DNA Damage Recognition And p53 In Cisplatin Resistance

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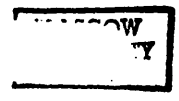

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ABBREVIATIONS

A-	Adenine.
AAS-	Atomic absorption spectroscopy.
ATP-	Adenine tri-phosphate.
AT-	Ataxia telangiectasia.
BICR-	Beatson Institute for Cancer Research.
C-	Cytosine.
CAT-	Chloromphenicol acetyl transferase.
CHO-	Chinese hamster ovary.
Cdk-	Cyclin dependent kinase.
^{o}C-	Degrees celsius.
dpm-	Disintegrations per minute.
DEP-	Dichloro(ethylenediamine)platinum(II).
DNA-	Deoxyribonucleic acid.
DRP-	DNA damage recognition proteins.
ELISA-	Enzyme linked immunosorbant assay.
FACS-	Fluorescence activated cell sorting.
G-	Guanine.
Gy-	Greys (a unit of radioactivity).
HMG-	High mobility group.
HPLC-	High performance liquid chromatography.
ID_{50}-	Drug dose capable of killing 50% of a cellular population.
ID_{20}-	Drug dose capable of killing 20% of a cellular population.
Kb-	Kilobases (a unit of nucleic acid strand length).
KDa-	Kilodaltons (a unit of protein mass).
M-	Molar.
mA-	Milliamps.
μg-	Microgram.
mg-	Milligram.
μl-	Microlitres.
ml-	Millilitres.
MW-	Molecular weight.
PAGE-	Polyacrylamide gel electrophoresis.
RNA-	Ribonucleic acid.
SDS-	Sodium dodecyl sulphate.
T-	Thymine.
TCA-	Trichloroacetic acid
XP-	Xeroderma pigmentosum.

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ABSTRACT

Cis-dichlorodiammineplatinum(II) (cisplatin) is widely used as an anti-cancer agent to treat various tumours particularly ovarian, testicular, bladder cancers and tumours of the head and neck. Although the precise mechanism of cisplatin-mediated cytotoxicity remains elusive, DNA damage is thought to be the therapeutically important interaction. Cisplatin resistance is an obstacle to effective treatment of cancer patients. Such resistance may arise due to a variety of intracellular changes including enhanced repair of DNA lesions caused by cisplatin and reduced susceptibility to DNA damage-induced apoptosis. The tumour suppressor protein, p53 is required for DNA damage-induced apoptosis in certain cell types.

Cellular levels of p53 protein are increased subsequent to DNA damage due to post-translational alterations in p53. Induction of p53 protein levels by 1μM cisplatin or transplatin peaks at 24 hours for the cisplatin sensitive human, ovarian adenocarcinoma cell line, A2780. Levels of p53 protein increase in the cisplatin resistant derivative, A2780cp70 for at least 48 hours following treatment with transplatin and at least 72 hours following treatment with cisplatin. Also, certain cisplatin resistant cell lines contain higher basal levels of p53 protein compared to the cisplatin sensitive parental cells from which they were derived. Results of Western blotting experiments show an 8 fold higher p53 concentration in the A2780cp70 cell line than A2780, the cisplatin sensitive parental line. This difference in p53 protein levels does not reflect a mutation of the *p53* gene, as direct PCR sequencing of *p53* from A2780 and A2780cp70 showed both lines to have a wild-type *p53* sequence. Furthermore, the p53 from A2780cp70 has less cross-reactivity with mutant-specific anti-p53 antibodies in immunoprecipitation experiments than cell lines with known mutations of the *p53* gene. No evidence for increased *MDM2* expression is observed in A2780cp70 suggesting that the p53 protein is not being stabilised by binding to the MDM2 protein in this cell line. Although increased levels of Hsp70 protein are observed in these cells, this may simply reflect a lack of transcriptional repression of the Hsp70 promoter in these cells due to the p53 being non-functional.

Altered p53-mediated transcriptional *trans*activation in A2780cp70 was examined by use of a luciferase reporter construct and by Northern blotting of endogenous genes, known to be transcriptionlly activated by p53. A2780cp70 cells have 10 fold lower *WAF-1* mRNA levels than A2780 cells indicating reduced p53 transcriptional activity in the resistant cell line. Introduction of a mutant p53 gene into A2780 cells also provokes a reduction in basal p53 levels with a 3 fold decrease detected. Reduced function of p53 in the A2780cp70 cell line would be consistent with reduced entry to the apoptotic pathway in these cells. This may be one reason why A2780cp70 cells are more cisplatin resistant than A2780 cells. The DNA damaging agents, cisplatin and transplatin, induced *WAF-1* mRNA levels 4 fold by 24 hours after drug removal in A2780 cells. Ionising radiation provoked a more rapid induction of 4.5 fold by 4 hours after drug removal in A2780 cells. This is consistent with induced p53 protein levels detected in A2780 after similar treatments. A2780cp70 cells, by comparison, showed only slight induction of *WAF-1* following treatment with these agents. Clonogenic assay data shows that a pretreatment with cisplatin does not alter sensitivity to a second cisplatin dose. Thus intracellular alterations evoked by cisplatin treatment are not involved in a protective response.

DNA damage recognition proteins (DRPs) have been proposed to have a role in drug resistance, possibly by an involvement in DNA repair although other functions are possible. Partial purification of a DRP which binds to cisplatin-damaged DNA was carried out. Using nuclear proteins, soluble in 2% tri-carboxylic acid for anion exchange chromatography and heparin column chromatography, this DRP was purified 183 fold. Using recombinant

HMG proteins and using anti-HMG protein antibodies to retard protein-DNA complexes in the gel mobility shift assay, the DRP activity was shown to be identical to HMG1. Evidence is also presented showing that p53 does not directly bind to regions of cisplatin-induced DNA damage. However this does not eliminate a possible link between DRP-mediated DNA damage recognition and the p53 response to DNA damage.

Chapter 1

CHAPTER 1: INTRODUCTION

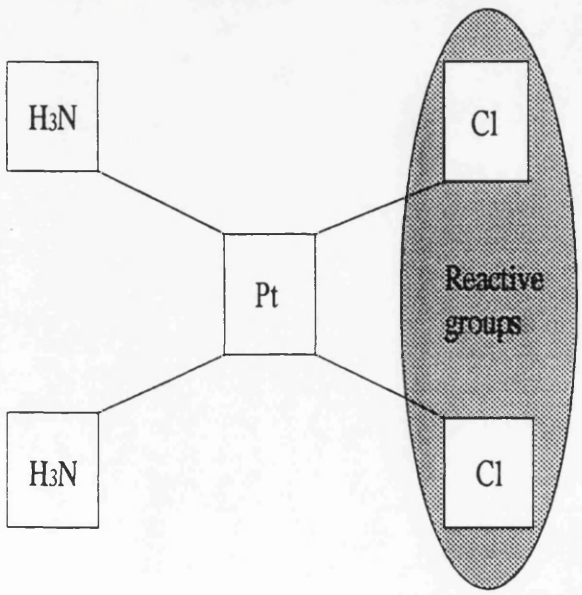
1.1 *CIS*-DIAMMINEDICHLOROPLATINUM II (CISPLATIN)

1.1.1 THE DISCOVERY OF CISPLATIN

Cisplatin was first synthesized in 1845 when it was known as "Peyrone's chloride" and its structure was elucidated in 1893 (Eastman, 1990). The toxic effects of cisplatin were first appreciated when an AC current was delivered through platinum electrodes to a culture of *Escherichia coli* bacterial cells. The cells stopped dividing and proceeded to form long filaments. At the electrodes, the responsible compound was found to be the planar cisplatin molecule (Rosenberg et al., 1965). Stereochemistry has important implications for these effects as the *trans* isomer does not inhibit division and merely functions as a bacteriocide. The structure of cisplatin, showing the reactive chloride groups is depicted below in figure 1.1.1. These chloride ions are stable in the extracellular environment but, on uptake to cells, the low intracellular chloride concentration causes them to be replaced by hydroxyl ions. This gives rise to a bifunctional, charged electrophile which is reactive with nucleophilic sites within the cell such as DNA, RNA and proteins.

FIGURE 1.1.1

The Chemical Structure of Cisplatin



1.1.2 CISPLATIN AS A CHEMOTHERAPEUTIC AGENT

Cisplatin was shown to have powerful anti-tumour activity when injected into mice with tumour implants. Platinum compounds were able to decrease the mass of Sarcoma 180 solid tumours and to increase the mean survival time of mice with leukemia L1210 compared to control mice (Rosenberg & VanCamp, 1969; Rosenberg & VanCamp, 1970). Cisplatin is now used in drug combination strategies to treat testicular, bladder, ovarian cancer and tumours of the head and neck (Loehrer & Einhorn, 1984).

1.2 POSSIBLE MECHANISMS OF CISPLATIN MEDIATED CELL DEATH

1.2.1 CISPLATIN INDUCED DNA DAMAGE

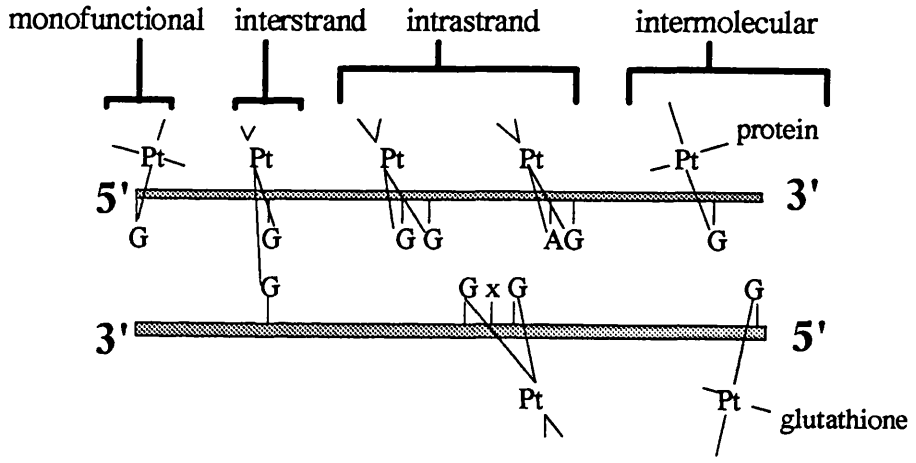
Approximately 1% of the total cellular platinum binds to the DNA, forming a range of adducts which have been detected *in vitro* and *in vivo*. These lesions, which are represented pictorially in figure 1.2.1 include monofunctional, intrastrand, interstrand and intermolecular adducts involving a second molecule such as glutathione (GSH) or protein. The majority of cisplatin interactions with DNA take the form of intrastrand cross-links with 65% between neighbouring guanines at the N^7 position, i.e 1,2 d(GpG), 25% are between adjacent adenine and guanine residues, i.e 1,2 d(ApG), and the remainder are between guanines separated by another nucleotide i.e 1,3 d(GpNpG) (Fichtinger-Schepman et al., 1984; Pinto & Lippard, 1985a; Eastman, 1986).

Such DNA damage could result in reduced viability of cells which are unable to repair lesions before the genome is replicated at S phase of the cell cycle. Thus rapidly dividing cells, such as one often observes in tumours, would be targeted more efficiently. Indeed there is evidence that cisplatin preferentially kills proliferating rather than quiescent rat thymocytes (Evans et al., 1994). Damaged DNA might result in aberrant separation of the

chromatids at M phase of the cell cycle, giving rise to daughter cells with an abnormal complement of chromosomes. It could also interfere with the progress of replicative enzymes along the DNA strand or with transcription of genes essential for cellular survival.

FIGURE 1.2.1

The Main Types of Cisplatin-DNA Adducts



Transplatin is stereochemically incapable of forming the main cisplatin DNA adduct, the 1,2 d(GpG) intrastrand crosslink. This may account for the differential toxicities and anti-tumour capacities of these two isomers (Pinto & Lippard, 1985a). Within CV-1 cells given a continuous 10μM dose, of cisplatin or transplatin, transplatin lesions increased rapidly for 6 hours, as measured by atomic absorption spectroscopy (AAS), then declined suddenly. On the other hand cisplatin adducts rose steadily throughout the course of the 48 hour experiment, indicating that cells are less proficient at repairing cisplatin-mediated damage (Ciccarelli et al., 1985). DNA damage is the most likely explanation for the cytotoxicity and anti-tumour activity of cisplatin and interactions with DNA would certainly have more drastic effects than

interactions of cisplatin with more transient intracellular molecules such as proteins and mRNA.

1.2.2 MUTAGENESIS

In certain instances, cisplatin mediated DNA damage may be incurred in essential, "housekeeping" genes. Such mutations might cause the gene product to be inactive or to be abnormally expressed. This would result in reduced viability of affected cells and so represents a further mechanism by which cisplatin could kill cells. One would expect the majority of such mutations to occur at nucleotide sequences rich in guanines adjacent to either other guanines or adenines. Studies using the Chinese hamster ovary (CHO) *aprt* gene have shown 5'AGG and 5'GAG sequences to be hotspots for cisplatin induced mutations and there is no apparent overlap with UV induced mutations (deBoer & Glickman, 1989).

1.2.3 INHIBITION OF DNA SYNTHESIS

Cisplatin may exert its effects by prevention of normal cellular functions such as DNA synthesis. DNA synthesis is impaired by cisplatin (Harder & Rosenberg, 1970) and this may be due to direct inhibition of polymerases. Progress of DNA polymerases has been shown to be halted *in vitro* by DNA intrastrand crosslinks (Pinto & Lippard, 1985b). An alternative means of DNA synthesis inhibition would be to block the cells in a cell cycle phase where DNA replication does not occur for example in G1 or G2 phases. Cisplatin has been shown to cause cell cycle blocks in both G2 (Sorenson & Eastman, 1988; Ormerod et al., 1994) and G1 phases (Brown et al., 1993).

1.2.4 APOPTOSIS

Apoptosis is a form of programmed cell death which exhibits certain distinct morphological features (Kerr et al., 1972). Cells become rounded, membrane blebbing can be detected, and in some cases, non-random DNA fragmentation to multiples of 180-200bp occurs and, probably as a result of this, the chromatin becomes condensed. This is rapidly followed by separation of the nucleus into discrete masses of condensed chromatin and, finally, fragmentation of the cell into several membrane bound vesicles known as apoptotic bodies which are then phagocytosed by other cells such as macrophages. The process of apoptosis is involved in cell turnover within tissues, tumour regression, focal elimination during embryonic development and it also seems to occur in response to toxic substances.

Apoptosis is a thermodynamically uphill process and requires the expression of certain gene products. The c-myc protein is required for activation-induced apoptosis of T-cell hybridomas (Shi et al., 1992). Deregulation of the *c-myc* gene causes apoptosis of rodent fibroblasts following serum withdrawal (Evan et al., 1992). Apoptotic induction by c-myc occurs via its interaction with the splice variant products of the *max* gene (Amati et al., 1993). Apoptosis in response to DNA damage seems to require p53 protein (Lowe et al., 1993a) although p53 independent apoptotic pathways have been reported (see section 1.4.7 for further details). Another important protein is bcl-2, a 25KDa integral, inner mitochondrial membrane protein, which antagonises apoptotic death (Hockenberry et al., 1990). It forms heterodimers *in vivo* with bax, a protein which promotes apoptosis (Oltvai et al., 1993). The bcl-2 protein has 23% amino acid homology with the product of the *ced9* gene from *Caenorrhabditus elegans* which suppresses the *ced3* and *ced4*-mediated programmed cell death in this worm species (Hengartner et al., 1994). The *C.elegans* cell death programme requires the *ced3* and *ced4* genes to effect the regulated demise of 131 of the 1090 cells present in the

developing worm. A mammalian homologue of the *ced3* gene has been found (Yuan et al., 1993) which encodes a 45KDa protein, the interleukin B converting enzyme (ICE). Other genes implicated in the apoptotic pathway include TRPM-2/SGP and Fas/APO-1 (for a review see Martin et al., 1994).

Apoptosis can be induced by anticancer drugs including cisplatin (for a review see Dive & Hickman, 1991). This has been demonstrated in CHO cell lines which were proficient (AA8) or deficient (UV41) in DNA excision repair (Barry et al., 1990). The UV41 cell line was 100 fold more sensitive to a 2 hour cisplatin dose. Treatment of either of these cell lines with a 90% lethal dose of cisplatin caused the DNA fragmentation, symptomatic of apoptosis, which was detected 48 hours after drug treatment and earlier for higher doses. The DNA fragmentation was followed by cell shrinkage and by loss of membrane integrity as measured by trypan blue exclusion. There appears to be a fairly linear relationship between drug dose and apoptosis for each of several cytotoxic drugs including cisplatin (Frankfurt et al., 1994).

Rat thymocytes, unlike hepatoma cells, did not seem to show sensitivity to cisplatin-induced apoptosis despite their extreme sensitivity to apoptosis induced by other conditions (Evans & Dive, 1993). However, this was probably due to 95% of the thymocyte population being quiescent. The minority sub-population of larger, proliferating thymocytes exhibited increased levels of apoptosis as detected by acridine orange staining of nuclei and agarose gel electrophoresis of fragmented DNA, upon treatment with a 2 hour, 50μM cisplatin dose when compared to both untreated controls and quiescent thymocytes (Evans et al., 1994). As no differences in either cisplatin intracellular levels (AAS) or cisplatin-DNA adducts were detected (ELISA), it may be that cells must be in a certain cell cycle phase in order to succumb to cisplatin-induced apoptosis or that DNA replication is necessary to couple the induced DNA damage to the apoptotic response.

1.3 MECHANISMS OF RESISTANCE TO CISPLATIN TREATMENT

1.3.1 THE ACQUISITION OF DRUG RESISTANCE

The development of drug resistance is a problem which has marred the progress of chemotherapy for many cancer sufferers. The ability to understand and hence modulate such resistance is one of the major goals of oncology today. In some large tumours drugs are not efficiently delivered to the cancerous cells. This is due to the extensive, collagen-enriched interstitium and the higher pressure and greater viscosity of the blood supply, which reduces blood flow and so, convection. Thus larger molecules which require the blood to "carry" them cannot reach cancerous cells as readily as small molecules which travel principally by diffusion (Jain, 1994). Therefore such tumours do not respond well to chemotherapy and so could be termed "resistant".

Certain cell types display increased tolerance of drugs than others and this is termed "innate" or "inherent" resistance. For example, bladder cells have a greater tolerance of cisplatin than testicular cells and this difference is maintained in cell lines derived from them (Walker et al., 1987). For instance testicular cell lines are generally 3- 5 fold more sensitive to cisplatin than bladder cell lines (Bedford et al., 1988 and many others). "Acquired" resistance on the other hand, is resistance which develops due to a phenotypic change in the affected cells. This can be mimicked *in vitro* by selection of spontaneous mutants by exposure of cell lines to high doses of the drug (Behrens et al., 1987; McLaughlin et al., 1991).

1.3.2 DRUG RESISTANCE DUE TO REDUCED INTRACELLULAR ACCUMULATION

One mechanism by which cells could become resistant to chemotherapeutic drugs is by decreasing the concentration of the drug in the cell either by lowering the uptake or by increasing efflux of the substance. A

classic example of such a mechanism is the MDR phenotype where resistance to a multitude of hydrophobic, natural product, cytotoxic drugs develops due to overexpression of the *MDR1* gene which encodes p-glycoprotein, a 170KDa membrane protein (Juliano & Ling, 1976). High levels of p-glycoprotein expression are sometimes detected in cancers derived from certain tissues. Increased p-glycoprotein expression has also been detected in acute leukemias, neuroblastomas ovarian and breast cancers which have relapsed after chemotherapy and in acute non-lymphocytic leukemia and chronic myelogenous leukemia. However the *MDR1* gene does not confer resistance to cisplatin (Bradley et al., 1988; Deuchars and Ling, 1988). A gene has been identified which expresses its product, the ATP binding MRP , at relatively high levels in non-p-glycoprotein mediated MDR cells (Cole et al., 1992; Slovak et al., 1993). MRP is a 190KDa membrane protein which is located principally at the endoplasmic reticulum (Krishnamacery & Center, 1993). Another protein has been described which, like MRP, is associated with MDR but not cisplatin resistance. This 110KDa protein is overexpressed in cells which have an ATP dependent defect in drug accumulation (Scheper et al., 1993).

Reduced accumulation of cisplatin has been reported for some cisplatin tolerant, human ovarian carcinoma cell lines although it does not appear to be a universal mechanism (Andrews et al., 1989; Schmidt and Chaney, 1993). A 200KDa protein, which displays increased expression in cisplatin resistant cell lines, has been detected by raising antibodies to the plasma membrane of murine thymic lymphoma cells (Kawai et al., 1990). The level of cisplatin resistance correlated with a decrease in intracellular concentration of the drug. Another manner by which cisplatin accumulation might be reduced, is by increased binding to extracellular proteins which would make cisplatin effectively impermeable to cells. Using a human cell line, NHIK3025, it has been demonstrated that the cytotoxicity of cisplatin can be decreased by binding to serum proteins (Melvik et al., 1992).

1.3.3 INACTIVATION OF CISPLATIN BY CELLULAR COMPONENTS

If the reactivity of cisplatin were obliterated by increased interaction with nucleophilic molecules such as glutathione (GSH) or metallothioneins before it was able to carry out its destructive effects within the cell, resistance might arise. GSH, with a concentration within cells of 0.5-10mM, is the main intracellular thiol and is reactive with cisplatin. An association between increased GSH levels and cisplatin resistant cell lines have been detected in certain cases (Andrews et al., 1989). Incubation of cisplatin with GSH reduced its toxicity. Exposure of CHO fibroblast cell lines to 5mM buthionine sulphoximine caused a 90% reduction in cellular GSH levels resulting in the cells becoming sensitized to cisplatin treatment (Spitz et al., 1993). However there is scant evidence for GSH levels being a causative factor in modulation of cisplatin toxicity *in vivo*.

Metallothioneins are proteins of 6-7KDa in size, which are thought to be involved in zinc homeostasis and the detoxification of heavy metals in cells. In primates there are at least five *MT-1*, one *MT-2* and one brain specific *MT-3* genes which encode such proteins. A correlation between elevation of the cellular metallothionein content and transient cisplatin resistance in a murine fibrosarcoma cell line has been observed (Eichholtz-Wirth et al., 1993). However this was not shown to be a cause-effect phenomenon and the transient nature of the resistance suggested that it was not due to a mutation or gene amplification event. Curiously, the cells used for these experiments became cisplatin resistant following irradiation yet exhibited no resistance to γ-radiation. Overexpression of metallothioneins in cisplatin resistant, human, carcinoma cell lines following exposure to heavy metals was associated with resistance to cisplatin, chlorambucil and melphalan and transfection of cells with the human metallothionein-II_A gene conferred resistance to these drugs (Kelley et al., 1988). However transfection with a mouse metallothionein gene

(MT-1) seemed to enhance cisplatin sensitivity, rather than resistance, of a CHO cell line (Koropatnick and Pearson, 1993).

An extensive study using three untreated ovarian, carcinoma cell lines including A2780 (a cell line which will be referred to in several subsequent sections), five A2780 derivatives selected for cisplatin resistance in vitro and five ovarian, carcinoma cell lines from patients who had been treated with, and acquired tolerance of cisplatin, has been performed (Schilder et al., 1990). There was no overall correlation between basal levels or inducibility of metallothionein II gene expression and cisplatin resistance.

1.3.4 INCREASED REPAIR OR TOLERANCE OF CISPLATIN-MEDIATED DNA DAMAGE

As DNA damage seems to be an important cytotoxic event following cisplatin treatment, enhanced repair of such lesions may represent the primary cause of cisplatin tolerance. In addition, increased tolerance of DNA adducts may occur for example, if DNA distortions are minimised due to binding of a protein which corrects the resultant kink in the DNA structure.

There are three classes of DNA repair and these are direct repair, recombinational repair and excision repair (Lindahl et al., 1982). Direct repair involves reversal of covalent modifications as occurs in the photoreactivation reaction of yeast where photolyase breaks the cyclobutane ring of pyrimidine dimers, caused by UV radiation, and restores the DNA to its native state. Recombinational repair happens when polymerases encounter DNA adducts. They cease replicating and initiate 1000bp beyond the adduct, where they continue, leaving a region of single stranded DNA which, in *Escherichia coli*, is filled using the RecA protein. Excision repair consists of several stages which are, in chronological order, damage recognition, formation of the preincision complex, incision of the phosphate backbone at both sides of the

adduct, excision of the abnormal nucleotide, repair synthesis, release of the post-incision complex and DNA ligation.

Cisplatin resistant cells show elevated DNA repair compared to sensitive cells as detected by loss of [^{3}H]-*cis*-DEP adducts (Eastman & Schulte., 1988) or by elevated [^{3}H] thymidine incorporation indicating increased repair synthesis (Lai et al., 1988). Cells which are sensitive to cisplatin are less able than resistant derivatives to repair a damaged chloramphenicol acetyl transferase (CAT) gene as measured by CAT activity in murine L1210 cells (Sheibani et al., 1989), human fibrosarcoma lines (Chu & Chang, 1990) and HeLa cells (Chao et al., 1991d). Gene specific, as opposed to total genome, repair of DNA interstrand crosslinks formed by cisplatin have been found to be increased in two cisplatin resistant cell lines when compared to their sensitive parentals (Zhen et al., 1992). This was demonstrated for the dihydrofolate reductase, γ-globin and *MDR1* genes using a denaturation-reannealing protocol then neutral gel electrophoresis, to separate crosslinked DNA from single stranded DNA, and subsequent Southern blotting. A comparison of sensitive SUSA bladder cells with the more resistant RT112 bladder cell line, using alkaline elution and ELISA, incorporating antibodies against cisplatin damaged DNA, revealed that although there was no overall difference in platinum adduct removal between these cell lines, SUSA cells had a defect in removal of intrastrand crosslinks involving two adjacent nucleotides (Bedford et al., 1988). This provides evidence that this is the main cytotoxic lesion formed by cisplatin and that repair of intrastrand crosslinks may be important in the development of cisplatin resistance.

1.3.5 OTHER FACTORS WHICH INFLUENCE CISPLATIN RESISTANCE

Altered expression of the *ras, fos and myc* oncogenes have been implicated in cisplatin resistance, as have alterations in activity of protein

kinase A, a cyclic AMP dependent enzyme which has a possible role in gene transcription, and protein kinase C inhibition or down regulation (for reviews see Andrews & Howell, 1990; Keith & Brown, 1991). Elevation of cyclic AMP has been shown to accentuate both cisplatin accumulation and sensitivity in 2008 cells probably by phosphorylation of a protein which modulates cisplatin influx or efflux. Also an increase in phosphorylation of 32KDa and 20KDa proteins in cisplatin resistant PC-9/CDDP cells compared to sensitive PC-9 human, lung, adenocarcinoma cells has been detected by SDS/PAGE after ^{32}P labelling, with no concurrent increase in protein levels (Nishio et al., 1992). The observation that tamoxifen increased the efficacy of a cisplatin combination therapy used against malignant melanoma cells, provoked a study demonstrating synergy between these two compounds in the T-289 melanoma cell line (McClay et al., 1992). No increase in uptake of a tritiated cisplatin analogue, dichloro(ethylenediammine)platinum II(DEP) could be determined by scintillation counting. ELISA showed no difference in metallothionein levels and, GSH levels, determined by a HPLC method, remained unaltered by tamoxifen presence. Also, tamoxifen had no affect on the repair of DEP-DNA adducts determined by the quantity of tritium in DNA samples prepared at various times after exposure of the cells to tritiated DEP. This suggests some novel form of cisplatin resistance is counteracted by tamoxifen. Thus the development of drug resistance is a complex process which cannot be accounted for by one, sole mechanism.

1.4 A ROLE FOR p53 PROTEIN IN CISPLATIN RESISTANCE ?

1.4.1 p53, THE TUMOUR SUPPRESSOR

The nuclear phosphoprotein, p53 was discovered by coimmunoprecipitation from SV40 transformed mouse cells, due to an association with SV40 large T antigen (Lane & Crawford, 1979). Association of p53 protein with viral proteins, such as SV40 large T antigen, adenovirus

E1B and the E6 proteins of HPV16 and 18 human papillomaviruses, indicates that it is an important regulatory protein whose function must be overcome in order to permit viral replication. Indeed, SV40 replication has been shown to be inhibited by wild type but not mutant p53 protein *in vitro* (Friedman et al., 1990). Several nuclear localisation sequences are located in the C terminal region of p53 protein (Shaulsky et al., 1990). Expression of p53 can be discerned in many multicellular eukaryotic species and the gene is located on the short arm of chromosome 17 at 17p13.1 in humans, a region which is often deleted in cancers. The sequence of the p53 protein has remained highly conserved throughout evolution indicating a critical function.

The wild type form is a tumour suppressor and mutations or deletions of the *p53* gene are one of the most frequent genetic alterations observed in human cancer as determined by immunohistochemistry, Western blotting and ELISA (Malkin et al., 1990; Srivastava et al., 1990; Brash et al., 1991; Hsu et al., 1991; Moll et al., 1992; Cunningham et al., 1992; Allred et al., 1993; Burns et al., 1993). Mutant *p53* can cooperate with *ras* to transform primary embryonic rat fibroblasts and there was some initial confusion as to which form of p53 protein represented the native, wild type (Finlay et al., 1988).

There does appear to be a common conformational change of p53 protein which occurs on mutation of the gene. This was suggested, as certain antibodies can bind a range of different mutant p53 proteins but not to the wild type protein (Gannon et al., 1990). Changes in p53 conformation have also been detected under different growth conditions (Milner and Watson., 1990). This protein has fundamental importance in the development of cancerous conditions and may, as will be discussed in subsequent sections, also have an effect on how well such diseases respond to chemotherapy and radiotherapy.

1.4.2 EVIDENCE FOR AN INVOLVEMENT OF p53 IN CISPLATIN RESISTANCE

DNA damaging agents such as cisplatin, mitomycin C, etoposide and UV radiation have been shown to induce levels of intracellular, particularly nuclear, p53 protein in cells of both human and murine descent (Maltzman & Czyzyk, 1984; Kastan et al., 1991; Brown et al., 1993; Fritsche et al., 1993). Such an increase has also been detected *in vivo* in response to UV by immunohistochemistry of paraffin wax-embedded human, forearm biopsies (Hall et al., 1993). This implicates p53 protein in the response of cells to DNA damage, possibly as a mediator of signal transduction to downstream events such as altered gene transcription, cell cycle arrest or the decision to enter the apoptotic pathway. Bone marrow or spleen cells from transgenic mice with *p53* mutations show an increased tolerance of ionizing radiation induced DNA damage compared to cells from wild type littermates (Lee and Bernstein., 1993). Mutant p53 protein enhances, and wild type p53 represses, transcription of the *MDR1* gene promoter placed upstream of a CAT reporter construct in SW13, human adrenocortical, carcinoma cells and NIH3T3 cells (Chin et al., 1992) and could theoretically alter transcription of drug resistance genes involved in cisplatin tolerance in a similar manner. Also cisplatin resistant cell lines selected using multiple exposures to cisplatin or one, single, chronic 15μM dose have an increased incidence of constitutive p53 protein elevation which cannot be accounted for by mutation of the p53 gene (Brown et al., 1993).

1.4.3. STABILIZATION OF p53 PROTEIN

The enhanced levels of p53 protein detected in cisplatin resistant cells, and following exposure of cells to DNA damaging agents, could arise by elevation of *p53* gene transcription. This is unlikely as drug induced p53 protein stabilization has been shown to occur in the absence of any increase in

p53 mRNA levels (Kastan et al., 1991; Fritsche et al., 1993). It is also independent of the cell cycle phase which cells are in when exposed to DNA damaging agents.

Certain types of *p53* gene mutations cause the p53 protein half life to increase from 15-45 minutes to 120-360 minutes, as determined by pulse chase experiments after transfection of cells with plasmids expressing *p53* (Finlay et al., 1988). It also shows increased binding to the heatshock protein Hsp70 from coimmunoprecipitation data and the region of p53 involved in this association has been elucidated (Hainaut & Milner, 1992; Lam & Calderwood, 1992). SV40 large T antigen is also able to prolong p53 longevity without increasing *p53* mRNA levels as can be seen by comparing pulse chase experiments in 3T3 cells and in the SV40 transformed cell line, SV3T3 (Oren et al., 1981) Binding to proteins such as Hsp70 and SV40 large T antigen may protect p53 from degradation by masking a protease recognition site on the p53 molecule. E6 viral proteins which complex with p53, on the other hand, destabilize p53 protein (Scheffner et al., 1990).

Other p53 interactive proteins include the phosphoprotein, MDM2 which inhibits p53 mediated transcriptional *trans*activation (Momand et al., 1992). It binds to a region from amino acids 18-23 of mouse and human p53 proteins, which differ in one residue within this peptide sequence (Picksley et al., 1994). There appear to be several forms of this protein derived by alternative mRNA splicing. Four naturally occurring forms have been found in mice, of which one was unable to bind p53 protein possibly due to its lacking an N-terminal region (Haines et al., 1994). These variant forms may react with p53 protein in different circumstances and thereby provide fine tuning of p53 function. Amplification of the MDM2 gene, found at the chromosomal region 12q13-14, is another frequent genetic alteration associated with cancerous disease (Oliner et al., 1992; Leach et al., 1993). Major 45KDa, 56KDa and

70KDa proteins, as well as many minor proteins from non-small cell lung carcinoma lysates have been found to interact with the conserved p53 conformational domain encompassing amino acids 115-295 and these associations were reduced by 40-80% after point mutations at codon 273 or 175 of the p53 sequence (Maxwell & Roth., 1993). Quantitative differences in various p53 binding proteins were detected between cell lysates of different origins.

There may be several pathways by which p53 protein levels can be induced. Although a reduced or delayed ionising radiation p53 induction is apparent for cells from at least four complementation groups of ataxia telangiectasia (AT), induction of p53 protein following treatment with UV-B light is normal (Khanna & Lavin, 1993). Patients who suffer from AT are highly cancer prone and hypersensitive to ionising radiation but not UV-B light. Induction of p53 protein levels in response to both agents was inhibited by calphostin A, a protein kinase C inhibitor and by the serine/threonine phosphatase inhibitors, okadaic acid and calyculin A, indicating a role of protein kinase C and serine/threonine phosphatases in the stabilization of p53 protein. Inhibitors of protein kinase A, tyrosine kinases or tyrosine phosphatases did not inhibit p53 induction.

Nuclear accumulation of p53 may arise due to excessive, unrepaired DNA damage in actively transcribed genes rather than due to damage which occurs randomly throughout the genome. Cells from patients with a defect in repair of transcribed genes for example, Cockayne's syndrome or xeroderma pigmentosum group A, showed induction of p53 protein at much lower doses of UV radiation than normal cells or cells with a defect in overall genome repair, such as those from xeroderma pigmentosum group C patients (Yamaizumi & Sugano, 1994).

1.4.4 INTRACELLULAR DEGRADATION OF p53 PROTEIN

Within cells, breakdown of proteins which have outlived their usefulness is carried out by lysosomal autophagy, various ATP dependent proteases or by the ubiquitin pathway. *In vitro*-translated p53 protein coimmunoprecipitates ubiquitin when ATPγS is used to inhibit HPV E6 protein-stimulated p53 breakdown, indicating an involvement of ubiquitin in p53 degradation (Scheffner et al., 1990). Also, using a cell free degradation system followed by SDS/PAGE, the ubiquitin pathway has been shown to be involved in degradation of *in vitro*-synthesized, radiolabelled p53 protein (Ciechanover et al., 1991). Degradation of p53 does not occur in the absence of ATP, it can be inhibited by depletion of E1, the ubiquitin activating protein, using immunoprecipitation and subsequently restored by addition of purified E1.

The degradation of proteins by the ubiquitin pathway involves several proteins. After activation of ubiquitin by E1, ubiquitin is transferred to a carrier protein, E2, E1 is recycled and then E2 conjugates ubiquitin to the protein to be destroyed, which has been recognised by the ubiquitin protein ligase, E3, of which there are four types. E2 is recycled at this point, multiple moieties of the 76 amino acid, ubiquitin become attached and ATP dependent degradation is carried out by the 26S protease complex. A free α amino group is important in the recognition of proteins for rapid turnover by this system, also the identity of the N-terminal residue plays a role in its recognition and t-RNA seems to be involved in covalently modifying substrates for binding (for reviews see Hershko, 1988; Ciechanover, 1989). Modulation of p53 recognition by the ubiquitin system may have implications for the p53 DNA damage response pathway.

1.4.5 HOW p53 MAY BE INVOLVED IN THE DEVELOPMENT OF DRUG RESISTANCE

DNA damage caused by cisplatin and other cytotoxic treatments results in increased wild type p53 protein in the nucleus of cells exposed to these agents. This may, under normal circumstances, cause cell cycle arrest in response to DNA damage which then affects the decision of cells to commence apoptosis. Alternatively, p53 may influence the entry of cells to the apoptotic pathway, independently of any cell cycle affects. P53 probably mediates its effects by altering transcription of relevant genes. An aberration in a component of the p53 DNA damage response pathway could thus result in drug resistance due to inappropriate survival of cells following drug exposure.

1.4.6 THE INDUCTION OF CELL CYCLE ARRESTS BY p53 PROTEIN

Overexpression of wild type p53 protein in osteosarcoma cell lines blocks growth of osteosarcoma (Diller et al., 1990) and colorectal carcinoma cell lines (Baker et al., 1990) as determined by differences in the colony forming ability of these cells. The transfection of wild type p53 protein into Li-Fraumeni syndrome cells with mutant p53 alleles restored normal cell cycle control (Yin et al., 1992). Wild type, but not mutant p53 protein blocks growth of both *S.pombe*, when overexpressed from a thiamine inducible promoter (Bischoff et al., 1992), and *S.cerevisiae*, when expressed from a galactose inducible promoter (Nigro et al., 1992). In the latter case this was shown to involve a G1 phase block.

A glioblastoma cell line, T98G, when transfected with a dexamethasone inducible p53 gene, exhibits a dexamethasome inducible G1 block and prevents entry to S phase following release from stationary growth by 86% (Mercer et al., 1990). Cycloheximide has been employed to demonstrate that protein synthesis is required in order to enter S phase of the cell cycle after release from a G1 phase cell cycle block resulting from

overexpression of wild type p53 from a dexamethasone inducible promoter (Lin et al., 1992). It was also shown that the p53 induced block corresponded with the mammalian restriction point as determined by expression of genes known to be transcribed just before and after this cell cycle event. Using Northern blotting, B-*MYB* and DNA polymerase α were found to be repressed but c-*FOS*, c-*JUN*, *JUN*-B and c-*MYC*, which are expressed earlier in the cell cycle, were not.

DNA damage induction of p53 protein levels by ionising radiation has been shown to correlate with acquisition of a G1 block in ML-1 and proliferating, normal bone marrow progenitor cells (Kastan et al., 1991). Also cycloheximide, a protein synthesis inhibitor, or caffeine, an inhibitor of phosphodiesterase which breaks down cAMP, were both shown to prevent the induction of p53 protein levels and G1 arrest. Cells which lack wild type p53 protein did not arrest in G1 phase of the cell cycle but did show a G2 arrest. Cell lines derived from AT patients, which do not induce p53 protein, after ionising radiation exposure had a defect in their ability to arrest in G1 in response to ionising radiation suggesting that the AT gene products may be upstream of p53 in the p53 mediated response to DNA damage (Kastan et al., 1992). In several cell lines, a strong correlation between ionising radiation-caused p53 inducibility, G1 arrest propensity and radiosensitivity has been demonstrated (McIlwrath et al., 1994). A direct relationship of p53 protein in causing the G1 arrest was shown because transfection of wild type *p53* into HL60 cells, which lack *p53*, resulted in acquisition of a G1 arrest in response to ionising radiation (Kuerbitz et al., 1992). Also, loss of G1 arrest in response to ionising radiation, occurred when a mutant *p53* gene was expressed in RKO cells which have endogenous, wild type *p53*. Similar experiments have been performed on A2780 cells (McIlwrath et al., 1994). Thus p53 protein is very important for normal, regulated cell growth in the cells of multicellular eukaryotic organisms.

1.4.7 p53 AND THE INDUCTION OF APOPTOSIS

Thymocytes from 4.5-7 week old mice whose *p53* genotype was either wild type, heterozygous or homozygous mutant were used to demonstrate an involvement of p53 in apoptosis. A wild type p53 function was necessary for radiation induced, but not glucocorticoid induced apoptosis as determined by electrophoresis of fragmented DNA (Lowe et al., 1993b). Ionising radiation-induced, p53-dependent apoptosis has also been reported for murine, intestinal crypt cells *in vivo* (Clarke et al., 1994). A requirement for p53 protein to carry out apoptosis in response to etoposide but not calcium influx, stimulated by use of a phorbol ester and a calcium ionophore, was demonstrated in a similar system using acridine orange staining of nuclear chromatin to assess the induction of apoptotic death (Clarke et al., 1993). Apoptosis in mouse embryo fibroblasts, resulting from treatment with anti-cancer agents such as 5-fluorouracil, etoposide, adriamycin or ionising radiation has been shown to require a functional p53 protein (Lowe et al., 1993a). The percentage of apoptotic nuclei in the embryonic mouse lens, resulting from a homozygous mutation of the retinoblastoma gene, was reduced by 94% in mice which were also homozygous mutants for the *p53* gene, indicating that wild type *p53* is involved in execution of the apoptotic programme in this situation (Morgenbesser et al., 1994). Prevention of *p53* expression by transfection of antisense *p53* mRNA into cells obtained from acute myeloblastic leukemia patients or a human erythroleukemia cell line, suppressed the growth factor deprivation-induced apoptosis which occurred in 21-54% of control cells (Zhu et al., 1994). It is apparent therefore, that p53 is involved in promoting apoptosis in certain situations, particularly in response to DNA damage and so it is likely to be influential in the response of cells to DNA damaging drugs such as cisplatin.

