad protein assay (section 2.4.1) which is based on the Bradford method, (Bradford, 1976). To be confident of these estimated protein concentrations a Coomassie Blue stained SDS-polyacrylamide gel of the cell extracts are visually examined to check the protein content. A Coomassie Blue stained gel is shown for equivalent concentrations of Ov1p and Ov1DDP, (**Figure 4.1c**). After transfer of the proteins from the gel to the nitrocellulose filter, the filter is stained with Ponceau-S (data not shown). This procedure allows one to identify equal transfer from the gel to the nitrocellulose filter. The increase in both the 50 and 100KD proteins in the Ov1DDP has consistently been found in independent prepared extracts.

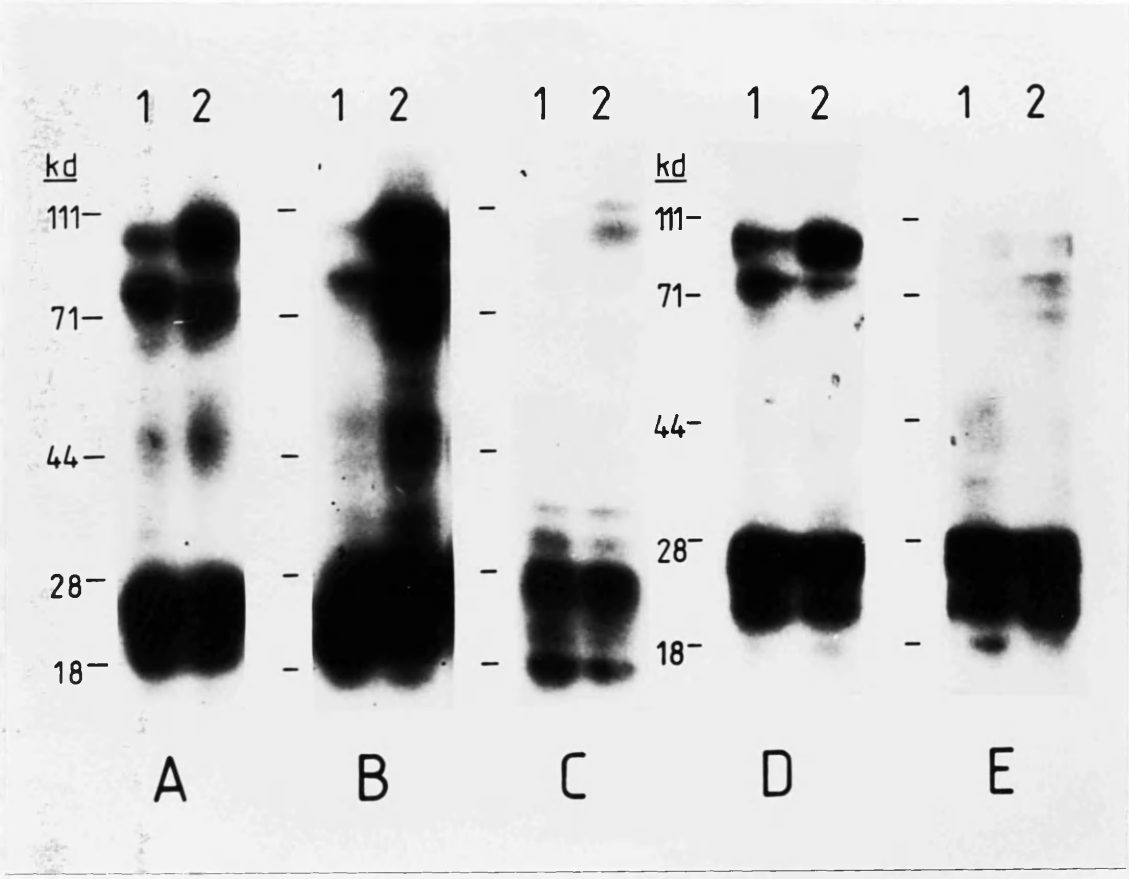
4.2.3 Competition experiments of the DDRPs in the South-Western Assay

The competition for binding of the DDRPs with a cold excess of unplatinated and platinated DNA can help to lead to an understanding in the binding specificity of the DDRPs. It is more difficult, however, to show that binding is competed in a dose dependent manner in the South-Western system as shown in the gel mobility shift system (**Figure 3.7**). These types of experiments should provide evidence that the proteins are binding to damaged DNA and are independent of the sequence used in their detection.

The DDRPs identified by incubating a South-Western blot of Ov1 and Ov1DDP with CDDP-ds-2αG7 are shown in **Figure 4.2a**. Again the increase in the 100KD and 50KD DDRPs is observed in the Ov1DDP cell extract. This particular South-Western analysis shows higher levels of a 70-80KD protein(s)

Figure 4.2

Competition of DDRPs with double -stranded DNA, platinated and unplatinated



South-Western blots were carried out under identical conditions as described in figure 4.1 and incubated with γ^{32}P ATP labelled double-stranded CDDP treated 2αG7 oligonucleotide in the presence of 3µg/ml poly (dI-dC): poly (dI-dC) and 0.25% non-fat milk proteins and coincubated with :

Panel A : no competitor.
Panel B : at least 2,400 fold excess of unplatinated double-stranded 2αG7.
Panel C : at least 2,400 fold excess of platinated double-stranded 2αG7.
Panel D : 2µg calf thymus DNA.
Panel E : 2µg platinated calf thymus DNA (representing at least a 2,000 fold excess of platinum DNA adducts.

Lane 1 Ov1p, Lane 2 Ov1DDP

Sizes in Kilodaltons estimated from molecular weight size markers.

than usually observed. This may represent modified or degraded forms of the 100KD protein. Coincubation of the blot with greater than 200 fold Molar excess of cold unplatinated double stranded 2αG7 has little effect on the binding of the DDRPs, **(Figure 4.2b)**. However competition for binding to the 25, 50, and 100KD DDRPs by the CDDP-ds-2αG7 occurs when the blot is coincubated with unlabelled CDDP-ds-2αG7, **(Figure 4.2c)** .

The binding of the CDDP-ds-2αG7 to the DDRPs cannot be competed for by an excess of ds-2αG7. However the binding can be competed for with an excess of CDDP-ds-2αG7. This suggests the identified DDRPs recognise DNA damaged with CDDP.

To determine if the binding of the DDRPs is independent of the sequence 2αG7 used, competition experiments were carried out using DNA of a totally unrelated sequence, calf thymus DNA, as a competitor for binding. The calf thymus DNA used in these experiments was from the same source used for the competition experiments described in chapter 3 (section 3.5). Coincubation of the blot with platinated calf thymus DNA, representing more than a 2000 fold excess of platinum adducts, competed for binding of the 50KD and 100KD DDRPs, **(Figure 4.2e)**. However coincubation with the same concentration of unplatinated calf thymus DNA did show some competition for binding to the 100KD and 50KD DDRPs, although competition for the 100KD DDRP was not as effective as that with platinated calf thymus DNA. Therefore, binding of CDDP-ds-2αG7 to the DDRPs can be competed more efficiently with CDDP treated DNA. Further, binding to the 50KD and 100KD proteins can be competed with an unrelated DNA sequence that contains platinum adducts. However the platinated calf thymus DNA is less efficient at competing for binding to the 25KD protein than the platinated 2αG7 double-stranded sequence.

4.2.4 DDRPs in Cell Extracts of Xeroderma pigmentosum Complementation Group E (XPE)

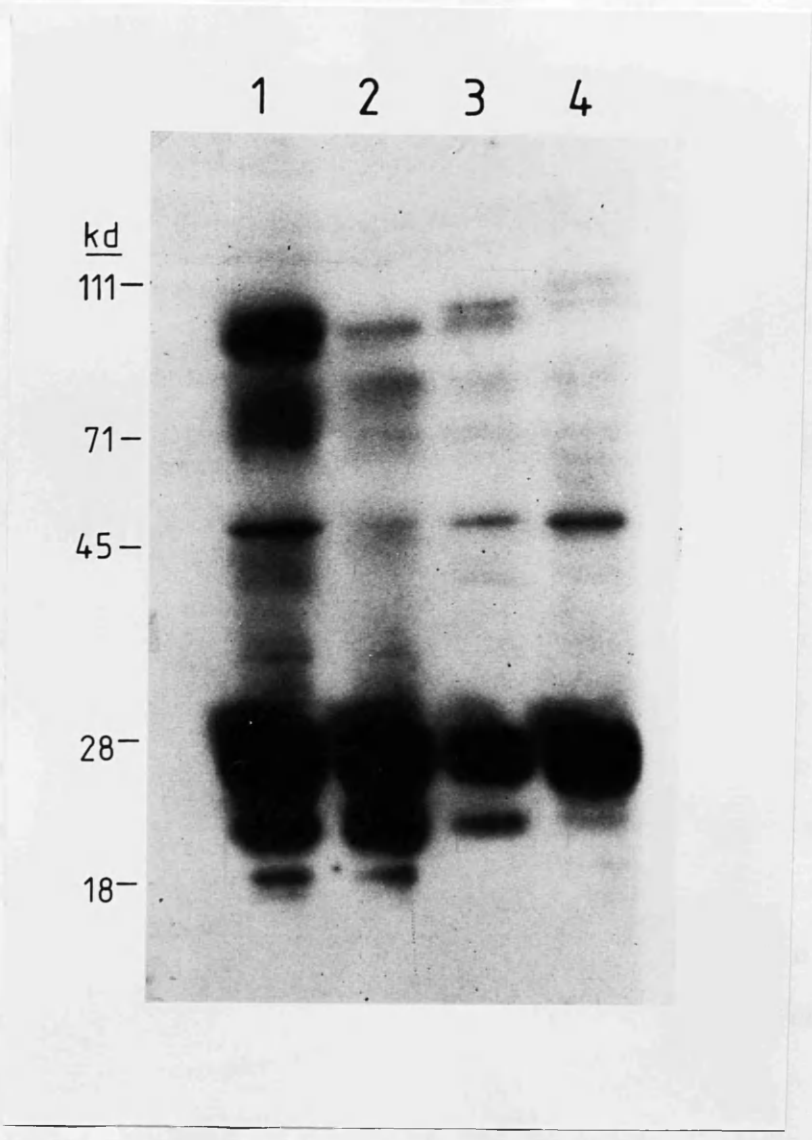
As introduced in chapter 3, a factor which binds to UV damaged probe DNA has been identified (Chu and Chang, 1988). Using gel mobility shift assays, it has been reported that the factor is found to be absent in cell extracts made from XPE. The factor is denoted XPE binding factor. The comparison was made with the XPE between Hela and other normal fibroblastic cell lines.

Using the same XPE cell line as used in these experiments, GM02415, the DDRPs of XPE cell extracts were compared to those in Hela and Ov1p and Ov1DDP cell lines, **(Figure 4.3)**, in a South-Western blot probed with ds-CDDP-2αG7. Three major DDRPs are present in the XPE cell extract of 25, 50 and 100KD. These do not appear to be different from those previously detected in the Ov1p and Ov1DDP cell extracts except they occur at different intensities. Hela cell extracts show DDRPs of molecular weight 25 and 50KD similar to the other extracts examined, but instead of a 100KD DDRP, factor(s) slightly larger are observed, (this result is repeatedly observed in different gels).

The deficiency of XPE binding factor in XPE cell extracts compared to Hela cell extracts was reported in gel shift assays. Presented in chapter 3 is the examination of DDRPs detected in the gel shift assay. Comparing the DDRPs binding to CDDP-ds-2αG7 of Hela with XPE cell extracts no detectable differences are observed, (section 3.8, fig.3.11).

Figure 4.3

South-Western analysis of DDRPs in cell extracts of XPE compared with Hela and ovarian tumour cell extracts



South-Western blot carried out under identical conditions as described in figure 4.1 and incubated with γ^{32}P ATP labelled CDDP treated double-stranded 2αG7 in presence of 3μg/ml poly (dI.dC): poly (dI.dC) and 0.25% non-fat milk protein.
Lane 1 Ov1DDP, Lane 2 Ov1p, Lane 3 XPE, Lane 4 Hela cell extracts.

Sizes in Kilodaltons estimated from molecular weight size markers.

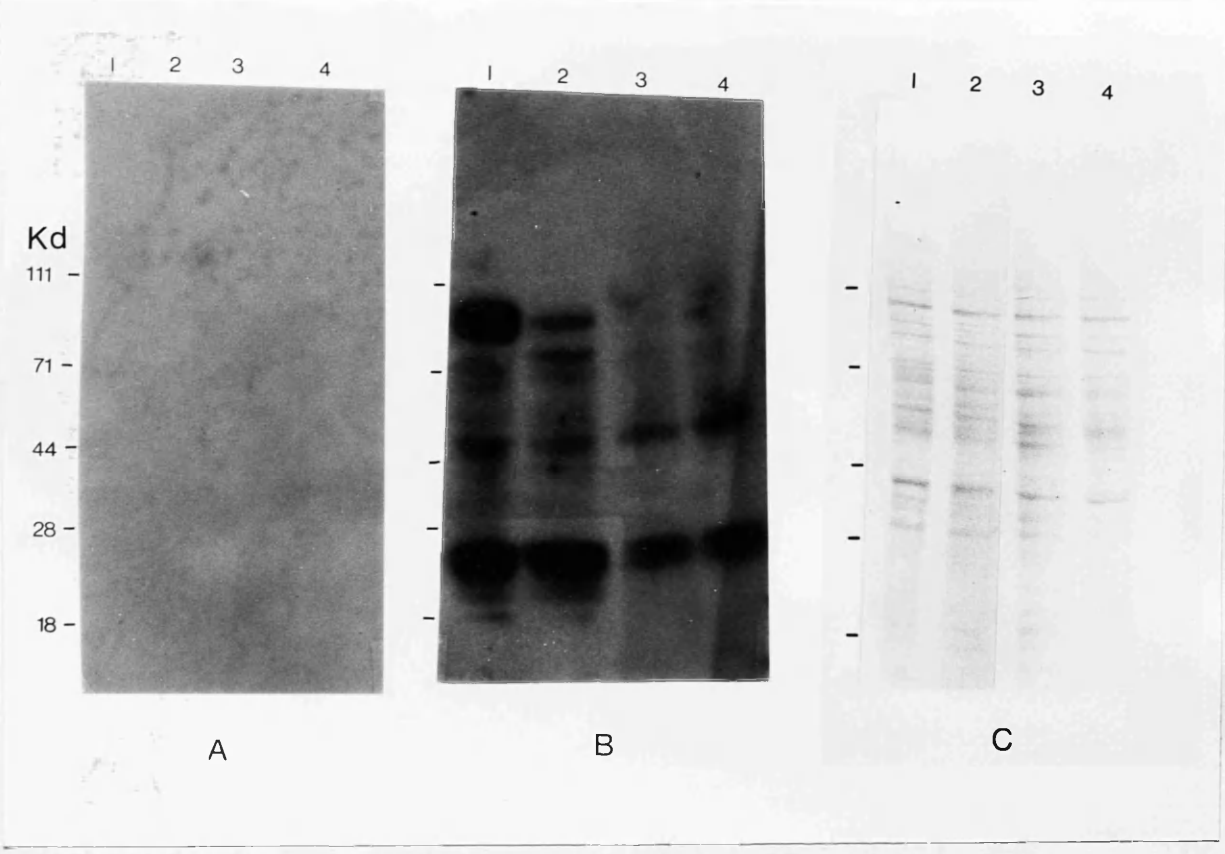
It therefore can be concluded that either by gel mobility shift assay or South-Western analysis the DDRPs detected by incubations with ds-CDDP-2αG7 may be different from those detected by Chu and Chang since the identified DDRPs are detected in extracts of XPE.

4.2.5 Examination of DDRPs in A2780 and A2780CP

CDDP resistance can occur by a number of different mechanisms. An increase in both a 50KD and 100KD protein has been demonstrated in the Ov1DDP, in the previous section. The DDRPs may be involved in the CDDP resistant phenotype of the Ov1DDP cell line. DDRPs in an independent human ovarian tumour cell line, A2780 were examined. A2780 has various CDDP resistant sublines derived from it (Behrens et al, 1987). The resistant A2780CP cell line used in these South-Western analysis is approximately 20 fold more resistant to CDDP than the parental A2780 cell line. Under conditions which show an increase in the 50 and 100KD DDRPs in the Ov1DDP cell extract, there is no detectable differences in the DDRPs of the A2780 resistant cell line, A2780CP when incubated with ds-CDDP-2αG7, **(Figure 4.4b)**. In both the A2780 cell lines, proteins of 25 and 50KD are detected, however the 100KD protein, which is detected in the Ov1DDP cell extract, is not detectable in the A2780CP. No binding to the unplatinated labelled ds-2αG7 oligonucleotide is observed under these conditions in the A2780 cell extract, **(Figure 4.4a)**. Equivalent amounts of protein from each cell line extract are loaded on the gel. A Coomassie Blue stained gel is shown as a check of protein concentrations, **(Figure 4.4c)**.

Figure 4.4

DDRPs recognising DNA damaged with CDDP in A2780 and A2780CP, analysed by South-Western blots



South-Western blots were carried out under identical conditions as described in figure 4.1 and incubated with γ^{32}P ATP labelled double-stranded oligonucleotide 2αG7 both platinated and unplatinated in the presence of 3μg/ml poly(dI.dC): poly(dI.dC) and 0.25% non-fat milk proteins.
Lane 1 Ov1DDP, Lane 2 Ov1p, Lane 3 A2780, Lane 4 A2780CP

Panel A : blot incubated with unplatinated oligonucleotide 2αG7.

Panel B : blot incubated with CDDP treated double-stranded 2αG7.

Panel C : Coomassie Blue stained gel of loaded protein concentrations.

4.2.6 SUBSTRATE SPECIFICITY OF DDRPs

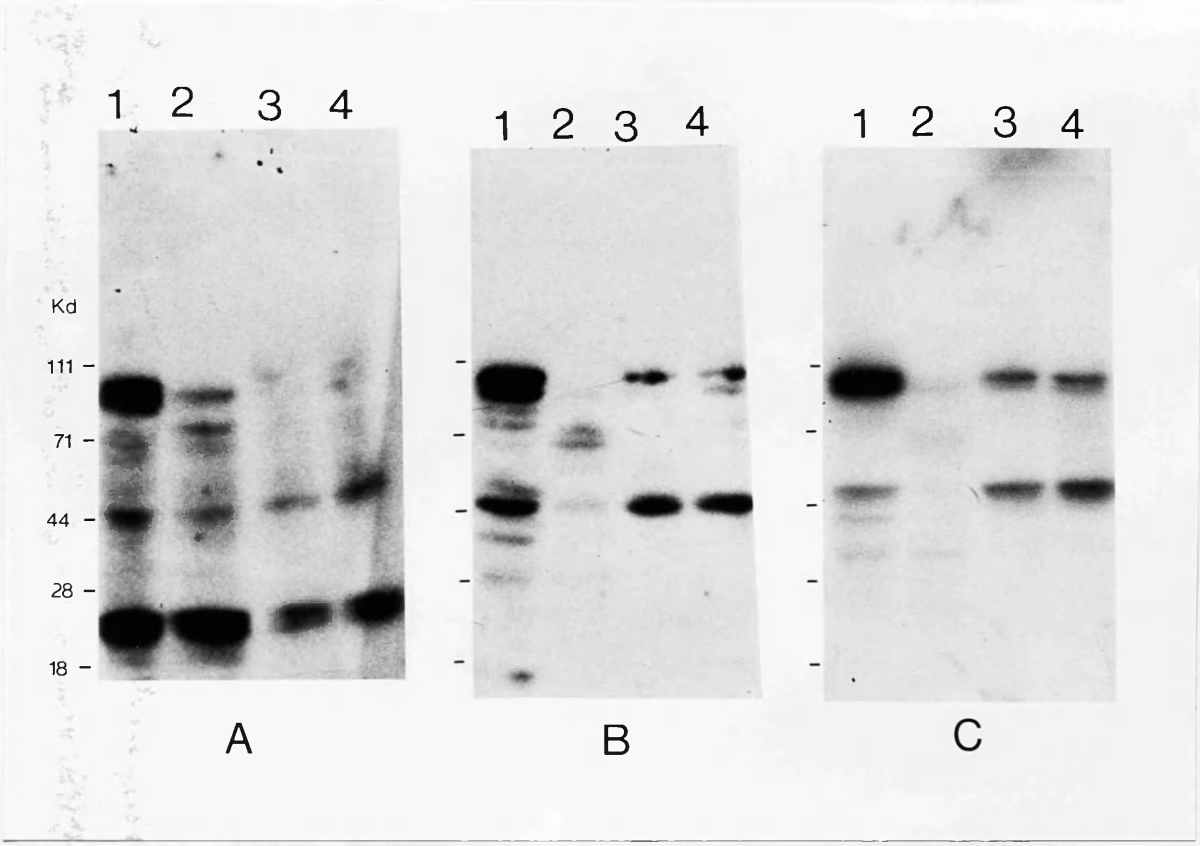
4.2.6.1 Binding of single-stranded oligonucleotides to protein extracts from the human ovarian cell lines

A South-Western blot of the two independent, A2780 and Ov1p, ovarian extracts, both sensitive and resistant sublines, was incubated with γ^{32}P ATP labelled single-stranded oligonucleotide 2αG7, **(Figure 4.5b)**. Proteins of approximate molecular weight 50KD and 100KD were detected in these lines. Increased binding to the γ^{32}P ATP single-stranded 2αG7 to the 50 and 100KD was again detected in the Ov1DDP cell line extract. This suggests that the DDRPs detected with the ds-CDDP-2αG7, may also recognise regions of single-strandedness, **(Figure 4.5a)**. A protein of approximate size 65KD is detected with the γ^{32}P ATP labelled single-stranded oligonucleotide in the Ov1p cell extract which is not detectable in the Ov1DDP cell extract. The 25KD protein observed with the ds-CDDP-2αG7 was not observed using the single-stranded 2αG7. Therefore the 25KD DDRP recognises double stranded DNA damaged with CDDP but not single-stranded DNA. Experiments incubating a South-Western blot with single-stranded oligonucleotide treated with CDDP does not show any difference in the binding as observed in the single-stranded probed blot, **(Figure 4.5c).**

It therefore may be postulated the DDRPs may be recognising regions of single-strandedness generated around CDDP adducts.

Figure 4.5

South-Western analysis of single-stranded binding proteins in human ovarian tumour cell line extracts



South-Western analysis was as described in figure 4.1. Blots were incubated in the presence of 3µg/ml poly (dI.dC) : poly (dI.dC) and 0.25 % non-fat milk proteins with
Panel A : γ^{32}P ATP labelled CDDP treated double-stranded 2αG7.
Panel B : γ^{32}P ATP labelled single-stranded 2αG7 oligonucleotide.
Panel C : γ^{32}P ATP labelled CDDP treated single-stranded 2αG7.

Lane 1 Ov1DDP, Lane 2 Ov1p, Lane 3 A2780, Lane 4 A2780CP

Sizes are in Kilodaltons estimated from molecular weight size markers.

4.2.6.2 Competition experiments with single-stranded DNA

From the previous set of experiments it has been suggested that the 50KD and 100KD protein detected in a ds-CDDP-2αG7 probed Western blot of Ov1 and Ov1DDP are binding to single-stranded regions in the DNA. The binding of the single-stranded oligonucleotide **(Figure 4.6a)** can be competed for with at least a 2000 fold Molar excess of single-stranded oligonucleotide **(Figure 4.6b)** If the DDRPs detected with the platinated double-stranded 2αG7 also recognise areas of single-strandedness then it may be hypothesized that competition of the binding of the DDRPs will occur upon incubation with an excess of single-stranded oligonucleotide.

Figure 4.6b in comparison to the other figures presented in this chapter shows many additional bands. These bands may be due to the degradation of the extract although on repeated experiments using the same extract less bands are detected. These additional bands may also represent modified forms of the major proteins detected. The binding of these additional bands may more likely be due to factors present in the extract which have lower affinities to the single-stranded oligonucleotide and may only be detected if the probe used is of higher specific activities. The washing conditions in the South-Western system is seven minutes at 4^0C, therefore any variations in the time of washes after incubation with the probe may cause variations in the bands detected.

