SOMATIC GENETIC ANALYSIS OF p53 FUNCTION AND CISPLATIN RESISTANCE

William Gallagher, BSc

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CRC Department of Medical Oncology
Faculty of Medicine
University of Glasgow
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For Catherine,
my wife and friend

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ABSTRACT

p53 modulates a number of cellular responses to genotoxic stress including cell cycle arrest and apoptosis. Loss of p53 function either by mutation or otherwise may alter the sensitivity of cells to DNA damage. Introduction of a dominant negative p53 mutant (codon 143, Val to Ala) has been shown to confer cisplatin resistance in the chemosensitive human ovarian A2780 carcinoma cell line. Induced resistance of A2780 cells to cisplatin was used as the selection strategy to isolate genetic suppressor elements (GSEs) from retroviral libraries expressing random fragments of human or murine TP53 cDNA (**Chapter 4**). Six GSEs were identified, encoding either dominant negative mutant peptides or antisense RNA molecules which corresponded to various regions within the TP53 gene. Both types of GSE induced cisplatin resistance when introduced individually into A2780 cells.

Expression of antisense GSEs led to decreased intracellular levels of p53 protein. One sense GSE induced loss of p53-mediated activities such as DNA damage induced G1 arrest and apoptosis. A synthetic peptide, representing the predicted amino acid sequence of this GSE, conferred resistance to cisplatin when introduced into A2780 cells and inhibited the sequence specific DNA binding activity of p53 protein *in vitro*. Overall, these results directly indicate that inactivation of p53 function confers cisplatin resistance in these human ovarian tumour cells. We have identified short structural domains of p53 which are capable of independent functional interactions and highlighted the efficacy of this approach to discriminate biological active p53 GSEs from a random fragment library. This work may have use in the rational design of low molecular mass modifiers of the p53 response.

Both G1 arrest and apoptosis induced by ionising radiation or cisplatin are dependent upon an intact p53-mediated DNA damage response pathway in the A2780 line. Evidence is presented for a defect in the p53-mediated DNA damage response pathway in cisplatin resistant derivatives of A2780 cells as determined by reduced DNA damage induced G1 arrest, apoptosis and transcription of certain target genes (**Chapter 3**). The basis of the p53 dysfunction in one of these resistant cell lines does not appear to be due mutation of the TP53 gene, overexpression of mdm2 protein, differences in the cell cycle related expression of p53 protein or gross changes in the pattern of p53 isoforms. Cisplatin resistant A2780 derivatives also acquire a microsatellite instability (RER+) phenotype, which is associated with defective mismatch repair (MMR) and loss of hMLH1 mRNA and protein expression. Defective MMR in these cisplatin resistant cells may lead to tolerance of DNA damage and reduced ability to engage both p53-dependent and -independent apoptotic pathways.

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DECLARATION

I, **William Matthew Gallagher**, declare that I am the author of this thesis, that all the references have been consulted by myself in the preparation of the manuscript, and that all the work described herein was performed by myself unless otherwise stated. This work has not been previously accepted for a higher degree.

William M. Gallagher

October, 1996

ABBREVIATIONS

APS	Ammonium persulphate
BrdUrd	Bromodeoxyuridine
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
cisplatin (or cDDP)	*Cis*-diamminedichloroplatinum(II)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRP	DNA damage recognition protein
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylene diamminetetra-acetic acid
ELISA	Enzyme-linked-immunoadsorbant assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein 5-isothiocyanate-conjugated
GSE	Genetic suppressor element
GSH	Glutathione
HMG	High mobility group
HRPL	Horseradish peroxidase-linked
HSP	Heat-shock protein
LTR	Long terminal repeat

M	Molar
mA	Milliamps
MAb	Monoclonal antibody
mg	Milligramme
min	Minute
ml	Millilitre
MMR	Mismatch repair
MOPS	4-(*N*-Morpholino)propane-sulphonic acid
MT	Metallothionein
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PI	Propidium iodide
PIPES	Piperazine-*N*-*N*-bis(2-ethane-sulphonic acid)
RER	Replication error
RNA	Ribonucleic acid
SDM	Standard deviation from the mean
SDS	Sodium dodecyl sulphate
SEM	Standard error from the mean

SSC	Simple sodium citrate
TEMED	*N,N,N',N'*-tetramethylethylenediammine
UV	Ultra violet
V	Volts

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CHAPTER 1

INTRODUCTION

1.1 p53-MEDIATED DNA DAMAGE RESPONSE PATHWAY

Resistance of tumour cells to radiation and chemotherapy remains a significant obstacle in the effective treatment of cancer. DNA is the primary target for many antitumour agents. A wealth of evidence has supported a role for p53 in the cellular response to DNA damage (see reviews: Ko and Prives, 1996; Shimamura and Fisher, 1996). DNA damage induced activation of p53 can result in either cell cycle arrest or apoptosis (Figure 1). Furthermore, p53 may also modulate DNA replication and DNA repair. In response to DNA damage, p53 protein accumulates and can activate (or repress) the transcription of a variety of target genes which may mediate its biological effects. Alternatively, p53 may directly interact with and effect critical proteins involved in the above processes in a transcriptionally-independent manner. The decision between p53-mediated arrest or apoptosis is at least partially influenced by cell type and environment. It must be noted that in addition to its response to genotoxic stress, p53 may mediate a more general response to suboptimal growth conditions (Donehower and Bradley, 1993). For example, both hypoxia and heat induce p53, as does starvation (Zhan *et al.*, 1993; Graeber *et al.*, 1994). In addition, ribonucleotide depletion has been shown to induce a p53-dependent G1 arrest in the absence of detectable DNA damage (Linke *et al.*, 1996).

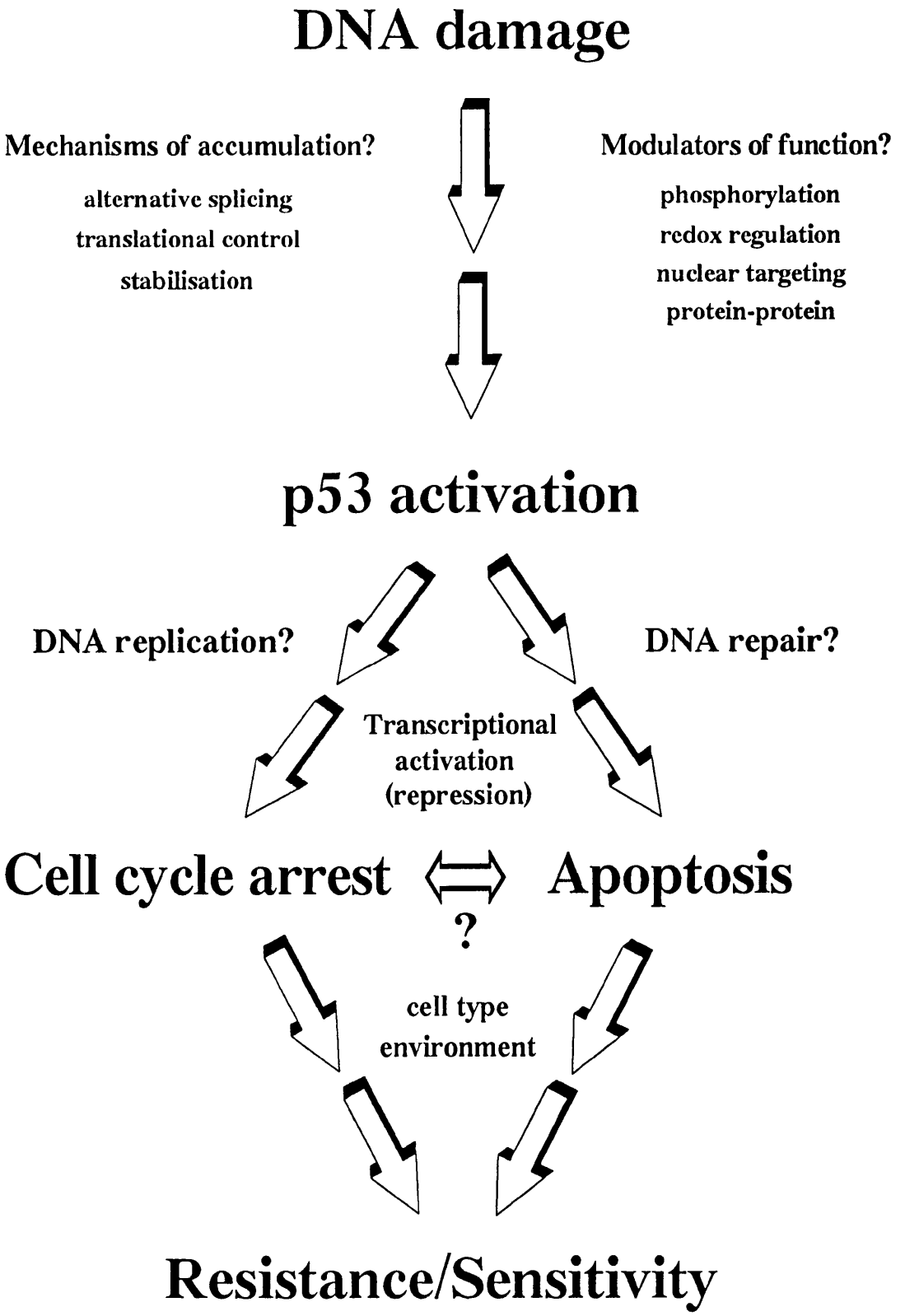


Figure 1. The p53-dependent DNA damage response pathway.

1.1.1 STRUCTURE AND FUNCTION

The human p53 protein is a complex molecule composed of 393 amino acids and with an ever increasing number of functional domains identified (see reviews: Deppert, 1994; Harris, 1996). The N-terminus (residues 1-42) contains a large number of acidic residues and harbours a transcriptional activation domain (Unger *et al.*, 1992). However, neighbouring sequences are also likely to contribute to the transcriptional activity of p53 (Chang *et al.*, 1995a). Adjacent to the N-terminus is a proline-rich region spanning amino acids 61-94 which represents a putative target site for SH3 domain binding proteins. In fact, recent evidence has suggested that this SH3-like domain within p53 may play a role in transcription-independent protein signalling and growth suppression (Walker and Levine, *unpublished observations*).

The sequence-specific DNA binding domain (residues 100-290) is located within the central core of p53 (Cho *et al.*, 1994). The majority of p53 missense mutations which occur in human tumours are clustered within this area, particularly within the conserved regions of homology II-V (Hollstein *et al.*, 1994). The C-terminus of p53 can function as an independent domain capable of binding non-specifically to different forms of DNA, including damaged DNA (Wang *et al.*, 1993; Lee *et al.*, 1995; Reed *et al.*, 1995). A flexible "linker" region (residues 290-310) connects the sequence-specific DNA binding domain to the oligomerisation domain which spans amino acids 320-360. X-ray crystal structures of both the sequence-specific DNA binding and oligomerisation domains have been resolved (Cho *et al.*, 1994; Jeffrey *et al.*, 1995). At the extreme C-terminus is a

stretch of 30 amino acids that is rich in basic residues and which negatively regulates the sequence-specific DNA binding activity of p53 (Hupp *et al.*, 1992).

1.1.2 THE SIGNAL

The focus of much current interest is the elucidation of how signals are transmitted from damaged DNA to activate the p53 pathway. One of the initial inducing signals for p53 accumulation in response to damage appears to be double strand breaks (DSBs; Nelson and Kastan, 1994). In fact, it has been predicted that even a single DSB is enough to induce p53 (Di Leonardo *et al.*, 1994). However, a signal transduction pathway linking the presence of DSBs to p53 accumulation has yet to be identified. The suggestion that a regulated kinase cascade may, at least in part, be responsible for controlling this process is based upon the observation that inhibitors of PKC and serine/threonine phosphatases can prevent radiation induced p53 responses (Khanna and Lavin, 1993). Triggering of the p53 response may require DNA metabolism, although either replication or repair may suffice (Cox and Lane, 1995).

Other lesions apart from DBSs have been demonstrated to activate the p53 arrest pathway (Huang *et al.*, 1996). Nuclear injection of linearised plasmid DNA, circular DNA with a gap of larger than 30 nucleotides or single-stranded circular phagemid was sufficient to induce a p53-dependent G1 arrest in normal human fibroblasts. However, both supercoiled and nicked plasmid DNA and circular DNA with a 25 nucleotide gap were ineffective. It was suggested that the difference in arrest potential of 25 and 30 nucleotide gaps is consistent with the potential involvement of the nucleotide excision

repair (NER) machinery in activating the arrest mechanism. Interestingly, dumbbell substrates containing single mismatched bases (which are recognised and corrected by the mismatch repair machinery) also produced a p53-dependent G1 arrest (Wahl *et al.*, *personal communication*).

1.1.3 MECHANISMS OF ACCUMULATION

Activation of p53 results in increased p53 protein levels which is generally agreed to occur through a post-transcriptional mechanism (Kastan *et al.*, 1991). Furthermore, p53 induction appears to be cell type specific (Midgley *et al.*, 1995). The ATM gene product, although not required for p53 induction, can influence the timing of this process (Enoch and Norbury, 1995). An alternatively spliced form of human TP53 mRNA has recently been identified in normal lymphocytes (Flaman *et al.*, 1996). This splice variant lacks a major portion of the C-terminus which may have significant implications for its activity. Intriguingly, p53 may also be negatively autoregulated by specifically inhibiting translation of its own mRNA (Mosner *et al.*, 1995).

Levels of p53 protein are usually quite low in normal cells, due in part to its rapid turnover. Ubiquitin-dependent proteolysis has been shown to play a significant role in the degradation of p53 protein (Maki *et al.*, 1996). After treatment with DNA damaging agents, the half-life of p53 becomes markedly extended (Matlzman and Czyzyk, 1984; Kastan *et al.*, 1991; Liu *et al.*, 1994). It is assumed that this increase in p53 stability represents the major mechanism for p53 accumulation after DNA damage. However, the molecular basis of p53 stabilisation has not been clarified but may result from changes in

post-translational modification, oligomerisation and binding to other proteins. Alteration of ubiquitin-dependent proteolytic degradation of p53 protein is another likely possibility. Interestingly, when complexed with DNA, p53 is resistant to ubiquitin-dependent proteolysis, whereas unbound protein undergoes rapid degradation (Molinari and Milner, 1995).

1.1.4 MODULATORS OF FUNCTION

In addition to increasing p53 protein levels, cells may respond to DNA damage by modulating the function of the protein in a variety of ways including phosphorylation, redox regulation, nuclear targeting and protein-protein association. The human p53 protein is multiply phosphorylated by a number of protein kinases at serines and threonines within its N- and C-termini *in vitro* and in cell lines (see review: Meek, 1994). Phosphorylation may affect p53 by altering turnover rate and activity. In fact, hyperphosphorylation of p53 using the serine phosphatase inhibitor okadaic acid increased the steady-state level of p53 protein (Zhang *et al.*, 1994). The sequence-specific DNA binding activity of p53 can be stimulated *in vitro* by phosphorylation using a number of different protein kinases (Hupp *et al.*, 1992; Takenaka *et al.*, 1995; Wang and Prives, 1995). Indeed, p53 has been shown to be phosphorylated by a UV damage inducible protein kinase (Milne *et al.*, 1995). However, conflicting results have emerged from studies which examined the effect of mutation of certain phosphorylation sites on p53 function (Ko and Prives, 1996). While some studies have demonstrated alterations in

p53 function as a result of mutation of phosphorylation sites, others have shown no effect. Therefore, the full relevance of p53 phosphorylation remains to be elucidated.

The sequence-specific DNA binding activity of p53 is subject to redox regulation. Oxidation of p53 inhibits DNA binding, whereas reduction favours it (Hupp *et al.*, 1993; Rainwater *et al.*, 1995). Furthermore, oxidation also abolishes the ability of p53 to block nuclear DNA replication *in vitro* (Cox *et al.*, 1995). The balance between oxygen radicals induced by genotoxic stresses such as ionising radiation and cellular reducing responses may have important implications for regulation of p53 function (Hainaut and Milner, 1993). Subcellular localisation can provide an important mechanism of regulating p53 function. Indeed, translocation of p53 protein to the nucleus was shown to be required for p53 to induce a cell cycle arrest following DNA damage (Shaulsky *et al.*, 1991). Cytoplasmic protein anchors may act to prevent p53 from entering the nucleus (Gannon and Lane, 1991). An ever increasing number of cellular proteins have been reported that bind to p53 protein. Some of these interactions may be intimately linked with regulation of p53 function. A prime example is the MDM2 oncogene which encodes a protein that can complex with p53 and inhibit its transcriptional activation ability (Momand *et al.*, 1992; Oliner *et al.*, 1993). Furthermore, MDM2 itself is a transcriptional target of p53 and is activated in response to radiation, thus implying an autoregulatory feedback loop between p53 and MDM2 (Wu *et al.*, 1993).

1.1.5 TRANSCRIPTIONAL ACTIVATION (REPRESSION)

A large body of evidence has indicated that the sequence-specific transcriptional activation function of p53 is a major component of its biological effects (Ko and Prives, 1996). Transcriptional activation of specific target genes by p53 may lead to a number of cellular outcomes including DNA repair, cell cycle arrest and apoptosis (explored in more detail in the following subsections). Molecules of p53 bind as tetramers to highly degenerate DNA motifs which are found generally in the control regions of target genes. These binding sites permit both position- and orientation-independent activation by p53. In addition, both p53 oligomerisation and DNA looping have been associated with the transcriptional activation function of p53 (Stenger *et al.*, 1994). Intriguingly, recent data has emerged which suggests that certain p53 mutants may be able to discriminate between various target gene promoters and therefore have differential effects on transcriptional activation of these genes (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996).

p53 also represses the transcription of a number of cellular genes in a manner which is independent of specific DNA recognition (Ko and Prives, 1996). Examples of genes whose expression is repressed by p53 include BCL-2, FOS and PCNA (Kley *et al.*, 1992; Jackson *et al.*, 1994; Miyashita *et al.*, 1994). As these genes lack obvious p53-binding sites in either promoter or enhancer regions, p53 is believed to act as a broad-range repressor. However, only those promoters which contain TATA boxes appear to be inhibited by p53 (Mack *et al.*, 1993). The C-terminus of p53 is required for transcriptional repression and interaction with the TATA-binding protein, TBP

(Horikoshi *et al.*, 1995; Shaulian *et al.*, 1995). Indeed, p53 may repress transcription through a squelching mechanism in which TBP is sequestered from promoter sites by interaction with p53 thus causing reduced initiation of transcription.

1.1.6 CELL CYCLE ARREST

The role of p53 in regulating the G1/S cell cycle checkpoint in response to DNA damage is well documented (Shimamura and Fisher, 1996). A major effector of the p53-mediated G1 arrest is thought to be the CIP1/WAF1 gene (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; El-Deiry *et al.*, 1994). The CIP1 gene is a transcriptional target for p53 and contains two p53-binding sites - one close to the TATA box and the second several kilobases upstream. The encoded protein p21 forms part of a quaternary complex found in normal cells along with cyclin, cyclin-dependent kinase (CDK) and PCNA (Xiong *et al.*, 1993). Induction of CIP1 expression by activated p53 was shown to inhibit the function of those cyclin/CDK complexes which are predominantly found during G1 phase of the cell cycle (Dulic *et al.*, 1994). Such inhibition results in the accumulation of hypophosphorylated Rb protein thus inducing a G1 arrest (Slebos *et al.*, 1994). In addition, CIP null cells displayed a defective G1 arrest in response to radiation treatment (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). It must be noted that expression of CIP1 is also regulated by p53-independent mechanisms (Michieli *et al.*, 1994; Macleod *et al.*, 1995). p53 has also been suggested to play a potential role in the control of the G2/M cell cycle checkpoint (Agarwal *et al.*, 1995; Stewart *et al.*, 1995).

1.1.7 APOPTOSIS

Substantial evidence has accumulated to suggest that p53 is a positive regulator of cell death, and particularly of apoptosis (Oren, 1994). Cells obtained from TP53 null mice were shown to be resistant to the induction of apoptosis by radiation and chemotherapy (Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Lowe *et al.*, 1993b). Indeed, the effectiveness of a variety of cancer therapies *in vivo* correlated with the ability to induce a p53-dependent apoptotic response (Lowe *et al.*, 1994). However, it must be noted that apoptosis can also occur by pathways independent of p53 (see review: Liebermann *et al.*, 1995). The BAX gene is a transcriptional target of p53 and encodes a protein with homology to the survival factor encoded by the BCL-2 oncogene (Miyashita and Reed, 1995). In addition, expression of BCL-2 is repressed by p53 (Miyashita *et al.*, 1994). Both bcl-2 and bax proteins can heterodimerise and the relative levels of these proteins (and possibly other family members) are critical to the regulation of the apoptotic process (White, 1996). Some studies have indicated that p53 may also have a transcription-independent function in apoptosis (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Recent evidence has suggested that, in response to DNA damage, p53 binding to the transcription-repair complex TFIIH either modulates DNA repair efficiency or triggers apoptosis (Wang *et al.*, 1996).

1.1.8 DNA REPLICATION AND REPAIR

p53 may have a direct impact on DNA replication and repair through its function as a transcriptional activator. The encoded products of two different target genes, CIP1 and GADD45, have been shown to interact with PCNA, a factor that is involved in both DNA replication and DNA repair (Flores-Rozas *et al.*, 1994; Smith *et al.*, 1994). The p21 protein has been shown to directly inhibit the function of PCNA in replication, although its function in repair is relatively unaffected (Li *et al.*, 1994; Waga *et al.*, 1994). p53 may also directly regulate DNA replication and repair in a manner independent of its transcriptional activation function. In this respect, p53 can bind to several proteins which may be involved in the above processes most notably RPA, several components of the TFIIH complex (p62, XPB and XPD) and the strand-specific repair factor CSB (Dutta *et al.*, 1993; Li and Botchan *et al.*, 1993; Wang *et al.*, 1995; Leveillard *et al.*, 1996 and references therein).

Direct inhibition of nuclear DNA replication by p53 has been demonstrated *in vitro* (Cox *et al.*, 1995; Miller *et al.*, 1995). A 3'→5' exonuclease activity has been recently described for p53 which may be involved in processes of damage avoidance and/or error correction during DNA replication and repair (Mummenbrauer *et al.*, 1996). Recent data by our group has suggested that p53 may function in a signalling pathway downstream of mismatch repair (Anthoney *et al.*, 1996). However, there is some evidence which argues against a role for p53 in DNA replication and repair. The interactions of p53 with RPA and TFIIH may relate to its function as a regulator of

transcription rather than DNA replication or repair. While deficiency of p53 function has been shown to result in reduced repair of cellular DNA in some cases (Ford and Hanawalt, 1995; Smith *et al.*, 1995; Wang *et al.*, 1995; Rafferty *et al.*, 1996), other studies have demonstrated that cells from TP53 null mice display normal rates of DNA repair as wild-type cells (Ishizaki *et al.*, 1994; Sands *et al.*, 1995).

1.1.9 CELL TYPE AND ENVIRONMENT

Cell type and environment seem to be major determinants of whether p53 responds to DNA damage by inducing cell cycle arrest or apoptosis (Bates and Vousden, 1996). Thymocytes entered apoptosis in response to DNA damage through a p53-dependent pathway (Clarke *et al.*, 1993; Lowe *et al.*, 1993b). However, irradiation of normal fibroblasts resulted in a prolonged p53-dependent G1 arrest (Di Leonardo *et al.*, 1994). Oncogenically transformed fibroblasts showed a shift in the DNA damage induced p53 response from cell cycle arrest to apoptosis (Lowe *et al.*, 1993a). Alterations in cellular environment, particularly the presence or absence of survival factors, resulted in a similar shift in the p53 response (Collins *et al.*, 1992; Canman *et al.*, 1995). Intriguingly, certain p53 mutants have been shown to maintain the ability to induce a G1 arrest but display impaired apoptotic functions (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996; Rowan *et al.*, 1996). These results demonstrate that cell cycle arrest and apoptosis mediated by p53 can be separated and suggest that these pathways are independent of each other. Overall, the decision to undergo cell cycle arrest or apoptosis in response to DNA damage will have important ramifications for the response of tumour cells to therapy.

1.2 CISPLATIN

1.2.1 CISPLATIN AS A CHEMOTHERAPEUTIC AGENT

Despite many recent advances in understanding the molecular basis of cancer, treatment of human cancer is still limited by the toxicity of chemotherapeutic agents and the development of intrinsic or acquired resistance to these drugs. *Cis*-diamminedichloroplatinum(II) or cisplatin has become the primary building block for regimens that cure patients with testicular carcinoma and produces high response rates in patients with non-small cell lung, bladder, and ovarian cancer among others (Rosenberg, 1985). However, the development of resistance to cisplatin in the clinic represents a major problem in long term treatment of these diseases. Elucidation of the molecular basis of cisplatin resistance may help in the design of rational approaches to circumvent this problem.

Cisplatin was initially discovered during a study into the effects of electric current on growing bacteria (Rosenberg *et al.*, 1965). It was observed that when an alternating current was delivered through platinum electrodes to a growing culture of *Escherichia coli*, the bacterial cells stopped dividing and grew into long filaments. Upon further investigation, a compound was identified which formed from the electrodes during electrolysis and inhibited cell division - the platinum coordination complex cis-$Pt(II)(NH_3)_2Cl_2$ or cisplatin. This compound was shown soon afterwards to have potent antitumour activity (Rosenberg *et al.*, 1969). Cisplatin is a bifunctional alkylating agent

which can form covalent adducts with many biological molecules including RNA, DNA and proteins. However, most data are consistent with the hypothesis that the major cytotoxic target of cisplatin is DNA (Fram, 1992). Perhaps, the most compelling evidence comes from experiments showing that both prokaryotic and eukaryotic cells deficient in DNA repair were hypersensitive to cisplatin (Beck and Brubaker, 1973; Chu, 1994).

But the type of DNA lesions primarily responsible for the cytoxicity and antitumour activity of cisplatin are not clearly established. Cisplatin forms a variety of DNA lesions including monofunctional adducts and both interstrand and intrastrand crosslinks (Zamble and Lippard, 1995). The most prevalent DNA adduct (>90%) formed by cisplatin is the 1,2-intrastrand crosslink, where platinum is covalently bound to the N^7 positions of adjacent purine bases. The chemotherapeutically inactive *trans* geometrical isomer of cisplatin cannot form the 1,2-intrastrand crosslink suggesting that this lesion may be responsible for the antitumour activity of cisplatin (Connors *et al.*, 1972). Furthermore, the 1,2-intrastrand crosslink was shown to be poorly repaired by NER *in vitro* and such inefficient repair may increase cellular toxicity (Szymkowski *et al.*, 1992). Formation of cisplatin-DNA adducts has important structural implications, e.g. in case of the 1,2-intrastrand crosslink, the DNA is unwound and bent towards the minor groove (Anin and Leng, 1990). Changes in DNA topology induced by cisplatin have the potential to affect the ability of the DNA to be used as a template for cellular processes such as DNA replication and gene transcription.

1.2.2 TUMOUR CELL LINES AS MODEL SYSTEMS

Cell culture model systems may be useful in the elucidation of the molecular mechanisms of both intrinsic and acquired drug resistance. Advantages of such experimental systems include ease of manipulation and precise control of external factors. Cell lines can be established from tumour cells which show intrinsic resistance to cisplatin. Acquired cisplatin resistance can be studied by making drug resistant variants of inherently sensitive cells. This process normally requires continuous exposure to increasing concentrations of cisplatin over a period which can encompass several years (Behrens *et al.*, 1987). A possible disadvantage in using multiple step selection is that these drug resistant variants are destined to show a variety of resistance mechanisms making the task of dissecting out each pathway more difficult. Single step selection with cisplatin has also proven effective in the isolation of drug resistant variants (McLaughlin *et al.*, 1991). Cell lines generated in this way would be expected to display a single mechanism of resistance. The resistance phenotype of either single or multiple step selected cell lines is generally stable for long periods in the absence of selective medium (Thompson and Baker, 1973).

An important consideration is how relevant such *in vitro* cell line models are to clinically observed drug resistance. Extrapolating results from *in vitro* systems can be difficult as the repeated high levels of cisplatin used for selection of resistant tumour cell lines often bare little resemblance to those used clinically and the large fold resistance frequently studied using certain *in vitro* models may be unrepresentative of the low levels

of resistance that can lead to treatment failure. Another more direct, albeit artificial, approach is genetic intervention of tumour cells i.e. overexpression or inactivation of genes which may play a role in the response of cells to cisplatin.