Neither cycloheximide or actinomycin D prevented ionising radiation induced, p53 dependent apoptosis in GHFT1 cells (Caelles et al., 1994). Thus, it has been proposed that rather than activating genes involved in the promotion of apoptosis, p53 protein acts by repression of those necessary for survival. This is supported by the finding that wild type p53 represses expression of the *bcl-2* gene (Myashita et al., 1994a & b), whose product is known to protect cells from apoptosis.

1.5 REGULATION OF TRANSCRIPTION BY p53 PROTEIN

1.5.1 THE CAPACITY OF p53 PROTEIN TO BIND DNA

An ability to regulate transcription of specific genes is one way by which p53 protein may mediate its affects and the first indication that this could be the case was the demonstration that p53 has affinity for DNA. Using DNase I footprinting, it has been shown that wild type human or murine p53 proteins, but not mutant p53, can bind to SV40 DNA adjacent to the SV40 origin of replication and this can be inhibited by SV40 large T antigen (Bargonetti et al., 1991). *In vitro* translated, wild type p53 proteins of mouse or human origin were shown to bind to calf thymus DNA, using electrophoresis of [^{35}S]-methionine labelled proteins eluted from a DNA-cellulose column (Kern et al., 1991a). Several mutant p53 proteins had reduced binding affinity and enhanced binding of both mutant or wild type varieties occurred when p53 protein molecules were treated with potato acid phosphatase to remove phosphate molecules which indicates that phosphorylation may restrict DNA binding by p53. A DNA sequence, $^{5'}$ACGTTTGCCTTGCCTGGACTTGCCTGGCCTTGCCTT$^{3'}$, which binds p53 specifically was isolated by incubation of ^{32}P end-labelled DNA fragments with lysates of cells overexpressing *p53* from a viral promoter (Kern et al., 1991b). Also a consensus sequence of nucleotides for p53 binding has been defined as $^{5'}$GGACATGCCCGGGCATGTC$^{3'}$ and this sequence is

capable of binding to p53 protein specifically, as determined by gel mobility shift analysis in the presence of competitor DNA (Funk et al., 1992). Thus there is much evidence for an intrinsic DNA binding capability of p53 protein and this is probably relevant to the function of this protein *in vivo.*

Deletion of the 47 C-terminal amino acids, but not the 70 N-terminal amino acids, obliterated the ability of murine p53 protein to bind to calf thymus DNA in the South-western binding assay, although in both cases the protein conformation was defined as "wild-type" due to affinity for the antibody PAb 246 (Foord et al., 1991). Recombinant, human p53 protein was shown to require a domain within the 90 carboxy-terminal amino acids in order to bind specifically to the p53 consensus sequence in the gel mobility shift assay, as deletion of this region abolished binding (Hupp et al., 1992). Thus a DNA binding domain of the p53 protein seems to reside at the C-terminal end of the protein, which is highly basic although it has since been suggested that there are several regions of p53 protein which may mediate DNA binding. A peptide encompassing amino acids 80-320 of p53 protein is able to bind DNA specifically, in the gel mobility shift assay, and forms only monomers as determined by gluteraldehyde crosslinking then SDS/PAGE, whereas a peptide representing amino acids 280-390 binds DNA non specifically and can form tetramers (Wang et al., 1993). This data, if relevant to p53 protein conformation *in vivo*, may have implications for whether p53 oligomerization is necessary for DNA binding to take place.

The ability of p53 protein to bind a consensus DNA sequence, in the gel mobility shift assay, has been shown to increase in response to several drugs such as hydrogen peroxide, actinomycin D, adriamycin, etoposide, camptothecin, 5-fluorouracil, mitomycin C, cisplatin and ionising radiation (Tischler et al., 1993). This lends further support to the theory that p53 has a role to play in mediating the effects of chemotherapy and radiation therapy.

1.5.2 THE ABILITY OF p53 PROTEIN TO ACTIVATE TRANSCRIPTION

The DNA binding affinity of p53 protein could indicate one of several physiological roles. For example it could be taken to suggest that p53 was involved in maintaining the structure of the DNA, or it could mean that p53 was associated with processes such as replication of the genome, repair of aberrant nucleotides or transcription of certain specific genes. The transcriptional control function of p53 was first examined by use of chimeric proteins formed by fusion of p53 segments to regions of DNA binding proteins. When placed in a p53 null background, this allowed the measurement of transcriptional control separately from DNA binding function and overcame the obstacle of having to find genes which respond to transcriptional control by p53.

The use of GAL4-p53 fusion proteins, containing the GAL4 DNA binding domains which do not activate transcription alone, and varying amounts of human p53 protein, showed that the first 73 N-terminal amino acids of p53 were sufficient to direct transcription in yeast (Fields and Jang, 1990). This was assessed by β-galactosidase activity, produced from the *lacZ* gene, in a yeast strain which lacked GAL4 and had an integrated *lacZ* gene with a GAL4-binding promoter. Transfection of plasmids containing the GAL4-p53 sequences into CHO cells along with CAT reporter constructs which direct transcription of CAT activity under control of GAL4 binding sites confirmed that such activity was also possible in mammalian cells. Also, the 73 amino terminal residues of p53 protein were as potent at activating transcription as the VP16 protein, a highly active transcriptional regulator, from herpes virus. A similar study using CHO cells demonstrated that whilst the N-terminal 160 amino acids can activate CAT 17 fold, the entire p53 protein sequence activates CAT 13 fold and the C-terminal 233 amino acids provide negligible activation of transcription (O'Rourke et al., 1990). As the N-terminal region shows greater activity than the whole molecule, there may

be regulatory regions also present in the p53 protein structure. The N terminus of p53 is 20% acidic and as such, resembles that of other transcription factors for example Fos, GAL4 and the glucocorticoid hormone receptor. This is followed by a proline rich stretch which is also characteristic of transcription factors, being found in CTF, Fos, Jun, Oct-2 and SRF which are all involved in transcriptional control.

Two transforming mutants of p53, when fused to GAL4 DNA binding domains and transfected into HeLa cells, were unable to activate transcription of a CAT reporter construct (Raycroft et al., 1990). This suggests that transcriptional control by p53 protein is required for a normal, untransformed phenotype and that the loss of this ability by p53 protein may be the event responsible for the unregulated growth of cells lacking wild type p53. Further experiments using a range of human and murine p53 fusion proteins to activate CAT reporter constructs, in HeLa, CHO and NIH 3T3 cell lines demonstrated that many mutant p53 proteins have lost the ability to activate transcription (Raycroft et al., 1991). One mutant p53 protein however, which is found in Li-Fraumeni syndrome sufferers, contains a substitution at amino acid 245 and retains the ability to activate the reporter gene.

After the discovery of a p53 DNA binding consensus sequence, it was possible to directly assess p53 transcriptional *trans*activation by using reporter gene constructs inserted downstream of this in a plasmid vector. These studies demonstrate that intact p53 protein is able to direct transcription of genes alone and the transcriptional activity reported for fusion proteins is not an artefactual result of using an artificial system. A 33bp sequence, which had been shown to bind p53, was able to direct transcription of β-galactosidase in *Saccharomyces cerevisiae* when wild type human or murine p53 was also expressed, from a different plasmid (Scharer & Iggo, 1992). Cotransfection of a CAT reporter system with a human p53 expression plasmid, into the human colorectal cancer cell line, HCT 116, showed that wild type, but not mutant,

p53 activates transcription from its binding site and that activity increases with increasing numbers of binding sites in the reporter construct (Kern et al., 1992). A mutated p53 binding site was unable to direct transcription. The luciferase gene has also been placed downstream of a p53 DNA binding consensus sequence or the "fragment A" p53 binding sequence and luciferase activity was observed on transfection of such constructs into a p53 null cell line, H1299. A requirement for the C-terminal DNA binding regions of p53 protein in order to permit transcriptional *trans*activation has been examined by the use of three human p53 deletion mutants incorporating amino acids 82-393, 160-393 or 1-326, and found to depend on the binding site used (Zhang et al., 1994a).

1.5.3 REGULATION OF p53 MEDIATED TRANSCRIPTIONAL *TRANS*ACTIVATION

Regulation of the DNA binding and transcriptional *trans*activation functions of p53 protein may be performed by interaction with proteins or by post translational modifications such as phosphorylation. Proteins which complex with wild type p53 such as SV40 large T antigen and mutant p53 are able to prevent site specific DNA binding by wild type p53 protein as measured by gel mobility shift analysis (Bargonetti et al., 1992). A CAT reporter construct whose expression was driven by several copies of the p53 binding motif, $^{5'}$TGCCT$^{3'}$, was used to show that wild type SV40 large T antigen or HPV-16 E6 oncoprotein were able to prevent p53 mediated transcriptional regulation probably by causing its rapid degradation before p53 was able to carry out transcriptional transactivation (Mietz et al., 1992). Native cellular proteins may operate in a similar way to modulate p53 activity. It has also been reported that E6 proteins from HPV-6 and HPV-11 are able to prevent p53 mediated transactivation of CAT activity and that they may also interact with p53 protein, albeit more weakly than the "high risk" HPV viruses

(Crook et al., 1994). These HPV viruses do not enhance degradation of the p53 molecule. MDM2 protein has been shown to inhibit p53 associated transcriptional control and, as mentioned in section 1.4.3, the different splice variants of this protein may be responsible for controlling p53 activity in a sophisticated manner.

It has been suggested that p53 requires activation for DNA binding, for example by CKII phosphorylation (Hupp et al., 1992). However mutation of the proposed CKII site on the p53 molecule did not alter its DNA binding affinity (Rolley and Milner., 1994). Reduced phosphorylation may be important as potato acid phosphatase activated DNA binding by p53 protein (Kern et al., 1991a). However, okadaic acid, a phosphatase inhibitor, was able to reduce p53 mediated transcriptional transactivation and yet increase DNA binding (Zhang et al., 1994b). Possibly such experiments are too crude to provide insights into the possibly contradictory effects of phosphate groups at different sites on the p53 molecule.

1.5.4 GENES WHICH BIND AND/OR BECOME *TRANS*ACTIVATED BY p53 PROTEIN

*Trans*activation of the transcription of specific genes by p53 probably occurs by p53 binding to a DNA site similar to the consensus sequence *in vivo*. This would then assist the binding of further proteins involved in the production of mRNA. A region from 3300-2800bp upstream of murine muscle creatine kinase (MCK) has been shown to respond to transcriptional *trans*activation by wild type murine p53 protein (Weintraub et al., 1991). A 50bp region within this element was identified as important for human and murine p53 binding by DNase I footprinting and, when placed upstream of a minimal promoter adjacent to a CAT gene, it conferred p53 responsiveness so CAT activity was induced by p53 (Zambetti et al., 1992). Wild type human and murine p53 proteins have been shown to activate transcription of a CAT

construct placed downstream of a human ribosomal gene cluster (RGC) DNA sequence *in vitro* using transcription factors from HeLa nuclear extracts (Farmer et al., 1992). The region of this element to which p53 binds has also been mapped by DNase I footprinting.

The *GADD45* gene, which is also induced by DNA damaging agents such as ionising radiation (Papathanasiou et al., 1991), has been shown to contain a p53 responsive element (Kastan et al., 1992). Normal lymphoblasts, fibroblasts and tumour cells with a wild type p53 gene showed between 2 fold and 9.9 fold induction of *GADD45* mRNA following a 20Gy ionising radiation dose whereas cells lacking a wild type p53 protein due to point mutation or deletion do not exhibit greater than 1.4 fold induction. This correlated with the ability of these cell lines to arrest in G1 phase of the cell cycle following irradiation. A 20bp DNA sequence with identity to the p53 consensus binding sequence in all but one nucleotide was found in the third intron of the *GADD45* gene and this fragment was shown to have affinity for p53 by gel mobility shift and by coimmunoprecipitation of a radiolabelled oligonucleotide containing the putative p53 binding site. *MDM2* mRNA is induced in fibroblast cells by ionising radiation so long as the endogenous *p53* gene is wild type, not mutated or deleted (Price and Park., 1994). Northern blotting shows that MDM2 transcription increases rapidly in cells containing a temperature sensitive mutant p53 protein when the temperature drops from 37.5°C to 32.5°C, where p53 is in its wild type conformation (Barak et al., 1993). This induction occurs even in the presence of cycloheximide, indicating a direct interaction with the p53 protein. A region downstream of exon 1 in the *MDM2* gene has p53 binding activity as demonstrated by its ability to direct p53 dependent transcription of a CAT gene (Juven et al., 1993). This may affect not only the level, but also the range, of *MDM2* transcripts. P53 protein was able to coimmunoprecipitate an end labelled oligonucleotide corresponding to this region.

The p53 protein is able to bind to a region from +22 to +67 nucleotides within its own gene in gel mobility shift assays and to direct transcription of a CAT reporter gene linked to the *p53* promoter (Deffie et al., 1993). A mutant p53 protein was found to lack this activity. The cyclin G gene also seems to respond to p53 protein. Mouse embryonic fibroblasts from a p53 deficient mouse showed a more than 10 fold reduction in the level of cyclin G mRNA when compared to embryonic fibroblasts from a wild type mouse (Okamoto and Beach, 1994). A fragment from 1.5kb upstream of the cyclin G gene was shown to have affinity for p53 by coimmunoprecipitation of the end labelled oligonucleotide and by gel mobility shift analysis. It was also able to carry out p53 mediated transcription of a luciferase gene when inserted upstream of it in a plasmid construct. This may have implications for the effects of p53 protein on cell cycle regulation, although the specific function of cyclin G has yet to be elucidated.

Another cell cycle associated gene which p53 regulates is *WAF-1* or *CIP-1* as it is otherwise known. This gene was identified by a subtractive hybridisation approach using dexamethasone inducible p53 protein in a colon cancer cell line (El-Deiry et al., 1993). It was also simultaneously discovered due to the association of the gene product, p21, with cyclin dependent kinase 2 (cdk2) when cDNA for the gene was expressed in yeast. This was detected using a system whereby binding of a protein to cdk2 activates transcription of *HIS3* and *lacZ* genes on a reporter plasmid (Harper et al., 1993). WAF-1 inhibits phosphorylation by cyclin A-cdk2, cyclin E-cdk2 and cyclin D1-cdk4 of proteins such as the *Rb* gene product, which behaves as a negative regulator of growth when hypophosphorylated. This may prove to be one manner by which p53 exerts its effects on the cell cycle as these cyclins are associated with the G1 to S phase transition. Induction of *WAF1* seems to occur during p53 mediated G1 arrest and apoptosis as demonstrated using a dexamethasone inducible *p53* gene, but not in cells with a mutant *p53* gene or cells which

undergo apoptosis by a p53 independent pathway (El-Deiry et al., 1994). During radiation induced G1 arrest, accumulation of cyclin A was blocked, cyclin E accumulated but was inactive as a kinase and the responsible molecule was shown to be the *WAF-1* gene product (Dulic et al., 1994). Removal of p21 from irradiated cell lysates, using a p21 specific antibody attached to protein A beads, prevented inhibition of cyclin E activity as measured by phosphorylation of histone H1. Cyclin E is normally activated in late G1 and is thought to be involved in making the commitment to enter S phase. Thus the p21, *WAF-1* gene product may be an important mediator of p53 function.

1.5.5 GENES WHICH ARE REPRESSED BY p53 PROTEIN

Certain genes respond to p53 transcriptional control by repressing the level of mRNA transcripts produced. Serum stimulation of the CAT gene, under control of the interleukin 6 (IL-6) promoter, in HeLa cells was repressed by transfection with wild type murine or human, but not mutant *p53* (Santhanam et al., 1991). Wild type mouse p53 has been shown to repress transcription from the SV40 enhancer promoter as determined by use of a CAT reporter gene in HeLa cells (Jackson et al., 1993). A temperature sensitive mutant p53 has been used to show that wild type p53 can repress transcription from *c-fos* as determined by Northern blotting and activation of a CAT gene downstream of the *c-fos* promoter (Ginsberg et al., 1991). Also data was presented showing that p53 repressed CAT constructs containing promoter regions from the *p53*, *c-jun*, β *actin* and *hsc70* genes but not the *MHC* promoter. This agrees with other data showing p53 mediated repression of the gene encoding Hsp70 protein. It does conflict however, with data showing an activation of the *p53* promoter by p53 protein (Deffie et al., 1993).

P53 protein can repress transcription from a minimal promoter probably by interfering with the action of the TATA binding protein (TBP).

TBP, immobilised on Affi-Gel beads to produce an affinity column, was used to show that wild type p53 protein has TBP binding activity (Seto et al., 1992). The p53 protein is thereby able to inhibit the transcriptional transactivation activity of TBP. This was demonstrated by heat inactivation of the transcription factor, TFIID, in HeLa extracts. Human TBP could partially rescue the defective transcription which resulted but this rescue capacity was negated by incubation of wild type p53 protein with TBP prior to inclusion in the transcription reaction. Another transcriptional regulatory protein which p53 binds is the CAAT binding factor (CBF) as was shown using an affinity column consisting of glutathione S transferase-CBF attached to glutathione agarose (Agoff et al., 1993). Coimmunoprecipitation was also used to demonstrate this interaction.

The *MDR1* gene has been shown, using CAT reporter constructs, to be activated 7-180 fold by mutant p53 protein but repressed by wild type p53 protein (Chin et al., 1992). CAT constructs and a temperature sensitive p53 mutant were used to demonstrate that p53 can repress transcription from the *Rb* gene (Shiio et al., 1992). Deletion of the *Rb* gene enabled the responsible *cis* acting element to be defined as the sequence, $^{5'}$GGAAGTGA$^{3'}$. Regions from both the amino and the carboxyl terminals of p53 were important for this transcriptional repression. Northern and Western blotting both demonstrate that wild type p53 protein enhances expression of *bax* and represses expression of *bcl-2* in a murine leukemia cell line transfected with a temperature sensitive mutant p53 protein which is in the mutant conformation at 37°C and is functionally wild type at 32.5°C (Miyashita et al., 1994a). Immunohistochemistry and Western blotting confirmed that levels of these two proteins are altered in a consistent manner in tissues from mice which are deficient in wild type p53. Also a 195bp DNA sequence from a region comprising -279 to -85bp upstream of the *bcl-2* transcriptional start site was

able to confer p53 dependent repression when inserted adjacent to a CAT reporter gene on a plasmid (Myashita et al., 1994b).

1.6 DNA DAMAGE RECOGNITION PROTEINS (DRPs) IN CISPLATIN RESISTANCE

1.6.1 THE DNA BINDING SIGNAL

In order for cells to respond to DNA damage either by repair of the damage or by apoptosis, the DNA adducts must be recognised. The p53 protein might directly bind to the damaged DNA and this might effect some alteration in p53 activity. Alternatively another protein may transduce the signal from the site of damage to the p53 molecule. For example, p53 protein has been shown to be phosphorylated by a DNA dependent protein kinase (Lees-Miller et al., 1990) and this modification occurs within a region which is thought to be important in *trans*-activation of transcription by p53 (Lees-Miller et al., 1992). Such a kinase could be the link between DNA damage and a p53 DNA damage response pathway. Proteins which recognise DNA adducts may be involved in the initial stages of such a pathway or may be part of an alternative DNA damage response mechanism.

Obvious roles for DNA damage recognition proteins (DRPs) in drug tolerance would be as repair proteins or proteins which can bind to both the site of DNA damage and a repair protein at once, thereby recruiting repair proteins to the region of damaged DNA. Also, proteins which bind to damaged DNA could be irrelevant proteins which serendipitously minimise the DNA distortion caused by the adduct thereby allowing the normal interaction of proteins involved in DNA replication and transcription. It is possible that certain DRPs are replicative or transcriptional proteins which are trapped on the DNA by the adduct. In which case they may be irrelevant to drug resistance although they may enhance toxicity of the drug by protecting the damage site from recognition by repair enzymes or they could be titrated

away from their own natural site of action and this might affect cisplatin toxicity. Such proteins may include the human upstream binding factor (hUBF), a 94-97KDa protein involved in transcription of ribosomal RNA (Treiber et al., 1994). Proteins which are involved in maintaining the chromatin structure such as histones could also become attached to the damage site. Although such an interaction may not be significant for drug resistance, gross alterations in the levels or activity of such proteins might influence the access of drugs to the DNA and so affect the levels of DNA damage. However such proteins would be unlikely to show specificity for cisplatin-damaged DNA.

1.6.2 THE DETECTION OF DRPs

Proteins which recognise cisplatin-damaged DNA were detected by gel mobility shift analysis in the human, ovarian carcinoma cell lines A2780 and 2008 but there was no increase of binding activity in cisplatin resistant derivatives of these cell lines compared to sensitive parentals (Andrews & Jones, 1991). Also, no differences in levels of cisplatin DRPs were detected between the CHO cell line AA8 and cisplatin hypersensitive derivatives UV4 and UV5. However, lower levels of DRP activity were detected in the kidney cell lines, MDCK and LLC-PK when compared to 2008 cells which are more cisplatin sensitive. DRPs of 26, 28 and 97KDa were detected with the same cisplatin-damaged probe on South-western blotting of 2008 cells and 2008/C13*, the cisplatin resistant derivative.

A cisplatin DRP of approximately 100KDa was identified in human HeLa cell extracts by South-western blotting (Toney et al., 1989). This DRP has apparent specificity for the intrastrand d(GpG) and d(ApG) adducts (Donahue et al., 1990). In order to isolate the gene encoding this DRP, a library of DNA fragments prepared from human B cells was screened by expression of these fragments from λ phage vector in *Escherichia coli*

bacterial cells. Out of three hundred and sixty thousand phage plaques, only two showed affinity for a radiolabelled, cisplatin damaged DNA probe. These two contained regions of the same gene and this was used as a probe in Northern blotting of RNA from HeLa, hamster V79 and murine L1210 cells. A mRNA species of 2.8kb was detected in each case. This gene was shown to be expressed in brain, heart , ileum, jejenum, kidney, liver, muscle and spleen by Northern blotting of tissues derived from baboon but there was no increase of mRNA expression on acquisition of cisplatin resistance as determined by Northern analysis of HeLa cells selected *in vitro* for cisplatin resistance (Bruhn et al., 1992).

A 125KDa UV-damage specific nuclear protein, UVBP1, has been purified from HeLa cells by FPLC and phosphocellulose chromatography (van Assendelft et al., 1993). It seems to be specific for the 6-4' (pyrimidine-2'-one) type of pyrimidine dimer rather than the cyclobutane form as it binds more efficiently to a substrate containing one UV damage site at a TC pair of nucleotides rather than at a TT, CT or CC sequence. Also DNA photolyase was unable to reduce DNA binding by this factor when incubated with the DNA before addition of UVBP1. This protein exhibited no cross reaction with cisplatin-damaged DNA.

1.6.3 DRPs WITH ALTERED EXPRESSION IN CELLS RESISTANT TO CHEMOTHERAPY AND/OR RADIOTHERAPY

DRPs with altered expression on development of cisplatin resistance are of particular interest as this indicates a possible direct involvement in cisplatin resistance. DRPs whose expression is altered when they become resistant to other DNA damaging agents are also interesting as there may be some degree of cross-talk between different DRPs. The DRPs concerned may not necessarily be exclusive to one form of DNA lesion. Also effects downstream of damage recognition may converge to a similar response mechanism. Even if DRP levels are not significantly altered between resistant

and sensitive cells, this does not mean that a DRP response mechanism is not involved in cisplatin resistance. If DRPs do not represent a rate limiting step in the damage response mechanism, it may be that factors involved in subsequent stages are altered in cisplatin resistant lines instead.

A cisplatin resistant HeLa derivative, selected by stepwise exposure to cisplatin doses up to 8μM, showed increased expression of two DRPs when compared to sensitive HeLa cells (Chao et al., 1991a). These proteins were 130KDa and 95KDa in size and were identified by South-western blotting with a cisplatin treated DNA probe. These proteins were inducible with a 4 hour, 3μM cisplatin dose and cross-reacted with UV damaged DNA. Also a 25KDa protein band was detected with reduced expression in the resistant cell line and after cisplatin treatment of the sensitive cell line. Using the gel mobility shift assay two bands of retardation activity were seen for cisplatin damaged DNA and a further two bands were seen for a UV damaged DNA probe. These DRPs have been further characterised and found to be mainly nuclear, protease sensitive and independent of RNA (Chao et al., 1992). Increased DRP activity has been found by other investigators for cisplatin resistant, compared to sensitive, HeLa and HT1080 cell lines (Chu & Chang, 1990).

A protein whose expression correlates with cisplatin sensitivity has been found in *Saccharomyces cerevisiae* (Brown et al., 1993). This protein is encoded by the *IXR1* gene and inactivation of the gene conferred increased resistance to cisplatin. The mutant strain was twice as resistant to a 2 hour cisplatin dose in the concentration range 50-1000μM but no difference in transplatin toxicity was detected. This was probably due to reduced accumulation of cisplatin induced DNA lesions as these appeared to be reduced to a third of the levels seen in cells which contained an active *IXR1* gene. This yeast species appears to have several cisplatin DRPs of 100, 80, 55 and 20KDa in size as detected by South-western blotting. The 80KDa protein

is missing from an *ixr1* strain and thus probably represents the *IXR1* gene product. The predicted amino acid sequence of this protein contains several long stretches of asparagine and glycine residues which is often seen for transcription factors and it has 56 identical residues to ABF2, the ARS-binding factor, in a 151 amino acid sequence.

A cisplatin resistant cell line contains increased levels of two UV specific DNA binding proteins by comparison to a cisplatin sensitive line using the gel mobility shift assay (Chao et al., 1991c). These cells also showed cross resistance to UV light compared to their parentals. The human ovarian carcinoma cell line, Ov1P contains four DRPs of 25, 48, 70 and 97KDa from Western blotting and the levels of the 97KDa and 48KDa proteins are elevated in a cisplatin resistant derivative, Ov1PDDP (McLaughlin et al., 1993). Several bladder and testicular cell lines were also examined for expression of these proteins as bladder cells are known to be more tolerant of cisplatin than testicular cells. Bladder cells expresses more 25KDa protein whereas testicular lines seem to express more 70KDa protein. Neither of these cell types expressed much 97KDa protein and the 25KDa DRP seemed to be present as a doublet rather than one discrete band.

South-western blotting of HeLa cells shows repression of a DNA binding protein along with induction of several DRPs in cisplatin resistant HeLa extracts compared to extracts from sensitive HeLa cells (Chao et al., 1991b). Also 2 dimensional gel electrophoresis showed reduction of at least four nuclear proteins in the resistant and cells levels of these were relatively enhanced in cells which had reverted to a cisplatin sensitive phenotype and which had reduced repair capacity.

1.6.4 DRPs ASSOCIATED WITH DNA REPAIR PROCESSES

An obvious reason why DRPs could be involved in cisplatin resistance is that they might be repair proteins or proteins which recruit repair proteins to sites of DNA damage so DRPs which are linked to repair processes are especially interesting. Cisplatin resistant HeLa cells selected by exposure to doses of cisplatin up to 8μM exhibit enhanced DNA repair activity as detected by their ability to repair a damaged CAT reporter gene and thus give rise to measurable CAT activity (Chao et al., 1991d). These cells also contained increased UV specific DRP activity as determined by an increase in the intensity of the band observed on gel mobility shift analysis. This factor did not cross react with cisplatin damaged DNA in competition experiments unless competitor was added at excessive levels, although another DRP was observed on gel mobility shift analysis using radiolabelled, cisplatin damaged DNA as a probe. Likewise this DRP activity could only be competed for by adding high levels of UV damaged probe. The increased repair in these cells was later shown to correlate with enhanced recognition and incision of DNA adducts but not elevated repair synthesis (Chao et al., 1993).

An absence of DRP activity has been found in cells from patients suffering from the disease xeroderma pigmentosum group E (Chu & Chang, 1988). There are seven complementation groups for this DNA repair deficiency syndrome, which have been defined by the use of cell fusion studies. HeLa cell nuclear, but not cytoplasmic, extracts were shown to give rise to two retarded bands when subjected to the gel mobility shift assay using UV damaged DNA and they also had affinity for cisplatin damaged DNA from competition of DRP activity with unlabelled, cisplatin-damaged DNA. The upper band had 10 fold higher affinity for damaged than undamaged DNA and the lower band had 100 fold greater affinity for damaged DNA. Both of these bands were absent from xeroderma pigmentosum

complementation group E cells but not from cells from the other complementation groups examined and so the binding activity was referred to as XPE binding factor.

It was postulated that the DRP activity which was missing in the human, xeroderma pigmentosum complementation group E cells might be a homologue of *Saccharomyces cerevisiae* photolyase. This was based on their similar affinities for DNA damaged by various agents, intracellular localisation, dependence on magnesium ions and their abundance relative to genome size (Patterson & Chu, 1989). Yeast nuclear extracts contained DRP activity which gave rise to two bands on gel mobility shift analysis, in a similar manner to that seen for HeLa nuclear extracts. This activity was not found for nuclear extracts from yeast cells mutant for the *phr1* gene. However the two bands differed in mobility between the two species and human DRP activity did not respond to light as yeast DRP did. When the yeast gel mobility shift assay was incubated in the presence of light, reduced DNA binding activity was detected compared to an assay incubated in the dark. This is consistent with dissociation of yeast photolyase from the DNA following light stimulated repair of DNA damage.

The photolyase enzyme uses visible light, absorbed through associated chromophores, as an energy source to repair pyrimidine dimers, a UV specific DNA lesion. Expression of this protein is induced by various DNA damaging agents as determined by use of a *PHR1-lacZ* fusion construct transfected into yeast cells and by Northern blotting (Sebastian et al., 1990). As yeast cells are more amenable to genetic manipulation than those of higher eukaryotes, elucidation of DNA damage response mechanisms within such organisms may provide information relevant to analogous mammalian pathways. The XPE binding factor showed increased expression in HeLa and HT1080 human fibrosarcoma cells selected for cisplatin resistance *in vitro* as compared to the original, sensitive cell lines (Chu & Chang, 1990). Resistant HT1080 cells

were also examined for DNA repair capacity by use of a cisplatin damaged CAT reporter construct. They displayed increased CAT activity, indicative of increased repair, compared to the parental cell line. A further DRP was also identified in HeLa nuclear extracts. This factor, named CCD due to affinity for cisplatin crosslinked DNA had specificity for cisplatin damaged DNA and so neither UV damaged or single stranded DNA could compete for binding by this factor on gel mobility shift analysis. This was unlike the XPE binding factor which possessed affinity for all three of these DNA types but not for double stranded DNA. A DRP which is inducible by UV, mitomycin C or aphidicolin and absent in xeroderma pigmentosum group E extracts, was observed using the gel mobility shift assay (Hirschfeld et al., 1990). This factor was observed in the monkey kidney cell line, CV-1 and may be homologous to the XPE binding factor.

Another DRP associated with xeroderma pigmentosum has been identified (Robbins et al., 1991). This 40-42KDa protein has 1000 fold greater affinity for UV damaged than undamaged DNA as determined by retention of DRP-radiolabelled DNA complexes on nitrocellulose filters. Cell extracts were assayed for DNA damage repair ability by incorporation of radiolabelled ATP into UV-damaged plasmid DNA followed by electrophoresis of plasmid DNA. This was used to demonstrate that deficient repair in xeroderma pigmentosum group A extracts could be complemented by addition of the purified 40-42KDa protein.

The product of the DNA repair gene, *ERCC1* has a helix-turn-helix motif indicating DNA binding capacity and this may represent another DRP. This gene appears to be expressed at 2.6 fold higher levels in patients with cisplatin resistant tumours compared to patients with cisplatin responsive tumours (Dabholkar et al., 1992). The *ERCC1* gene was identified due to its ability to complement repair deficiency in UV sensitive CHO cells. The *ERCC1* has some degree of homology to the *RAD10* gene from yeast which is

involved in DNA repair (van Duin et al., 1986). There is therefore, a possible role for DRPs in cisplatin resistance and as such they warrant further investigation.

1.7 AIMS OF THIS THESIS

The aims of this thesis are to molecularly define some of the possible events leading from cisplatin-induced DNA damage to cell cycle arrest and cell death.

1) In this thesis an attempt will be made to examine differences in p53 protein expression in cisplatin sensitive and resistant cells.

2) Alterations in p53 transcriptional activity in these experimental models will also be characterised as this may have relevance to DNA damage induced apoptosis and the development of cisplatin resistance.

3) A scheme for purification of a cisplatin DRP, which is active in the gel mobility shift assay, will be developed using extracts from human ovarian carcinoma cell lines.

4) The cisplatin DRP mentioned in 3) will be characterised in order to discover its identity and thus any possible relevance of this protein to cisplatin resistance.

Chapter 2

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

The following section lists routinely used materials. Less frequently used materials are described in the appropriate figure legends.

2.1.1 CHEMICALS

All chemicals were of the highest available quality and were obtained from BDH Chemicals Ltd, Gibco BRL, Pharmacia LKB or Sigma Chemicals except the following;

Acrylamide	Severn Biotech, Kidderminster.
Phenol	Rathburn, Walkerburn, UK.

2.1.2 RADIOCHEMICALS

(α^{32}P)dCTP and (γ^{32}P)dATP for labelling probes were obtained from Amersham International plc.

2.1.3 EQUIPMENT

Routine equipment which would be an integral part of any laboratory is not listed.

1. Electroblotting System: Milliblot SDE, Millipore, Watford.

2. Gel Tanks: tanks for agarose & acrylamide gels were from IBI Ltd, Cambridge & Biorad Labs Ltd, Watford, Hertfordshire.

3. Hybridisation membranes: Hybond-N, Amersham International. Nitrocellulose 0.45um, Schleicher & Schuell via Anderman Lab Supplies, Kingston-upon-Thames. Immobilin-P, Millipore, Bedford.

4. Hybridisation Oven & Bottles: Hybaid Ltd, Middlesex.

5. Laser densitometer: autoradiographs were analysed at a SUN workstation using a Molecular Dynamic Densitometer and PDI Quantitation 1 software.

6. Chromatographic protein separations were performed on FPLC equipment from Pharmacia Ltd or on a "Waters" HPLC system from Millipore Ltd.

2.1.4 RESTRICTION ENDONUCLEASES & OTHER ENZYMES

Restriction Enzymes

The majority of restriction endonucleases (RE) were from Pharmacia Ltd, Boeringer Mannheim Corporation or Northumbria Biologicals. Bulk ordering of RE's by the Department meant that the same "brand" of RE could not be used throughout this work.

Other Enzymes

T4 Polynucleotide kinase	Northumbria Biologicals

2.1.5 SIZE MARKERS

DNA size markers; Hind III digested phage λ,BRL

RNA size markers; 0.24 - 9.5kb RNA ladder, BRL

Protein size markers; Prestained standards, 14,300-200,000, BRL.

2.1.6 BUFFERS, SOLUTIONS & MEDIA

Where mentioned, autoclaving was carried out at 121°C for 20 minutes.

General Buffers & Solutions

TMS

10mM Tris (pH 7.5)

5mM $MgCl_2$

8.6% sucrose

RNA Lysis Buffer

0.3M Na acetate

0.5% SDS

5mM EDTA. Made to pH8 with 10M NaOH then autoclaved.

TAE(1x) pH8

40mM Tris base

2mM EDTA

20mM NaCl

20mM Na Acetate

TBE(1x) pH8

89mM Tris borate

89mM Boric acid

2.5mM EDTA

BLOTTO (1x)

50mM Tris (pH 7.5)

50mM NaCl

1mM EDTA

Spacer gel Buffer

0.5M Tris

4% SDS

Running Buffer

1.5M Tris

4% SDS

Storage Buffer (SB)

50mM NaCl

20mM Hepes

5mM $MgCl_2$

0.1mM EDTA

20% glycerol

1mM DTT (added fresh)

TE

10mM Tris

1mM EDTA

High Salt Lysis Buffer

500mM NaCl

1% NP-40

50mM Tris (pH 7.5)

1mM DTT (added fresh)

protease inhibitors 1x (added fresh)

Protease Inhibitors (100x)

0.1mg/ml aprotinin

0.1 mg/ml pepstatin

0.1 mg/ml chymostatin

0.05M benzamidine

0.05M PMSF

0.1 mg/ml leupeptin

These were stored at -70^{o}C

TNE-50 (10x)

10mM Tris pH 7.5

50mM NaCl

1mM EDTA

1mM DTT (added fresh)

Tank Buffer

0.26M Tris

2% glycine

0.5% SDS

Phosphate Buffered Saline (PBS)

0.8% NaCl

0.115% $Na_2H\ PO_4$

0.02% KCl

0.02% $KH_2\ PO_4$

Denaturation Buffer (for Southern blotting)

0.5M NaOH

1.5M NaCl

Neutralisation Buffer (for Southern blotting)

1M Tris

1.5M NaCl

pH to 7.4 with HCl

Formaldehyde Gel Running Buffer

0.1M MOPS (pH 7)

40mM Na acetate

5mM EDTA

Loading Buffers

Dye Mixture

30% w/v Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol

Formaldehyde Gel Loading Buffer (for Northern blotting)

50% glycerol

1mM EDTA (pH 8)

0.25% bromophenol blue

0.25% xylene cyanol FF

Acrylamide Gel Loading Buffer

80% Formamide

1 x TBE

1mM EDTA

0.1% Xylenecyanol

0.1% Bromophenol blue

Western Loading Buffer(x5)

0.25M Tris (pH 8)

10% SDS

10mM EDTA

50% glycerol

0.25% bromophenol blue

0.25% xylene cyanol FF

Transfer solutions

Phosphate buffer "Genescreen"(20x), used to transfer both RNA & DNA.

0.5M Na_2HPO4

0.5M NaH_2PO4

Transfer Buffer (for proteins)

48mM Tris base

39mM Glycine

0.037% (v/v) SDS

20% methanol

Hybridisation Solutions

Hybridisation mix (for Southern and Northern blotting)

50mM Pipes

100mM NaCl

50mM Na_2PO_4

50mM NaH_2PO_4

1mM EDTA

5% SDS

South-western Binding Buffer

30mM Hepes

10mM $MgCl_2$

2mM $MnCl_2$

0.25% marvel

Tissue culture media

RPMI

88 ml RPMI-1640 (10x) from Gibco BRL

800ml sterile distilled water

26.6ml 7.5% $Na(CO_3)_2$

10ml 100mM Na pyruvate

10ml 200mM L-glutamine

1ml 1M NaOH

100ml foetal calf serum

Dulbecco's Modified Eagles media (DMEM)

50ml Dulbecco's MEM (10X) from Gibco BRL

400ml sterile distilled water

5ml 100mM Na pyruvate

5ml 200mM L-glutamine

25ml 7.5% $Na(CO_3)_2$

Special Liquid Medium (Gibco BRL)

500ml Special Liquid Medium

50ml 200mM L-glutamine

50ml foetal calf serum

2.1.7 CELL LINES

A2780 A human, ovarian adenocarcinoma cell line obtained from R.F. Ozols and T.C. Hamilton, Fox Chase Cancer Center, Philadelphia.

A2780cp70 A cisplatin resistant derivative of A2780.

For a more complete description of A2780 and A2780cp70 see Behrens et al., 1987.

Ov1P A human, ovarian adenocarcinoma cell line obtained from J. Benard, Institut Gustav Roussy, France.

Ov1PDDP A cisplatin resistant line derived from Ov1P.

For further information concerning Ov1P and Ov1PDDP see Teyssier et al., 1989.

2.1.8 MONOCLONAL AND POLYCLONAL ANTIBODIES AND IMMUNOLOGICAL REAGENTS

Monoclonal Antibodies

PAb 419 A control antibody reactive against SV40 large T antigen (Harlow et al., 1981).

PAb 240 A p53 mutant-specific antibody.

PAb DO-1 A pantropic anti-p53 antibody.

The three antibodies listed above were used for immunoprecipitation of p53 protein. For a more detailed explanation of these reagents see Vojtesek et al, 1992.

AB-2 A pantropic anti-p53 antibody derived from mouse and purchased from Oncogene Science, Cambridge, used as a primary antibody on Western blots at a 1 in 1000 dilution.

Anti-HMG1 This was a gift from Dr P. Billings, University of Pennsylvania.

Anti-HMGI This was donated by Dr R. Reeves, Washington State University.

IF2 An anti MDM2 antibody from Oncogene Science, Cambridge which was used at a 1 in 1000 dilution for Western blots.

Anti-PCNA This was obtained from Boeringer-Mannheim, Germany and was used at a in 1000 dilution as a control antibody for Western-blotting.

Anti-Hsp70 This was obtained from Sigma, Dorset and was used at a 1 in 1000 dilution for Western blots.