Competition of DDRPs detected in Ov1 and Ov1DDP, with an excess of cold ds-CDDP-2αG7 competes well for the 50KD and 100KD DDRPs, **(Figure 4.7b)**. Competition of the 25KD DDRP occurs but to a lesser extent than of the other two identified DDRPs. Upon coincubation of the ds-CDDP-2αG7 probed blot of Ov1 and Ov1DDP with excess of cold single-stranded 2αG7, competition for binding of the 50KD and 100KD protein occurs, **(Figure 4.7c)**, but to a lesser extent than with excess of ds-CDDP-2αG7, **(Figure 4.7b)**. This therefore suggests that the 50KD and 100KD DDRPs may have an affinity for CDDP damaged DNA as well as single-stranded DNA.

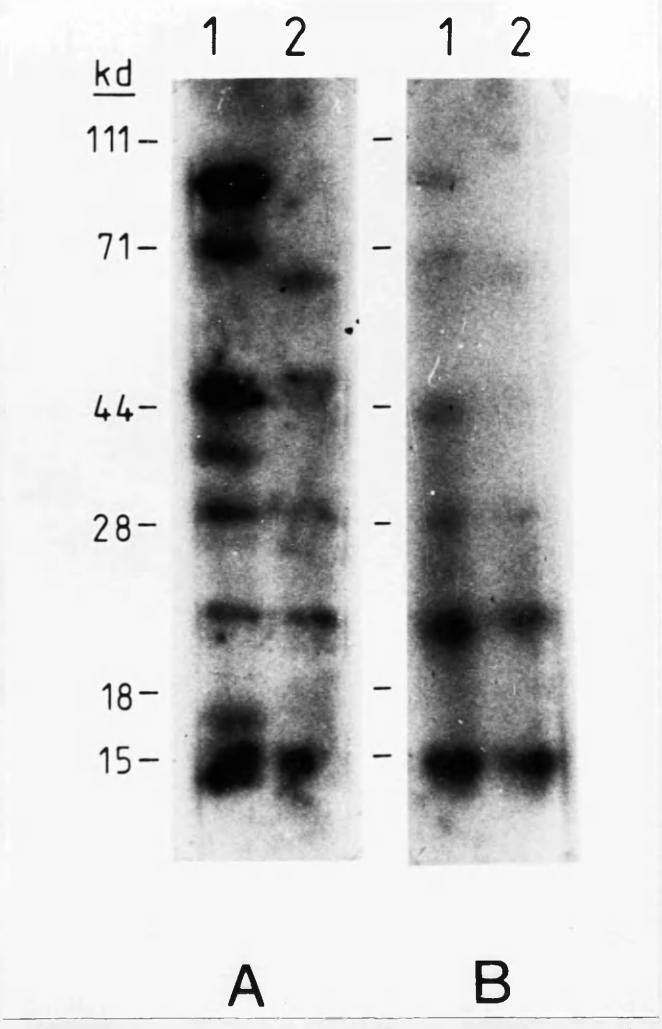
4.2.6.3 Binding of DDRPs to mismatch oligonucleotides

It has been demonstrated in the previous section that two DDRPs of 50KD and 100KD are detected in the ovarian tumour cell extracts by incubation with single-stranded DNA. These DDRPs are of similar size to those detected with platinated oligonucleotide. Evidence in the literature has suggested CDDP adducts in DNA to behave as the centre of a directed bend with generation of single-stranded regions over some of the DNA (Sundquist et al, 1986; Anin and Leng, 1990). Anin and Leng reported CDDP adducts to locally denature over 2 base pairs and distort over 4-5 base pairs. This therefore leads to a disruption over 6-7 base pairs of the DNA duplex.

To test whether CDDP adduct induced disruption of the duplex could be mimicked by mismatches in the DNA, oligonucleotides with different mismatches were generated. The mismatches were generated at the region of the oligonucleotide to which the majority of CDDP is thought normally to react i.e the sequence of guanines. Mismatches of 3 base pairs (3bpMM) and 7 base pairs (7bpMM) were generated in the 2αG7 sequence (refer to table 2.1 for sequence). **Figure 4.8** demonstrates that upon incubation under similar conditions which detects binding of DDRPs to CDDP-ds-2αG7 **(Figure 4.8a)**, there is no detectable binding of the mismatch oligonucleotides in the cell extracts of Ov1 and Ov1DDP **(Figure 4.8b, c)**. However upon incubation with an excess of either 3 base pair mismatch or 7 base pair mismatch, there is preliminary evidence to suggest binding to the 25, 50, 100KD DDRPs is competed for **(Figure 4.8d, e)**. It is unlikely that this competition is caused by single-stranded sequences in the oligonucleotide population, as binding is found not to occur to either the 3bpMM or the 7bpMM sequence, so suggesting that the oligonucleotides are properly reannealed. It is not entirely clear as to whether this competition is true and these experiments would have

Figure 4.6

Competition of single-stranded DDRPs detected in the South-Western system



South-Western analysis was under identical conditions as described in figure 4.1. DDRPs detected in all panels by incubation of the blot with γ^{32}P ATP single-stranded oligonucleotide 2αG7. The blots were coincubated with :

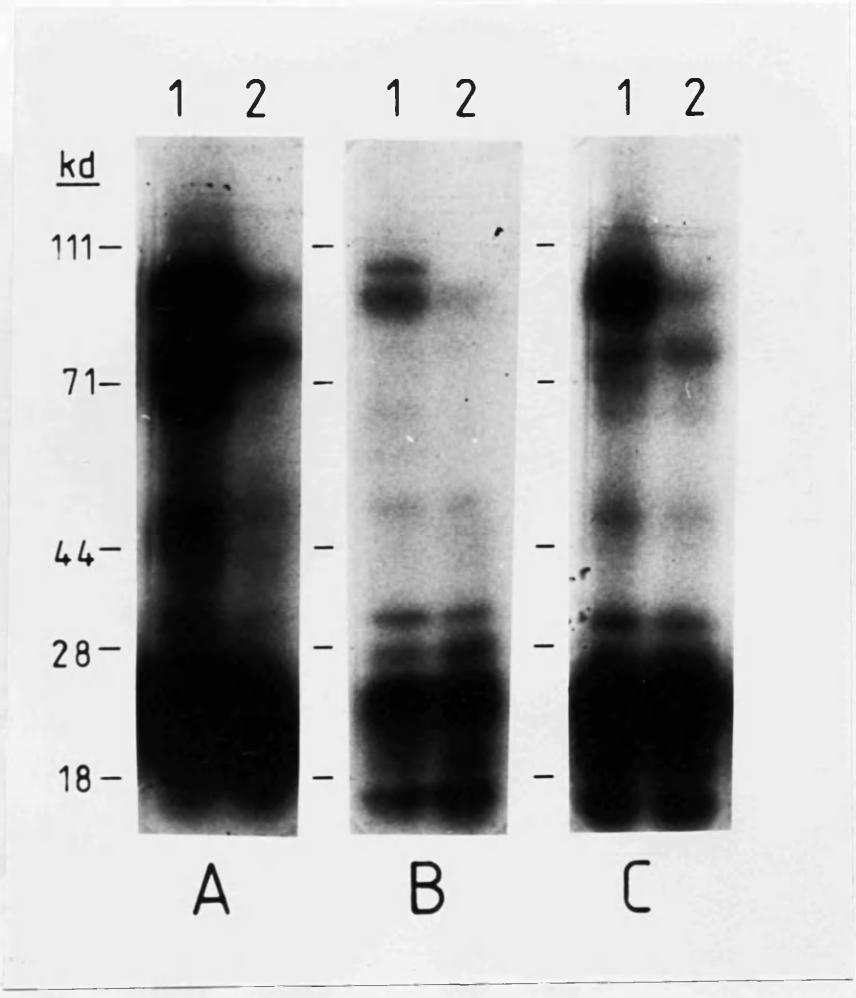
Panel A : no competitor.
Panel B : a 2000 fold excess of single-stranded oligonucleotide.

Lane 1 Ov1DDP, Lane 2 Ov1p

Sizes are in Kilodaltons estimated from molecular weight size markers.

Figure 4.7

Competition of DDRPs, detected with platinated double-stranded 2αG7, with both double-stranded CDDP treated 2αG7 and single-stranded 2αG7



South-Western analysis was as described in figure 4.1. DDRPs detected by incubating blots with γ^{32}P ATP labelled double-stranded CDDP treated 2αG7. Blots were coincubated with :

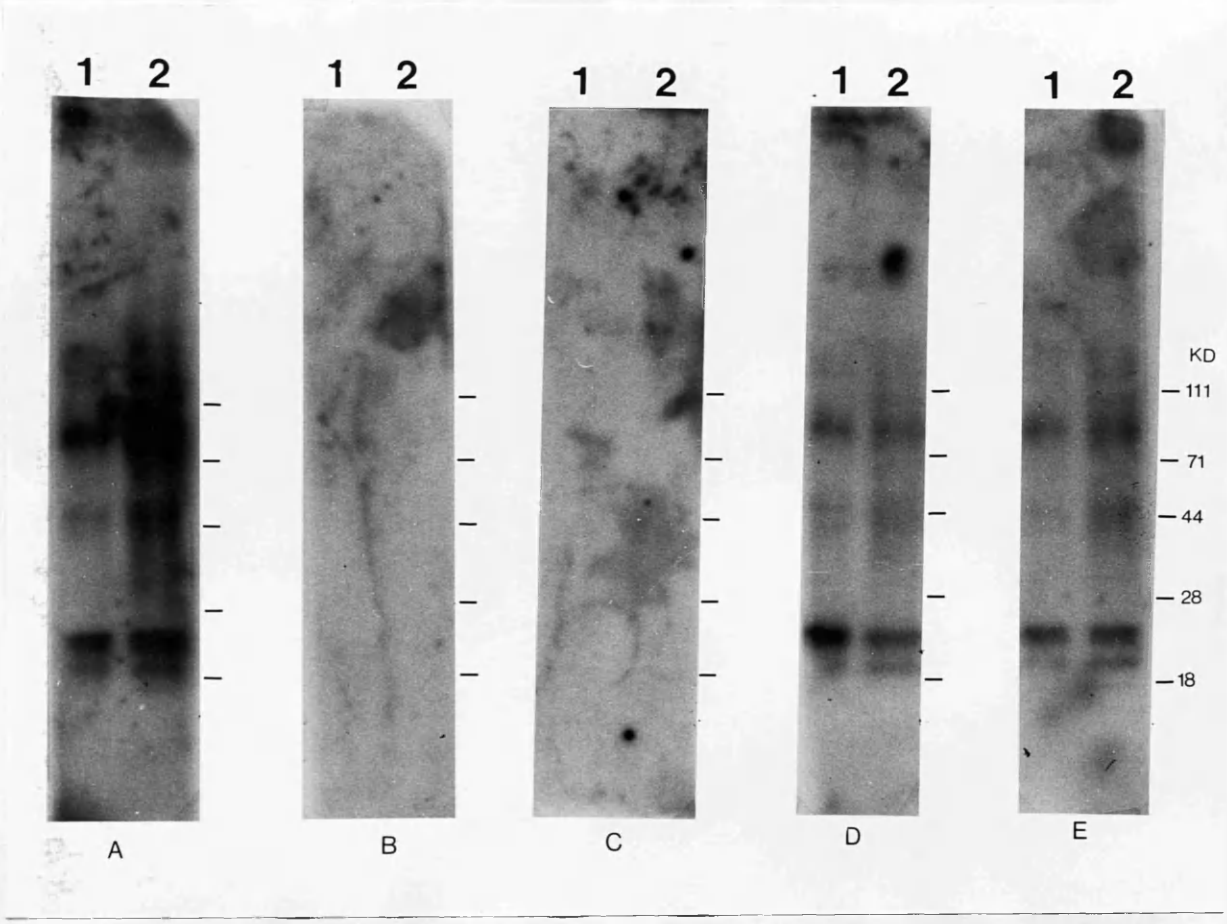
Panel A : no competitor.
Panel B : at least a 2000 fold excess of CDDP treated double stranded oligonucleotide 2αG7.
Panel C : At least a 2000 fold excess of single-stranded 2αG7 oligonucleotide.

Lane 1 Ov1DDP, Lane 2 Ov1p

Sizes are in Kilodaltons estimated from molecular weight size markers.

Figure 4.8

DDRPs binding to mismatch oligonucleotides



South-Western analysis was as described in figure 4.1. DDRPs detected by incubating blots with (a) γ^{32}P ATP CDDP 2αG7 in panels A, D, E (b) γ^{32}P ATP 3bpMM and 7bpMM in panels B and C. Two of the panels were coincubated with a 2000 fold excess of ; Panel D, 3bpMM and panel E, 7bpMM oligonucleotide.

Lane 1 = Ov1p, Lane 2 = Ov1DDP

Sizes are in Kilodaltons estimated from molecular weight size markers.

to be repeated using different extracts. It was shown previously in **Figure 4.2** that binding was not competed for with an excess of double-stranded 2αG7.

DDRPs can therefore bind to platinated double-stranded DNA, single-stranded DNA, but are not detected, using similar conditions, binding to DNA containing mismatches. However preliminary evidence suggests that binding of the DDRPs detected by CDDP-ds-2αG7 can be competed for with the mismatch oligonucleotides, however not to as great an extent as competition with the CDDP treated DNA **(Figure 4.2).**

4.2.6.4 Summary of competition experiments

The following table is a summary of the previous described competition experiments. It summarises the DDRPs detected by CDDP-ds-2αG7 and their competition either by double stranded oligonucleotide ± CDDP, by single-stranded DNA ± CDDP or by mismatch oligonucleotides.

COMPETITOR	25KD	50KD	100KD
ds-2αG7	-	-	-
ds-CDDP-2αG7	++	++	++
Calf thy DNA	+/-	+/-	+/-
CDDP Calf thy DNA	+	++	++
ss DNA	-	+	+
CDDP ssDNA	-	+	+
3bpMM	+/-	+/-	+/-
7bpMM	+/-	+/-	+/-

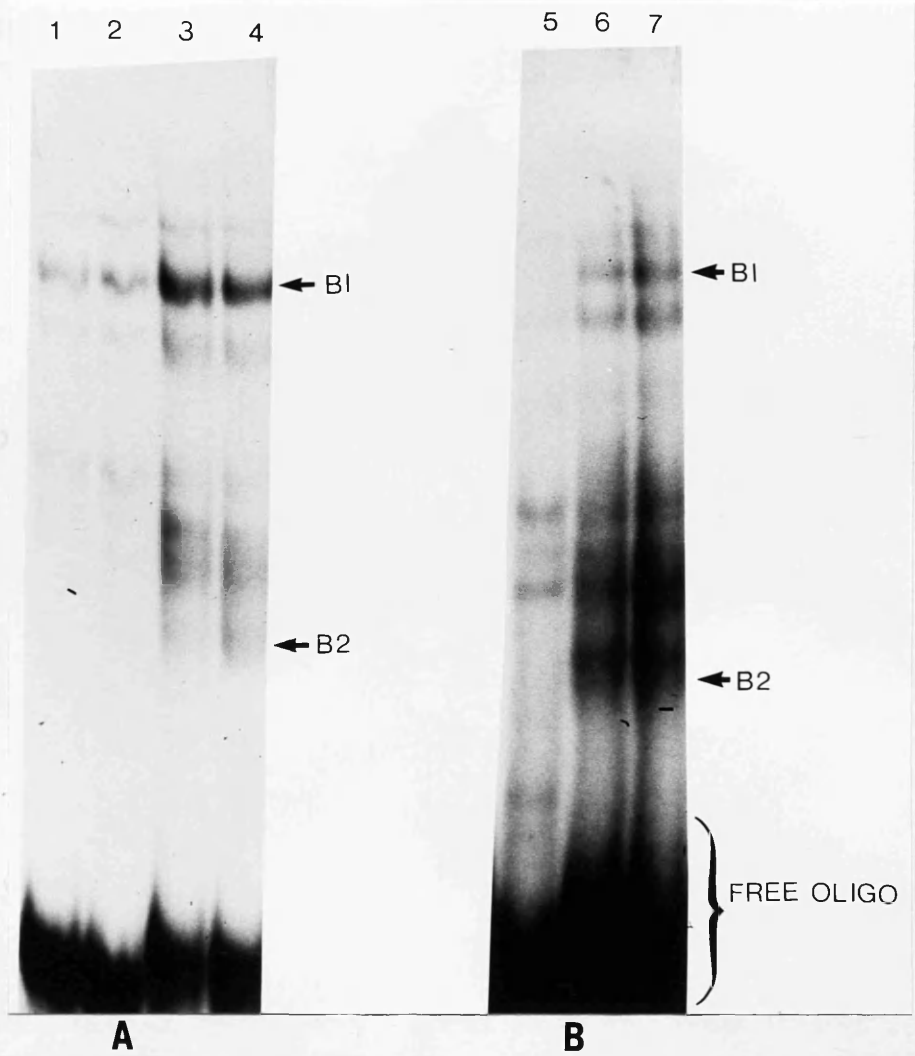
4.2.7 DDRPs in A2780, A2780CP, Ov1p and Ov1DDP - Detection by gel mobility shift assays

The gel mobility shift assay is a very different system from the South-Western technique, therefore it is very difficult to determine whether the DDRPs detected by South-Western analysis are different from those detected by gel mobility shift assays.

Two different groups, using gel mobility shift assays, have reported an increase in a binding factor recognising UV damaged DNA in CDDP resistant cells, (Chu and Chang, 1990, Chao et al, 1991[a]). Presented in the previous set of experiments there is found to be an increase in a 50KD and 100KD DDRP detected by South-Western analysis in a human ovarian cell line resistant to CDDP, Ov1DDP. Both the Ov1 and A2780 cell lines with their resistant sublines have been analysed on gel mobility shift assays. Under the conditions described, section 2.5.1, neither A2780CP or Ov1DDP show any differences in the binding factors detected (B1 and B2) in the gel mobility shift assay, **(Figure 4.9)**.

Figure 4.9

Detection of DDRPs recognising DNA damaged with CDDP by gel mobility shift assay



γ^{32}P ATP labelled non-CDDP and CDDP treated 2αG7 incubated with two different human ovarian tumour cell extracts. Both CDDP sensitive and CDDP resistant counterparts of each line examined. Binding conditions as described in gel mobility shift assay, section 2.5.1.
Lanes 1, 2 A2780; Lanes 3, 4 A2780CP; Lanes 5, 6 Ov1p; Lane 7 Ov1DDP. Lanes 1, 2, 5 incubated with unplatinated 2αG7, lanes 3, 4, 6, 7 incubated with platinated 2αG7.

4.2.8 Summary of DDRPs detected by different probes

PROBE	EXTRACT	25	50	65	100kd
DS	A2780	-	-	-	-
	A2780CP	-	-	-	-
	Ov1	-	-	-	-
	Ov1DDP	-	-	-	-
DS CDDP	A2780	+	+	-	-
	A2780CP	+	+	-	-
	Ov1	+	+	-	+
	Ov1DDP	+	++	-	++
ss	A2780	-	+	-	+
	A2780CP	-	+	-	+
	Ov1	-	-	+	-
	Ov1DDP	-	++	-	++
ssCDDP	A2780	-	+	-	+
	A2780CP	-	+	-	+
	Ov1	-	-	+	-
	Ov1DDP	-	++	-	++

4.2.9 Investigation of levels of DDRPs upon pretretment with CDDP

Many repair functions are induced *de novo* in damaged bacterial cells as a part of the SOS pathway which is believed to facilitate the recovery of bacterial cells from the toxic effects of DNA damaging agents (review, Little and Mount, 1982). Recent studies have indicated that inducible phenomena associated with DNA processing occur in mammalian cells exposed to carcinogens or tumour promoters (Elespuru, 1987). Hirschfeld and co-workers have demonstrated that upon pretreatment of primate cells with ultra-violet light, higher levels of DNA damage recognition proteins recognising UV damage can be detected (Hirschfeld et al, 1990). Another study by Glazer and co-workers have demonstrated the inducibility of DNA binding proteins upon pretreatment of Hela cells with DNA damaging agents (Glazer et al, 1989) It was therefore of interest to examine if there was any significant change in the levels of the DDRPs recognising CDDP damage by pretreatment of the cells with CDDP.

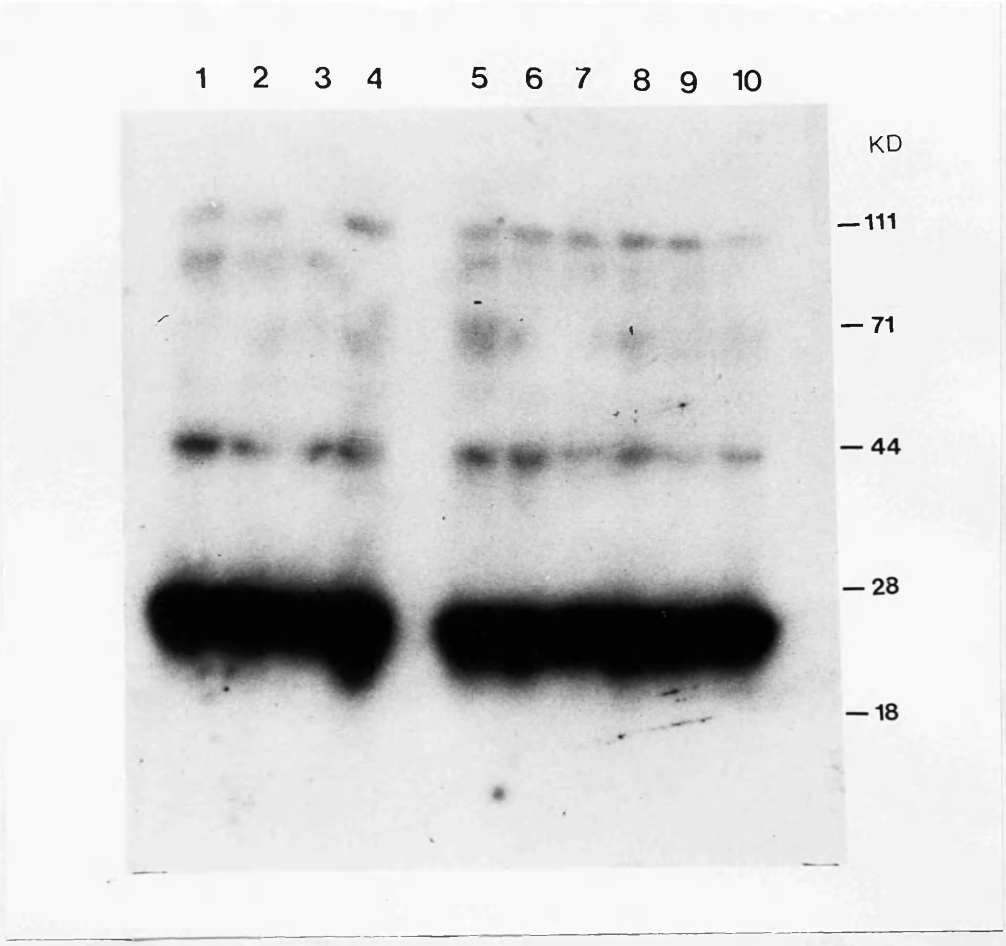
After a 1 hour exposure to CDDP the cells were allowed to grow in drug free medium for various periods of time prior to a cell free salt extraction of the proteins. **Figure 4.10** shows a Western blot of the A2780 and A2780CP probed with ds-CDDP-2αG7 with extracts made after different time periods in drug free medium. Conditions are identical to those detecting the change in the DDRPs of the Ov1DDP cell extract **(Figure 4.1)**. It can be seen however that over a time period of 24 hours for the A2780 and 28 hours for the A2780CP after the CDDP pretreatment, there was no significant increase detectable in the DDRPs recognising CDDP damage. A short exposure of this gel did not show any significant difference in the 25KD DDRP.

Extracts from the Ov1 and Ov1DDP cell line were made 24 hours after a 1 hour CDDP treatment. **Figure 4.11** demonstrates that under conditions as described previously, there was no significant increase in the levels of the DDRPs in these cell extracts. It can therefore be concluded that under the conditions described, an increase in

the levels of the DDRPs, upon pretretment with CDDP, does not occur in either of the ovarian cell line extracts.