1.2.3 MOLECULAR MECHANISMS OF RESISTANCE

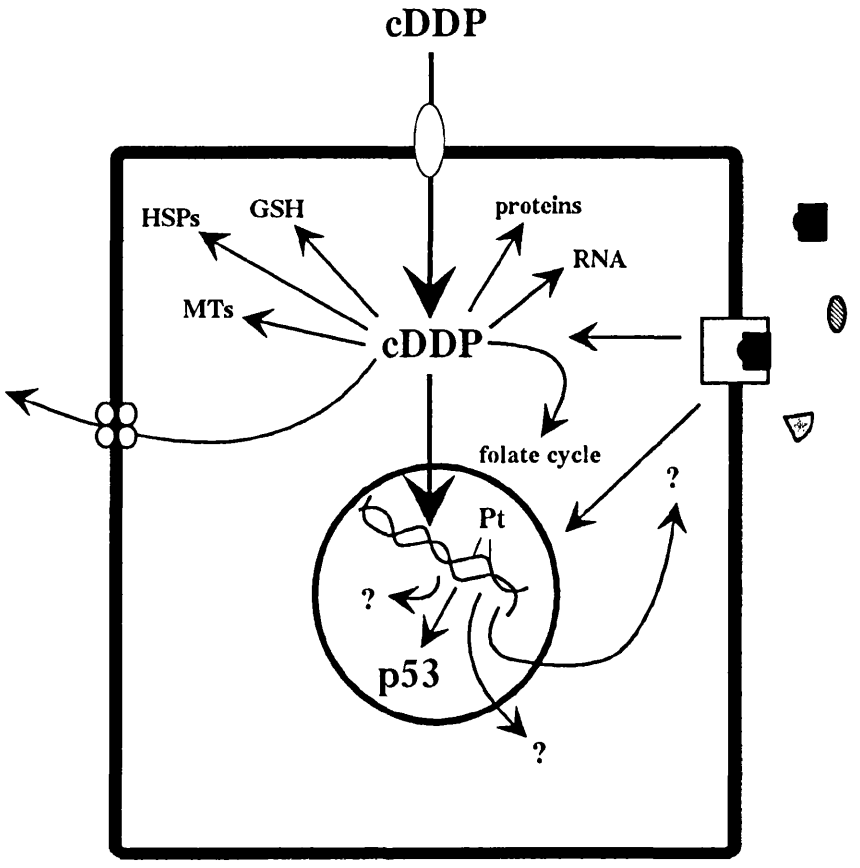
Investigations at the cellular level have shown cisplatin resistance to be due to one or more of a number of mechanisms including decreased drug accumulation, increased cytoplasmic detoxification, alterations in signal transduction pathways and aberrant DNA damage induced responses (Figure 2; Ishida *et al.*, 1995). Decreased intracellular cisplatin accumulation, resulting from reduced influx or increased efflux of the drug, has been observed in many cisplatin resistant tumour cell lines (Andrews and Howell, 1990). However, the causative molecular mechanism(s) underlying decreased cisplatin accumulation is as yet unclear. A number of reports have indicated that cisplatin enters cells through passive diffusion (Gale *et al.*, 1973; Ogawa *et al.*, 1975). Nevertheless, it is unlikely that changes in the passive permeability of the plasma membrane is responsible for the decreased cisplatin accumulation because this would effect both influx and efflux.

ACCUMULATION

INFLUX
EFFLUX

DETOXIFICATION

GSH
MTs
HSPs



SIGNAL TRANSDUCTION

PKA/PKC
ras
fos

DNA DAMAGE

REPAIR
TOLERANCE
CELL CYCLE ARREST
APOPTOSIS

Figure 2. Molecular mechanisms of cisplatin resistance.

Evidence suggesting the existence of an active transporter for cisplatin has been increasing. In one particular study, a 200kDa membrane glycoprotein was identified in a cisplatin resistant derivative of the murine R1.1 lymphoma cell line, which is distinct from the multidrug resistance-associated P-glycoprotein (Kawai *et al.*, 1990). Elevated levels of the 200kDa glycoprotein correlated with reduced cisplatin accumulation in this resistant line. Determination of the exact contribution of the 200kDa glycoproten to cisplatin resistance will require further experimentation (Bernal *et al.*, 1990). *In vivo* studies of cisplatin resistance have also been performed. Human ovarian 2008 carcinoma cells were made resistant to cisplatin (1.4-2.2 fold) as xenografts in athymic mice (Andrews *et al.*, 1990). These resistant cells showed a 28% decrease in cisplatin accumulation.

Intracellular detoxification of cisplatin may occur by binding to sulfhydryl peptides, small thiol-rich proteins or heat-shock proteins. Cisplatin, being an electrophilic molecule, is extremely reactive toward sulfur-containing nucleophiles. Glutathione (GSH), the principal cellular sulfhydryl peptide, has many important functions including protection from oxidative stress, drug metabolism and intracellular detoxification (Meister and Anderson, 1983). Although levels of GSH are commonly increased in cisplatin resistant tumour cell lines, the role of GSH in mediating resistance to cisplatin remains unclear (Andrews and Howell, 1990; Ishida *et al.*, 1995). Elevated GSH levels were only observed in resistant cells selected by continuous exposure to cisplatin *in vitro*. It is possible that the increased GSH levels may be due to a general stress response to drug treatment and are not directly involved in inducing cisplatin resistance.

Metallothioneins (MTs) are small, thiol-rich proteins important in binding and detoxifying heavy metals (Hamer, 1986). Elevated levels of MTs have been observed in certain cisplatin resistant cell lines (Kelly *et al.*, 1988; Kasahara *et al.*, 1991). But, examination of MT levels in ovarian tumours before and after chemotherapy revealed that MT content was not a major determinant of tumour sensitivity (Murphy *et al.*, 1991). Heat-shock proteins (HSPs) are a unique set of highly conserved proteins, induced by exposure to heat and a variety of other types of stress, which are presumed to confer a protective function to the cell (Parsell and Lindquist, 1993). 2008 cells selected for cisplatin resistance by chronic *in vitro* exposure expressed increased levels of HSP60 mRNA and protein (Kimura *et al.*, 1993). In fact, the level of HSP60 mRNA expression may be a valuable prognostic factor for epithelial ovarian cancer treated with platinum compounds (Kimura *et al.*, 1992).

The folate cycle provides an important source of deoxythymidylate molecules which are essential for DNA synthesis and repair (Voet and Voet, 1990). A number of cisplatin resistant cell lines have exhibited alterations in folate metabolism (Andrews and Howell, 1990). Human ovarian A2780 carcinoma cells made resistant to cisplatin (3.2 fold) by discontinuous exposure showed 2.5 fold increases in thymidine kinase, thymidylate synthase and dihydrofolate reductase activities (Scanlon *et al.*, 1989a). Similar changes were observed with cisplatin resistant human colon HCT8 carcinoma cells (Scanlon *et al.*, 1989b). Data from clinical samples suggested that changes in folate metabolism may arise in cisplatin resistant tumours also (Scanlon *et al.*, 1989a).

Cell growth and differentiation can be initiated by signal cascades which are commonly altered in human cancer (Cantley *et al.*, 1991). Chemotherapeutic agents have been shown to encroach upon on these signal transduction pathways which may lead to the evolution of drug resistant clones (Brunton and Workman, 1993). Several components of cellular signalling pathways have been implicated in cisplatin resistance mechanisms including ras and fos among others (Ishida *et al.*, 1995). Overexpression of a mutant c-Ha-RAS gene conferred cisplatin resistance in NIH 3T3 cells, which was associated with an impairment of intracellular drug accumulation and an increase in metallothionein content (Isonishi *et al.*, 1991). Increased expression of FOS has been observed in cisplatin resistant tumour cell lines and in patients (Scanlon *et al.*, 1989a). A FOS ribozyme has also been shown to completely reverse cisplatin resistance in certain tumour cell lines, possibly through downregulation of AP-1 responsive genes such as thymidylate synthase, topoisomerase I and metallothionein (Scanlon *et al.*, 1991). In contrast, activation of both PKA and PKC enhanced sensitivity of certain tumour cells to cisplatin (Christen *et al.*, 1994).

A variety of resistance mechanisms may operate after cisplatin induced DNA damage including enhanced DNA repair, increased damage tolerance, aberrant cell cycle arrests and reduced apoptosis (Brown *et al.*, 1995). DNA repair plays an integral role in the molecular mechanism of cisplatin action (Zamble and Lippard, 1995). Cells derived from Xeroderma pigmentosum patients, which are deficient in NER, were shown to display hypersensitivity to cisplatin (Plooy *et al.*, 1985). Moreover, it has been shown that both the 1,2- and 1,3- intrastrand crosslinks are repaired by the NER pathway

(Huang *et al.*, 1994). However, repair of the 1,2-intrastrand crosslink is relatively inefficient in comparison to other platinum-DNA lesions (Szymkowski *et al.*, 1992). Enhanced DNA repair has been demonstrated in a variety of tumour cell line models with either intrinsic or acquired resistance to cisplatin (Sheibani *et al.*, 1989; Parker *et al.*, 1991). Interestingly, increased gene-specific repair interstrand crosslinks has been observed in certain cisplatin resistant human ovarian cancer cell lines (Zhen *et al.*, 1992). It has been suggested that the increase in DNA repair that accompanies cisplatin resistance may be due to altered expression of proteins involved in NER such as ERCC1 and PCNA (Haneda *et al.*, 1991; Dabholkar *et al.*, 1992).

Proteins, termed DNA damage recognition proteins (DRPs), have been identified which specifically bind to cisplatin-DNA adducts and are overexpressed in cisplatin resistant tumour cells (McLaughlin *et al.*, 1993; Chu *et al.*, 1994). Subsequent studies revealed that such DRPs contain HMG-domains which recognise DNA structural elements, such as the bends in cisplatin-modified DNA (Zamble and Lippard, 1995). HMG-domain proteins may serve as recognition elements for repair and act by recruiting other proteins in the NER complex to sites of DNA damage. An alternative hypothesis is that HMG-domain proteins bind tightly to cisplatin-DNA adducts and prevent access of the repair complex to the site of damage. Increased tolerance of cisplatin-DNA adducts may play an important role in cisplatin resistance of human tumour cells (Parker *et al.*, 1991; Mamenta *et al.*, 1994). Enhanced replicative bypass (the ability to synthesize DNA past the site of DNA damage) has been proposed as a potential mechanism leading to adduct tolerance.

DNA damaging agents can induce arrest of proliferating cells at several points in the cell cycle. Such cell cycle arrests have been proposed to allow sufficient time for repair of potentially lethal damage (Hartwell, 1992; Lane, 1992). However, it is also possible that these arrests may be prerequisite events necessary for programmed cell death mechanisms. Cisplatin induces a number of cell cycle perturbations including a slow down in S-phase transit and a dose-dependent arrest in G2 (Demarcq *et al.*, 1992; Ormerod *et al.*, 1994). In fact, the presence of a G2 arrest has been shown to be crucial in allowing cell death to occur by apoptosis in response to cisplatin (Eastman, 1990). Therefore, loss of a cisplatin induced G2 arrest in cisplatin selected cell lines may be a possible mechanism of increased resistance. Data concerning the existence of a cisplatin induced G1 arrest is controversial (Brown *et al.*, 1995). Some cell cycle studies on cisplatin treatment of mammalian cells exhibit no evidence of a G1 arrest induced by cisplatin (Demarcq *et al.*, 1992; Ormerod *et al.*, 1994).

In contrast, work from our laboratory showed that A2780 cells, which possess wild-type p53 and a functional ionising radiation induced G1 arrest, arrested at G1 after exposure to cisplatin (Brown *et al.*, 1993). In fact, this cisplatin induced G1 arrest was lost after transfection of a dominant negative p53 mutant (codon 143, Val to Ala) into these cells, which is known to abrogate an ionising radiation induced G1 arrest (McIlwrath *et al.*, 1994; Vasey and Brown, *personal communication*). Such mutant p53 transfectants were more resistant to ionising radiation and cisplatin than vector alone controls (McIlwrath *et al.*, 1994; Vasey *et al.*, in press). Loss of a radiation induced G1 arrest and aberrant p53 protein expression have been observed in certain human ovarian

tumour cell lines selected for resistance to cisplatin (Brown *et al.*, 1993). These observations suggested that loss of p53 function may be associated with acquisition of cisplatin resistance. However, genetic inactivation of p53 in human breast MCF-7 cancer cells increased sensitivity to cisplatin (Fan *et al.*, 1995). These cells also displayed a reduced ionising radiation induced G1 arrest and it has been proposed that the increase in cisplatin sensitivity is due to reduced DNA repair. These apparently contradictory results may be explained by cell type or environmental differences between these model systems (see subsection 1.1.9 for more details).

Reduced apoptosis in response to DNA damage has been postulated as one of the molecular mechanisms leading to increased cisplatin resistance (Brown *et al.*, 1995). Cisplatin has been shown to induce apoptosis in a variety of cell systems (Evans and Dive, 1993; Gorczyca *et al.*, 1993a). As mentioned, the G2 arrest induced by cisplatin may be intimately linked with the ability of a cell to undergo apoptosis. However, the mechanism controlling the decision to enter apoptosis at G2 has still to be established. Intriguingly, cisplatin has been shown to induce rapid apoptosis in proliferating, but not quiescent, thymocytes (Evans *et al.*, 1994). Consequently, it has been proposed that for cisplatin induced cell death to occur, either progression is required through specific points in the cell cycle prior to entry into apoptosis or proliferation/DNA replication are necessary to convert DNA damage induced by cisplatin into a form that activates an apoptotic response (Brown *et al.*, 1995).

Immature thymocytes from TP53 null mice and dominant negative p53 mutant (codon 143, Val to Ala) transfectants of A2780 cells both displayed a reduced ability to

undergo apoptosis in response to cisplatin (Vasey *et al.*, in press). Introduction of wild-type p53 into human TP53 null lung cancer cells increased sensitivity to cisplatin (Fujiwara *et al.*, 1994). Notably, an increased level of cisplatin induced apoptosis was observed in these cells. Recent evidence has suggested an association between the development of cisplatin resistance, mutation of the TP53 gene and reduced BAX expression in ovarian cancer (Perego *et al.*, 1996). Furthermore, exogenous expression of BCL-2 in A2780 cells resulted in protection from cisplatin induced apoptosis (Eliopoulos *et al.*, 1995). Cisplatin has been shown to induce the expression of interleukin 1β-converting enzyme (ICE), a mammalian homologue of the *Caenohabiditis elegans* cell death gene CED-3 (Kondo *et al.*, 1995). Interestingly, the induction of ICE by cisplatin was independent of p53. In summary, cisplatin can induce apoptosis through both p53-dependent and -independent pathways.

1.3 GENETIC SUPPRESSOR ELEMENTS

1.3.1 CONCEPT AND BACKGROUND

Genetic suppressor elements (GSEs) are short DNA fragments that induce a biological effect when expressed in cells by encoding either dominant negative mutant peptides or antisense RNA molecules (Roninson *et al.*, 1995). GSEs function to inhibit the activity of the gene from which they are derived. Both types of GSE may be generated by random fragmentation of the target gene(s) and identified by functional selection for a desired phenotype in a suitable cellular system. Selection of GSEs from a single gene allows the identification of functional protein domains. In this way, efficient and specific modulators of gene function may be developed. GSE selection from multigene libraries may identify genes that are involved in the selectable cellular phenotypes, e.g. drug resistance.

Igor Roninson and co-workers initially used bacteriophage λ as a model system to determine the efficacy of random fragment selection as an approach to isolate GSEs (Holzmayer *et al.*, 1992). Bacteriophage λ efficiently lyses infected *E. coli* cells, thus providing a convenient strategy for selecting GSEs that would interfere with phage infection. λ DNA was randomly fragmented with DNaseI and cloned into a bacterial plasmid expression vector. An expression library of λ DNA fragments was transformed into *E. coli* cells. The presence of the random fragment expression library resulted in increased protection from λ-induced lysis. Plasmids extracted from surviving cells were individually tested for the ability to render *E. coli* resistant to λ upon re-transformation.

Sequence analysis of 51 GSEs isolated by this selection indicated that they were derived from eleven different regions of the λ genome and encoded either specific peptides or antisense RNA molecules. Investigation of these GSEs revealed some previously unknown functions of bacteriophage λ, including suppression of the cellular λ receptor by an uncharacterised gene of the phage. These experiments unveiled the power of the GSE approach for exposing new biological information, even in a very thoroughly investigated system.

1.3.2 GSE SELECTION IN MAMMALIAN CELLS

The methodology for conducting GSE selection in mammalian cells is shown in Figure 3. The cDNA of interest is randomly fragmented using DNaseI and cloned into a retroviral expression vector system. The resulting random fragment expression library is transfected into a packaging cell line which produces infectious virus. Viral supernatant is collected and used to infect a cell line of interest. Selection of infectant cells for the desired phenotype results in enrichment of biologically active GSEs. Putative GSEs are isolated and sequenced by PCR amplification using primers spanning the cloning site. Verification of a particular phenotypic effect of a GSE must be shown by independent re-introduction into the target cell line.

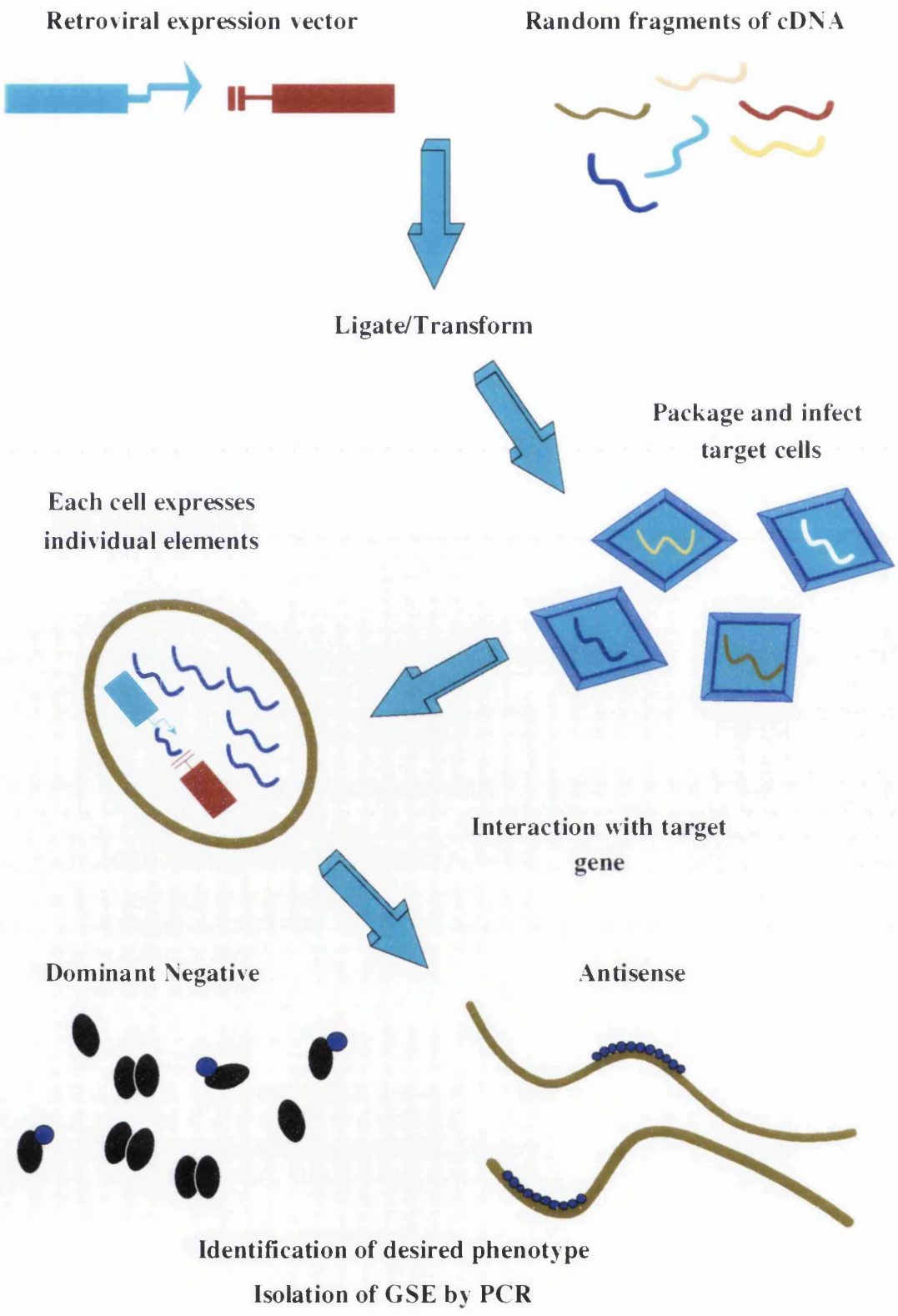


Figure 3. Generalised approach for the isolation of genetic suppressor elements.

Retroviral transduction has a number of advantages over more commonly used DNA transfection procedures, primarily the relatively high efficiency of stable gene transfer and that most of the recipient cells acquire only a single copy of the integrated provirus (Coffin *et al.*, 1996). One drawback of using retroviral transduction with complex libraries is the problem of maintaining equal representation of different clones in the library. Therefore, it is important to check for even distribution of proviral inserts in every infected cell population.

The first application of GSE selection in mammalian cells was performed using drug resistance as the selectable cellular phenotype (Gudkov *et al.*, 1993). A total of twelve sense- and antisense-oriented GSEs were isolated from human topoisomerase II cDNA which induce resistance in HeLa cells to a number of topo II-interactive drugs, including etoposide. Most of the predicted peptides encoded by the sense-oriented GSEs were clustered in a region involved in topo II dimerisation and topo II-DNA interactions. Consequently, such GSEs are thought likely to interfere with drug induced cleavable complex formation (Schneider *et al.*, 1990). Frameshift mutants of two sense-oriented GSEs were unable to induce etoposide resistance in HeLa cells indicating that these GSEs acted through topo II-derived peptides (Gudkov *et al.*, 1993). Expression of one antisense-oriented GSE resulted in a decreased level of topo II protein. These results demonstrated that random fragment selection could be efficiently utilised in mammalian cells to isolate biologically active GSEs.

This approach was extended to isolate GSEs from a complex population of mammalian genes (Gudkov *et al.*, 1994; Kirschling *et al.*, 1994). Normalised cDNA

libraries were prepared from mouse NIH 3T3 and human HeLa cells. The first GSE selection was carried out with the NIH 3T3 library using etoposide (Gudkov *et al.*, 1994). Three different GSEs were isolated, one of which encoded antisense RNA for a heavy chain of the motor protein kinesin. Although not previously implicated in drug response, kinesin was subsequently shown to be downregulated in several etoposide resistant cell lines. Therefore, GSE selection exposed a natural mechanism for etoposide resistance in mammalian cells. A number of GSEs have been isolated from the HeLa library which confer cisplatin resistance (Kirschling *et al.*, 1994). Some of these GSEs are derived from unknown genes and others from genes not previously associated with drug resistance. Other selectable cellular phenotypes have been employed apart from drug resistance to isolate GSEs, including the immortalisation of primary embryo fibroblasts (Roninson *et al.*, 1995). In summary, random fragment libraries obtained from total cellular DNA can be used to isolate GSEs derived from previously unknown genes and which confer different selectable phenotypes.

1.4 AIMS

This thesis has two main objectives:

1. To characterise the p53-mediated DNA damage response pathway in cisplatin resistant derivatives of the human ovarian A2780 carcinoma cell line (**Chapter 3**).

2. To identify genetic suppressor elements derived from TP53 cDNA which confer resistance to cisplatin when introduced into A2780 cells (**Chapter 4**).

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 Gibco BRL, BDH Chemicals, Pharmacia LKB, Severn Biotech, Rathburn or Sigma Chemicals.

2.1.2 RADIOCHEMICALS

(α^{32}P)dCTP used for labelling DNA probes was obtained from Amersham International.

2.1.3 EQUIPMENT

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

Cell counter	Coulter Electronics
Confocal microscope	BioRad
Electrophoresis tanks	IBI
	Cambridge
	BioRad

Electroblotting system	Millipore
FACScan flow cytometer	Becton Dickenson
Film processor	Kodak
Hybridisation membranes	Amersham International Millipore
Hybridisation oven and bottles	Hybaid
Laser densitometer	Molecular Dynamics
Liquid scintillation analyzer	Packard
PCR machines	Hybaid Perkin Elmer
Sequencer	Applied Biosystems
UV transilluminator/imager	Appligene

2.1.4 RESTRICTION ENDONUCLEASES AND OTHER ENZYMES

Restriction endonucleases and other enymes were obtained from Gibco BRL and Promega.

2.1.5 SIZE MARKERS

DNA size markers

Phage λ (HindIII)	Gibco BRL
100bp DNA ladder	Gibco BRL

RNA size markers

0.24-9.5kbp RNA ladder	Gibco BRL

Protein size markers

29.5-214kDa prestained	Gibco BRL

2.1.6 BUFFERS, SOLUTIONS AND MEDIA

Where mentioned, autoclaving was carried out at 121^0C for 20 minutes.

Blocking solution

5% Carnation non-fat milk powder

50mM Tris HCl pH 7.5

50mM NaCl

1mM EDTA

0.01% Tween-20

1mM DTT

Cacodylate buffer

0.2M Potassium cacodylate

2.5mM Tris HCl pH 6.6

2.5mM $CoCl_2$

0.25mg/ml BSA

5U Terminal transferase

0.5nmoles Biotin-dUTP

DEPC treated water (0.1%)

999ml Double distilled water

1ml DEPC

Leave at 37^0C for 24h

Autoclaved solution

DNA loading dye

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol

DNaseI buffer (10x)

500mM Tris HCl pH 7.5

100mM $MnCl_2$

10mg/ml BSA

High salt lysis buffer

500mM NaCl

1% NP-40

50mM Tris pH 7.5

Protease inhibitors (1x)

Hybridisation buffer

50mM PIPES

50mM NaH_2PO_4

50mM Na_2HPO_4

100mM NaCl

1mM EDTA

5% SDS

Made with DEPC treated water

L-broth

1% Tryptone

0.5% Yeast extract

1% NaCl

MOPS RNA running buffer (10x)

0.2M 4-(N-Morpholino)propane-sulphonic acid

0.05M Na Acetate pH 7.0

0.01M Na_2EDTA

Made with DEPC treated water

pH to 7 with glacial acetic acid

Autoclaved solution

PBT

PBS

0.1% Tween-20

1.0% BSA fraction V

Phosphate buffered saline (PBS)

0.8% NaCl

0.115% Na_2HP0_4

0.02% KCl

0.02% KH_2PO_4

Protease inhibitors (100x)

0.1mg/ml Aprotinin

0.1mg/ml Pepstatin

0.1mg/ml Chymostatin

0.05M Benzamidine

0.05M PMSF

0.1mg/ml Leupeptin

Stored at -70^{0}C

RNA loading dye

95% Formamide

20mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol

0.1 Volume of 50% glycerol

RPMI

88ml RPMI-1640 (10x)

800ml Sterile distilled water

26.6ml 7.5% $Na(CO_3)_2$

10ml 100mM Na Pyruvate

10ml 200mM L-glutamine

3.5ml 1M NaOH

100ml Foetal calf serum

5ml Penicillin/Streptomycin (50mg)

Running gel buffer

1.5M Tris base

0.4% SDS

pH to 8.9 with concentrated HCl

Running gel (8%)

8ml Running gel buffer

8.5ml 30% Acrylamide, 0.8% bis-acrylamide

3.2ml 1% Polyacrylamide

12.3ml Distilled water

120µl 10% APS

15µl TEMED

Spacer gel buffer

0.5M Tris base

0.4% SDS

pH to 6.7 with concentrated HCl

Spacer gel (4%)

3ml Spacer gel buffer

1.6ml 30% Acrylamide, 0.8% bis-acrylamide

1.2ml 1% Polyacrylamide

4.8ml Distilled water

180µl 10% APS

15µl TEMED

Special liquid medium

500ml Special liquid medium

50ml 200mM L-glutamine

50ml Foetal calf serum

2.5ml Penicillin/Streptomycin (25mg)

SSC (20x)

3M NaCl

0.3M Tri-sodium citrate

Tank buffer (5x)

0.25M Tris base

0.5M Glycine

0.5% SDS

TBE (1x)

89mM Tris borate

89mM Boric acid

2.5mM EDTA

Transfer buffer

48mM Tris base

39mM Glycine

0.038% SDS

20% Methanol

Trypsin

360ml Sterile distilled water

40ml 10x PE

40ml Trypsin (2.5% stock)

Wash buffer

1x SSC

5% SDS

Western loading dye

4% SDS

50% Spacer gel buffer

19mM EDTA

20% Glycerol

5% β-mercaptoethanol

0.25% Bromophenol blue

2.1.7 CELL LINES

A2780

A human ovarian adenocarcinoma cell line derived from an untreated patient received from R. F. Ozols and T. C. Hamilton, Fox Chase Cancer Centre, Philadelphia.

A2780/CP70

A cisplatin resistant derivative of A2780 cells isolated after two years selection with multiple exposures to the drug. For a more complete description of A2780 and A2780/CP70 cell lines see Behrens *et al.*, 1987. Both cell lines have previously been shown to express only wild-type p53 (Brown *et al.*, 1993).

A2780/MCP1-9

Independent multiple step selected cisplatin resistant derivatives of A2780 (Anthoney *et al.*, 1996).

PA317

An amphotropic virus packaging line derived from NIH/3T3 TK^- cells by cotransfection with retrovirus packaging construct DNA (pPAM3) and the herpes simplex thymidine kinase gene (Miller and Buttimore, 1986).

2.1.8 ANTIBODIES AND IMMUNOLOGICAL REAGENTS

The following is a list of the primary antibodies used for Western blotting and immunocytochemistry.

Antigen	**Antibody**	**Isotype**	**Company**
mdm2	IF2	Mouse IgG_{2b}	Oncogene Science
p53	DO-1	Mouse IgG_{2a}	Oncogene Science
p53	PAb122	Mouse IgG_{2b}	Pharmingen
cyclin E	HE12	Mouse IgG_1	Pharmingen
cyclin B1	GNS-1	Mouse IgG_1	Pharmingen
vinculin	VIN-11-1	Mouse IgG_1	Sigma
BrdUrd	Bu20a	Mouse IgG_1	Dako

Anti-mouse IgG horseradish peroxidase-linked (HRPL) rabbit antibody (Amersham) was used as a secondary agent in Western blotting studies; whereas anti-mouse IgG fluorescein 5-isothiocyanate-conjugated (FITC) goat antibody (Sigma) was used in immunocytochemistry approaches.

2.1.9 PLASMIDS AND MOLECULAR PROBES

The following is a list of the plasmids used for GSE library construction and Northern blotting, respectively. Molecular probes were derived from DNA fragments obtained using the indicated restriction enzymes.