Polyclonal Antibodies

CM1 A polyclonal anti-p53 antibody from rabbit which was used as a primary antibody for Western blotting at a 1 in 200 dilution. See Midgely et al., 1992 for further details.

Immunological Reagents

Anti-mouse Ig Horseradish peroxidase conjugate (from sheep). This was obtained from Amersham International and was used at a 1 in 5000 dilution in Western immunoblots.

Anti rabbit Ig Horseradish peroxidase conjugate (from mouse). This was obtained from Dako Ltd, Aylesbury. and used at a 1 in 150 dilution as a secondary antibody for Western blotting.

2.1.9 MOLECULAR PROBES

WAF-1 This 12.4 kb cDNA fragment was used for Northern blotting and was derived from the plasmid, pCEP-WAF-1-S (El-Deiry et al., 1993).

GAPDH This 7.2 kb cDNA probe was obtained by digestion of the construct, pCRII GAPDH (a gift from M. Walker, BICR) , and was used to normalize for loading on Northern blots. For further information on GAPDH see Tso et al., 1985.

Both of these probes were prepared by digestion of the plasmid, using the appropriate restriction enzymes. The DNA fragments were separated out by electrophoresis through an 0.8% agarose , 1x TBE gel containing 0.25μg/ml ethidium bromide at 150v for 2 hours using 1x TBE as a buffer. A fragment of the expected size was visualised under UV and the appropriate agarose slice was cut from the gel. The slice was sealed, along with 400μl 1x TBE, in a piece of dialysis tubing which had been pre-boiled for 10 minutes in autoclaved water. This was then placed in a tank of 1x TBE and subjected to electrophoresis at 50V for 75 minutes. The current was reversed for 1 minute then reversed again for 30 seconds. The 1x TBE, containing the DNA,

was removed from the tubing, the DNA was recovered by ethanol precipitation and resuspended in TE.

2αG7 This 54bp DNA probe was used in platinated and non-platinated forms in the gel mobility shift assay. For a more detailed description see K. McLaughlin, PhD thesis, Glasgow University, also McLaughlin et al., 1993.

2.2 EXTRACTION OF NUCLEIC ACIDS & PROTEINS

All work with RNA & DNA was carried out using autoclaved solutions & where appropriate DEPC treated solutions & equipment. Disposable gloves & plasticware were used throughout. DNA & RNA quantitation was performed using a combination of visual assessment on a 0.8% agarose, 1x TBE, 0.25mg/ml ethidium bromide gel when run against known standards and spectrophotometrically using O.D.260.

2.2.1 RNA EXTRACTION

Monolayer cultures were lysed in the flasks with TRIzol™, BRL. The extraction procedure followed the manufacturers instructions.

2.2.2 DNA EXTRACTION

Cell lines were lysed with 0.3M Na Acetate equilibrated phenol pH7.6. 10mls of phenol was used per 175cm^2. An equal volume of RNA lysis buffer and chloroform isoamyl alcohol was added & the samples mixed for 20 minutes. The samples were then spun at 3000g for 20 minutes at room temperature. The upper aqueous phase was collected & precipitated with an equal volume of isopropanol at 4°C for at least one hour. The DNA was spun down at 3000g for 20 minutes at 4°C, airdried then resuspended in sterile water. This procedure simultaneously extracts DNA & RNA.

2.2.3 EXTRACTION OF NUCLEAR PROTEINS FROM CELL LINES

This protocol was carried out at 4°C and was adapted from Emerson and Felsenfeld., 1984. Cells were harvested during exponential growth, washed with RPMI, then ice cold PBS, osmotically swollen with a TMS wash and lysed with 0.25% TRITON X-100. NaCl was added to 0.3M, followed by spinning at 3,500g for 20 minutes. To the supernatant, 1/5 volume Glycerol Brj 35 and DTT to 1M was added, it was dialysed against storage buffer overnight and pelleted. The supernatent was collected and stored at -70°C. Protein estimation was carried out using the Biorad kit method.

2.2.4 LYSIS OF MONOLAYER CELLS

Unless otherwise stated, cells were washed with PBS, then lysed at 4°C for 10 minutes using high salt lysis buffer supplemented with 1x protease inhibitors (section 2.1.6). The lysate was spun at 12000g for 10 minutes and the supernatant stored at -20°C. Protein content was estimated using the Biorad kit method and by comparison of gels stained with coomasie stain (0.2% coomasie brilliant blue R250 in a 50:50:7 v/v ratio of methanol:H_2O:glacial acetic acid) then de-stained using a 25:68:7 v/v ratio of methanol:H_2O:glacial acetic acid.

2.3 PREPARATION OF ^{32}P RADIOLABELLED PROBES

With the exception of end labelled 2αG7 (see 2.3.1), all other probes were separated from unincorporated ^{32}P-labelled nucleotides using disposable Sephadex containing "NICK" columns from Pharmacia.

2.3.1 PREPARATION OF PROBES USING AGAROSE GELS

10 picomoles of oligonucleotide were incubated with 12 units of T4 polynucleotide kinase and 1.85 MBq of ^{32}P γ-ATP in One-Phor-All-Buffer-Plus at 37°C for 45 minutes. Samples were then electrophoresed on a 1X

TBE,0.8% agarose, non-denaturing gel for 90 minutes at 150V. The gel was exposed to Kodak XOMAT-AR film and the developed film was used as a template to remove the labelled oligonucleotide. This gel fragment was placed in a Spinex tube with 400µl distilled H_2O overnight. The contents of the Spinex tube were spun down to separate the oligonucleotide and the gel fragment.

2.3.2. RANDOM PRIMING OF dsDNA

^{32}P labelled dsDNA probes were produced with the aid of the "Prime-it" random primer kit from Stratagene. Between 50ng and 100ng of template DNA was used and random primed using a Stratagene-recommended protocol.

2.4 SEPARATION & HYBRIDISATION OF NUCLEIC ACIDS

2.4.1 DIGESTION, SEPARATION & SOUTHERN TRANSFER OF DNA

Separation and transfer of DNA was essentially as described by Southern., 1975. 20µg of genomic DNA was digested for at least 16 hours in a total volume of 150µl using 150 units of restriction enzyme. The digested DNA was then ethanol precipitated with 0.1 volume of 3M Na Acetate and 1 volume of isopropanol at -20°C for 1 hour. The DNA was pelleted by centrifugation at 13,000g for 15 mins followed by air-drying and resuspension in 25µl of sterile water. Resuspension continued overnight at 37°C and then transferred to 4°C until use (usually within one week). 5µl of Dye Loading Buffer was added to the DNA and this was run on a 0.8%-1.2% TAE gel. Gels were run overnight at 50V using a buffer recirculation system. The next morning, gels were stained with ethidium bromide for 20 minutes to allow polaroid photography on a UV transilluminator. DNA was denatured for 20 minutes in 1.5M NaCl/0.5M NaOH followed by neutralization in 3M NaCl/0.5M Tris-HCl pH7.0 for 30 minutes. The gel was then rinsed in Genescreen buffer (1x) and transferred onto either Genescreen or Hybond N

membranes overnight using Genescreen buffer. The membranes were exposed to UV for 6 seconds to fix the DNA onto the membrane.

2.4.2 SEPARATION & NORTHERN TRANSFER OF RNA

Methodology for separation and transfer of RNA followed instructions in Sambrook et al., 1989. 0.8% (w/v) agarose gels were prepared by dissolving 2.4g of agarose in 187.5mls of water then cooling to 60°C. 54mls of 37% formaldehyde plus 59mls of gel running buffer (5x) were then added, mixed and immediately poured.To 20µg of total RNA in 5µl, 10µl formamide, 7µl formaldehyde and 2µl of RNA gel running buffer were added . The samples were heated to 65°C for 10 minutes then chilled on ice before addition of 2µl RNA gel loading buffer and subsequent loading onto the gel. Gels were electrophoresed for 3 hours at 200V. The gels were photographed then soaked in 50mM NaOH for 20 minutes. They were rinsed with RNAse free water and soaked in Genescreen (1x) for 45 minutes prior to transfer onto Hybond N using Genescreen as the transfer buffer. Membranes were rinsed & exposed to UV as previously described for DNA transfer.

2.4.3 HYBRIDISATION OF SOUTHERN AND NORTHERN BLOTS

Hybridisations in hybridisation buffer (recipe in section 2.1.6) were carried out at 65°C (for a minimum of 16 hours) using a Hybaid oven and Hybaid roller bottle system.

2.4.4 WASHING FILTERS

Following non-formamide hybridisation, membranes were washed at 65°C using 1 x SSC plus 5% SDS for at least 90 minutes with a minimum of 3 changes of wash buffer.

2.4.5 AUTORADIOGRAPHY

Following washing, filters were blotted dry, wrapped in clingfilm and exposed to Kodak AR film in a film cassette with fast tungstate intensifying screens. Loaded film cassettes were held at -70°C until developed.

2.5 GEL SEPARATION & IMMUNODETECTION OF PROTEINS

2.5.1 SAMPLE PREPARATION

Protein extracts were prepared & quantified as detailed in section 2.2.3, 2.2.4 or 2.6.5. 50–200μg of total protein was loaded per lane in western loading buffer after boiling for 3 minutes to facilitate denaturation .

2.5.2 GEL & RUNNING CONDITIONS

Denaturing protein gels were cast and run in the Protean gel tank system (Biorad).

8% acrylamide gels were prepared as follows;

8.6ml acrylamide (30% acrylamide with 0.8% bis acrylamide)

1.6ml polyacrylamide (1%)

8ml running buffer

12.2ml H_2O

90μl ammonium persulphate (10%)

20μl temed (from Biorad)

This mixture was poured leaving a 3-4 cm gap at the top for the stacking gel;

1.6ml acrylamide (30% acrylamide with 0.8% bis acrylamide)

1.2ml polyacrylamide (1%)

3ml spacer gel buffer

6.2ml H_2O

250μl ammonium persulphate (10%)

20μl temed

The proteins were electrophoresed through the stacking gel at 200v then the gel was run overnight at 50v in 1x Tank buffer.

2.5.3 WESTERN TRANSFER OF PROTEINS BY ELECTROBLOTTING (Kyhse-Andersen et al., 1984)

Electroblotting was performed using a millipore semi-dry electroblotter. The Immobilin-P, Millipore membrane was wetted in methanol then transfer buffer; 6 sheets of 3M Whatmans filter paper was sandwiched next to the anode and cathode with the membrane and gel layered in between. Transfer took place over 1 hour at 200mA. The gel was then stained in Coomasie stain overnight and destained as described in section 2.2.4 . This allowed a visual assessment to be made of the evenness of the transfer and the integrity of the proteins.

2.5.4 SILVER STAINING OF PROTEIN GELS

Proteins were separated on a 5-15% acrylamide concentration gradient gel, pre-fixed with 50% methanol, 10% acetic acid for 1 hour, fixed for 30 minutes in 10% gluteraldehyde and then washed in H_2O for 2 hours. They were sensitized with 1M DTT for 1 hour, stained with silver nitrate for 1 hour, rinsed with water and developed using 3% sodium carbonate, 0.05% formaldehyde. The reaction was stopped with 5% acetic acid.

2.5.5 IMMUNODETECTION OF PROTEINS ON WESTERN BLOTS (adapted from Harlow and Lane.,1988)

Chemiluminescence method

Blocking, and all antibody dilutions, were in 1X Blotto, 5% Marvel, 0.01% Tween-20, 1mM DTT. Washes were in PBS,0.01% Tween-20. All procedures were carried out at 4°C. Block was applied overnight followed by incubation with primary antibody overnight. The first wash in PBS, 0.01%

Tween-20 was for 25 minutes with 3 changes of buffer. The second antibody, was applied for at least 2 hours. The second wash, for 25 minutes, had a further 3 buffer changes. The membrane was then incubated with a chemiluminescense substrate (ECL kit from Amersham) as per manufacturers instructions and exposed to radiographic film.

2.5.6 SOUTH-WESTERN TRANSFER OF PROTEINS (Bowen et al., 1980)

This protocol was essentially the same as for Western blotting (sections 2.5.1-4) with the following exceptions: Samples were electrophoresed through a 5-15% acrylamide concentration gradient gel; proteins were transferred onto nitrocellulose membranes(blocking solution did not contain tween-20). After blocking, the membrane was washed twice with 1x TNE-50 then placed in south-western binding buffer with 10μg/ml of poly (dI.dC):(dI.dC) and radiolabelled oligonucleotide ($2x10^4$ d.p.m./ml) for 90 minutes; membranes were washed for 9 minutes in 30mM Hepes (pH 7.5), 0.25% marvel. Then autoradiography was carried out as in section 2.4.5.

2.6 PROTEIN SEPARATION TECHNIQUES

2.6.1 TCA TREATMENT

TCA was added to crude nuclear extracts (see section 2.2.3) to give a final concentration of 2%. This was incubated on ice for 30 minutes then spun in the microfuge at 12000g for 15 minutes. The clear supernatant was removed and β-mercaptoethanol added to 10mM.

2.6.2 ANION EXCHANGE CHROMATOGRAPHY

Samples were de-salted using a sephadex G25 column. Buffer A was 0.1M NaCl, 0.02M Tris (pH 7.6); buffer B was 2M NaCl, 0.02M Tris (pH7.6). The MonoQ™ column (from Pharmacia) was washed with 100% buffer A, 0% buffer B then 100% buffer B,0% buffer A and finally, 100% buffer A, 0% buffer B. A gradient of 0% buffer B to 100% buffer B was set up. Proteins

were eluted at 25-50% buffer B. Fraction volume was 2ml, collected at a speed of 1ml/minute.

2.6.3 HEPARIN COLUMN CHROMATOGRAPHY

Buffer A was 0.1M NaCl, 0.02M Tris (pH 7.6); buffer B was 2M NaCl, 0.02M Tris (pH 7.6). The heparin-sepharose column (Pharmacia) was washed with 100% buffer A, 0% buffer B, then 100% buffer B, 0% buffer A and finally 100% buffer A, 0% buffer B. Samples were prepared as for 2.6.2. A gradient of 0% buffer B to 100% buffer B was set up. Proteins were eluted at 25-50% buffer B. Fraction volume was 1ml collected at a speed of 1ml/minute.

2.6.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Buffer A was 0.1% TFA (tri-fluoro acetic acid) in H_2O (pH 0.5); buffer B was 0.1% TFA in acetonitrile:H_2O at a v/v ratio of 95:5 (pH 2.11). Buffers were degassed using helium. The Biorad Hipore butyl C_4 silica-based reverse phase column (30nm pore, 4.6x250mm dimensions was equilibrated for 1 hour then purged. A gradient of 15% buffer B to 50% buffer B over 60 minutes was set up. Buffer B then remained at 50% for 30 minutes. After the run was completed, buffer B rose to 100% over 30 minutes. A zinc lamp (214nm filter) was used for monitoring. Fraction volume was 1ml collected at a speed of 1ml/minute.

2.6.5 SEPARATION OF SPECIFIC PROTEINS BY IMMUNOPRECIPITATION (Harlow and Lane., 1988)

Monolayer cells were washed twice with PBS. 700µl of high salt lysis buffer was added per 175cm^2 flask, cells were scraped off, mixed, left on ice for 30 minutes and mixed every 10 minutes then spun at 11000g for 30 minutes at 4°C. 15µl of protein G was added to the supernatant and this was

rotated at 4°C for at least 40 minutes. After spinning at 11000g for 5 minutes at 4°C, 1μg of antibody was added to the supernatant and this was left overnight at 4°C. 20μl of protein G was added and after 40 minutes, it was spun at 11000g for 1 minute at 4°C, the pellet washed three times with high salt lysis buffer then resuspended in 1x western loading buffer. This was boiled for 8 minutes at 11000g and the supernatant stored at -20°C.

2.7 TISSUE CULTURE TECHNIQUES

2.7.1 GENERAL TECHNIQUES

Asceptic manipulations were performed using sterilised glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown at 37°C as monolayers in supplemented RPMI (Rosswell Park Memorial Institute) medium in the presence of 5% CO_2. They were stored by freezing $1x10^6$ cells/ml, along with 10% di-methyl sulphoxide (DMSO), at -70°C then they were maintained in liquid nitrogen. For RPMI supplements see page 49.

2.7.2 CLONOGENIC ASSAY

10^3 cells were used per $75cm^2$ flask. Following drug treatment colonies were grown for 10-14 days then stained with 1x Giemsa stain (BDH Ltd.) for 10 minutes and rinsed.

2.7.3 TRANSFECTION OF PLASMID DNA INTO CELL LINES

All solutions were filtered before use. 10^6 cells per $75cm^2$ flask were incubated with appropriately supplemented Special Liquid Medium. 5μg plasmid DNA and 1μg G418 resistance marker plasmid, pHSG272, per sample were used, to which 2.5M CaCl2 (Rf 1.401) was added giving a final concentration of 0.2M. This was added dropwise to an equal volume of 280mM NaCl, 50mM Hepes, 0.5mM $Na_2H\ PO_4$. (final pH 7.12) whilst air was bubbled through. This was incubated at room temperature for 30 minutes,

mixed, added to cells and incubated at 37°C for 16-20 hours. After 48 hours, transfectants were selected with 1mg/ml G418 and maintained in 400μg/ml G418.

2.8 GEL MOBILITY SHIFT ASSAY (Garner and Revzin., 1981)

2.8.1. REACTION CONDITIONS

2μg of nuclear extract was incubated with ^{32}P end labelled oligonucleotide ($5x10^3$ d.p.m.), 2μg poly(dI.dC):(dI.dC) made up to 20 μl with storage buffer, for 30 minutes on ice.

2.8.2. GEL RUNNING CONDITIONS

The gel was pre-electrophoresed at 200V for 30 minutes. Reactions were then electrophoresed (at 200V for 2-3 hours) through the "retardation gel":

20ml acrylamide (30% acrylamide with 0.8% bis acrylamide)

4ml TBE (10x)

56ml H_2O

300μl APS (10%)

30μl temed

The buffer used was 0.5% TBE and dye mixture was placed in the end lane only. Once the dye front had reached the bottom of the gel, the gel was sandwiched between 3mm Whatman paper and clingfilm and dried under vacuum then subjected to autoradiography as detailed in section 2.4.5.

2.9 LUCIFERASE ASSAY

2.9.1 PREPARATION OF CELL LYSATES

All solutions were equilibrated to room temperature. 10^6 cells were lysed with 250μl of 25nM Tris phosphate (pH 7.8), 2mM DTT, 2mM diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% TRITON X-100. After 15 minutes they were scraped off and the lysate was spun briefly. The supernatant was removed to a separate tube.

2.9.2 ASSAY OF LYSATES FOR LUCIFERASE ACTIVITY

To the cell lysates, 1.5 volumes of 20mM tricine, 1mM $(MgCO_3)_4$ $MgCOH_2.5H_2O$, 2.7mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270μM coenzyme A, 470μM luciferin, 530μM ATP (final pH 7.8) was added then light production was measured for 1 minute in a luminometer.

2.10 MICROBIOLOGICAL TECHNIQUES

2.10.1 BACTERIAL CULTURE

Bacteria were maintained on Luria agar (1.5% agar in L-broth) plates at 4°C and stored for long periods in 50% glycerol in L-broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at -20°C.

2.10.2 TRANSFORMATION OF BACTERIAL CULTURES WITH PLASMID DNA

100μl of competent bacteria were added to 10-50ng of transforming DNA and left on ice for 30 minutes. 5 minutes after a 37°C heat shock, 1ml L-broth was added and the samples were vortexed before incubation for 1-1.5 hours at 37°C. Appropriate dilutions were plated on selective medium (L-agar, 100μg/ml) using glass beads to distribute transformed bacteria. Plates were incubated at 37°C.

2.10.3 RECOVERY OF PLASMID DNA FROM BACTERIAL CULTURES

5ml of a 10ml culture was used to innoculate 500ml of L-broth containing 100mg/ml ampicillin (for selection) and this culture was grown to stationary phase overnight. The Quiagen Maxi Preparation kit, Quiagen Inc. was used according to manufacturers recommendations to obtain plasmid DNA.

Chapter 3

CHAPTER 3: THE ROLE OF P53 PROTEIN IN CELLULAR RESISTANCE TO CISPLATIN

INTRODUCTION

3.1.1 WHY ONE SHOULD SUSPECT THAT p53 PROTEIN IS RESPONSIBLE FOR CISPLATIN RESISTANCE

DNA damaging agents such as cisplatin induce p53 protein levels via post translational means (Maltzman & Czyzyk, 1984; Fritsche et al., 1993) and this is inhibited by caffeine (Kastan et al., 1991). Also, when normal human skin is exposed to sunlight, p53 protein levels rise over a twenty four hour period (Hall et al., 1993). This evidence lends support to the theory that p53 is a component of a physiological DNA damage response pathway. Overexpression of wild type p53 protein *in vivo* has been reported for the normal tissue of an individual with a familial susceptibility to breast cancer (Barnes et al., 1992). Another *in vivo* situation where p53 protein levels are temporarily raised without mutation is in the cytoplasm of normal lactating breast tissue (Moll et al., 1992). Thus there are circumstances where p53 protein concentration is enhanced *in vivo* and application of our *in vitro* knowledge to clinical scenarios would not be totally contrived. There are several possible means by which p53 could affect the cellular tolerance of cisplatin and these will be discussed in the ensuing sections.

3.1.2 G1 ARREST AS A POSSIBLE MECHANISM BY WHICH p53 PROTEIN COULD MODULATE SENSITIVITY TO CISPLATIN

One hypothesis proposed was that raised p53 protein might cause cells to remain longer in G1 phase of the cell cycle thereby allowing more time for repair of DNA damage before DNA replication at S phase. DNA damage-induced p53 protein causes G1 arrest of cells (Kastan et al., 1991) and this appears to be dependent on expression of a wild-type *p53* gene (Kuerbitz et

al., 1992). Patients with the disease ataxia telangiectasia (A-T), who exhibit radiosensitivity and susceptibility to cancer, have altered induction of p53 and GADD45 in response to DNA damage (Kastan et al., 1992). Therefore, the A-T genes and GADD45 are implicated as elements of this pathway. There is a correlation between the ability to arrest in G1 phase of the cycle in a p53 dependent manner after DNA damage, and the level of radiosensitivity in cell lines from a wide variety of tissues (McIlwrath et al., 1994) Suppression of growth is observed when a glioblastoma cell line conditionally overexpresses wild type p53 (Mercer et al., 1990) and p53 acts as a control protein in osteosarcomas (Diller et al., 1990). Even in the yeast, *Schizosaccharomyces pombe*, overexpression of wild type p53 from a plasmid inhibits growth (Bischoff et al., 1992). However, the observation that there is a loss of, rather than an increase in, the G1 checkpoint when A2780cp70 cells become resistant to cisplatin invalidates this as the method by which this cell line achieves resistance (Brown et al., 1993). The G1 arrest following DNA damage is one p53 mediated property which is amenable to quantitative analysis and so it provides us with a measure of p53 function.

3.1.3 GENE AMPLIFICATION AS A POSSIBLE MECHANISM FOR p53 INDUCED DRUG RESISTANCE

Introduction of wild type p53 to cells with mutant p53 alleles results in inhibition of gene amplification (Yin et al., 1992). A defect in such a mechanism could give rise to amplification of drug resistance genes, leading to increased probability of cells acquiring a drug resistant phenotype.

3.1.4 p53 DEPENDENT APOPTOSIS AND DRUG RESISTANCE

At high concentrations, chemotherapeutic drugs cause apoptosis (Dive & Hickman, 1991; Eastman, 1990) and this may link p53 to drug resistance. Some cells, e.g. M1 clone S6 myeloid leukemic cells, require p53

for progression to an apoptotic-like cell death (Yonish-Roach et al., 1993). There is no p53-inducible growth arrest in these cells but they are particularly vulnerable to cell death when in the G1 phase of the cell cycle. A fault in the p53 dependent apoptotic pathway would permit the survival of cells which would otherwise be eliminated following DNA damage and thereby allow resistance to drugs such as cisplatin. As described in section 1.4.7, genetic inactivation of p53 in mice causes increased resistance to DNA damaging agents such as ionising radiation and topoisomerase inhibitors (Lowe et al., 1993b; Clarke et al., 1994).

3.1.5 THE IMPLICATIONS FOR DRUG RESISTANCE OF ALTERED TRANSCRIPTIONAL CONTROL BY p53

The functions performed by p53 leading to drug resistance could involve p53 protein acting directly on other regulatory or structural components of the cell. Alternatively p53 could bind to and alter the transcription of genes conferring drug resistance. Transcription of *mdr1*, encoding a transmembrane pump known as p-glycoprotein, which when overexpressed causes MDR, is induced by a mutant p53 containing an arginine to histidine substitution at amino acid 175. (Chin et al., 1992). However there seems to be scant evidence for a correlation between *p53* mutations and p-glycoprotein overexpression *in vivo* at least in the case of advanced myelodysplastic syndromes (MDS), a clinically relevant situation for p-glycoprotein abnormalities (Preudhomme et al., 1993). Also p53 can function as a transcription factor when expressed in yeast (Scharer & Iggo, 1992). Site specific binding of DNA by p53 has been demonstrated (Kern et al., 1991; Zambetti et al., 1992) as has its intrinsic transcriptional *trans*activation activity (Raycroft et al., 1990; Raycroft et al., 1991; Kern et al., 1992; Farmer et al., 1992). Using GAL4-p53 fusion proteins transcriptional *trans*activation has

been found to be associated with the N-terminal portion of p53 protein (Hupp et al., 1992). This arrangement is fairly common amongst transcription factors.

p53 mediated repression of the human *hsp70* promoter (Agoff et al., 1993) and the SV40 enhancer promoter (Jackson et al., 1993) has been reported. This also involves the N-terminus of p53. Proteins which have p53-binding affinity, such as SV40 large T antigen, HPV E-6 and mutant p53, can inhibit site specific binding to DNA (Bargonetti et al., 1992) and transcriptional activation by p53. The protein, p53 has been implicated in transcriptional *trans*activation of *MDM2* (Barak et al., 1993; Juven et al., 1993; Price and Park., 1994), a putative transcriptional control protein in its own right and *WAF-1* (El-Deiry et al., 1993) which may have a role in cell cycle control. If the major function of p53 protein were to enhance transcription of inherent, cellular "suicide genes", perturbation of the p53 gene cascade would result in inappropriate cell survival and drug resistance. Also if p53 were conformationally altered it might activate transcription of genes which when aberrantly expressed could inactivate cisplatin, pump cisplatin out of a cell, prevent entry of cisplatin to the cell or repair DNA damage more efficiently. Alteration of p53-mediated transcriptional *trans*activation is another means by which p53 functional activity can be assessed and this has been made use of in section 3.4 in order to compare p53 activity between cell lines.

3.1.6 AIMS OF THE WORK PRESENTED IN THIS CHAPTER

1) To examine the sensitivity of the cells used to cisplatin or transplatin.

2) To examine differences in p53 protein expression between cisplatin sensitive and resistant cell lines and to investigate possible mechanisms leading to any differences observed.

3) To examine differences between cisplatin sensitive and resistant cells in p53 transcriptional *trans*activation activity as determined by the level of expression of downstream targets such as the *WAF-1* gene.

4) To examine whether there is any alteration in cisplatin sensitivity of the cells induced by prior cisplatin treatment during the time when one would expect p53 protein levels to have risen.

Cellular sensitivity assays performed in this chapter employ the clonogenic assay. This assay provides a very direct measure of cell survival. Also it has the advantage over other sensitivity assays in that one does not confuse growth arrested cells with dead cells.

Much use will be made in this section of a model system comprising a highly chemosensitive, human, ovarian, tumour cell line, A2780 and a more cisplatin tolerant derivative, A2780cp70. These cell lines are interesting because of their origin. Ovarian carcinomas are frequently treated with cisplatin regimes and sometimes develop cisplatin resistance. Using resistant and sensitive pairs such as A2780cp70 and A2780 is more relevant to acquisition of drug tolerance and patient relapse than the study of cells from tissues which have innate cisplatin resistance.

The p53 protein could theoretically be involved in cisplatin resistance due to one or all of the mechanisms outlined in section 3.1.1. A fuller comprehension of how p53 protein becomes stabilised and how its activity is altered in cisplatin resistant cells may increase our understanding of drug resistance. The knowledge gleaned from this research may ultimately assist in modulation of cellular sensitivity or at least help in the diagnosis of which tumours will respond to chemotherapy.

RESULTS

3.2 STABILISATION OF p53 PROTEIN IN CISPLATIN RESISTANT CELL LINES

3.2.1 A COMPARISON OF DRUG SENSITIVITIES OF THE A2780 CELL LINE AND ITS CISPLATIN RESISTANT DERIVATIVE, A2780cp70

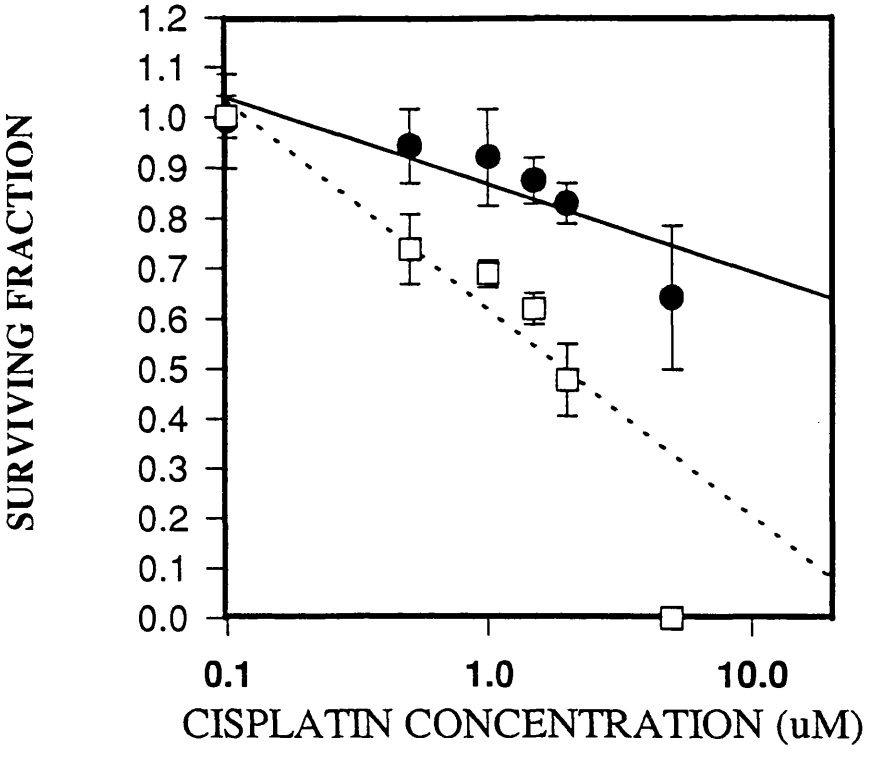
Several experiments, which will be described in subsequent sections, involve comparisons of the cisplatin sensitive A2780 cell line and its resistant counterpart, A2780cp70 following treatment with 1μM cisplatin for 1 hour. It was therefore of interest to compare the sensitivities of these two cell lines to a 1 hour cisplatin exposure. Also their sensitivities to a 1 hour dose of the *trans* isomer (transplatin), which lacks anti-tumour activity, were examined. As the ID_{50} value for A2780 cells treated with cisplatin for 24 hours is 0.6μM (McLaughlin et al., 1991), cisplatin doses of this order were chosen. Transplatin was anticipated to be less toxic than cisplatin so higher concentrations of this were used to treat the cells.

A2780 or A2780cp70 cells were seeded out at 10^3 per $75cm^2$ flask, left overnight and then given a 1 hour exposure to either cisplatin (0, 0.5, 1, 1.5, 2 or 5μM) or transplatin (0, 0.5, 1, 1.5, 2, 5, 10, 15 or 20 μM). Three replicates were performed at each dose and the results presented are the average of two such experiments. The cells were allowed to grow for 10 days, after which time they were stained with Giemsa stain, the colonies were counted and surviving fractions at the various doses were determined. The data obtained is represented graphically in figure 3.2.1.

As one can see, A2780cp70 is significantly more resistant to both cisplatin (fig. 3.2.1a) and transplatin (fig. 3.2.1b). Transplatin however, appears to be considerably less toxic to either of the two cell lines than cisplatin. The ID_{50} value determined for A2780 cells treated with cisplatin for 1 hour is 2μM (table 3.2.1). As one would expect, this value is higher than those calculated for a 24 hour drug exposure as cells will have less total

FIGURE 3.2.1 (legend overleaf)

a) CISPLATIN SENSITIVITIES OF A2780 AND A2780cp70



b) TRANSPLATIN SENSITIVITIES OF A2780 AND A2780cp70

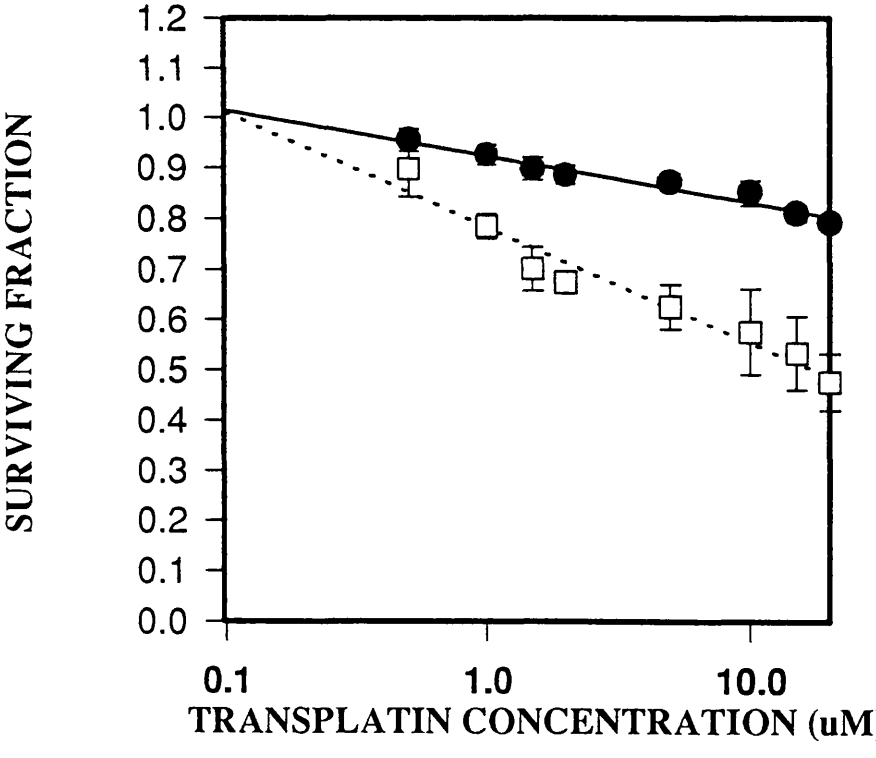


FIGURE 3.2.1

A2780cp70 (black circles, solid lines) or A2780 (white squares, dotted lines) cells were seeded out at 10^3 cells/75cm^2 flask in RPMI medium, cultured overnight, then treated with either cisplatin (a) or transplatin (b) for 1 hour. Doses were 0, 0.5, 1, 1.5, 2 or 5 μM for cisplatin and 0, 0.5, 1, 1.5, 2, 5, 10, 15, or 20 μM for transplatin. Cells were cultured for a further 10 days in drug free RPMI medium then stained with Giemsa stain and the colonies were counted. Each point is the average of at least 3 flasks/ experiment from 2 separate experiments. Surviving fractions are calculated from the ratio of number of colonies at a given dose /number of colonies at 0μM of drug. Error bars shown represent standard deviations.

TABLE 3.2.1 Survival Of A2780 and A2780cp70 Cells After A 1 Hour Exposure To Cisplatin Or Transplatin

Cell Line	Drug	ID50 (μM)	ID20 (μM)	Fold Resistance (from ID20 values)	Percentage Survival at 1μM	Degree of Resistance to 1μM
A2780	cisplatin	2	0.5	1	73	1
A2780cp70	cisplatin	>5	2.7	6	92	1.3
A2780	transplatin	16	1	1	80	1
A2780cp70	transplatin	>20	17	17	96	1.2

accumulated cisplatin and therefore are less likely to be subject to its toxic effects.

The concentrations of drugs used were not high enough to determine ID_{50} values for A2780cp70 with any degree of accuracy so fold resistance was calculated from the ID_{20} concentrations. Using these values, A2780cp70 cells were found to be approximately 6 fold resistant to cisplatin and 17 fold resistant to transplatin as compared to A2780 cells (table 3.2.1). The A2780cp70 cell line has previously been calculated to be 4 fold more resistant to cisplatin than A2780 cells (Brown et al., 1993) but this figure was estimated from ID_{50} rather than ID_{20} concentrations and a 24 hour exposure time was used.

Certain experiments, which will be described later, involved a 1μM, 1 hour drug dose so it seemed relevant to determine the relative toxicity of drugs at this dose. The degree of resistance of A2780cp70, compared to A2780 cells is 1.3 for cisplatin and 1.2 for transplatin, following a 1μM, 1hour drug treatment (table 3.2.1). The percentage survival of cells after such drug doses was 73% for A2780 and 92% for A2780cp70 in the case of cisplatin and on exposure to transplatin 80% of A2780 cells and 96% of A2780cp70 cells survived. It is important to know whether the majority of a cell population will survive a particular drug treatment as this affects the yield of lysate, protein, DNA or RNA obtained in an experiment. As the majority of A2780 and A2780cp70 cells do survive a 1μM, 1 hour dose of either cisplatin or transplatin, it is feasible to prepare various cellular extracts after such a treatment in order to examine responses to these compounds at the intracellular level. The data presented in this section confirm that A2780cp70 cells are more resistant to both cisplatin and transplatin than their parental line A2780 and that a 1μM dose of either drug can be used without compromising experimental viability.

3.2.2 INDUCTION OF p53 PROTEIN BY CISPLATIN AND TRANSPLATIN

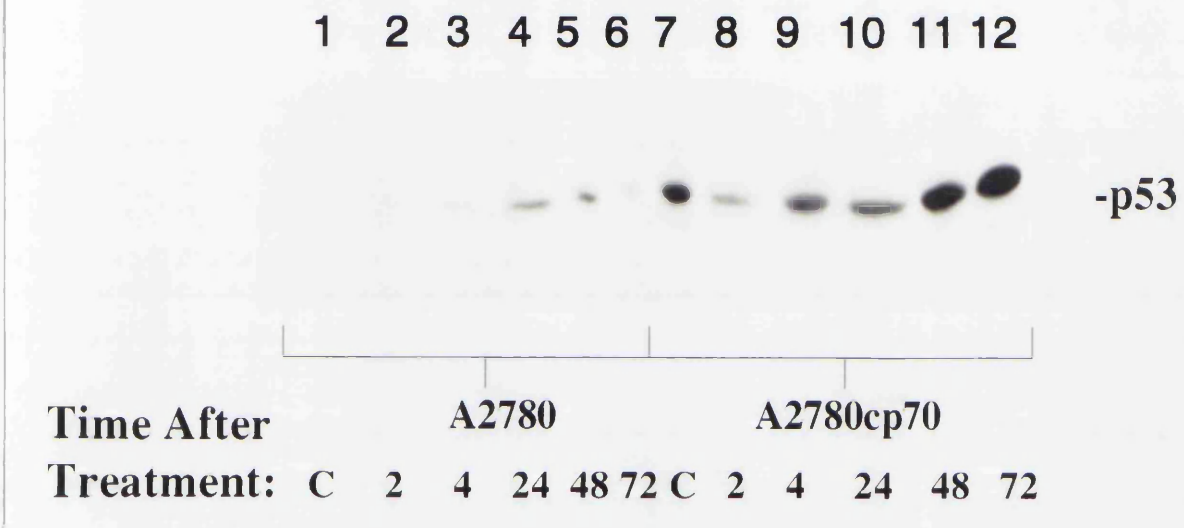
It has been reported that p53 protein levels increase in response to a variety of DNA damaging agents (Kastan et al., 1991; Fritsche et al., 1993; Maltzman & Czyzyk, 1984). Due to its potential role in a DNA-damage response pathway, p53 is a likely target for alteration on acquisition of cisplatin resistance. Thus it seemed interesting to compare both basal and induced levels of p53 protein in cisplatin resistant and sensitive cell lines. This was carried out using either cisplatin or transplatin for induction. The effects of transplatin were interesting as this substance differs from cisplatin in that it is unable to form 1,2 d(GpG) adducts which represent the major cisplatin adduct.

A2780 and A2780cp70 cells were seeded out at 10^6 cells per $75cm^2$ flask. The following day they were given a 1μM dose of either cisplatin or transplatin for a period of 1 hour or a medium change for control cells. After this they were rinsed with PBS then incubated in drug-free medium. The cells were lysed in their flasks at 2, 4, 24, 48 or 72 hours after removal of the drug. The cell lysates were subjected to Western blotting and the blots probed with an anti-p53 antibody. Then relative p53 protein levels were quantified by laser densitometry after correction for protein loading.