Figure 4.10

South-Western analyses of DDRPs in cell extracts of cell lines A2780 and A2780CP pretreated with CDDP

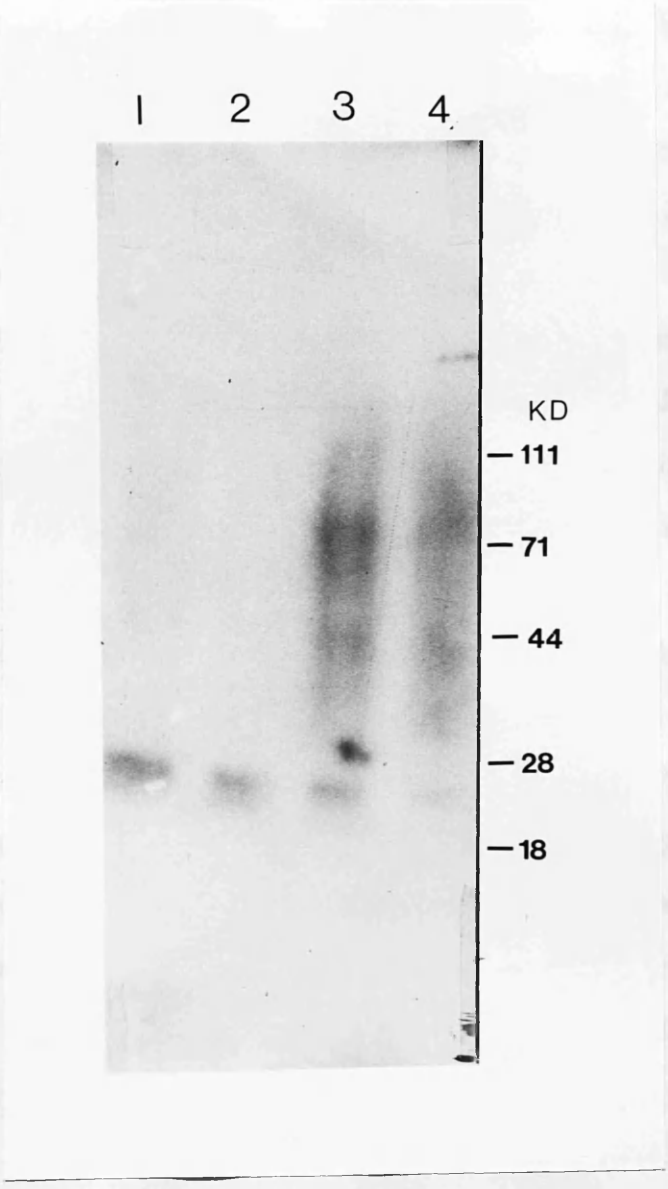


South-Western analyses was as described in figure 4.1. The DDRPs were detected by incubation with CDDP-ds-2αG7 The extracts were made from A2780 and A2780CP pretreated with CDDP for 1 hour with different recovery periods prior to extraction. Lanes 1, 2, 3, 4 A2780, 5, 6, 7, 8, 9, 10 A2780CP. Extracts made from A2780, lanes 1=0hours, 2=1hour, 3=4hours, 4=24hours after the 1hour CDDP treatment. Extracts made from A2780CP, lanes 5=0hours, 6=1hour, 7=2hours, 8=4hours, 9=24hours, 10=28hours after the 1hour CDDP treatment.

Sizes are in Kilodaltons.

Figure 4.11

South-Western analyses of DDRPs in cell extracts of cell lines Ov1 and Ov1DDP pretreated with CDDP



South-Western analyses was as described in figure 4.1. The DDRPs were detected by incubation with CDDP-ds-2αG7. The extracts were made from Ov1 and Ov1DDP pretreated with CDDP for 1 hour with a 24 hour recovery period. Lane 1, 2 = Ov1, lane 3, 4= Ov1DDP. Lanes 1 and 3, extracts were made directly after the 1hour CDDP treatment, lanes 2 and 4 extracts made 24 hours after the 1 hour CDDP treatment.

4.3 DISCUSSION

The work carried out in this chapter has attempted to identify the presence of mammalian proteins which bind to DNA damaged with CDDP. Unlike the previous chapter, chapter 3, which concentrates on the identification of DNA damage recognition proteins (DDRPs) by gel mobility shift assays, the South-Western technique is used.

Proteins which bind to UV damaged DNA have been detected by the gel mobility shift assay, (Chu and Chang, 1988; Hirschfeld et al, 1990; Chao et al, 1991[a]). The identification of proteins binding to CDDP damaged DNA have also been reported from gel mobility shift assays, (Donahue et al, 1990; Andrews and Jones, 1991). Little work has been carried out using South-Western analysis although it has been reported that factors of both 100KD and 28KD bind to DNA modified with either CDDP or $Pt(en)Cl_2$, (Toney et al, 1989). There is also a recent report showing South-Western data on the detection of proteins recognising CDDP damage present in kidney tubule cells and human ovarian tumour cells (Andrews and Jones, 1991).

The advantage of using the South-Western technique, is that it involves using an SDS polyacrylamide gel to separate the proteins. Sodium dodecyl sulphate (SDS) is an ionic detergent which is used in the SDS-PAGE system as an agent which will dissociate all proteins into their individual polypeptide subunits. The proteins which are identified in the South-Western blot probed with CDDP damaged DNA are individual proteins unlike the identified complexes in the gel mobility shift assay. The gel mobility shift assay uses a "native" gel system therefore the factors identified may represent complexes of proteins recognising and binding to the damaged DNA. It is therefore very difficult to compare results between chapter 3 and the present chapter since the gel systems used are very different and the binding conditions to damaged DNA have been optimised for each system, leading to different reaction conditions for each.

Using the South-Western system, this chapter presents DDRPs binding to ds-CDDP-2αG7 oligonucleotide of 25, 50, and 100KD in human ovarian cell line extracts of Ov1p and A2780. The role of the DDRPs in the recognition of CDDP damage has not yet been defined. If they are involved in a DNA repair pathway then the question of their involvement in resistance to CDDP becomes a necessary one to address. Reported work from gel shift assays has shown an overexpression of the XPE binding factor in CDDP resistant Hela and HT1080 fibrosarcoma cell lines, (Chu and Chang, 1990). A separate group also reported overexpression of two nuclear factors that recognise UV modified DNA in CDDP resistant Hela cells, (Chao et al, 1991[a]). It has been shown in the previous section that both the 50KD and 100KD DDRPs identified in the Ov1p cell line extract are overexpressed in the resistant subline Ov1DDP. Ov1DDP has been shown to be approximately five times more resistant to CDDP than the Ov1p cell line by the MTT assay. Before any correlation can be made on the observed increase in DDRPs of the Ov1DDP cell line with pathways of DNA repair, DNA repair levels in these cell lines must be measured. Both the studies reported in the literature of the increase in DRPs demonstrated their resistant cell lines to have an enhanced repair capacity (Chu and Chang, 1990; Chao et al, 1991[a]).

Competition for binding of the DDRPs with platinated and unplatinated oligonucleotide and calf thymus DNA were studied to determine the specificty of binding. This set of experiments showed that DDRPs in a South-Western system can be competed with DNA containing CDDP adducts, both with the same oligonucleotide 2αG7 and DNA of an unrelated sequence, calf thymus DNA. Binding to double-stranded unplatinated oligonucleotide 2αG7 does not occur under the conditions used to detect DDRPs binding to CDDP damaged DNA. There is no detectable competition of the DDRPs when incubated with an excess of double-stranded oligonucleotide. It was shown that binding to the 25KD is less efficiently competed for with platinated calf thymus DNA compared to the platinated double-stranded oligonucleotide 2αG7. This

may be due to the number of adducts or types of adducts induced in these DNA, (analysis of the DNA has been described in chapter 3, sections 3.3 and 3.5). This set of experiments suggest that the 25, 50, and 100KD DDRPs are binding to DNA damaged with CDDP but are independent of sequence. Before a statement can be made about the type of damage the DDRPs are binding to, competition experiments would have to be carried out in which the DNA used as the competitor is damaged with different damaging agents, e.g. ultra-violet radiation , mitomycin C.

In comparison to the overexpression of the 50KD and 100KD DDRPs detected in the CDDP resistant subline, Ov1DDP, in the South-Western study, no difference was observed in the gel mobility shift system under the binding conditions described. This therefore emphasises that comparisons between gel shift systems and South-Western systems should not be made because conditions both of the gels and binding are very different. A recent study of another CDDP resistant ovarian cell line showed no differences in DDRPs in gel mobility shift assay but did detect proteins of similar size to those identified in this chapter, (Andrews and Jones, 1991). Although not shown in detail the South-Western analysis carried out in this separate resistant ovarian cell line, showed apparent differences in the DDRPs of the resistant line compared to those in the sensitive line.

CDDP has been shown to bind to DNA and cause a disruption of the DNA duplex over 4-5 base pairs, (Anin and Leng, 1990). Work using the anti-nucleoside antibodies in the probing for local denaturation of calf thymus DNA has shown that CDDP binding to DNA causes a single-stranded region around the adduct, (Sundquist et al, 1986). It is possible that DDRPs do not recognise specific adducts but recognise a distortion in the native double-stranded DNA generating areas of single-strandedness. It has been reported that the bacteriophage T4 gene 32 protein which is involved in DNA replication (Huberman et al, 1971), recombination (Kozinski and Felgenhauer, 1967) and repair of UV damage (Krisch and vanHoule, 1976) binds to single-stranded DNA (Jensen

et al, 1976). Studies showed that the gene 32 protein which normally shows a low affinity for double-stranded DNA binds more efficiently to double-stranded DNA modified with CDDP (Toulme et al, 1983), therefore suggesting that CDDP may be causing areas of single-strandedness which are recognised by the gene 32 protein. A report on the absence of a single-stranded DNA binding activity in cell lines from xeroderma pigmentosum complementation group A, may suggest an involvement of single-stranded binding proteins in excision repair (Kuhnlein et al, 1983). Recent evidence has suggested a role for the replication protein human SSB in human DNA excision repair. Antibodies which have been generated towards the 70KD and 34KD subunits of SSB (Kenny et al, 1990) were included in a cell free system that can carry out nucleotide excision repair *in vitro* (Wood et al, 1988). It was found that on addition of the antibodies to the system, DNA excision repair was inhibited (Coverley et al, 1991). On reintroduction of hSSB into the repair system, excision repair was initiated.

Results presented in this chapter have shown that proteins detected by incubating a South-Western blot of ovarian cell extracts with single-stranded oligonucleotide have identified binding proteins of approximate sizes 50KD and 100KD. It has been found that the proteins detected by the single-stranded oligonucleotide are also increased in the CDDP resistant Ov1DDP cell line. The size of the DDRPs recognising the CDDP damaged double-stranded DNA is very similar to those detected with the single-stranded oligonucleotide. An increase in DDRPs of approximate sizes 50KD and 100KD can be seen in the Ov1DDP cell line both when either the platinated double-stranded oligonucleotide or single-stranded oligonucleotide (platinated or not) is used as the probe in the South-Western blot. DDRPs recognising CDDP damaged DNA can be competed for with single-stranded DNA although not to as great an extent than seen with the platinated calf thymus DNA. This may seem curious that binding to the single-stranded oligonucleotide only detects proteins of approximate size 50KD and 100KD when there are many known DNA single-stranded binding proteins. Binding of

these two proteins under the denaturing conditions used suggest that they function as a single species whereas as other single-stranded binding proteins will only function as part of a complex. Experiments using other single-stranded DNA would prove useful both in the competition for binding and also to detect if the same DDRPs would be detected. Before anything can be concluded on whether the DDRPs have an affinity for single-stranded DNA as well as CDDP treated double-stranded DNA, experiments must be carried out in looking at the competition of binding of the 50 and 100KD proteins recognising single-stranded DNA with an excess of double-stranded DNA, both CDDP treated and not. The unplatinated oligonucleotide 2αG7 shows no binding of DDRPs therefore suggesting that in the reannealed stock of oligonucleotide prior to CDDP treatment, only a small amount, if any, is single-stranded. The 25KD DDRP detected by South-Western analysis using CDDP-ds-2αG7 shows no detectable binding to single-stranded DNA.

It was of interest to determine whether the DDRPs which are detecting damage by CDDP would detect base pair mismatches in a DNA duplex. Mismatches generate disruptions in the DNA duplex. Binding of the DDRPs was not detected using the mismatch oligonucleotides however, preliminary evidence may suggest that binding to the 25, 50, 100KD DDRPs was competed for upon incubation with an excess of either the 3 base pair mismatch (3bpMM) oligonucleotide or the 7 base pair mismatch (7bpMM) oligonucleotide. Competition for binding to the DDRPs by the mismatch oligonucleotides is not as efficient as competition with CDDP treated oligonucleotide. It is unlikely that in the reannealed population of oligonucleotides that single-strands are a major problem, as there is no detection of DDRPs binding to the labelled 3bpMM or 7bpMM. This set of experiments is very preliminary and would have to be repeated with different extracts before anything definitive can be said.

Proteins which bind to either UV damaged DNA or CDDP damaged DNA have been identified (Chu and Chang, 1988; Hirschfeld et al, 1990; Chao et al, 1991[a];

Donahue et al, 1990; Andrews and Jones, 1991; Toney et al, 1989). Chu and Chang reported that the DRPs they identified recognising UV damaged DNA, were absent in cell line extracts of Xeroderma Pigmentosum Complementation Group E (XPE). They denoted this factor the XPE binding factor. They have suggested that because the factor is absent in XPE cell line extracts and recognises UV damage, it is a likely candidate to participate in a DNA repair system. There is a recent study that confirms the absence of the XPE binding factor in this particular cell line, however it shows the presence of the XPE binding factor in other cell lines derived from totally unrelated individuals with XPE (Kataoka and Fujiwara, 1991). Therefore the absence of the binding proteins is not always commonly observed in all the different XPE cell strains. The DDRPs discussed in this chapter were examined in the XPE cell strain in which a deficiency was shown both by Chu and Chang, and Kataoka and Fujiwara. Either by gel mobility shift assay (chapter 3, section 3.8, figure 3.11) or South-Western analysis, the identified DDRPs are present in the XPE extract. The DDRPs detected in the South-Western system in the XPE cell line extract are of similar size to those detected in the Ov1p cell lines. The presence of the DDRPs in the XPE cell extract, in both the systems described in this thesis, may suggest a different protein is being assayed. The differences could be due to the different extraction protocols used. It may also be due to the differences in the probes used for their detection and the binding conditions of the different assays which means that different factors may be detected in each of the systems.

Throughout this chapter there has been presentation of the results suggesting that both a 50KD and 100KD protein are increased in a CDDP resistant human ovarian cell line, Ov1DDP. It was therefore important to include in this study another set of independent human ovarian cancer cell lines, A2780 and A2780CP which are well studied human ovarian cancer cell lines. Work has been carried out in these cell lines suggesting that increased DNA repair may be an important mechanism by which the

A2780 cells become resistant to CDDP (Lai et al, 1988). The A2780 cell lines were studied in the South-Western system. It was found that upon examination of the DDRPs only proteins of 25KD and 50KD were present under the conditions which normally reflect the differences in the binding to the 50KD and 100KD DDRPs in the Ov1 cell lines. There was no detectable changes in either of the identified DDRPs in the A2780CP cell line extract. It may be that changes do occur in the A2780 DDRPs but under the binding conditions used, they are not detectable for the A2780 cell lines. It may however be argued that the DDRPs have no involvement in the CDDP resistant phenotype of the A2780CP subline and CDDP resistance may occur by some other mechanism.

Upon pretreatment of cells with CDDP prior to nuclear extraction, the levels of the DDRPs detecting CDDP damage were shown to remain constant both in the A2780 cell line and the Ov1p cell line. Evidence from Hirschfeld and co-workers have demonstrated an increase in damage recognition protein levels upon pretreatment with UV irradiation (Hirschfeld et al, 1990) therefore suggesting that the damage recognition complex identified in this study is inducible unlike the DDRPs presented in this chapter.

In summary the results presented in this chapter have identified factors recognising DNA damaged with CDDP of 25, 50 and 100KD. It has been found that the 50KD and 100KD DDRPs show increased levels in a CDDP resistant ovarian cell line whilst the 25KD DDRP remains constant between the sensitive parental line and the CDDP resistant subline. A study of the DDRPs with single-stranded DNA detects DNA binding proteins of similar size to those identified by the platinated double-stranded oligonucleotide. It has also been presented that the single-stranded binding proteins are increased in the Ov1DDP cell line. This has led to the suggestion that the DDRPs recognising CDDP damaged double-stranded DNA may be identifying with areas of single-strandedness generated around the adducts.

CHAPTER 5

ISOLATION OF CDDP RESISTANT OVARIAN CANCER CELL LINES BY A SINGLE STEP SELECTION

5.1 INTRODUCTION

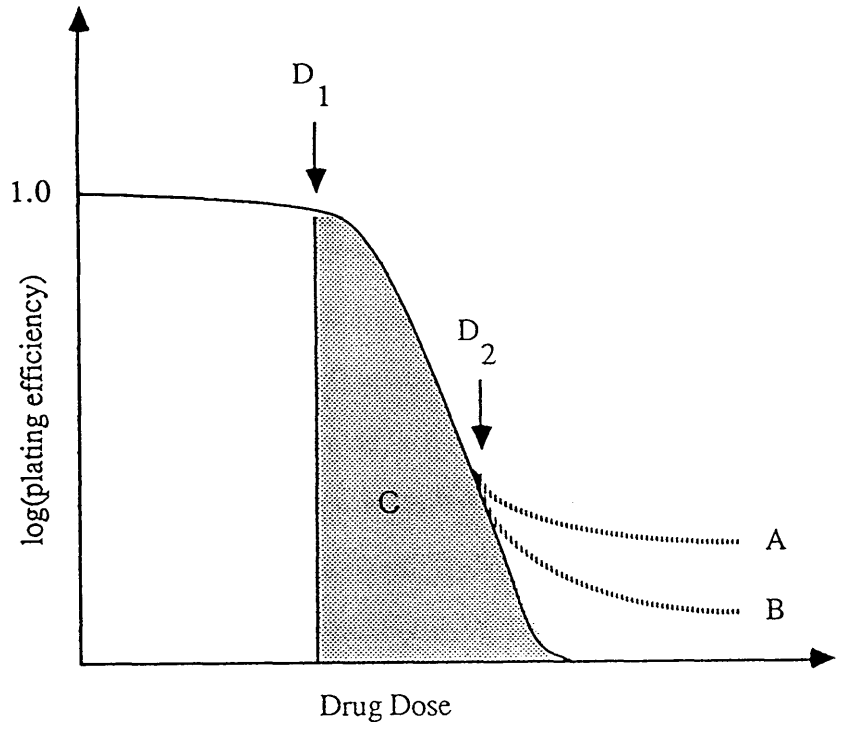
It has been suggested that drug resistant tumour cells may arise during the development of a tumour and that they may be the reason for treatment failure during chemotherapy. Goldie and Coldman, (1984), have described a mathematical model for progression to resistance based on theoretical considerations of mutation frequencies and the assumption that drug resistant mutants exist or are induced in the tumour cell population. In this model, the mutation rate is considered as a measure of the probability of occurrence of a new phenotype per cell division. It is therefore obvious that the frequency of mutants in the tumour will be directly related to the size of the tumour population being considered. The larger the tumour population the greater the probability that a drug resistant mutant will have appeared. Conversely, within a small population of tumour cells there will be a significant probability that no resistant variants will as of yet occurred. The model is based upon the principles of the Luria-Delbruck fluctuation analysis, (Luria and Delbruck, 1943), which measures the probability of a sub-population not containing a particular mutant phenotype. Thus the fluctuation analysis provides a means of testing the stochastic nature of generation of a given phenotype.

An understanding of the mechanism of CDDP resistance is necessary for the development of a treatment regimen. An effective, although not perfect, way to attain this goal, is through studies done in a cell culture model; partly because established cell lines are readily demonstrated to be resistant to drugs. A variety of drug selection protocols have been used to examine mutations in mammalian cells, (Thacker, 1985). The most reliable and quantitative selection procedures have used a single step selection protocol that allows the identification of those rare cells that are drug resistant, (Thompson and Baker , 1973).

Generally cell lines which are made resistant to CDDP *in vitro* have been derived by multiple exposures to the drug. These cell lines cannot however be used to quantitate mutation frequencies and factors which affect this frequency. It may also be expected that cells selected by multiple exposures to drug will exhibit multiple mechanisms of resistance. It is therefore important to derive cell lines which are resistant to the drug by a single step exposure to the drug. This will allow the frequency of variants to be calculated and may yield a range of resistant clones for analysis which may be more relevant to resistance ocurring *in vivo*.

CDDP resistant cells have previously been isolated from the human ovarian carcinoma cell line A2780 using repeated selection protocols, (Behrens et al, 1987). Three A2780 cell lines have been isolated for resistance to CDDP by this method (the three sublines have an increasing resistance varying from 7.3-39 times more resistant to CDDP than the sensitive parental A2780 cell line , Behrens et al, 1987). CDDP resistant A2780 cell lines isolated by multiple exposures to CDDP are therefore available, however it was important to examine if CDDP resistant clones could be isolated by a single step selection protocol.

The choice of concentration at which the CDDP resistant clones are isolated, is very important. The concentration of drug is chosen upon analysis of the survival of the cells to a particular range of concentrations. The dose response of wild type cells to a selecting drug can be carried out in two ways. It can either be measured in terms of growth rate or plating efficiency in the presence of the drug. In most cases a plating assay is preferable because it allows an evaluation of population heterogeneity throughout the dose range. The plating efficiency measurement is also a more sensitive indicator of the minimum dose necessary to kill the wild-type cells and to distinguish any unusual resistant clones. The typical dose response curve in a plating assay with increasing concentration of drug in the medium can be demonstrated with the hypothetical survival curve shown below.

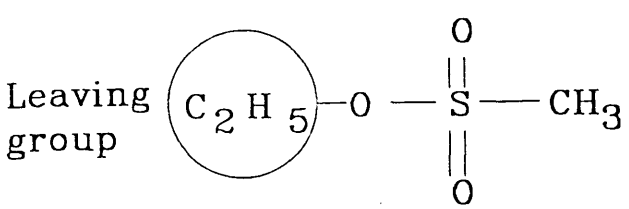


For drug concentrations above a threshold dose of D_1, the plating efficiency declines over a narrow window of drug concentration. For doses higher than D_2, if the curve tails off, as in the dashed lines "A" and "B", discrete surviving colonies may reflect the presence of a sub-population of cells. It is therefore important to pick a concentration of drug at which the colonies selected would be from area "A" or"B" but not from the steeper part of the curve, area "C" (represented by shaded area of the diagram). At area "C" of the curve, colonies selected will not ordinarily represent resistant cells; rather, they probably reflect the statistical nature of colony formation. We examined the dose response curve of A2780 to CDDP over a range of concentrations in the hope that a resistant sub-population of A2780 cells would be found. The resistant sub-population was isolated as clones of the A2780 cells picked at a specific CDDP concentration.

One important question concerning such drug resistant variants is whether or not they they represent gene mutations in specific genes. In cases where the gene or enzyme conferring resistance is unknown, the mutational origin of drug resistance has been implied by mutagens inducing an increase in the frequency of resistant cells and by the stable retention of the resistant phenotype in the absence of selecting drug over a number of cell doubling times, (Thompson and Baker, 1973).

In order to provide evidence for a mutational origin of the isolated clones, the effect of the chemical mutagen ethyl methane sulphonate, EMS, on CDDP resistant variant frequency has been examined. The type of mutation which would cause cells to become resistant to CDDP is not known. It is therefore necessary to choose a mutagen which has been shown to cause various types of mutations. This will therefore increase the chance of the CDDP resistant phenotype being induced by pretreatment with such an agent.