Plasmid	**cDNA**	**Probe fragment**	**Reference**
pC53-SN3	Human TP53		Baker *et al.*, 1990
pMT-P53-T	Murine TP53		Arai *et al.*, 1986
pCEP-WAF-1-S	Human CIP1	NotI (2100bp)	El-Deiry *et al.*, 1993
pFL4-MDM2	Human MDM2	HindIII (870bp)	Oliner *et al.*, 1992
pCRII-GAPDH	Human GAPDH	EcoRI (720bp)	Tso *et al.*, 1985
pLNCX	Retroviral Vector		Miller and Rosman, 1989

The pLNCX retroviral expression vector plasmid was used in the construction of the random fragment p53 DNaseI GSE libraries (section 2.10). A schematic representation of an integrated LNCX provirus containing a fragment of TP53 cDNA is displayed in Figure 4.

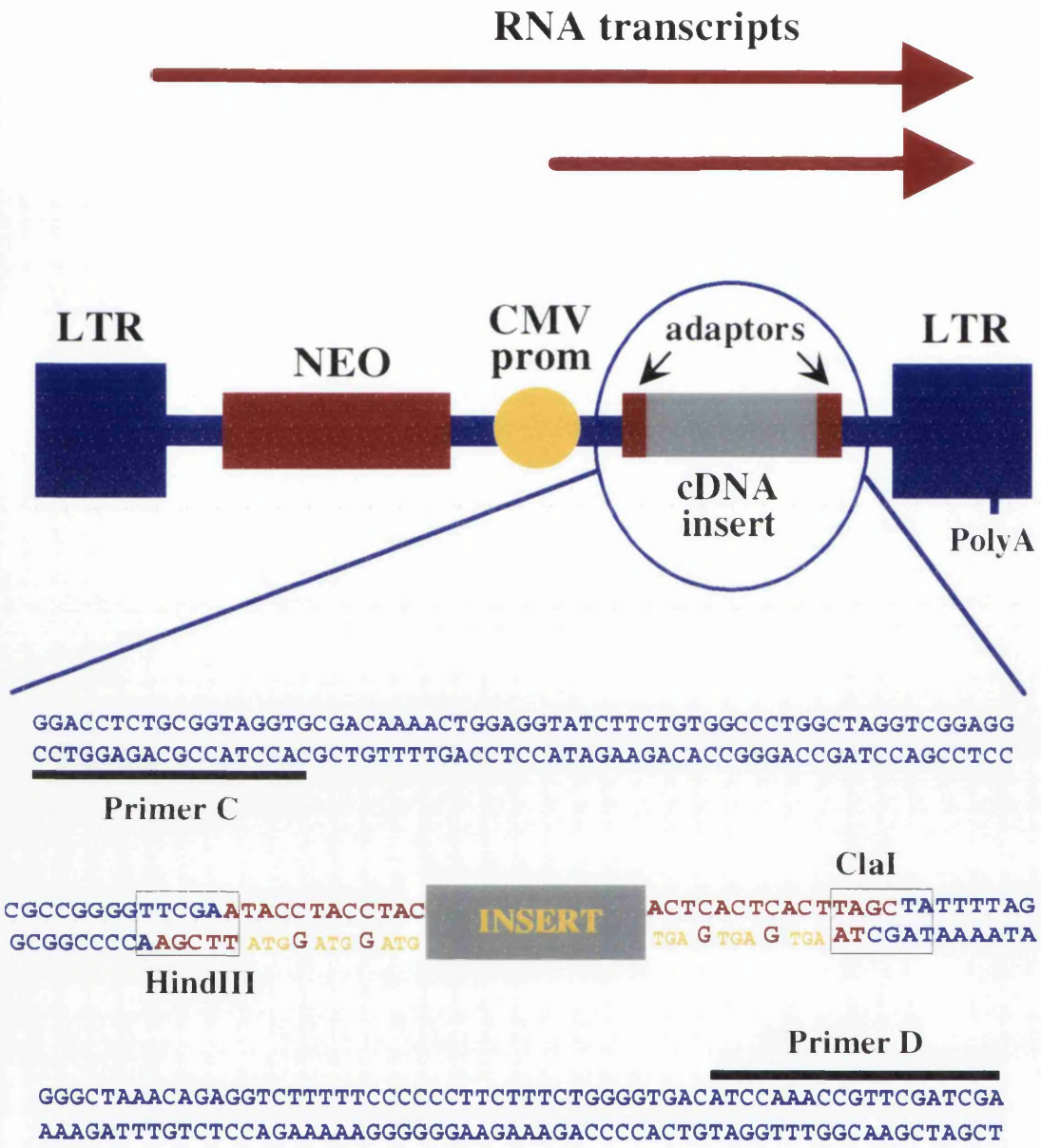


Figure 4. An integrated LNCX provirus containing a fragment of TP53 cDNA.

The LNCX vector carries the NEO (G418 resistance) gene transcribed from the long terminal repeat (LTR) promoter, and the cDNA insert transcribed from the cytomegalovirus (CMV) promoter. Adaptor-derived sequences are displayed in red, with translation initiation and termination codons shown in yellow. Sequences used as PCR primers for isolation and sequencing of the inserts are underlined.

2.1.10 OLIGOMERS

The following is a list of oligomers used in the generation and characterisation of the genetic suppressor element libraries.

Primer A	5'-TACCGAATTCAAGCTTATGGATGGATG-3'
Primer B	5'-CATCCATCCATAAGCTTGAATTC-3'
Primer C	5'-TGAGTGAGTGAATCGATGGATCC-3'
Primer D	5'-TATAGGATCCATCGATTCACTCACTCA-3'
Primer E	5'-CCTGGAGACGCCATCCAC-3'
Primer F	5'-AGCTAGCTTGCCAAACCTA-3'

2.2 EXTRACTION OF NUCLEIC ACID AND PROTEIN

All work with RNA and DNA was carried out using autoclaved solutions and where appopriate DEPC treated solutions and equipment. Disposable gloves and plasticware were used throughout. DNA and RNA quantitation was performed using a combination of visual assessment on a 1% agarose, 1x TBE, 0.25μg/ml ethidium bromide gel when run against known standards and spectrophotometrically using OD_{260nm}. Extraction protocols were followed as per the manufacturers instructions.

2.2.1 RNA EXTRACTION

Total RNA was extracted from monolayer cultures using TRIzol reagent (Gibco).

2.2.2 DNA EXTRACTION

Genomic DNA was extracted from monolayer cultures using the Nucleon I kit (Scotlab). Plasmid DNA was extracted from large (50-500ml) and small (1-2ml) scale bacterial cell cultures using the Maxi (Qiagen) and SpinBind (Flowgen) kits, respectively.

2.2.3 PROTEIN EXTRACTION

Unless otherwise stated, cells were washed with PBS, then lysed at 4^0C for 10min using high salt lysis buffer supplemented with protease inhibitors. The lysate was spun at 12,000g for 10min and the supernatant stored at -70^0C. 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

2.3.1 RANDOM PRIMING OF DOUBLE-STRANDED DNA

(α^{32}P)dCTP labelled dsDNA probes (50-100ng) were produced with the aid of the Prime-It kit (Stratagene).

2.3.2 NICK COLUMN PURIFICATION

Radiolabelled probes were separated from unincorporated ^{32}P-labelled nucleotides using disposable NICK columns (Pharmacia).

2.3.3 DETERMINATION OF SPECIFIC ACTIVITY OF PROBE

The specific activity of radiolabelled probes was determined by taking a small aliquot (5µl) and measuring the number of disintegrations per minute using a liquid scintillation analyzer.

2.3.4 DENATURATION OF PROBES

Probes were denatured before hybridisation by adding 0.1 volume of 3M NaOH and leaving for 5min at room temperature. After a 5min incubation on ice with 0.05 volume of 1M tris HCl pH 7.5, 0.1 volume of 3M HCl was added and left for a further 5min.

2.4 SEPARATION AND HYBRIDISATION OF RNA

2.4.1 SEPARATION AND NORTHERN TRANSFER OF RNA

Methodology for separation and transfer of RNA emulated instructions detailed in Sambrook *et al.*, 1989. 1.2% (w/v) agarose gels were prepared by dissolving 2.4g of agarose in 150ml of water then cooling to 60^{0}C. 19.5ml of 10x MOPS RNA running buffer and 36ml of 37% formaldehyde were added and the mixture immediately poured.

To 20μg of total RNA in 12μl, 25μl formamide, 8μl formaldehyde and 5μl 10x MOPS RNA running buffer were added. The samples were heated to 65^0C for 15min and chilled on ice before addition of 5μl RNA gel loading buffer. After electrophoresis for 4h at 200V, the gels were imaged by an UV transilliuminator and soaked in 50mM NaOH for 20min. They were rinsed with DEPC treated water and soaked in 20x SSC for 45min prior to transfer onto Hybond-N membranes using 20x SSC as the transfer buffer. Membranes were rinsed and exposed to UV for 6min to allow fixation.

2.4.2 HYBRIDISATION OF BLOTS

Pre-hybridisation of blots was carried out at 65^0C using hybridisation buffer.

2.4.3 WASHING

Following hybridisation, filters were washed at 65^0C using wash buffer until the background level of radioactivity was reduced sufficiently.

2.4.4 AUTORADIOGRAPHY

Following washing, blots were blotted dry, wrapped in clingfilm and exposed to Kodak AR film in a film cassette with tungstate intensifying screens. Loaded film cassettes were held at -70^0C until developed.

2.5 SEPARATION AND IMMUNODETECTION OF PROTEINS

2.5.1 SAMPLE PREPARATION

Protein extracts for one-dimensional gel electrophoresis were prepared and quantified as detailed in section 2.2.3. 50μg of total protein was loaded per lane in western loading dye after boiling for 3min to facilitate denaturation. Cell extracts were prepared for two-dimensional gel electrophoresis using the following protocol. After two washes with PBS, 2 x 10^5 cells were harvested directly into 240μl loading buffer [9.49M urea, 4% NP-40, 5.5% ampholytes pH 3-10/2D optimised (Millipore), 0.1% DTT, 0.003% bromophenol blue].

2.5.2 ONE DIMENSIONAL GEL ELECTROPHORESIS

Solubilised protein, including size markers, were separated by SDS-polyacrylamide gel electrophesis on an 8% running gel after passing through a preliminary 4% stacking gel both suspended in 1x tank buffer.

2.5.3 TWO DIMENSIONAL GEL ELECTROPHORESIS

Isoelectricfocusing gels [9.5M urea, 2% NP-40, 2% ampholytes pH 3-10, 4% polyacrylamide (acrylamide/bis 30.8%T, 2%C), 0.05% APS] were overlayed [0.5M urea, 0.2% NP-40, 0.1% ampholytes pH 3-10, 5mM DTT, 0.7M 2-mercaptoethanol], and prefocused to 1,500V, then loaded (50μl), and run overnight to 18,000Volt hours,

before being equilibrated [0.3M Tris base, 75mM Tris HCl, 3% SDS, 50mM DTT, 0.001% bromophenol blue] and run out on polyacrylamide slab gels [10% polyacrylamide (acrylamide/bis 30.8%T, 2.6%C), 0.37M Tris buffer pH 8.8, 0.1% SDS, 0.05% TEMED, 0.025% APS, overlayed with butan-2-ol] at 16W/gel.

2.5.4 WESTERN TRANSFER BY ELECTROBLOTTING

Electroblotting was performed using a semi-dry electroblotter. Immobilin-P membrane was immersed in methanol and then transfer buffer. Six sheets of 3M Whatman filter paper was sandwiched adjacent to the anode and cathode with the membrane and gel layered in between. Transfer took place over 1h at 200mA. The gel was stained in Coomasie stain overnight and destained as described in section 2.2.3. This allowed a visual assessment to be made of the eveness of the transfer and the integrity of the proteins.

2.5.5 IMMUNODETECTION OF PROTEINS ON WESTERN BLOTS

Membranes were incubated with blocking solution at 4^0C for 4h, probed overnight in the same buffer with primary antibody and washed with 0.1% Tween-20 in PBS. Blots were incubated in blocking solution with anti-mouse IgG HRPL rabbit antibody, then washed again in 0.1% Tween-20 in PBS, after which bound complexes were visualised by enhanced chemiluminescence (Boehringer Mannheim).

2.5.6 ELISA ANALYSIS OF p53 PROTEIN LEVELS

For ELISA analysis, a sandwich immunoassay was used to measure p53 protein levels in cell extracts as described previously (Vojtesek *et al.*, 1992) with anti-p53 MAb DO-1 as the solid-phase reagent and polyclonal rabbit antisera to p53 for the detection of captured proteins. Bound complexes were detected using peroxidase-conjugated swine antiserum to rabbit immunoglobulin (Dako) and visualised with tetramethylbenzidine; the results were then monitored by changes in OD_{450nm}.

2.6 IMMUNOCYTOCHEMISTRY

2.6.1 GENERAL IMMUNOFLUORESCENCE TECHNIQUE

Cells were seeded at 10^6 cells/10cm plate and grown for 3-4 days. Fixation was achieved 24h later when the cells were rinsed with PBS and resuspended in 80% ethanol (precooled to -20^{0}C). The samples were centrifuged, washed with PBS and treated with 0.25% Triton X-100 in PBS for 5min on ice. After addition of 5ml PBS and centrifugation, the cells were incubated overnight at 4^{0}C in the presence of mouse monoclonal primary antibody which was diluted to 5μg/ml in PBT. Cells were washed and incubated for 30min with anti-mouse IgG FITC goat antibody diluted 1:40 in PBT. Controls were prepared as described above, except that a mouse isotype specific

antibody was used instead of the primary antibody. Cells were analysed via flow cytometry and confocal microscopy.

2.6.2 CELL CYCLE ASSAY

DNA synthesis was assessed by incorporation of bromodeoxyuridine (BrdUrd) and flow cytometric analysis essentially as previously described (Kastan *et al.*, 1991). Exponentially growing cells were plated at $5x10^5$/10cm plate in growth medium and incubated for 3-4 days. Cells were either irradiated with γ-rays from a ^{60}Co source or exposed to cisplatin for 1h. At various times after treatment, growth medium was replaced by medium containing 10μM BrdUrd and the cells were incubated for 4h at 37^0C. The cells were fixed with ethanol, washed with PBS and resuspended in 1ml 2N HCl for 30min at room temperature. The partially denatured cells were washed twice in PBS and once in PBT and then incubated with an anti-BrdUrd MAb Bu20a. The cells were washed with PBT and incubated with anti-mouse IgG FITC goat antibody. After a further wash in PBT, cells were analysed via flow cytometry.

2.6.3 APOPTOSIS ASSAY

Apoptotic cells were detected as previously described (Gorczyca *et al.*, 1993b). Exponentially growing cells were treated with DNA damaging agents and harvested 72h later. Monolayer cells were trypsinised, combined with suspension cells and fixed in 1% formaldehyde for 15min on ice. The cells were resuspended in PBS and finally stored in 70% ethanol at 4^0C prior to detection of non-random DNA strand breaks. Cells were

rehydrated in PBS and aliquots of 10^6 cells were incubated for 30min at 37^0C with cacodylate buffer containing terminal transferase and biotin-dUTP. After washing in PBS, cells were incubated for 30min at room temperature in the dark with 4x SSC and 0.1% Triton X-100 containing 5% Marvel and 5μg/ml FITC-avidin. After a further wash in PBS and 0.1% Triton-X, cells were analysed via flow cytometry.

2.6.4 FLOW CYTOMETRY

After FITC staining, cells were washed with PBS and resuspended in 10μg/ml of propidium iodide (PI) in PBS and stored at 4^0C until analysis. Cellular fluorescence was measured using a FACScan flow cytometer. The red (PI) and green (FITC) emissions from each cell were separated and quantified using the standard optics of the FACScan.

2.6.5 CONFOCAL MICROSCOPY

Small aliquots of cell suspension (20μl) were taken after secondary antibody staining and placed onto microscope slides. After dehydration, the slides were mounted with Vectashield anti-fade mountant H-1000 (Vector Labs) with 0.3μg/ml PI added. Analysis of slides was performed on a laser scanning confocal microscope equipped with a krypton/argon ion laser. For FITC and PI, 488/568nm line excitation and dual channel 522 and 585nm emission filters were used. Image analysis was performed using BioRad software.

2.7 TISSUE CULTURE TECHNIQUES

2.7.1 GENERAL TECHNIQUES

Aseptic manipulations were performed using sterilised glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown at 37^0C as monolayers in supplemented RPMI medium (Rosswell Park Memorial Institute) in the presence of 5% CO_2. Cells were frozen at a concentration of 10^6/ml with 10% di-methyl sulphoxide (DMSO) at -70^0C. After 24h, samples were transferred to liquid nitrogen.

2.7.2 CLONOGENIC ASSAY

Cells were seeded at 10^3/10cm plate and incubated at 37^0C for 24h. Following cisplatin treatment, colonies were grown for 10-14 days then stained with Giemsa (BDH) for 10min and rinsed with H_2O.

2.7.3 MTT ASSAY

Cells were seeded in 96-well plates at 500 cells per well and incubated for 3 days. After cisplatin treatment (24h), medium was replenished 3 times every 24h. Relative cell numbers were measured using 3-(4,5-dimethiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide staining as described (Plumb *et al.*, 1989).

2.7.4 TRANSFECTION OF CELL LINES WITH PLASMID DNA

Cells were seeded at 10^6/T75 flask and incubated at 37^0C in appropriately supplemented SLM for 24h. Medium was replenished 3h before transfection. Plasmid DNA (20μg/sample) was introduced into the cells using a standard calcium phosphate procedure (Wigler *et al.*, 1977). After a 24h incubation, the medium containing the DNA precipitate was removed and fresh medium added for another 24h. The transfectant cells were selected with 750μg/ml G418.

2.7.5 INFECTION OF CELL LINES WITH VIRAL SUPERNATANT

Cell lines to be infected were seeded at 10^6/T75 flask and incubated at 37^0C for 24h. Viral supernatant collected from transfected packaging cells was passed through a 0.45μm sterile filter (Gelman Sciences) and used to infect target cells. Superinfection was achieved by repeated addition of virus. After a 24h incubation, the cells were placed under selective conditions.

2.7.6 LIPOSOME-MEDIATED PEPTIDE TRANSFER

Synthetic peptides (1 or 10μM final concentration) were transfected into A2780 cells using the cationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl] *N,N,N*-trimethylammoniummethylsulfate: Boehringer Mannheim) at 6μg/ml final concentration (Chen *et al.*, 1993). Lipid and peptide mixtures were incubated for 15min at room temperature, before being added to fresh medium and directly loaded onto cells.

2.8 MICROBIOLOGICAL TECHNIQUES

2.8.1 BACTERIAL CULTURE

Bacteria were maintained either in liquid culture or on plates under selective conditions. Cells were stored for long periods in 15% glycerol in L-broth at -70^{0}C.

2.8.2 TRANSFORMATION OF BACTERIA WITH PLASMID DNA

50-100μg of plasmid DNA was added to 100μl of MAX efficiency DH5α competent cells (Gibco) and left on ice for 30 minutes. After a 5min heat-shock at 37^{0}C, 900μl of L-broth was added and the samples vortexed before incubation at 37^{0}C for 1h. Appropriate dilutions were plated on selective medium [1.5% L-agar, 100μg/ml ampicillin] using sterile hockey sticks. Plates were incubated overnight at 37^{0}C.

2.9 GENERAL MOLECULAR BIOLOGY TECHNIQUES

2.9.1 GENERAL PURIFICATION OF DNA

DNA was purified using either of two methods depending upon downstream applications - ethanol precipitation or the Wizard DNA clean-up kit (Promega). The former procedure required the addition of 0.1vol 3M sodium acetate, 2.5vol EtOH and 1μl glycogen to a sample containing DNA. After precipitation and isolation, the DNA was washed with 70% EtOH and resuspended in H_2O.

2.9.2 ELECTROELUTION OF DNA FROM AGAROSE

DNA fragments were isolated from agarose gels by electroelution into dialysis bags using a previously described protocol (Sambrook *et al.*, 1989).

2.9.3 REMOVAL OF PRIMERS FROM PCR PRODUCTS

Primer dimers were removed from PCR products using the Wizard PCR preps kit (Promega).

2.9.4 STANDARD PCR PROTOCOL

Each standard PCR reaction consisted of 100-1000ng template DNA, 1x PCR reaction buffer (contains Mg^{+}), 0.8mM dNTP, 1μg each primer, 5U Taq DNA polymerase with the volume adjusted to 100μl. Reactions conditions were as stated in the appropriate sections.

2.9.5 AUTOMATED DNA SEQUENCING

Samples were sequenced using the PRISM dye terminator cycle sequencing ready reaction kit (ABI). Purified PCR products (300-500ng) were mixed with 3.2pmoles of each primer (E and F) in separate reactions and the volume made up to 12μl with H_2O. 8μl of terminator ready reaction mix was added to each sample before undergoing the following PCR protocol:

Preheat to 96^0C
96^0C x 15sec
50^0C x 1sec
60^0C x 4min
25 cycles
Soak 4^0C

Excess dye terminators were removed using ethanol precipitation and samples were loaded in appropriate buffer on an automated DNA sequencer and run overnight. Sequence information was examined using an ABI data analysis program (version 1.2.1).

2.10 GENERATION OF RANDOM FRAGMENT p53 PLASMID LIBRARIES

2.10.1 RANDOM DNaseI DIGESTION OF PLASMID DNA

Plasmids containing human and murine TP53 cDNA were randomly fragmented by digestion with DNaseI as previously described (Holzmayer *et al.*, 1992). Each reaction sample consisted of 5μl DNA (5-10μg), 2.6μl 10x DNaseI buffer and 4μl DNaseI with the volume adjusted to 30μl. Reactions were incubated for 30min at 16^0C and stopped by the addition of 4μl 500mM EDTA pH 8.0 to a final concentration of 5mM. Samples were purified using the Wizard DNA clean-up kit.

2.10.2 REPAIR OF FRAGMENTED DNA

The fragment termini were repaired with T4 DNA polymerase and Klenow fragment of DNA polymerase I. T4 DNA polymerase (10U) was added to 20μl DNaseI digested DNA, 4μl 10x One-Phor-All PLUS buffer (Pharmacia) and 2μl 10mM dNTP with the volume adjusted to 40μl. Enzyme reactions were incubated for 30min at 37^0C. Klenow-PolI (10U) was added, left for a further 15min at the same temperature and samples were purified as before.

2.10.3 BLUNT-END LIGATION OF ADAPTORS

Adaptors were prepared by denaturation and annealling of oligomer sets: adaptor1 - primers A and B; adaptor2 - primers C and D. 100μg of each primer were mixed and the volume adjusted to 1000μl with H_20. Samples were incubated for 10min at 90^0C. After a further 30min incubation at 70^0C, samples were allowed to cool down overnight to room temperature. DNA fragments (100ng) were then ligated with an excess amount of each adaptors (500ng) using T4 DNA ligase (2U), 4μl 5x T4 DNA ligase buffer and 2μl 10mg/ml BSA with the volume adjusted to 20μl. Reactions were left at room temperature overnight and purified as before.

2.10.4 PCR AMPLIFICATION OF ADAPTOR LIGATIONS

Adaptor ligations (5μl aliquots) were amplified using the standard PCR protocol (section 2.9.3) with primers A and D. Reaction conditions were as follows:

94^0C x 3min
94^0C x 1min
56^0C x 1min
72^0C x 1min
25 cycles
Soak 4^0C

PCR products were purified using the Wizard PCR preps kit.

2.10.5 RESTRICTION DIGEST OF VECTOR AND INSERTS

Both vector and inserts were sequentially digested using HindIII and ClaI. The initial reaction consisted of 5μg DNA, 10μl 10x React1 buffer (Gibco) and ClaI (50U) with the volume adjusted to 100μl. Samples were incubated for 4h at 37^0C. After purification, 50μl digested DNA was mixed with 10μl 10x React2 buffer and HindIII (50U) with the volume adjusted to 100μl. Samples were purified as before.

2.10.6 LIGATION OF INSERTS TO RETROVIRAL VECTOR

Random fragment inserts were ligated to the retroviral vector plasmid, pLNCX, using T4 DNA ligase. Reaction samples consisted of 1-20ng insert, 50-100ng vector, 2μl 5x T4 DNA ligase buffer, 1μl 10mg/ml BSA and T4 DNA ligase (1U) with the volume adjusted to 10μl. Vector alone controls were included also.

2.10.7 PCR AMPLIFICATION OF INSERT/VECTOR LIGATIONS

Insert/vector ligations (1µl aliquots) were amplified using the standard PCR protocol with primers E and F. Reaction conditions were as follows:

94^0C x 3min
94^0C x 1min
60^0C x 1min
72^0C x 1min
25 cycles
Soak 4^0C

2.10.8 GENERATION AND SCREENING OF LIBRARY CLONES

Samples were transformed into competent bacteria and plasmid DNA prepared. Plasmid libraries were generated which comprised of 70,000 and 40,000 independent recombinant clones (>90% inserts as determined by HindIII/ClaI digestion and PCR amplification) from human and murine TP53 cDNA plasmids, respectively.

2.11 IDENTIFICATION OF GSEs CONFERRING CISPLATIN RESISTANCE

2.11.1 LIBRARY TRANSDUCTION

PA317 cells were transfected with library DNA or insert-free pLNCX plasmid. Viral supernatant was collected from stable G418-resistant transfectants and used to infect A2780 cells.

2.11.2 CISPLATIN SELECTION

Stable A2780 infectants were seeded at 10^6/10cm plate, exposed to 10-20μM cisplatin for 24h and left for 10-14 days (plating efficiency controls were included). Resistant colonies were picked and expanded.

2.11.3 ISOLATION OF PUTATIVE GSEs FROM GENOMIC DNA

Proviral inserts were recovered from the genomic DNA of cisplatin resistant A2780 infectants by PCR with primers E and F. Reaction conditions were as follows:

94^0C x 3min
94^0C x 1min
60^0C x 1min
72^0C x 1min
35 cycles
Soak 4^0C

2.11.4 SUBCLONING OF PUTATIVE GSEs AND IDENTIFICATION

PCR products were re-cloned into the pLNCX vector plasmid maintaining original orientation of the insert. Subsequent to cloning, inserts were sequenced in both directions. Putative GSEs were compared against sequences present in the GenEMBL database using the GCG package. Sequence alignments of p53 GSEs versus human TP53 cDNA are shown in Appendix I. Plasmids containing p53 GSEs were transfected into A2780 cells, G418-resistant colonies were isolated and subjected to cisplatin resistance assays.

2.12 PEPTIDE STUDIES

2.12.1 PEPTIDE SYNTHESIS

Two peptides were chemically synthesized and purified >95% by HPLC (Affiniti Research). PEP1-F corresponded to the 17 amino acid sequence predicted by the short sense-orientated element GSE1. A control peptide was created which comprised of the same amino acids but in reverse order.

PEP1-F	H-MDVPPGSTKRALPNMSE-OH
PEP1-R	H-ESMNPLARKTSGPPVDM-OH

2.12.2 GEL MOBILITY SHIFT ASSAY

A 25µl reaction volume contained DNA binding buffer [20% glycerol, 25mM HEPES pH 7.6, 50mM KCl, 0.1% Triton X-100, 1mM DTT and 1mg/ml BSA] mixed with combinations of Baculovirus-expressed human p53 protein (Hansen *et al.*, 1996), radiolabelled oligonucleotide probe (comprised of the p53 DNA binding consensus site: El-Deiry *et al.*, 1992) and synthetic peptide. After incubation on ice for 30min, reaction products were processed by using native gel electrophoresis as indicated previously (Hupp *et al.*, 1992).

CHAPTER 3

DEFECTIVE p53-MEDIATED DNA DAMAGE RESPONSE PATHWAY IN CISPLATIN RESISTANT OVARIAN TUMOUR CELLS

3.1 INTRODUCTION

It has previously been demonstrated that expression of a dominant negative p53 mutant (codon 143, Val to Ala) in the human ovarian A2780 carcinoma cell line confers increased resistance to a variety of DNA damaging agents, including ionising radiation and cisplatin (McIlwrath *et al.*, 1994; Vasey *et al.*, in press). These mutant p53 transfectants also lost the ability to undergo either ionising radiation or cisplatin induced G1 arrest and apoptosis (McIlwrath *et al.*, 1994; McIlwrath, Vasey and Brown, *personal communication*). Therefore, both of these phenotypes are dependent upon an intact p53-mediated DNA damage response pathway in this particular cell line.

A number of cisplatin resistant A2780 derivatives have been isolated by multiple step selection with increasing concentrations of cisplatin (Behrens *et al.*, 1987; Anthoney *et al.*, 1996). This chapter investigates the role of p53 in the acquisition of cisplatin resistance in these cells. Various components of the p53-mediated DNA damage response pathway were studied in these cisplatin resistant cells. These include analysis of DNA damage (ionising radiation and cisplatin) induced cell cycle arrests and apoptosis, p53 protein accumulation and ability of p53 to transcriptionally activate certain target genes. All observations were compared with the sensitive parental A2780 cell line. Ionising radiation induced DNA damage provided a "direct" measure of p53 function in these cisplatin resistant cells without the problems associated with the determination of initial platinum-DNA adducts after cisplatin treatment.