On average, there was 8 fold more p53 protein in non-induced A2780cp70 cells than the cisplatin sensitive A2780 parental line (lanes 1 and 7 of figs 3.2.2 and 3.2.3). Following cisplatin treatment (fig.3.2.2), p53 protein in the A2780 cell line increased from control levels (lane 1), to peak with a 9 fold induction at the 24 hour time point (lane 4). It then decreased again to approach basal levels by 72 hours post-treatment, where a 1.6 fold induction was observed (lane 6). In the case of A2780cp70, the quantity of p53 protein increased steadily from control levels (lane 7) to a 4 fold induction at 24 hours. It then, unlike for the A2780 line, continued to rise and was still on the

FIGURE 3.2.2

a) A Western Blot Showing p53 Protein Induction by Cisplatin



b) Relative Levels of p53 Protein

Lane:	1	2	3	4	5	6	7	8	9	10	11	12
Relative p53 protein levels:	1	1.4	3	9	7	1.6	8	8	20	31	57	59

A2780 (lanes 1-6) and A2780cp70 (lanes 7-12) cells were seeded out at $10^6/75cm^2$ flask and grown in RPMI medium overnight. Cells were then either given a medium change (lanes 1 & 7) or treated with 1μM cisplatin for 1 hour and allowed to recover for 2 (lanes 2 & 8), 4 (lanes 3 & 9), 24 (lanes 4 & 10), 48 (lanes 5 & 11) or 72 (lanes 6 & 12) hours. High salt lysis buffer was used to lyse the cells and large cell debris was removed by spinning in a microfuge. Protein concentration was determined by the Biorad kit assay, 50μg loaded/lane and proteins were separated on an 8% polyacrylamide gel. Western blotting (section 2.5) was carried out using Ab-2, an anti-p53 antibody, from Oncogene Science as primary antibody at a 1/1000 dilution. Anti-mouse Ig horseradish peroxidase conjugate from Amersham International was used as secondary antibody at a 1/5000 dilution. Protein sizes were determined by comparison to prestained size markers from BRL. Relative p53 levels were estimated by densitometry.

increase 72 hours after treatment, when p53 protein level was 7 fold higher than control levels (lane 12). Cisplatin can therefore induce p53 protein accumulation in A2780 and A2780cp70 cell lines, but although the former achieves maximum levels by the 24 hour timepoint, the latter still has increasing levels 72 hours after drug withdrawal.

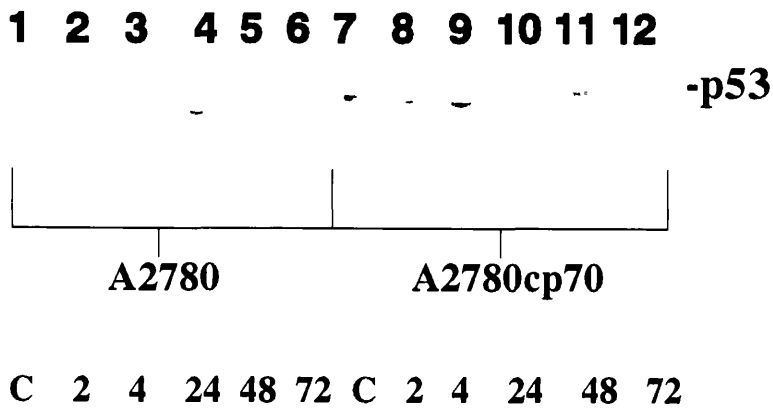
A similar situation was observed on treatment with transplatin (fig. 3.2.3). In A2780 cells induction occurred to a comparable degree to that seen for cisplatin. An exception was that the peak p53 protein level detected 24 hours after transplatin removal was induced 14 fold (lane 4) which is somewhat higher than seen for cisplatin.

In the cisplatin resistant A2780cp70 cell line, induction with transplatin did not seem to occur to the same extent as one sees for cisplatin. By the 24 hour time point, p53 protein levels had doubled whereas following cisplatin induction, they would have quadrupled. A 2 fold peak induction was seen at 48 hours after which levels declined to a 1.3 fold induction at 72 hours. Levels of p53 protein were still rising at this timepoint following cisplatin treatment. This difference may be accounted for by the lower toxicity of transplatin resulting in the cells attaining confluency more rapidly and thus switching off expression of p53. An alternative explanation is that p53 production is no longer being induced by transplatin due to repair of the transplatin-DNA adducts. Cisplatin-induced DNA damage accumulates for up to 48 hours but the number of adducts formed by transplatin only increase for 6 hours after treatment and then begin to recede (Ciccarelli et al., 1985).

Transplatin is therefore able to induce p53 protein within A2780 and A2780cp70 cell lines in a similar manner to cisplatin, but not always to the same degree. In both experiments there was approximately 8 fold more uninduced p53 protein in the resistant line than in the sensitive cells.

FIGURE 3.2.3

a) A Western Blot Showing p53 Protein Induction by Transplatin



b) Relative Levels of p53 Protein

Lane:	1	2	3	4	5	6	7	8	9	10	11	12
Relative p53 protein levels:	1	1.4	3	14	7	1.2	8	10	16	17	20	10

A2780 (lanes 1-6) and A2780cp70 (lanes 7-12) cells were seeded out at 10^6/75cm^2 flask and grown in RPMI medium overnight. Cells were then either given a medium change (lanes 1 & 7) or treated with 1μM transplatin for 1 hour and allowed to recover for 2 (lanes 2 & 8), 4 (lanes 3 & 9), 24 (lanes 4 & 10), 48 (lanes 5 & 11) or 72 (lanes 6 & 12) hours. High salt lysis buffer was used to lyse the cells and large cell debris was removed by spinning in a microfuge. Protein concentration was determined by the Biorad kit assay, 50μg loaded/lane and proteins were separated on an 8% polyacrylamide gel. Western blotting (section 2.5) was carried out using Ab-2, an anti-p53 antibody, from Oncogene Science as primary antibody at a 1/1000 dilution. Anti-mouse Ig horseradish peroxidase conjugate from Amersham International was used as secondary antibody at a 1/5000 dilution. Protein sizes were determined by comparison to prestained size markers from BRL. Relative p53 levels were estimated by densitometry.

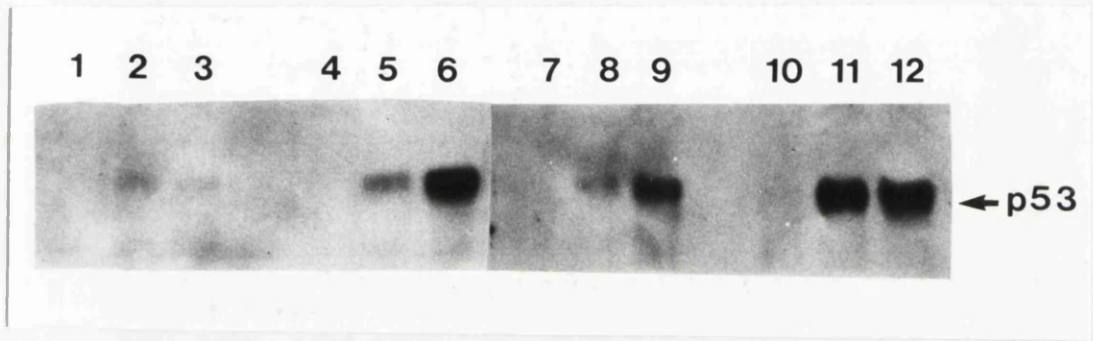
3.2.3 THE p53 STATUS IN CISPLATIN RESISTANT AND SENSITIVE CELL LINES

Ov1P is a human, ovarian adenocarcinoma cell line and Ov1PDDP is a more cisplatin tolerant derivative (Teyssier et al., 1989). As in the case of A2780 and A2780cp70, the resistant cell line contains more p53 protein than the sensitive parental. Also several cisplatin resistant clones were selected by exposure of A2780 cells to 15 μM cisplatin (McLaughlin et al., 1991) and many of these exhibit increased p53 protein as compared to randomly selected A2780 clones (Brown et al., 1993). Although this does not suggest that increased p53 protein is the sole cause for cisplatin resistance, it does identify p53 as a potential contributory factor in the acquisition of cellular resistance to the drug.

Increased intracellular p53 protein is frequently indicative of a mutant p53 gene (Bartek et al., 1990). The p53 status within the cisplatin sensitive cell lines A2780 and Ov1P was compared to that of their resistant partners, A2780cp70 and Ov1PDDP respectively, by immunoprecipitation using antibodies specific for p53 in the mutant conformation. A semiconfluent 175cm^2 flask (2x10^7 cells) of each cell line was harvested using high salt lysis buffer. The lysate was precleared to reduce background, then divided into three aliquots of equal volume which were each subjected to immunoprecipitation using one of three antibodies, PAb DO-1 which reacts with all forms of p53 protein, PAb 240 which binds to p53 protein in the mutant conformation and PAb 419, a control antibody reactive with SV40 large T antigen (Vojtesek et al., 1992). The immunoprecipitated p53 protein was then subjected to Western blot analysis (fig. 3.2.4).

No p53 protein was precipitated by the control antibody (lanes 1, 4, 7 and 10). An increased amount of p53 protein was immunoprecipitated with PAb DO-1 from the resistant lines, A2780cp70 (lane 6) and Ov1PDDP (lane 12) than from the sensitive parental lines, A2780 (lane 3) and Ov1P (lane 9)

FIGURE 3.2.4 Immunoprecipitation Of p53 Protein From Cisplatin Sensitive And Resistant Cell Lines



Immunoprecipitation (section 2.6.5) was performed followed by separation of proteins on an 8% polyacrylamide gel and Western blotting (section 2.5) of p53 protein from A2780 (lanes 1-3), A2780cp70 (lanes 4-6), Ov1P (lanes 7-9) and Ov1PDDP (lanes 10-12). Extracts were immunoprecipitated with the antibodies PAb DO-1 (lanes 3, 6, 9 & 12), PAb 240 (lanes 2, 5, 8 & 11) and PAb 419 (lanes 1, 4, 7 & 12). PAb DO-1 is a pantropic anti-p53 antibody, PAb 240 is specific for mutant p53 and PAb 419 is an irrelevant control antibody. For Western blotting, the primary antibody was CM1 at a 1/200 dilution and the secondary antibody was anti rabbit Ig horseradish peroxidase from Dako, Aylesbury, at a 1/150 dilution. Protein sizes were determined by comparison to prestained size markers from BRL.

consistent with increased levels of p53 protein in these cell lines. The mutant-specific 240 antibody precipitates detectable amounts of p53 protein from both resistant lines but, whilst this antibody reacts with a similar level of p53 protein to the pantropic DO-1 antibody in the Ov1PDDP line, substantially less p53 is reactive with the 240 antibody compared to the DO-1 antibody in the case of the A2780cp70 cell line.

A2780cp70, like the A2780 cell line from which it was selected, has a wild type p53 genotype as determined by direct sequencing of PCR products derived from cDNA (Brown et al., 1993). Examination of the entire coding sequence of the p53 gene in the Ov1P and Ov1PDDP cell lines revealed no difference between them although both possessed a cystine to tyrosine substitution at codon 126 (Brown et al., 1993). From this data, one can see that the increased p53 protein observed in cisplatin resistant cells cannot be explained by mutation in the p53 gene, thus some other cellular constituent which interacts with p53 must be involved.

3.2.4 ANALYSIS OF POSSIBLE MEDIATORS OF p53 PROTEIN ACCUMULATION IN CISPLATIN RESISTANT CELL LINES

The raised p53 protein levels observed in cisplatin resistant cell lines are not due to a mutant p53 gene, so it was of interest to examine other factors which could influence p53 protein stability. This section contains an initial analysis of possible mechanisms leading to increased p53 protein accumulation in the resistant A2780cp70 cell line.

The protein, p53, is phosphorylated by at least four protein kinases (Milne et al., 1992a). These could modulate the half life of p53 by altering its ability to be recognised by proteases. In order to examine the effect of phosphorylation on p53 protein stability, nuclear extracts were prepared from A2780 and A2780cp70 cell lines in the presence or absence of the phosphatase inhibitors β-glycerol, sodium orthovanadate, sodium fluoride,

okadaic acid and microcystin LR. These extracts were subjected to electrophoresis then Western blotting and the blot was probed for p53 immunoreactivity (fig. 3.2.5).

The p53 protein levels in the A2780 line are so low as to be undetectable (lanes 1, 2, 3 and 4). This is not unexpected as basal p53 protein concentration in the nucleus of this cell line, and most other cell lines with a wild type p53 genotype, are generally fairly low. There is much more nuclear p53 protein in the A2780cp70 cell line, with three apparent isoforms, but no obvious difference in p53 protein quantity between extracts prepared in the absence (lanes 5 and 7) or presence (lanes 6 and 8) of phosphatase inhibitors. This does not however eliminate the possibility that phosphorylation is involved in controlling p53 protein accumulation. Possibly kinases as opposed to phosphatases are responsible for targeting p53 protein for degradation. These, being more specific than phosphatases, would be more difficult to inhibit. Or perhaps the phosphatase inhibitors were simply not allowed sufficient time to cause any noticeable affect on p53 protein levels. Also there do not appear to be any differences between phosphorylation patterns of p53 protein as determined by 2 dimensional gel electrophoresis of p53 isoforms (L. Gallagher, CRC Dept. Medical Oncology, Glasgow University *personal communications*). It is interesting to note that in the nuclear extracts of these two cell lines, differences between p53 protein levels seem to be even more extreme than for total cell lysates. This argues against nuclear exclusion of p53 protein being the manner in which p53 activity is disrupted in A2780cp70 cells.

Proteins which interact with p53 protein might protect it from degradation by masking a site recognised by proteases. The nuclear transcription factor, MDM2 binds to p53 and prevents p53 mediated transcriptional *trans*activation (Momand et al., 1992). An attempt was made to probe Western blots of A2780 and A2780cp70 lysates with a monoclonal anti-

FIGURE 3.2.5 Levels Of p53 Protein In Nuclear Extracts Prepared In The Presence Or Absence Of Phosphatase Inhibitors

Nuclear extracts were prepared (section 2.2.3) from A2780 (lanes 1-4) and A2780cp70 (lanes 5-8) cell lines in the presence (lanes 2, 4, 6 & 8) or absence (1, 3, 5 & 7) of the phosphatase inhibitors β-glycerol, sodium orthovanadate, sodium fluoride, okadaic acid and microcystin LR. Extracts were subjected to electrophoresis on an 8% polyacrylamide gel then Western blotting (section 2.5). Crude cell lysate from HT29 cells (20μg) was used as a positive control (lane 9). The blot was probed for p53 immunoreactivity using AB2 from Oncogene Science at a 1/1000 dilution as a primary antibody. Anti-mouse Ig horseradish peroxidase from Amersham was used at a 1/5000 dilution as a secondary antibody. Protein sizes were determined by comparison to prestained size markers from BRL.

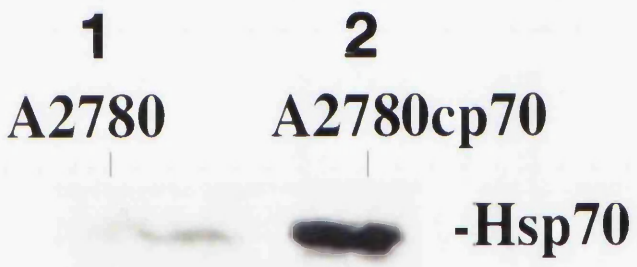
MDM2 antibody, IF2. However, no binding by this antibody was detected for either cell line, probably because MDM2 is normally expressed at quite low levels and the antibody may be intended for use with cell lines which overexpress this protein. The level of MDM2 mRNA is lower in A2780cp70 cells than A2780 cells as will be detailed in section 3.3.3 and this may have implications for steady state levels of p53 protein.

The heatshock protein, Hsp70 has been shown to bind p53 protein, particularly mutant p53 which it then sequesters out of the nucleus of the cell and into the cytoplasm (Pinhasi-Kimhi et al., 1986; Martinez et al., 1991). It can prolong the half-life of p53 protein and so an increase in Hsp70 protein concentration could potentially be responsible for the enhanced p53 protein levels detected in A2780cp70 cells. It therefore seemed worthwhile to investigate differences between Hsp70 levels in A2780 and A2780cp70 cells.

Each of these two cell lines were seeded out at 10^6 cells per $75cm^2$ flask. After 24 hours the cells were lysed with high salt lysis buffer and the lysates were electrophoresed through an 8% polyacrylamide gel then assessed for Hsp70 protein levels by Western blotting then probing of the blot with an anti-Hsp70 antibody (fig. 3.2.6a). Lane 1 contains lysate prepared from A2780 cells and lane 2 contains lysate from A2780cp70 cells. The relative level of p53 protein contained in each of the cell lines was estimated after correction for protein loading (fig.3.2.6b) and there was found to be 4 fold more Hsp70 in the cisplatin resistant A2780cp70 cells than the sensitive A2780 line. Thus in this example of cisplatin sensitive and resistant counterparts at least, there is more Hsp70 protein in the resistant derivative. This provides some clues as to why there may be a greater accumulation of p53 protein in resistant than sensitive cell lines although it is not necessarily the only, or even the major, causative factor. It is possible that the increased p53 protein may be stabilising the Hsp70 protein, but wild type p53 has been shown to negatively regulate

FIGURE 3.2.6

a) **A Western Blot Showing Hsp70 Protein Levels in A2780 and A2780cp70 cell lines**



b) **Relative Levels of Hsp70 Protein**

Lane:	1	2
Relative Hsp70 protein levels:	1	4

A2780 (lane 1) and A2780cp70 (lane 2) cells were seeded out at 10^6/75cm^2 flask and grown in RPMI medium overnight. High salt lysis buffer was used to lyse the cells and large cell debris was removed by spinning in a microfuge. Protein concentration was determined by the Biorad kit assay, 50μg loaded/lane and proteins were separated on an 8% polyacrylamide gel. Western blotting (section 2.5) was carried out using, an anti-Hsp70 antibody, from Sigma as primary antiboby at a 1/1000 dilution. Anti-mouse Ig horseradish peroxidase conjugate from Amersham International was used as secondary antibody at a 1/5000 dilution. Protein sizes were determined by comparison to prestained size markers from BRL. Relative p53 levels were estimated by densitometry.

Hsp70 protein levels, at least at the transcriptional level (Agoff et al., 1993). If p53 functional activity was inactivated in A2780cp70 cells, increased Hsp70 protein could be due to reduced repression of transcription.

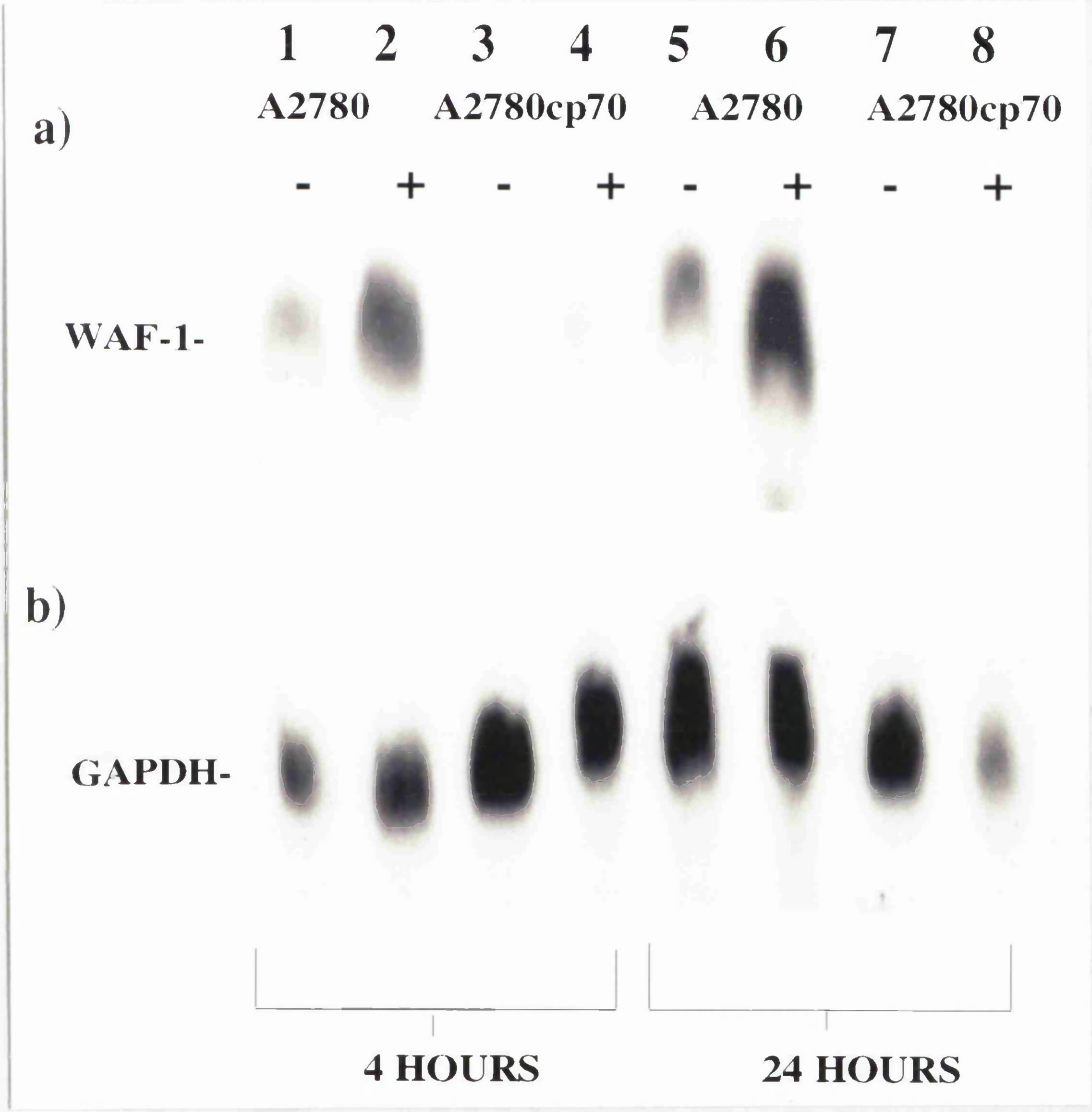
3.3 ARE THERE DIFFERENCES IN p53 ACTIVITY BETWEEN CISPLATIN SENSITIVE AND RESISTANT CELL LINES ?

3.3.1 INDUCTION OF *WAF-1* mRNA BY VARIOUS DNA DAMAGING AGENTS IN CISPLATIN RESISTANT AND SENSITIVE CELLS

As there are noticeable differences between p53 protein concentration in certain cisplatin sensitive and resistant cell lines, it seemed important to find out whether there were also differences in p53 protein activity. An alteration in p53 protein activity could affect downstream events which might have a bearing on whether cells became resistant to cisplatin. WAF-1, also known as CIP-1 or p21, is a 21KDa nuclear protein whose expression is induced on transcriptional *trans*activation by p53 (El-Deiry et al., 1993). As such, *WAF-1* mRNA levels provide a convenient way of measuring differences in p53 mediated transcriptional *trans*activation activity. Differences in *WAF-1* mRNA levels between resistant and sensitive cells were quantified as was the induction of *WAF-1* mRNA by cisplatin.

Alteration in *WAF-1* mRNA levels in response to cisplatin was assessed by seeding out $4x10^6$ A2780 or A2780cp70 cells per $175cm^2$ flask. These flasks were left overnight then given a 1μM, 1 hour treatment with cisplatin or, in the case of control cells, a medium change to drug-free RPMI medium. At timepoints of 4 hours and 24 hours post-treatment, RNA was extracted from the cells, and this was used for Northern blotting. The resultant blot was probed with *WAF-1* DNA (fig.3.3.1a) and then with *GAPDH* DNA (fig.3.3.1b) in order to control for RNA loading. These probes hybridized to RNA of the expected sizes, 2.4Kb and 1.2Kb respectively.

FIGURE 3.3.1 Induction Of *WAF-1* mRNA By Cisplatin



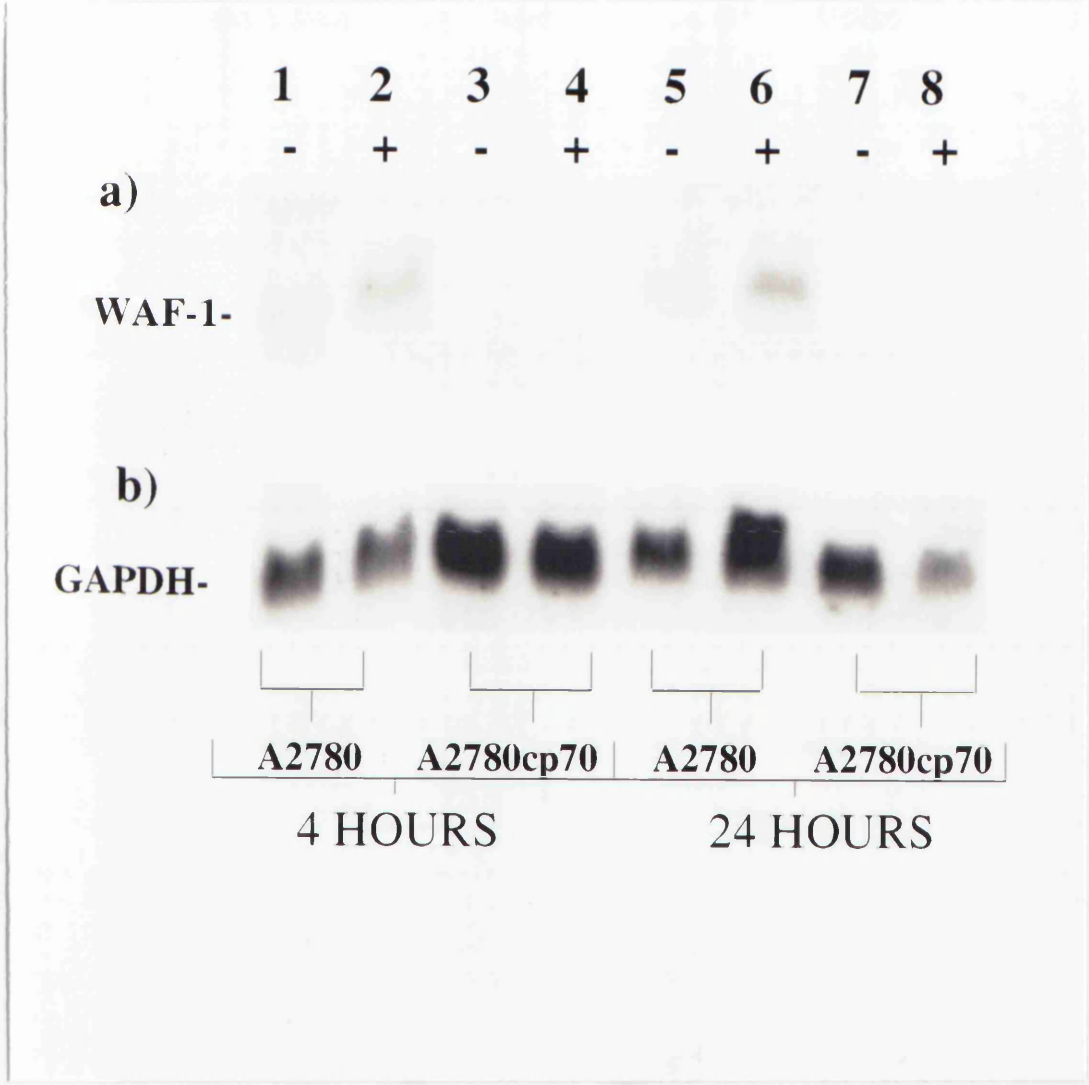
A2780 (lanes 1, 2, 5 & 6) and A2780cp70 (lanes 3, 4, 7 & 8) were seeded at 4x 10^6 cells/ 175cm^2 flask and grown overnight in RPMI medium. Cells were given a medium change (lanes 1, 3, 5 & 7) or treated with 1μM cisplatin for 1 hour (lanes 2, 4, 6 & 8) and grown for a further 4 (lanes 1-4) or 24 (lanes 5-8) hours in drug-free RPMI. Total RNA was extracted with TRIzol™ and Northern blotting carried out (section 2.4) using *WAF-1* and *GAPDH* DNA probes. RNA sizes were determined by comparison to 0.24-9.5kb size markers from Gibco, Paisley.

Basal levels of *WAF-1* mRNA were higher in A2780 (lanes 1 and 5) than A2780cp70 (lanes 3 and 7) indicating greater p53-mediated transcriptional *trans*activation capacity in the sensitive cell line in spite of its lower p53 protein level. Following induction with cisplatin, *WAF-1* mRNA levels increased 1.3 fold after 4 hours (lane 2) and 4 fold by the 24 hour timepoint (lane 6) in A2780 cells (table 3.3.1). In the A2780cp70 cell line there was a slight induction of 1.2 fold at 4 hours (lane 4) but none 24 hours after cisplatin removal (lane 8). Thus not only are basal *WAF-1* mRNA levels lower in the resistant cells, but they are also less inducible with cisplatin.

A similar experiment was performed using 1μM transplatin rather than cisplatin and the Northern blot obtained is depicted in figure 3.3.2. Again one sees more *WAF-1* mRNA in uninduced A2780 (lanes 1 and 5) than A2780cp70 cells (lanes 3 and 7). Transplatin treatment of cells caused induction of *WAF-1* to a similar degree as was seen following a cisplatin dose of the same concentration. In A2780 cells, *WAF-1* was induced 1.8 fold after 4 hours (lane 2) and 4 fold 24 hours after transplatin treatment (lane 6). With A2780cp70 cells there was some induction but not to such an extent as for the sensitive parental line. At the 4 hour timepoint, a 1.1 fold induction was recorded (lane 4) and this had risen to 1.3 fold by 24 hours after drug withdrawal (lane 8). Therefore, one can observe similar effects on *WAF-1* mRNA levels after transplatin treatment as following cisplatin treatment of A2780 and A2780cp70 cells. That is, levels of *WAF-1* mRNA increase by 4 hours after drug treatment and rise to a more noticeable extent by the 24 hour timepoint in the sensitive A2780 cell line. In the resistant line, there is less *WAF-1* mRNA in uninduced cells and induction is not so striking.

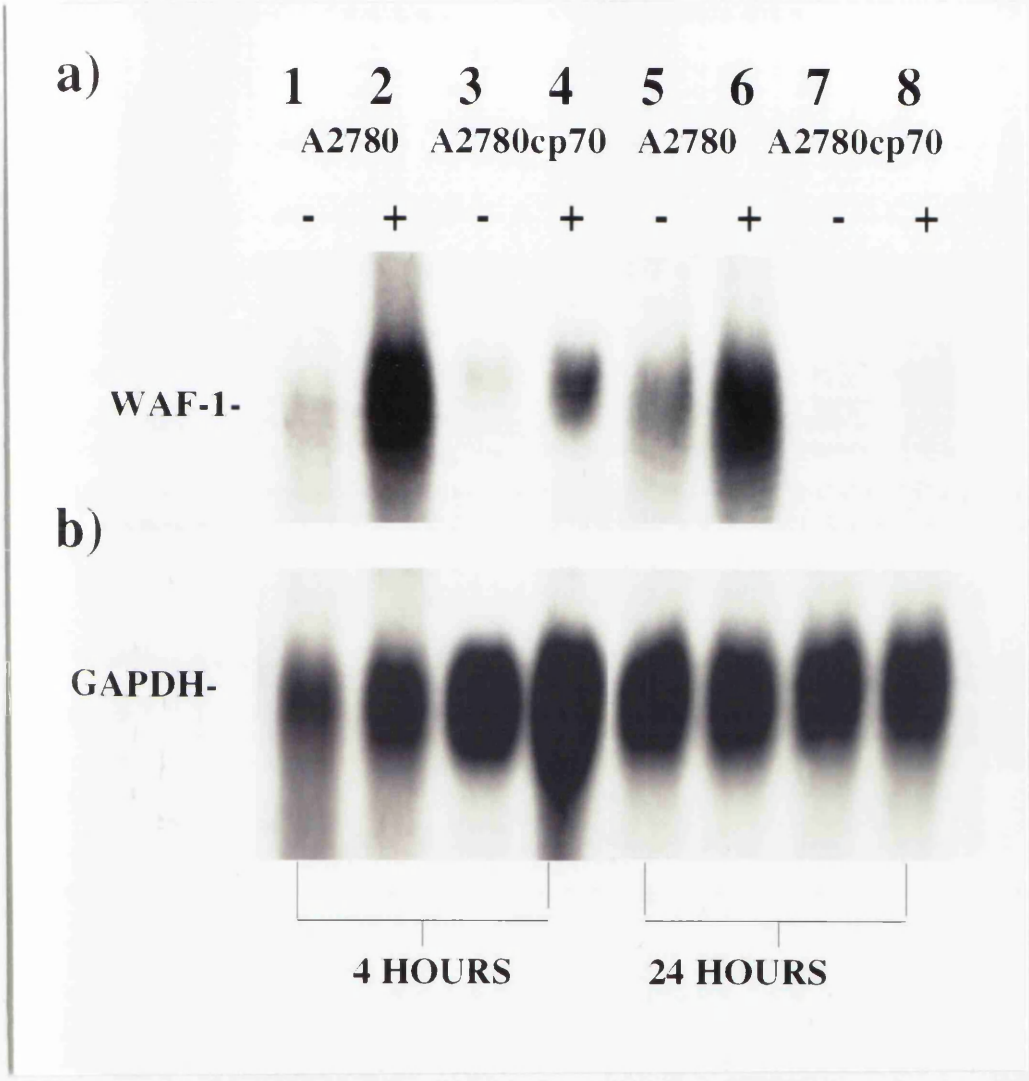
Ionising radiation is also capable of inducing an increase in *WAF-1* RNA in these two cell lines (fig. 3.3.3). Again, $4x10^6$ A2780 or A2780cp70 cells per $175cm^2$ flask were grown up overnight. These were either exposed to 2Gy ionising radiation from a ^{60}cobalt source or in the case of control cells,

FIGURE 3.3.2 Induction Of *WAF-1* mRNA By Transplatin



A2780 (lanes 1, 2, 5 & 6) and A2780cp70 (lanes 3, 4, 7 & 8) were seeded at 4x 10^6 cells/ 175cm^2 flask and grown overnight in RPMI medium. Cells were given a medium change (lanes 1, 3, 5 & 7) or treated with 1μM transplatin for 1 hour (lanes 2, 4, 6 & 8) and growN for a further 4 (lanes 1-4) or 24 (lanes 5-8) hours in drug-free RPMI. Total RNA was extracted with TRIzol™ and Northern blotting carried out (section 2.4) using *WAF-1* and *GAPDH* DNA probes. RNA sizes were determined by comparison to 0.24-9.5kb size markers from Gibco, Paisley.

FIGURE 3.3.3 Induction Of *WAF-1* mRNA By Ionising Radiation



A2780 (lanes 1, 2, 5 & 6) and A2780cp70 (lanes 3, 4, 7 & 8) were seeded at 4x 10^6 cells/ 175cm^2 flask and grown overnight in RPMI medium. Cells were either exposed to 2Gy ionising radiation from a $cobalt^{60}$ source or left untreated (lanes 1, 3, 5 & 7) and growN for a further 4 (lanes 1-4) or 24 (lanes 5-8) hours in drug-free RPMI. Total RNA was extracted with TRIzol™ and Northern blotting carried out (section 2.4) using *WAF-1* and *GAPDH* DNA probes. RNA sizes were determined by comparison to 0.24-9.5kb size markers from Gibco, Paisley.

left untreated. Then RNA was prepared either 4 hours or 24 hours later and used for Northern blotting. At the 4 hour timepoint, there was 4.5 fold more *WAF-1* RNA in A2780 cells which have been exposed to ionising radiation (lane 2) than control A2780 cells (lane 1) and at the 24 hour timepoint there was a 4 fold induction (lanes 5 and 6). In A2780cp70 cells *WAF-1* mRNA was induced 2.4 fold at 4 hours (lanes 3 and 4) and 1.9 fold at 24 hours after irradiation (lanes 7 and 8). When ionising radiation is used to treat these cell lines one can observe induction of *WAF-1* mRNA in both cell lines, although not so strongly in the cisplatin resistant A2780cp70 line which also has lower uninduced levels of *WAF-1* mRNA. The induction is more immediate than that seen with transplatin or cisplatin and this may reflect the fact that there is no time lost on transport into the cells as is the case for cytotoxic drugs.

In this section we have seen how *WAF-1* mRNA expression, which is induced via transcriptional *trans*activation by p53 protein, increases once cells have been treated with DNA damaging agents. These agents cause a rise in intracellular p53 protein so one would expect to see a concurrent increase in p53 activity as measured by its ability to activate transcription of a dependent gene, namely *WAF-1*. A summary of *WAF-1* induction data is provided in table 3.3.1.

In summary, differences in p53 mediated transcriptional *trans*activation between the cisplatin sensitive, A2780 cell line and A2780cp70, the resistant cell line selected from A2780 by multiple cisplatin exposures, were detected. A2780 cells contain 10 fold more basal *WAF-1* mRNA than A2780cp70 cells (table 3.3.2) although they have less p53 protein, indicating that the p53 protein in the resistant line is somehow less active as a transcriptional regulator. Also *WAF-1* mRNA is not so powerfully induced in the resistant line. Alteration in the transcriptional *trans*activation properties of p53 might be due to changes in the sequence of the binding site

TABLE 3.3.1 Induction Of *WAF-1* mRNA By DNA Damaging Agents

Cell Line	Drug/Radiation Dose	Fold Induction of WAf-1 mRNA at 4 Hours	Fold Induction of WAF-1mRNA at 24 Hours
A2780	cisplatin (1μM, 1 hour)	1.3	4
A2780cp70	"	1.2	1
A2780 +vector	"	1.9	4
A2780 +mutant p53	"	1.7	3.5
A2780	Transplatin (1μM, 1 hour)	1.8	4
A2780cp70	"	1.1	1.3
A2780	Ionising Radiation (2Gy)	4.5	4
A2780cp70	"	2.4	1.9

TABLE 3.3.2 Basal *WAF-1* mRNA Levels In A2780 And A2780cp70

Cell Line	Relative Level of WAF-1 mRNA
A2780cp70	1
A2780	10

TABLE 3.3.3 Basal *WAF-1* mRNA Levels In A2780 Transfected With A Mutant *p53* Gene

Cell Line	Relative Level of WAF-1 mRNA
A2780+mutant p53	1
A2780+ vector	3

TABLE 3.3.4 Basal *MDM2* mRNA Levels

Cell Line	Relative Level of MDM2 mRNA
A2780cp70	1
A2780	4
ZR-75	1.5

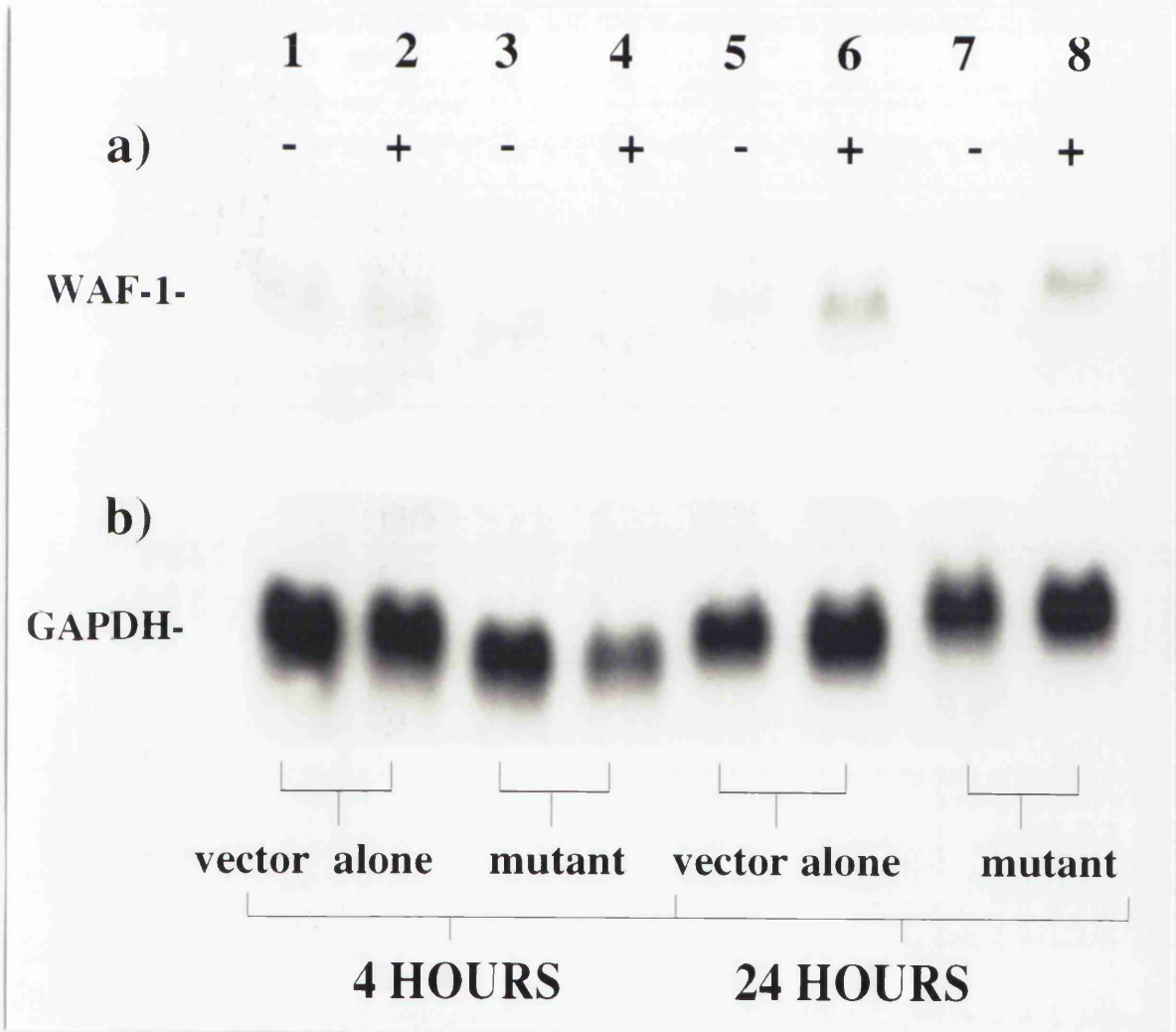
at the p53 responsive gene or because of altered post-transcriptional modifications to p53 protein resulting from mutant modifying proteins.