EMS is a monofunctional ethylating agent that has been found to be mutagenic in a wide variety of genetic test systems from viruses to mammals, (for review, Sega, 1984). Genetic data obtained using microorganisms suggest that EMS may produce GC to AT and AT to GC transition mutations (Prakash and Sherman, 1973). There is also some evidence that EMS can cause base pair insertions or deletions (Huang and Baker, 1976) as well as more extensive intragenic deletions (Schalet, 1977). In higher organisms there is evidece that EMS is able to break chromosomes (Abbondandolo, 1982). EMS therefore seemed a good choice of mutagen for use in the single step selection system. The structural formula of EMS is shown below :



To further substantiate the evidence of the clones being of a mutational origin they were subsequently assayed for CDDP resistance after prolonged periods of growth in non-selective media.

Studies in the modulation of CDDP resistance are becoming an important area of research. *In vitro* studies suggest mechanisms of resistance to CDDP could include increased glutathione levels in tumour cells, (Waud, 1987, Hromas et al, 1987, Hamilton et al, 1985), and a capacity for the resistant lines to have an increase in DNA repair, (Masuda et al, 1990). It has therefore been the topic of many studies to modulate resistance by decreasing the glutathione levels or to inhibit the capacity for DNA repair. Buthionine sulfoximin (BSO) inhibits the γ glutamyl synthetase enzyme, an enzyme important in the metabolism of glutathione. BSO has been shown to decrease glutathione levels in cell lines resistant to CDDP resulting in increased sensitivity of the tumour cells to CDDP treatment, (Masuda et al 1990). As discussed in greater depth in the previous chapters increased DNA repair has been reported to be included in the way in which tumour cells can become resistant to CDDP. It has been reported that the major CDDP adducts 1,2 d(GpG) and 1,2 d(ApG) are removed from DNA by the nucleotide excision repair pathway in bacteria (Beck et al, 1985; Husain et al, 1985) and in mammalian cells (Fraval and Roberts, 1979). This pathway requires DNA polymerase activity for restoration of functional DNA. If DNA polymerases are components of a multi-enzyme repair complex or catalyze a rate-limiting reaction in the excision repair process, one might expect the inhibition of DNA polymerase activity to enhance DNA repair. Aphidicolin, an inhibitor of DNA polymerase α, has been shown to increase the cytotoxicity of CDDP in CDDP resistant A2780CP cells, (Masuda et al, 1988). These studies with BSO and aphidocolin have involved specific CDDP resistant lines which have been isolated by multiple rounds of CDDP selection. If resistance modifiers are to be used clinically at the time of first treatment in order to eliminate any resistant sub-populations, then it is important to know how generally effective they are against

resistant mutants in the tumour cell population. In order to assess this, the effect of aphidicolin and BSO on the frequency of cells surviving the single step selection with CDDP in a population of A2780 cells not previously exposed to CDDP, has been examined.

This chapter analyses the DDRPs from the single isolated resistant clones in comparison to clones of the A2780 sensitive parental cell line.

RESULTS

5.2 SELECTION OF CDDP VARIANTS

5.2.1 Dose Response of A2780 cells to CDDP

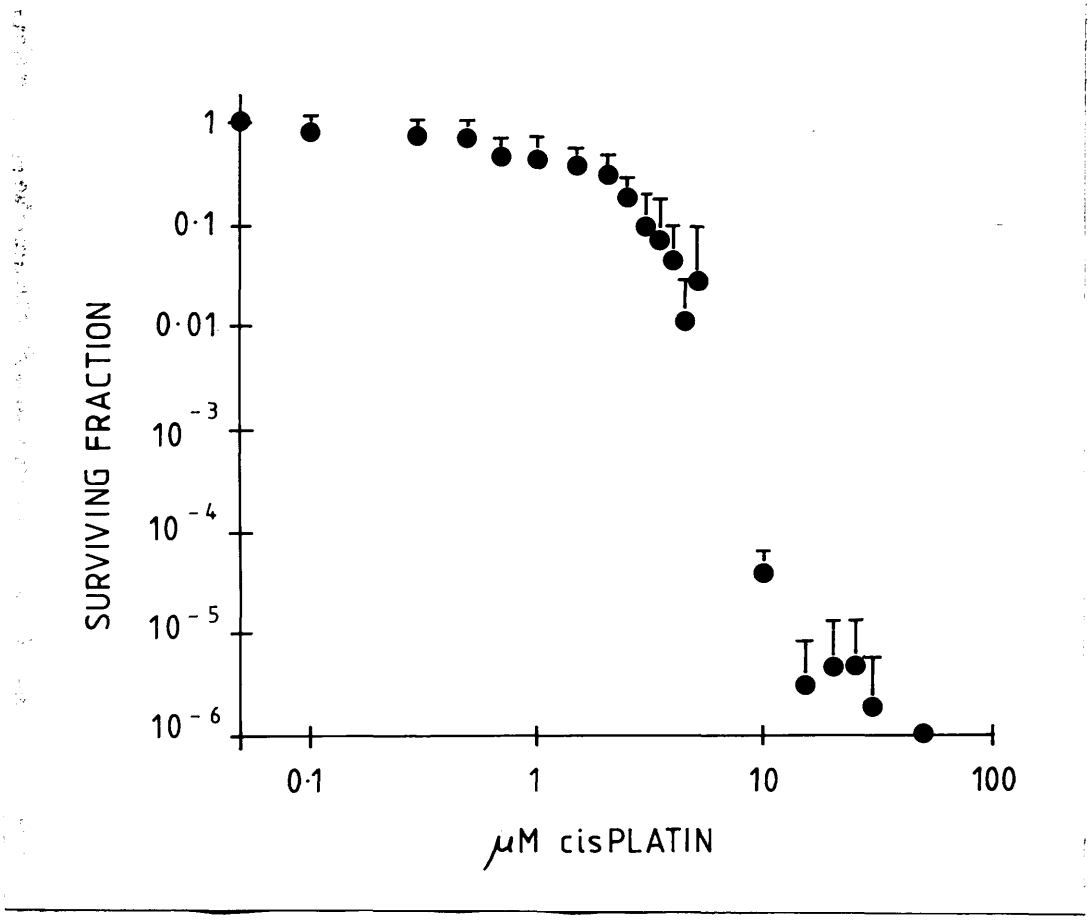
Before CDDP resistant colonies of A2780 could be isolated by a single step selection, a dose response curve of the A2780 over a wide range of CDDP concentration had to be examined.

The surviving fraction of clonogenic cells of the human ovarian tumour cell line A2780 after exposure to various concentrations of CDDP ranging from 0-50 μM is shown in **Figure 5.1.** The concentration of CDDP giving 50% survival (ID_{50} value) is 0.6μM which is comparable with the ID_{50} observed by others using clonogenic assays of A2780 cells (Behrens et al, 1987). At concentrations of CDDP between 15-30μM, a surviving fraction in the range of $2\text{-}5x10^{-6}$ is observed. This appears as a tail in the survival curve, **(Figure 5.1)**, suggesting that a subpopulation of cells exist which are resistant to this concentration of CDDP. It is therefore reasonable to choose a concentration between 15-30μM for the isolation of resistant variants. Selection of the cells with 15μM CDDP, the concentration chosen for the selection of the CDDP resistant variants, gives a spontaneous frequency of colonies surviving of 3.2×10^{-6} surviving colonies per viable cell (refer to **Table 5.1** for figures).

A fluctuation analysis was then employed to calculate the frequencies, (Luria and Delbruck, 1943). This type of analysis of the data eliminates frequency values being affected by daughter colony formation. The equation used for the calculation was :

Figure 5.1

Dose Response Curve for 24h CDDP Treatment in the A2780 Cell Line



Dose response curve for 24h CDDP treatment in the A2780 cell line. Error bars shown are 95% confidence limits.

$$m = -\frac{\ln P_0}{N}$$

m = mutation frequency

P_0= probability of no mutations

N = number of cells

Using a fluctuation analysis, a spontaneous frequency of colonies surviving 15μM CDDP selection of 1.7×10^{-6} per viable cell is observed. The frequency of colonies surviving 15μM CDDP is comparable to known mutation frequencies of other mammalian drug resistant lines (Thompson and Baker, 1973).

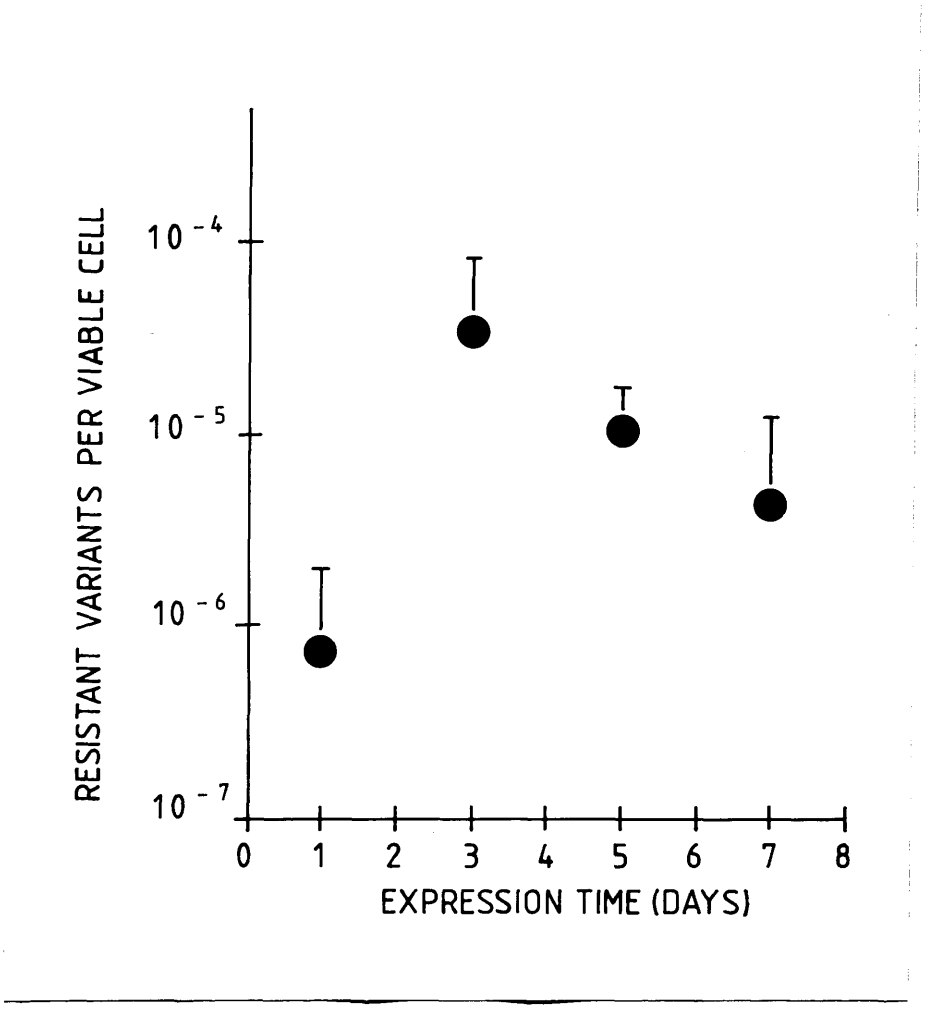
5.2.2 Pretreatment of A2780 with EMS Prior to CDDP Selection

A large population of A2780 cells (5×10^6 cells) were treated with EMS (section 2.6.2.2). After the EMS treatment the cells were left for various periods of time before the selection protocol began.

It has generally been well documented that after treatment of cells with a mutagen, the cells must be maintained for a period under non-selective conditions to permit recovery from sub-lethal damage, fixation of any mutations and expression of mutant genes which would lead to a drug resistant phenotype, (Thompson and Baker 1973). **Figure 5.2** shows an increase in frequency of resistant colonies between days 1 and 3 and supports a time dependant delay in the expression of a mutatant phenotype thus arguing against any increase in the frequency being due to the selection of EMS resistant

Figure 5.2

Effect of Pretreatment of the A2780 Cell Line with EMS prior to CDDP Selection



The number of colonies per viable cell surviving selection with 15μM CDDP when assayed at the time shown after exposure to 2.5mg/ml EMS. Error bars shown are 95% confidence limits.

clones. After the three day period of expression there is a reduction in the frequency at which the variants occur. Similar reduction in mutant frequency at longer expression times have been observed for other drug resistant selection systems, (Thompson and Baker, 1973), and suggests that the CDDP resistant mutants may be selected against during non-selective growth conditions.

The resistant colony frequency 3 days after EMS treatment represents at least a ten fold increase compared to the spontaneous frequency, **(Table 5.1)**. Thus EMS can induce CDDP resistant clonogenic cells, supporting a mutational basis for the resistance.

5.2.3 Maintenance of CDDP Resistant Phenotype

If the cells maintain a drug resistant phenotype in the absence of CDDP selection, this would also support a stable genetic alteration leading to the drug resistant phenotype. After at least 40 generations of growth in non-selective media, the ID_{50} values to CDDP of various clones independantly isolated after selection in 15μM CDDP were assessed using a short term sensitivity assay, the MTT assay, (Mosmann, 1983). Six out of eight clones assayed showed a significant increase in level of resistance to CDDP, with up to a seven fold increase in resistance to CDDP in comparison to the sensitive parental A2780 cell line, **(Figure 5.3)**. This stable retention of a drug resistant phenotype over a period of six months growth in non-selective media is further support for a mutational basis of CDDP resistance.

Table 5.1

EMS induction of CDDP resistant mutants

Treatment	Viability[a]	Mutant frequency[b]
None	1.0	$3.2 \times 10^{-6} \pm 1.5 \times 10^{-6}$ (40)
EMS[c]	0.22	$3.4 \times 10^{-5} \pm 2.5 \times 10^{-5}$ (10)

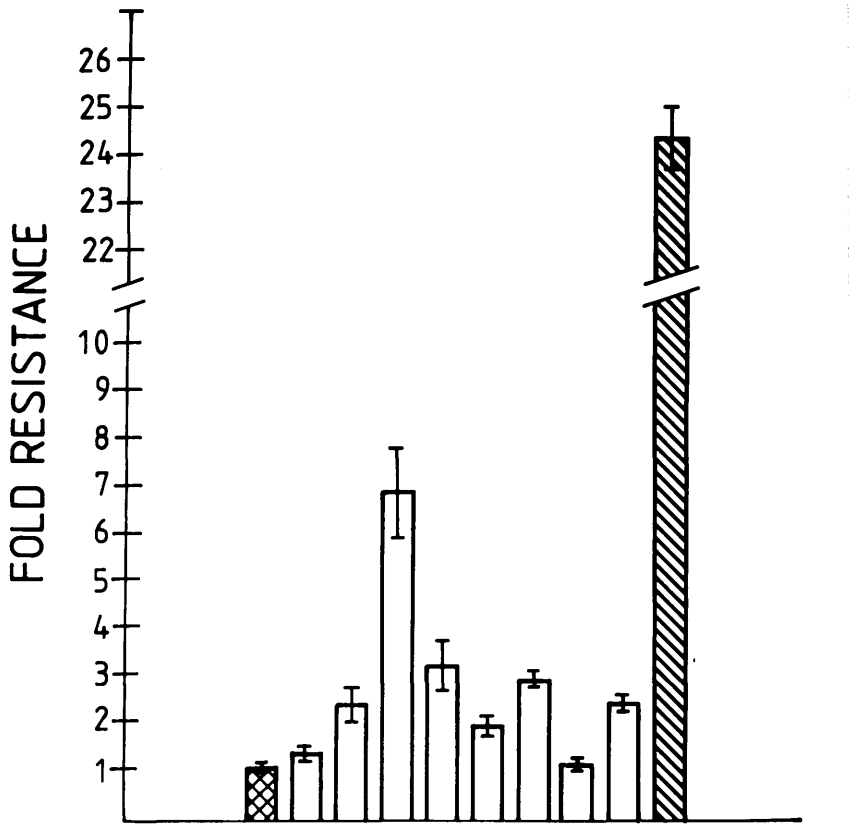
(a) Viability is expressed as fraction of A2780 plating efficiency.

(b) The number of colonies surviving selection with 15μM CDDP per viable cell, with 95% confidence limits shown. Numbers in parenthesis are number of experiments.

(c) 2.5mg/ml EMS for 2 hours. Data shown are for 3 day expression time prior to CDDP selection.

Figure 5.3

Sensitivity to CDDP of Randomly Selected Independant Clones Surviving 15μM CDDP



The sensitivities were measured by MTT assay. The parental A2780 cell line had an average ID_{50} of 0.37μM using the MTT assay. Resistance factors were calculated as the fold increase in the ID_{50} of the lines to CDDP compared to the parental A2780 cell line.

- A2780
- Isolated clones
- A2780CP (multiple selection)

5.3 Modulation of CDDP Resistance with BSO and Aphidicolin

Previous studies suggest that buthionine sulfoximin (BSO), (Hamilton et al 1985), and aphidicolin, (Masuda et al 1990), increase the CDDP cytotoxicity in resistant lines by depleting glutathione levels and inhibiting polymerase α levels respectively. The CDDP resistant lines used in these studies were isolated after multiple exposures to CDDP hence making it unclear how representative these studies would be to all types of resistant mutants surviving CDDP treatment.

Table 5.2 shows the effect of pre-exposure of a population of A2780 cells to either BSO or aphidicolin prior to CDDP selection. Analysis of **Table 5.2** shows that pretreatment with BSO reduces the cell viability in clonogenic assays of A2780 cells as has been previously reported, (Louie et al, 1985). Aphidicolin also markedly reduced the cell viability, in contrast to a previous report, (Masuda et al, 1988). Although the fraction of experiments with zero colonies increases in both the BSO and aphidicolin studies there is no significant difference in the mutant frequency, when the cell viability is taken into account. Both the aphidicolin prior to CDDP treatment and BSO prior to CDDP seem only to have a synergistic toxic effect on the cells with no difference in the frequency of variants surviving.

5.4 Analysis of Isolated Clones on South-Western System.

Having shown differences in an ovarian cancer cell line which is resistant to CDDP in damage recognition proteins, the single step selection clones were examined on a South - Western blot for these proteins.

Table 5.2

Effect of BSO and aphidocolin on CDDP resistant mutation frequency

Modifier	Viability[(a)]	Fraction of experiments with zero colonies	Mutant[(b)] frequency	Mutant[(c)] frequency
None	1.0	34/68	5.2×10^{-6} (1.1×10^{-6})	1.7×10^{-6}
2.5μg/ml Aphidicolin	0.38	22/30	4.5×10^{-6} (2.4×10^{-6})	2.0×10^{-6}
5μg/ml Aphidicolin	0.17	21/23	1.3×10^{-6} (9×10^{-7})	1.3×10^{-6}
25μM BSO	0.41	26/30	2.6×10^{-6} (1.9×10^{-6})	8.7×10^{-7}
50μM BSO	0.20	21/25	8.3×10^{-6} (4.8×10^{-6})	2.1×10^{-6}

(a) Viability is expressed as fraction of A2780 plating efficiency
(b) The number of colonies surviving selection with 15μM CDDP per viable cell. Numbers in parenthesis are the standard error of the mean
(c) The mutant frequency calculated using fluctuation analysis

From **Figure 5.4** it can be seen that from various clones isolated after CDDP selection, no obvious difference in the specific proteins recognising the CDDP adducts was seen. These clones were previously shown to be resistant to CDDP by use of the MTT assay (**Figure 5.3**).

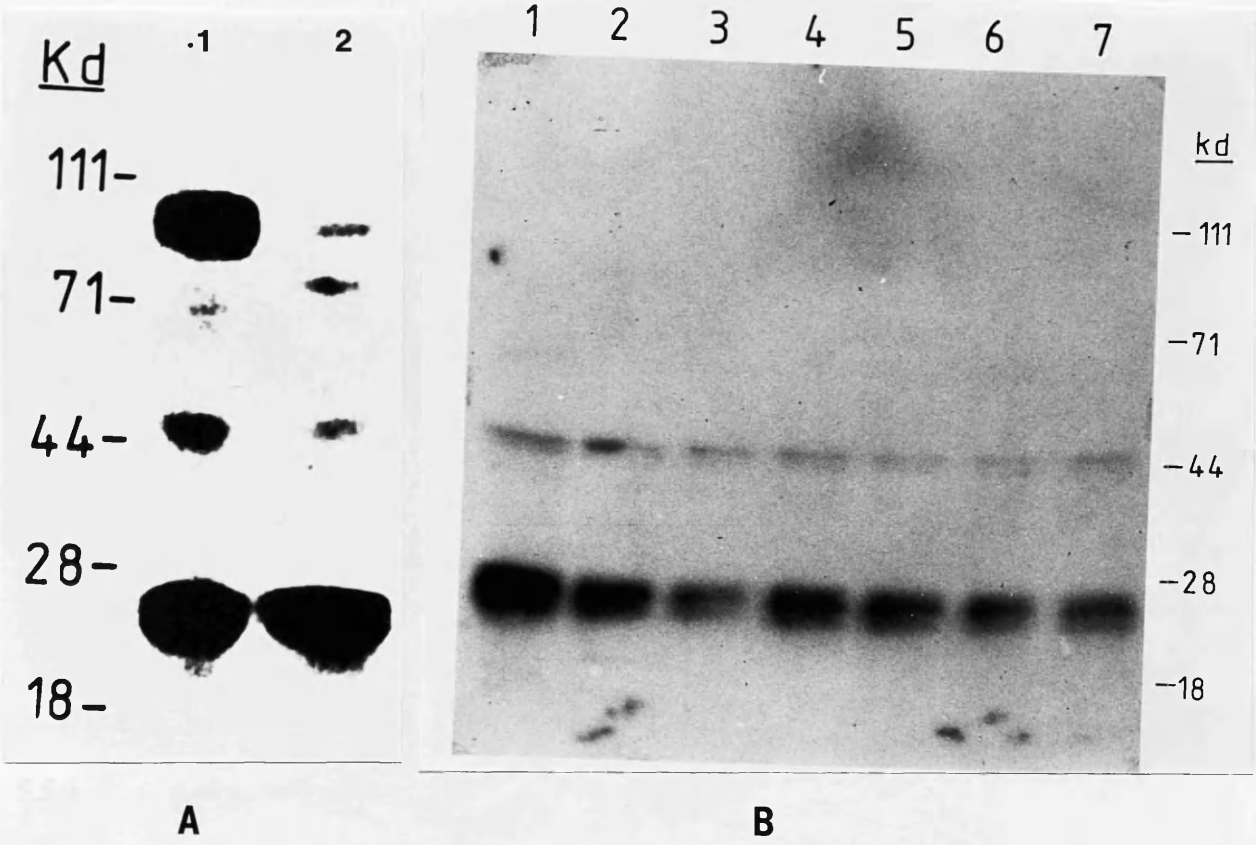
The 100KD protein which is increased in the Ov1DDP cell line, (**Figure 5.4a**), is not detectable in any of the A2780 cell lines or isolated resistant clones, (**Figure 5.4b**). The proteins which seems to be in common within the ovarian cell lines are the 25KD and 50KD proteins but these do not show any difference in the lines resistant to CDDP so suggesting that is not the important protein involved in the mechanism of resistance.

A2780 was plated out in drug free medium at a low cell density and clones of the cell line were picked and expanded. These clones were included on the South-Western blot, (see **Figure 5.4**). It would be possible to identify clones resistant to CDDP by the simple method of picking the clones. If, however, the mutation rate is considered of a cell becoming resistant, then hypothetically a sample of 10^6 clones would have to be examined before one drug resistant mutant would be found. The CDDP in the system is therefore simply acting as a selecting agent. It can be seen that there is no difference in the DDRPs in these picked clones.

In comparing the sensitive A2780 cell line with its resistant counterpart isolated by multiple exposures in the South - Western system no major differences in the specific DDRPs are found. Therefore the resistance phenotype of the A2780 cell lines selected for resistance to CDDP either by multiple exposures to the drug or by a single step selection is not explained in part by an increase of the DDRPs as is described in chapter 4 with the ovarian Ov1DDP cell line.