3.2 REDUCED G1 ARREST AND APOPTOSIS

The effects of DNA damage on cell cycle progression may be analysed by flow cytometry in proliferating cells (Kastan *et al.*, 1991). Exponentially growing A2780 cells were treated with either ionising radiation or cisplatin and analysed 24h later by flow cytometry (Figure 5). After exposure to 2Gy γ-radiation, a decrease was observed in the proportion of A2780 cells which labelled positively after a 4h pulse of BrdUrd (Figure 5). These cells have entered S-phase from the G1-phase of the cell cycle and the observed decrease suggests an inhibition of DNA synthesis caused by a block at the G1/S boundary (Kastan *et al.*, 1991). Cells also accumulated in G2/M-phase of the cell cycle indicating a G2 arrest. It has been previously suggested that cisplatin does not induce a G1 arrest in mammalian cells (Demarcq *et al.*, 1992; Ormerod *et al.*, 1994). However, treatment of A2780 cells with 20μM/1h cisplatin induced both G1 and G2 arrests (Figure 5). Specifically, a notable reduction was observed in the number of cells entering S-phase from G1 after cisplatin treatment (i.e. those cells with a 2N DNA content). In addition, there was a simultaneous increase in the number of S-phase cells with a 4N DNA content. These data are consistent with a decrease in the G1/S transition, an increased period of transit time through S-phase and a subsequent G2 delay.

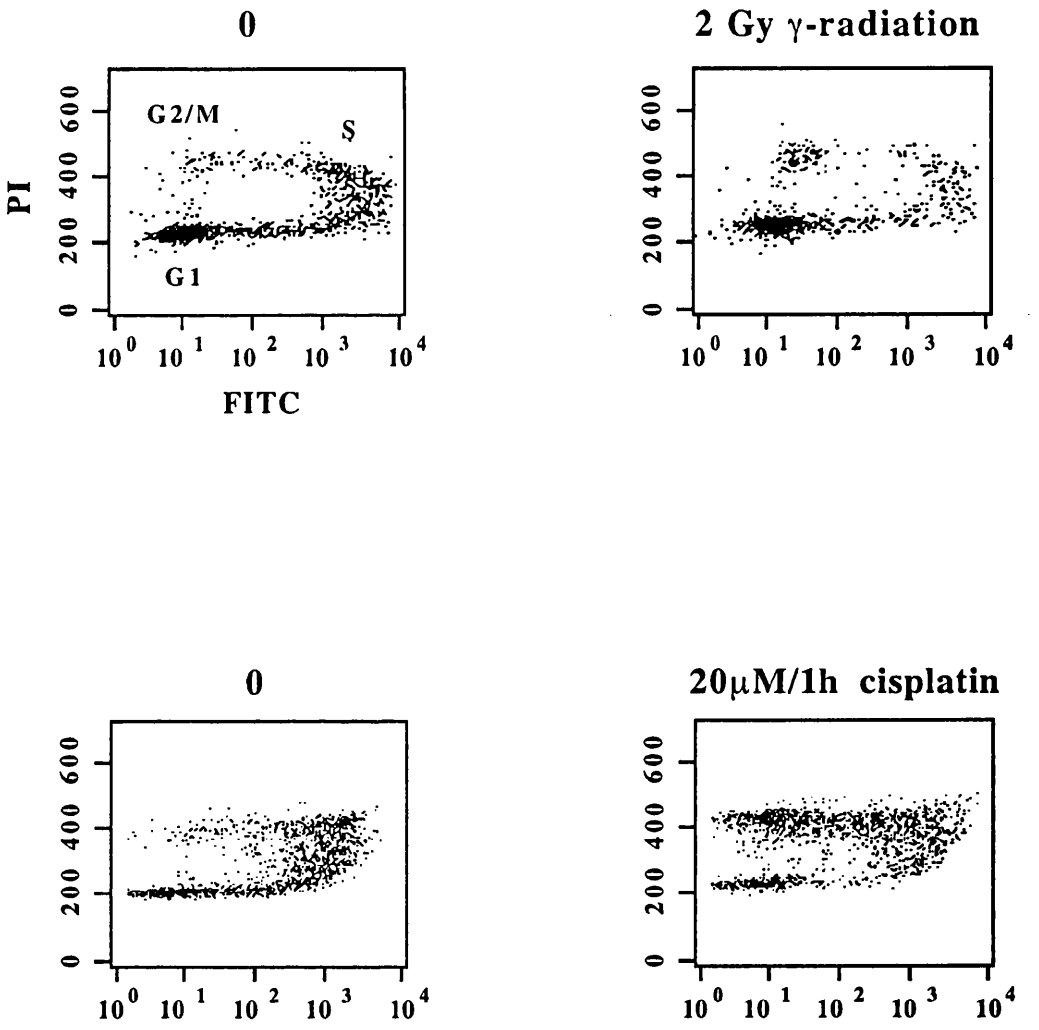


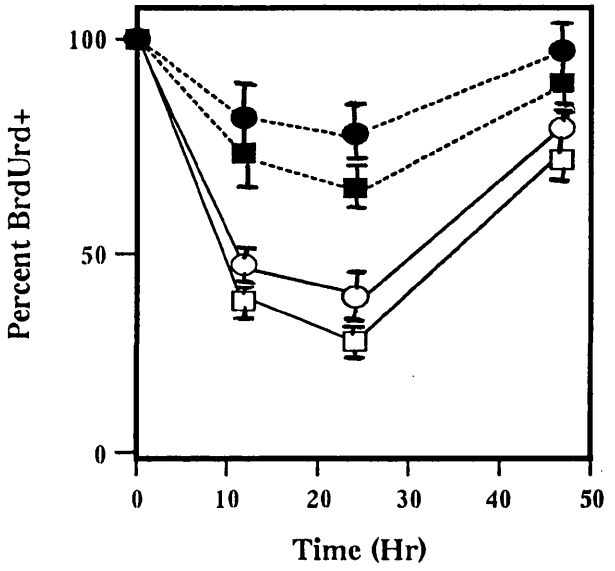
Figure 5. DNA damage induced cell cycle arrests in A2780 cells.

Cell cycle analysis was accomplished by flow cytometric measurement of cells after bromodeoxyuridine (BrdUrd) incorporation during a 4h pulse and propidium iodide (PI) staining. Increase in FITC represents fluorescent labelling of an antibody against BrdUrd. At least 20,000 events were counted in each sample. Cells were treated with DNA damaging agents at indicated doses and left for 24h before analysis.

Time course studies were performed to further characterise these cell cycle arrests in A2780 cells and a cisplatin resistant derivative. The ionising radiation induced G1 arrest in the A2780 cell line appeared maximal 24h post treatment and had disappeared by 48h, indicating the reversible nature of the cell cycle arrest (Figure 6A). The degree of G1 arrest also increased with the dose of γ-radiation. The A2780/CP70 cell line has previously been isolated from A2780 cells after two years selection with multiple exposures to cisplatin (Behrens *et al.*, 1987). A2780/CP70 cells are approximately 5 fold more resistant to cisplatin than the parental line as determined by clonogenic assay (Brown *et al.*, 1993). These cisplatin resistant cells also show cross-resistance to a number of DNA damaging agents including ionising radiation and doxorubicin (Brown *et al.*, 1993; Vasey *et al.*, in press).

The A2780/CP70 cells displayed a reduced G1 arrest in response to radiation treatment compared to the parental line (Figure 6A). These results are in agreement with previously published data and suggest that the p53-mediated DNA damage response pathway is dysfunctional in these cisplatin resistant cells (Brown *et al.*, 1993). The G2 arrest induced by ionising radiation was also dose-dependent and reversible in A2780 cells with maximal accumulation of cells in G2/M-phase by 12h (Figure 6B). However, the A2780/CP70 cells showed no significant difference in ability to arrest in G2 than the parental line. A similar lack of correlation has been noted between radiation induced G2 arrest and radiosensitivity in a panel of human tumour cell lines (McIlwrath *et al.*, 1994).

A. Total S-phase



B. G2/M-phase

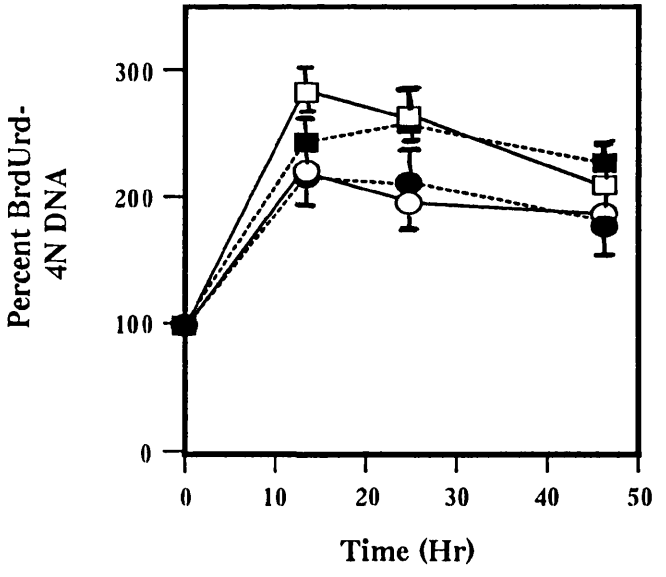


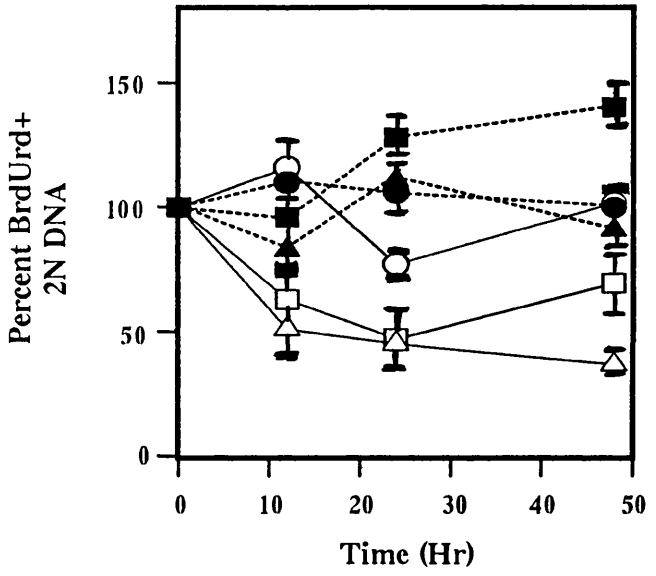
Figure 6. Cell cycle arrests induced by ionising radiation.

The number of radiation treated cells with a 2N/4N DNA content and BrdUrd+ (A) or 4N DNA content and BrdUrd- (B) was expressed as a percentage of untreated controls at each time point. Each point represents the mean of at least two independent determinations (>20,000 events counted). Open symbols and solid line, A2780; closed symbols and broken line, A2780/CP70. Cells were treated with γ-radiation at doses of 2Gy (circles) or 4Gy (squares).

The symbols and bars refer to the mean and range of values, respectively.

A cisplatin dose-dependent and reversible reduction was observed in the percentage of BrdUrd positive A2780 cells with a 2N DNA content (Figure 7A). Maximal inhibition of DNA synthesis in A2780 cells occurred 24h post treatment. Treatment of A2780/CP70 cells with cisplatin at either 20μM or 40μM for 1h did not induce a decrease in the percentage of cells entering S-phase. This indicates that the A2780/CP70 cells have an abrogated G1 arrest in response to cisplatin, although intact in the parental line. These results again suggest that the A2780/CP70 cells have a defective p53-mediated DNA damage response pathway. It has been proposed that the G2 arrest is necessary for cisplatin to mediate its cytotoxic effects (Eastman, 1990). The A2780 cells also showed a marked G2 arrest after exposure to cisplatin which was reversible and occurred maximally 24h post treatment (Figure 7B). On the other hand, the A2780/CP70 cells demonstrated little or no G2 arrest after cisplatin treatment. These observed differences between the ionising radiation and cisplatin induced G2 arrests will be discussed in Chapter 5.

A. Early S-phase



B. G2/M-phase

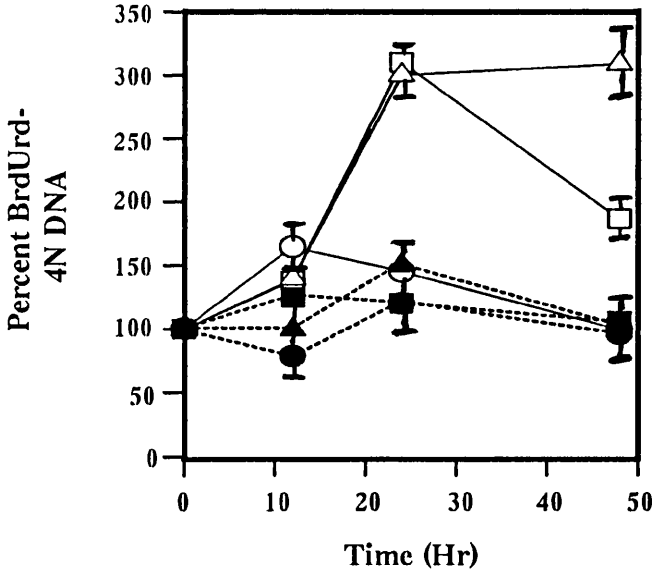


Figure 7. Cell cycle arrests induced by cisplatin.

The number of cisplatin treated cells with a 2N DNA content and BrdUrd+ (A) or 4N DNA content and BrdUrd- (B) was expressed as a percentage of untreated controls at each time point. Each point represents the mean of at least two independent determinations (>20,000 events counted). Open symbols and solid line, A2780; closed symbols and broken line, A2780/CP70. Cells were treated with cisplatin for 1h at doses of 10μM (circles), 20μM (squares) or 40μM (triangles).

The symbols and bars refer to the mean and range of values, respectively.

The multi-agent resistance phenotype observed in A2780/CP70 cells may be due to an inability to undergo DNA damage induced apoptosis (Wyllie, 1993; Enoch and Norbury, 1995). DNA strand breaks generated during apoptosis can be detected by end-labelling of the DNA with biotin-dUTP using terminal transferase and detection of labelled cells with fluorescein-avidin (Gorczyca *et al.*, 1993b). This technique is referred to as TUNEL. The proportion of cells staining positively by TUNEL may be quantified using flow cytometry. Treatment of A2780 cells with either 2Gy γ-radiation or 40μM/1h cisplatin induced a high rate of apoptosis 72h later (Figure 8). Indeed, time course studies have shown this to be the time point where maximal apoptosis occurs in response to DNA damage (McIlwrath, *personal communication*). However, the A2780/CP70 line displayed a reduced level of apoptosis using similar treatments compared to the parental cells. It has previously been shown that A2780/CP70 cells form 2 fold less platinum-DNA adducts than the parental line when treated at equivalent cisplatin exposures (Johnson *et al.*, 1994). At cisplatin exposures which induce comparable levels of initial DNA damage, the resistant cells still showed markedly reduced levels of apoptosis (data not shown). These results further support the notion of a dysfunctional p53-mediated DNA damage response pathway in the A2780/CP70 line.

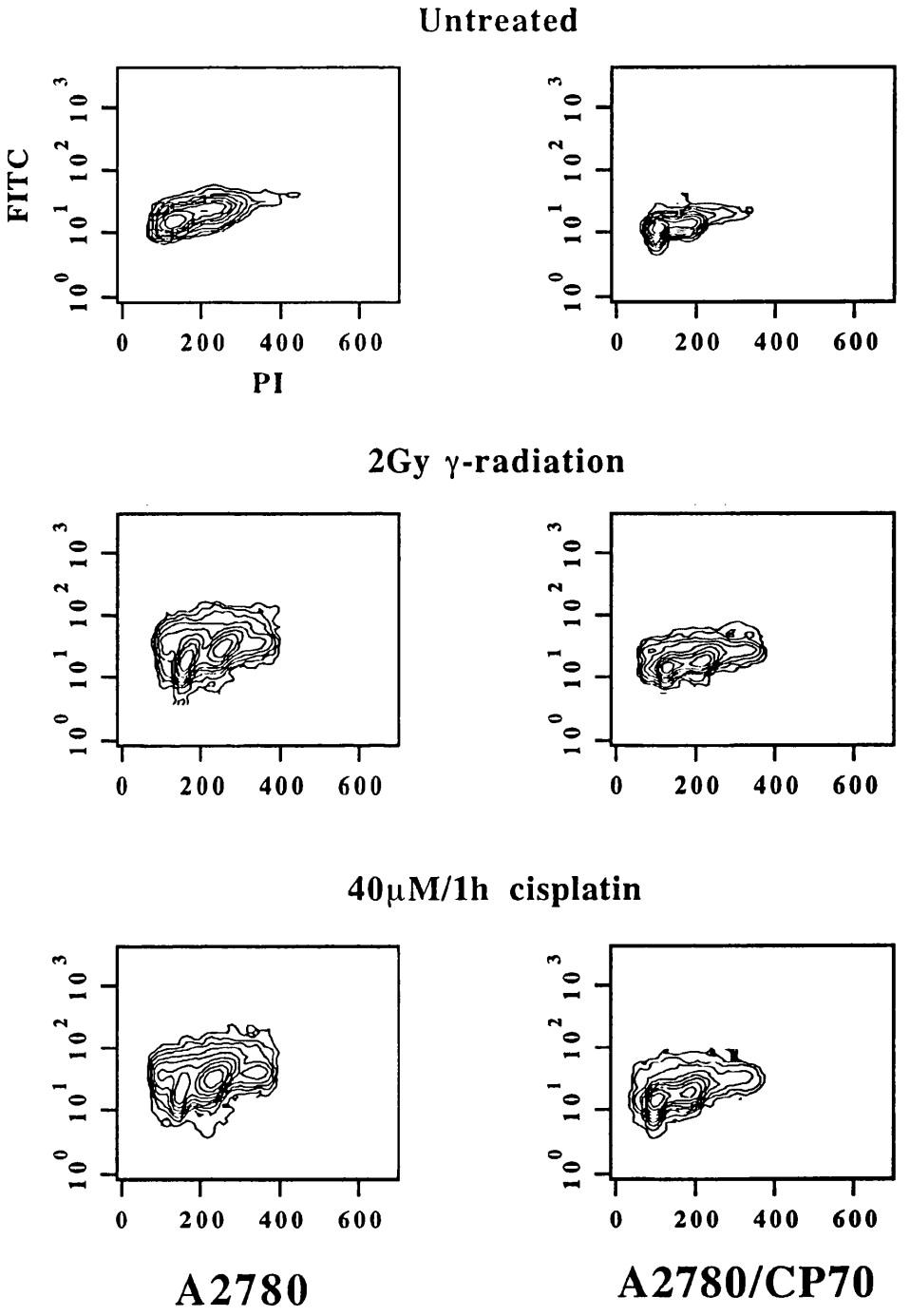


Figure 8. Apoptosis induced by ionising radiation and cisplatin.

Flow cytometric analysis of DNA strand breaks. Increase in FITC represents end-labelling of the DNA with biotin-dUTP using terminal transferase and detection of labelled cells with fluorescein-avidin. At least 15,000 events were counted in each sample. Cells were treated with DNA damaging agents at indicated doses and left for 72h before analysis. The apparent subpopulation of cells with >4N DNA content represented less than 5% of total cell number and may be an artifact of the TUNEL procedure.

3.3 INCREASED ACCUMULATION OF p53 PROTEIN

It has previously been shown that A2780/CP70 cells have elevated constitutive levels of p53 protein compared to the parental A2780 cells, but that both lines express wild-type TP53 mRNA (Brown *et al.*, 1993). The results presented in the previous section indicate that the DNA damage response pathway controlled by p53 is abrogated in the cisplatin resistant A2780/CP70 cells. Therefore, the following work attempts to elucidate the mechanism of p53 dysfunction in these cells. It also investigates the kinetics of p53 accumulation by ionising radiation and cisplatin. The data presented in Figure 9 was obtained from Dr. Robert Brown, CRC Department of Medical Oncology. This figure illustrates the time course of p53 protein accumulation in A2780 and A2780/CP70 cells after treatment with either ionising radiation or cisplatin. Exposure of A2780 cells to 2Gy γ-radiation induced increased cellular accumulation of p53 protein (Figure 9A and C). The p53 protein levels maximally increased in the A2780 cells by 4h and returned to control amounts by 48h. These kinetics of induction by ionising radiation are similar to those observed in other wild-type p53 expressing cells (Kastan *et al.*, 1991). The A2780/CP70 cells showed increased constitutive levels of p53, but also an increase in p53 accumulation after irradiation. However, the p53 protein level was still increased in the A2780/CP70 line at 48h after treatment. Elevated accumulation of p53 protein has been detected in the A2780/CP70 line as late as 96h after treatment (data not shown).

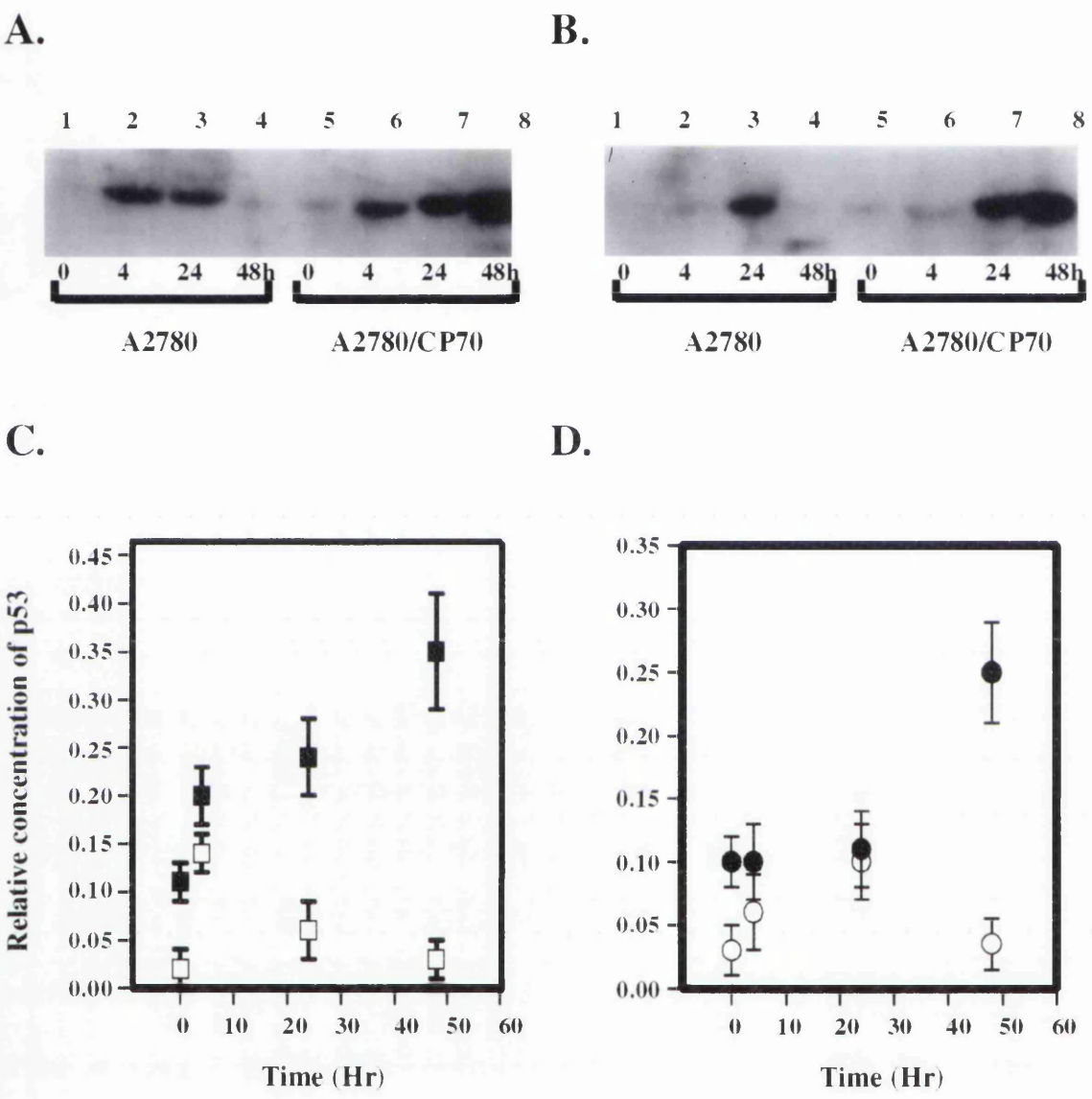


Figure 9. Time course of p53 protein accumulation in response to DNA damage.

The following data was obtained from Dr. R. Brown, CRC Department of Medical Oncology, Glasgow.
(**A**, **B**) Western blot analysis of p53 protein levels in cell extracts using an anti-p53 MAb (clone DO-1). The correct size of p53 protein (53kDa) was determined by using a 29.5-214kDa protein ladder (Gibco BRL). (**C**, **D**) ELISA analysis of p53 protein levels in cell extracts using an anti-p53 MAb (clone DO-1). Values shown are the $O.D._{450nm}$ of bound complexes visualised with tetramethylbenzidine (100µg of total protein cell extract). The ELISA assays were linear over this range. Values represent the mean of 16 independent determinations and error bars represent the SDM. Cells were treated with either γ-radiation (**A**, **C**) or cisplatin (**B**, **D**) at doses of 2Gy or 1µM/1h, respectively. Extracts were prepared after treatment at time points indicated.

Maximal accumulation of p53 protein in the A2780 cells occurred 24h after treatment with 1μM/1h cisplatin (Figure 9B and D). The significance of this delayed accumulation of p53 protein in response to cisplatin is discussed in Chapter 5. Again, increased accumulation of p53 protein was observed in the A2780/CP70 cells compared to the parental line. However, the increased accumulation of p53 protein in A2780/CP70 cells after either ionising radiation or cisplatin treatment does not correlate with increased p53 activity (as determined by DNA damage induced G1 arrest and apoptosis), even though these cells express wild-type p53.

Immunocytochemical analysis revealed information on the subcellular localisation of p53 protein in A2780 and A2780/CP70 cells (Figures 10 and 11). These studies employed the use of an isotype specific antibody (IgG_{2b}) in place of the primary antibody against p53 for staining control purposes. Increased levels of cytoplasmic p53 protein are often associated with dysfunctional p53 (Bartek *et al.*, 1990). However, the elevated p53 protein observed in the A2780/CP70 line was nuclearly localised as determined by confocal microscopy (Figure 10). Following DNA damage, p53 can only function to arrest the cell cycle if the protein is located in the nucleus (Shaulsky *et al.*, 1991). The A2780 and A2780/CP70 cells displayed nuclear accumulation of p53 protein in response to either 2Gy γ-radiation or 1μM/1h cisplatin (Figures 10 and 11), which is consistent with both lines expressing wild-type p53. It is of interest to note that the accumulated p53 protein was expressed heterogenously between cells in these clonally derived lines.

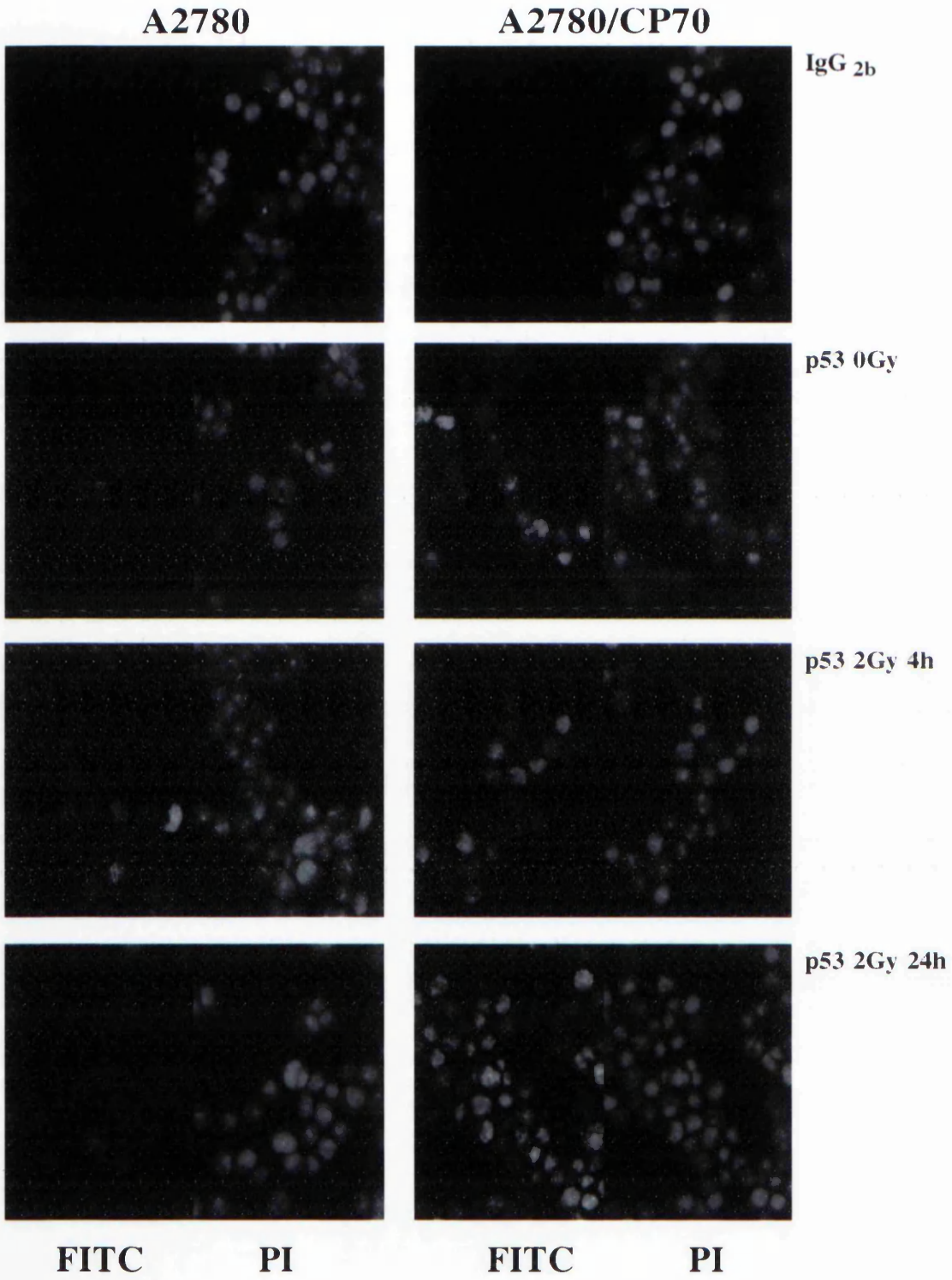


Figure 10. Nuclear accumulation of p53 protein - ionising radiation.