3.3.2 ALTERATION OF BASAL AND INDUCED *WAF-1* mRNA LEVELS IN A2780 CELLS FOLLOWING TRANSFECTION WITH A MUTANT *p53* GENE

In order to demonstrate dependence of *WAF-1* mRNA levels on the presence of active p53 protein, induction of *WAF-1* mRNA in the presence of a mutant p53 protein was analysed. Dominant p53 mutants bind wild type p53 forming heterodimers which abrogate wild type p53 activity (Milner & Medcalf, 1991). A2780 cells which had been transfected with the plasmid pC53-SCX3 (Baker et al., 1990), which expresses mutant *p53* with a valine to alanine substitution at codon 143, or control cells transfected with the vector alone were set up at a concentration of $4X10^6$ cells per $175cm^2$ flask. The following day, experimental cells were treated for 1 hour with 1μM cisplatin and control cells were given a medium change to drug-free medium. RNA was extracted at 4 hours and 24 hours after drug removal and used for Northern blotting. The blot was probed for *WAF-1* and *GAPDH* (fig. 3.3.4).

Introduction of the mutant p53 gene into A2780 cells reduced uninduced *WAF-1* mRNA 3 fold (table 3.3.3) compared to cells containing the vector alone. This difference was not so profound as the difference between A2780 and A2780cp70 cell lines where the sensitive line contains 10 times the level of *WAF-1* mRNA as the resistant line. Also there was very little difference in *WAF-1* induction between cells containing mutant *p53* and vector alone controls. Cells transfected with the vector alone exhibited a 1.9 fold induction at 4 hours after cisplatin treatment (fig. 3.3.4, lanes 1 and 2) and a 4 fold induction 24 hours after drug removal (lanes 5 and 6) whereas those with the mutant p53 protein showed a 1.7 fold induction after 4 hours (lanes 3 and 4) and a 3.5 fold induction after 24 hours (lanes 7 and 8). This data is

FIGURE 3.3.4 Induction Of WAF-1 mRNA In The Presence Of A Mutant p53 Gene



A2780 cells transfected with vector alone (lanes 1, 2, 5 & 6) and A2780 cells transfected with a mutant p53 expressing plasmid (lanes 3, 4, 7 & 8) known as pC53-SCX3 (Baker et al., 1990) were seeded at 4x 10^6 cells/ 175cm^2 flask and grown overnight in RPMI medium. Cells were treated with 1μM cisplatin for 1 hour and grown for a further 4 (lanes 1-4) or 24 (lanes 5-8) hours in drug-free RPMI. Total RNA was extracted with TRIzol^{TM} and Northern blotting carried out (section 2.4) using *WAF-1* and *GAPDH* DNA probes. RNA sizes were determined by comparison to 0.24-9.5kb size markers from Gibco, Paisley.

summarised in table 3.3.1.

Decreased *WAF-1* mRNA was observed in A2780 cells when a mutant p53 was introduced. This indicates that *WAF-1* expression in these cells is p53 dependent. The presence of mutant p53 does not entirely abolish *WAF-1* mRNA nor does it effectively prevent its induction by cisplatin. This is probably due to residual wild-type p53 protein which is not complexed to the mutant form.

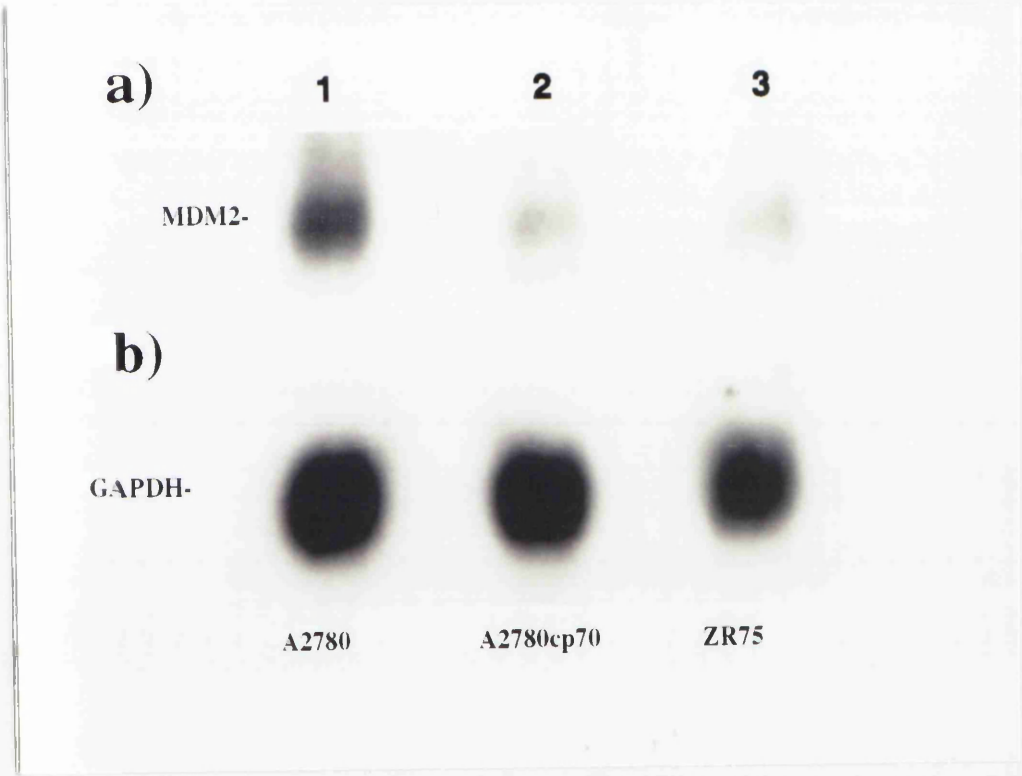
3.3.3 DIFFERENTIAL EXPRESSION OF *MDM2* mRNA IN CISPLATIN RESISTANT AND SENSITIVE CELL LINES

Expression of the *MDM2* gene is also transcriptionally *trans*activated by p53 and so, differences in *MDM2* mRNA levels between A2780 and A2780cp70 cells were also interesting as being indicative of p53 protein activity. RNA was prepared from the cell lines A2780, A2780cp70 and ZR-75 (a breast tumour derived cell line which overexpresses MDM2 protein). The RNA obtained was analysed by Northern blotting (fig. 3.3.5). After correction for loading, there was found to be 1.5 fold more *MDM2* mRNA in ZR-75 cells than A2780cp70 cells and 4 fold more in A2780 cells than A2780cp70 cells (table 3.3.4). This reduced amount of *MDM2* in A2780cp70 cells, compared with its parental line, provides further circumstantial evidence for a lower level of transcriptional control by p53 protein in these cisplatin resistant cells. It also suggests that these effects are not limited to WAF-1.

3.3.4 THE USE OF A LUCIFERASE REPORTER SYSTEM TO MEASURE TRANSCRIPTIONAL CONTROL BY p53 PROTEIN

In order to measure p53 mediated regulation of transcription more directly, an attempt was made to use a reporter gene downstream of a known p53 binding site. The plasmid pGUP.PA.8 (fig. 3.3.6a), which contains the gene encoding firefly luciferase, was used either alone or with the fragment A

FIGURE 3.3.5 Levels Of *MDM2* mRNA In A2780. A2780cp70 And ZR-75 Cell Lines



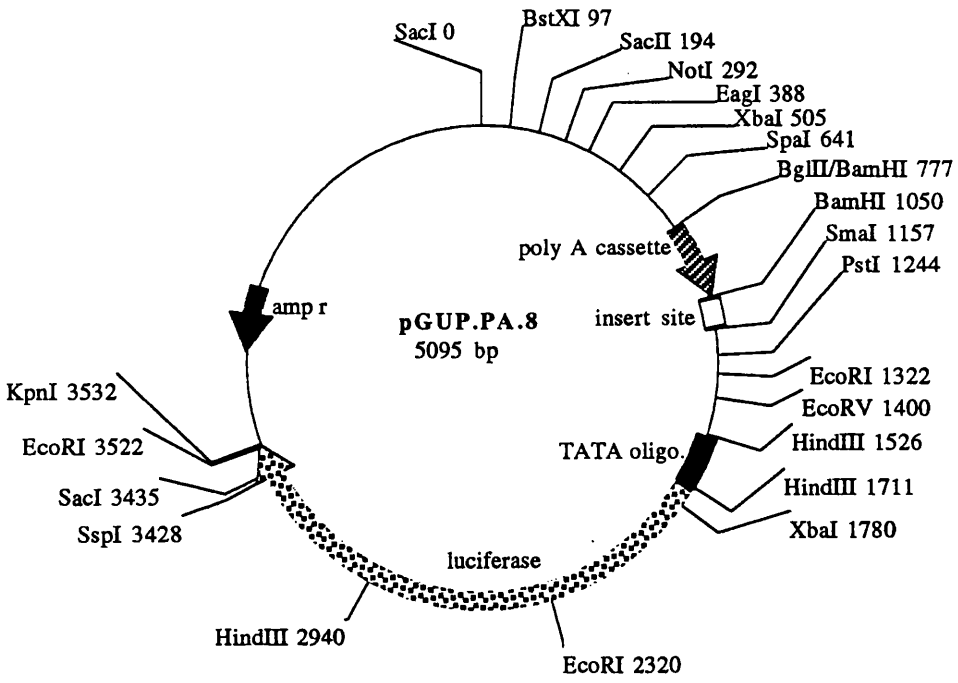
A2780 (lane 1), A2780cp70 (lanes 2) and ZR-75 cells were grown to semi-confluency in RPMI medium. Total RNA was extracted with TRIzol^{TM} and Northern blotting carried out (section 2.4) using an *MDM2* DNA probe. RNA sizes were determined by comparison to 0.24-9.5kb size markers from Gibco, Paisley. This work was performed in collaboration with L.Gallagher, CRC Department of Medical Oncology, Glasgow University.

sequence (fig. 3.3.6b) inserted upstream of the luciferase gene i.e Frag.A-pGUP.PA.8 (Funk et al., 1992). Fragment A is a 36 bp fragment of a human genomic clone known as CBE772 to which p53 binds specifically (Seto et al., 1990). Luciferase enzyme activity involves oxidation of beetle luciferin via an intermediate, luciferyl-CoA in this case, with subsequent chemiluminescent photon production.

Either Frag.A-pGUP.PA.8 or the vector alone, pGUP.PA.8, were stably transfected into A2780cp70 cells along with a plasmid known as pHSG272 (Brady et al., 1984) which contains a selectable marker, the G418 resistance plasmid. An attempt was also made to transfect these plasmids into A2780 cells, however this failed due to the susceptibility of this line to the toxic effects of calcium phosphate. Transfectants were selected for G418 resistance and Southern blotting was employed to conclude whether transfection had occurred successfully. A clone which had been transfected with Frag.A-pGUP.PA.8 (FA) and one which contained the vector alone (V) were then used in induction experiments with DNA damage-inducing agents.

The clones FA and V were seeded out at 10^6 cells per $75cm^2$ flask and left overnight. They were then given a 1 hour, 1μM dose of cisplatin or a change of growth medium for control cells. At 4, 24, or 48 hours later the cells were lysed and lysates were later assayed for luciferase activity (light production) in a luminometer using an empty tube as a control in order to estimate background luminescence. The results of this experiment, after subtraction of background activity, are depicted graphically in figure 3.3.7. Cells containing the vector alone showed approximately background levels of activity whether untreated (V-) or treated with cisplatin (V+) at all 3 timepoints. Cells containing Frag.A-pGUP.PA.8 have above background levels of luciferase activity before cisplatin treatment (FA-) at the timepoints examined and levels are induced by cisplatin treatment with the greatest induction 4 hours after cisplatin removal when luciferase activity is induced 4

FIGURE 3.3.6a) The Plasmid, pGUP.PA.8



b) The Sequence Of Fragment A

5'ACGTTTGCCTTGCCTGGACTTGCCTGCACTTGCCTGGCCTTGCCTT3'

c) The Geneticin Expression Plasmid, pHSG272

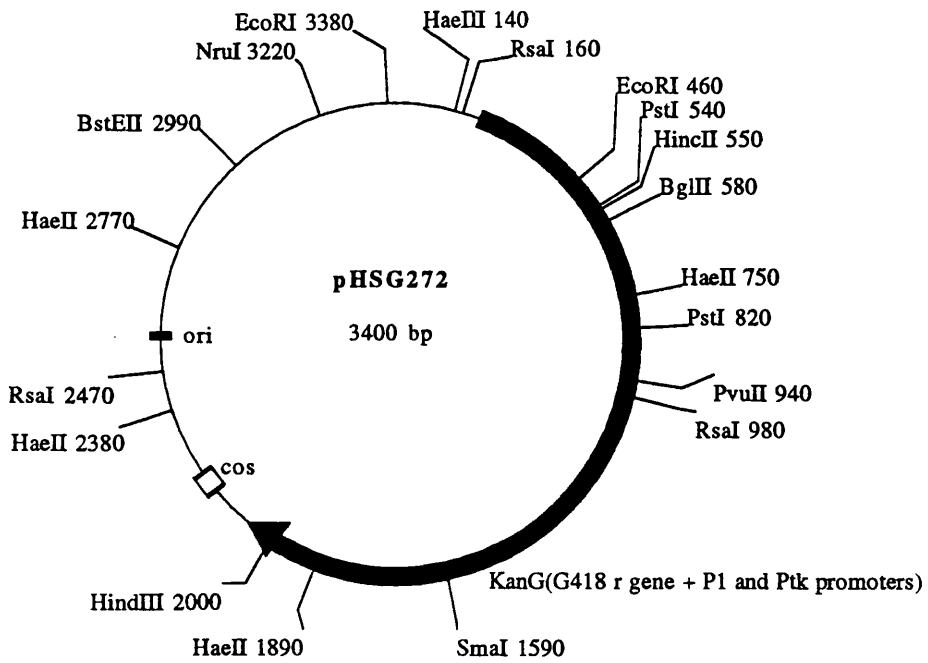


FIGURE 3.3.7 Induction Of Transcription By Cisplatin In A2780cp70 Cells

(legend overleaf)

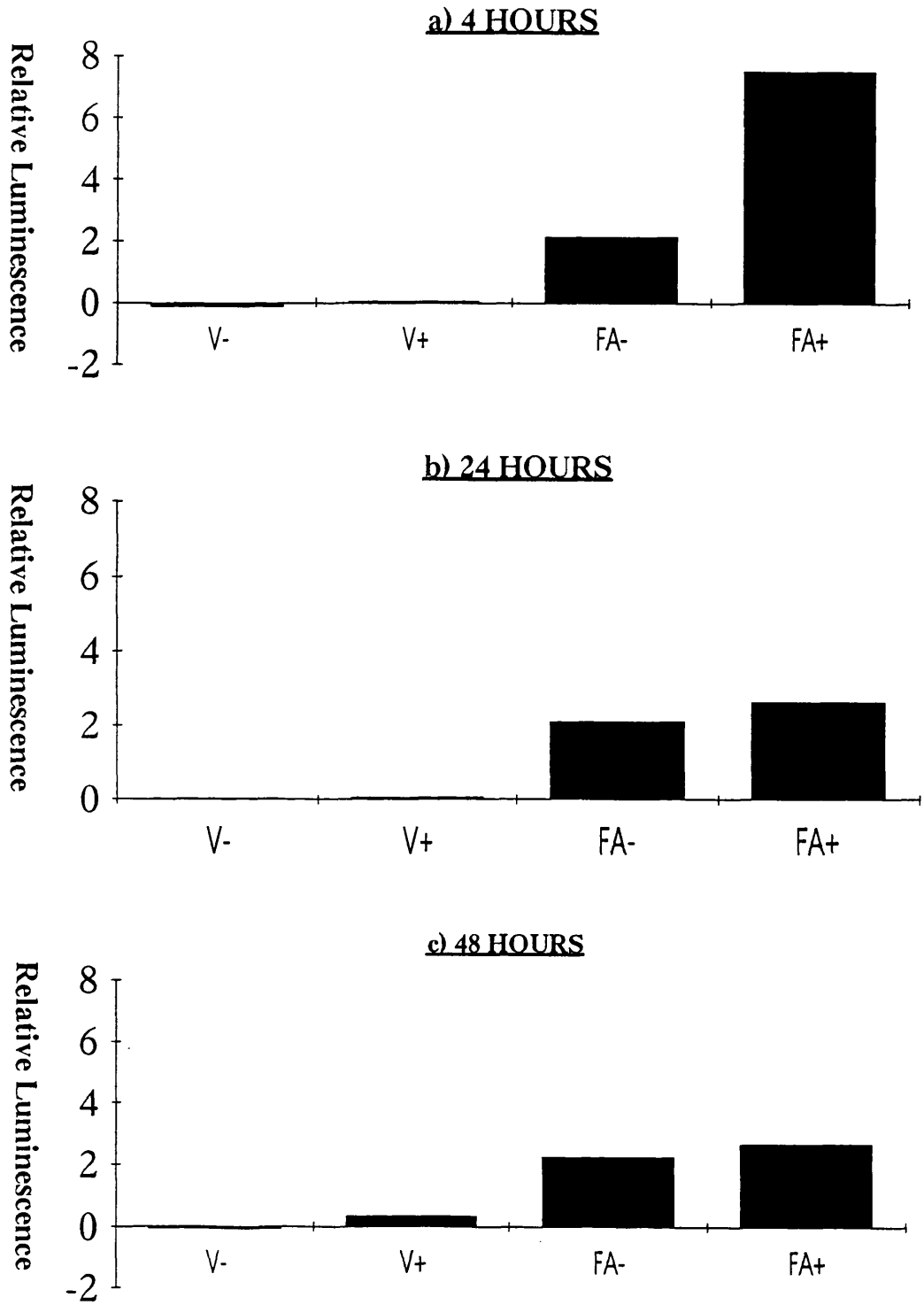


FIGURE 3.7.7

Induction Of Transcription By Cisplatin In A2780cp70 Cells

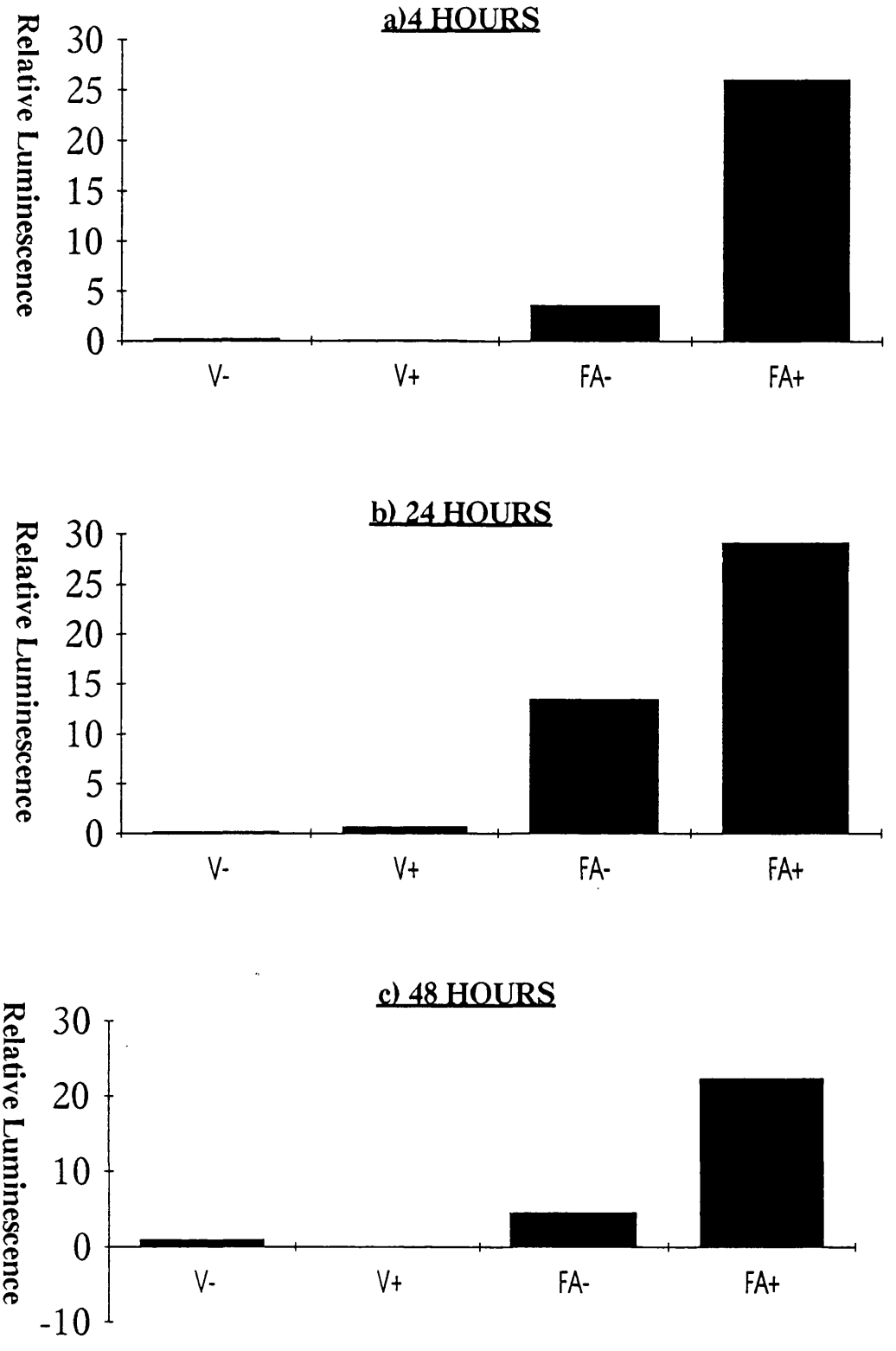
A2780cp70 cells transfected with the vector, pGUP.PA.8 alone (V) or containing fragment A DNA sequence (FA) were seeded out at 10^6 cells/ $75cm^2$ flask and grown overnight in RPMI medium. They were then either given a medium change to fresh medium (-) or given a 1μM dose of cisplatin for 1 hour (+). At 4 hours (a), 24 hours (b) and 48 hours (c) after drug removal cells were lysed, and assayed for luciferase activity in a luminometer (section 2.9). Relative luminescence is the luminescence/ sample tube after subtraction of the luminescence/ blank tube.

FIGURE 3.7.8

Induction Of Transcription By Transplatin In A2780cp70 Cells

A2780cp70 cells transfected with the vector, pGUP.PA.8 alone (V) or containing fragment A DNA sequence (FA) were seeded out at 10^6 cells/ $75cm^2$ flask and grown overnight in RPMI medium. They were then either given a medium change to fresh medium (-) or given a 1μM dose of transplatin for 1 hour (+). At 4 hours (a), 24 hours (b) and 48 hours (c) after drug removal cells were lysed, and assayed for luciferase activity in a luminometer (section 2.9). Relative luminescence is the luminescence/ sample tube after subtraction of the luminescence/ blank tube.

FIGURE 3.3.8 Induction Of Transcription By Transplatin In A2780cp70 Cells (legend on previous page)



fold (fig. 3.3.7a). As luciferase activity must be directly proportional to the quantity of luciferase enzyme which is in turn, directly proportional to the level of transcription from the luciferase plasmid, this shows that p53 transcriptional *trans*activation activity can be assessed using a reporter system such as this and that p53 protein activity can be induced by cisplatin.

A similar experiment was performed with transplatin rather than cisplatin and the results of this are shown in figure 3.3.8. Again there is little luciferase activity displayed by cells transfected with the vector alone either with (V+) or without a drug dose (V-). Cells containing the Frag.A-pGUP.PA.8 plasmid showed some luminescence activity in the absence of a transplatin dose (FA-) and showed an induction of this luciferase activity after transplatin treatment (FA+) with the greatest induction at the 4 hour timepoint (fig. 3.3.8a) when luciferase activity was 7 fold greater in treated than untreated cells. This again demonstrates how p53 mediated transcriptional *trans*activation can be induced by a DNA-damaging agent.

It is interesting to note that in both cases of induction with transplatin or with cisplatin, the p53 mediated transcriptional transactivation activity was induced to a greater extent at the 4 hour timepoint than the 24 or 48 hour timepoints. This suggests that p53 activity peaks earlier than p53 protein levels which are still increasing until at least 48 hours after drug treatment in this line.

3.3.5 ARE CELLS WHICH HAVE BEEN GIVEN ONE CISPLATIN DOSE MORE RESISTANT TO A SECOND CISPLATIN TREATMENT?

One might wonder whether increased p53 protein levels, or other such factors which alter on treatment with DNA-damaging agents, would serve to prepare cells for further assault with cytotoxic substances. Such a situation

FIGURE 3.3.9 The Effect Of Cisplatin Pretreatment On The Sensitivity Of A2780 Cells To A Second Cisplatin Dose (legend overleaf)

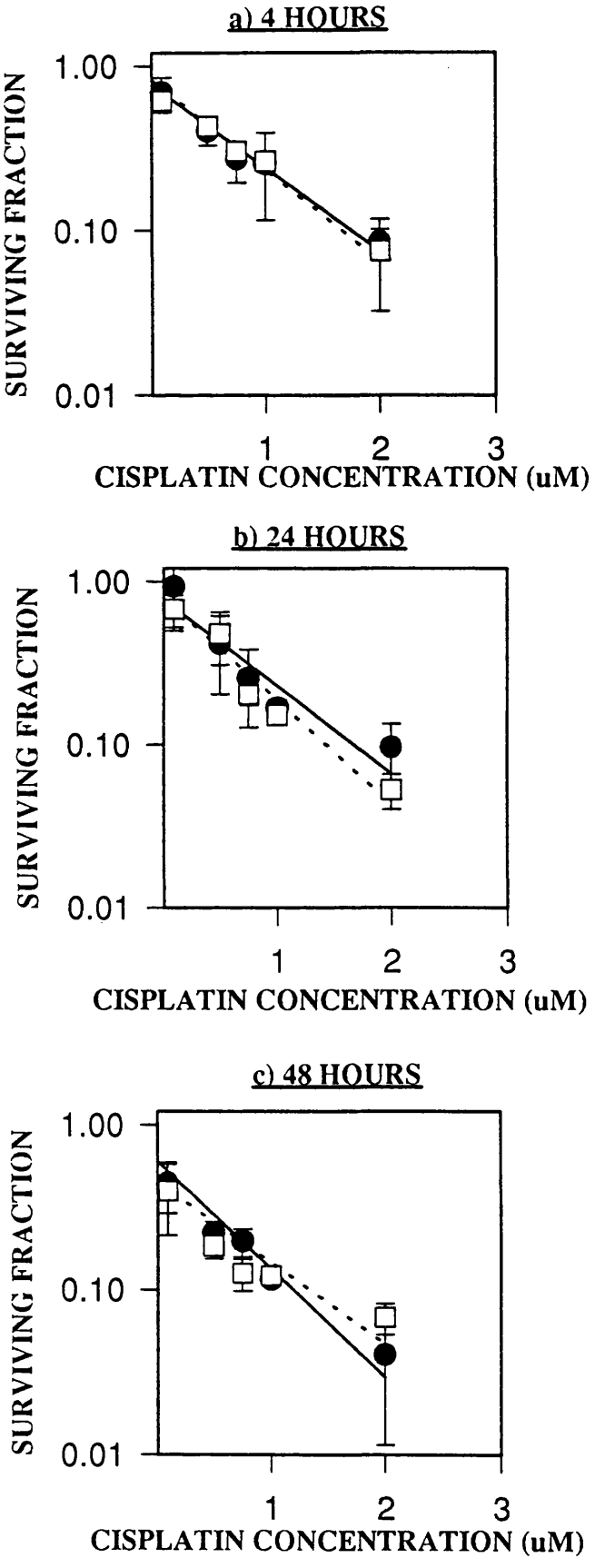


FIGURE 3.3.9 (previous page) The Effect Of Cisplatin Pretreatment On The Sensitivity Of A2780 Cells To A Second Cisplatin Dose

A2780 cells were seeded out at 10^3 cells/75cm^2 flask in RPMI medium, cultured overnight, then treated with 1μM cisplatin for 1 hour (black circles, solid lines) or given a medium change (white squares, dotted lines). 4 (a), 24 (b) and 48 (c) hours later, a second cisplatin dose of 0, 0.5, 0.75, 1 or 2 μM was given for 24 hours. Cells were cultured for a further 10 days in drug free RPMI medium then stained with Giemsa stain and the colonies were counted. Each point is the average of at least 2 flasks/ experiment from 2 separate experiments. Surviving fractions are calculated from the ratio of number of colonies at a given dose /number of colonies at 0μM of drug. Error bars shown represent standard deviations.

FIGURE 3.3.10 (overleaf) The Effect Of Cisplatin Pretreatment On The Sensitivity Of A2780cp70 Cells To A Second Cisplatin Dose

A2780cp70 cells were seeded out at 10^3 cells/75cm^2 flask in RPMI medium, cultured overnight, then treated with 1μM cisplatin for 1 hour (black circles, solid lines) or given a medium change (white squares, dotted lines). 4 (a), 24 (b) and 48 (c) hours later, a second cisplatin dose of 0, 0.5, 1, 2, 4 or 10 μM was given for 24 hours. Cells were cultured for a further 10 days in drug free RPMI medium then stained with Giemsa stain and the colonies were counted. Each point is the average of at least 2 flasks/ experiment from 2 separate experiments. Surviving fractions are calculated from the ratio of number of colonies at a given dose /number of colonies at 0μM of drug. Error bars shown represent standard deviations.

FIGURE 3.3.10 The Effect Of Cisplatin Pretreatment On The Sensitivity Of A2780cp70 Cells To A Second Cisplatin Dose (legend on previous page)

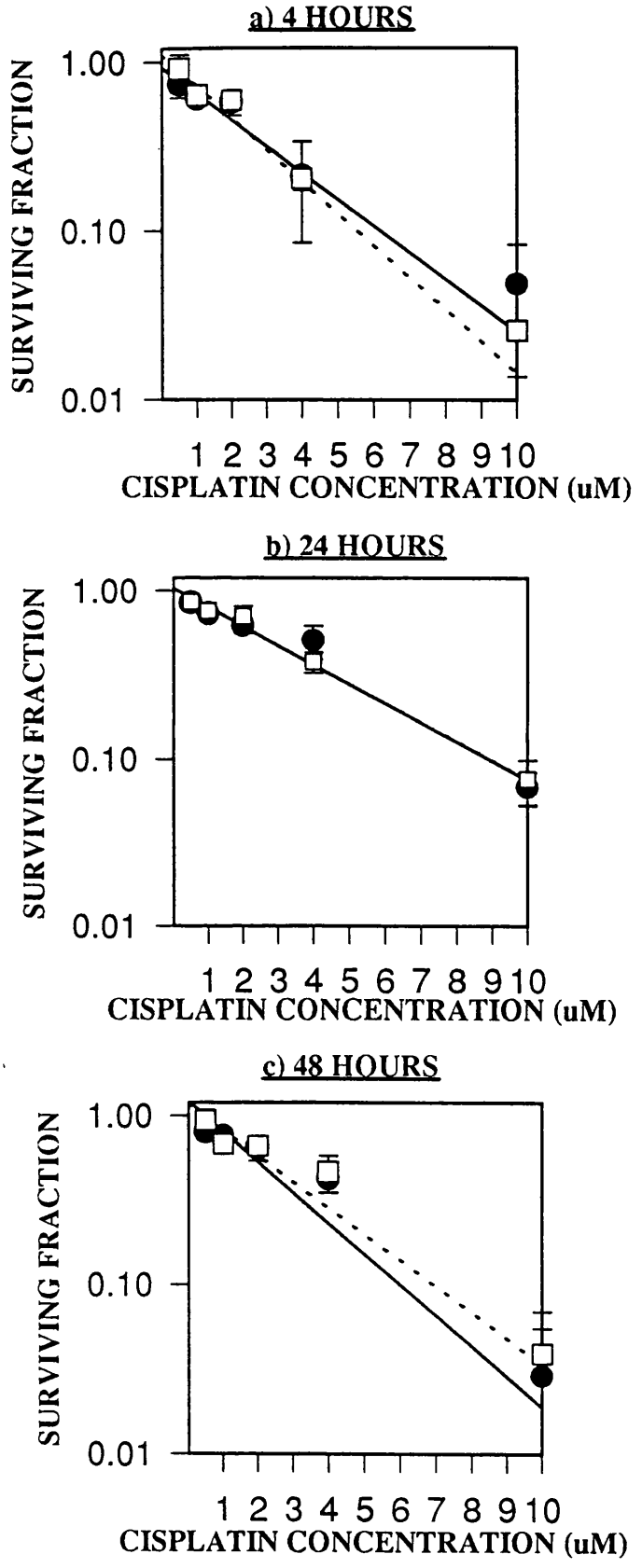


TABLE 3.3.5 The Effect Of Pretreating Cells With Cisplatin On Their Sensitivity To A Second Cisplatin Dose

Cell Line	Time Between Pretreatment and Second Cisplatin Dose (in hours)	ID_{50} of Pretreated Cells (μM)	ID_{50} of Control Cells (μM)	Fold Difference
A2780	4	0.62	0.62	1
A2780	24	0.69	0.57	+1.2
A2780	48	0.28	0.2	+1.4
A2780cp70	4	3.3	3.6	-1.1
A2780cp70	24	4	4	1
A2780cp70	48	4.3	4.5	-1.1

would be analogous to the immune response system where a pool of "memory cells" are retained following infection in order to provide a more rapid response when the organism is invaded a second time by a similar foreign body.

In order to ascertain the validity of this theory, A2780 or A2780cp70 cells were either pretreated with 1μM cisplatin for 1 hour or, in the case of control cells, given a medium change. They were then allowed to recover for either 4, 24 or 48 hours before their cisplatin sensitivity was determined by clonogenic assay involving a second exposure to cisplatin for 24 hours. The range of cisplatin doses used for A2780 cells was lower (0, 0.5, 0.75, 1, 1.5 or 2 μM) than for A2780cp70 cells (0, 0.5, 1, 2, 4 or 10). The data obtained for A2780 cells is depicted in figure 3.3.9 and that for A2780cp70 cells in figure 3.3.10. Pretreated cells are denoted by black circles and solid lines whereas control cells are represented by white squares and dotted lines. There appears to be no significant difference between pretreated and control cells and this is borne out by comparisons of ID_{50} values (table 3.3.5). At the 4 hour timepoint there is no difference in ID_{50} value for A2780 cells and a 1.1 fold decrease in A2780cp70 cells when pretreated with cisplatin. At 24 hours after pretreatment, A2780 cells show a 1.2 fold increase and A2780cp70 cells show no difference and at the 48 hour timepoint A2780 cells exhibit a rise of 1.4 fold and A2780cp70 cells a 1.1 fold difference in favour of control cells. Bearing in mind the confidence limits of this experiment, these small alterations in cell survival are not significant. Pretreating cells with cisplatin does not significantly protect them from a second cisplatin dose.

3.4 DISCUSSION

3.4.1 STABILISATION OF p53 PROTEIN IN CISPLATIN RESISTANT CELL LINES

Increased levels of p53 protein are seen following cisplatin treatment of the A2780 and A2780cp70 cell lines. Higher amounts of this protein are also found in cells selected for cisplatin resistance in vitro e.g. A2780cp70 compared to A2780 cells, from which they originate. This suggests that p53 protein could be instrumental in causing cisplatin resistance. Using ID_{20} values obtained from clonogenic assay the A2780cp70 cell line was found to be 6 fold resistant to a 1 hour cisplatin dose and 17 fold resistant to a 1 hour treatment with transplatin when compared to the parental line, A2780. A 4 fold cisplatin resistance was previously found using a 24 hour dose clonogenic assay (Brown et al., 1993) and a 39 fold resistance recorded using a soft agar assay (Behrens et al., 1987). This demonstrates the importance of specifying the duration of drug exposure and the assay used when quoting data concerning drug sensitivity. Also more importantly, a 1 hour, 1μM dose of either cisplatin or transplatin did not kill more than 30% of a population of either cell line. Therefore an adequate yield of various extracts could be prepared following such treatments and there was not a significant danger of selecting out a more resistant subpopulation of cells.

Induction of p53 protein occurred following transplatin or cisplatin exposure in A2780 cells, where peak levels were detected 24 hours after cisplatin treatment. In A2780cp70 cells cisplatin-induced p53 protein was still rising 72 hours after drug removal but a drop in p53 induction was seen after 72 hours for transplatin. This discrepancy may be accounted for by the observation that transplatin-DNA adducts accumulate for only 6 hours compared to 48 hours for cisplatin-DNA adducts (Ciccarelli et al., 1988). Another possible explanation could be that transplatin, being less toxic, permits overcrowding of cells which attain a quiescent state and switch off

expression of the p53 gene. To control for this, lysates could be prepared at the same time but drug treatments given at different times prior to this. Alternatively, cells could be treated with equitoxic drug doses.

As *p53* mutations frequently cause enhanced stabilization of the p53 protein, it was important to investigate the p53 genotype in the cell lines used. A common conformation for mutant p53 proteins has been proposed due to the ability of the antibody PAb 240 to bind to several different mutant p53 proteins (Gannon et al., 1990). Immunoprecipitation with this mutant specific antibody, followed by Western blotting of precipitated proteins, showed that although some p53 was precipitated from the cisplatin resistant cell line A2780cp70 this was not as much as for the pantropic DO-1 antibody. Ov1PDDP contained a p53 protein which was precipitable to equal degrees with both antibodies. The implication that A2780cp70 contained a wild type p53 gene whereas Ov1PDDP contained a mutant p53 gene was borne out by direct PCR sequencing of the entire *p53* cDNA (Brown et al., 1993).

The p53 protein stabilisation attained in these cisplatin resistant lines A2780cp70 and Ov1PDDP, is not the result of a mutant p53 gene. Induced p53 protein levels do not correlate with induced mRNA levels (Kastan et al., 1991; Fritsche et al., 1993). Other possible causes are differences in post translational modification or binding to a protein which protects it from recognition by the ubiquitin protein degradation system. No difference was detected between p53 protein concentration in A2780cp70 nuclear extracts which had been prepared in the presence or absence of phosphatase inhibitors. This may indicate that insufficient time was allowed for these inhibitors to exert their effects also phosphorylation of p53 at different sites may have opposing affects on stability or activity.

Phosphorylation does appear to be important in the control of p53 protein activity. There are at least four protein kinases active against p53 as demonstrated by fractionation of SV3T3 cells (Milne et al., 1992a). Serine 389

of p53 protein is phosphorylated by casein kinase II (Meek et al., 1990; Herrman et al., 1991). Also a casein kinase like enzyme which separates from casein kinase I (CKI) itself on a phosvitin-sepharose affinity column, phosphorylates serines 4, 6 and 9 of murine p53 protein. Within the nuclear localisation domain of human p53, at serines 312-323, serine 315 is phosphorylated by $p34^{cdc2}$ (Addison et al., 1990). Both interphase and M-phase forms of the enzyme are active and p53 is probably phosphorylated at this site throughout most of the cell cycle except G1 (Bischoff et al., 1990), although two to three fold more ^{32}P is incorporated into p53 protein during G1 than either G2, S or M phases. Mitogen activated protein (MAP) kinase has also been shown to have activity against p53 protein (Milne et al., 1994) as has the c-jun kinase, JNK1 (Milne et al., in press). A DNA activated protein from HeLa cells phosphorylates p53 (Lees-Miller et al., 1990) at serines 15 and 37 in the *trans*activation domain of human p53 (Lees-Miller et al., 1992) and serines 7, 9 and 18 of mouse p53 protein (Wang & Eckhardt., 1992).