Figure 5.4

Identification of CDDP-DNA damage binding proteins present in A2780 clones



South-Western analysis of individual clones of A2780 either selected by 15μM CDDP or by seeding A2780 at a low cell density and picking individual clones (CDDP isolated clones shown to be resistant by MTT assay). Damage recognition proteins are identified by incubation of 100μg protein blotted on a nitrocellulose filter with probed CDDP-2αG7. Panel A; Lane 1= Ov1DDP, Lane 2= Ov1p. Panel B; Lane 1= A2780, Lane 2= A2780CP (Behrens et al 1987, Lanes 3 & 4 clones isolated from A2780 seeded out at low cell number; lanes 5-7 clones selected for resistance to CDDP by selection with 15μM CDDP. ID_{50} values of CDDP selected clones examined are Lane 5 = 0.39μM, Lane 6= 0.97μM, Lane 7= 2.4μM. ID_{50} value of A2780 = 0.37μM (Lane 1) - ID_{50} values measured by MTT assay.

5.5 DISCUSSION

Selection of the CDDP variants, generated variants at a frequency of $3.2x10^{-6}$ surviving colonies per viable cell. This frequency was also calculated by the use of the Luria-Delbuck fluctuation analysis. A frequency of colonies surviving 15μM CDDP selection of $1.7x10^{-6}$ per viable cell is observed using this analysis.

The frequency of variants surviving CDDP was calculated by two seperate methods. Whichever method is used to analyse the data, values are generated which are comparable to each other. Since the Luria-Delbruck fluctuation analysis is used in the data analysis, one can compare calculated frequencies knowing that there is little effect from the formation of satellite colonies. Values in the range of $1.7\text{-}3.2x10^{-6}$ surviving colonies per viable cell are observed and these values are comparable with known mutation frequencies of drug resistant mutants in mammalian cells, (Ling,1982).

This leads to the question, are the cells surviving CDDP, mutants, or are they simply variants which will survive CDDP in a random manner without representing stable genetic changes.

5.5.1 Are CDDP Variants of Mutational Origin?

In the CDDP resistant cells derived in vitro, we do not as of yet know which gene is conferring resistance, we are therefore unable to identify a mutation in a specific gene. The mutational origin of drug resistance can however be inferred from circumstantial evidence such as known chemical mutagens inducing an increase in the frequency of resistant cells and by the stable retention of the resistant phenotype in the absence of selecting drug over a number of doubling times of the cells.

Pretreatment of A2780 cells with EMS prior to selection with CDDP causes a ten fold increase in CDDP resistant colonies three days after EMS exposure (**Figure 5.2**)

The increase in frequency of resistant colonies between days 1 and 3 support a time dependant delay in the expression of a mutant phenotype and argues against the EMS cytotoxicity selecting for EMS resistant clones that are cross-resistant to CDDP. The reduction in frequency from day 5 onwards suggests that CDDP resistant mutants may be selected against during non-selective growth conditions. Similar reduction in mutant frequency at longer expression times have been observed for other drug resistant selection systems, (Thompson and Baker, 1973). EMS can thus induce CDDP resistant mutants supporting a mutational basis for resistance.

Six out of eight of the isolated clones, assayed by MTT, showed a significant increase in the level of resistance to CDDP. As great as seven fold difference to CDDP was observed on comparison to the sensitive parental A2780 cell line. The clones which did not show a decrease in the sensitivity may simply be representing variant cells which are not mutational in origin but may have occurred due to selective growth conditions at the time of selection. The stable retention of a drug resistant phenotype over a period of six months growth in non-selective media is further support for a mutational basis of CDDP resistance.

The isolated clones show an increase in resistance as great as seven fold, however upon comparison with the A2780CP derived by multiple exposures to CDDP, this fold resistance is not of the same magnitude, **(Figure 5.3).** The A2780CP cell line was derived *in vitro* by multiple rounds of exposure to CDDP over increasing concentrations. It is therefore likely that the "high" resistance to CDDP requires multiple changes since within the series of selection steps each step may yield resistant variants. Mutants with multiple mechanisms of resistance are highly unlikely to be selected in a single step using a high concentration of drug since simultaneous multiple alterations would be required for such a mutant to survive this selection and based on known mutation frequencies the probability of this occurring would decrease for each clone with increasing mechanisms of resistance. Since the clones have been isolated in a single

exposure to CDDP, it is most likely that their resistance is due to a single type of mutation leading to a sub-population of cells which are resistant to the selecting concentration of CDDP. The isolated clones are therefore an ideal subpopulation to study the mechanism(s) of resistance to CDDP.

5.5.2 Modulation of CDDP Resistance with BSO and Aphidocolin

Studies with BSO in different cell lines have been controversial. Andrews and colleagues have found that depleting GSH did not change the character of CDDP resistance in two ovarian cancer cell line, COLO 316 and 2008 cells (Andrews et al, 1989). Additionally depleting GSH did not overcome CDDP resistance in a human head and neck squamous carcinoma cell line, (Holden et al, 1985). Other studies, however found that GSH content was elevated in the resistant daughter cell line of A2780 and that depleting GSH reversed this resistance, (Hamilton et al, 1985). All of these above studies were carried out in cells made resistant to CDDP by multiple exposures to CDDP. The disagreement in the observations from each study could be due to each different cell line having a different mechanism(s) of resistance to CDDP.

Few studies have used single step selection protocols with chemotherapeutic drugs to examine the frequency of resistant mutants occurring in a cell population. Barranco et al, (1990), have shown that after only one exposure to an LD_{99} dose of melphalan, the surviving population in a human gastric carcinoma cell line was ten times more resistant to melphalan treatment one week later: this resistance eventually increased almost 50-fold after the cells had received the tenth weekly treatment. The initial resistance expressed to melphalan at the time of second treatment was preceded by a two fold elevation in GSH values. BSO partially reversed the expression of resistance to melphalan by inducing a 60% reduction in GSH content. It was therfore of interest to look at the effect of BSO on the frequency at which the CDDP selected variants occur.

Pretreatment of A2780 with a concentration of BSO of 25μM has previously been shown to deplete glutathione levels by 70-90% with minimal toxicity, (Louie et al, 1985). Pretreatment with BSO at a concentration of 50μM has been shown to deplete GSH in a seperate ovarian cancer cell line again with little cytotoxicity, (Green et al, 1984), and also in a gastric carcinoma cell line, (Barranco et al, 1990). Pretreatment of the monolayer of A2780 with seperate concentrations of 25μM and 50μM prior to CDDP selection of the resistant variants and maintenance of the BSO through the CDDP selection was presumed to deplete the GSH and keep the cells in a depleted state.

Analysis of the study with pretreatment with BSO prior to CDDP selection, shows that there is no significant difference in the frequency of variants surviving. On examining viabilities of the A2780 at both concentrations of BSO there is a toxic effect to the cells.

For a definitive conclusion to be drawn on the effect of depletion of GSH by BSO, it would be necessary to measure the GSH content to show that GSH is depleted and the cells are maintained in the depleted state. It has however been previously shown that the concentrations used, deplete GSH content in A2780 cells with minimal toxicity. From our studies the pretreatment with BSO and the continual exposure to BSO during CDDP selection has a toxic effect. At these concentrations of BSO minimal toxicity has been observed previously, (Hamilton et al, 1985). The difference could be due to pretreatment with BSO and then maintenance during the CDDP selection. The CDDP selection in our system is of a much higher and stringent selection, therefore BSO could be having a synergistic toxic effect with CDDP on the A2780 cells. This synergy could further be investigated by the method of Momparler, (1979), which evaluates drug synergy by comparing the survivng fraction produced by drugs individually, with that observed when both drugs are used simultaneously. Synergy is established if the surviving fraction for two drugs is less than the product of the surviving fractions of the drugs individually.

It therefore can only be concluded that pretreatment of the A2780 cells at concentrations of BSO of 25μM and 50μM have a toxic effect on the cells and therefore no conclusion can be drawn on the frequency at which the variants occur with BSO pretreatment.

Previous investigations have suggested a role for DNA polymerase α in the mechanism of resistance to CDDP. Studies have shown that pretreatment of CDDP resistant cells with aphidicolin have increased the cytotoxicity of these cells to CDDP. Work has been carried out in both the A2780 and A2780CP cell lines and it has been shown that aphidicolin causes an increase in the cytotoxicity of A2780CP to CDDP but not of the parental A2780 cell line. These reports also verify that aphidicolin is not cytotoxic by itself, (Masuda et al, 1988). We found treatment of A2780 cells with aphidicolin prior to single step selection with CDDP to have a toxic effect in synergy with CDDP on the cell population. We treated A2780 cells, 1 hour prior to CDDP selection, with either 2.5μg/ml or 5μg/ml aphidicolin (both these concentrations have previously been reported to be non-toxic to A2780 cells (Masuda et al, 1988). The 1 hour exposure to aphidicolin has been reported to show an effect on the cytotoxicity of A2780CP cells to CDDP, (Masuda et al, 1988), by increasing the cytotoxicity by about two fold.

In this work by Masuda, where there was shown to be an effect on the CDDP cytotoxicity in resistant cells, the experiments were carried out in the same manner as they did for the repair replication experiments, in which inhibition of replication was a major factor in the design of the protocol (Masuda et al, 1988). Cells were grown to heavy confluence, switched to arginine deficient medium with 2.5% bovine serum albumin for three days and then exposed to drug. Only after drug treatment were cells split and seeded into tissue culture dishes at low density in complete medium. While this routine enables Masuda and co-workers to perform repair replication experiments, it does so by imposing severe growth restraints on the cells. This near-total growth arrest may

account for the lack of cytotoxicity of aphidocolin they observed. In quiescent cells only about 5% of the DNA polymerase activity is polymerase α, (Kornberg and Gefter,1974). Cells would not be expected to be killed by a DNA polymerase α or δ inhibitor in the absence of appreciable replication or repair. If the A2780 CP cells have a higher level of polymerase α (or δ) that is involved in repair but not required for replication, inhibition of polymerase activity by aphidicolin could restore the sensitivity of these cells to CDDP.

The studies described in this chapter, although following the 1 hour pre-exposure to aphidicolin during CDDP selection, as above, do not inhibit replication. Using the concentrations of aphidicolin, as previously, seem to have a toxic effect upon the cells therefore analysis of the figures of variant frequency becomes very difficult.

Therefore in conclusion, both pretreatment of A2780 with either BSO or aphidicolin prior to CDDP selection causes a toxic effect on the cells, being in diasgreement with previous reports. There can therefore be no final conclusions drawn on the effect of reported modifiers to CDDP on the variant frequency.

5.5.3 DDRPs in single step selection clones

The single step selection clones are an ideal model for the examination of mechanism(s) of resistance to CDDP. Having analysed an ovarian cancer cell line which is resistant to CDDP in a previous chapter and shown that the 100KD DDRP was increased, the single step selection clones were studied on a South-Western.

The 100KD DDRP which is increased in the Ov1DDP cell line, is not detectable in any of the A2780 cell lines or isolated resistant clones. The DDRPs which seems to be in common within the ovarian cell lines is the 25KD and 50KD proteins, but these do not show any detectable difference in the lines resistant to CDDP so suggesting that they are not involved in the mechanism of resistance in this line.

A difference is observed in the 100KD DDRP in the CDDP resistant Ov1DDP cell line but not in the A2780CP resistant line or the resistant isolated clones suggesting that the development of the resistant phenotype in these separate pairs of ovarian cancer cell lines is by different mechanisms.

After this work of the single step selection of resistant A2780 clones was in progress, the study of the DDRPs in various cell lines was initiated. At the time of choice of A2780 as a model system for the single step selection variants nothing was known about the DDRPs. On reflection however, since there is found to be no difference in the DDRPs in the CDDP resistant A2780 cell line which was derived by multiple exposure to CDDP but with a difference being seen in the resistant Ov1, it may have proved more fruitful if the single step selection system had been carried out in the Ov1 cell line. It would be of interest to generate a single step mutant of the Ov1 to examine the DDRPs and compare them to the increase in the DDRPs in the Ov1DDP - the ovarian cell line derived by multiple exposures to CDDP. It is now appropriate to take these resistant clones and study other mechanisms of resistance as described in chapter 1.

CHAPTER 6

GENERAL DISCUSSION

6.1 DAMAGE RECOGNITION PROTEINS (DRPs)

The following discussion will use two abbreviations;

DRPs - Damage recognition proteins

DDRPs - CDDP damage recognition proteins

The DRPs will be used in the general context of proteins binding and recognising damage. The DDRPs will be used in the context of those damage recognition proteins identified in the work in this thesis recognising CDDP damage.

A number of studies in the literature have reported the identification of damage recognition proteins present in mammalian cells (Lehmann and Kirk-Bell, 1978; Feldberg and Grossman, 1976; Huang et al, 1975; Moranelli and Lieberman, 1980; Tsang and Kuhnlein, 1982; Chu and Chang, 1988; Toney et al, 1989; Hirschfeld et al, 1990; Chao et al, 1991[a]; Donahue et al, 1990; Andrews and Jones, 1990). Few of the proteins identified so far have been shown to directly recognise CDDP adducts. Introduced in chapter 3, various assays have been employed in the study of such proteins, including partial purification of DRPs by phosphocellulose column techniques, gel mobility shift assays and South-Western analyses. It is not entirely clear as to whether these techniques are identifying similar proteins or whether a broad spectrum of proteins are being identified. Eventual purification of these proteins will lead to characterisation of each individual protein and comparisons can then be made between them.

Some of the mammalian mutants which have been isolated by their sensitivity to UV light are shown also to be sensitive to CDDP. Both xeroderma pigmentosum cell lines and CHO mutants have been shown to be defective in the incision step of the removal of bulky adducts (Setlow et al, 1969; Thompson et al,1982). It has therefore been suggested that CDDP adducts in mammalian cells, may be recognised and removed in a similar way to UV adducts, for which evidence suggests an excision repair pathway.

Few studies have presented direct evidence on damage recognition proteins recognising CDDP damage. Chu and Chang suggested that the DRPs they identified recognising UV damage, may also be important in the recognition of CDDP adducts (Chu and Chang, 1988). CDDP, unlike UV, forms a variety of adducts in the DNA. Donahue and co-workers therefore tried to identify if damage recognition proteins were rcognising specific adducts. They presented evidence to suggest that the recognition proteins they identified, bound specifically to 1,2d(GpG) and 1,2d(ApG) cross-links (Donahue et al, 1990) which have previously been demonstrated to be the main adducts formed upon reaction with DNA both in vitro and *in vivo* (Eastman, 1986), (further discussion in section 6.3.2). Recently, during the progress of this research, a report suggested the ability of damage recognition proteins, present in ovarian carcinoma and kidney tubule cells, to bind directly to CDDP damaged DNA (Andrews and Jones, 1991). Toney and co-workers have reported on the isolation of cDNAs by screening a human B-cell library with CDDP damaged DNA (Toney et al, 1989). Therefore reports are beginning to provide evidence that damage recognition proteins exist in mammalian cells which recognise and bind to damaged DNA.

Presented in the preceding chapters of this thesis, DDRPs recognising CDDP damage have been directly identified in mammalain cells, both by gel mobility shift assays and South-Western analyses. Evidence has been presented that these proteins are different to those identified in the gel mobility shift assay recognising UV damage (Chu and Chang, 1988). It is important to point out, that it is not clear, as to whether the protein complexes identified by gel mobility shift assays are the same as those identified by South-Western analyses. The South-Western system as described in chapter 4 detects damage recognition proteins under entirely different binding and gel conditions compared to the gel mobility shift assay. In the South-Western system the proteins are separated on a denaturing gel and therefore any secondary structure, as well as complexing of the proteins, will be altered. This therefore suggests that in detection of

these proteins, using this system, secondary structure or complexing are not a critical factor. However in the gel shift system the proteins have the ability to run as a complex, as the gel conditions are non-denaturing. The proteins detected by this system may therefore be present as a complex, or the secondary structure of the proteins may prove important in the recognition of the damage. To try to compare the proteins detected in both systems some characterisation is necessary. Identification of the DNA-protein complex by cross-linking it with a cross-linking agent in the gel shift system will allow one to define the specific location of the complex. This can then be isolated and run in a second dimension, followed by silver staining of the proteins which will allow one to determine the molecular weight of the polypeptide(s). This will allow the comparison of the molecular weights of the polypeptides detected between the gel shift system and the South-Western system. In theory this may prove a fruitful approach, however in practice low levels of the specific factor or other factors which migrate at a similar position to the cross-linked DNA-complex may cause problems. An easier way perhaps, to compare the DDRPs in the two systems, is by purification steps. Fractionation of the protein extract and then analyses of each individual fraction on both the gel mobility shift assay and the South-Western assay may allow one to compare any differences or similarities in binding in each of the fractions.

6.2 ROLE OF DDRPs

If proteins are present in mammalian cells which recognise damage in the DNA then the question of what is the function of this recognition step becomes an issue. Is this step merely a signal for the initiation of other cellular processes to occur? The following discussion on DDRPs will concentrate mainly on speculative roles of the damage recognition proteins identified in this thesis recognising and binding to CDDP damage.

6.2.1 Repair pathway

Evidence has been presented in the literature to suggest that an increased DNA repair capacity may be an important mechanism of resistance to CDDP (Andrews and Howell, 1991). Cell lines, resistant to CDDP, which have shown to have an increase in DNA repair capacity have demonstrated an increase in DNA damage recognition proteins (Chu and Chang, 1990; Chao et al, 1991[a]). A cell strain of XPE has shown the absence of a damage recognition protein which has been found in normal human fibroblasts (Chu and Chang, 1988). It is believed that CDDP adducts are removed by an excision repair pathway. Certain mammalian cell lines defective in an excision repair pathway e.g. XP and CHO mutants, are hypersensitive to the effects of both UV and CDDP damage. This has led to the link that the DNA damage recognition proteins may be repair proteins with functions analogous to the uvrABC present in *E.coli*. It therefore seems apt to propose that the DDRPs presented in this thesis, which recognise and bind to CDDP damage, and of which two show an increase in expression in a CDDP resistant cell line, may function in a repair pathway. The main criticism of this hypothesis with these results is that, the CDDP resistant ovarian tumour cell line which demonstrated the increase in the 50KD and 100KD DDRPs has not been fully characterised for its repair capacity. It therefore is not known as to whether the CDDP resistant Ov1DDP cell line shows any difference in repair capacity compared to the parental CDDP sensitive cell line. At this stage it is therefore only speculative as to whether the DDRPs are involved in the repair of CDDP adducts.

Proteins involved in the excision repair pathway in *E.coli* have other functions as well as the initial recognition of the damage. The uvrABC complex will cause nicks at the area surrounding the bulky adducts in the presence of ATP. It therefore may provide further evidence for the DDRPs identified in this thesis being

involved in repair processes, if such activities as a nicking activity in the presence of an energy source could be demonstrated.

At this stage it is therefore only speculative as to whether the DDRPs are involved in the repair of CDDP adducts They may however be as easily involved in any one of the following which will be discussed in turn :

(1) Protection protein

(2) Signal transduction

(3) CDDP adducts mimicking cellular structures

6.2.2 Protection protein

In the theory of evolution some mutations would lead to survival of a fitter species. Cells may therefore have evolved proteins which protect damage leading to mutations , from its own repair machinery. The protein recognising CDDP damage may therefore have been involved in the protection of certain mutations. Specific adducts formed by CDDP may therefore be recognised and protected by such proteins, allowing the adducts to have a killing effect on the cell. The level of DDRPs, may vary in tumour cells compared to normal cells providing a means for CDDP to be selective in the killing of tumour cells in comparison to normal cells.

6.2.3 Signal transduction

CDDP may bind to a damage recognition protein which in turn may function as a receptor in a signal transduction pathway which will lead to a chain of events eventually ending in cell death. This idea suggests that the cytotoxicity of CDDP may not only be due to a single event but a complex pathway of events which will eventually

lead to cell death. The secondary pathways may include events leading to cell death via apoptosis or inhibition of replication. The DDRPs may act as a signal which inhibits the synthesis of any of the enzymes involved in replication and thus would lead to cell death. CDDP along with other anticancer drugs has been shown to activate apoptosis. The DDRPs could merely be a signal which causes the arrest of cells in G_2 (comparable to the RAD 9 gene in *Saccharomyces cerevisiae*)

6.2.4 CDDP adducts mimicking cellular structures

The normal function of the damage recognition proteins may be an involvement in the regulation of tumour cell gene expression - the protein may be an example of a transcription factor. If specific CDDP adducts were to mimic natural regulatory sequences of genes, the titration of the recognition proteins from these important sequences would result in the regulation of gene expression in the tumour cell being destroyed. Normal cells in which such gene expression may be less critical, would prove to be less vulnerable to the drug. This would give the CDDP a selective killing mode in tumour cells compared to normal cells.

It therefore can be concluded that DDRPs recognising CDDP adducts have been identified in this thesis but the pathway which is followed after damage recognition is not clear cut. Evidence in the literature on enhanced DNA repair capacity in CDDP resistant cells showing an increased level of damage recognition proteins and work showing that a cell line deficient in excision repair demonstrating an absence in a recognition protein is suggestive that these reported damage recognition proteins are involved in repair (Chao et al, 1991[a]; Chu and Chang, 1990). These proteins in the literature all recognise UV damage, therefore a direct comparison has still to be made on recognition proteins recognising CDDP adducts with the cells ability for DNA repair.

6.3 SUBSTRATE SPECIFICITY

This thesis has presented damage recognition protein(s) recognising CDDP damage in mammalian cells. It however should not be assumed that the DDRPs will only recognise CDDP damage. The protein identified by Chu and Chang recognising UV damage was also shown to recognise CDDP damage. The recognition protein identified in *E.coli* has been reported to have a broad range of substrates which it recognises. It is therefore more likely that a cell will recognise different types of damage. The following will discuss substrates that the DDRPs may recognise.

6.3.1 Single-strandedness

CDDP in binding to DNA has been reported to form regions of single-strandedness (Sundquist et al, 1986). Reports from work carried out in bacteriophage T4 have suggested a protein, gene 32 protein, which normally binds to single-stranded DNA, to bind to double-stranded DNA modified with CDDP (Toulme et al, 1983). This therefore suggests that in the bacterial system at least, a protein may be present, which recognises areas of single-strandedness which can be caused by a local opening of several bases when DNA reacts with CDDP. Recent evidence has suggested the involvement of human single-stranded binding proteins, normally involved in eukaryotic DNA replication, also to be involved in excision repair (Coverley et al, 1991). The authors have suggested the single-stranded binding protein to either be involved in a recognition step of the damage or to be involved in the recruitment of DNA polymerase onto incised or gapped DNA. The results presented in the previous chapters suggest that two of the three DDRPs recognising CDDP damage may be single-stranded binding proteins. Antibodies have now been isolated for different subunits of the hSSBs (Kenny

et al, 1990). Experiments using these antibodies in the gel shift system are beginning to provide evidence that band B1 may represent hSSB (personal communication, Dr.C.Clugston, Dept. Medical Oncology, University of Glasgow). The generation of areas of single-strandedness may prove to be an important structural determinant in the type of damage recognised by the DDRPs.

CDDP is known to bind to DNA and generate a disruption over 4-5 base pairs (Anin and Leng, 1990). Presented in chapter 4, oligonucleotides with 3 base pair and 7 base pair mismatches were used as substrates in an attempt to define if the DDRPs simply bind to areas of disruption or whether it is some type of structures the CDDP generates which is recognised. Mismatch correction catalysed *in vitro* by human cell extracts has been reported (Glazer et al, 1987). Although the individual steps in the process have not been defined, proteins recognising G.T, A.C, T.T, T.C mispairs have been reported in human cells (Jiricny et al, 1988; Stephenson and Karran, 1989). Under the conditions which detects binding to the platinated oligonucleotides in the ovarian tumour cells, binding to the mismatch oligonucleotides does not occur. This therefore suggests that the DDRPs being detected in the previous chapters recognise some structural motif generated by the CDDP and not only areas of base pair disruption.