Immunocytochemical analysis of p53 protein expression was accomplished by confocal microscopy using an anti-p53 MAb (clone PAb122). An isotype specific antibody (IgG_{2b}) was used in place of the primary antibody for staining control purposes. Cells were treated with 2Gy γ-radiation and fixed at the time points indicated. Each image was divided into signals derived from either FITC (left) or PI (right) staining.

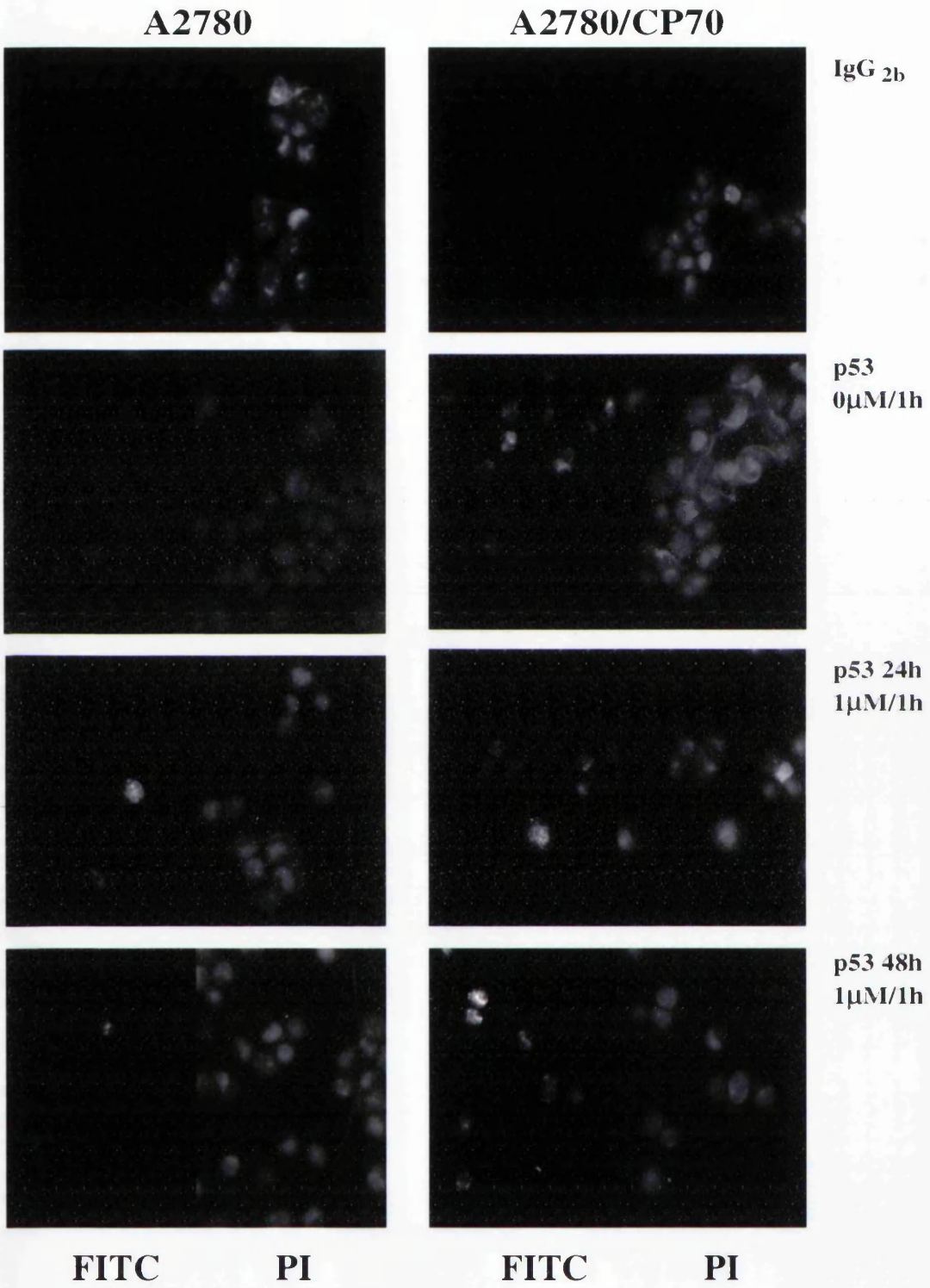


Figure 11. Nuclear accumulation of p53 protein - cisplatin.

Immunocytochemical analysis of p53 protein expression was accomplished by confocal microscopy using an anti-p53 MAb (clone PAb122). An isotype specific antibody (IgG_{2b}) was used in place of the primary antibody for staining control purposes. Cells were treated with 1μM cisplatin/1h and fixed at the time points indicated. Each image was divided into signals derived from either FITC (left) or PI (right) staining.

Flow cytometry was used to determine the percentage of the total cell population which stained p53 positive and provide information on the cell cycle related expression of p53 protein in response to ionising radiation and cisplatin (Figures 12 and 13). Figure 12 illustrates that a small proportion of A2780 cells (4%) were p53 positive compared to A2780/CP70 cells (37%). The percent of cells with detectable p53 increased maximally in the A2780 line 4h after irradiation and had mostly returned to control levels by 24h. The majority of A2780/CP70 cells (83%) stained positively for p53 4h after irradiation and maintained this increased level at 24h. Maximal accumulation of A2780 cells which were p53 positive was observed 24h after cisplatin treatment (Figure 13). A sustained cisplatin induced accumulation of A2780/CP70 cells staining positively for p53 was detected (60% by 48h). These results are in general agreement with the Western blot and ELISA studies described in Figure 9. There were no apparent differences between A2780 and A2780/CP70 cells regarding the cell cycle related expression of p53 protein in response to either ionising radiation or cisplatin (Figures 12 and 13). These results are comparable with a previous study showing UV induced accumulation of p53 protein in primary human fibroblasts which was independent of the cell cycle (Yamaizumi and Sugano, 1994).

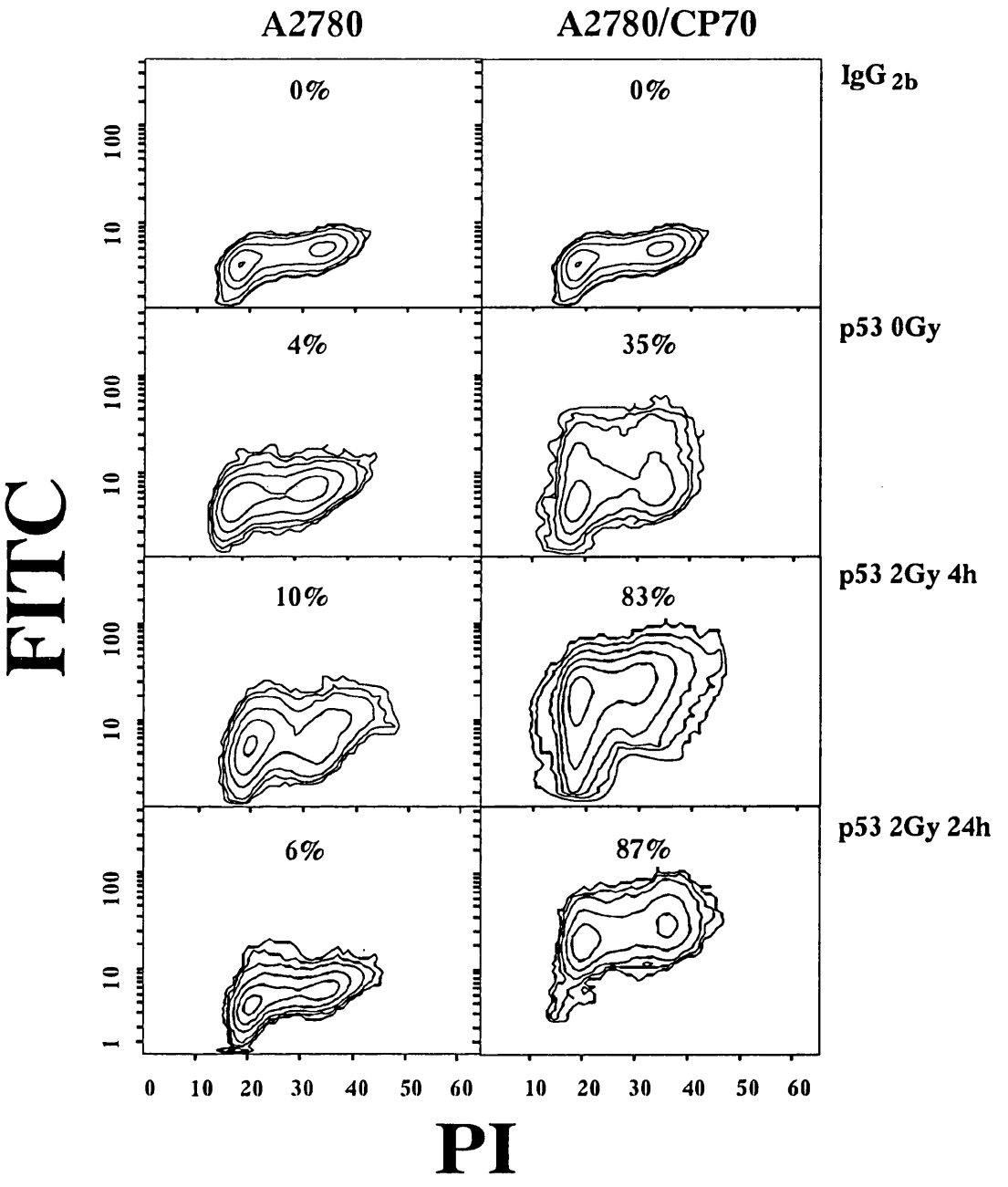


Figure 12. Cell cycle related expression of p53 protein - ionising radiation

Immunocytochemical analysis of p53 protein expression was accomplished by flow cytometry using an anti-p53 MAb (clone PAb122). An isotype specific antibody (IgG_{2b}) was used in place of the primary antibody for staining control purposes. Cells were treated with 2Gy γ-radiation and fixed at the time points indicated. Percentages refer to the number of cells within the total population which were p53+ as determined by an increase in FITC staining above control levels. Each value represents the mean of at least two independent determinations (>20,000 events counted).

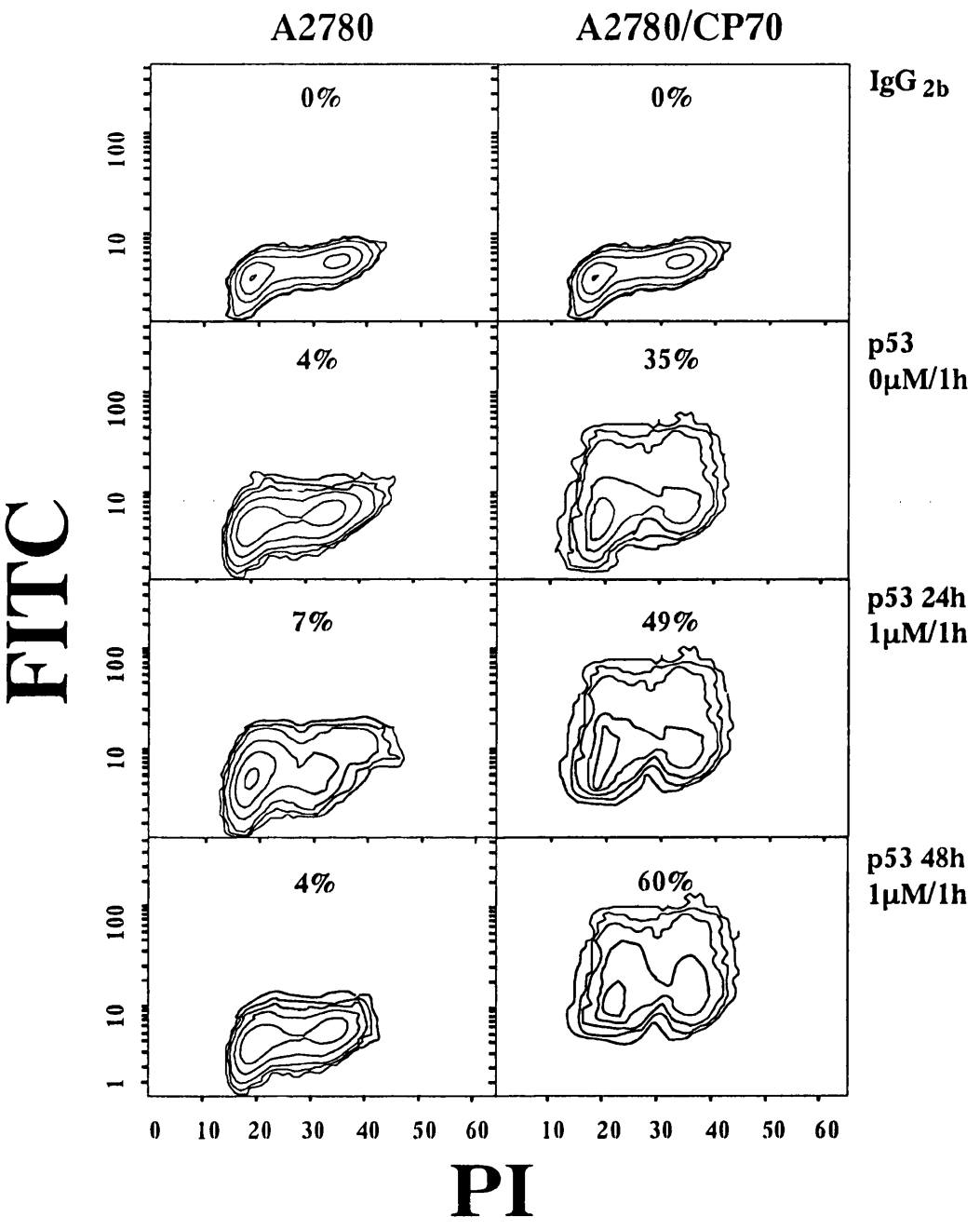


Figure 13. Cell cycle related expression of p53 protein - cisplatin.

Immunocytochemical analysis of p53 protein expression was accomplished by flow cytometry using an anti-p53 MAb (clone PAb122). An isotype specific antibody (IgG_{2b}) was used in place of the primary antibody for staining control purposes. Cells were treated with 1μM cisplatin/1h and fixed at the time points indicated. Percentages refer to the number of cells within the total population which were p53+ as determined by an increase in FITC staining above control levels. Each value represents the mean of at least two independent determinations (>20,000 events counted).

3.4 LOSS OF p53 TRANSCRIPTIONAL ACTIVITY

If the reduced DNA damage induced G1 arrest and apoptosis observed in the cisplatin resistant A2780/CP70 cells was due to a defective p53 pathway, a reasonable prediction would be that these cells also had reduced p53 transcriptional activity. Transcription of the CIP1 gene is partially regulated by p53 and is necessary for radiation induced G1 arrest (El-Deiry *et al.*, 1994). Levels of CIP1 mRNA in both A2780 and A2780/CP70 cells were quantified by hybridisation of a Northern blot with a CIP1 cDNA probe (Figure 14). This data was obtained in collaboration with Dr. Angela Edlin, CRC Department of Medical Oncology. The A2780/CP70 cells contained 3-4 fold reduced CIP1 mRNA compared to the parental A2780 cells. Treatment of A2780 cells with either 2Gy γ-radiation or 1μM/1h cisplatin induced CIP1 mRNA (Figure 14A and B). Based on densitometric scanning of relative hybridisation signals, ionising radiation treatment induced a 4.5 fold increase in CIP1 mRNA by 4h. Cisplatin treatment showed little CIP1 induction at 4h (1.4 fold), but did increase CIP1 mRNA by 4 fold at 24h. These results are consistent with the delayed induction of p53 protein levels by cisplatin in the A2780 cells (Figures 9B and D, 11 and 13). Ionising radiation induced CIP1 mRNA 2 fold at 4h in the A2780/CP70 cells, however this is markedly reduced compared to the induced CIP1 mRNA levels in the A2780 cells. In addition, little or no induction of CIP1 mRNA by 1μM/1h cisplatin was observed in the A2780/CP70 cells. The reduced transcription of the CIP1 gene in response to either ionising radiation or cisplatin is again consistent with abrogated p53 function in the A2780/CP70 line.

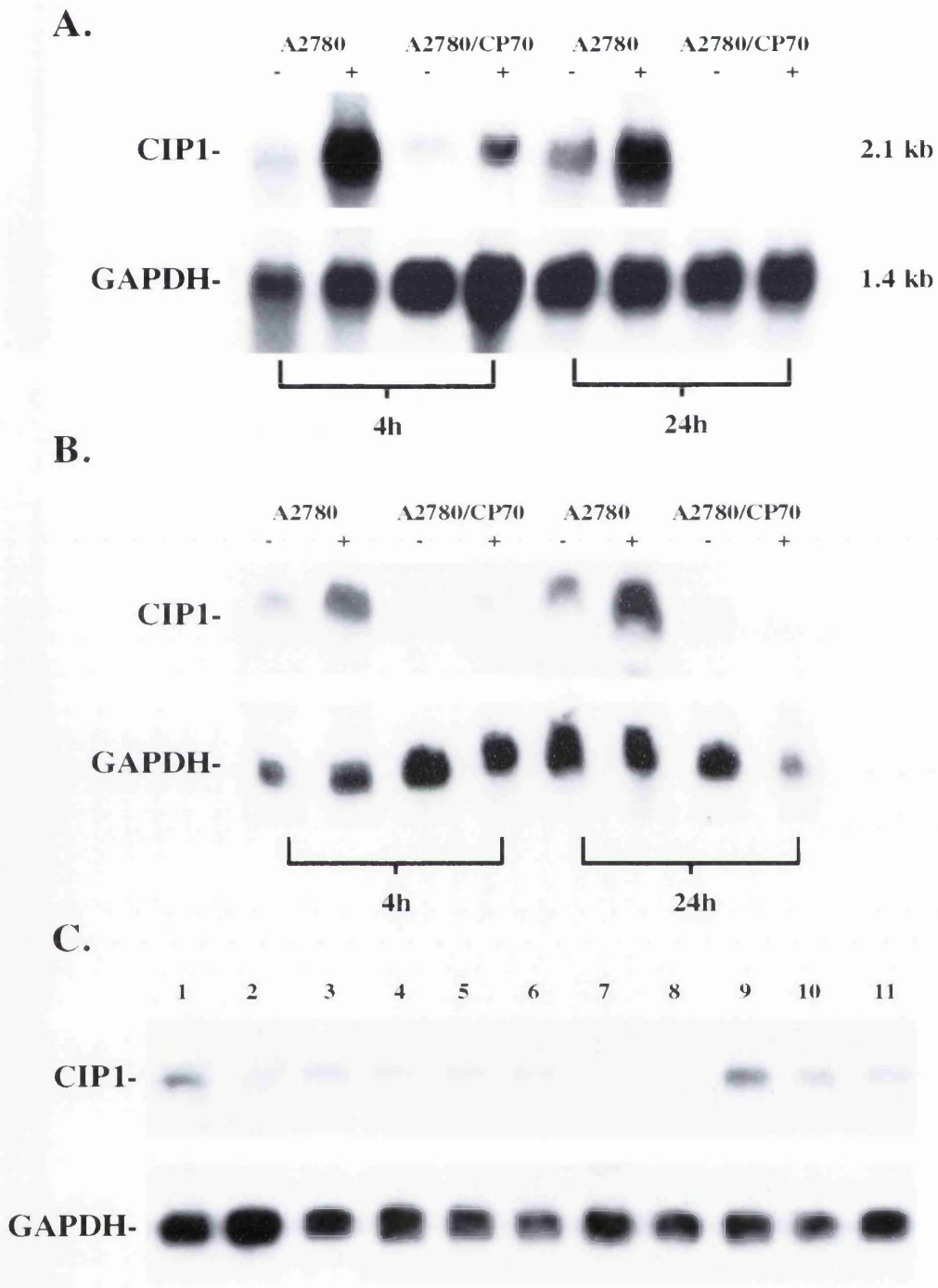


Figure 14. Expression of CIP1 mRNA in cisplatin resistant cells.

Northern blot of total RNA was hybridised with CIP1 and GAPDH probes. RNA was extracted from cells at the indicated time points after treatment (+) with either (**A**) 2Gy γ-radiation or (**B**) 1μM cisplatin/1h. Untreated control (-) samples are also shown. (**C**) Constitutive CIP1 mRNA levels within A2780/MCP lines. Lane 1, A2780; lane 2, A2780/CP70; lanes 3-11, A2780/MCP1-9. Correct size of transcripts was confirmed by comparison with size markers. *The data for **A** and **B** was obtained in collaboration with Dr. Angela Edlin, CRC Department of Medical Oncology.*

Nine independent cisplatin resistant A2780 lines (MCP1-9) have recently been isolated by repeated exposure to increasing concentrations of cisplatin up to a final concentration of 15μM (Anthoney *et al.*, 1996). Characterisation of these cell lines has been performed mainly by Dr. Alan Anthoney, CRC Department of Medical Oncology. All of these lines have been shown to be stably resistant (1.3-4.7 fold) to cisplatin compared to the parental A2780 line (Table 1). The majority of the multiply selected cisplatin resistant lines showed reduced CIP1 mRNA levels (Figure 14C and Table 1). These resistant lines also appeared deficient in DNA damage induced G1 arrest and apoptosis responses which is consistent with loss of p53 function being a general phenomenon in acquired cisplatin resistance (Table 1). Western blot analysis revealed that the resistant lines contained varying constitutive levels of p53 protein. Whereas some lines (A2780/MCP1, A2780/MCP3 and A2780/MCP5) exhibited elevated levels of p53 protein compared to the parental A2780 line, many had no detectable difference, and none had the enhanced levels to the extent observed in the A2780/CP70 line.

The next stage of the study was to determine the nature of the p53 dysfunction in A2780/CP70 cells. It has been proposed that mdm2 overexpression may constitute an alternative pathway to mutational inactivation of p53 (Meltzer, 1994). Indeed, mdm2 protein has been shown to complex with p53 and inhibit its transcriptional activation ability (Momand *et al.*, 1992; Oliner *et al.*, 1993). However, there was no evidence of mdm2 protein overexpression in the A2780/CP70 line as determined by immunocytochemistry (Figure 15B).

Table 1. Characteristics of A2780 cisplatin resistant cell lines.

Cell line	Fold Resistance[a]	IR G1 arrest[b]	% Apoptosis[c]	p53 protein[d] level	CIP1 mRNA[e] level	RER status[f]	Ectopic cyclin B1[g] expression
A2780	1.0	+	22	+	1.0	-	2
A2780/CP70	4.9	-	1	+++++	0.3	+	18
A2780/MCP1	1.3	-	4	++	0.5	+	1
A2780/MCP2	3.3	-	2	+	0.6	+	2
A2780/MCP3	4.7	-	1	+++	0.3	+	1
A2780/MCP4	4.1	-	1	+	0.6	+	14
A2780/MCP5	2.1	+/-	8	++	0.3	+	1
A2780/MCP6	2.6	-	7	+	0.5	+	1
A2780/MCP7	3.1	+	20	+	1.0	-	4
A2780/MCP8	3.6	+/-	1	+	0.7	+	2
A2780/MCP9	1.6	+/-	1	+	0.6	+	1

a. Calculated from the clonogenic surviving fraction of cells after exposure to 20μM/1h cisplatin relative to parental A2780 cells. Each value is the mean of five independent experiments. b. Presence of a functional ionising radiation induced G1 arrest similar to that accomplished by A2780 cells is indicated by a positive sign. c. The percent of total cells undergoing apoptosis 72h after treatment with 40μM/1h cisplatin as determined by flow cytometric measurement of DNA strand breaks. Values shown are the mean of duplicate experiments. d. Relative levels of p53 protein as detected by Western blot analysis using 50μg of protein cell extracts. Approximate levels are indicated. In all cases of elevated p53 protein this was identified as being localised within the nucleus. e. Relative levels of CIP1 mRNA were determined by Northern blot analysis. Standarisation was achieved using a GAPDH cDNA probe. f. Presence of a RER+ phenotype as determined by defects in mismatch repair and resulting microsatellite instability indicated by a positive sign. g. The percent of total cells with a 2N DNA content which were cyclin B1 postive as determined by immuncytochemistry. *The data for a-c and f was obtained from Dr. Alan Anthoney, CRC Department of Medical Oncology.*

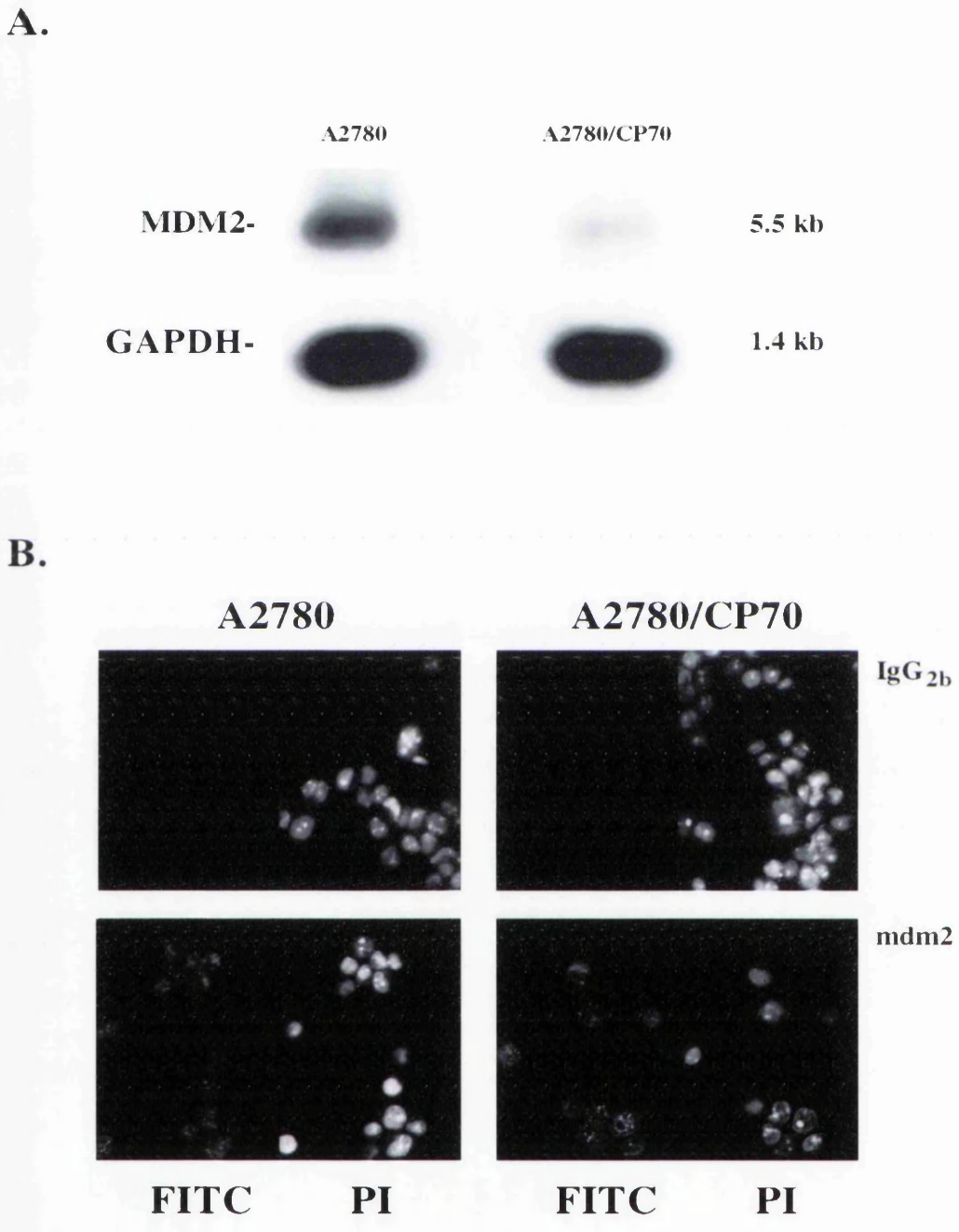


Figure 15. Constitutive levels of MDM2 mRNA and protein.

(**A**) Northern blot of total RNA was hybridised with MDM2 and GAPDH probes. RNA was extracted from untreated exponentially growing cells. Correct size of transcripts was confirmed by comparison with size markers. (**B**) Immunocytochemical analysis of mdm2 protein expression was accomplished by confocal microscopy using an anti-mdm2 MAb (clone IF2). An isotype specific antibody (IgG_{2b}) was used in place of the primary antibody for staining control purposes. Each image was divided into signals derived from either FITC (left) or PI (right) staining.

Attempts at quantification of mdm2 protein levels in either cell line by Western blot analysis proved unsuccessful. The MDM2 gene is itself a transcriptional target of p53 (Barak *et al.*, 1993; Perry *et al.*, 1993). Reduced levels of MDM2 mRNA (4 fold) were observed in the A2780/CP70 cells compared to the parental line (Figure 15A). These results add weight to the assertion that the p53 protein in the A2780/CP70 cells, despite having wild-type TP53 sequence, is functionally inactive for transcriptional activation. Considering that transcription of both the CIP1 and MDM2 genes was reduced in the A2780/CP70 cells, the defect in the p53-mediated DNA damage response pathway was assumed not to lie downstream of p53.