A change in activity of one of these kinases could profoundly alter p53 protein stability or activity. This is borne out by the observation that mutation of the serine 15 phosphorylation site within human p53 protein to an alanine residue reduces the ability of p53 to mediate a G1 phase block (Fiscella et al., 1993). A mutation of the CKII site of mouse p53 at serine 386, prevented p53 mediated growth suppression activity as measured by inhibition of colony formation in SV3T3 and SV40 transformed baby hamster kidney cells (Milne et al., 1992b). Also, it has been shown that okadaic acid can reduce transcriptional *trans*activation by p53 protein (Zhang et al., 1994b). This is in agreement with the finding that potato acid phosphatase enhances DNA binding by p53 (Kern et al., 1991a). It might be possible that a signal which serves to activate p53 for transcriptional *trans*activation could also be responsible for its subsequent destruction thus ensuring dispersal of p53 protein after carrying out its effects. This idea seems attractive in view of the

characteristics of p53 protein in A2780cp70 cells where it has greater stability but lower activity than in the cisplatin sensitive counterpart, A2780.

Unfortunately it was not possible to obtain results from an investigation into MDM2 protein levels in A2780 and A2780cp70 cells. This would have been an interesting area for experimentation as this 95KDa protein has affinity for p53 protein which can stabilize p53 protein and prevent p53 mediated transcriptional *trans*activation (Momand et al., 1992). An increase in MDM2 levels or affinity for p53 protein, again would account for both the reduced activity and increased concentration of p53 protein in A2780cp70 cells relative to the parental line. The reduced levels of *mdm2* mRNA in A2780cp70 cells would suggest that this theory is unlikely to prove correct but there is some evidence for regulation of MDM2 protein levels at the translation stage so decreased mRNA would not necessarily correlate with decreased MDM2 protein (Landers et al., 1994).

The comparatively high levels of the heatshock protein Hsp70 which one detects in the A2780cp70 line could explain why p53 protein exhibits reduced activity in this line as this protein has the ability to bind p53 protein (Pinhasi-Kimhi et al., 1986). It can therefore remove it to the cytoplasm of the cell (Martinez et al., 1991), away from the transcription sites which p53 protein *trans*activates. However this seems an unlikely explanation for A2780cp70 cells, bearing in mind the levels of p53 protein seen for nuclear extracts of this cell line. The region of p53 protein responsible for this interaction has been narrowed down to the 28 amino acids at the N-terminus (Hainaut & Milner, 1992; Lam & Calderwood, 1992). The high levels of Hsp70 protein which one sees in A2780cp70 cells may result from reduced p53 activity, as p53 is able to repress the Hsp70 promoter (Agoff et al., 1993). This indicates increasing evidence of a reduced p53 activity in A2780cp70 cells relative to A2780 parental cells.

Heatshock proteins are induced following various forms of cellular stress including heat exposure. The Hsp70 protein is serum stimulated and is normally expressed in late G1/early S phase of the cell cycle. Certain members of the heatshock protein family have been postulated to be involved in cisplatin resistance. For example, three isoforms of Hsp25 are induced by cisplatin in Erlich ascites tumour cells (Oesterreich et al., 1991). Also overexpression of Hsp60 is associated with reduced survival in ovarian carcinoma patients and both Hsp60 mRNA and protein can be induced by cisplatin (Kimura et al., 1993). Cisplatin and hyperthermia only seem to have an additive rather than synergistic effect against ovarian carcinoma cells *in vitro* (Kimura & Howell, 1993).

3.4.2 ALTERED ACTIVITY OF p53 PROTEIN IN CISPLATIN RESISTANT CELL LINES

Despite the problem that the overall function of p53 in cells is not known, several measurable activities of p53 protein have been found. These include its ability to activate transcription from certain promoters (Weintraub et al., 1991; Farmer et al., 1992; Zambetti et al., 1992; Okamoto & Beach, 1994). One promoter which responds to transcriptional *trans*activation by p53 protein is *WAF-1* (El-Deiry et al., 1993). I observed an induction of *WAF-1* mRNA expression using various DNA damaging agents. This is consistent with similar data from other laboratories (El-Diery et al., 1994). Induction of this promoter was more rapid following treatment with 2Gy ionising radiation than after exposure to cisplatin or transplatin, probably due to its more direct penetration of cells. This would be expected as p53 protein itself is induced with faster kinetics using this DNA damaging agent, showing peak p53 protein level after 8 hours rather than 24 hours after cisplatin treatment. Also 10 fold lower uninduced levels of *WAF-1* mRNA were detected in the cisplatin resistant A2780cp70 cell line than the sensitive A2780 cells

suggesting that p53 protein has lower activity in the resistant cells. Transfection of a mutant *p53* gene into A2780 cells has the effect of causing a 3 fold reduction in the basal level of *WAF-1* mRNA however it does not eradicate *WAF-1* expression or prevent *WAF-1* induction. This could be due to non-p53 mediated transcription from the *WAF-1* promoter which has been shown to be induced by serum or certain growth factors in fibroblasts from mice which lack the *p53* gene (Micheili et al., 1994). Alternatively it might be caused by the mutant p53 being unable to bind to all wild type p53 protein within the cells. Also activation of p53 protein for DNA binding might result in post-translational modification which prevents the formation of heterodimers with mutant p53 protein due to the binding site on wild type p53 protein being obscured. The use of cells lacking any p53 gene would be required in order to demonstrate conclusively that *WAF-1* mRNA transcription and its induction by DNA damaging drugs relies on p53 protein activity.

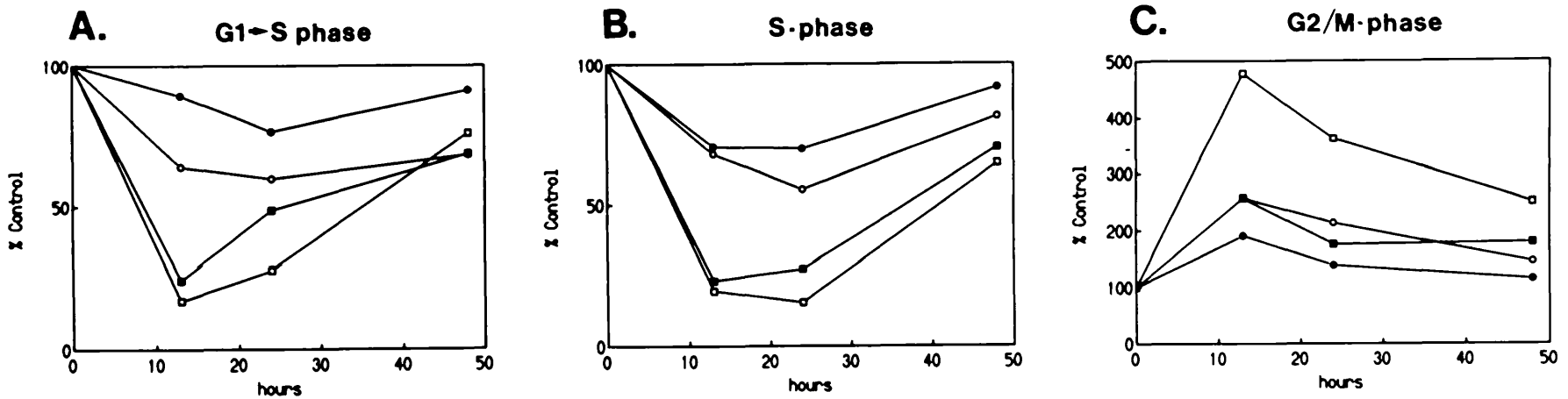
Reduced *MDM2* mRNA expression in A2780cp70 cells compared to A2780 cells is interesting as it provides evidence that decreased expression from p53 responsive promoters is not restricted to *WAF-1*. This negates the explanation that reduced activation of transcription could result from a mutated p53 binding site upstream of the coding region as it would be unlikely for this to occur at two p53 binding sites at once. The implications of reduced *MDM2* expression were discussed in the previous section.

A luciferase expression system can be utilised to measure p53 mediated transcriptional *trans*activation following stable transfection with the plasmids, pGUP.PA.8 or Frag.A.pGUP.PA.8. Some drug-induced expression of luciferase from the p53 responsive DNA sequence, fragment A, was observed in A2780cp70 cells however, relative luminescence levels were not very high and a direct comparison with A2780 was not possible. This is congruous with other data which suggest that A2780cp70 has reduced p53

activity with respect to its parental line, A2780. The data obtained by Northern blot analysis is perhaps more meaningful as it involves activation of an endogenous gene rather than exogenously transfected DNA.

Another measure of p53 activity is the ability to cause arrest in the G1 phase of the cell cycle in response to DNA damage inducing agents. This can be measured by flow cytometric analysis of BUdR incorporation along with propidium iodide staining. An example of an experiment which used the G1 arrest in response to 2Gy or 4Gy ionising radiation to measure the differential p53 activity between A2780 and A2780cp70 cells is provided in figure 3.4.1 (taken from Brown et al., 1993). In this figure, A2780 cells are represented by squares, A2780cp70 by circles. Also the open symbols signify cells treated with 4Gy and the closed symbols cells given a 2Gy dose. Following irradiation there was a rapid drop in the percentage of A2780 cells entering S phase (fig. 3.4.1a) and the percentage of cells in S phase (fig. 3.4.1b) compared to untreated control cells. Also the fraction of cells responding to DNA damage with a G1 block, rose when the radiation dose was increased. In the case of A2780cp70 cells, there was not such a pronounced drop in the fraction of cells entering S phase or actually in S phase. Thus transition from the cisplatin sensitive cell line, A2780 to resistant A2780cp70 cells involved loss of the capacity to arrest in G1 phase of the cell cycle after exposure to DNA damage inducing agents. This loss of G1 arrest in A2780cp70 cells is symptomatic of reduced p53 activity. Transfection of a mutant *p53* gene into A2780 cells abrogates G1 arrest in response to ionising radiation (McIlwrath et al., 1994). It also prevents G1 and G2 arrests in response to cisplatin treatment.

There does appear to be a link between the ability to arrest in G1 phase of the cell cycle in response to DNA damage, as determined by flow cytometric analysis after treatment with ionising radiation, and the sensitivity



This figure is taken from Brown et al., 1993. The percentage of cells in each cell-cycle phase is shown as a percentage of the cell cycle distribution in untreated control cells at each time point. Each time point shown represents the mean of 2 independent experiments. The cell-cycle populations were assessed by flow cytometric analysis of BUdR incorporation during a 4-hour pulse at the times shown, together with propidium iodide staining. a) Percentage of control cells that have recently entered S-phase from G1; b) percentage of cells in S phase (variable DNA content with BUdR incorporation); c) percentage of cells in G2/M phase (4N DNA content with no BUdR incorporation). Square symbols, A2780; circles, A2780cp70. Filled symbols, cells treated with 2Gy; open symbols, cells treated with 4Gy.

of cells to DNA damaging agents (O'Conner et al., 1993; McIlwrath et al., 1994). However there are likely to be other mechanisms which predominate in different cell types which explains why some investigators (Slichenmeyer et al., 1993) do not find such a correlation.

It has been shown that transfection of a mutant p53 gene with a valine to alanine substitution at codon 143 into A2780cp70, but not A2780 cells, raises cisplatin sensitivity 2-3 fold compared to vector alone controls (Brown et al., 1993). This suggests that possession of a wild type p53 protein is relevant to drug resistance in this cell line at least, despite its reduced activity. It is feasible that some factor of a DNA damage response pathway, which incorporates p53 protein, has been mutated in this cell line so that p53 protein is no longer able to recognise it. The mutant p53 protein conformation might be altered in such a way as to restore affinity for this component and "mend" the "broken link" in the damage response pathway.

As p53 protein is required for apoptosis in certain circumstances (Lowe et al., 1993a; Lowe et al., 1993b; Yonish-Roach et al., 1993; Clarke et al., 1993; Lee & Bernstein, 1993), reduced p53 activity might in turn reduce the frequency of drug-induced apoptosis thereby permitting greater cell survival after DNA damage. Indeed there does appear to be reduced susceptibility to apoptosis, as determined by DNA fragmentation 72 hours after exposure to γ-ray radiation, in A2780cp70 cells when compared to A2780 cells (A.McIlwrath, CRC Department of Medical Oncology, Glasgow University *personal communications*).

Chapter 4

CHAPTER 4: DNA DAMAGE RECOGNITION PROTEINS AS POTENTIAL MEDIATORS OF CISPLATIN RESISTANCE

4.1 INTRODUCTION

4.1.1 THEORETICAL ROLES OF DNA DAMAGE RECOGNITION PROTEINS IN DRUG RESISTANCE

In the previous chapter, I have shown that a signal pathway leads from cisplatin-induced DNA damage to p53 induction. DNA damage might be recognised directly by p53 but it is likely that the signal is transduced by other factors. Proteins which bind to lesions of the DNA i.e damage recognition proteins are likely candidates for this task. They are of interest anyway as they could be involved in alternative DNA damage signal pathways. They may prove to be repair proteins or proteins which minimize damage-related DNA distortions and thereby be capable of counteracting cisplatin-induced DNA damage. However they could simply be trapped because the lesion prevents their dissociation from, or progression along, the DNA and may have a function unrelated to cisplatin DNA damage metabolism.

An example of DRP involvement in cell survival after DNA damage is provided by the Uvr endonuclease-mediated excision pathway. This occurs in the prokaryotic organism, *Escherichia coli* when the DRP, UvrA binds as a dimer to UvrB and this multimeric complex associates with UV-induced DNA adducts. Then the UvrC protein becomes associated with and nicks the damaged DNA facilitating its removal (Orren & Sancar, 1989). The UvrA protein is not involved in incision and functions as a DRP in order to recruit the other proteins to the damage site.

4.1.2 DNA DAMAGE RECOGNITION PROTEINS FOUND USING THE GEL MOBILITY SHIFT ASSAY

The gel mobility shift assay operates on the principle that DNA-binding proteins will complex to and retard the progress of radiolabelled DNA through a non-denaturing polyacrylamide gel. This results in the formation of further "bands" on exposure of the gel to photographic film. UV specific DRPs have been detected by gel mobility shift analysis in HeLa cells (Chao et al., 1991c; van Assendelft et al, 1993), and CV-1 monkey cells. DRPs which have affinity for cisplatin-modified DNA have been found in extracts from human HeLa cells (Chu & Chang, 1988; Chao et al, 1991c). Also cisplatin-DRP activity has been detected using this technique in CHO hamster cells, 2008 human ovarian, MDCK and LLC-PK kidney tubule cells (Andrews & Jones, 1991) and in *S.cerevisiae* yeast cells (Patterson & Chu, 1989).

Using a cisplatin-damaged, 54 base-paired oligonucleotide (2αG7) in the gel mobility shift assay with nuclear extracts prepared from human, ovarian tumour cell lines, two complexes designated "B1" and "B2" were detected (K.McLaughlin PhD thesis, Glasgow university). Binding of 2αG7 to the damage recognition proteins which form B1 and B2 can be competed out using platinated calf thymus DNA but not unplatinated calf thymus DNA demonstrating their specificity. Similar complexes can be seen for human, murine and feline extracts so the proteins involved appear to be conserved amongst mammals. Complex B1 has been found to involve human single stranded binding protein (hssB), also known as RPA and RFA, which participates in DNA replication (Clugston et al., 1992). However no difference in levels of hSSB between the cisplatin sensitive cell lines, Ov1P, SuSa, A2780 and RT112 and their resistant counterparts could be detected by Western blotting. The protein, hSSB, has been demonstrated to be required for human DNA excision repair in a cell free system using HeLa cell extracts

(Coverley et al., 1991). In this chapter the B2 complex will be analysed and its identity elucidated.

4.1.3 DNA DAMAGE RECOGNITION PROTEINS FOUND USING SOUTH WESTERN BLOTTING

South-western blotting involves the separation of proteins on a denaturing concentration gradient gel followed by blotting of the proteins onto a nitrocellulose membrane. Proteins attached to the membrane are then examined for their DNA hybridization capacity by incubation in the presence of a radiolabelled oligonucleotide (see section 2.5.6 for further details). From HeLa cells, DNA-damage inducible DRP proteins of 130KDa and 95KDa have been detected by South-western blotting (Chao et al., 1991a). Also in HeLa cell extracts a cisplatin-DRP doublet of 40-42KDa (Robbins et al., 1991) and an 81KDa cisplatin DRP (Toney et al., 1989; Bruhn et al., 1992) have been found using this modified Western blotting procedure. Using this method DRP activity has also been seen for the IXR1 yeast protein (Brown et al., 1993) and proteins of 26, 28 and 97KDa in the 2008 ovarian carcinoma cell line (Andrews & Jones, 1991).

When South-western blotting was employed to identify cisplatin-DNA DRPs in nuclear extracts from the human ovarian carcinoma cell lines A2780, Ov1P and their cisplatin resistant derivatives, there seemed to be four such proteins of 97KDa, 70KDa, 48KDa and 25KDa (K. McLaughlin, PhD thesis, Glasgow University). The 97KDa and 48KDa proteins exhibit increased expression in the cisplatin resistant Ov1PDDP cell line compared to the cisplatin sensitive Ov1P cells from which they were selected. However there is no difference in detectable levels of these proteins between A2780 cells and the cisplatin resistant derivative, A2780cp70. This does not however rule out the possibility of differential activity of these proteins as DNA damage signal transducers. None of the four proteins could be induced by cisplatin exposure

(McLaughlin PhD thesis, Glasgow university). These proteins could be identical to those observed using the gel mobility shift assay or they may be entirely different proteins with novel damage recognition sites exposed by denaturation. One might wonder whether the 70KDa protein detected on South-western blots could represent the 70KDa hSSB subunit, however this seems unlikely as the 70KDa DRP was unable to bind to single-stranded DNA on South-western blotting. The hSSB protein probably needs to be in a complex, constituting 70KDa, 34KDa and 13KDa subunits, in order to show DNA binding capacity. This would not happen in South-western blotting as the denaturing conditions cause separation of the components of multimeric protein complexes.

4.1.4 WHAT ARE HMG PROTEINS AND COULD THEY BE RESPONSIBLE FOR B2 COMPLEX FORMATION?

HMG (High Mobility Group) proteins are all less than 30KDa and include HMG1 (28KDa), HMG2 (27KDa), HMG 14 and 17 (10-20 KDa) , HMG 20 also known as ubiquitin and HMGI/Y, the 10KDa splice variants which differ in 11 amino acids which are lacking in HMGY (Eckner & Birnstiel, 1989). They interact with DNA and are soluble in 2% TCA (Einck & Bustin, 1985). This latter property is not the case for most other proteins. Also as HMG1 has been shown to interact with cruciform DNA (Bianchi et al., 1989), it seemed feasible that such proteins might have affinity for other structural distortions such as cisplatin-damaged DNA. HMG proteins contain a high proportion of acidic and basic residues (Einck & Bustin., 1985). They have a well conserved primary sequence and can be extracted from nuclei and chromatin with 0.35M NaCl.

The B2 complex detected by gel retardation analysis of cisplatin treated oligonucleotides could be formed by a protein containing a "HMG box", a region of homology to HMG proteins. Examples of such proteins are

the transcriptional regulators, LEF-1 (Giese et al., 1991) and hUBF (Jantzen et al., 1990). The *ACP2* gene from the yeast, *Saccharomyces cerevisiae* has 19% amino acid homology with HMG1 (Haggren & Kolodrubetz, 1988). In fact, members of the HMG superfamily can be found in organisms as diverse as plants, yeast and animals (Laudet et al., 1993).

4.1.5 AIMS OF THIS CHAPTER

1) In this chapter a purification scheme for the protein involved in B2 complex formation will be developed using the gel mobility shift assay to determine DRP activity.

2) The identity of the B2 forming protein will be determined.

3) The protein, p53 will be shown to have no intrinsic DRP activity as measured by the gel mobility shift assay.

RESULTS

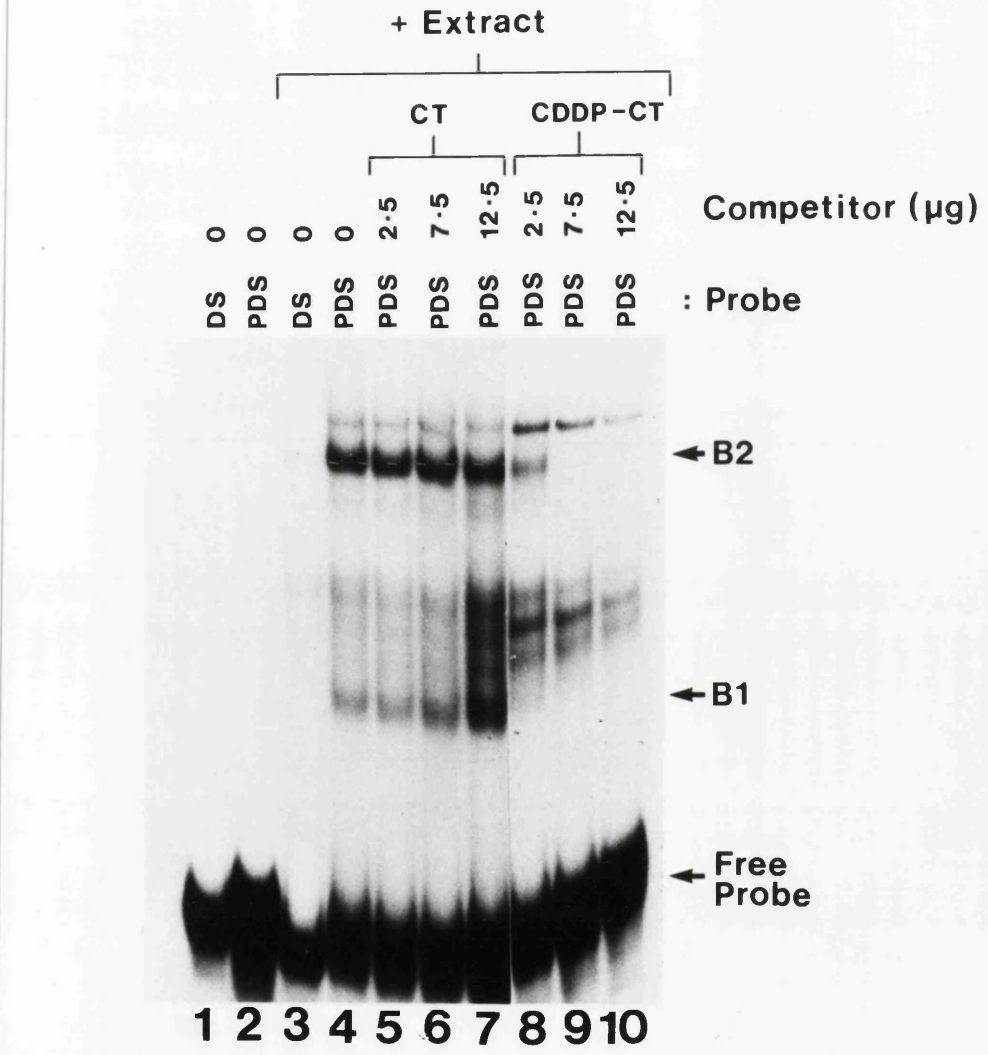
4.2 METHOD DEVELOPMENT FOR PURIFICATION OF A DNA DAMAGE RECOGNITION PROTEIN

4.2.1 USE OF THE GEL MOBILITY SHIFT ASSAY TO ASSESS DNA BINDING ACTIVITY

The gel mobility shift assay has often been used to detect proteins which bind to DNA either non-specifically or to DNA of a particular shape or sequence. An example (taken from K.McLaughlin., Phd. thesis, Glasgow University) is given in figure 4.2.1 of a typical gel mobility shift assay used to examine proteins which have affinity for cisplatin-damaged DNA. Lanes 1 and 2 were control lanes, loaded with radio-labelled oligonucleotide alone. A single band representing free oligonucleotide probe can be seen in these lanes. Lanes 3-10 were each loaded with reaction products obtained by incubating 1μg of HeLa cell nuclear extract with the radioactive oligonucleotide, 2αG7. In lane 3, unplatinated 2αG7 (DS) was used and no complex observed. When 2αG7 that had been platinated (PDS), and had thus acquired cisplatin-DNA adducts, was used instead (lane 4), 2 other major bands were seen which were named B1 and B2. This chapter is concerned with analysing the B2 complex. The B1 complex has been shown to contain hSSB protein (Clugston et al., 1992).

Lanes 5, 6 and 7 were loaded with similar components to lane 4 but with the inclusion of increasing amounts of "cold" calf thymus (CT) DNA which had no effect on formation of either complex. When "cold" calf thymus DNA containing cisplatin-DNA adducts was used as a competitor (lanes 8-10), there was a disappearance of both B1 and B2 complexes. This demonstrates that the proteins involved in formation of these complexes have specific affinity for cisplatin-damaged DNA.

FIGURE 4.2.1 An Example Of A Gel Mobility Shift Assay



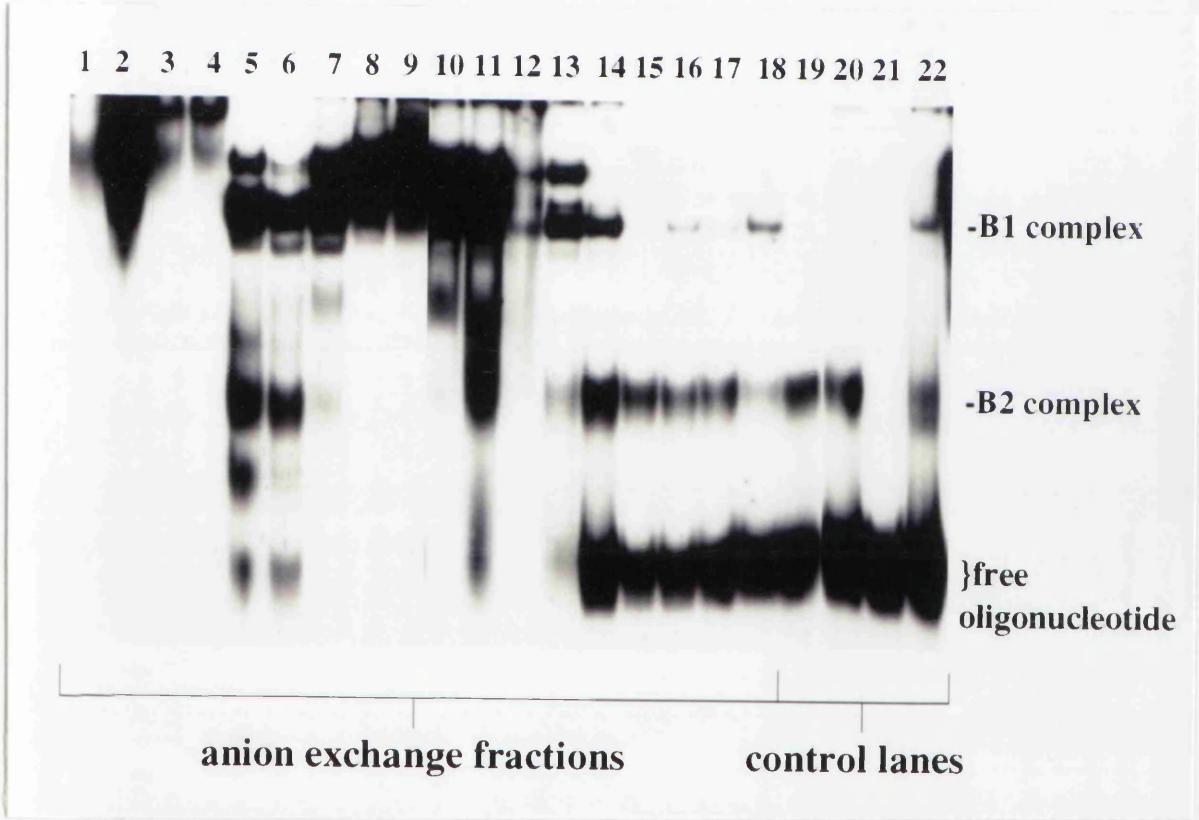
This example of a gel mobility shift assay (section 2.8) is adapted from K. M^{c}Laughlin PhD thesis, Glasgow University. 5x 10^{3} d.p.m. of 2αG7 either treated with cisplatin (lanes 2 & 4-10) or untreated (lanes 1 & 3) was incubated on ice for 30 minutes in a final volume of 20µl. 1µg HeLa nuclear extract (section 2.2.3) was included in lanes 3-10. Untreated calf thymus DNA was included in lanes 5 (2.5µg), 6 (7.5µg) & 7 (12.5µg). Cisplatin treated calf thymus DNA was included in lanes 8, (2.5µg), 9 (7.5µg) & 10 (12.5µg). Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

4.2.2 ASSESSING THE VALIDITY OF ANION EXCHANGE CHROMATOGRAPHY AS A METHOD OF DRP PURIFICATION

Anion exchange chromatography was tried out as a method for separating the protein involved in B2 complex formation from other nuclear constituents. So as to quantify B2 complex formation, the arbitary units "ba" (binding activity) will be used, where 1ba is equal to the binding activity found in 8.6mg of crude extract. A large scale nuclear extract (196 mg total) was prepared from Ov1PDDP cells (see section 2.2.3) and this had a specific activity of 0.116ba/mg and a protein concentration of 8.6mg/ml. Anion exchange chromatography was carried out as described in section 2.6.2 using 172mg of this crude nuclear extract as a starting material, a total of 32 fractions were collected. An equal volume (3µl) of each fraction was assayed by comparison to extracts with known B2 complex-forming ability using the gel mobility shift assay (section 2.8). Lanes 1-18 contain fractions 3-7 then 20-32, a negative control which lacks cellular extract was contained in lane 21 and positive controls of Ov1PDDP TCA supernatant and crude nuclear extract were loaded on lanes 20 and 22 respectively. Fractions were chosen for inclusion in this assay based on the elution profile obtained during anion exchange chromatography.

Most B2 complex forming activity is eluted in fractions 27-32 (lanes 13-18) with peak activity in fraction 28 (lane 14). The total protein content of these 6 fractions was estimated as 16.8mg and the total activity was 9ba as quantified by comparison to crude nuclear extract. Therefore specific activity of these 6 fractions once pooled, was 0.54ba/mg indicating a 5 fold purification. Use of an anion exchange column can be used to separate the B2 complex forming activity although it is not especially efficient.

FIGURE 4.2.2 A Gel Mobility Shift Assay Of Anion Exchange Fractions



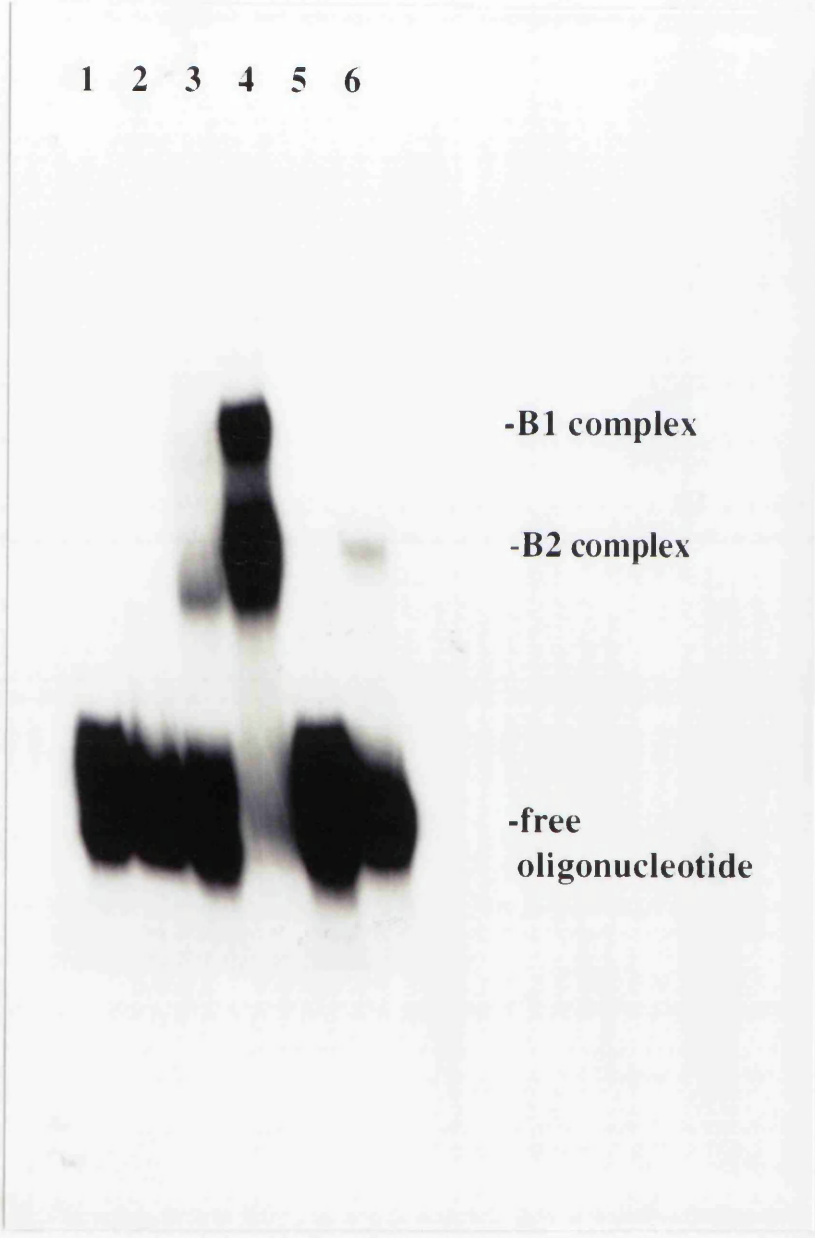
The proteins in 172mg of crude Ov1PDDP nuclear extract (section 2.2.3) were separated using a gradient of 100% buffer A (0.1M NaCl, 0.02M Tris -pH 7.6) to 100% buffer B (2M NaCl, 0.02M Tris-pH 7.6) on a MonoQ^{TM} column (section 2.6.2). Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated for 30 minutes on ice in a final volume of 20µl. 3µl of fractions 3-7 then 20-32 were included (lanes 1-18). 3µl of an anion exchange fraction with known DRP activity was included in lane 19 as a positive control. 3µl of 2% TCA soluble Ov1PDDP nuclear proteins (lane 20) and 3µl of crude, nuclear extract (lane 22) were also used as positive controls. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

4.2.3 THE USE OF TCA TREATMENT AS AN INITIAL CLEAN UP STAGE FOR DRP PURIFICATION

Due to an apparent similarity in certain properties between the B2 complex forming protein and the HMG class of proteins, which are soluble in 2% TCA unlike most proteins, solubility in TCA was examined as a possible method of separation. TCA treatment of nuclear extracts was carried out as described in section 2.6.1 and proteins were analysed for DRP activity by gel mobility shift assay. The results of such an assay using a TCA treated A2780cp70 nuclear extract are depicted in figure 4.2.3. Lanes 1 and 2 are control lanes with lane 1 containing unplatinated 2αG7 and lane 2 containing platinated 2αG7. No complex is detected in these lanes. In lanes 5 and 6, 1μl of TCA supernatant was incubated with unplatinated or platinated oligonucleotide respectively. Binding was only seen when platinated oligonucleotide was included indicating that this binding activity was specific for cisplatin-damaged DNA. In lanes 3 and 4, 1μl of resuspended TCA precipitate was included instead of supernatant. It was expected that the pelleted proteins would represent all other nuclear proteins except for the TCA soluble varieties and so no B2 complex forming activity would be present. On the contrary 12 fold more activity was seen in lane 4 compared to lane 6 but due to the concentrated nature of the pelleted proteins, this accounts for a fraction of total nuclear activity. Some non-specific binding to unplatinated oligonucleotide was seen in lane 3 but this had a lower mobility than the cisplatin-damage specific activity observed with the supernatant in lane 6. Thus most, but not all of this DRP is found in the TCA soluble fraction of a nuclear extract.

When 17.2mg of crude nuclear Ov1pDDP extract was treated with TCA, the supernatant had an affinity for cisplatin-damaged DNA of 6.4ba/mg representing a 55 fold purification when compared to crude nuclear starting material (table 4.2.1). The DRP activity of this supernatant displayed a

FIGURE 4.2.3 DRP Activity In A TCA Supernatant



The proteins in a crude A2780cp70 nuclear extract (section 2.2.3) were treated with 2% TCA (section 2.6.1) and TCA soluble proteins (2ml) separated out by spinning in a microfuge. The pellet was resuspended in 20µl. Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 either treated with cisplatin (lanes 2, 4 & 6) or untreated (lanes 1, 3 & 5) was incubated for 30 minutes on ice in a final volume of 20µl. 1µl of TCA pellet was included in lanes 2 & 3 and 1µl of TCA soluble proteins were included in lanes 5 & 6. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

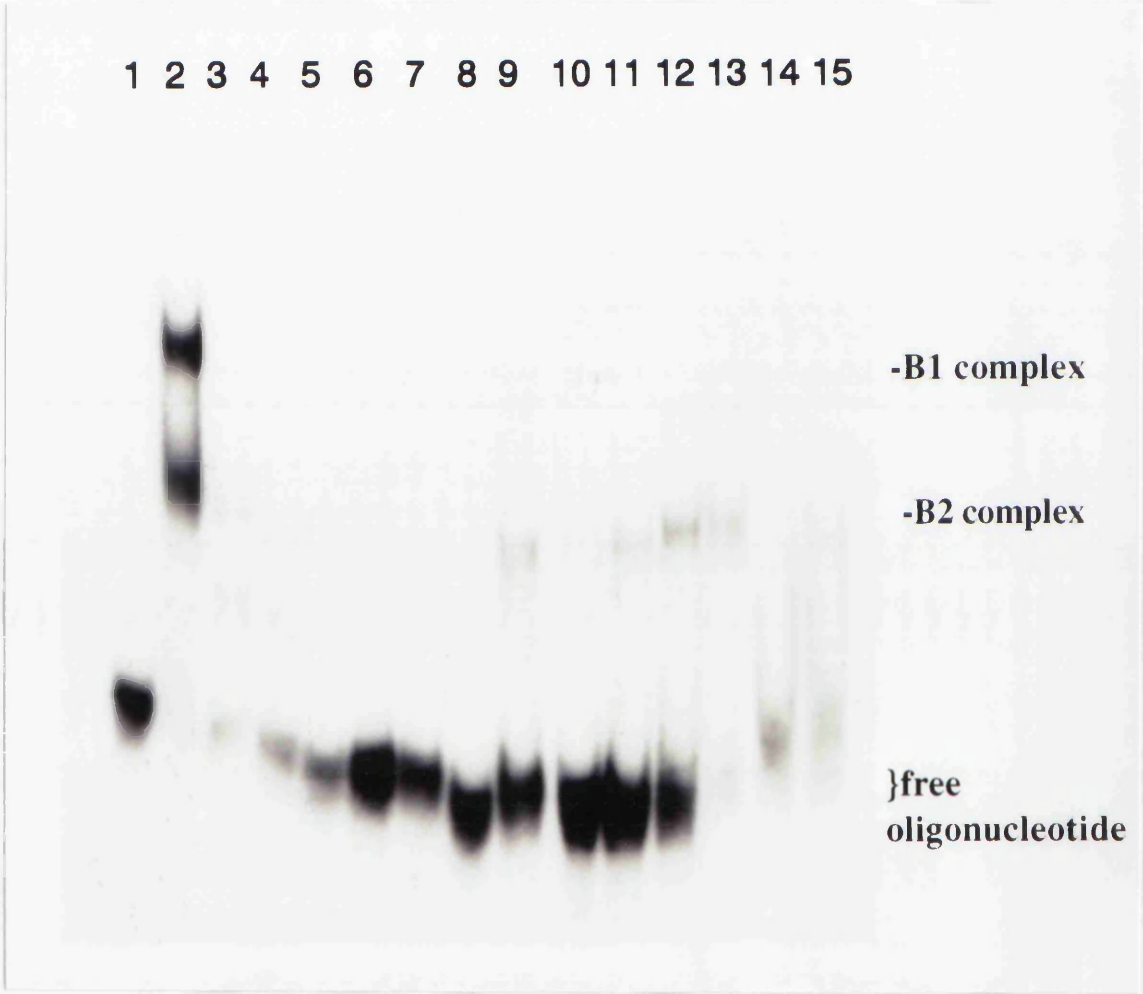
similar mobility (fig. 4.2.2 lane 20) to that of crude nuclear material (fig. 4.2.2 lane 22) and to the anion exchange fraction used as a positive control (lane 19), when these extracts were concomitantly subjected to gel mobility shift analysis. Also one can discern no B1 complex formation by the TCA supernatant. When 4.5μg of this TCA supernatant was loaded and run on a 20% polyacrylamide gel only 2 bands close to the 18KDa and 28KDa size markers were discernible following coomasie and silver staining. TCA treatment of nuclear extract was thus selected as the initial stage in the purification scheme and Ov1PDDP TCA supernatant was subjected to further separation protocols.

4.2.4 USING CHROMATOGRAPHIC TECHNIQUES TO FURTHER PURIFY A DRP FROM OTHER TCA SOLUBLE PROTEINS

The Ov1PDDP TCA supernatant detailed in section 4.2.3 was loaded onto an anion exchange column and proteins were eluted as described in section 2.6.2. Fractions were examined for activity by gel mobility shift assay (fig. 4.2.4). Lane 1 contains platinated oligonucleotide alone, lane 2 contains platinated 2αG7 incubated with crude nuclear Ov1PDDP extract as a positive control and lanes 3-15 contain fractions 1-13. Activity was found to be in lanes 7-13, which when pooled had a specific activity of 7.5ba/mg giving a 64 fold purification compared to crude extract. These 5 pooled fractions were then used for a further purification strategy, heparin column chromatography.