6.3.2 Specific adducts as substrates

Both CDDP and TDDP are known to bind to DNA and cause a spectrum of adducts. TDDP cannot form intra-strand cross-links between adjacent nucleotides and this has therefore led to the suggestion that the d(GpG) and d(ApG) adducts formed uniquely by CDDP are responsible for its cytotoxic activity (Pinto and Lippard, 1985). It has therefore been suggested that if these adducts are the important lesions, there may be a damage recognition protein present in mammalian cells recognising the specific type of adduct. Donahue and co-workers have identified a damage recognition protein

recognising specifically 1,2 intrastrand d(GpG) and d(ApG) cross-links formed only by CDDP (Donahue et al, 1990). If this DDRP were involved in repair then it may be hypothesized that tumour cells contain lower levels of the DDRP recognising these major adducts than normal cells. This would allow CDDP to be selective in its mode of cell killing. The other adducts formed both by CDDP and TDDP will not be repaired and thus will be cytotoxic to both cell types. It would be important to determine therefore if the DDRPs identified in the previous chapters recognising CDDP damage, are recognising specific types of adducts or structures which are acting as a signal for the binding of the DDRPs.

6.3.3 Recognition of other damage by the DDRPs

It may be likely that the DDRPs also recognise adducts induced by UV irradiation, since it has been reported that cells hypersensitive to UV are also hypersensitive to CDDP, therefore suggesting that both these damaging agents are recognised by a similar pathway. Other agents which induce bulky damage or are cross-linkers e.g mitomycin C (MMC) may also be recognised by the DDRPs. There can therefore be no conclusions definitively drawn on the damage the DDRPs are recognising. It will be necessary to carry out experiments in both the assays described in this thesis, to determine whether DNA damaged with other types of agents will compete for the binding of the DDRPs recognising the CDDP damage. From the results presented in this thesis it can only be said that the DDRP binds to CDDP damage. It however cannot be concluded that the DDRPs are specific for CDDP adducts.

6.4 INVOLVEMENT OF DDRPs IN CDDP RESISTANCE

DNA repair has been reported to be involved in the generation of the CDDP resistant phenotype (refer to section 1.9). If the DDRPs are the first step in the recognition of damage, prior to recruitment of other repair enzymes, then it is reasonable to hypothesise that cell lines resistant to CDDP may exhibit increased levels of DDRPs. Chu and Chang have recently shown that CDDP resistant Hela and HT1080 cells have increased levels of DRPs recognising UV damage (Chu and Chang, 1990). Another report has demonstrated that a Hela cell line resistant to CDDP shows increased levels of DRPs recognising UV damage (Chao et al, 1991a). In both these reports the increased levels of DRPs have been correlated with an enhanced capacity for DNA repair in the CDDP resistant cell lines. Evidence therefore exists to suggest that increased levels of DRPs recognising UV damage are present in CDDP resistant cell lines which have shown to have an enhanced capacity for DNA repair.

There is however, at present no direct evidence in the literature on the increase of DRPs recognising CDDP damage in CDDP resistant cell lines. Presented in chapter 4 of this thesis, a difference is seen in the proteins binding to a CDDP damaged DNA probe, by South-western analyses in a CDDP resistant ovarian tumour cell line, Ov1DDP. Little is known about this pair of cell lines, unlike the extensive research reported in the literature in the A2780 cell line. Before any conclusions can be drawn as to whether the increase in DDRPs are involved in the mechanism of repair leading to resistance, some studies in the examination of DNA repair would have to be undertaken in this pair of cell lines. For conclusive results on DDRPs being involved in DNA repair, the proteins would have to be cloned and reintroduced into DNA repair defective mutants. If the mutants returned to their wild type phenotype, then the DDRPs can be said to be involved in DNA repair.

To determine if the increase in the DDRPs occurred frequently, another pair of ovarian tumour cell lines, A2780, sensitive and resistant counterparts, were analysed for the DDRPs. The results presented in this thesis on the analysis of these cell lines show that under the binding conditions used to detect the difference in the Ov1 cell lines, no difference was apparent. This therefore suggests that the DDRPs do not play a role in the mechanism of resistance to CDDP in this ovarian tumour cell line. Many other mechanisms of resistance have been suggested for the way in which a tumour cell population becomes resistant to CDDP (see section 1.8). The A2780 cell line is therefore likely to express its resistance through any one of these mechanisms or a combination, although the work presented here demonstrates the DDRPs are unlikely to be a major factor in the generation of CDDP resistance in A2780 cells.

6.4.1 DDRPs in single step selection clones

Most studies on the mechanisms of resistance reported in the literature, have been carried out in cell lines in which their resistance was derived by multiple exposures to the drug. This method of isolation of CDDP resistant lines is more likely to generate cell lines exhibiting multiple mechanisms of resistance. These lines would therefore provide difficulties in the examination of independent mechanisms of resistance generating the CDDP resistant phenotype. It was therefore important to derive cell lines which were resistant to CDDP by a single-step exposure to the drug. This type of isolation of a CDDP resistant clone may yield a range of resistant clones for analysis which may be more relevant to resistance occurring *in vivo*.

Chapter 6 presents the isolation of such resistant clones from the A2780 tumour cell line and the frequency at which they occur in the population. These clones allowed the analysis of the DDRPs in the generation of the CDDP resistant phenotype without the influence of other factors. Of the clones examined there was no detectable

difference in any of the DDRPs, under the binding conditions that demonstrate the difference in the 50KD and 100KD in the Ov1DDP. This work on the isolation of the single step selection clones was initiated at the beginning of the project prior to any difference being shown in the DDRPs of the CDDP resistant cell line Ov1DDP. On reflection, it may have proved more beneficial to isolate clones by single step selection to CDDP of the Ov1p sensitive parental cell line. The isolated resistant clones, although not showing any change in DDRPs, may express their resistant phenotype by other mechanisms of resistance and thus may provide excellent models for the examination of the phenotypes. The single step selection clones and the resistance mechanisms generated within them are more likely to be clinically relevant than the mechanisms of resistance to CDDP defined in cell lines made resistant to CDDP by multiple exposures to the drug.

6.5 CLINICAL RELEVANCE OF DDRPs

6.5.1 Use of *in vitro* assays for measurements in tumours

The mechanisms of resistance studies so far in the literature have all been reported from work carried out in cell lines. It is therefore not entirely clear as to how much relevance these studies have *in vivo*. The assays used in cell lines to study resistance e.g UDS, plasmid reactivation, accumulation studies cannot be applied to tumour material. We are therefore left with the question of whether the mechanisms of resistance studied *in vitro* have any relevance to those studied *in vivo*. The type of *in vitro* assays presented in this thesis can however be applied to tumour material thus allowing one to determine the importance of DDRPs *in vivo* and not just in *in vitro* derived cell lines.

6.5.2 Involvement of DDRPs in tumorigenecity

It has been reported that CDDP may be selective in the killing of tumour cells by the cell's differential ability to repair certain types of adducts (Ciccarelli et al, 1985). It has generally been hypothesized that tumour cells are less likely to be involved in repair mechanisms than normal cells. In the development of a cell line from a tumour it may be hypothesized that it has very low levels of repair, therefore if the identified DDRPs were involved in DNA repair, it may be suggested that tumour cells express lower levels of the DRPs than normal wild-type cells. The resistant Ov1DDP cell line in these terms may be showing levels of DDRPs comparable with wild-type normal cells. The DDRPs may therefore per se not be involved in the development to resistance but in the development of tumour cells. If this hypothesis were true, then an explanation for no differences occurring in the DDRPs of A2780 may be in the responsiveness of the tumour *in vivo*.

6.5.3 Modulation of resistance

If tumours become resistant to chemotherapeutic drugs, modulation becomes an issue. Studies in cell lines resistant to CDDP have suggested modulation through the DNA repair pathways by agents such as aphidocolin or through the GSH pathway by BSO (refer to section 5.1). Problems would occur with these type of modulators *in vivo*. The aphidicolin and BSO would not only affect the tumour cells but also cause effects on the normal cells which could prove cytotoxic to the cells, therefore not only killing the tumour cells but also the normal cells. If the DDRPs are involved in a recognition step prior to other events occurring and are important in mechanisms of resistance to CDDP *in vivo*, they may prove a potentially important target in the modulation of CDDP resistance occurring in tumours. Both systems described in this thesis may prove potentially

important in the screening of compounds for modulation of binding of the DDRPs if they were proven to be important in the CDDP resistant phenotype.

6.6 CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has presented proteins which recognise and bind to CDDP damaged DNA. The role of these recognition proteins has not yet been defined. It has been suggested that they could be involved in a DNA repair role, however there is no evidence to suggest that they are not involved in protection mechanisms, signal transduction pathways or as regulatory proteins of tumour cell gene expression. The DDRPs are not likely to recognise CDDP only, but some structural motif which is generated by CDDP and also other damaging agents. CDDP has been reported to bind to DNA and generate single-stranded regions of DNA around the adducts. Evidence is presented to suggest that the DDRPs may be recognising these regions of single-strandedness.

The involvement of the DDRPs in the generation of the CDDP resistant phenotype in an ovarian tumour cell has been presented. If the DDRPs are important *in vivo* then they may provide a potential target for modulation.

It is necessary for the DDRPs to be isolated and characterised before any definitions can be made on its role both *in vitro* and *in vivo*. Once the protein has been characterised, antibodies can be raised which may eventually have clinical applications if the DDRPs are demonstrated to be important *in vivo*.

REFERENCES

Abbondandolo,A., Dogliotti,E., Lohman,P.H.M., Berends,F. (1982) Molecular dosimetry of DNA damage caused by alkylation, I. Single-strand breaks induced by ethylating agents in cultured mammalian cells in relation to survival. Mutation Research 92, 361-377

Andrews,P.A., Murphy,M.P., Howell,S.B. (1989) Characterization of cisplatin-resistant COLO 316 human ovarian carcinoma cells. Eur.J.Can.Clin.Oncol. 25(4), 619-625

Andrews,P.A., Howell,S.B. (1990) Cellular pharmacology of CDDP : perspectives on mechanisms of acquired resistance. Cancer Cells Vol 2 No.2, 35-43

Andrews,P.A., Jones,J.A. (1991) Characterization of binding proteins from ovarian carcinoma and kidney tubule cells that are specific for cisplatin modified DNA. Cancer Communications 3(1), 1-10

Anin,M.F., Leng,M. (1990) Distortions induced in double-stranded oligonucleotides by the binding of cis- and trans-diammine-dichloroplatinum(II) to the d(GTG) sequence. N.A.R.18(15), 4395-4400

Arends,M.J, Morris,R.G. and Wylie,A.H. (1990) Apoptosis; the role of the endonuclease. Am.J.Pathol.136, 593

Barranco,S.C., Townsend,C.M.,Jr., Weintraub,B., Beasley,E.G., MacLean,K.K., Shaeffer,J., Liu,N.H., Schellerberg,K. (1990) Changes in glutathione content and resistance to anticancer agents in human stomach cancer cells induced by treatment with melphalan *in vitro*. Cancer Res.50, 3614-3618

Beck and Brubaker (1973) Effect of cis-platinum(II) diamminedichloride on wild type and deoxyribonucleic acid repair deficient mutants of *Escherichia coli*. J.Bacteriol.116, 1247-1252

Beck,D.J., Popoff,S., Sancar,A., Rupp,W.D. (1985) Reactions of the UVRABC excision nuclease with DNA damaged by diamminedichloroplatinum(II). N.A.R.13, 7395-7412

Bedford, P., Fichtinger-Schepman, A.M.J., Shellard, S.A., Walker, M.C., Masters, J.R.W., Hill,B. (1988) Differential repair of platinum-DNA adducts in human bladder and testicular tumour continuous cell lines. Cancer Research 48, 3019-3024

Behrens,B.C., Hamilton,T.C., Masuda,H., Grotzinger,K.R., Whang-Peng,J., Louie,K.G., Knutsen,T., M^{c}Koy,W.M., Young,R.C., Ozols,R.F. (1987) Characterization of a cis-diamminedichloroplatinum (II) resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Can.Res.47, 414-418

Benard,J., DaSilva,J., DeBlois,M.C., Boyer,P., Duvillard,P., Chiric,E. (1985) Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and nude mice. Can.Res. 45, 4970-4979

Bianch, V., Nuzzo, F., Abbondandolo, A., Bonatti, S., Capelli, E., Fiori, R., Giulotto, E., Mazzaccaro, A., Stefanini,M., Zaccaro, L., Zantedeschi, A., Levis, A.G. (1982) Scintillometric determination of DNA repair in human cell lines - A critical appraisal. Mutation Research 93, 447-463

Boer,J.G.D.,Glickman,B.W. (1989) Sequence specificity of mutation induced by the anti-tumour drug cisplatin in the CHO aprt gene. Carcinogenesis 10, 1363-1367

Bohr, V.A., Phillips, D.H., Hanawalt, P.C. (1987) Heterogeneous DNA damage and repair in the mammalian genome. Cancer Research 47, 6426-6436

Bootsma,D., Westerveld,A., Hoeijmakers,J.H.J. (1988) DNA repair in human cells; from genetic complementation to isolation of genes. Cancer Surveys 7,303-315

Bowen,B., Steinberg,J., Laemmeli,U.K., Weintraub,H. (1980) The detection of DNA binding proteins by protein blotting. Nucleic Acid Research 3,1-20

Bradford.M.(1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248

Bradley,G.,J uranka,P.F., Ling,V. (1988) Mechanisms of multidrug resistance. Biochemica Biophysica Acta 948, 87-128

Brouwer,J., van de Putte,P., Fichtinger-Schepman,A.J., Reedijk,J. (1981) Base-pair substitution hotspots in GAG and GCG nucleotide sequences in *Escherichia coli* K-12 induced by cis-diamminedichloroplatinum(II). P.N.A.S.(USA) 78, 7010-7014

Burnouf, D., Gauthier, C., Chottard, J., Fuchs, R.P. (1990) Single d(ApG)/cis-diamminedichloroplatinum (II) adduct-induced mutagenesis in *Escherichia coli*. P.N.A.S.(USA) 87, 6087-6091

Burnouf,D., Daune,M., Fuchs,R.P.P. (1987) Spectrum of cisplatin induced mutations in *Escherichia coli*. P.N.A.S.(USA) 84, 3758-3762

Busch,D., Greiner,C., Lewis,K., Ford,R., Adair,G., Thompson,L.H. (1989) Summary of complementation groups of UV sensitive CHO cell mutants isolated by large scale screening. Mutagenesis 4, 349-354

Campbell,J.L. (1986) Eukaryotic DNA replication. Ann.Rev.Biochem.55,733-771

Caron,P.R., Kushner,S.R., Grossman,L. (1985) Involvement of helicase II (uvrD gene product) and DNA polymerase I in excision mediated by the uvrABC protein complex. P.N.A.S.(USA) 82, 4925-4929

Caron,P.R., Grossman,L. (1988)[a] Involvement of a cryptic ATPase activity of UvrB and its proteolysis product, UvrB* in DNA repair. Nucleic Acid Research 16, 9651-9662

Caron.P.R., Grossman,L. (1988)[b] Incision of damaged vs nondamaged DNA by the *Escherichia coli*. Nucleic Acids Research 16, 9641-9650

Chao,C.C.K., Huang,S.L., Huang,H., Lin-Chao,S. (1991)[(a)] Cross-resistance to UV radiation of a cisplatin-resistant human cell line; overexpression of cellular factors that recognize UV-modified DNA. Molecular and Cellular Biology 11(4), 2075-2080

Chao,C.C.K., Lee,Y.L., Cheng,P.W., Lin-Chao,S. (1991)[(b)] Enhanced host cell reactivation of damaged plasmid DNA in Hela cells resistant to cis-Diamminedichloroplatinum(II). Cancer Research 51, 601-605

Chu,G., Berg,P. (1987) DNA cross-linked by cisplatin: a new probe for the DNA repair defect in xeroderma pigmentosum. Mol.Biol.Med 4, 227-290

Chu,G., Chang,E. (1988) Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. Science 242, 564-567

Chu,G., Chang,E. (1990) Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. P.N.A.S.(USA) 87, 3324-3327

Ciccarelli,R.B., Solomon,M.J., Varshavsky,A., Lippard,S.J. (1985) *In vivo* effects of cis- and trans-Diamminedichloroplatinum(II) on SV40 chromosomes: differential repair, DNA-protein cross-linking, and inhibition of replication. Biochemistry 24, 7533-7540

Cleaver,J.E., Kramer,K.H. (1989) Xeroderma Pigmentosum. In The Metabolic Basis of Inherited Disease, vol.11, C.R.Scriver, A.L.Beaudet, W.S.Sly, and D.Valle, eds (New York: M^{C}Graw-Hill), pp2949-2971

Coverley,D., Kenny,M., Munn,M., Rupp,W.D., Lane,D.P., Wood,R.D. (1991) Requirement for the replication protein SSB in human DNA excision repair. Nature 349,538-541

Cox,B.S., Game,J.C. (1974) Repair systems in *Saccharomyces*. Mutation Research 20, 257-264

Cox,M.M., Lehman,I.R. (1987) Enzymes of general recombination. Ann.Rev.Biochem.56, 229-62

Dijt,F.J., Fichtinger-Schepman,A.J., Berends,F., Reedijk,J. (1988) Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibrobalsts. Cancer Research 48, 6058-6062

Donahue,B.A., Augot,M., Bellon,S.F., Treiber,D.K., Toney,J.H., Lippard,S.J., Essigman,J.M. (1990) Characterisation of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. Biochemistry 29, 5872-5880

Doolittle,R.F., Johnson,M.S., Husain,I., VanHouten,B., Thomas,D.C, Sancar,A. (1986) Domainal evolution of a prokaryotic DNA repair protein and its relationship to active-transport proteins. Nature 323, 451-453

Dresler,S.L., Lieberman,M.W. (1983) Reqirement of ATP for specific incision of ultraviolet-damaged DNA during excision repair in permeable human fibroblasts. J.Biol.Chem.258,12269-12273

Eastman,A. (1982) Comparison of the interaction of cis- and trans-diamminedichloroplatinum(II) with DNA by a simple filter binding assay. Biochem.Biophys.Res.Commun. 105, 869

Eastman,A. (1986) Reevaluation of interaction of cis-Dichloro(ethylenediamine)platinum(II) with DNA. Biochemistry 25, 3912-3915

Eastman,A. and Schulte,N. (1988) Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). Biochemistry 27, 4730-4734

Eastman,A. (1990) Activation of programmed cell death by anticancer agents; cisplatin as a model system. Cancer Cells 2, 275-280

Eisen,A., Camerini-Otero,R.D. (1988) A recombinase from Drosphila melanogaster embryos. P.N.A.S.(USA) 85, 7481-7485

Elespuru,R.K. (1987) Inducible responses to DNA damage in bacteria and mammalian cells. Environ.Mol.Mutagen 10,97-116

Erickson,L.C., Zwelling,L.A., Ducore,J.M., Sharkey, N.A., Kohn,K.W. (1981) Differential cytotoxicity and DNA cross-linking in normal and transformed human fibroblasts treated with cis-diamminedichloroplatinum(II). Cancer Research 41, 2791-2794

Fairman,M.P., Stillman,B. (1988) Cellular factors required for multiple stages of SV40 DNA replication. EMBO.J. 7, 1211-1218

Feldberg,R.S., Grossman,L. (1976) A DNA binding protein from human placenta specific for ultraviolet damaged DNA. Biochemistry 15(11), 2402-2408

Fichtinger-Schepman,A.J., Lohman,P.H.M., Reedijk,J. (1982) Detection and quantification of adducts formed upon interaction of diamminedichloroplatinum(II) with DNA by anion exchane chromatography after enzymatic degredation. N.A.R. 10,5345-5356

Fichtinger-Schepman,A.J., van der Veer,J.L., denHartog,J.H.J., Lohman,P.H.M., Reedijk,J. (1985) Adducts of the antitumour drug cis-Diamminedichloroplatinum(II) with DNA; formation, identification, and quantitation. Biochemistry, 24, 707-713

Fichtinger-Schepman,A.M.J., Lohman,P.H.M., Berends,F., Reed,E. and VanOosterom, A.T. (1986) Interaction of the antitumour drug cisplatin with DNA *in vitro* and *in vivo*. In; Schmahl and Kaldser,J.E (eds) The geneticity of alkylating cytostatic drugs,83-99. IARC Sc.Publ. Lyon,France; International agency for research on cancer.

Fichtinger-Schepman,A.M.J., VanOosterman,A.T, Lohman,P.H.M. (1987) cis-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients; quantitative immuno-chemical detection of the adduct induction and removal after a single dose of cis-Diamminedichloroplatinum(II). Cancer Research 47, 3000-30004

Fichtinger-Schepman,A.J., Baan,R.A., Berends,F. (1989) Influence of the degree of DNA modification on the immunochemical determination of cisplatin-DNA adduct levels. Carcinogenesis 10, 2367-2369

Fram,R.J., Cusick,P.S., Wilson,J.M., Marinus,M.G. (1985) Mismatch repair of cis-Diamminedichloroplatinum(II)-Induced DNA damage. Molecular Pharmacology,28, 51-55

Fraval, H.N., Roberts, J.J. (1979) Excision repair of cis-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Research 39 (5), 1793-1797

Friedberg,E.C. (1988) Deoxyribonucleic acid repair in the yeast *Saccharomyces cerevisiae*. Microbiological Reviews Mar,1988, 70-102

Garner,M.M, Revzin,A. (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions : applications to components of Escherichia coli lactose operon regulatory system. NAR Vol 9 No. 13, 3047-3060

Gilman, A. (1963) The initial clinical trials of nitrogen mustard. Am.J.Surgery 105, 574-578

Glazer, P.M., Sarkar, S.N., Chisholm,G.E., Summers, W.C. (1987) DNA mismatch repair detected in human cell extracts. Molecular and Cellular Biology 7(1); 218-224

Glazer,P.M., Greggio,N.A., Methreall,J.E., Summers,W.C. (1989) UV-induced DNA-binding proteins in human cells. P.N.A.S.(USA) 86, 1163-1167

Goldie,J.H., Coldman,A.J. (1984) The genetic origin of drug resistance in neoplasms : Implications for systemic therapy. Can.Res.44, 3643-3653

Green,J.A., Vistica,D.T., Young,R.C., Hamilton,T.C., Rogan,A.M., Ozols,R.F. (1984) Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. Can.Res.44, 5427-5431

Grossman.L., Yeung.A.T. (1990) The uvrABC endonuclease of *Escherichia coli.* Photochem.Photobiol.51, 749-755

Hamilton,T.C., Winker,M.A., Louie,K.G., Batist,G., Berens,B.C., Tsuro,T., Grotzinger,K.R., M^{c}Koy,W.M., Young,R.C., Ozols,R.F. (1985) Augumentation of adriamycin, melphalan and cisplatin cytotoxicity in drug resistant and sensitive human ovarian cancer cell lines by buthionine sulphoximine-mediated depletion of glutathione. Biochem. Pharmacol. 34, 2583-2586

Hansson,J., Wood,R. (1989) Repair synthesis by human cell extracts in DNA damaged by cis- and trans-diamminedichloroplatinum(II). Nucleic Acid Research 18,35-40

Hansson,J., Grossman,L., Lindahl, T., Wood,R.D. (1990) Complementation of the xeroderma pigmentosum DNA repair synthesis defect with Escherichia coli UvrABC proteins in a cell free-system. Nucleic Acid Research 18, 35-40

Harder,H.C. and Rosenberg,B. (1970) Inhibitory effects of anti-tumour platinum compounds on DNA, RNA and protein synthesis in mammalian cells *in vitro.* Int.J.Cancer 6, 207

Haynes,R.H., Kunz,B.A. (1981) DNA repair and mutagenesis in yeast, p.371-414. In J.Strathern,E.W.Jones and J.R.Broach (ed.), The molecular biology of the yeast *Saccharomyces.* Life Cycle and inheritance. Cold Spring Harbor Laboratory,Cold Spring Harbor,New York

Hickson,I.D., Harris,A.L. (1988) Mammalian DNA repair - use of mutants hypersensitive to cytotoxic agents. TIGS 4(4), 101-106

Hirschfeld,S., Levine,A.S., Ozato,K., Protic,M. (1990) A constitutive damage-specific DNA-binding protein is synthesised at higher levels in UV-irradiated DNA. Molecular and Cellular Biology 10(5), 2041-2048

Hoeijmakers,J.H.J., vanDuin,M., Westerveld,A., Yasui,A., Bootsma,D. (1986) Identificaton of DNA repair genes in the human genome. Cold Spring Harbor Symp.Quant.Biol 51, 91-101

Holden, S.A., Teicher,B.A., Cucchi,C.C., Frei,E. (1985) Cross-resistance patterns and the mechanism of resistance of a human head and neck squamous carcinoma cell line resistant to cis-diamminedichloroplatinum(II). Proc.Am.Assoc.Cancer Research 26, 261

Hollander,M.C., Fornace,A.J.(jr) (1989) Induction of fos RNA by DNA-damaging agents. Cancer Research 49, 1687-1692

Howle,J.A. and Gale,G.R. (1970) cis-dichlorodiammineplatinum(II). Persistent and selective inhibition of deoxyribonucleic acid synthesis *in vivo*. Biochem.Pharmacol.19, 2757

Hoy,C.A., Thompson,L.H., Mooney,C.L., Salazar,E.P. (1985) Defective DNA cross-link removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. Cancer Research 45, 1737-1743

Hromas R.A., Andrews.P.A., Murphy M.P.,B urns C.P. (1987) Glutathione depletion reverses CDDP resistance in murine L1210 Leukemic cells. Cancer Letters,34, 9

Huang,A.T., Riddle,M.M., Koons,L.S. (1975) Some properties of a DNA-unwinding protein unique to lymphocytes from chronic lymphocyte leukemia. Cancer Research 35, 981-986

Huang,S.L., Baker,B.S. (1976) The mutability of the Minute loci of *Drosophila melanogaster* with ethylmethane-sulfonate. Mutation Research 34, 407-414

Huberman,J.A., Kornberg,A., Alberts,B. (1971) Stimulation of T4 bacteriophage DNA polymerase by the protein product of T4 gene 32. J.Mol.Biol.62, 39-52.