3.5 PATTERN OF p53 PROTEIN ISOFORMS

It has been suggested that phosphorylation of p53 protein may be one of the signals leading from DNA damage to p53 activation (Milne *et al.*, 1995). Therefore, a defect in phosphorylation of p53 would be a likely candidate for the p53 dysfunction observed in the cisplatin resistant cells. Using 2D-PAGE, charged isoforms of p53 protein can be identified (Merrick *et al.*, 1995). Such isoforms have been shown to represent specific phosphorylated forms of p53 protein (Merrick *et al.*, 1995). Twelve major isoforms of p53 protein were consistently observed in both A2780 and A2780/CP70 cells having isoelectric points between 5.6 and 6.8 (Figure 16).

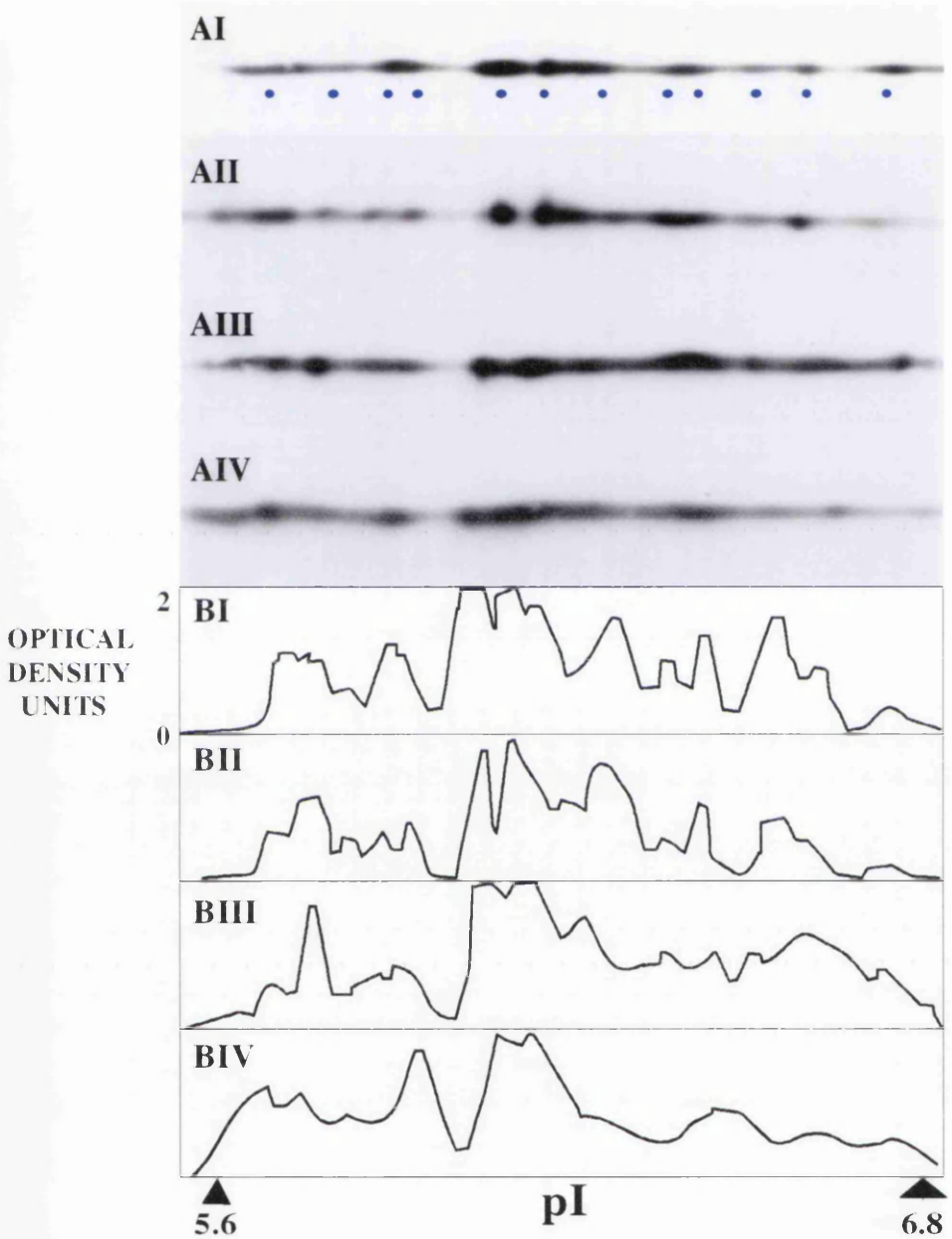


Figure 16. Spectrum of p53 protein isoforms revealed by 2D-PAGE.

The p53 protein isoforms were separated by 2D-PAGE, transferred by Western blotting onto an Immobilin-P membrane and detected immunologically using an anti-p53 MAb (clone DO-1). Enhanced chemiluminescence and autoradiography allowed the visualisation of protein-antibody complexes. Image capture and profile analysis was achieved using PDQuest software. The above data was compiled from a single experiment, although similar results were observed in two other independent experiments. A: images; B: profiles. A2780 (I-0Gy) and (III-4Gy 6h); A2780/CP70 (II-0Gy) and (IV-4Gy 4h). pI refers to the isoelectric point of the protein isoforms detected. The *2D-PAGE was carried out by Lynn McGarry, Beatson Institute.*

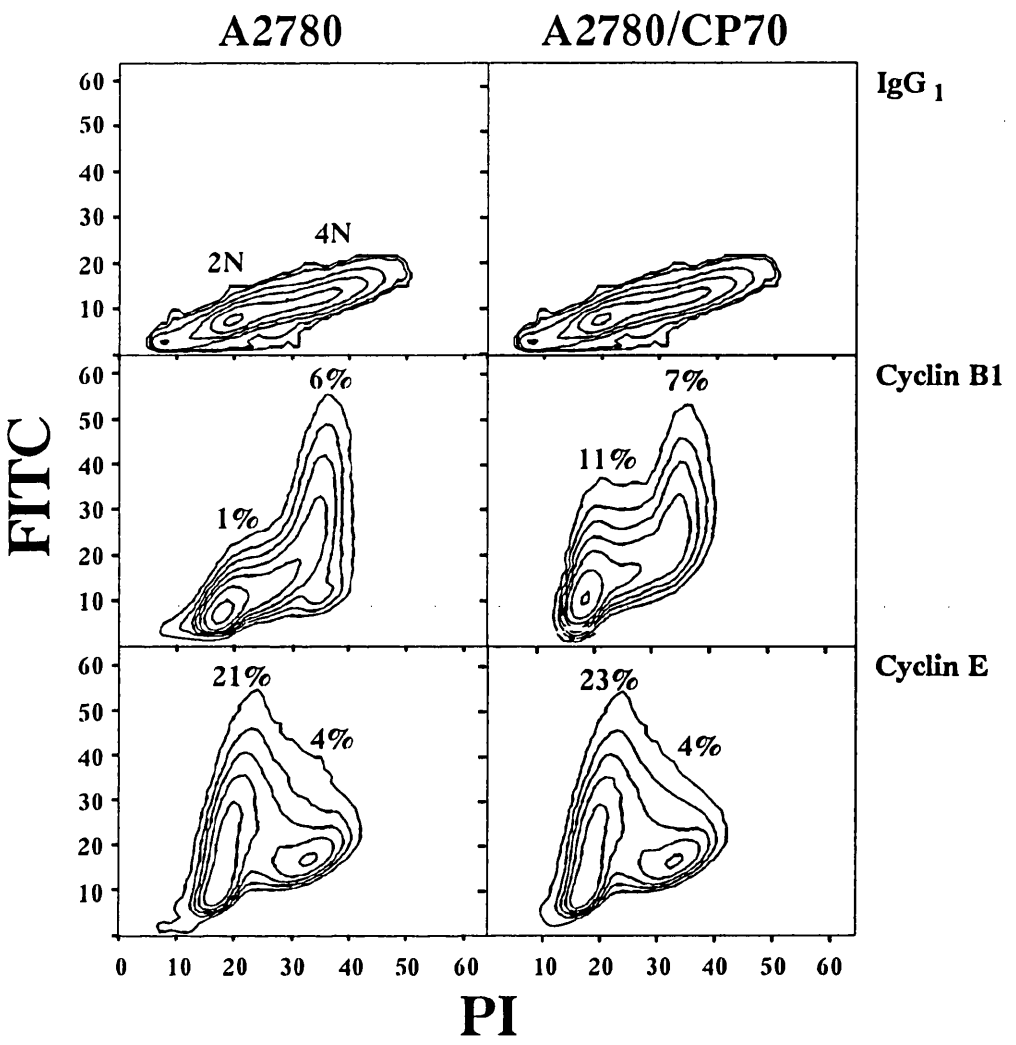
The number and charge of p53 isoforms observed are comparable with those previously characterised by others (Merrick *et al.*, 1995). The most cathodic isoform (pI ~ 6.7) is assumed to represent the unphosphorylated backbone of p53. Profile analysis revealed no gross differences in the pattern of p53 isoforms between both A2780 and A2780/CP70 cells either on a constitutive basis or after exposure to ionising radiation. Although there may be very specific alterations in the pattern of p53 isoforms, the data does not provide any clear evidence for a defect in the phosphorylation of the p53 protein in A2780/CP70 cells.

3.6 UNSCHEDULED CYCLIN PROTEIN EXPRESSION

The following work presents data on a potentially interesting abnormality found in the cisplatin resistant A2780/CP70 cells and does not have any direct relevance to the primary aim of Chapter 3 (i.e. to investigate the role of p53 in acquired cisplatin resistance). Cyclins are key components of the cell cycle machinery (Baserga, 1990; Norbury and Nurse, 1992). They are expressed selectively in different phases of the cell cycle and function by activating one of several CDKs, each of which phosphorylates a distinct group of proteins that in turn causes advancement of the cell through a particular stage of the cell cycle. Cyclin B1 (a G2 cyclin) is required for entrance of the cell into mitosis and its expression is confined mainly to the G2/M-phase cell population. Cyclin E (a G1 cyclin) controls progress into S-phase and maximal expression of this protein occurs during the G1-S transition.

Unscheduled expression of cyclin B1 and E proteins have been observed in a variety of leukaemic and solid tumour cell lines (Gong *et al.*, 1994). Such ectopic expression of cyclin proteins may indicate defective cell cycle regulatory mechanisms. The cell cycle related expression of both cyclin B1 and E proteins was examined in A2780 and A2780/CP70 cells. A2780 cells exhibited a normal pattern of cyclin B1 protein expression, i.e. peaking during G2/M-phase (Figure 17). However, a significant subpopulation of A2780/CP70 cells (11%) with a 2N DNA content were cyclin B1 positive compared to the parental line (1%). No evidence of aberrant expression of cyclin E protein was observed in either A2780 or A2780/CP70 cells. In both cell lines, cyclin E protein was maximally expressed in late G1 cells and its content progressively declined during S-phase. Ectopic expression of cyclin B1 was only observed in two cisplatin resistant lines, one of which includes A2780/CP70 (Figure 17B and Table 1). This suggests that unscheduled expression of cyclin B1 may not be a general phenomenon in cells which acquire a cisplatin resistant phenotype.

A.



B.

	Cyclin B1		Cyclin E	
	A2780	A2780/CP70	A2780	A2780/CP70
2N FITC+ (%)	2	18	34	38
4N FITC+ (%)	22	21	12	11

Figure 17. Cell cycle related expression of cyclin B1 and E protein.

(A) Immunocytochemical analysis of cyclin protein expression by flow cytometry using anti-cyclin B1 (clone GNS-1) and anti-cyclin E (clone HE12) MAbs. An isotype specific antibody (IgG_1) was used in place of the primary antibody for staining control purposes. Percentages refer to the number of cells within the total population which were cyclin B1 or E positive as determined by an increase in FITC staining above control levels. Each value represents the mean of at least two independent determinations (>20,000 events counted). (B) Summary of cyclin B1 and E results. The number of cells with either 2N or 4N DNA which stained positive for cyclin expression was expressed as a percentage of the total number of cells with the same DNA content.

3.7 SUMMARY

Evidence has been presented for a defect in the p53-mediated DNA damage response pathway in cisplatin resistant derivatives of A2780 cells as determined by reduced DNA damage induced G1 arrest, apoptosis and transcription of certain target genes. The basis of the p53 dysfunction in one of these resistant cell lines (A2780/CP70) does not appear to be due to overexpression of mdm2 protein, differences in the cell cycle related expression of p53 protein or gross changes in the pattern of p53 isoforms. In addition, previous sequencing of the entire TP53 cDNA from this line revealed no mutations (Brown *et al.*, 1993). The proposed mechanism of p53 dysfunction in these cisplatin resistant cells is discussed in Chapter 5.

A possible limitation of this work was the reliance on indirect measures of p53 function such as transcription activation of endogenous p53 target genes. It is conceivable these genes are regulated by other factors within the cell apart from p53. In particular, expression of CIP1 has been shown to be regulated by p53-independent mechanisms (Michieli *et al.*, 1994; Macleod *et al.*, 1995). However, reporter constructs using specific p53-responsive promoters would allow direct assessment of the transcriptional activation-dependent functions of p53 only. Ionising radiation was useful in evaluating the p53 response, where the degree of platinum-DNA adduct formation after cisplatin treatment was an unknown entity (as in A2780/MCP1-9 lines).

CHAPTER 4

IDENTIFICATION OF p53 GENETIC SUPPRESSOR ELEMENTS WHICH CONFER RESISTANCE TO CISPLATIN

4.1 INTRODUCTION

As mentioned in the previous chapter, transfection of a dominant negative p53 mutant (codon 143, Val to Ala) into the A2780 cell line confers increased resistance to cisplatin (Vasey *et al.*, in press). In the present study, libraries of recombinant retroviruses, expressing random fragments of human or murine TP53 cDNA plasmids, were introduced into A2780 cells and the resulting clones selected on the basis of cisplatin resistance. It was predicted that cisplatin selection would enrich for GSEs which could abrogate the endogenous p53-mediated DNA damage response pathway. The primary aim was to identify small molecules (dominant negative peptides or antisense RNA) derived from p53 which allow modification of the p53 response to genotoxic stress.

4.2 RANDOM FRAGMENT p53 EXPRESSION LIBRARIES

The construction and utilisation of the random fragment p53 expression libraries is outlined as follows. Plasmids containing human and murine TP53 cDNA were randomly digested with DNaseI. Each fragment was ligated to asymmetrical adaptors which provide translational initiation and termination sites and allow for oriented cloning. Following PCR amplification using primers derived from the adaptor sequences, these fragments were inserted between HindIII/ClaI sites within the pLNCX retroviral expression vector plasmid. Libraries of recombinant plasmid clones were generated and transfected into an amphotropic virus packaging cell line. Viral supernatants (containing the p53 recombinant retroviruses) were collected and used to infect A2780 cells. Figure 4 displays the structure of a hypothetical integrated LNCX provirus containing a fragment of TP53 cDNA.

A detailed description regarding the generation of the random fragment p53 plasmid libraries can be found in Chapter 2 (section 2.10). The initial step in this process was the random fragmentation of human and murine TP53 cDNA plasmids. Both plasmids were partially digested with serial dilutions of DNaseI (Figure 18A). Fragments between 50 and 500bp were isolated (Figure 18A, lane 4) and rendered blunt-ended as previously described (Holzmayer *et al.*, 1992). Synthetic adaptors were ligated to these fragments using T4 DNA ligase and subsequently amplified by PCR using primers derived from the adaptor sequences (Figure 18B, lanes 1-4). Self-ligated adaptors were removed by a purification step (Figure 18B, lanes 5-8). Amplified random fragments were digested with HindIII/ClaI and inserted into the corresponding cloning site within the pLNCX vector plasmid using T4 DNA ligase. Integration of these random fragments into the vector was determined by PCR amplification using primers spanning the cloning site (Figure 18C).

PCR amplification of vector alone ligations produced a DNA fragment of 165bp as predicted (Figure 18C, lane 9; Miller and Rosman, 1989). Larger DNA fragments (>200bp) were also observed in PCR products obtained from insert/vector ligations (Figure 18C, lanes 1-8). This indicates that the random fragments had successfully integrated into the vector cloning site. The vector alone PCR product was undetectable in certain ligations, particularly those reactions with a high insert to vector ratio (Figure 18C, lanes 7-8). These ligations were predicted to have negligible amounts of non-integrated vector and therefore ideal for the generation of recombinant plasmid libraries.

Competent bacterial cells were transformed with DNA from such ligations and selected on the basis of ampicillin resistance.

Plasmid DNA was isolated from pools of transformed bacteria (>1000 clones each). Random fragment inserts were amplified from these plasmid libraries by PCR using primers spanning the cloning site (Figure 18D). The majority of the resulting PCR products ranged in size from 250 to 600bp (Figure 18D, lanes 1-10). No discrete PCR products were observed indicating that no particular sequences were over-represented within the plasmid libraries. In total, libraries of 70,000 and 40,000 plasmid clones were constructed which contained 50-500bp fragments from human and murine TP53 cDNA plasmids, respectively (Table 2). Over 90% of the plasmid clones within both libraries were predicted to contain recombinant inserts (Table 2 and Figure 19).

Table 2. Random fragment p53 plasmid libraries.

Species origin	Number of clones[a]	Recombinants[b]
Human	70,000	>90%
Murine	40,000	>90%

a. The number of plasmid clones was calculated by plating small aliquots of the bacterial cell cultures and counting the number of transformed colonies. b. Plasmid DNA was extracted from >50 independent bacterial cell clones. The percentage of recombinants within these plasmid clones was determined by either HindIII/ClaI digestion (data not shown) or PCR amplification using primers spanning the cloning site (Figure 19).

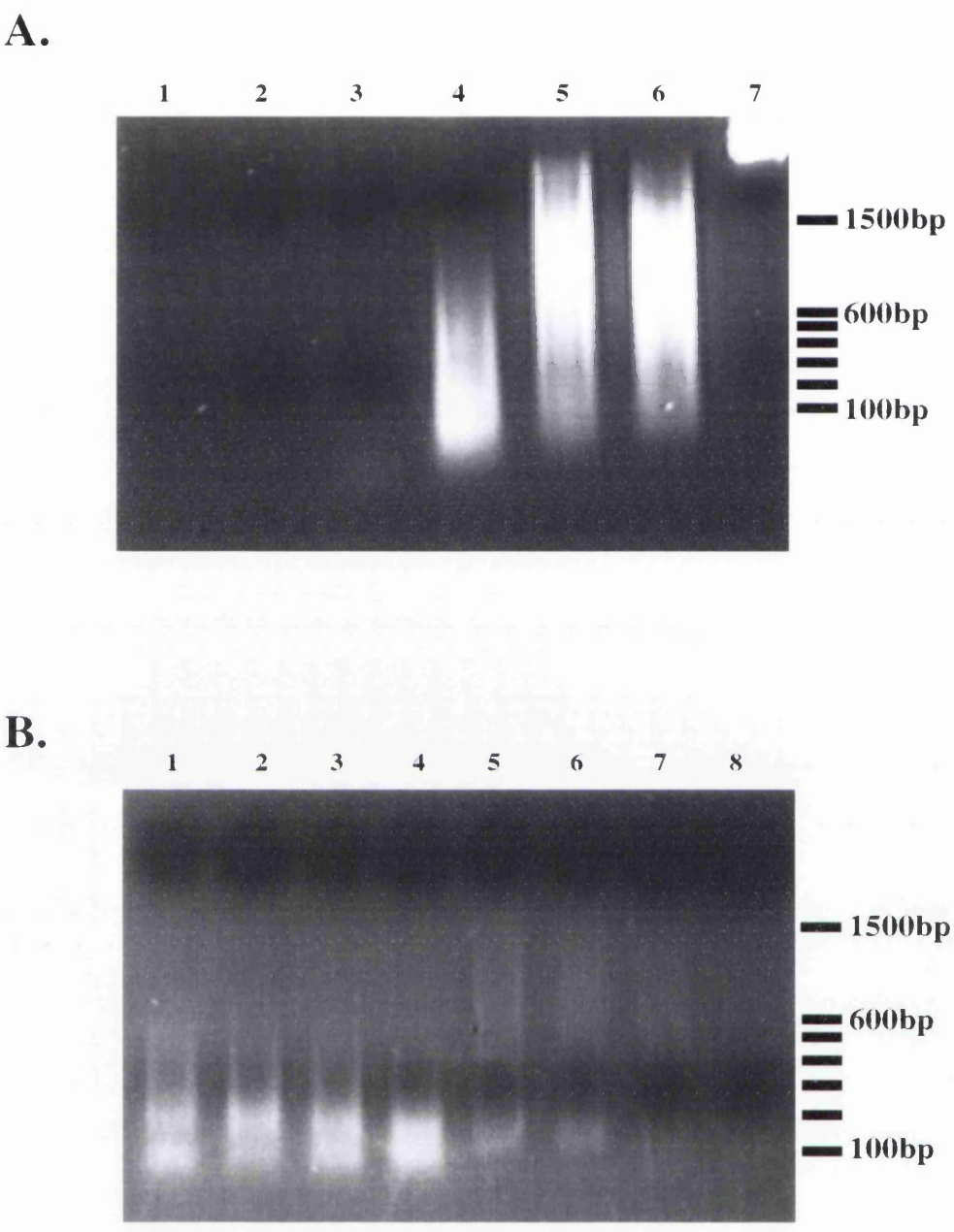
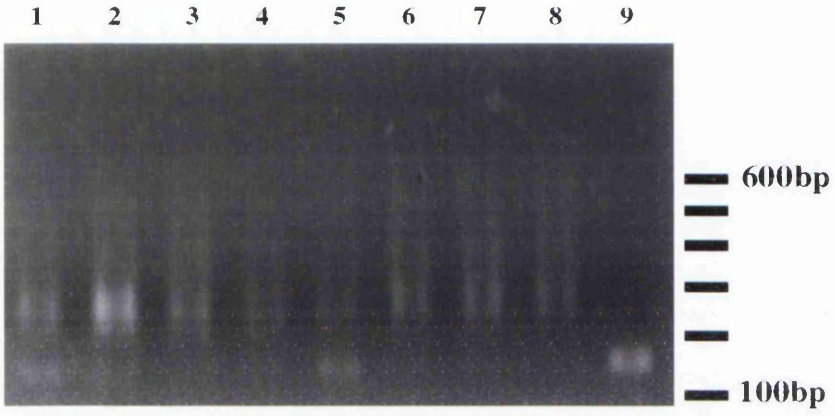


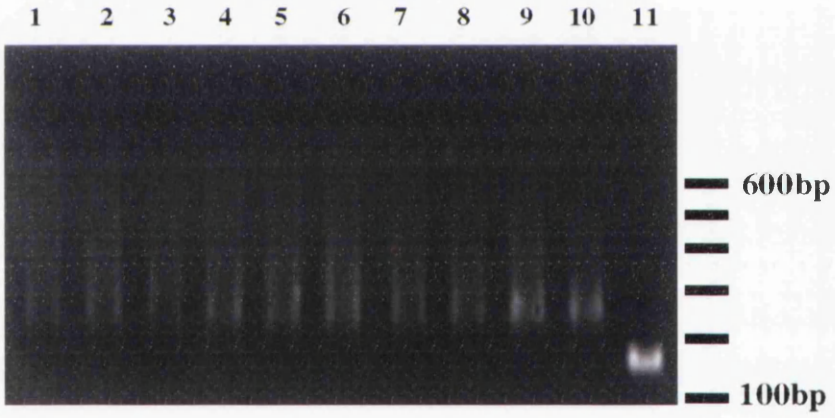
Figure 18. Construction of random fragment p53 plasmid libraries.

(**A**) Random fragmentation of human TP53 cDNA plasmid. Plasmid DNA (10μg) was incubated with serial dilutions of DNaseI for 30min. Lanes 1-6, 10 fold serial dilutions of enzyme with initial reaction containing 4U; lane 7, no enzyme added. (**B**) PCR amplification of adaptor ligations. Synthetic adaptors (500ng) were ligated to random DNA fragments (100ng) using T4 DNA ligase (2U) and subsequently amplified by PCR using primers derived from the adaptor sequences. PCR products were purified using the Wizard PCR preps kit. Lane 1-4, PCR products from adaptor ligations before purification; lanes 5-8, PCR products from adaptor ligations after purification.

C.



D.



(**C**) PCR amplification of insert/vector ligations. Random DNA fragments were digested with HindIII/ClaI and inserted into the corresponding cloning site within the pLNCX vector plasmid using T4 DNA ligase (1U). Ligation reactions were subsequently amplified by PCR using primers spanning the cloning site. Lanes 1 and 5, 1ng insert; lanes 2 and 6, 5ng insert; lanes 3 and 7, 10ng insert, lanes 4 and 8; 20ng insert; lane 9; no insert. Lanes 1-4, 100g vector; lanes 5-9, 50ng vector. (**D**) PCR amplification of random fragment p53 plasmid libraries. Plasmid DNA was isolated from pools of bacteria (>1000 clones each) transformed with certain insert/vector ligations and subsequently amplified by PCR using primers spanning the cloning site. Lanes 1-10, plasmid from different pools of transformed bacteria; lane 11, vector alone plasmid.

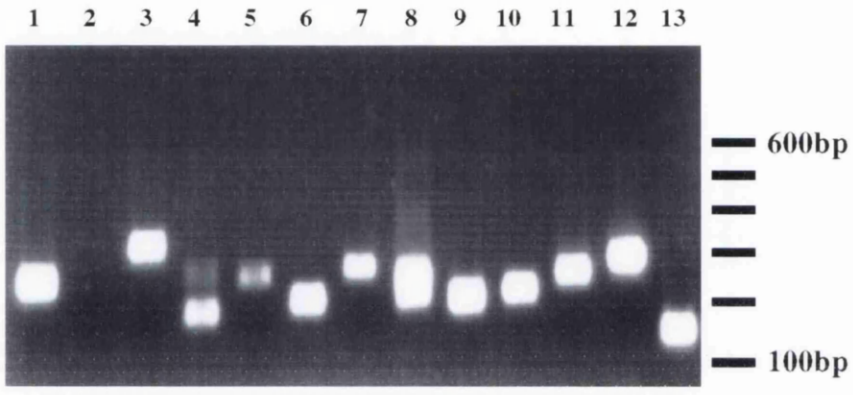


Figure 19. Screening of recombinant plasmid library clones.

PCR amplification of individual plasmid clones from recombinant libraries using primers spanning the cloning site. PCR products from all of these plasmid clones contained DNA fragments of >165bp (which is obtained using the vector alone plasmid), indicating the presence of recombinant inserts. Lanes 1-12, individual plasmid clones; lane 13, vector alone plasmid.

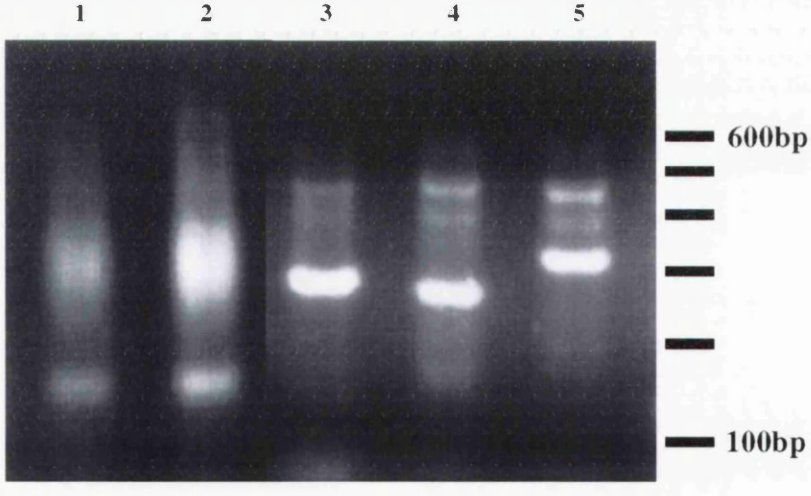


Figure 20. Cisplatin selection of A2780 infectants.

PCR amplification of proviral inserts from genomic DNA using primers spanning the cloning site. Genomic DNA was isolated from A2780 infectants before (lanes1-2) and after (lanes 3-5) cisplatin selection. Lanes 1 and 3-4, murine p53 library; lanes 2 and 5, human p53 library.

Both plasmid libraries were transfected into the murine amphotropic virus packaging PA317 cell line (Miller and Buttimore, 1986). Transfection efficiencies were in the range 100 to 500 CFU/10^6 viable cells/μg vector. A total of 2,500 stable PA317 transfectants were isolated from each library. Retroviral particles derived from these transfectants formed the basis of the random fragment p53 expression libraries. A2780 cells were infected with either the human or murine p53 recombinant retroviruses. Viral titres ranged from 50 to 300 CFU/10^6 viable cells/ml. A total of 10,000 stable A2780 infectants were isolated from each library. Most of the infected clones were expected to contain only one copy of the integrated provirus (Coffin, 1996).

4.3 ISOLATION OF p53 GSEs WHICH CONFER RESISTANCE TO CISPLATIN

Pools (>1000 clones) of stable A2780 infectants (p53 libraries or vector alone) were subjected to cisplatin selection. It has been shown that cisplatin resistant mutants can be isolated from A2780 cells using a single step selection protocol (McLaughlin *et al.*, 1991). Treatment of vector alone A2780 infectants with 12.5μM cisplatin for 24h gave a frequency of 2 x 10^{-5} surviving colonies/viable cell. Pools of A2780 cells infected with differing populations of either human or murine p53 recombinant retroviruses were selected at the same drug exposure. Frequencies of surviving colonies/viable cell were observed in the range 7 to 56 x 10^{-5}, representing a 4 to 29 fold increase in the number of cisplatin resistant colonies induced by the p53 recombinant libraries.