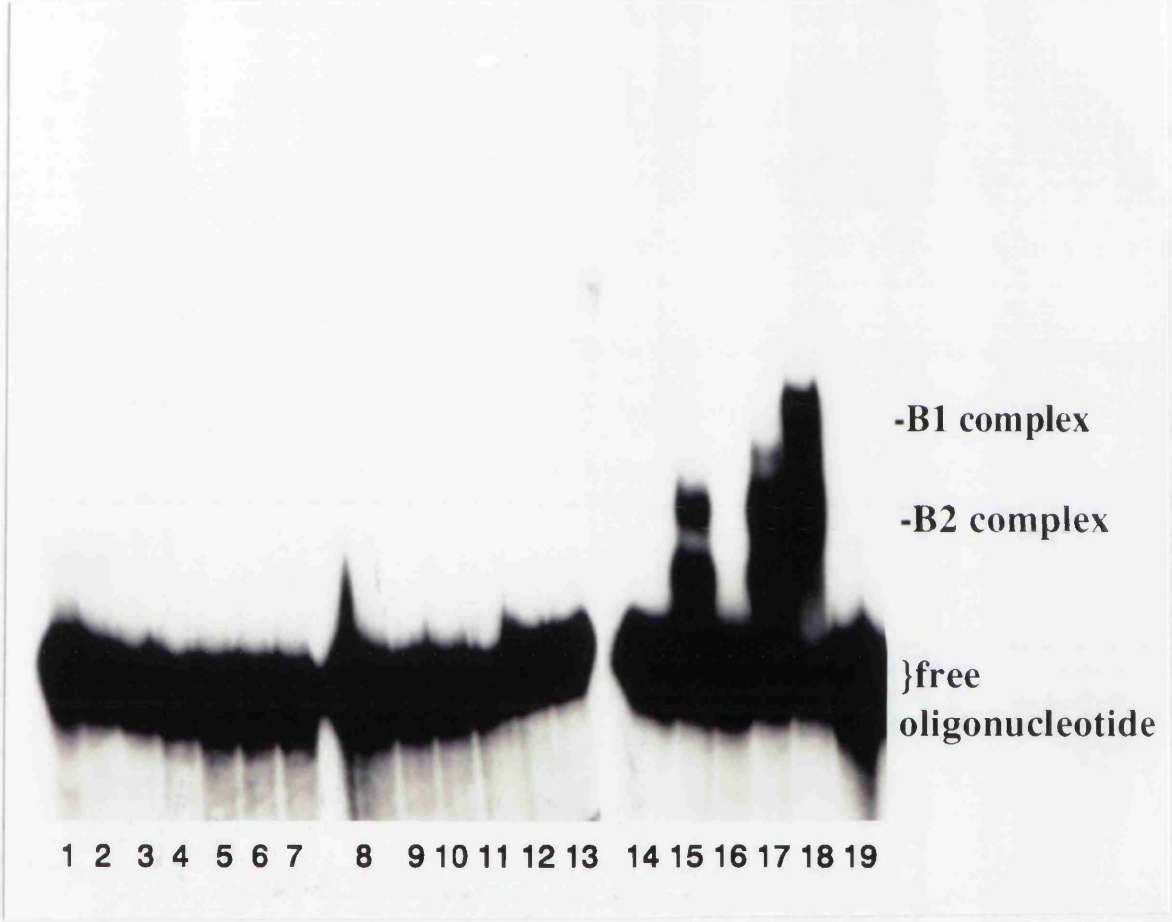
Heparin column separation was performed as recorded in section 2.6.3 using pooled anion exchange fractions of an Ov1PDDP nuclear TCA supernatant. 16 fractions were collected and analysed by gel mobility shift assay (fig. 4.2.5 lanes 1-16) using an extract-free incubation as a negative control (lane 19), also positive controls of Ov1PDDP TCA supernatant (lane 17) and crude nuclear extract (lane 18). Specific activity was 21.2ba/mg thus use of this scheme resulted in a 183 fold purification.

FIGURE 4.2.4 DRP Activity In TCA Treated Anion Exchange Fractions



The proteins in 17.2mg of crude Ov1PDDP nuclear extract (section 2.2.3) were treated with 2% TCA (section 2.6.1) and TCA soluble proteins separated out by spinning in a microfuge. Proteins were further separated using a gradient of 100% buffer A (0.1M NaCl, 0.02M Tris -pH 7.6) to 100% buffer B (2M NaCl, 0.02M Tris-pH 7.6) on a MonoQ^{TM} (Pharmacia) anion exchange column (section 2.6.2). Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated for 30 minutes on ice in a final volume of 20µl. Lane 1 was loaded with a negative control which contained no cell extract, 3µl of Ov1PDDP nuclear extract was included in a positive control reaction (lane 2) and 3µl of fractions 1-13 were included in lanes 3-15. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

FIGURE 4.2.5 DRP Activity In Fractions Which Have Been TCA Treated Then Purified By Anion Exchange And Heparin Column Chromatography



Anion exchange fractions of 2% TCA soluble, Ov1PDDP nuclear proteins, which had DRP activity (figure 4.2.4) were separated further by heparin-sepharose column (Pharmacia) chromatography (section 2.6.3) using a gradient of 100% buffer A (0.1M NaCl, 0.02M Tris -pH 7.6) to 100% buffer B (2M NaCl, 0.02M Tris (pH 7.6). Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated on ice for 30 minutes in a final volume of 20µl. 5 µl of fractions 1-16 were included in reactions loaded on lanes 1-16 respectively. Also, as positive controls 1µl of 2% TCA soluble, Ov1PDDP nuclear extract and 1µl of crude nuclear extract were included in a reactions (lane 17 and lane 18 respectively). Lane 19 was a negative control lane containing no cell extract. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

TABLE 4.2.1 Partial Purification of A DRP

Purification Stage	Total Activity units (ba) defined below	Protein Concentration (mg/ml)	Protein Content (mg)	Specific Activity (ba/mg)	Fold Purification
crude nuclear	2	8.6	17.2	0.116	1
TCA supernatant	1.91	0.15	0.3	6.4	55
anion exchange	1.49	0.02	0.2	7.5	64
heparin column	0.38	0.018	0.018	21.2	183

4.3 CHARACTERISATION OF A DAMAGE RECOGNITION PROTEIN (DRP)

4.3.1 ANALYSING THE SPECIFICITY OF THE B2 COMPLEX-FORMING ACTIVITY FOR CISPLATIN DAMAGED DNA

It was important to know that the activity responsible for the B2 band was specific for cisplatin-damaged DNA rather than being a more general DNA binding protein as this affects how relevant the activity is to cisplatin resistance. To confirm specificity, partially purified DRP activity obtained by HPLC separation of TCA soluble proteins (see section 4.3.2 for details) were examined by inclusion of competitor oligonucleotides in a gel mobility shift assay (section 2.8).

The results of this assay are presented in figure 4.3.1. Lane 1 contains platinated 2αG7 incubated with a known B2 complex-forming activity as a positive control. Lanes 2-21 were all loaded with 1μl of pooled positive HPLC fractions from a TCA soluble portion of an Ov1PDDP nuclear extract. No competitor was included in lanes 2,3 and 15 and so the retarded band observed in these lanes represents the full B2 complex-forming capacity of this semi-pure extract. Either 100ng, 500ng or 5μg of "cold" calf thymus DNA were included in the incubations loaded on lanes 4, 5 and 6 respectively. The B2 band seen in each of these lanes does not appear to be diminished by the presence of calf thymus DNA . This indicates that the DNA binding activity has a preference for the cisplatin treated, radiolabelled DNA as opposed to the "cold" DNA without damage. Lanes 7, 8 and 9 were loaded with incubations containing 100ng, 500ng or 5μg respectively of "cold", UV treated DNA. Some reduction of the B2 band can be detected in lane 9 indicating that B2 forming activity also has some degree of affinity for UV mediated DNA damage.

This competition is not nearly so dramatic as that seen on inclusion of cisplatin-treated, "cold" calf thymus DNA. Although 2ng of this (lane 10)

FIGURE 4.3.1 Specificity Of Semi-Pure DRP Activity For Cisplatin-Damaged DNA (legend overleaf)

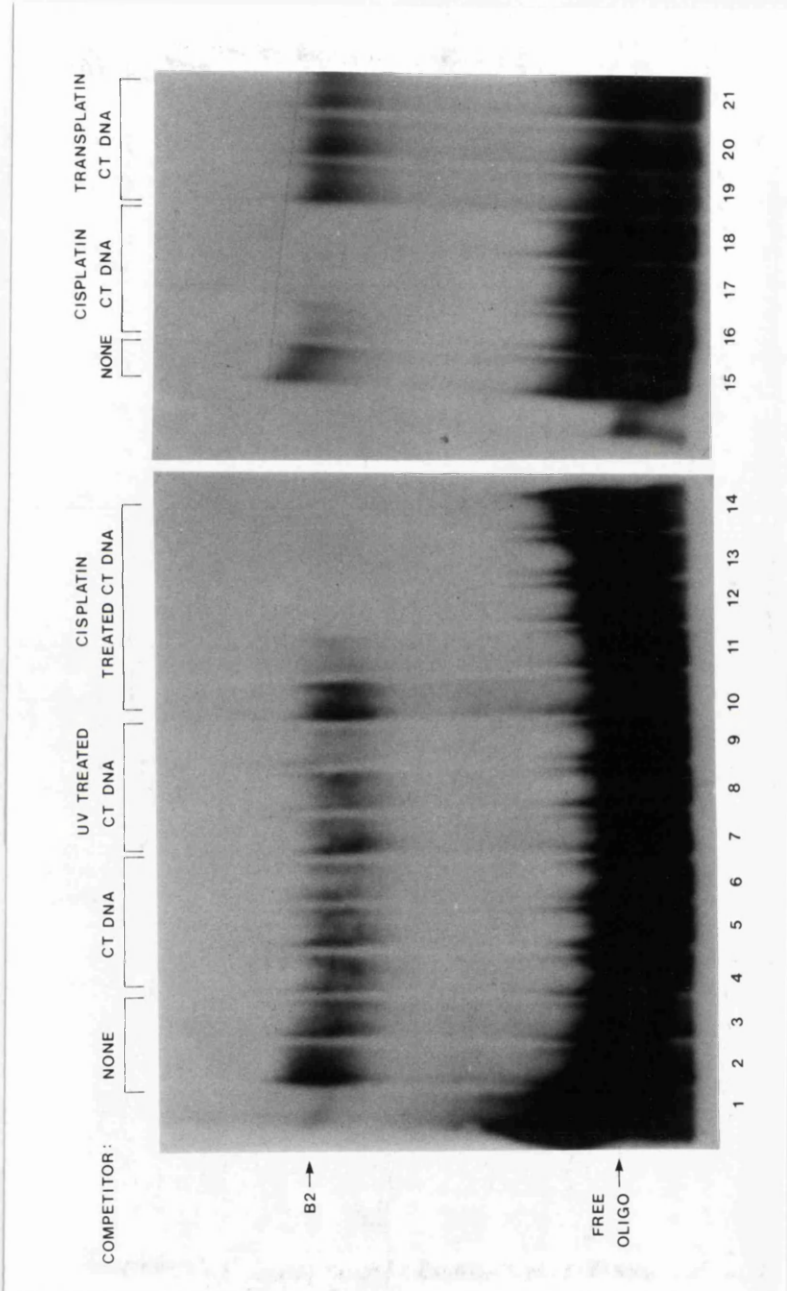


FIGURE 4.3.1 Specificity Of Semi-Pure DRP Activity For Cisplatin-Damaged DNA

2% TCA soluble, Ov1PDDP, nuclear proteins were separated by reverse phase HPLC (section 2.6.4) on a Biorad Hipore butyl C_4 silica-based column (30nm pore, 4.6x250mm dimensions using a Waters HPLC unit (Millipore, Herts). A zinc lamp with a 214nm filter was used for monitoring. A gradient was set up of 15%-50% then 50%-100% buffer B (0.1% trifluoroacetic acid in acetonitrile:H_2O at a v/v ratio of 95:5 -pH 2.11) was set up. Buffer A was 0.1% trifluoroacetic acid in H_2O (pH 0.5). Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated on ice for 30 minutes in a final volume of 20µl. HPLC fractions with DRP activity were pooled and 1µl included per reaction (lanes 2-21). An anion exchange fraction was included as a positive control (lane 1). Competitors were 100ng (lane 4), 500ng (lane 5) or 5µg (lane 6) of calf thymus (CT) DNA, 100ng (lane 7), 500ng (lane 8) or 5µg (lane 9) of UV-treated CT DNA, 2ng (lane 10), 20ng (lanes 11 & 16), 100ng (lane 12 & 17), 500ng (lanes 13 & 18) or 5µg (lane 14) of cisplatin-treated CT DNA and 20ng (lane 19), 100ng (lane 20) or 500 ng (lane 21) of transplatin-treated CT DNA. No competitor was included in reactions loaded in lanes 1-3 or 15. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film. **This work was carried out in collaboration with Dr Donald Bisset, CRC Dept. of Medical Oncology.**

produces no marked effect on the B2 band intensity, when 20ng of cisplatin-damaged DNA are included (lanes 11 and 16), there is a sharp decrease in B2 forming activity. When 100ng (lanes 12 and 17), 500ng (lanes 13 and 18) or 5μg (lane 14) of "cold", cisplatin-damaged DNA are used as competitor, no apparent B2 complex can be discerned which suggests that virtually all the damage recognition activity is competed out by this DNA due to its higher concentration in these incubations. DNA treated with the trans isomer is ineffective as a competitor (lanes 19-21) demonstrating how specific this affinity is for cisplatin-induced DNA damage. Thus the B2 complex forming, damage recognition protein shows a preference for cisplatin-treated DNA rather than untreated, transplatin-treated or UV-treated DNA.

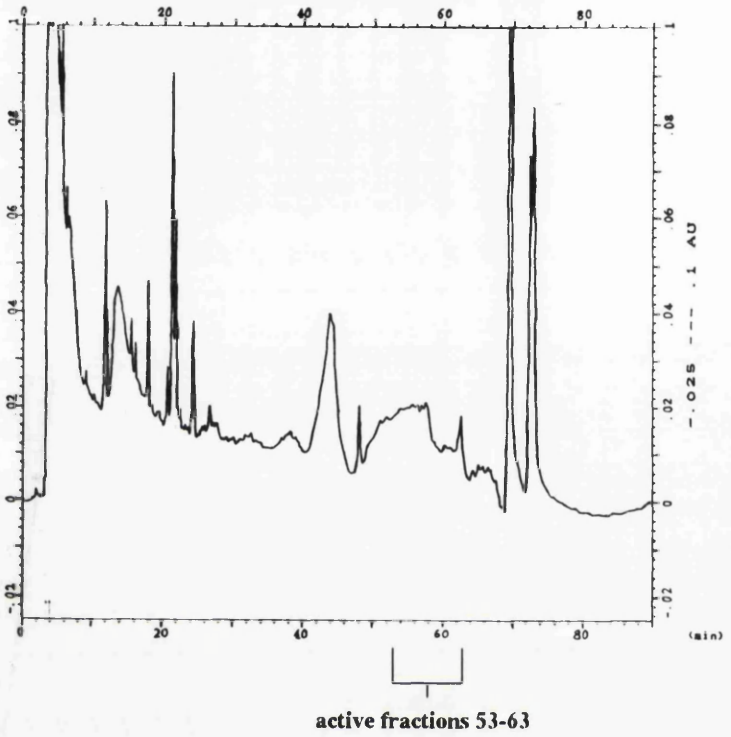
4.3.2 IS THE B2 COMPLEX-FORMING DAMAGE RECOGNITION PROTEIN A MEMBER OF THE HMG CLASS OF PROTEINS?

As discussed in section 4.1, there are certain similarities between the damage recognition protein responsible for the B2 complex seen in gel mobility shift assays and members of the HMG class of proteins. Reverse phase HPLC can be used to separate out HMG proteins from each other (Elton & Reeves., 1986). Therefore this seemed to be an appropriate point to begin analysis into the identity of the B2 complex-forming protein.

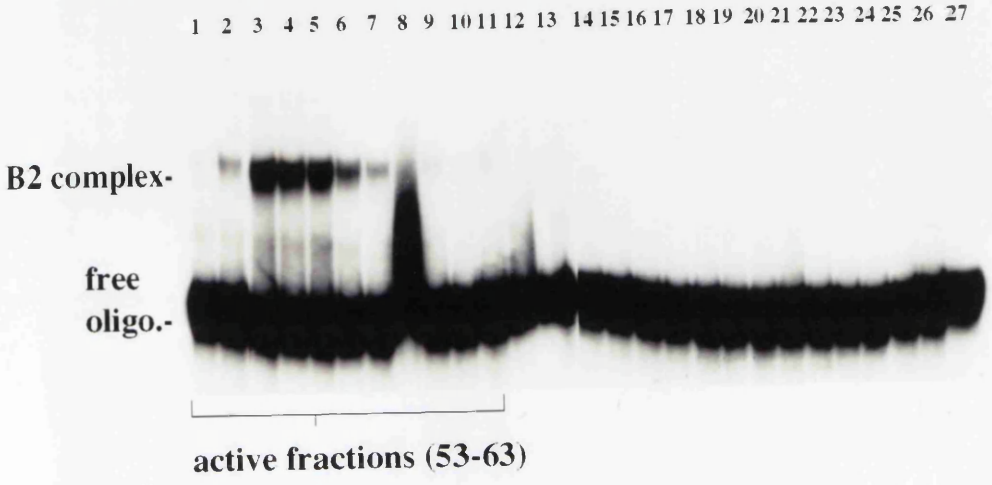
HPLC separation of 37.3μg of TCA soluble proteins from an Ov1PDDP nuclear extract was done as described in section 2.6.4 and fractions were assayed for cisplatin-DNA damage affinity by gel mobility shift analysis as detailed in chapter 2.8. The elution profile is given in figure 4.3.2a and the result of the subsequent assay of fractions is shown in figure 4.3.2b. The cisplatin-DNA damage recognition activity seen was presumed to be identical to that found in the starting material. However a more objective investigation into this activity was given in figure 4.3.1.

FIGURE 4.3.2 HPLC Separation of TCA Soluble Proteins (legend overleaf)

a) HPLC Trace of a TCA Supernatant Prepared from an Ov1P/DDP Nuclear Extract



b) Gel Retardation Analysis of the Ov1p/DDP Fractions Obtained by HPLC



HPLC Separation of TCA Soluble Proteins

TCA soluble, Ov1PDDP, nuclear proteins were separated by reverse phase HPLC (section 2.6.4) on a Biorad Hipore butyl C_4 silica-based column using a Waters HPLC unit (Millipore, Herts.). Monitoring was carried out using a zinc lamp with a 214nm filter. A gradient was set up of 15%-50% then 50%-100% buffer B (0.1% trifluoroacetic acid in acetonitrile:H_2O at a v/v ratio of 95:5 -pH 2.11) was set up. Buffer A was 0.1% trifluoroacetic acid in H_2O (pH 0.5). The trace obtained is shown (a). Gel mobility shift analysis was carried out (section 2.8): 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated for 30 minutes on ice in a final volume of 20µl. Fractions 53-79 (10µl of one fraction/reaction) were included in reactions (lanes 1-27 respectively). Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film (b).

From comparison to similar HPLC traces, the 2 large peaks seen between fractions 58 and 66 of the elution profile bore some resemblance to HMG1 and HMG2 peaks whereas the region representing DRP activity (fractions 53-63) had a similar appearance to a HMGI peak. This indicated that the DRP under investigation might be identical to HMGI.

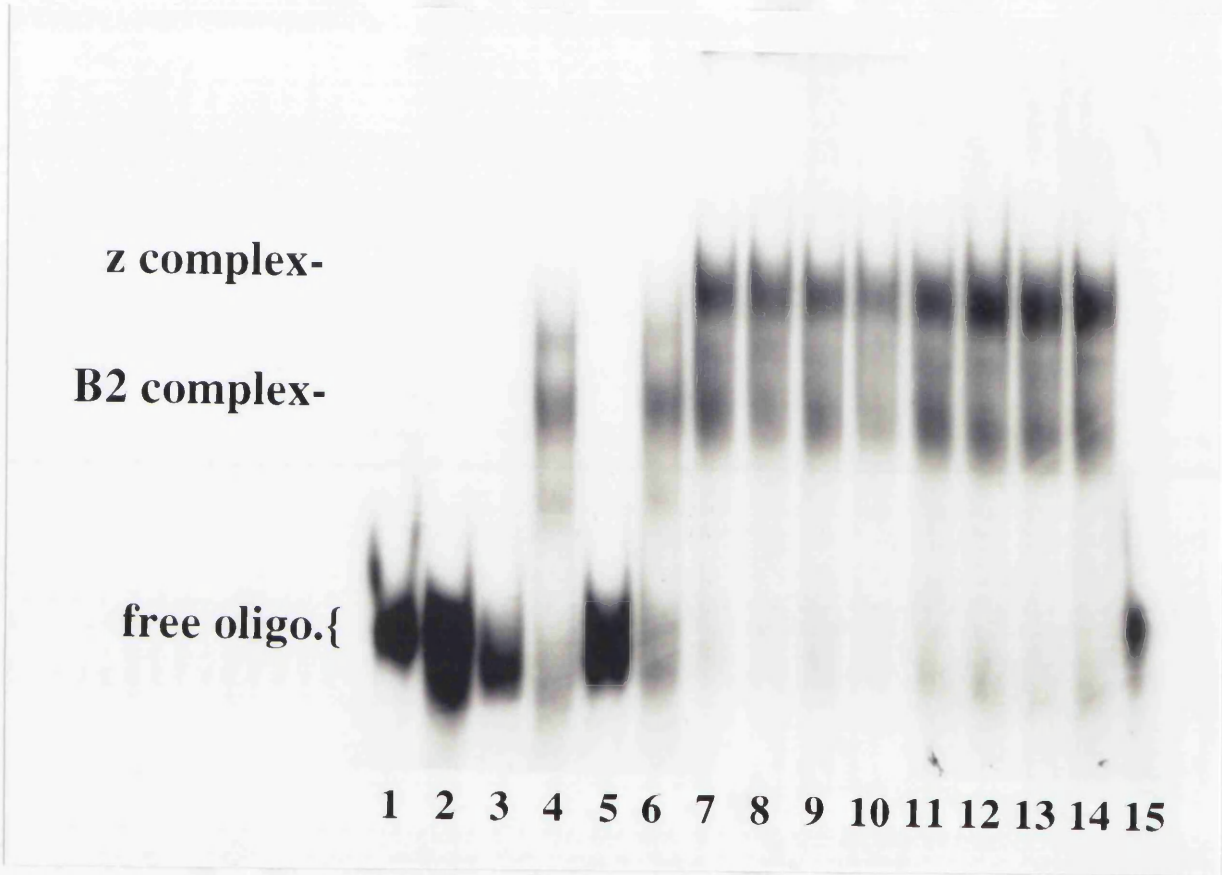
4.3.3 IS HMGI RESPONSIBLE FOR THE B2 COMPLEX DETECTED BY GEL MOBILITY SHIFT ANALYSIS?

Due to the result obtained in the previous section, it seemed reasonable to further investigate whether the B2 complex was caused by HMGI binding to cisplatin-damaged DNA. In order to do this, anti-HMGI antibodies were used to examine whether they could bind to the complex, reducing its mobility and causing a supershift of the complex to a region further up on the gel.

Prior to gel mobility shift analysis (chapter 2.8), 1.5μg of TCA soluble proteins (per lane) were incubated for 30 minutes either alone, with preimmune serum or with anti-HMGI antibody then for a further 30 minutes with protein A. Also 500ng of recombinant HMGI were used in a gel mobility shift incubation. The products of these incubations were loaded and run on a gel (section 2.8.2) and results of this are shown in figure 4.3.3.

Lanes 1, 2 and 3 were negative control lanes containing unplatinated, platinated 2αG7 DNA or unplatinated 2αG7 incubated with TCA soluble proteins respectively. Lane 4 contained 1.5μg of TCA supernatant which had been incubated with platinated 2aG7, giving rise to a B2 band, as a positive control. Lanes 5 and 6 were essentially the same as lanes 3 and 4 but with the inclusion of protein A which made no difference to the number or intensity of any bands observed. Lanes 7-14 all contained 1.5μg of TCA soluble nuclear proteins incubated with platinated 2αG7. Of these, lanes 11 and 13 also contained preimmune serum, lanes 7 and 9 contained preimmune serum and

FIGURE 4.3.3 HMGI Does Not Bind To Cisplatin-Damaged DNA (1)



1.5µg/lane of 2% TCA soluble, Ov1PDDP, nuclear proteins were incubated alone (lanes 3 & 4), with 25µl of protein A (Amersham International) for 30 minutes (lanes 5 & 6), with 2µl of either preimmune serum (lanes 11 & 13) or anti-HMGI (lanes 12 & 14) for 30 minutes or with 25µl protein A for 30 minutes followed by a further 30 minutes with either 2µl of preimmune serum (lanes 7 & 9) or 2µl of anti-HMGI (lanes 8 & 10). Gel mobility shift reactions were then set up (section 2.8): 5x 10^3 d.p.m. of 2αG7 either untreated (lanes 1, 3 & 5) or treated with cisplatin (lanes 2, 4 & 6-15) was incubated for 30 minutes in a final volume of 45µl. Also 500ng of HMGI protein in 5µl of TMS buffer was included in a gel mobility shift reaction in the absence of cell extract (lane 15). All incubations were carried out on ice. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film. Anti-HMGI and pre-immune serum were each stored at a 1/500 dilution in PBS, 0.02% sodium azide. All incubations were carried out on ice.

FIGURE 4.3.4 HMGI Does Not Bind To Cisplatin-Damaged DNA (2)

0.8μg/lane of 2% TCA soluble, Ov1PDDP, nuclear proteins were incubated alone (lanes 3 & 4), with 1μl of preimmune serum (lane 5) or with 1μl (lane 6), 0.1μl (lane 7), 0.01μl (lane 8) or 0.001μl (lane 9) of anti-HMGI for 30 minutes. Gel mobility shift reactions were then set up (section 2.8): 5x 10^3 d.p.m. of 2αG7 either untreated (lanes 1, 3 & 10) or treated with cisplatin (lanes 2, 4-9 & 11-13) was incubated for 30 minutes in a final volume of 20μl. Also 500ng of HMGI protein/reaction was included in gel mobility shift reactions without cell extract and either without antibody (lanes 10 & 11), with 1μl preimmune serum (lane 12) or with 1μl anti-HMGI (lane 13). All incubations were carried out on ice. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film. Anti-HMGI and pre-immune serum were each stored at a 1/500 dilution in PBS, 0.02% sodium azide.

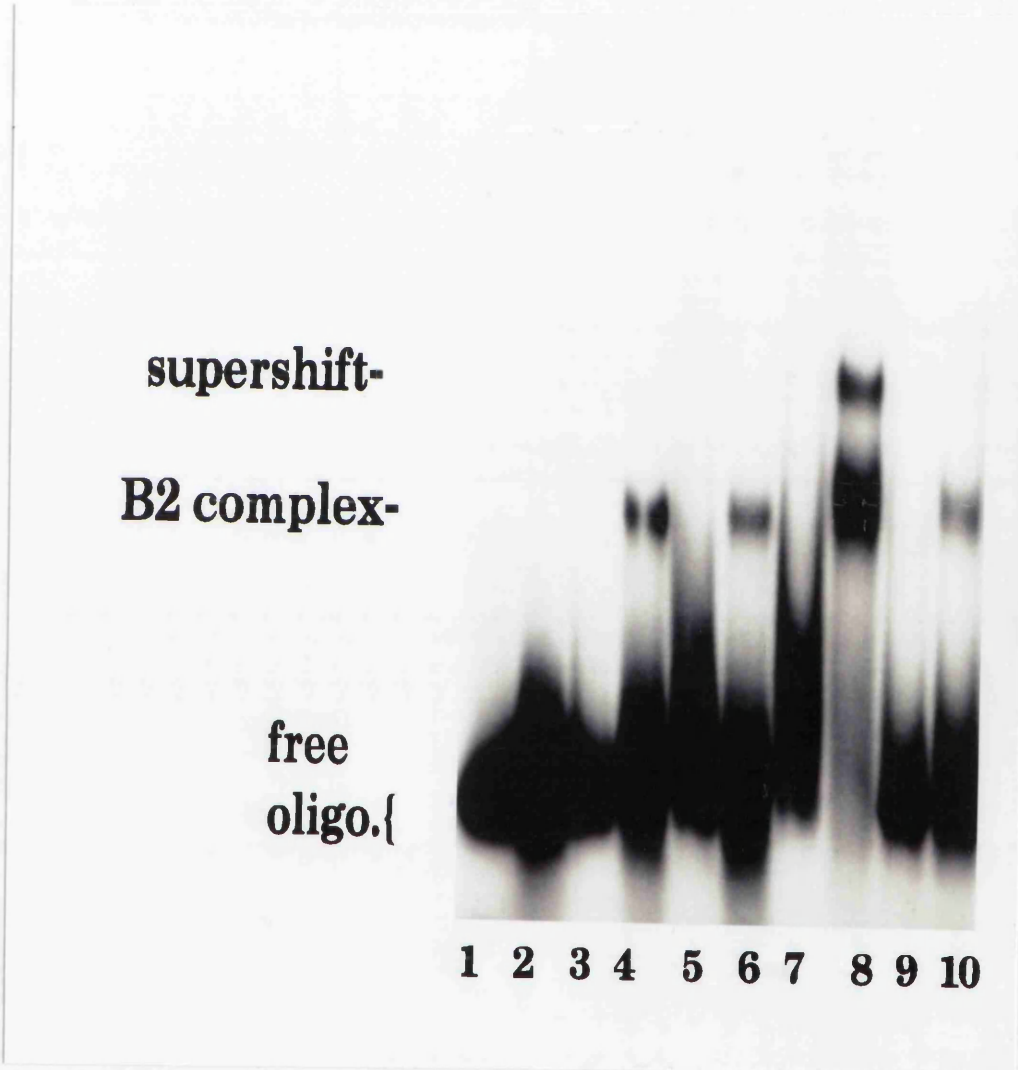
protein A, lanes 12 and 14 contained anti-HMGI and lanes 8 and 10 contained anti-HMGI along with protein A. There was no difference in the number of retarded bands seen in any of these lanes, although they all possessed a band not seen for TCA supernatant alone (lane 4). This band has been designated the "z complex". As there was no difference in the number of complexes seen for incubations including preimmune serum or anti-HMGI, as the B2 complex is not diminished in any of these lanes and as recombinant HMGI alone is unable to retard the platinated oligonucleotide (lane 15), one must conclude that the B2 complex does not involve HMGI.

The results of a similar experiment are depicted in figure 4.3.4. Again no shift of 2αG7 oligonucleotide DNA was detected for negative control lanes (1-3) but a B2 complex was seen for the positive control, lane 4, which contained an incubation of 0.75μg of TCA soluble proteins with platinated 2αG7. When preimmune serum (lane 5) or anti-HMGI (lane 6) were incubated with TCA supernatant before addition of 2αG7, the z complex was seen again and this disappeared on dilution of anti-HMGI (lanes 7-9). Recombinant HMGI was examined for its capacity to retard platinated oligonucleotide by incubation with unplatinated 2αG7 (lane 10), platinated 2αG7 (lane 11), platinated 2αG7 and preimmune serum (lane 12) or platinated 2αG7 and anti-HMGI (lane 13). Although the entire free oligonucleotide band seems to have been shifted upwards in each of these lanes, it is streaky, forms no discrete band indicating no specific affinity and there is no evidence for recombinant HMGI being capable of forming a B2 complex. Thus HMGI does not seem to be the protein involved in B2 complex formation.

4.3.4 HMG1 IS INVOLVED IN FORMATION OF THE B2 COMPLEX

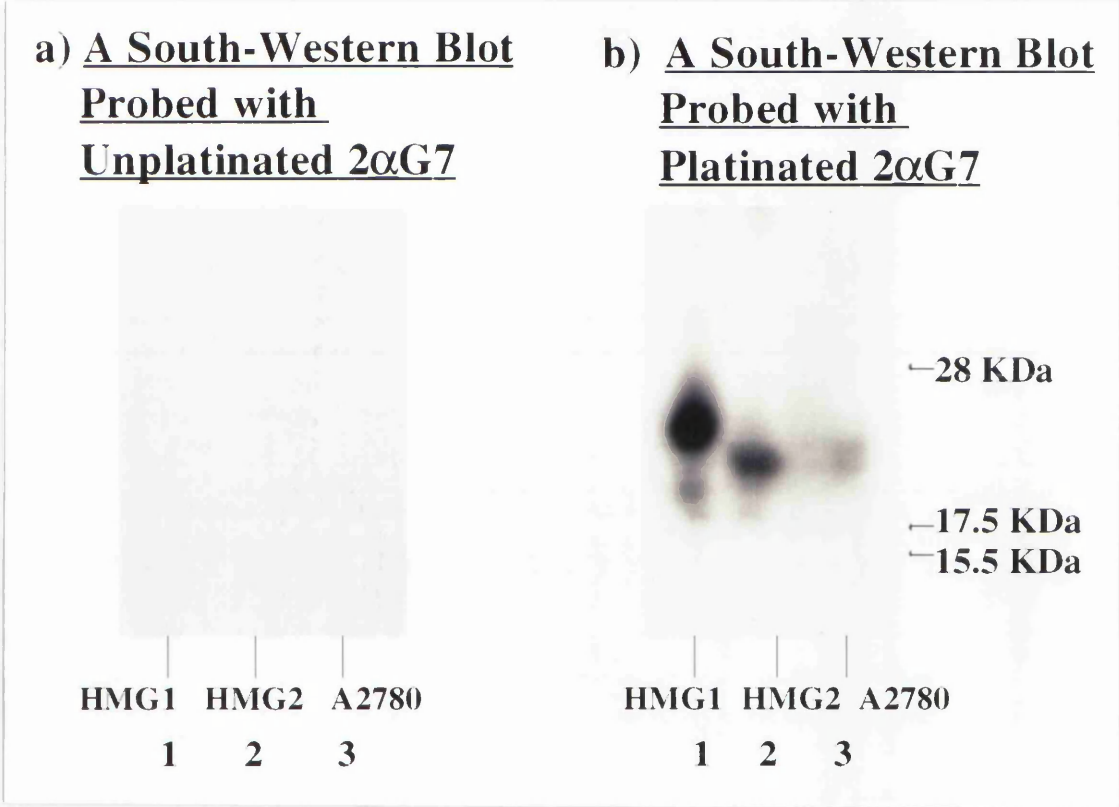
To examine whether HMG1 might be responsible for the B2 complex which one detects with the gel mobility shift assay, a similar experiment to

FIGURE 4.3.5 HMG1 Binds To Cisplatin-Damaged DNA



0.75µg/lane of 2% TCA soluble, Ov1PDDP, nuclear proteins were incubated with 1µl of anti-HMGI for 30 minutes on ice before addition of radiolabelled DNA (lanes 7 & 8). Gel mobility shift reactions were then set up (section 2.8): 5x 10^3 d.p.m. of 2αG7 either untreated (lanes 1, 3, 5, 7 & 9) or treated with cisplatin (lanes 2, 4 , 6, 8 & 10) was incubated for 30 minutes in a final volume of 20µl. 0.75mg of 2% TCA soluble nuclear extract was included in reactions without antibody (lanes 3 & 4) and with 1µl anti-HMG1 added at the same time as DNA (lanes 5, 6). Also 10µg of HMG1 protein was included in gel mobility shift reactions (lanes 9 & 10). All incubations were carried out on ice. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

FIGURE 4.3.6



South-western blotting (section 2.5.6) was carried out on 1μg of HMG1 (lane 1), 1μg of HMG2 (lane 2) and 50 μg of A2780 nuclear extract (lane 3): Proteins were separated on a 5-15% polyacrylamide gradient gel and transferred to a nitrocellulose filter which was probed with $2x10^4$ d.p.m/ml of either untreated (a) or cisplatin-treated 2αG7 (b). Protein sizes were estimated using prestained molecular weight standards, BRL.

those described in section 4.3.3 was carried out using an anti-HMG1 antibody and recombinant HMG1. Results of this are presented in figure 4.3.5. Lanes 1, 2 and 3 are negative control lanes loaded with unplatinated 2αG7, platinated 2αG7 or unplatinated 2αG7 which had been incubated with 0.75μg of TCA soluble proteins. Lane 4, the positive control lane, was loaded with an incubation of 0.75μg of TCA soluble proteins and platinated 2αG7 and this clearly demonstrates B2 complex formation. When anti-HMG1 was included in the incubation at the same time as 2αG7, no additional complex was detected (lane 6). However, when anti-HMG1 was incubated with the proteins for 30 minutes prior to 2αG7 being added, a supershift of the B2 complex was perceived (lane 8). Also 1μg of recombinant HMG1 was able to retard the platinated 2αG7 (lane 10) but not the unplatinated version (lane 9). This data suggests that HMG1 is the protein responsible for the B2 complex attained by gel mobility shift analysis of nuclear proteins.

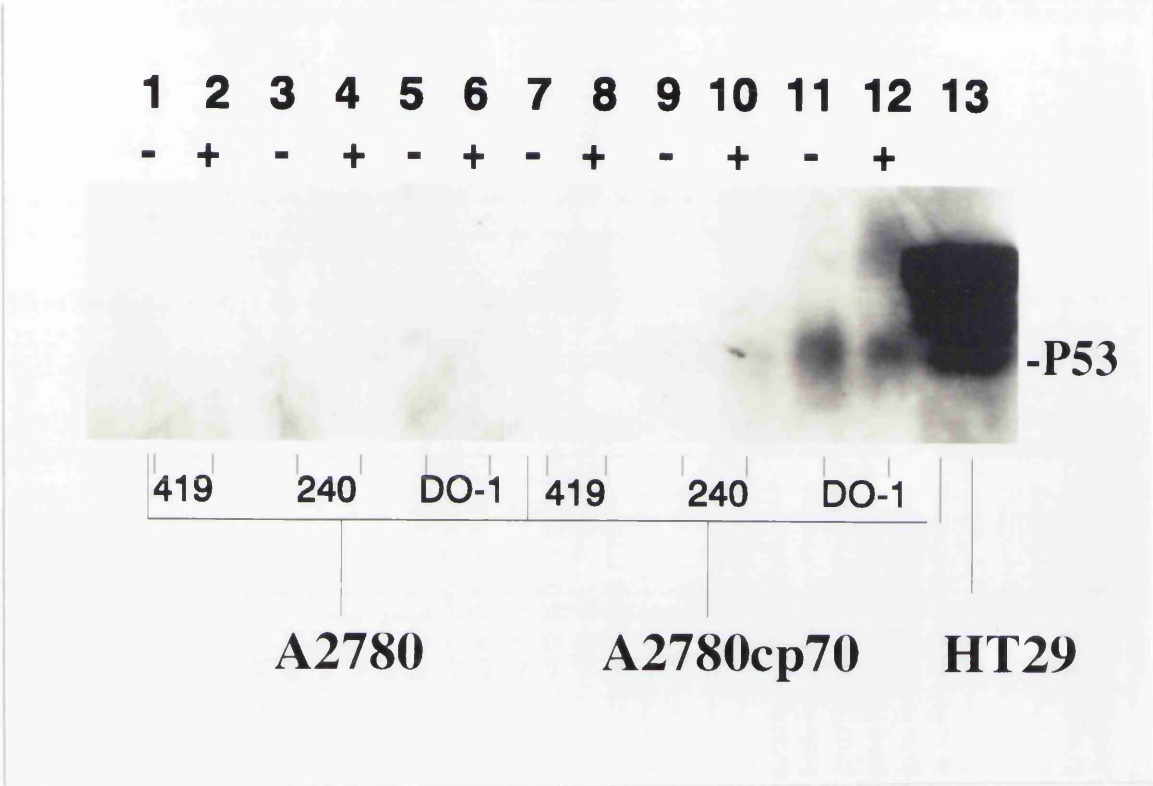
Recombinant HMG1 and HMG2 proteins were also examined for their affinity for platinated DNA by South-western blotting (section 2.5.6). Either 1μg HMG1, 1μg HMG2 or 50μg crude A2780 nuclear extract were electrophoresed through a polyacrylamide gel, blotted onto nitrocellulose and the blot was probed for binding by unplatinated 2αG7 (fig. 4.3.6a) then re-probed with platinated 2αG7. No binding was detected with the unplatinated oligonucleotide but a faint band was seen in the 25KDa region for A2780 extract (lane 3 of fig. 4.3.6b) and a more intense band observed for HMG1 (lane 1) and HMG2 (lane 2). The HMG1 band has slightly slower mobility than the HMG2 band or the band in the A2780 lane but small differences in mobility between nuclear extract proteins and recombinant proteins could be accounted for by post translational modification. This data suggests that HMG1 and HMG2 are able to bind specifically to platinated DNA under denaturing conditions.