Husain,I., Chaney,S.G., Sancar,A. (1985) Repair of cis-platinum-DNA adducts by ABC excinuclease *in vivo* and *in vitro*. J.Bacteriology 163 (3), 817-823

Jensen,D.E., Kelly,R.C., vonHippel,P.H. (1976) DNA "melting" proteins. II. Effects of bacteriophage T4 gene 32-protein binding on the conformation and stability of nucleic acid structures. J.Biol.Chem.251, 7215-7228

Jiricny,J., Hughes,M., Corman,N., Rudkin,B. (1988) A human 200kDa protein binds selectively to DNA fragments containing G.T mismatches. P.N.A.S.(USA) 85, 8860-8864

Johnson,R.T., Elliot,G.C., Squires,S., Joysey,V.C. (1989) Lack of complementation between xeroderma pigmentosum complementation group D and H. Human Genetics 81, 203-210

Jones, J.C., Zhen, W., Reed, E., Parker, P.,Sancar, A., Bohr, V.A. (1991) Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J.Biol.Chem.266(11), 7101-7107

Kartner,N. and Ling,V. (1989) Multidrug resistance in cancer. Scientific American 260(3), 44-51

Kataoka,H., Fujiwara,Y. (1991) UV damage-specific DNA-binding protein in xeroderma pigmentosum complementation group E. Biochem.Biophys.Res.Comm.175, 1139-1143

Kawai, K., Amatani, N., Georges, E., Ling, V. (1990) Identification of a membrane glycoprotein overexpressed in murine lymphoma sublines resistant to cis-Diamminedichloroplatinum (II). J.Biol.Chem. 265 (22), 13137-13142

Kelley,S.L., Basu,A., Teicher,B.A., Hacker,M.P., Hamer,D.H., Lazo,J.S. (1988) Overexpression of metallothionein confers resistance to anticancer drugs. Science 241,1813

Kenny,M.K., Schlegel,U., Furneaux,H., Hurwitz,J. (1990) The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. J.Biol.Chem. 265(13), 7693-7700

Kerr,J.F.R., Wylie,A.H. and Currie,A.R. (1972) Apoptosis. A basic biological phenomenon with wider implications in tissue kinetics. Br.J.Cancer 26, 239

Kociba,R.J., Sleight,S.D. and Rosenberg,B. (1970) Inhibition of Dunning ascitic leukemia and Walker 256 carcinosarcoma with cis-diamminedichloroplatinum. Cancer Chemother.Rep.54, 325-328

Kohn,K.W., Grimek-Ewig, R.A. (1973) Alkaline elution analysis, a new approach to the study of DNA single-strand interruptions in cells. Cancer Research 33, 1849-1853

Kornberg,T., Gefter,M.L. (1974) Deoxyribonucleic acid polymerase 3 (*Escherichia coli* K12). Methods in enzymology 29, 22-26

Kozinski,A.W., Felgenhauer,Z.Z. (1967) Molecular recombination in T4 bacteriophage deoxyribonucleic acid II. Single-strand breaks and exposure of uncomplemented areas as a prerequisite for recombination. J.Virol. 1, 1193-1202

Kraker,A.J. and Moore,C.W. (1987) Characterization of reduced transport and DNA repair in Pt-resistant murine leukemia cells in vitro (abstr.). Proc.Am.Assoc.Cancer Res.28, 317

Kraker,A.J. and Moore,C.W. (1988) Elevated DNA polymerase beta activity in a cis-diamminedichloroplatinum(II) resistant P388 murine leukemia cell line. Cancer Lett.38, 307-314

Krisch,H.M., vanHouwe,G. (1976) Stimulation of the synthesis of bacteriophage T4 gene 32 protein by ultraviolet light irradiation. J.Mol.Biol. 108, 67-81

Kuhnlein,U., Tsang,S.S., Lokken,O., Tong,S., Twa,D. (1983) Cell lines from xeroderma pigmentosum complementation group A lack a single-stranded-DNA-binding activity. Bioscience Reports 3, 667-674

Kyhse -Andersen,J. (1984) Electroblotting of multiple gels : A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J.Biochemical and Biophysical Methods 10(3-4), 203-209

Lai,G., Ozols,R.F., Smyth,J.F., Young,R.C., Hamilton,T.C. (1988) Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. Biochem.Pharmacol. 37, 4597-4600

Lasko,D.D., Tomkinson,A.E., Lindahl,T. (1990) Eukaryotic DNA ligases. Mutation Research 236, 277-287

Lehman, A.R., Kirk-Bell,S. (1978) DNA binding proteins in xeroderms pigmentosum. Exp.Cell Res. 114,197-201

Leopold, W., Batzinger, R.P., Miller, E.C., Miller,J.A., Earhart, R.H. (1981) Mutagenicity, tumorigenicity, and electrophilic reactivity of the stereisomeric platinum(II) complexes of 1,2-Diaminocyclohexane. Cancer Research 41, 4368-4377

Leopold,W.R., Miller,E.C., Miller,J.A. (1979) Carcinogenecity of antitumour cis-Platinum(II) coordination complexes in the mouse and rat. Cancer Research 39, 913-918

Lindahl,T. (1974) An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. P.N.A.S.(USA)71, 3649-3654

Lindahl,T. (1982) DNA repair enzymes. Ann.Rev.Biochem.51, 61-87

Lindahl,T. and Sedgwick.B. (1988) Regulation and expression of the adaptive response to alkylating agents. Ann.Rev.Biochem. 57, 133-157

Ling.V.(1982) Genetic basis of drug resistance in mammalian cells. Drug and Hormone Resistance in Neoplasia. CRC Press, Miami Vol.1, 1-19

Linn,S. (1991) How many pols does it take to replicate nuclear DNA. Cell 66, 185-187

Lippard,S.J., Ushay,H.M., Merkel,C.M., Poirer,M.C. (1983) Use of antibodies to probe the stereochemistry of antitumour platinum drug binding to deoxyribonucleic acid. Biochemistry, 22, 5165-5168

Little ,J.W. and Mount,D.W. (1982) The SOS regulatory system of *Escherichia coli.* Cell 29, 11-22

Loehrer,P.J., Einhorn,L.H. (1984) Cisplatin. Ann.Intern.Med.100, 704-713

Louie,K.G., Behrens,B.C., Kinsella,T.J., Hamilton,T.C., Grotzinger,K.R., M^{c}Koy,W.M., Winker,M.A., Ozols,R.F.(1985). Radiation survival parameters of antineoplastic drug-sensitive and resistant human ovarian cancer cell lines. Cancer Res.48, 5713-5716

Luria,S.E and Delbruck,M. (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics,28, 491-495

MacInnes, M.A., Mudgett, J.S. (1990) Cloning of the functional human excision repair gene ERCC-5; potential gene regulatory features conserved with other human repair genes. Prog.Clin.Biol.Res. 340A, 265-74

Masuda,H., Ozols,R.F., Lai,G.M., Fojo,A., Rothenberg,M., Hamilton,T.C. (1988) Increased DNA repair as a mechanism of acquired resistance to cis-Diamminedichloroplatinum(II) in human ovarian cancer cell lines. Cancer Res.48, 5713-5716

Masuda,H., Tanaka,T., Matsuda,H., Kusaba,I. (1990). Increased removal of DNA bound platinum in a human ovarian cancer cell line resistant to cis-Diamminedichloroplatinum(II). Cancer Res.50, 1863-1866

Mis,J.R.A. and Kunz,B.A. (1990) Analysis of mutations induced in the SUP4-o gene of Saccharomyces Cerevisiae by cis-diamminedichloroplatinum(II). Carcinogenesis 11, 633-638

Mitchell,P.J.,Tijan,R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245, 371-378

Momparler,R.L., Vesely,J., Momparler,L.F, Rivard,G.E. (1979) Synergistic action of 5-aza-2'-deoxycytidine and 3-deazauridine on L1210 leukemic cells and EMT6 tumour cells Cancer Research 39(10), 3822-3827.

Moranelli,F.,Lieberman,M.W. (1980) Recognition of chemical carcinogen-modified DNA by a DNA-binding protein. P.N.A.S.(USA) 77, 3201-3205

Mosmann,T. (1983) Rapid coloimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J.Imm.Methods,65, 55-63

Oh,E.Y., Claassen,L., Thiagalingam,T., Mazur,S., Grossman,L. (1989) ATPase activity of the UvrA and UvrAB protein complexes of the *Escherichia coli* UvrABC endonuclease. Nucleic Acid Research 17, 4145-4157

Orren,D.K.,Sancar,A. (1989) The (A)BC excinuclease of *Escherichia coli* has only the uvrB and uvrC subunits in the incision complex. P.N.A.S.(USA) 86, 5237-5241

Ozols,R.F., Young,R.C. (1984) Chemotherapy of ovarian cancer. Semin.Oncol.,11, 251-263

Ozols,R.F., Hamilton,T.C., Reed,E., Poirer,M.C., Masuda,H., Lai,G., Young,R.C. (1988) High dose cisplatin and drug resistance; clinical and laboratory correlations. In Platinum and other metal coordination compounds in cancer chemotherapy. (ed.M.Nicolini), 197-206

Patterson,M., Chu,G. (1989) Evidence that xeroderma pigmentosum cells from complementation group E are deficient in a homolog of yeast photolyase. Molecular and Cellular Biology 9(11), 5105-5112

Pinto,A.L. and Lippard,S.J. (1985)[a] Sequence-dependent termination of *in vitro* DNA synthesis by cis and trans-diamminedichloroplatinum(II) P.N.A.S.(USA) 82, 4616-4619

Pinto,A.L., Lippard,S.J. (1985)[b] Binding of the antitumour drug cis-diamminedichloroplatinum(II) (Cisplatin) to DNA. B.B.A. 780, 167-180

Plooy, A.C., van Dijk, M.,Lohman, P.H. (1984) Induction and repair of DNA cross-links in Chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity, and antitumour activity. Cancer Research 44, 2043-2051

Plooy,A.C.M., Fichtinger-Schepman,A.J., Schutte,H.H., vanDijk,M., Lohman,P.H.M. (1985)[a] The quantitative detection of various Pt-DNA adducts in Chinese hamster ovary

cells treated with cisplatin: application of immunochemical techniques. Carcinogenesis 6; 561-566

Plooy,A.C.M., vanDijk,M., Berends,F., Lohman, P.H.M. (1985)[b] Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with cis-Diamminedichloroplatinum(II). Cancer Research 45, 4178-4184

Plumb,M., Frampton,J., Wainwright.H., Walker,M., Macleod.K., Goodwin.G., Harrison.P. (1989) GATAAG; a *cis*-control region binding an erythroid-specific nuclear factor with a role in globin and non-globin gene expression. Nucleic Acid Research 17(1), 73-92

Plumb.J.A., Milroy.R.and Kaye.S.B. (1989) Effects of the pH Dependance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Can.Res.49, 4435-4440.

Poirer,M.C., Lippard,S.J., Zwelling,L.A., Ushay,H.M., Kerrigan,D., Thill,C.C., Santella,R.M., Grunberger,D., Yuspa,S.H. (1982) Antibodies elicited against cis-diammimedichloroplatinum (II) DNA adducts formed *in vitro* and *in vivo*. P.N.A.S.(USA) 79, 6443-6447

Poll,E.H.A., Abrahams,P.J., Arwett,F., Erikson,A.W. (1984) Host cell reactivation of cis-diamminedichloroplatinum (II)-treated SV40 DNA in normal human, Fanconi anaemia and xeroderma pigmentosum fibroblasts.

Popoff,S.C., Beck,D.J., Rupp,.D. (1987) Repair of plasmid DNA damaged *in vitro* with cis- or trans-diamminedichloroplatinum(II) in *Escherichia coli*. Mutation Research 183, 129-137

Prakash,L., Sherman,F. (1973) Mutagenic specificity: reversion of iso-1-cytochrome *r* mutants of yeast. J.Mol.Biol. 79,65-82

Rawlings, C.J., Roberts, J.J. (1986) Walker rat carcinoma cells are exceptionally sensitive to cis-diamminedichloroplatinum(II) (cisplatin) and other difunctional agents but not defective in the removal of platinum-DNA adducts. Mutation Research 166, 157-68

Reed,E., Ormond,P., Bohr,V.A., Budd,J., Bostick-Bruton,F. (1989) Expression of the human DNA repair gene ERCC-1 relates to cisplatin drug resistance in human ovarian cancer cells. Proc.Am.Assoc.Can.Res. 30, 488

Reed,E., Gupta-Burt,S., Litterst,C.L., Poirer,M.C. (1990) Characterization of the DNA damage recognized by an antiserum elicited against cis-diamminedichloroplatinum(II)-modified DNA. Carcinogenesis 11, 2117-2121

Reslova,S. (1971) The induction of lysogenic strains of *Escherichia coli* by cis-dichloro-diammine platinum(I). Chem.Biol.Interact 4, 66

Rice,J.A., Crothers,D.M., Pinto.A.L., Lippard,S.J. (1988) The major adduct of the antitumour drug cis-diamminedichloroplatinum(II) with DNA bends the duplex by ~40^0 toward the major groove. P.N.A.S.(USA) 85, 4158-4161

Roberts, J.J., Pascoe, J.M. (1972) Cross-linking of complementary strands of DNA in mammalian cells by antitumour platinum compounds. Nature 235, 282-284

Roberts,J.J. and Thomson,A.J. (1979) The mechanism of action of antitumour platinum compounds. Progress in N.A.R and Molecular Biology 22, 71-133

Rosenberg,B., vanCamp,L., Krigas,T. (1965) Inhibition of cell divisiom in *Escherichia coli* by electrolysis products from a platinum electrode. Nature 205, 698-699

Rosenberg,B., VanCamp,L., Trosko,J.E., and Mansour,V.H. (1969) Platinum compounds, a new class of potent antitumour agents. Nature 222, 385-386

Rosenberg, B., VanCamp,L. (1970) The successful regression of large solid sarcoma 180 tumours by platinum compounds. Cancer Research 30, 1799-1802

Royer-Pokora, B., Gordon,L.K., Haseltine, W.A. (1981) Use of exonuclease III to determine the site of stable lesions in defined sequences of DNA: the cyclobutane pyrimidine dimer and cis and trans dichlorodiammine platinum II examples. Nucleic Acids Research 9, 4595-4609

Rupp,W.D., Wilde,C.E.III, Reno,D.L., Howard-Flanders,P. (1971) Exchanges between DNA strands in ultraviolet-irradiated *Escherichia coli*. J.Mol.Biol.61, 25-44

Salles,B., Lesca,B. (1982) Induction of recA protein in *Escherichia coli* by three platinum (II) compounds. Biochem.Biophys.Res.Comm.105, 202-208

Salles,B., Butour,J.L., Lesca,C. and Macquet,J.P. (1983) cis-$Pt(NH_3)_2Cl_2$ and trans-$Pt(NH_3)_2Cl_2$ inhibit DNA synthesis in cultured L1210 leukemia cells. Biochem.Biophys.Res.Comm.112, 555-563

Sancar,A., Franklin,K.A., Sancar,G.B. (1984) *Escherichia coli* DNA photolyase stimulates uvrABC excision nuclease *in vitro*. P.N.A.S.(USA) 81, 7397-7401

Sancar,A. and Sancar,A. (1988) DNA repair enzymes. Ann.Rev.Biochem. 57, 29-67

Sancar,G.B.,Smith,F.W. (1988) *PHR1* photolyase stimulates excision repair in *Saccharomyces Cerevisiae* but inhibits *E.coli* excision repair. J.Cell.Biochem.Suppl. 12A, 298

Scanlon,K.J.,Newman,E.M.,Lu,Y.,Priest,D.G. (1986) Biochemical basis for cisplatin and 5-fluoro-uracil synergism in human ovarian carcinoma cells. P.N.A.S.(USA) 83, 8923-8925

Scanlon,K.J.,Kashani-Sabet,M.,Miyachi,H.,Sowers,.C.,Rossi,J. (1989)[a] Molecular basis of cisplatin resistance in human carcinomas; model systems and patients. Anticancer Research 9, 1301-1312

Scanlon,K.J., Kashni-Sabet,M., Sowers,L.C. (1989)[b] Overexpression of DNA replication and repair enzymes in cisplatin resistant human colon carcinoma HCT8 and their circumvention by azidothymidine. Cancer Comm. 1, 269-275

Schalet,A. (1977) Further evidence of EMS-induced rearrangements and a storage effect in *Drosophila melanogaster*. Genetics 86, s55 (abstract)

Seeberg,E. (1976) Incision of ultraviolet-irradiated DNA by extract of *E.coli* requires three different gene products. Nature 263, 524-526

Seeberg,E. (1978) Reconstitution of an *Escherichia coli* repair endonuclease activity from the seperated $uvrA^+$ and $uvrB^+$ / $uvrC^+$ gene products. P.N.A.S.(USA) 75, 2569-2573

Seeberg.E. (1981) Multiprotein interactions in strand cleavage of DNA damaged by UV and chemicals. Prog.Nucleic Acid Res.Mol.Biol.26, 217-226

Sega.G.A.(1984) A review of the genetic effects of ethylmethanesulfonate. Mutation Res,134, 113-142

Setlow,R.B., Regan,J.D., German,J., Carrier,W.L. (1969) Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA. P.N.A.S.(USA) 64, 1035-1040

Sheibani,N., Jennerwein,M.M., Eastman,A. (1989) DNA repair in cells sensitive and resistant to cis-diamminedichloroplatinum(II); host cell reactivation of damaged plasmid DNA. Biochemistry 28, 3120-3124

Sherman,S.E. and Lippard,S.J. (1987) Structural aspects of platinum anticancer drug interactions with DNA. Chem.Rev.87, 1153-1181

Sklar,M.D. (1988) Increased resistance to cis-diamminedichloroplatinum(II) in NIH 3T3 cells transformed by ras oncogenes. Cancer Research 48, 793-797

Soo Seo,Y., Lee,S.H., Hurwitz,J. (1991) Isolation of a DNA helicase from Hela cells requiring the multisubunit human single-stranded DNA-binding protein activity. J.Biol.Chem.266(20), 13161-13170

Sorenson,C.M. and Eastman,A. (1988) Influence of cis-Diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. Cancer Research 48, 6703-6707

Stephenson,C., Karran,P. (1989) Selective binding to DNA base pair mismatches by proteins from human cells. J.Biol.Chem. 264(35), 21177-21182

Sundquist,W.I., Lippard,S.J., Stollar,B.D. (1986) Binding of cis- and trans-Dichloroplatinum (II) to deoxyribonucleic acid exposes nucleosides as measured immunochemically with anti-nucleoside antibodies. Biochemistry 25, 1520-1524

Talley,R.W. (1970) Chemotherapy of a mouse reticulum cell sarcoma with platinum salts. Proc.Am.Assoc.Cancer Res. 11, 78

Teicher,B.A., Holden,S.A., Kelley,M.J., Shea,T.C., Cucchi,C.A., Rosowsky,A., Henner,W.D., Frei.E. (III) (1987) Characterisation of a human squamous carcinoma cell line resistant to cis-diamminedichloroplatinum(ii). Cancer Research 47, 388-393

Teyssier,J., Benard,J., Rerre,D., DaSilva,J., Betten-Renaud,L. (1989) Drug related chromosomal changes in chemoresistant human ovarian carcinoma cells. Cancer Genetics and Cytogenetics, 39, 35-43

Thacker.J. (1985) The molecular nature of mutation in cultured mammalian cells : a review. Mutation Res.,15, 113-142

Thomas ,D.C., Kunkel,T.A., Casna,N.J., Ford,J.P., Sancar,A. (1986) Activities and incision patterns of ABC excinuclease on modified DNA containing single-base mismatches and extrahelical bases. J.Biol.Chem.261, 14496-14505

Thompson,L.H., Baker,R.M.,(1973) Isolation of mutants of cultured mammalian cells, in "Methods in Cell Biology",vol.6, Prescott,D.M.,Ed.,Academic Press, New York 209-281

Thompson,L.H., Rubin,J.S., Cleaver,J.E., Whitmore G.F, Brookman,K. (1980) A screnning method for isolating DNA repair deficient mutants of CHO cells. Somatic Cell Genetic 6, 391-405

Thompson,L.H.,Brookman,K.W.,Dillehay,L.E.,Mooney,C.L., Carrano, A.V. (1982) Hypersensitivity to mutation and sister chromatid exchange induction in CHO cell mutants defective in incising DNA containing UV lesions. Somatic Cell Genetic 8, 759-773

Toney,J.H., Donahue,B.A., Kellett,P.J., Bruhn,S.L., Essigman,J.M., Lippard,S.J. (1989) Isolaton of cDNAs encoding a human protein that binds selectively to DNA modified by the anticancer drug cis-diamminedichloroplatinum(II). P.N.A.S.(USA) 86, 8328-8332

Tothill,P., Matheson,L.M., Smyth,J.F. (1990) Inductively coupled plasma mass spectrometry for the determination of platinum in animal tissues and a comparison with atomic absorption spectrometry. J.Anal.Atomic Spec. 5, 619-622

Toulme,J.J., Behmoaras,T., Guigues,M., Helene,C. (1983) Recognition of chemically damaged DNA by the gene 32 protein from bacteriophage T4. EMBO.J.3, 505-510

Troelstra,C., Odijk,H., de Wit,J., Westerveld,A., Thompson,L.H., Bootsma,D, Hoeijmakers,J.H.J. (1990) Molecular cloning of the human DNA excision repair gene ERCC-6. Molecular and Cellular Biology 10(11), 5806-5813

Tsang,S.S., Kuhnlein,U. (1982) DNA-binding protein from Hela cells that binds preferentially to supercoiled DNA damaged by ultraviolet light or N-Acetoxy-N-Acetyl-2-Aminofluorene. B.B.A.697, 202-212

Tsuruo,T., Iida,H., Tsukagoshi,S., Sakurai,Y. (1981) Overcoming of Vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of Vincristine and Vinblastine by Verapamil. Cancer Research 41, 1967-1972

Ullah,S., Husain,I., Carlton,W., Sancar,A. (1989) Human nucleotide excision repair in vitro: repair of pyrimidine dimers, psoralen and cisplatin adducts by Hela cell-free extract. Nucleic Acid Research, 17(12), 4471-4484

Van Houten.B. (1990) Nucleotide excision repair in *Escherichia coli.* Microbiological Reviews Mar.1990, 18-51

vanDuin,M., Dewit,J., Odijk,H., Westerveld,A., Yasui,A., Koken,M.H.M., Hoeijmakers,J.H.J. (1986) Molecular characterization of the human excision repair gene ERCC-1; cDNA cloning and amino acid homology with the yeast DNA repair gene RAD 10. Cell 44,913-923