Genomic DNA was isolated from A2780 infectants before and after cisplatin selection. Integrated proviral elements were amplified by PCR using primers spanning the cloning site (Figure 20). The PCR products from A2780 infectants before cisplatin selection showed a continuous distribution in size range from 250 to 600bp (Figure 20, lanes 1-2). This indicates that representation of the random fragments was maintained after retroviral transduction. However, an enrichment for specific DNA fragments was observed after cisplatin selection of the A2780 infectants (Figure 20, lanes 3-5).

These discrete PCR-amplified fragments from cisplatin selected A2780 infectants were recloned into the pLNCX vector plasmid in the same position and orientation as the primary construct. Sequence analysis revealed that subcloned inserts were derived from TP53 cDNA (sequence alignments are shown in Appendix I). It is worth noting that less than 20% of the original plasmid libraries contained p53 inserts (the remaining inserts being from the vector constructs). These observations show that cisplatin selection of A2780 cells infected with either library enriched for p53-derived GSEs.

The orientation and position of the six p53-derived GSEs isolated are shown in Table 3 and Figure 21. The four antisense-oriented GSEs were clustered around the central region of the TP53 gene (nucleotides 361-680). The sense-oriented element GSE1 was predicted to encode a short 17 amino acid peptide containing 11 amino acids of the human p53 protein (300-310) plus amino acids from the outlying adaptor sequences. GSE1 is derived from an uncharacterised "linker" region found between the sequence-specific DNA binding and oligomerisation domains and is adjacent to a cyclin dependent kinase site (Bischoff *et al.*, 1990).

Table 3. Position and orientation of p53-derived GSEs.

GSE	Origin	Orientation	Position		Size	
			N	A	N	A
GSE1	Human	Sense	898-930	300-310	33	11
GSE2	Human	Antisense	362-463		102	
GSE3	Murine	Antisense	560-680		121	
GSE4	Human	Antisense	487-555		69	
GSE5	Murine	Antisense	558-610		53	
GSE6*	Murine	Sense	751-837	251-279	87	29

Sequence comparisons were made relative to human TP53 cDNA (Appendix I). N=nucleotides. A=amino acids. *This putative GSE was enriched after cisplatin selection but was not reintroduced into A2780 cells to confirm its activity.

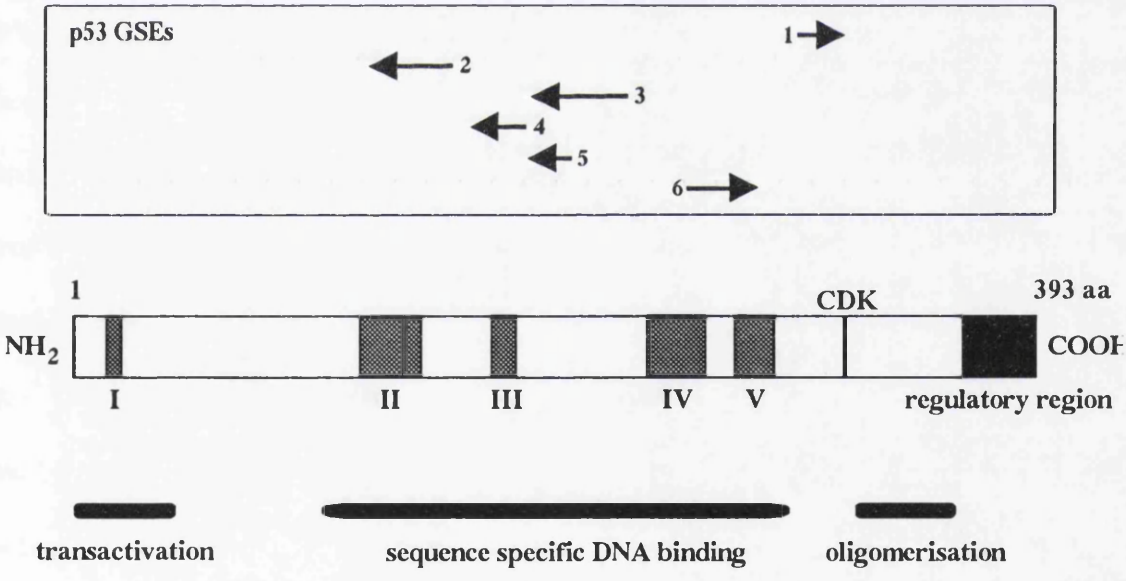


Figure 21. Schematic representation of human p53 showing position of GSEs.

Sense and antisense-oriented elements are shown by forward and reverse arrows, respectively. Some of the known functional sites within human p53 are indicated including the transactivation, sequence-specific DNA binding and oligomerisation domains (for a more complete description see reviews: Deppert, 1994; Harris, 1996). CDK=cyclin-dependent kinase site. Conserved regions of homology I-V shown as hatched boxes and C-terminal negative regulatory domain denoted by a solid box.

The predicted peptide sequence from the putative element GSE6 (amino acids 251-279) was localised to a portion of the sequence-specific DNA binding domain (Cho *et al.*, 1994). Interestingly, most of the GSEs derived from murine TP53 cDNA converged upon areas which contain highly conserved regions of homology (II-V) (Soussi *et al.*, 1990). This might be expected considering the GSEs of murine origin were selected in a human cell background.

The ability of the molecularly isolated GSE clones to confer cisplatin resistance was evaluated by both colony formation and growth inhibition assays. A2780 cells were independently transfected with individual plasmid clones (GSE1-5) and pools of transfectant colonies isolated (>50 colonies for each GSE). Table 4 shows the relative levels of resistance to cisplatin for A2780 GSE transfectants. GSE-carrying cells were more resistant than cells with insert-free provirus (1.2-2.9 fold by clonogenic assay). These levels are comparable with the 2-3 fold increase in cisplatin resistance observed by clonogenic assay when a dominant negative p53 mutant (codon 143, Val to Ala) is introduced into the same cell line (Vasey *et al.*, in press). The higher fold resistance measurements obtained using the MTT cell viability assay (1.5-8.2 fold) may reflect cytostatic effects on cell growth induced by cisplatin. These results demonstrate that the isolated p53 GSEs conferred cisplatin resistance in A2780 cells. It is important to note that these phenotypic effects were observed with mass populations of transfectant cells, thus obviating the problems associated with clonal variability (although possibly reducing the potential fold resistance measurements which may have been observed in clonal isolates).

Table 4. Characteristics of A2780 GSE transfectants.

GSE	Plating efficiency[a]	Fold resistance		Relative p53 protein[d] level
		Clonogenic assay[b]	MTT assay[c]	
VECTOR	0.14	1.0	1.0	1.0
GSE1	0.14	2.0 (0.1)	4.1 (0.7)	1.5
GSE2	0.17	1.2 (0.3)	1.5 (0.2)	0.8
GSE3	0.13	2.9 (0.6)	8.2 (0.9)	0.5
GSE4	0.15	1.9 (0.4)	3.2 (0.9)	0.4
GSE5	0.14	2.2 (0.6)	4.5 (0.2)	0.4

The figures in parenthesis refer to the SEM. Calculation of resistance and p53 protein levels were made relative to vector alone A2780 transfectants. a. Plating efficiencies were calculated from the fraction of seeded cells which formed colonies. There were no apparent differences between vector alone controls and GSE transfected populations with respect to population doubling times. b. Calculated from the surviving fraction of cells after exposure to 20μM cisplatin for 1h. Each value is the mean of four independent determinations. c. Calculated using IC_{50} values obtained from at least two experiments. d. Relative level of p53 protein as detected by Western blot analysis and standardised with vinculin expression. Each value is the mean of at least two determinations.

4.4 ABROGATION OF p53-MEDIATED DNA DAMAGE RESPONSE PATHWAY

The next step was to establish which mechanism the isolated p53 GSEs acted through. Ultimately, the inhibitory activity of antisense RNA is mediated by downregulation of protein expression (Takayama and Inouye, 1990; Denhardt, 1992). Western blot analysis revealed that the antisense GSE transfectants (GSE3-5) contained lower levels of p53 protein (0.4-0.5 fold) relative to vector alone cells (Table 4 and Figure 22).

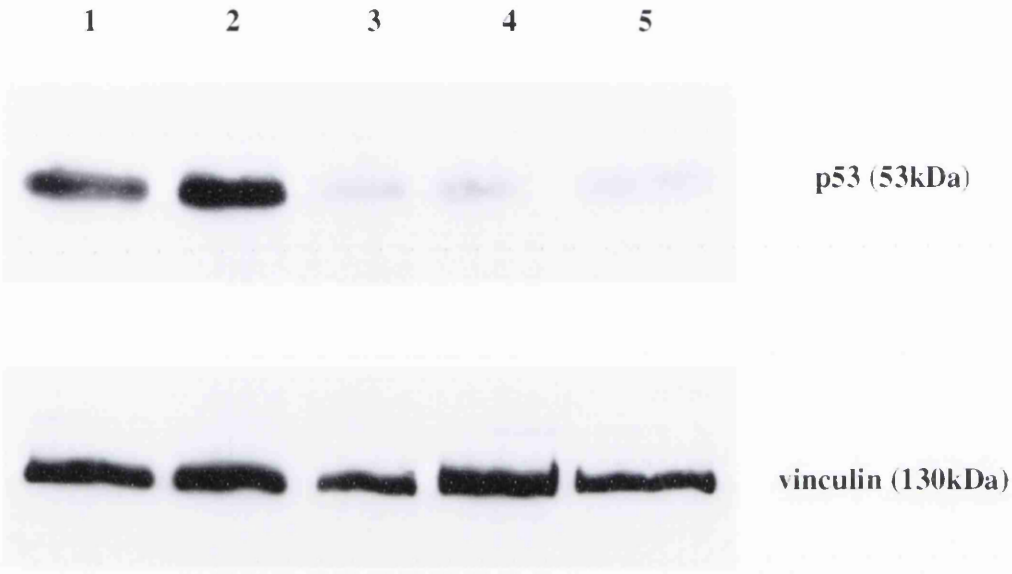


Figure 22. Levels of p53 protein in A2780 GSE transfectants.

Western blot analysis of p53 protein in cells transfected with GSEs by using an anti-p53 MAb (clone DO-1). Control for loading was provided by using an anti-vinculin MAb (clone VIN-11-5). Sizes were determined by using a 29.5-214kDa protein ladder (Gibco BRL). Lane 1, A2780 pLNCX transfectants; lane 2, A2780 GSE1 transfectants; lane 3-5, A2780 GSE3-5 transfectants.

These results suggest that the induction of cisplatin resistance in A2780 cells by antisense GSEs was due to loss of p53 function through reduced levels of the protein. It is worth noting that reintroduction of GSE2 did not significantly affect cisplatin resistance or the level of p53 protein in A2780 cells (Table 4). This may represent either inadequate expression in transfectant cells or the biological ineffectiveness of this particular GSE. The induced resistance to cisplatin and reduction in the steady-state level of p53 protein caused by expression of the antisense p53 GSEs further confirms the association between loss of p53 function and cisplatin resistance in these human ovarian tumour cells (Brown *et al.*, 1993; Eliopoulos *et al.*, 1995).

The sense-oriented GSEs were not anticipated to modulate p53 protein synthesis but work by encoding dominant negative mutant peptides (Roninson *et al.*, 1995). These peptides may inactivate p53 function in a variety of ways, perhaps by interference with modular activities such as sequence-specific DNA binding or oligomerisation (Herskowitz, 1987). GSE1-carrying cells contained increased levels of p53 protein (1.5 fold) compared to vector alone controls (Table 4). This may reflect stabilisation of endogenous p53 protein through interaction with the GSE1-derived peptide.

It was important to address what effect introduction of GSE1 into A2780 cells had on DNA damage induced G1 arrest and apoptosis. Radiation induced G1 arrest is dependent on wild-type p53 function in A2780 cells (McIlwrath *et al.*, 1994). Vector alone A2780 transfectants displayed a functional G1 arrest after irradiation, as measured by inhibition of DNA synthesis (Figure 23A and Table 5). This inhibition was partially abrogated in a pool of GSE1 transfectants, suggesting that the p53 protein was

inactivated in these cells, albeit marginally. Quantitation of cisplatin induced apoptosis was achieved using fluorescent (FITC) end-labelling of DNA strand breaks and flow cytometric detection as previously described (section 3.2). After cisplatin treatment, a 2.3 fold reduction was observed in the fraction of FITC-positive GSE1 transfectants compared to vector alone controls (Figure 23B and Table 5). This demonstrates that GSE1-carrying cells engaged apoptosis less readily than vector alone cells in response to cisplatin. Overall, these results suggest that p53 function is revoked by the sense-oriented GSE. No dissociation of cell cycle arrest and apoptosis activities was observed with this GSE.

Table 5. G1 arrest and apoptosis in A2780 GSE1 transfectants.

GSE	S-phase cells[a]			Apoptosis cells[b]		
	C	Treated	IR_S	C	Treated	Cis_A
VECTOR	45	20	45 (3)	1	17	16 (5)
GSE1	44	27	61 (1)	1	8	7 (2)

Principles of analysis, treatments and timepoints as outlined in Figure 23. C refers to untreated control samples. Each value represents the mean of four independent determinations with at least 15,000 events counted in each sample. The figures in parenthesis refer to the SEM. a. The percentage of total cells which were synthesising DNA and, therefore, incorporating BrdUrd. IR_S refers to radiation induced inhibition of DNA synthesis and was calculated using the number of treated cells in S phase expressed as a percentage of control samples. b. The percentage of total cells which were undergoing apoptosis. Cis_A refers to cisplatin induced apoptosis and was calculated after deduction of the basal apoptosis level as determined in untreated controls.

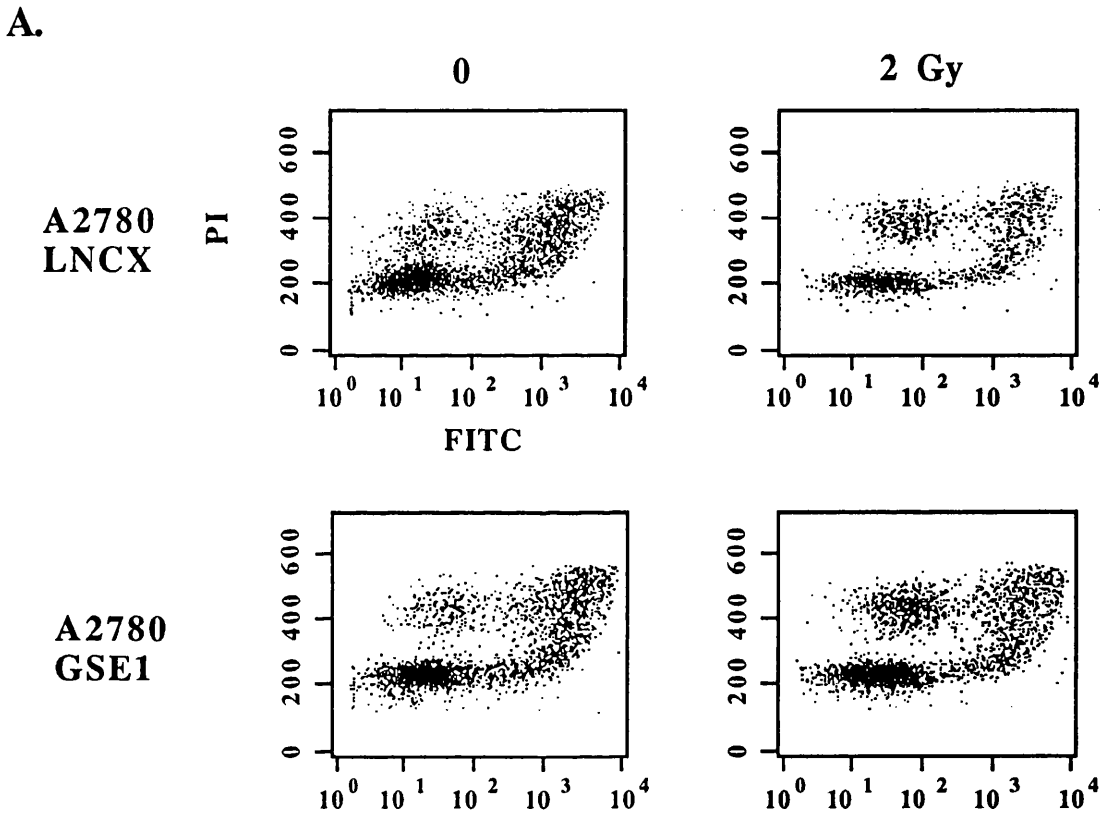
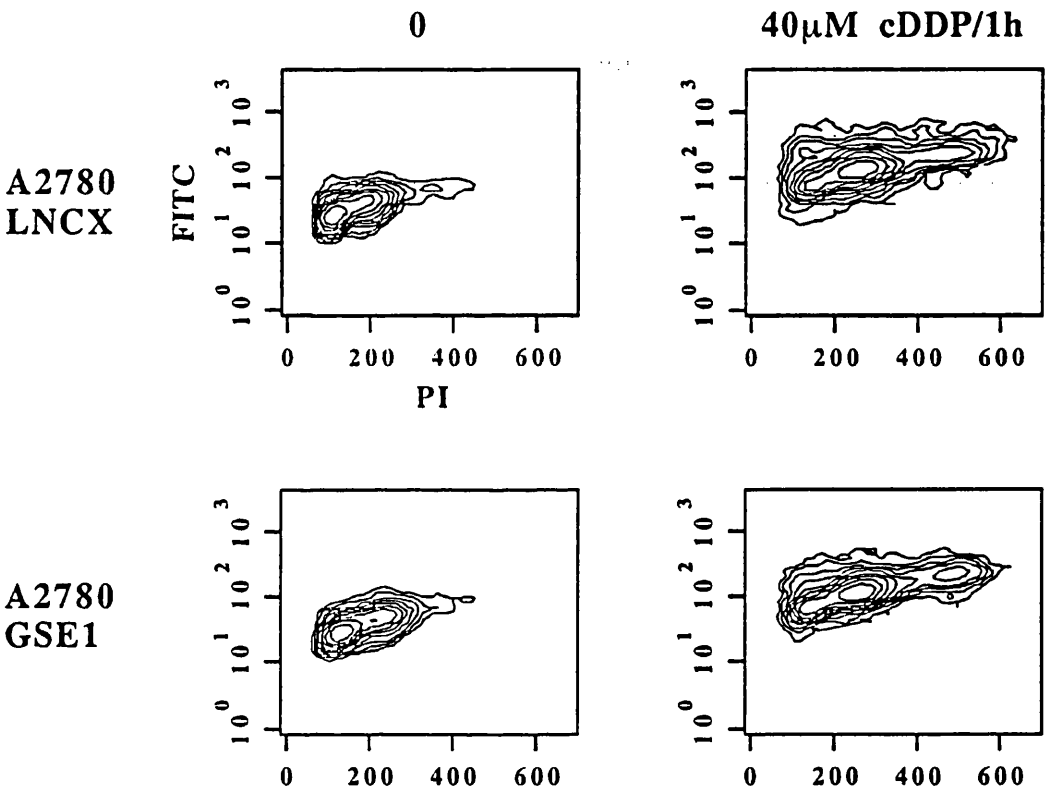


Figure 23. DNA damage induced responses in A2780 GSE1 transfectants.

(A) Radiation induced cell cycle arrests. Cell cycle analysis was accomplished by flow cytometric measurement of BrdUrd incorporation during a 4h pulse and PI staining as previously described (section 3.2). Cells were treated with 2Gy γ-radiation and analysed 24h later.

B.



(B) Cisplatin induced apoptosis. Flow cytometric analysis of DNA strand breaks as previously described (section 3.2). Increase in FITC represents fluorescent labelling of strand breaks. DNA was stained with PI. Cells were treated with 40μM cisplatin for 1h and analysed 72h later.

4.5 BIOLOGICAL ACTIVITY OF GSE1-ENCODED PEPTIDE

The results presented in the previous two sections indicate that introduction of GSE1 into A2780 cells conferred cisplatin resistance by abrogation of the endogenous p53-mediated DNA damage response pathway. To determine whether this effect was due to inhibition by the predicted p53-derived peptide encoded by GSE1, a peptide (PEP1-F) was synthesized which represented the entire 17 amino acid sequence anticipated by sequence analysis (which includes 3 amino acids at both termini derived from the adaptor sequences). A control peptide (PEP1-R), which has the same amino acid composition but in reverse order, was also synthesized. The control peptide did not show any significant homology to any presently known peptide sequences in the SwissPROT database.

Although small peptide molecules are commonly highly flexible, some basic structural information can be extrapolated from the primary structure. The basic structural characteristics of PEP1-F are displayed in Figure 24. The presence of two adjacent proline residues (Pro4 and Pro5) may have important structural implications in providing a high degree of constraint to the peptide in this region (Figure 24A). In fact, there is a strong possibility of a turn in the peptide between Pro5 and Gly6 (Figure 24B). Another turn may exist between Pro13 and Asn14. The central portion of the peptide appears to be quite hydrophilic and flexible (Figure 24C and D) and is likely to be on the surface of the molecule (Figure 24E). More detailed information on peptide structure would require NMR and circular dichromism studies.

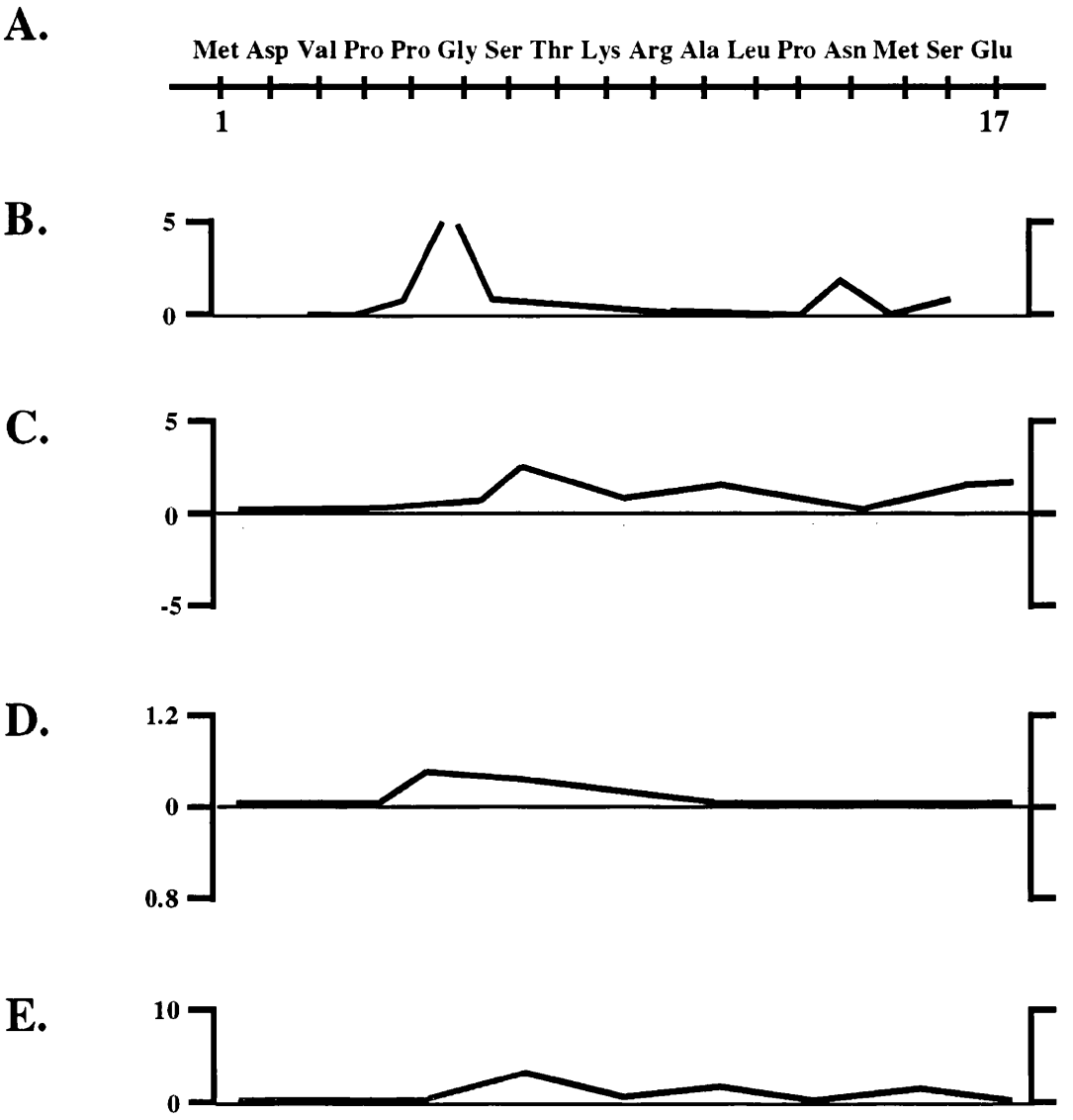


Figure 24. Structural characteristics of PEP1-F.

The PEPTIDESTRUCTURE and PEPPLOT programs within the GCG package were used to determine various structural characteristics of PEP1-F. The peptide was too short to reasonably predict any secondary structures apart from turns. Both turns and quantitative site- and residue-specific attributes such as hydrophilicity, surface probability and flexibilty are shown as continuous curves. An increase above indicated thresholds suggests a greater probability of a section of peptide adopting a particular structure or attribute. (**A**) Primary structure of PEP1-F. Residues are designated by 3 letter codes and numbered 1-17 starting from the N-terminus. Amino acids shown in blue are derived from p53. (**B**) Regions of the peptide molecule typically found in turns. (**C**) Hydrophilicity. (**D**) Flexibility. (**E**) Surface probability.

Peptides may be introduced into cells by a variety of methods including cationic liposome-mediated transfer or conjugation to a carrier molecule (e.g. Penetratin: Derossi *et al.*, 1994). Cationic lipids (e.g. DOTAP: Chen *et al.*, 1993) require interaction with negatively charged molecules in order to form liposomes. The presence of two acidic amino acids (Asp2 and Glu17) within PEP1-F suggested that such lipids could be effective at introducing the peptide into A2780 cells. PEP1-F induced resistance to cisplatin when introduced into A2780 cells by liposome-mediated transfer (Figure 25A). The control peptide did not modulate the sensitivity of A2780 cells to drug treatment. The effect on cisplatin resistance mediated by PEP1-F was dependent upon dose and time of administration. IC_{50} values from individual experiments (MTT cell viability assay used) were compared using a paired Student's t-test. A significant effect on resistance (RF=2.9; p value < 0.05) was demonstrated only when 10μM peptide was added 24h after cisplatin exposure (Figure 25A, right graph). Little or no effect was observed with 1μM nor when 10μM peptide was added at the same time as cisplatin treatment (Figure 25A, top and left graph, respectively). Maximal accumulation of p53 protein and transcription of CIP1 mRNA in A2780 cells occurs 24h after treatment with cisplatin (sections 3.3 and 3.4). This may be the critical time point for inhibition of p53 function with PEP1-F.

Wild-type p53 protein has a cryptic sequence-specific DNA binding function which can be activated *in vitro* by a variety of modifications including either deletion or phosphorylation of the C-terminal negative regulatory domain (Hupp *et al.*, 1992, Hupp *et al.*, 1995; Hansen *et al.*, 1996). PEP1-F inhibited binding of Baculovirus-expressed

human p53 protein to its consensus site in a dose-dependent manner (Figure 25B, top panel). Maximal inhibition was observed with a 10 fold excess of peptide compared to p53 protein. The control peptide appeared to stimulate DNA binding at high concentrations. Neither peptide affected the ability of a mutant p53 protein (Δ30), which has the last 30 C-terminal amino acids deleted (Hupp *et al.*, 1992), to bind specifically to its consensus site (Figure 25B, bottom panel). Small basic peptides have been shown to activate the latent sequence-specific DNA binding function of p53 (Hupp *et al.*, 1995). It is possible that the stimulatory activity of the control peptide at high concentrations may be due to non-specific involvement of its two basic amino acid residues (Lys9 and Arg10).