4.3.5 AN INVESTIGATION INTO WHETHER p53 HAS AFFINITY FOR CISPLATIN-INDUCED DNA DAMAGE

In order to examine whether p53 or proteins which bind to p53 have affinity for cisplatin-damaged DNA, immunoprecipitated p53 protein was analysed for DNA damage recognition capacity. Also in the hope of co-precipitating any proteins responsible for transducing the DNA damage signal to p53 protein and thus causing induction of p53 protein levels, lysis was carried out with and without a prior cisplatin treatment. Semi-confluent A2780 or A2780cp70 cells were lysed with high salt lysis buffer in 175cm^2 flasks immediately after a 1hour, 1μM cisplatin dose or a medium change in the case of control cells. Lysates were split into 3 equal portions and each of these was used for immunoprecipitation reactions (section 2.6.5) with one of the following antibodies, PAb419 (a control antibody), PAb240 (reactive with mutant p53 protein) or PAb DO-1 (reactive against both mutant and wild-type p53 protein).

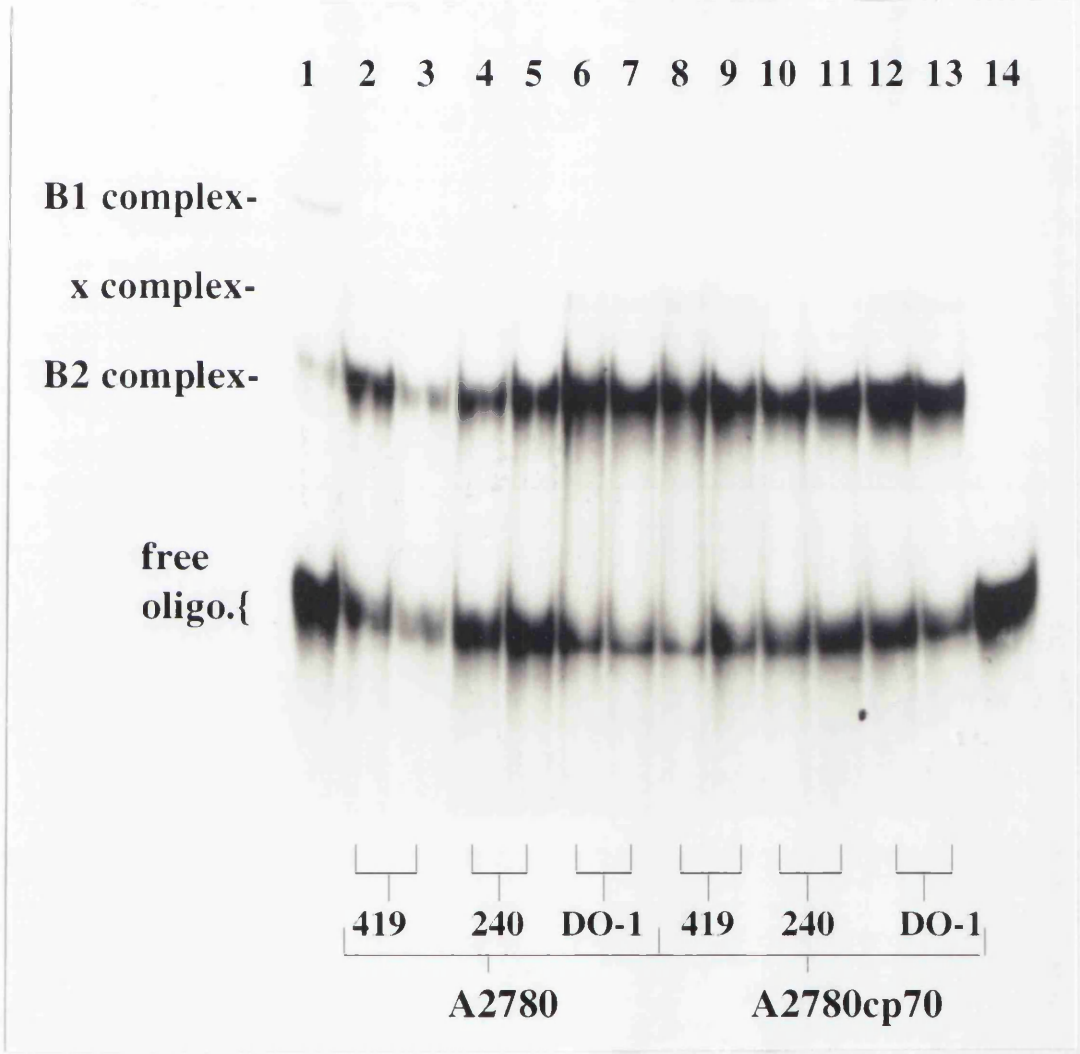
Immunoprecipitated proteins were separated and examined by Western blotting as described in chapter 2.5. The results of this are shown in figure 4.3.7. No p53 immunoreactivity was detected for A2780 cells (lanes 1-6). This was expected as A2780 has very low levels of p53 protein. No p53 was detected for A2780cp70 proteins immunoprecipitated with the control antibody (lanes 7 and 8). Some p53 protein was precipitated from A2780cp70 cells by the mutant specific antibody (lanes 9 and 10) but not as much as with the pantropic DO-1 antibody (lanes 11 and 12). There was no difference between levels of p53 protein precipitated from cisplatin untreated and treated cells (lane 9 compared to lane 10 and lane 11 compared to lane 12 respectively) as p53 protein levels had not been given sufficient time to respond to the DNA damage. Thus detectable levels of p53 protein were precipitated from A2780cp70 cells by PAb 240 and PAb DO-1 but not PAb419 or from the A2780 cell line.

FIGURE 4.3.7 Immunoprecipitation Of p53 Protein



Immunoprecipitation (section 2.6.5) was performed on cells which had been given a 1μM, 1 hour cisplatin dose an hour beforehand (lanes 2, 4, 6, 8, 10 & 12) or control cells (lanes 1, 3, 5, 7, 9 & 11) followed by separation of proteins on an 8% polyacrylamide gel. Western blotting (section 2.5) of immunoprecipitated p53 protein from A2780 (lanes 1-6), A2780cp70 (lanes 6-12) and 100μg of crude cell lysate from HT29 cells (lane 13) as a positive control was carried out. Extracts were immunoprecipitated with the antibodies PAb DO-1 (lanes 5, 6, 11 & 12), PAb 240 (lanes 3, 4, 9 & 10) and PAb 419 (lanes 1, 2, 7 & 8). PAb DO-1 is a pantropic anti-p53 antibody, PAb 240 is specific for mutant p53 and PAb 419 is an irrelevant control antibody. For Western blotting, the primary antibody was CM1 at a 1/200 dilution and the secondary antibody was anti rabbit Ig horseradish peroxidase from Dako, Aylesbury, at a 1/150 dilution. Protein sizes were determined by comparison to prestained size markers from BRL.

FIGURE 4.3.8 DRP Activity Of p53 Immunoprecipitates



Gel mobility shift analysis (section 2.8) was carried out: 5x 10^3 d.p.m. of 2αG7 treated with cisplatin was incubated for 30 minutes in a final volume of 20µl. 8µg of Ov1P nuclear extract was included as a positive control (lane 1). Immunoprecipitated proteins from A2780 (lanes 2-7) and A2780cp70 (lanes 8-13) cells were included. A 1 hour, 1µM cisplatin dose had been given to certain cells, 1 hour before immunoprecipitation (lanes 3, 5, 7, 9, 11 & 13). Extracts were immunoprecipitated with PAb 419 (lanes 2, 3, 8 & 9), PAb240 (lanes 4, 5, 10 & 11) or PAb DO-1 (lanes 6, 7, 12 & 13). All incubations were carried out on ice. Reaction products were separated out by non-denaturing polyacrylamide gel electrophoresis. The gel was dried and exposed to photographic film.

The immunoprecipitated proteins were then assessed for the ability to bind platinated 2αG7 DNA by gel mobility shift assay (section 2.8). The results of this experiment are depicted in figure 4.3.8. Lane 1 contained the positive control, an incubation of a crude nuclear Ov1P extract with known DRP activity and platinated 2αG7 oligonucleotide. Lane 14 contained the platinated oligonucleotide alone as a negative control. All of the immunoprecipitates obtained contained a DRP with similar mobility to the B2 complex and another DRP activity designated the "x complex". These proteins have probably been precipitated as background proteins, due to their relative abundance, by binding to the G protein used in the immunoprecipitation protocol. No difference in DRP bands can be discerned between those precipitates which showed p53 protein immunoreactivity (lanes 10-13) and those which did not (lanes 2-10). Therefore this experiment provides no evidence for a cisplatin-DNA damage recognition capacity inherent to p53 protein or any proteins tightly complexed to p53 protein.

4.4 DISCUSSION

4.4.1 PURIFICATION OF A DNA DAMAGE RECOGNITION PROTEIN

Progress was made in developing a purification scheme for separating the protein responsible for the B2 complex seen on gel mobility shift analysis, from other nuclear components. TCA treatment was the most efficient stage, giving a 55 fold purification fold with respect to crude nuclear extract. The subsequent anion exchange chromatography and heparin column chromatography gave a final 183 fold purification compared to crude, nuclear extract. A heparin column was used as it is thought to mimic DNA in shape and should therefore be relevant to purification of a DNA binding protein. In retrospect, the use of reverse phase HPLC and custom made cisplatin-damaged DNA affinity columns may have been worth further investigation as possible purification stages. However, once the protein of interest had been identified as a HMG protein, purification was no longer a priority. Partially purified activity retains specificity for cisplatin damaged DNA as shown by competition gel mobility shift assay. At times faint bands could be observed either just above or below the main B2 complex. These may be other DRPs which are not usually detected due to their low intracellular concentration, degradation products or multimers of the major B2 forming protein or bands formed when oligonucleotide is bound by the protein and released as it progresses through the gel.

4.4.2 THE DISCOVERY THAT THE B2 COMPLEX-FORMING ACTIVITY IS A HMG PROTEIN

TCA treatment followed by reverse phase HPLC indicated that HMG 1, 2, 14 and 17 were unlikely candidates for the DRP involved in B2 complex formation, from comparison with other HPLC traces of HMG proteins. However this can only be used as a rough guide-line rather than firm evidence as different cell lines will obviously give varying HPLC traces. Antibodies

specific for a particular HMG protein were utilised to show that the protein was a genuine HMG protein. Using an anti HMGI antibody in the gel mobility shift assay did not result in a supershift of the B2 complex. Therefore this antibody did not recognise the protein involved. An extra band was seen on inclusion of either preimmune or immune serum and this was presumed to represent another DRP native to the preimmune serum rather than a supershift of the B2 complex.

An antibody which recognises HMG1 however, was able to give a supershift of the B2 complex when included in a gel mobility shift assay. As no preimmune serum was available this is not conclusive alone but taken with the fact that recombinant HMG1 protein displayed affinity for cisplatin-damaged DNA this suggests that HMG1 is the protein which causes B2 complex formation. Furthermore HMG1 has been shown to have specific binding activity for 1,2-intrastrand crosslinked d(GpG), d(ApG) adducts and 1,3-d(GpTpG) adducts (Pil & Lippard., 1992). HMG 1 and 2 can be purified using a combination of ion exchange chromatography and cisplatin-damaged DNA affinity chromatography (Hughes et al., 1992) and HMG2 is able to bind DNA damaged by cisplatin, carboplatin or iproplatin (Billings et al., 1992). Also human cDNA clones encoding a protein of predicted MW 81KDa which contains a HMG box comprising 75 amino acids with 47% homology to HMG1 have been isolated (Bruhn et al., 1992). This protein, named SSRP1 recognises cisplatin induced DNA distortions. The murine homologue of SSRP1 is thought to be involved in immunoglobulin recombinational signalling (Shirakata et al., 1991). Other proteins which posses a HMG box and have cisplatin-DNA damage affinity are the 80KDa yeast protein encoded by the *Ixr1* gene (Brown et al., 1993) and the human transcriptional control protein hUBF (Treiber et al., 1994). This latter protein exhibits a similar affinity for cisplatin-damaged DNA as it does for its natural target, the ribosomal RNA promoter. It has been suggested therefore that cisplatin

adducts may titrate hUBF away from its site of action thereby representing another mechanism by which cisplatin mediates cellular toxicity.

HMG1 and 2 are thought to be involved in maintaining chromatin structure but a requirement for these proteins in transcription of class II genes such as trout protamine, human B-globin, adenovirus 2 major late promoter and herpes simplex virus (HSV) thymidine kinase has been demonstrated (Singh et al., 1990). Also by binding to cruciform structures caused by negative supercoiling, HMG1 alters the DNA conformation. This may allow RNA polymerase to continue along its route thereby permitting transcription to occur. Possibly, tolerance of cisplatin could be attained in a similar manner if HMG1 were able to bend cisplatin damaged DNA to resemble undamaged DNA in shape. The ability of both HMG1 and HMG2 to recognise cisplatin-damaged DNA under the denaturing conditions of South-western blotting lends support to the theory that the 25KDa protein observed in human, nuclear extracts using this technique, is also involved in B2 complex formation.

4.4.3 NO CISPLATIN-DRP ACTIVITY CAN BE DISCERNED FOR P53 PROTEIN

Gel mobility shift analysis of immunoprecipitated p53 protein suggests that p53 does not have inherent cisplatin-damage recognition capabilities nor do proteins tightly complexed to p53 protein. If they did, when p53 specific antibodies were used in the gel mobility shift assay, one would expect to see additional bands to those where immunoprecipitates of the irrelevant antibody, PAb419 were used. This does not happen but such findings do not eliminate the possibility that DRPs bind p53 transiently or that several intermediate signal transducers convey the DNA-damage message to p53 protein which then becomes stabilized. Also the gel mobility shift assay may not be sensitive enough to detect proteins with DRP activity that are expressed at a low level.

Chapter 5

CHAPTER 5: GENERAL DISCUSSION

5.1 DNA DAMAGE RESPONSE PATHWAYS AND CISPLATIN SENSITIVITY

5.1.1 DNA DAMAGE RESPONSE IN *E.COLI*

The bacterium, *E.coli* is able to repair DNA damage which could prove toxic, by various means , the best characterized being the "ABC exinuclease". In this response pathway, a dimer of UvrA forms, which then binds to a UvrB molecule and the complex reacts with DNA. UvrA dissociates from the DNA and it has been postulated that the UvrB which is bound to the DNA permits UvrC association (Orren & Sancar, 1989). UvrC nicks the DNA strand and excised nucleotides are then replaced using UvrD, polymerase I and DNA ligase. This response pathway is effective in removal of a variety of DNA adducts including cisplatin induced d(GpG) lesions. Mutant strains of E.coli such as *uvrA6*, *uvrB5* or *uvrC34* which are defective in excision repair of cisplatin-damaged plasmid DNA, are also highly sensitive to cisplatin but not transplatin (Beck et al., 1988). It would seem that DNA damage response is important in determining cisplatin sensitivity in this organism.

5.1.2 DNA DAMAGE RESPONSE IN *S.CEREVISIAE*

Many genes with an involvement in DNA damage repair have been found for the yeast *S.cerevisiae* by the use of mutants which were hypersensitive to UV or ionising radiation. These genes have been categorized into three epistasis groups on the basis of whether double mutants were synergysticaly sensitive. The RAD 3 epistasis group is involved in nucleotide excision repair. The RAD 52 group is responsible for recombinational repair and the RAD 6 group of gene products carry out post-replication repair. For a review of the genes involved in DNA repair in *S. cerevisiae* see Freidberg et

al., 1988. Also in *S.Cerevisiae*, an enzyme known as photolyase exists which catalyses the removal of pyrimidine dimers from the DNA in a light dependent reaction. The gene encoding the apoenzyme, *PHR1*, is induced in response to certain DNA-damaging agents (Sebastian et al., 1990). Also yeast photolyase has been demonstrated to have DRP activity (Patterson & Chu, 1989). Again DNA repair is vital to the survival of this organism after exposure to DNA damaging agents.

5.1.3 DNA DAMAGE RESPONSE IN HIGHER EUKARYOTES

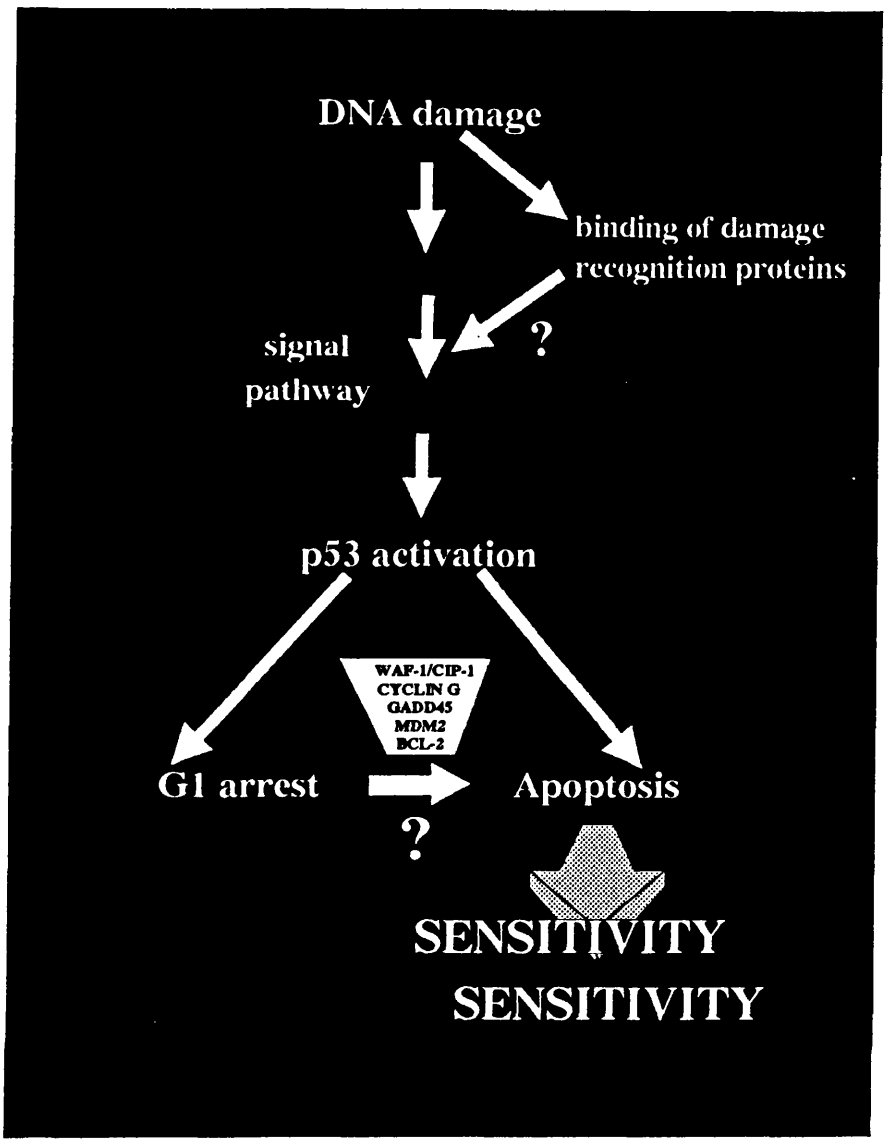
In multicellular organisms such as humans, the survival of individual cells is no longer a priority. Indeed unwarranted cell survival would be detrimental to the health of the organism. Thus DNA damage response pathways in multicellular organisms would probably have additional functions to those of single-celled organisms. As well as repairing DNA damage, it would be beneficial to the organism to have a means of aborting cellular survival in the incidence of excessive damage.

Analysis of diseases such as xeroderma pigmentosum provides some clues concerning the DNA damage response in humans. Extreme sensitivity to UV light, pigmentation abnormalities and a high incidence of cancer are characteristic symptoms of this autosomal, recessive disorder. XP cells are deficient in DNA repair including repair of cisplatin mediated lesions as measured by reactivation of a cisplatin-damaged CAT reporter construct (Chu & Berg, 1987). Cell lines derived from xeroderma pigmentosum patients are also more sensitive to cisplatin than normal human cells (Plooy et al., 1985). Thus sensitivity to cisplatin in eukaryotic cells also correlates with cellular repair capacity and one could extrapolate from this that resistant cells would have particularly efficient DNA repair mechanisms. Enhanced repair has been reported for certain cisplatin resistant cell lines. For instance the cisplatin resistant human, ovarian adenocarcinoma line A2780cp70 cells been shown to

incorporate 2-3 fold more ^{3}H thymidine, a measure of unscheduled DNA synthesis, than their more sensitive parental cell line, A2780 (Lai et al., 1988).

In response to insurmountable DNA damage, mammalian cells are proposed to undergo apoptosis and a possible pathway by which this may occur is represented below in figure 5.1.1. If cells were able to by-pass this pathway in some way, then resistance to DNA-damage inducing agents such as cisplatin would arise.

FIGURE 5.1.1. A Hypothetical DNA Damage Response Pathway



In this mechanism, DNA damage such as that elicited by cisplatin would cause changes in a signal pathway culminating in induction and activation of p53 protein and subsequent p53-dependent apoptosis. The response pathway may involve DRPs in the initial stages of DNA-damage signal transduction. The signal pathway could also have elements in common with repair pathways. DRP binding to DNA could stimulate activation of enzymes or kinases within the cell, which at a low level would enhance repair and once a threshold level were surpassed an apoptotic death programme would be initiated. The products of the AT genes could also be involved in early stages as cells from AT patients show a delayed or reduced induction of p53 protein following DNA damage (Kastan et al., 1992). The final events in the DNA response pathway may be carried out by the products of genes subject to p53-mediated transcriptional control.

5.2. p53, THE DNA DAMAGE RESPONSE PATHWAY AND CISPLATIN RESISTANCE

5.2.1 p53 STABILIZATION FOLLOWING DNA DAMAGE

Induced levels of p53 protein causes cells to arrest in G1 phase of the cell cycle in response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992). This may allow time for DNA repair before the genome is replicated at S phase in cells which are only mildly damaged. It could also allow accumulation of signaling proteins involved in the promotion of apoptosis in severely damaged cells. Perhaps such proteins would be titrated out or destroyed if cell cycling were permitted to continue.

In this thesis, Western blotting data is presented showing an induction of p53 protein in the human, ovarian, adenocarcinoma line A2780 and its cisplatin resitant derivative A2780cp70 by both cisplatin and transplatin. Peak p53 levels were attained 24 hours after drug removal in both cell lines. The

1μM, 1 hour dose of these drugs did not result in less than 70% survival, as determined by clonogenic assay, so was not overly toxic to the cells. Basal levels of p53 protein were 8 fold higher in the resistant line.

High levels of p53 protein are often observed when the *p53* gene is mutated (Bartek et al., 1990). Immunoprecipitation of p53 from A2780cp70 was greater with a pantropic antibody (PAb DO-1) than a mutant specific antibody (PAb 240). This was not the case for another cisplatin resistant human ovarian adenocarcinoma cell line, Ov1PDDP, from which equal levels of p53 were precipitated by the two antibodies. Direct PCR sequencing confirmed that *p53* in the A2780 and A2780cp70 cell lines was wild-type whereas in the Ov1PDDP line and its cisplatin sensitive parental, Ov1P, there was a mutation at codon 126 giving a cystine to tyrosine substitution. Other situations where p53 protein levels are enhanced in the absence of a mutant *p53* gene have been reported. For instance, high p53 protein levels have been detected in the normal tissue of a patient suffering from a familial susceptibility to breast cancer (Barnes et al., 1992). Also unusually high p53 protein concentration has been observed in the cytoplasm of normal, lactating breast tissue (Moll et al., 1992) and in normal human skin after exposure to UV light (Hall et al., 1993).

Induction of p53 protein by DNA damaging agents, in the absence of a concommitent *p53* mRNA increase, has been observed (Kastan et al., 1991; Fritsche et al., 1993). As p53 protein stabilisation is generally carried out by post-translational means, p53 protein stability in A2780 and A2780cp70 nuclear extracts prepared in the presence or absence of phosphatase inhibitors was examined but no difference was detected between these conditions. The levels of other cellular proteins known to interact with p53 were also examined. Hsp70 has been shown to bind p53 and may remove it from its nuclear site of action (Pinhasi-Kimhi et al., 1986; Martinez et al., 1991). Western blotting with a Hsp70 specific antibody showed 4 fold higher levels

of Hsp70 protein in A2780cp70 cell lysates than A2780 lysates. However the observation that nuclear extracts of A2780cp70 cells contained extremely high levels of p53 protein would suggest that Hsp70 is not responsible for stabilising p53 by removing it to the cytoplasm. The altered level of Hsp70 protein in the A2780cp70 cell line compared to the parental A2780, is probably due to reduced p53 protein activity since p53 has been shown to repress transcription from the Hsp70 promoter (Ginsberg et al., 1991; Agoff et al., 1993). Induction of certain heatshock proteins has been implicated in cisplatin resistance. This does not however include Hsp70 as neither Hsp70 protein (Oestereich et al., 1991) or the mRNA (Vikhanskaya et al., 1993) are induced by cisplatin exposure.

The high levels of p53 protein detected in A2780cp70 cells may be due to interaction with some other nuclear protein which may also prevent p53 from interacting with its natural targets. By interacting with the N-terminal region of p53, another protein would prevent recognition of the amino terminal residues by the ubiquitin protein degradation system which is probably responsible for the rapid turnover of p53 protein (Scheffner et al., 1990; Ciechanover et al., 1991). Binding to the N-terminal region would also directly interfere with p53 mediated transcriptional *trans*activation as N-terminal regions are responsible for this activity (O'Rourke et al., 1990; Fields & Jang, 1990; Jackson et al., 1993). The MDM2 protein is a possibility but the reduced *MDM2* mRNA detected in A2780cp70 cells relative to A2780 cells would argue against such an involvement. Interactions of p53 with other cellular proteins has been observed (Maxwell & Roth, 1993) and any one of these may be involved in stabilising p53 protein in A2780cp70 cells. Post translational modifications of p53 protein may be necessary to activate p53 and to stimulate p53 degradation and this may involve subtle changes in phosphorylation. Also association of p53 protein with other intracellular molecules such as RNA could be involved in its recognition by proteases.

5.2.2 DISRUPTION OF p53 MEDIATED APOPTOSIS IN CISPLATIN RESISTANCE?

Apoptosis has been reported to be involved in the cyotoxicity and anti-tumour activity of cisplatin and circumvention of the apoptotic machinery may result in cisplatin resistance (Eastman, 1990; Barry et al, 1990; Dive & Hickman, 1991; Evans et al., 1994; Frankfurt et al., 1994; Ormerod et al., 1994). p53 is required for apoptosis in certain cell types and in response to certain stimuli particularly DNA-damaging agents (Yonish-Roach et al., 1993; Lowe et al., 1993a & b; Clarke et al., 1993; Morgenbesser et al., 1994; Zhu et al., 1994). This may be due to p53-mediated cell cycle arrest or may be an entirely different function of p53. Both p53 induced cell cycle arrest and apoptosis are likely to involve the ability of p53 to modulate transcription of certain genes. These transcriptional control functions are summarised in table 5.2.1.

It has been demonstrated that p53 is able to induce apoptosis in the absence of transcriptional activation thereby indicating that transcriptional repression may be of greater importance in p53 stimulated apoptosis (Caelles et al., 1994). Thus the ability of p53 protein to inhibit *bcl-2* transcription (Myashita et al., 1994a & b) provides an attractive explanation for the effects of p53 on apoptosis. Bcl-2 is involved in preventing apoptosis (for a review see Korsmeyer, 1992) and so, reduction in the levels of this protein caused by p53 mediated transcriptional repression would allow apoptosis to take place. Likewise, perturbation of p53 activity would allow cells to avoid apoptosis and become resistant to cisplatin. Also, p53 transcriptionally transactivates the *bax* gene, whose product interacts with bcl-2 and promotes apoptosis (Miyashita et al., 1994a). The decision to enter apoptosis may depend on whether bcl-2 or bax levels predominate and p53 seems to be directly responsible for regulating the balance between these two proteins.

TABLE 5.2.1 Genes Which Respond To p53-Mediated Transcriptional Regulation

Gene or promoter	Activated by wild-type p53	Repressed by p53 wild-type	Gene product function	Reference
hRGC	+	-	ribosomes for protein translation	Farmer et al (1990)
MCK	+	-	murine muscle creatine kinase	Weintraub et al (1991)
cyclin G	+	-	? (probably cell cycle control)	Okamoto & Beach (1994)
p53	+	-	tumour suppressor	Deffie et al (1993)
GADD45	+	-	DNA-damage induced	Kastan et al (1992)
MDM2	+	-	possible transcription factor	Juven et al (1993)
WAF-1	+	-	inhibits cyclin dependent kinases	El-Deiry et al (1993)
bax	+	-	promotes apoptosis	Myashita et al (1994)
minimal promoter	-	+	none	Seto et al (1992)
hsp70	-	+	"heatshock" stress response protein	Ginsberg et al (1991) ;Agoff et al (1993)
c-fos	-	+	AP-1 mediated transcription in response to serum stimulation	Ginsberg (1991)
c-jun	-	+	"	"
p53	-	+	tumour suppressor	"
B-actin	-	+	cellular structural protein	"
IL6	-	+	interleukin 6, a cytokine	Sanantham et al (1991)
mdr1	-	+	p-glycoprotein membrane pump	Chin et al (1992)
Rb	-	+	tumour suppressor	Shiio et al (1992)
bcl-2	-	+	antagonizes apoptosis	Myashita et al (1994)

5.2.3 ALTERED p53 ACTIVITY IN CISPLATIN RESISTANT CELLS

Transfection of a p53 responsive luciferase construct into A2780cp70 cells, demonstrated that although there was very little p53 transcriptional *trans*activation by p53 in this cell line, p53-mediated transcription could still be induced by DNA damage inflicted using 1μM cisplatin or transplatin for 1 hour. Transcriptional *trans*activation by p53 can be determined by Northern blot analysis of p53 responsive genes such as *WAF-1* relative to a control gene such as *GAPDH*. Induction of *WAF-1* mRNA in A2780 and A2780cp70 cell lines has been examined in this thesis. Cisplatin or transplatin doses of 1μM for 1 hour caused induction of *WAF-1* mRNA which was detectable 4 hours after drug treatment and which was increased further up to a 4 fold induction by 24 hours after drug removal. Treatment with ionising radiation evoked a more rapid response with a 4.5 fold peak induction 4 hours after exposure. This is consistent with data from elsewhere showing induction of p53 DNA binding activity after DNA damage (Tischler et al., 1993) and induction of WAF-1 mRNA by DNA damage (El-Deiry et al., 1994). Also it demonstrates that p53 activity is not impaired when p53 protein levels are induced in response to DNA damage. *WAF-1* mRNA basal levels were 10 fold lower in the cisplatin resistant cell line A2780cp70 than in the sensitive line A2780. This indicates that there is reduced p53-mediated transcriptional control in the resistant cell line.

Transfection of A2780 cells with a mutant p53 expression plasmid, pC53-SCX3 (Baker et al., 1990) did not severely alter *WAF-1* inducibility by p53, however there was a reduction in basal levels of *WAF-1* expression by 3-fold compared to A2780 cells transfected with the vector alone. Thus transfection of A2780 cells with mutant p53 gives a *WAF-1* mRNA reduction similar to, but not as strong as that seen for A2780cp70 compared to A2780 cells. Also a 4 fold reduction in levels of mRNA from another p53 responsive

gene, *MDM2* were detected in the cisplatin resistant, A2780cp70 cells compared to A2780 cells.

This reduced p53 activity in the cisplatin resistant cell line A2780cp70, compared to the sensitive line from which it was derived, may allow survival of these cells following DNA damage levels which would be toxic to sensitive cells. Reduced p53 transcriptional regulation activity may affect other p53 functions such as inhibition of cell cycle progression in response to DNA damage and p53-dependent apoptosis particularly if one considers that these effects are transduced by genes downstream of p53 in the response pathway. Examination of the functions of p53 responsive genes summarised in table 5.2.1 would indicate that this may indeed be the case.

A2780cp70 cells also have a reduced capacity to arrest in G1 phase of the cell cycle in response to ionising radiation doses of 2Gy or 4Gy as determined by FACS analysis. This is consistent with reduced p53 activity in cisplatin resistant cells. Also recent data shows that there is a greater induction of non-random DNA fragmentation after ionising radiation exposure in the A2780 cells than the cisplatin resistant derivative, A2780cp70. This is indicative of reduced DNA damage-induced apoptosis in the resistant cell line which may permit resistance of these cells to DNA damaging agents such as cisplatin (A.McIlwrath, CRC Dept. of Medical Oncology, *personal communications*).

It would be interesting to determine how this reduced activity and enhanced level of p53 protein is attained. As mentioned in section 5.2.1, this may be mediated by mutation of a p53 post-translational modifying enzyme or altered expression of a p53 interactive protein. Perturbation of the p53 DNA damage response pathway by mutation of the p53 gene or by transfection of mutant p53 into cells has been examined elsewhere. A significant correlation between the ability to arrest in G1 phase of the cell cycle and the degree of sensitivity to DNA damage induced by ionising radiation has been detected in various cell lines (McIlwrath et al., 1994). Also transfection of mutant p53 into

A2780 cells reduced both the ability to arrest in G1 phase of the cell cycle in response to ionising radiation induced DNA damage and the cellular sensitivity to such damage. Thymocytes from transgenic mice with a mutant *p53* gene were more sensitive to ionising radiation induced apoptosis than thymocytes from wild-type littermates, demonstrating the importance of a wild-type *p53* gene for DNA damage induced apoptosis (Lee & Bernstein, 1993). Although DNA damage sensitivity of some cell lines seems to be indifferent to changes in the p53 signal pathway other mechanisms may predominate in different cell types. The lack of correlation between G1 arrest and sensitivity following DNA damage in colorectal carcinoma cell lines with wild-type or mutant *p53* genes (Slichenmeyer et al., 1993) may be related to these cell lines expressing particularly high levels of Bcl2 protein and being more intrinsically resistant to cisplatin. Analysis of several Burkitts lymphoma and lymphoblastoid cell lines showed that there was a significant correlation between p53 status, ability to arrest in G1 phase of the cell cycle in response to DNA damage and sensitivity to ionising radiation (O'Conner et al., 1993). Even so, certain exceptions exist such as the P3HR1 Burkitt's lymphoma cell line which is sensitive to DNA damage but lacks wild-type p53 or a radiation induced G1 arrest. Some of this data is summarised in table 5.2.2.

Also in this thesis the sensitivity of A2780 and A2780cp70 cells to a second cisplatin treatment after an initial pretreatment with 1μM cisplatin for 1 hour was investigated. It was reasoned that a pretreatment might elicit changes in cellular compnents such as p53 protein which might protect the cells from further DNA damage or prime them for apoptosis in response to further DNA damage. No difference in sensitivity was detected between pretreated and control cells when the second dose was given at 4, 24 or 48 hours following the first dose. These are times when one would expect p53 protein to be induced following the first cisplatin exposure so it would seem

that the induced p53 protein levels have no bearing on sensitivity to further DNA damage elicited after p53 induction.

TABLE 5.2.2 A Summary Of Data From our Laboratory And Others Showing The Correlation Between p53 Activity And DNA-Damage Sensitivity

(ND= not determined)

Cell line	p53 status	G1 arrest after DNA damage	Sensitivity to ionising radiation	Waf-1 expression level
A2780 (human , ovarian adenocarcinoma) .	wild-type	+++	+++	++++
A2780cp70 (a cisplatin resistant derivative of A2780).	wild-type	-	+	+
A2780 + pC53-SCX3 (a mutant p53 expressing plasmid-Baker et al, 1990).	mutant p53 from plasmid	-	+	++
SW480 (human, colorectal carcinoma-Slichenmeyer, 1993).	mutant	-	++	ND
RKO (human, colorectal carcinoma-Slichenmeyer, 1993).	wild type	+++	++	ND
RKO +HPV E6 (Slichenmeyer, 1993).	functionally null due to HPV E6 expression.	-	++	ND
WMN, AG876, SHO (Burkitt's lymphoma-O'Conner et al, 1993).	wild-type	+++	+++	ND
JLP119, EW36 """.	wild-type	+	+	ND
AKAU, ST486 """.	wild type/mutant	-	+	ND
CA46, Ramos, SG568, Namalwa, MC116, HWL, JD38 """.	mutant	-	+	ND
P3HR1 """.	mutant	-	+++	ND

5.3 DRPs IN DNA DAMAGE RESPONSE AND CISPLATIN RESISTANCE

5.3.1 HOW DRPs MAY AFFECT CISPLATIN SENSITIVITY OF CELLS

As mentioned in section 5.1.3, DRPs may be interlinked with the p53 apoptotic pathway or they may be involved in a separate response pathway concerned with DNA repair. In either case, alteration in their activity or intracellular concentration might alter cisplatin-induced cytotoxicity. Even if DRPs themselves are not the rate limiting stage in a DNA damage response pathway, they merit investigation as possible response pathway constituents. Alternatively they may be irrelevant proteins which are trapped by the cisplatin molecule and are unable to dissociate from the DNA. This too may have an effect on cell survival if cisplatin lesions impede the normal function of the DRP.

Data concerning partial purification of a DRP which binds to cisplatin-damaged DNA giving rise to the "B2 complex" in the gel mobility shift assay has been presented in this thesis. A strategy was developed using treatment of nuclear extracts with 2% TCA followed by anion exchange and heparin column chromatography. At each stage, binding activity was assessed by gel mobility shift analysis. This led to a final purification fold of 183. Other purification stages which may have proved useful in purifying the DRP further are HPLC and affinity chromatography using cisplatin modified DNA immobilised on cellulose. Specificity of partially purified DRP activity for cisplatin-damaged DNA over UV damaged or untreated DNA was demonstrated by competition gel mobility shift analysis.

5.3.2 HMG PROTEINS AND CISPLATIN RESISTANCE

In this thesis the protein responsible for the "B2 complex" seen on gel mobility shift analysis was examined for identity with members of the HMG

class of proteins. Antibodies reactive with HMGI were unable to give rise to a supershift of the "B2 complex" on gel mobility shift analysis, however antibodies reactive with HMG1 were. Furthermore, recombinant HMG1 bound to cisplatin-damaged DNA in the gel mobility shift assay, retarding the DNA to a similar extent as one would observe for the "B2 complex" itself. Both HMG1 and HMG2 were able to bind cisplatin-damaged DNA in the South-western binding assay. This is consistent with data from other laboratories demonstrating an affinity of HMG1 and HMG2 for cisplatin-damaged DNA (Pil & Lippard., 1992; Billings et al., 1992; Hughes et al., 1992). DRPs with HMG-homologous regions (HMG boxes) have also been reported. These include the 81KDa SSRP1 (Bruhn et al., 1992), the human ribosomal upstream binding factor, hUBF (Treiber et al., 1994) and the IXR1 protein of *S.cerevisiae* (Brown et al., 1993).

It is feasible that HMG proteins have a role to play in cisplatin resistance. They may reduce the DNA distortion caused by the cisplatin-DNA adduct allowing increased tolerance of cisplatin damage. They may prevent recognition by a DNA damage response pathway leading to apoptosis. The mouse homologue of SSRP1 is involved in recombinational control (Shirataka et al., 1991) and so, HMG protein interaction with cisplatin-damaged DNA may have implications for recombinational repair.

5.3.3 ARE DRPs INVOLVED IN THE p53-MEDIATED DNA DAMAGE RESPONSE PATHWAY?

It was of interest to determine whether p53 is able to recognise cisplatin-damaged DNA. If p53 or proteins which interact with p53 were able to bind to cisplatin adducts, one could envisage a situation where cisplatin damage was directly transduced to the p53 mediated apoptotic response pathway. In this thesis, p53 immunoprecipitates were assessed for cisplatin-recognition by the gel mobility shift assay. Cisplatin treated cells were also

used in immunoprecipitation reactions in order to determine whether DRPs would be induced to interact with p53 protein immediately after cisplatin treatment. No difference in DRPs precipitated with p53 specific antibodies or a control antibody could be discerned. This does not eliminate the possibility that DRPs are involved in the p53 DNA damage response, however. DRPs may become altered in conformation upon binding to cisplatin damage and this may result in modification of an intermediary protein which transduces the DNA damage distress signal to p53 protein.

5.4 THE FUTURE FOR CISPLATIN CHEMOTHERAPY

The indication that an altered p53 response pathway prevents apoptosis in response to DNA damage has depressing implications as p53 mutations or deletions are common occurances in cancer (Srivastava et al., 1990; Malkin et al., 1990; Cunningham et al., 1992; Allred et al., 1993; Burns et al., 1993). Thus, mutations of the *p53* gene would not only be instrumental in causing cancer but would also prevent response to chemotherapy. A greater understanding of the apoptotic mechanisms and how p53 activity is modulated *in vivo* will eventually lead to means of modulating the cytotoxicity of cisplatin. The use of the genetic supressor element strategy (Gudkov et al., 1993) may show which domains of p53 are involved in prevention of cisplatin resistance and may lead to the discovery of more genes which determine efficacy of chemotherapy. A deeper knowledge of the signal transduction events linking DNA damage to p53 induction is required. Also the role of DNA repair in influencing susceptibility of cells to apoptosis should be addressed. Meanwhile hope of circumventing cisplatin resistance is provided by the synergistic effect of tamoxifen on cisplatin activity (McClay et al., 1992).

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