Vogl,S.E., Pagano,M., Kaplan,B.H., Greenwald,E., Arseneau,J., Bennett,B. (1983) Cisplatin based chemotherapy for advanced ovarian cancer. Cancer,51, 2024-2030

Voigt,J.M., VanHouten,B.,S ancar,A., Topal,M.D. (1989) Repair of O^6-methylguanine by ABC excinuclease of *E.coli in vitro*. J.Biol.Chem.264, 5172-5176

Walker,G.C. (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiological Reviews 60-93

Waud.W.R. (1987) Differential uptake of cis-diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukemia cells. Cancer Res.47, 6549-6555

Weber,C.A., Salazar,E.P., Stewart,S.A., Thompson,L.H. (1988) Molecular cloning and biological characterisation of a human gene, ERCC2, that corrects the nucleotide excision repair defect in CHO UV5 cells. Molecular and Cellular Biology 8(3), 1137-1146

Weber,C.A., Salazar,A.P., Stewart,S.A., Thompson,L.H. (1990) ERCC2; cDNA cloning and molecular characterisation of a human nucleotide excision repair gene with high homology to yeast RAD 3. EMBO J.9, 1437-1447

Weeda.G., VanHam,R.C.A., Vermeulen,W., Bootsma,D., Van der Eb,A.J., Hoeijmakers,J.H.J. (1990)[a] A presumed DNA helicase encoded by ERCC-3 is involved in the human repair disorders xeroderma pigmentosum and cockayne's syndrome. Cell 62, 777-791

Weeda,G., vanHam,R.C.A., Masurel,R., Westerveld,A., Odijk,H., de Wit,J., Bootsma,D., van der Eb,A.J., Hoeijmakers,J.H.J. (1990)[b] Molecular cloning and biological characterization of the human excision repair gene ERCC-3. Molecular and Cellular Biology 10(6), 2570-2581

Weinert,T.A. and Hartwell,L.H. (1988) The RAD 9 gene controls the cell cycle response to DNA damage in *Saccharomyces Cerevisiae*. Science 241, 317-322

Weinfeld,M., Gentner,N.E., Johnson,L.D., Paterson,M.C. (1986) Photoreversal-dependent release of thymidine and thymidine monophosphate from pyrimidine dimer-containing DNA excision fragments isolated from ultraviolet-damaged human fibroblasts. Biochemistry 25,2656-2664

Welsch,C.J. (1971) Growth inhibition of rat mammary carcinoma induced by cis-platinum diammino-dichloride-II. J.National Cancer Institute 47, 1071

Westerveld,A., Hoeijmakers,J.H.J, VanDuin,M., Wit,J., Odijk,H., Pastink,A., Wood,R.D., Bootsma,D. (1984) Molecular cloning of a human DNA repair gene. Nature 310, 425-429

White,C.I., Sedgwick, S.G. (1987) Repair of UV-irradiated plasmid DNA in *Saccharomyces Cerevisiae*: Inability to complement mutational defects in excision repair by *in vitro* treatment with *Micrococcus luteus* UV endonuclease. Mutation Research 183, 161-167

Winker,M.A., Louie,K.G., Batist.G., Berens,B.C., Tsuro, T., Hamilton,T.C. (1985) Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug resistant and

sensitive human ovarian cancer cell lines by buthione sulphoximine mediated depletion of glutathione. Biochem.Pharmacol.34, 2583-2586

Wold,M.S., Kelly,T. (1988) Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. P.N.A.S.(USA) 85, 2523-2527

Wood, R.D., Robins, P., Lindahl,T. (1988) Complementation of the xeroderma pigmentosum defect in cell free extracts. Cell 53, 97-106

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Advances in Brief

Single Step Selection of *cis*-Diamminedichloroplatinum(II) Resistant Mutants from a Human Ovarian Carcinoma Cell Line[1]

Karen McLaughlin, Imogen Stephens, Nancy McMahon, and Robert Brown[2]

Department of Medical Oncology, Cancer Research Campaign, Alexander Stone Building, CRC Beatson Laboratories, Garscube Estate, Bearsden, Glasgow G61 1BD, United Kingdom

Abstract

We have shown that *cis* diamminedichloroplatinum-(II) (DDP) resistant mutants can be isolated from the human ovarian carcinoma cell line A2780 using a single-step selection protocol with DDP. DDP resistant colonies were calculated to be present at a frequency of 1.7×10^{-6}/viable cell using a fluctuation analysis. The mutational origin of these surviving colonies is inferred by the fact that their frequency is increased by treatment of the A2780 cells with the chemical mutagen ethyl methanesulfonate, with a maximum frequency observed after a 3-day expression time. Independently isolated clones maintain, in the absence of selection, a DDP resistant phenotype up to 7-fold more resistant than the parental A2780 cells. The resistance modifiers aphidicolin and buthionine sulfoximine have no effect on the frequency of DDP resistant mutants. Therefore neither of these drugs appears to have an effect on increasing the sensitivity of DDP resistant mutants existing in a cell population prior to DDP exposure.

Introduction

DDP[3] is an effective chemotherapeutic agent against a variety of tumor types (1). However, in many cases initial response is followed by relapse and failure of tumors subsequently to respond to chemotherapy. This has led to the suggestion that cells within the tumor have acquired specific cellular resistance mechanisms, causing these cells to have a selective advantage during treatment and thus to eventually predominate in the tumor (2). A variety of mechanisms of resistance to DDP have been suggested in cell lines selected *in vitro* for resistance (3–5). Most of these lines have been isolated after prolonged exposure to DDP, leading to the possibility of multiple mechanisms occurring together. It is not clear if these resistant cell lines will have mechanisms relevant to clinical resistance acquired during chemotherapy. Goldie and Coldman (6) have described a mathematical model for progression to resistance based on theoretical considerations of mutation frequencies and the assumption that drug resistant mutants exist or are induced in the tumor cell population. Using multiple exposure selection protocols it is not possible to measure the frequency of resistant variants in a cell population prior to DDP exposure or factors affecting this frequency.

A variety of drug selection protocols have been used to examine drug resistance mutations in mammalian cells (7, 8). The most reliable and quantitative selection procedures have used single step selection protocols that allow the identification of those rare cells that are drug resistant (8, 9). One important question concerning such drug resistant variants is whether or not they represent gene mutations in specific genes. In cases where the gene or enzyme conferring drug resistance is unknown, the mutational origin of drug resistance has been inferred by mutagens inducing an increase in the frequency of resistant cells and by the stable retention of the resistant phenotype in the absence of selection (8, 9).

DDP is used widely in the treatment of ovarian cancers (10). DDP resistant cells have previously been isolated from the human ovarian carcinoma cell line A2780 using repeated selection protocols (11). We have used this line to assess whether DDP resistant lines can be isolated using a single-step selection protocol. In order to provide evidence for a mutational origin, the effect of the chemical mutagen EMS on DDP resistant variant frequency has been examined. Isolated lines surviving DDP have subsequently been assayed for DDP resistance after prolonged periods of growth in nonselective media. Drugs which inhibit specific drug resistance mechanisms have been suggested as a means of increasing response of resistant tumors to chemotherapy or of increasing the efficacy of chemotherapy at first treatment (12). Aphidicolin (13) and BSO (14) have been suggested to increase the DDP cytotoxicity in resistant lines by inhibiting polymerase α mediated DNA repair and depleting glutathione levels, respectively. The DDP resistant lines used in these studies were isolated after multiple selections with DDP; therefore it is unclear how representative these lines are of all possible resistant mutants that survive DDP treatment. If resistance modifiers are to be used clinically at the time of first treatment in order to eliminate any resistant subpopulations, then it is important to know how generally effective they are against resistant mutants in the tumor population. In order to assess this, we have examined the effect of aphidicolin and BSO on the frequency of cells surviving the single-step selection with DDP in A2780 cells not previously exposed to DDP.

Materials and Methods

Cell Lines and Routine Culture Conditions. A2780, an ovarian carcinoma cell line derived from an untreated patient, and A2780CP, a DDP resistant line produced by exposure of the A2780 line to multiple increasing concentrations of DDP (11), were the kind gifts of Drs. R. F. Ozols and T. C. Hamilton, Fox Chase Cancer Centre, Philadelphia, PA. Both cell lines were maintained as monolayers in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum and 0.005 unit/ml penicillin at 37°C in a 5% CO_2-95% air atmosphere. All lines were free of *Mycoplasma* contamination.

Drug Selection. A2780 cells were plated at 2×10^6 cells/75-cm^2 flask for 5–50 μM DDP concentrations or at 10^3 for 0–5 μM DDP concentration. After 24 h the cells were treated with various doses of DDP for 24 h, washed twice with phosphate-buffered saline, and incubated in RPMI for 2 weeks; surviving colonies of more than 100 cells were then counted. The A2780 cells had a plating efficiency of about 20% under these conditions. The frequency of resistant colonies was calculated as

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: DDP, *cis*-diamminedichloroplatinum(II); EMS, ethyl methanesulfonate; BSO, buthionine sulfoximine; ID_{50}, concentration giving 50% survival; GSH, glutathione.

surviving colony number per viable cell or using fluctuation analysis (15) based on the number of cultures containing no surviving colonies. This latter analysis avoids any uncertainties due to daughter colony formation.

Mutagen Exposure Prior to DDP Selection. Cells (5×10^6) were treated with 2.5 mg/ml EMS (Sigma) for 2 h in serum free medium. This concentration of EMS gave 22% survival of the A2780 cells. At various times after the exposure to EMS the cells were selected at 2×10^6/75-cm^2 flask in 15 μM DDP as described above.

Aphidicolin and BSO Treatment. Aphidicolin (Sigma; 2.5 and 5 μg/ml) was added 1 h prior to DDP and maintained during the DDP selection. These are conditions previously shown to inhibit removal of DNA bound platinum in A2780 cells treated with DDP (13). For BSO treatment, BSO (Sigma) was added 24 h prior to DDP and maintained during the DDP selection. Concentrations of BSO used (25 and 50 μM) were previously shown to deplete glutathione levels in A2780 cells and increase the sensitivity of A2780CP cells to DDP in short term cell viability assays (14).

MTT Drug Sensitivity Assays. Cells in exponential growth were plated out at 10^3/well in medium in 96-well plates and incubated for 3 days. Cells were then incubated for 24 h in medium containing 10^{-4} to 6.4×10^{-9} M DDP and subsequently for 4 days in drug free medium. Metabolically active cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (Sigma) as a measure of cell viability as described previously (16).

Results and Discussion

Selection of DDP Variants. The surviving fraction of clonogenic cells of the human ovarian cell line A2780 after exposure to different concentrations of DDP is shown in Fig. 1. The DDP ID_{50} was 0.6 μM which is comparable with the ID_{50} observed by others using clonogenic assays of A2780 cells (11, 17). At concentrations of DDP between 15 and 30 μM a surviving fraction in the range 2 to 5×10^{-6} was observed. This appears as a tail in the survival curve suggesting that a subpopulation of cells are resistant to this concentration of DDP (Fig. 1). Selection of the cells with 15 μM DDP (Table 1) gives a frequency of 3.2×10^{-6} surviving colonies/viable cell. Using a fluctuation analysis (15) a spontaneous frequency of colonies surviving 15 μM DDP selection of 1.7×10^{-6}/viable cells was observed. This is a frequency comparable with known mutation frequencies for drug resistant mutants in mammalian cells (18). This questions whether the cells surviving DDP are mutants or simply variants which will survive DDP in a stochastic manner but do not represent stable genetic changes.

If the cells surviving 15 μM DDP are mutational in origin, then the frequency of these resistant cells should be increased with known mutagens. The requirement for an expression time of cells after mutagen treatment to allow phenotypic expression of resistance mechanisms has been well documented (9). The A2780 cells were treated with the chemical mutagen, EMS, and at varying times after exposure selected with 15 μM DDP. A maximum increase in DDP resistant colonies was observed 3 days after EMS exposure (Fig. 2). The increase in frequency of resistant colonies between days 1 and 3 supports a time dependent delay in the expression of a mutant phenotype and argues against the EMS cytotoxicity selecting for EMS resistant clones that are cross-resistant to DDP. The reduced frequency observed at later time points suggests that DDP resistant mutants may be selected against during nonselective growth conditions. Similar reductions in mutant frequency at longer expression times have been observed for other drug resistance selection systems (9). The resistant colony frequency 3 days after EMS treatment represents at least a 10-fold increase compared to the spontaneous frequency (Table 1). Thus EMS

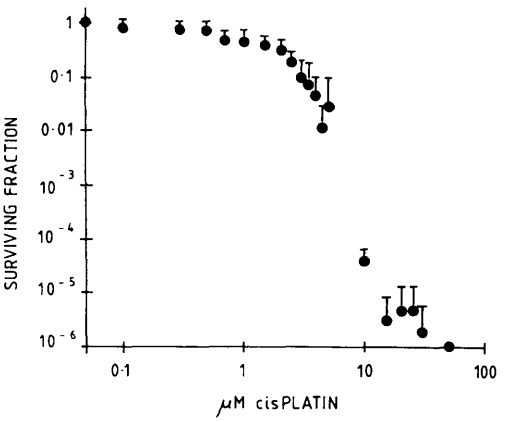

Fig. 1. Fraction of A2780 cells surviving 24-h exposure to the shown concentration of DDP. *Bars*, 95% confidence limits.

Table 1 *EMS induction of DDP resistant mutants*

Treatment	Viability[a]	Mutant frequency[b]
None	1.0	$3.2 \pm 1.5 \times 10^{-6}$ (40)
EMS[c]	0.22	$3.4 \pm 2.5 \times 10^{-5}$ (10)

[a] Viability is expressed as fraction of A2780 plating efficiency.
[b] The number of colonies surviving selection with 15 μM DDP per viable cell, with 95% confidence limits shown. Numbers in parentheses, number of experiments.
[c] EMS (2.5 mg/ml) for 2 h. Data shown are for 3-day expression time prior to DDP selection.

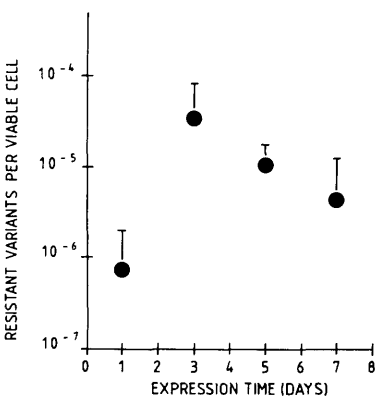

Fig. 2. Number of colonies surviving selection with 15 μM DDP per viable cell when assayed at the time shown after 2-h exposure to 2.5 mg/ml EMS. *Bars*, 95% confidence limits.

can induced DDP resistant clonogenic cells, supporting a mutational basis for the resistance.

If the cells maintain a drug resistant phenotype in the absence of DDP selection this also would support a stable genetic alteration leading to the drug resistant phenotype. After at least 40 generations of growth in nonselective media, the ID_{50}s to DDP of clones independently isolated after selection in 15 μM DDP were assessed using a short-term sensitivity assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Most of the clones assayed showed a significant increase in level of resistance to DDP, up to 7-fold greater than the parental A2780 cells (Fig. 3). This stable retention of a drug resistant phenotype is further support for a mutational basis of DDP resistance. However, the clones did not show as great an increased resistance as the A2780CP cell lines, which had been isolated by multiple rounds of DDP selection.

Treatment with BSO and Aphidicolin. We have shown that DDP resistant colonies can be isolated from the human ovarian

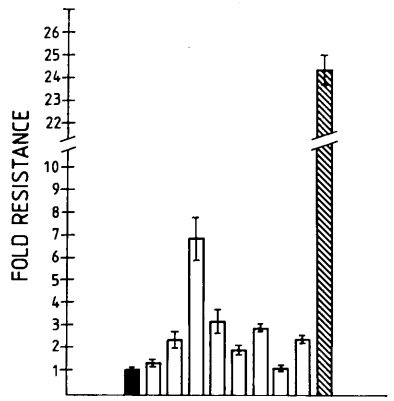

Fig. 3. Sensitivity to DDP of randomly selected independent clones surviving 15 μM DDP were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay (16). The parental A2780 cell line had an average ID_{50} of 0.37 μM using the 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Resistance factors were calculated as the fold increase in the ID_{50} of the lines to DDP compared to the parental A2780 cell line. ■, A2780; ▨, A2780CP; □, DDP selected clones. *Bars*, 95% confidence limits.

Table 2 *Effect of BSO and aphidicolin on DDP resistant mutant frequency*

Modifier	Viability[a]	Fraction of experiments with zero colonies	Mutant frequency[b]	Mutant frequency using fluctuation analysis
None	1.0	34/68	5.2×10^{-6} (1.1×10^{-6})	1.7×10^{-6}
2.5 μg/ml aphidicolin	0.38	22/30	4.5×10^{-6} (2.4×10^{-6})	2.0×10^{-6}
5 μg/ml aphidicolin	0.17	21/23	1.3×10^{-6} (9×10^{-7})	1.3×10^{-6}
25 μM BSO	0.41	26/30	2.6×10^{-6} (1.9×10^{-6})	8.7×10^{-7}
50 μM BSO	0.20	21/25	8.3×10^{-6} (4.8×10^{-6})	2.1×10^{-6}

[a] Viability is expressed as fraction of A2780 plating efficiency.
[b] The number of colonies surviving selection with 15 μM DDP per viable cell. Numbers in parentheses, SEM.

cell line A2780 by a single exposure to DDP. The low frequency of these resistant cells, their induction by treatment with the mutagen EMS, and the stable retention of the drug resistant phenotype support their mutational origin. Subpopulations of cells mutated at genes conferring resistance to chemotherapeutic drugs have been suggested to be the cause of eventual chemotherapy treatment failure for many types of tumors. A variety of possible resistance mechanisms have been suggested for cells resistant to DDP (2–5). Depletion of intracellular GSH by treatment with BSO, a potent inhibitor of γ-glutamyl cysteine synthetase, has been shown to increase the sensitivity of DDP resistant A2780CP cells (13, 19). Increased DNA repair levels have been shown in DDP resistant cells. Aphidicolin, an inhibitor of DNA polymerase α, has also been shown to increase the cytotoxicity of DDP in DDP resistant A2780CP cells (17). These studies have involved specific DDP resistant lines which have been isolated by multiple rounds of DDP selection. The effect of these resistance modifiers on the frequency of DDP resistant cells in a population prior to DDP selection has thus far not been studied.

Table 2 shows the effect of exposure of cells to aphidicolin or BSO prior to DDP selection. As shown BSO reduces the cell viability in clonogenic assays of the A2780 cells, as has previously been reported (19). Aphidicolin also markedly reduced the cell viability of A2780, in contrast to a previous report (17). Although both the BSO and aphidicolin treatments increase the number of experiments without DDP resistant colonies, when account is made of the reduction in viability there are no significant differences in the mutant frequencies. Therefore neither BSO nor aphidicolin has an effect on increasing the sensitivity of DDP resistant mutants existing in the cell population at time of exposure to DDP, although there is a combined cytotoxic effect.

Few studies have used single-step selections with chemotherapeutic drugs to examine the frequency of resistant mutants occurring in a cell population. Barranco *et al.* (20) have shown that a single exposure to melphalan (99% lethal dose) increased 10- to 50-fold resistance to the same agent 1 week later. The authors suggest that this is due to increased GSH levels in the cells and showed that BSO can partially reverse the resistance to melphalan. At present we are examining mechanisms of resistance to DDP in the single-step selection clones isolated, including alterations in drug accumulation, drug inactivation, and repair of induced lesions. However, since BSO has no effect on the frequency of DDP resistant mutants, this would suggest that GSH levels are not involved in resistance of these lines.

Both BSO and aphidicolin have previously been reported to increase the sensitivity of DDP resistant A2780 cells (13, 14, 17). These cells were selected after multiple exposure to DDP and may represent a different mutant type than that isolated by the single-step procedure used in the present study or may represent only a small subgroup of all possible mutant types. Which of the several possible mechanisms of DDP resistance have the highest probability of being selected for by DDP in human tumors is unclear. However, the type of single-step selection used in the present study will provide a means of examining the efficacy of modulators of DDP resistance to reduce the DDP resistant mutant frequency in cells not previously exposed to DDP.

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References

1. Loehrer, P. J., and Einhorn, L. H. Cisplatin. Ann. Intern. Med., *100:* 704–713, 1984.
2. Rosenberg, B. Fundamental studies with cisplatin. Cancer (Phila.), *55:* 2303–2316, 1984.
3. Andrews, P. A., and Howell, S. B. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells, *2:* 35–43, 1990.
4. deGraeff, A., Slebos, R. J. C., and Rodenhuis, S. Resistance to cisplatin and analogues: mechanisms and potential clinical implications. Cancer Chemother. Pharmacol., *22:* 325–332, 1988.
5. Kelley, S. L., and Rozencweig, M. Resistance to platinum compounds: mechanisms and beyond. Eur. J. Cancer Clin. Oncol., *25:* 1135–1140, 1989.
6. Goldie, J. H., and Coldman, A. J. A mathematical model for relating the drug sensitivity of tumours to their spontaneous mutations rate. Cancer Treat. Rep., *63:* 1727–1733, 1979.
7. Simmovitch, L. On the nature of hereditable variation in cultured somatic cells. Cell, *7:* 1–11, 1976.
8. Thacker, J. The molecular nature of mutations in cultured mammalian cells: a review. Mutat. Res., *15:* 431–442, 1985.
9. Thompson, L. H., and Baker, R. M. Isolation of mutants of cultured mammalian cells. Methods Cell Biol., *6:* 209–281, 1973.
10. Vogl, S. E., Pagano, M., Kaplan, B. H., Greenewald, E., Arseneau, J., and Bennett, B. Cisplatin based chemotherapy for advanced ovarian cancer. Cancer (Phila.), *51:* 2024–2030, 1983.
11. Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Lonie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., and Ozols, R. F. Characterisation of a *cis*-diamminedichloroplatinum(II) resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res., *47:* 414–418, 1987.
12. Brown, R., and Kaye, S. B. Drug resistance and the problem of treatment failure. *In:* B. A. J. Ponder and M. J. Waring (eds.), The Science of Cancer

Treatment, pp. 55–82. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1990.
13. Masuda, H., Tanaka, T., Matsuda, H., and Kusaba, I. Increased removal of DNA bound platinum in a human ovarian cancer cell line resistant to *cis*-diamminedichloroplatinum(II). Cancer Res., *50:* 1863–1866, 1990.
14. Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Berens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C., and Ozols, R. F. Augmentation of Adriamycin, melphalan and cisplatin cytotoxicity in drug resistant and sensitive human ovarian cancer cell lines by buthionine sulfoximine-mediated depletion of glutathione. Biochem. Pharmacol., *34:* 2583–2586, 1985.
15. Luria, S. E., and Delbruck, M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics, *28:* 491–495, 1943.
16. Mosman, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J. Immunol. Methods, *65:* 55–63, 1983.
17. Masuda, H., Ozols, R. F., Lai, G-M., Fojo, A., Rothenberg, M., and Hamilton, T. C. Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. Cancer Res., *48:* 5713–5716, 1988.
18. Ling, V. Genetic basis of drug resistance in mammalian cells. *In:* N. Bruchovsky and J. H. Goldie (eds.), Drugs and Hormone Resistance in Neoplasia. Boca Raton, FL: CRC Press, 1982.
19. Louie, K. G., Behrens, B. C., Kinsella, T. J., Hamilton, T. C., Grotzinger, K. R., McKoy, W. M., Winker, M. A., and Ozols, R. F. Radiation survival parameters of antineoplastic drug-sensitive and resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res., *45:* 2110–2115, 1985.
20. Barranco, S. C., Townsend, C. M., Jr., Weintraub, B., Beasley, E. G., MacLean, K. K., Shaeffer, J., Liu, N. H., and Schellerberg, K. Changes in glutathione content and resistance to anticancer agents in human stomach cancer cells induced by treatment with melphalan *in vitro*. Cancer Res., *50:* 3614–3618, 1990.