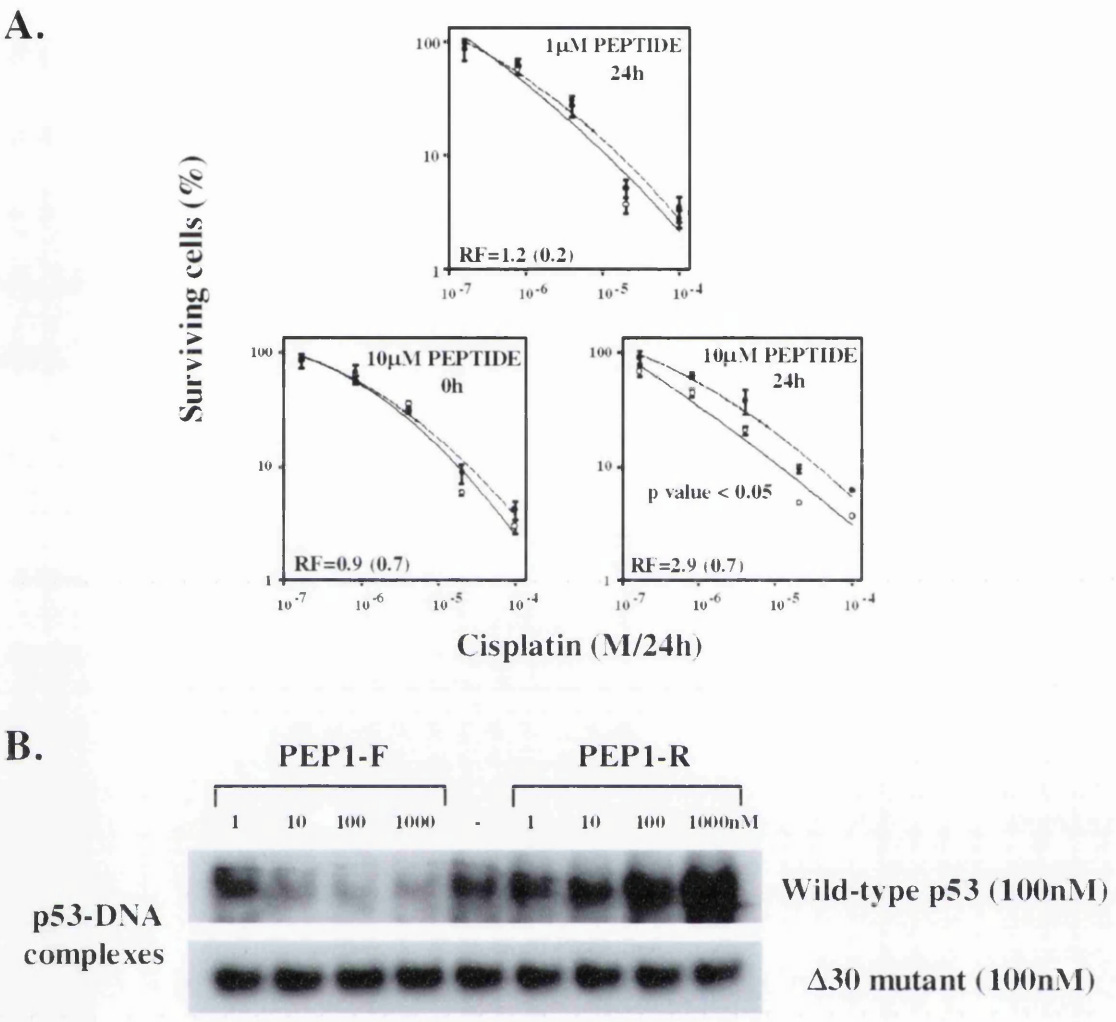


Figure 25. Biological activity of PEP1-F.

(**A**) Cisplatin resistance of A2780 cells transfected with synthetic peptides was determined by MTT analysis. Peptides were added to a final concentration of either 1 or 10μM using liposome-mediated transfer at the same time as cisplatin treatment or 24h later. Cells were exposed to peptides for 24h before washing. PEP1-R, solid line and open circles; PEP1-F, broken line and closed circles. Cell viability was determined relative to optical density readings at 570nm. Curve fitting was achieved using SigmaPlot 1.02 (Jandel Corporation). Each point represents the mean of four independent determinations with the error bars referring to the SEM. Fold resistance measurements (RF) were calculated using IC_{50} values obtained from at least three experiments. The figures in parenthesis refer to the SEM. All calculations were made relative to control peptide A2780 transfectants. A paired Student's t-test was used to compare IC_{50} values from individual experiments. (**B**) Gel mobility shift assay showing activated p53-DNA complexes. All lanes contained excess radiolabelled DNA probe and p53 protein - top (wild-type) and bottom (Δ30 mutant) panel. Synthetic peptides (PEP1-F and -R) were added to final concentrations indicated.

4.6 SUMMARY

Induced resistance of A2780 cells to cisplatin was used as the selection strategy to isolate GSEs from retroviral libraries expressing random fragments of human or murine TP53 cDNA. Six GSEs were identified, encoding either dominant negative mutant peptides or antisense RNA molecules which corresponded to various regions within the TP53 gene. Both types of GSE induced cisplatin resistance when introduced individually into A2780 cells. Expression of antisense GSEs led to decreased intracellular levels of p53 protein. One sense GSE induced loss of p53-mediated activities such as DNA damage induced G1 arrest and apoptosis. A synthetic peptide, representing the predicted amino acid sequence of this GSE, conferred resistance to cisplatin whe introduced into A2780 cells by liposome-mediated transfer and inhibited the sequence-specific DNA binding activity of p53 protein *in vitro*.

A possible limitation of this work was that the level of GSE expression remained undetermined. RT-PCR would be ineffective at measuring the extent of antisense GSE expression as two transcripts are produced from the proviral element (Figure 4). Therefore, the only alternative would be Northern blot analysis using the antisense GSE DNA as a probe. Determination of the expression level of the sense-orientated elements would require the generation of antibodies against the predicted peptides, followed by Western blot analysis. An approach to alleviate clonal variability of GSE expression is discussed in the next chapter. Finally, it must also be realised that less than 25% of the human or murine p53 recombinant libraries were screened and that other GSEs may await identification.

CHAPTER 5

DISCUSSION

5.1 CELL CYCLE ARRESTS, APOPTOSIS AND p53

Chapter 3 examined the ability of two DNA damaging agents, ionising radiation and cisplatin, to induce cell cycle arrests and apoptosis in the human ovarian A2780 carcinoma cell line and a series of cisplatin resistant derivatives. The primary advantage of this model system is that an isogenic group of cell lines was used (i.e. resistant lines generated from one sensitive precursor), thus obviating the problems associated with cell type variability. A variety of cell cycle perturbations induced by cisplatin have been reported including a decrease in the G1/S transition, delayed transit through S-phase and a G2 arrest (Demarcq *et al.*, 1992; Brown *et al.*, 1993; Ormerod *et al.*, 1994 and references therein). After cisplatin treatment of A2780 cells, it was observed that fewer cells entered S-phase from G1 which is consistent with a block at the G1/S boundary (section 3.2, Brown *et al.*, 1993). Furthermore, there was an increase in the number of late S-phase cells which indicated a slow-down in progression through this cell cycle stage.

Some studies have suggested that S-phase delay is the most important cytokinetic event after cisplatin treatment (Jackel and Kopf-Maier, 1991; Vaisman, Varchenko and Chaney, *unpublished observations*). However, rigorous examination of S-phase delay in the A2780 cell line and its cisplatin resistant derivatives was not carried out. The observation of a cisplatin induced G1 arrest in A2780 cells contrasts with the work of other investigators using human lung A549 carcinoma cells (wild-type p53 status) and murine L1210 leukaemic cells (unknown p53 status) which had suggested that cisplatin

does not induce a G1 arrest in mammalian cells (Demarcq *et al.*, 1992; Ormerod *et al.*, 1994). Such differences may be due to cell type variability in the p53 response to DNA damage (subsection 1.1.9).

Loss of p53 function in A2780 cells by introduction of a dominant negative p53 mutant (codon 143, Val to Ala) conferred resistance to multiple agents, including ionising radiation and cisplatin (McIlwrath *et al.*, 1994; Vasey *et al.*, in press). Indeed, both G1 arrest and apoptosis induced by either ionising radiation or cisplatin are dependent upon an intact p53-mediated DNA damage response pathway in this particular cell line (McIlwrath *et al.*, 1994; McIlwrath, Vasey and Brown, *personal communication*). A cisplatin resistant A2780 derivative, A2780/CP70, displayed a reduced ability to undergo G1 arrest and apoptosis after exposure to either ionising radiation or cisplatin (section 3.2). Furthermore, the A2780/CP70 cell line showed loss of p53 transcriptional activity, both on a constitutive basis and in response to DNA damage (section 3.4). Similar observations were noted in a series of recently isolated cisplatin resistant A2780 derivatives (Table 1; Anthoney *et al.*, 1996). Although the above experiments utilised "indirect" measures of p53 function, taken together the results indicate that the p53-mediated DNA damage response pathway is defective in these cisplatin resistant lines. The proposed mechanism of p53 dysfunction in these resistant lines is discussed in the next section.

Failure to undergo apoptosis in response to DNA damage is presumed to be the crucial event leading to acquired cisplatin resistance in these resistant lines. Cisplatin has been demonstrated to induce apoptosis in a number of cell systems (Evans and Dive *et*

al., 1993; Gorczyca *et al.*, 1993). Thymocytes from TP53 null mice are resistant to the induction of apoptosis by cisplatin, as are dominant negative p53 mutant (codon 143, Val to Ala) transfectants of A2780 cells (Vasey *et al.*, in press). Recent data has shown overexpression of bcl-2 protein in A2780/CP70 cells (Eliopoulis *et al.*, 1995). In addition, introduction of a BCL-2 gene into A2780 cells resulted in protection from cisplatin induced apoptosis (Eliopoulis *et al.*, 1995). These results are comparable with another study which demonstrated an association between the development of cisplatin resistance, mutation of the TP53 gene and reduced BAX expression in an alternative ovarian carcinoma cell line model system (Perego *et al.*, 1996). Therefore, p53-dependent regulation of downstream effectors of apoptosis such as BCL-2 and BAX may be important in determining the response of these cells to cisplatin.

It has been suggested that the G2 arrest induced by cisplatin is a prerequisite for cell death and that essential events occur during G2 which are necessary for death to occur by apoptosis (Eastman, 1990). The A2780/CP70 line displayed an abrogated G2 arrest in response to cisplatin but not ionising radiation (section 3.2). Indeed, the other cisplatin resistant A2780 derivatives have shown a reduced cisplatin induced G2 arrest also (Brown *et al.*, *personal communication*). Overall, the above results suggest that these resistant cells lose, at least partially, the ability to undergo cisplatin induced apoptosis through both p53-dependent and -independent pathways. A possible effector of p53-independent cisplatin induced apoptosis is the mammalian homologoue of the *C. elegans* cell death gene CED-3, known as ICE (Kondo *et al.*, 1995).

Cisplatin has been shown to induce apoptosis in proliferating, but not quiescent, thymocytes (Evans *et al.*, 1994). In contrast, other DNA damaging agents such as ionising radiation and etoposide appear to induce apoptosis independent of proliferation status. It has been proposed that DNA replication is necessary for conversion of cisplatin adducts into lesions capable of inducing apoptosis (Brown *et al.*, 1995). Consistent with this hypothesis was the observation of slower kinetics of p53 protein accumulation and CIP1 mRNA induction by cisplatin compared to that for ionising radiation (sections 3.3 and 3.4). The slower cisplatin induced responses are similar to those previously reported for UV damage induced p53 reponses (Khanna and Lavin, 1993; Lu and Lane, 1993). UV treatment of cells modifies DNA by causing thymine dimer formation but may also cause DNA strand breaks during NER or replication of the damaged DNA template (Nelson and Kastan, 1994). Therefore, both cisplatin and UV may share some components of the p53-mediated DNA damage response pathway not utilised by other agents.

5.2 MECHANISM of p53 DYSFUNCTION IN CISPLATIN RESISTANT CELLS

The cisplatin resistant A2780/CP70 cells have elevated levels of p53 protein, which is associated with dysfunctional p53-mediated reponses to DNA damage, yet they have wild-type TP53 mRNA sequence (Brown *et al.*, 1993). The p53 protein in these cells does not appear to be in a conformation often adopted by certain mutant forms of p53, as defined by conformation specific antibodies (Brown *et al.*, 1993). DNA damage was still able to induce increased p53 accumulation in these resistant cells, albeit with different kinetics to the parental line (section 3.3). In this respect, levels of p53 protein were sustained in A2780/CP70 cells as late as 96h after DNA damage. There is no evidence for overexpression of mdm2 protein in these cells, indeed mRNA levels of MDM2 were decreased along with other p53-responsive genes such as CIP1 (section 3.4). This suggests that the defect in the p53-mediated DNA damage response pathway is not downstream of p53.

However, no apparent differences were observed between A2780 and A2780/CP70 cells regarding the expression of p53 protein through the cell cycle (section 3.3). It must be considered that cell synchronisation may have revealed subtle changes in the cell cycle related expression of p53 protein in these cells. 2D-PAGE revealed no gross differences in the pattern of p53 protein isoforms between A2780 and A2780/CP70 cells (although again minor alterations may be present which were not resolved by this system). The p53 status of the remaining cisplatin resistant lines (A2780/MCP1-9) is unknown at present. Therefore, it is unclear whether these resistant lines have the same type of p53 dysfunction as that observed in A2780/CP70 cells.

Evidence is accumulating to suggest that cisplatin resistant A2780 derivatives also frequently acquire a microsatellite instability (RER+) or mutator phenotype (Table 1; Anthoney *et al.*, 1996), which is associated with defective mismatch repair (MMR) and loss of hMLH1 mRNA and protein expression (Drummond *et al.*, 1996; Brown *et al.*, *personal communication*). It is possible that the loss of p53 function in these cisplatin resistant cells is caused directly by the mutator phenotype. Direct sequestration of p53 may occur due to binding to DNA damage which may be tolerated in the resistant RER+ lines. In support of this hypothesis, it has recently been demonstrated that p53 is capable of binding to insertion/deletion mismatches (Lee *et al.*, 1995). Furthermore, the observation of increased nuclear p53 accumulation in some of the RER+ lines would be consistent with the concept of p53 sequestration (section 3.3).

Alternatively, it is possible that certain MMR proteins are necessary for coupling of DNA damage to the p53-mediated DNA damage response pathway. In fact, the MutSα component of the MMR complex can specifically recognise the cisplatin 1,2 intrastrand crosslink (Duckett *et al.*, 1996). Moreover, dumbbell substrates containing single mismatched bases have been shown to induce a p53-dependent G1 arrest (Wahl *et al.*, *personal communication*). A connection has also been demonstrated between the MMR system and the G2 arrest induced by certain alkylating agents (Hawn *et al.*, 1995). It is proposed that any agent that induces DNA mispairs (or signals to the MMR system) will cause a G2 arrest in MMR-proficient cells but not in MMR-deficient cells. Therefore, the loss of the cisplatin induced G2 arrest in A2780/CP70 cells (and possibly the other resistant lines also) may be due to the MMR defect (section 3.2). In summary,

defective MMR in these cisplatin resistant cells may lead to tolerance of DNA damage and reduced ability to engage both p53-dependent and -independent apoptotic pathways.

5.3 CLINICAL RELEVANCE OF p53 DYSFUNCTION

p53 appears to be an important determinant of response to cancer therapy in a wide spectrum of tumour types (Lowe, 1995). A large number of studies have associated TP53 mutations within the same tumour type with poor patient prognosis, hence, a reduced probability that therapeutic intervention will be effective (Chang *et al.*, 1995b and references therein). Patients with acute lymphoblastic leukaemia displayed substantially reduced survival and response to reinduction therapy on relapsing with TP53 mutations (Diccianni *et al.*, 1994). Similarly, in cases where TP53 mutations occurred in low-grade bladder tumours, patients showed a high probability of relapse after surgery and adjuvant therapy (Yoshimura *et al.*, 1995). A large study incorporating 316 consecutively presented breast cancers indicated that TP53 mutations were associated with worse prognosis and reduced effectiveness of adjuvant therapy (Bergh *et al.*, 1995). Interestingly, mutations of TP53 are not associated with chemoresistance in Burkitt's lymphoma (Preudhomme *et al.*, 1995), but Burkitt's lymphoma lines with TP53 mutations are more resistant to anticancer agents when treated in culture. This apparent discrepancy may be explained by the level of residual p53 activity in tumours compared with cell lines: Burkitt's lymphoma with TP53 mutations typically retain a normal TP53 allele, whereas the cell lines do not (O' Connor *et al.*, 1993; Fan *et al.*, 1994). Therefore,

careful consideration must be given to results from *in vitro* experiments before extrapolation to the *in vivo* clinical situation.

The observation that defective p53 function correlates with cisplatin resistance in the A2780 cell line model system may have important clinical relevance (Chapter 3). Many cisplatin sensitive tumour types express wild-type p53 (Peng *et al.*, 1993). Some recent reports have suggested a clinical correlation between the presence of wild-type p53 and sensitivity to platinum based chemotherapy in ovarian cancer (Al-Azraqi *et al.*, 1994; Righetti *et al.*, 1996). In addition, elevated levels of p53 protein, as detected by immunohistochemistry, have been correlated with resistance to chemotherapy in ovarian cancer, although this elevated p53 protein is again not explained in all cases by TP53 mutations (Righetti *et al.*, 1996). Thus, the phenotype of the A2780/CP70 cells (increased accumulation of p53 protein without mutation of the gene) may be relevant to clinically resistant ovarian tumours. Specifically, the absence of TP53 mutation in cells does not ensure that the p53-mediated DNA damage response pathway is intact (Vogelstein and Kinzler, 1992). This problem clearly complicates studies investigating the association between TP53 mutation and drug resistance.

If p53 function is an important determinant of sensitivity to DNA damaging agents in certain tumour types, several means of overcoming or modulating resistance can be envisaged. The most apparent is the utilisation of drugs which act in a p53-independent manner or are more effective in cells with defective p53. Dominant negative p53 mutant (codon 143, Val to Ala) transfectants of A2780 cells, while resistant to cisplatin, retain sensitivity to a number of agents including taxol and camptothecin

(Vasey *et al.*, in press). Intriguingly, recent evidence has demonstrated that loss of p53 function in human and murine fibroblasts confers increased sensitivity to taxol (Wahl *et al.*, 1996). If resistance is due to loss of p53 function then approaches which restore p53 function may increase drug sensitivity. Combination therapy using a p53-expressing adenovirus and cisplatin restored chemosensitivity to transplanted tumours harboring p53 mutations, producing substantial and sustained tumour regression (Fujiwara *et al.*, 1994). Interestingly, a study using retrovirus-mediated transfer of wild-type p53 into tumours of patients with lung cancer has recently been undertaken (Roth *et al.*, 1996). Such gene replacement therapies are predicted to work by inducing tumour cell death through either direct killing or bystander effects (Rosenfeld and Curiel, 1996). However, strategies such as these are limited by the method of gene transfer used and the expression level of the therapeutic transgene required. In addition, any adverse effects of the transgene on normal cells must be minimised.

Strategies are also being developed which target cells with mutant or defective p53. For example, the altered protein encoded by mutant TP53 alleles could serve as an antigen to direct an immune response against the tumour (Wiedenfeld *et al.*, 1994). Another approach is to use modified adenoviruses to selectively destroy p53-deficient cells. An attenuated adenovirus deleted in the E1B gene has been shown to replicate and be oncolytic predominantly in cells with defective p53 (McCormick *et al.*, *unpublished observations*). It is hypothesised that this may provide a means of targeting p53 defective tumours without affecting normal cells, and could effectively lyse cells which acquire resistance due to defective p53. A Phase I trial using the E1B deleted-adenovirus for

treatment of head and neck tumours is currently taking place within the CRC Department of Medical Oncology, Glasgow.

Several investigators have suggested the possibility of modulating p53 function using small peptides or peptidomimetic drugs (Anderson and Tegtmeyer, 1995; Hupp *et al.*, 1995). In particular, the feasibility of using small molecule approaches for restoring p53 function was based upon the observation that short basic peptides derived from the C-terminus of p53 activated sequence-specific DNA binding to its consensus site (Hupp *et al.*, 1995). Reactivation of mutant p53 using such molecules may lead directly to suppression of tumour growth, but could also modulate the sensitivity of the tumour cells to DNA damage. Finally, making certain cells more resistant to the adverse effects of chemotherapy may prove useful in the effective treatment of human cancer. For example, the generation of drug resistant bone marrow may facilitate the development of aggressive chemotherapeutic regimens that might otherwise be lethal due to marrow toxicity (Banerjee *et al.*, 1994; Flasshove *et al.*, 1995).

5.4 CISPLATIN RESISTANCE p53 GSES

Loss of p53 function by introduction of a dominant negative p53 mutant (codon 143, Val to Ala) into the A2780 cell line has been shown to induce resistance to cisplatin (Vasey *et al.*, in press). This phenomenon has been utilised during the selection of random TP53 cDNA fragments which confer cisplatin resistance in A2780 cells (Chapter 4). A number of GSEs have been isolated from both human and murine TP53 cDNA which are in either sense- or antisense-orientation (section 4.3). Each type of GSE induced cisplatin resistance when introduced individually into A2780 cells (section 4.3). Levels of resistance were comparable with those previously observed when a dominant negative p53 mutant (codon 143, Val to Ala) is expressed in the same cell line (Vasey *et al.*, in press). It is noteworthy that GSEs derived from murine TP53 cDNA acted in a human cellular context. This may reflect the high degree of evolutionary conservation found in the TP53 gene and/or the potent biological activity of the isolated GSEs (Gudkov *et al.*, 1993).

All of the antisense-oriented GSEs were derived from the central portion of the TP53 gene (spanning nucleotides 361-680) and this seemingly nonrandom distribution of biological active antisense elements has been previously observed by others (Gudkov *et al.*, 1993). Antisense oligonucleotides which target p53 expression are currently being used in clinical trials (Bishop *et al.*, 1996). Such agents are directed against a variety of regions within the TP53 gene, including exon 10 and the translation initiaton codon (Bayever *et al.*, 1994; Bi *et al.*, 1994). However, some concern has been expressed that

the phenotypic effect of some antisense oligonucleotides may be unrelated to the gene they have been designed to target (Barton and Lemoine, 1995). Expression of GSEs encoding antisense RNA reduced the intracellular level of p53 protein in A2780 cells confirming their inhibitory activity on p53 expression (section 4.4).

Sense-oriented GSEs are of particular interest as indicators of protein domains that are capable of independent functional interactions (Roninson *et al.*, 1995). It was anticipated that GSE1 would encode a 17 amino acid peptide, containing amino acids 300-310 of the human p53 protein which is localised to a previously uncharacterised "linker" region (Table 3 and Figure 21). When expressed in A2780 cells, this GSE reduced p53-mediated activities such as cell cycle arrest and apoptosis in response to DNA damage (section 4.4). An alternative study has utilised etoposide resistance and focus formation as selection strategies for the isolation of various sets of GSEs from rat TP53 cDNA (Ossovskaya *et al.*, 1996). Interestingly, GSE1 overlaps the region where the most powerful class of GSEs were observed in this independent study. The isolation of GSE1 allows a more precise identification of the region responsible for inhibition of p53 activity in this class of GSEs.

Liposome-mediated transfer of a synthetic peptide, comprised of the predicted amino acid sequence of GSE1, into A2780 cells conferred resistance to cisplatin (section 4.5). The effect on cisplatin resistance mediated by the peptide was dependent upon dose and, significantly, time of administration. The proposed critical time point (24h after cisplatin treatment) coincides with optimum activation of p53 (as defined by maximal p53 accumulation and transcription of CIP1) in A2780 cells (section 3.3 and 3.4). Therefore,

a narrow "window of opportunity" may exist for inhibition of p53 function with the peptide. Although stability of the peptide is unknown, rapid degradation would necessitate prompt interaction of the peptide with its target (presumably activated p53 protein).

The peptide also inhibited the sequence-specific DNA binding activity of human p53 protein *in vitro* (section 4.5). It is proposed that the peptide achieves its inhibitory activity by switching activated wild-type p53 protein to a latent conformation (Figure 26; Hupp *et al.*, 1992). An alternative scenario is that the peptide may interfere with oligomerisation of the p53, consequently reducing the number of p53 protein tetramers with the ability to bind to its consensus site. In light of the requirement for the C-terminal regulatory domain of p53, the peptide may interact with this area in order to mediate its inhibitory effects.

Both sense- and antisense-oriented GSEs induced cisplatin resistance in A2780 cells (which are permissive for p53-dependent apoptosis) through abrogation of the p53-mediated DNA damage response pathway by dominant negative interference or reduction in p53 protein levels, respectively. These results again indicate a strong association between loss of p53 function and cisplatin resistance in these chemosensitive human ovarian tumour cells. The efficacy of random fragment selection as a combinatorial approach to isolating biologically active p53 GSEs was also demonstrated. In particular, this is the first example of GSE selection being used to define the sequence of a biologically active synthetic peptide. In conclusion, the work presented in this study may have use in the rational design of low molecular mass modifiers of the p53 response.

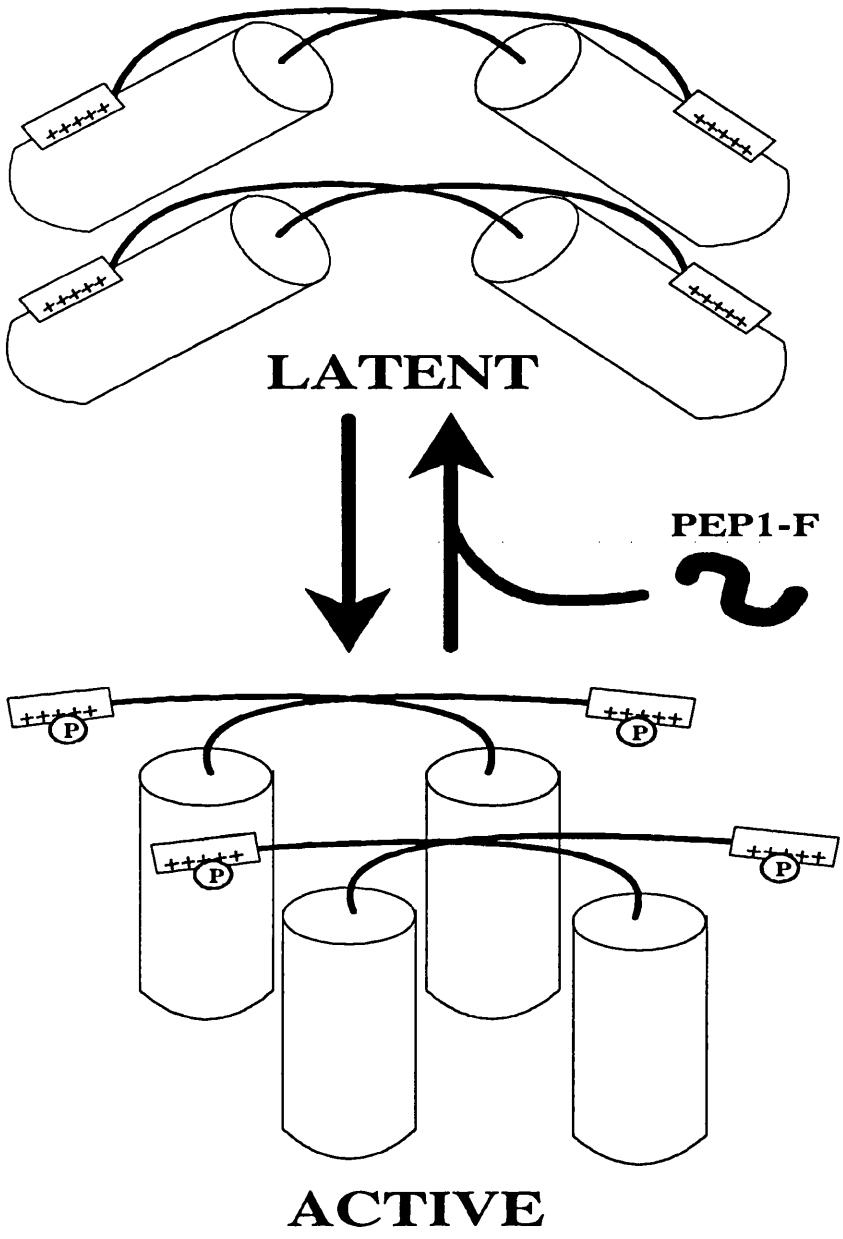


Figure 26. Proposed mechanism of p53 protein inhibition by PEP1-F.

The p53 protein tetramer exists in either a latent or active state for sequence-specific DNA binding. Phosphorylation of the C-terminal regulatory domain (indicated by a rectangle with basic residues represented as positive symbols) may convert the latent tetramer to an activated tetramer through a concerted transition of subunits. PEP1-F may mediate its inhibitory activity by reversing this conformational switch, possibly through interaction with the C-terminal regulatory domain. The oligomerisation and linker domains are shown by a black line and the rest of the p53 molecule by a cyclinder.

5.5 FUTURE PROSPECTS WITH p53 GSEs

The problem of clonal variability in the GSE experiments may be avoided by using an inducible gene expression system. Such an inducible system based on the regulatory elements of the Tn10-specified tetracycline-resistance operon of *E. coli* has been described (Gossen and Bujard, 1992). This inducible system may be used to examine the phenotypic consequences of switching GSEs on and off in a stringently controlled manner. An obvious advantage of using the tetracycline-regulated gene expression system is that as the regulatory elements are prokaryotic in origin, control of inducible transcriptional activity is not affected by endogenous control elements. Furthermore, and potentially of great interest, experimental control of the degree of promoter activity, by titration of tetracycline concentration present, may allow examination of possible quantitative effects of GSE function.

Further examination of the biological activity of the GSE1-encoded peptide can be imagined. The observation that PEP1-F inhibited the sequence-specific DNA binding activity of p53 *in vitro* may have important consequences for the effect of this peptide on the ability of p53 to transcriptionally activate certain target genes. The induction of cisplatin resistance in A2780 cells after transfer of the peptide may be due to loss of p53-mediated activities such as DNA damaged induced G1 arrest and apoptosis. Experiments which assess these points may be limited by the mechanism of peptide transfer used. Delivery of the peptide may be optimised by coupling to a carrier molecule such as Penetratin (Derossi *et al.*, 1994). It must be considered that the addition of this 16 amino

acid sequence may have adverse effects on the activity of the PEP1-F peptide. Functional mapping of the peptide should also be performed to determine the minimal region necessary for activity. Biotin conjugation, subsequent incubation with cell extracts and separation using streptavidin beads may allow isolation of proteins which interact with the peptide.

Depending upon genetic context, introduction of dominant negative p53 mutants can have apparently contradictory effects on cellular cisplatin resistance. For example, inactivation of wild-type p53 function in MCF7 cells using a dominant negative p53 mutant (codon 143, Val to Ala) conferred increased sensitivity to cisplatin (Fan *et al.*, 1995). Similar sensitisation effects have been observed when the same dominant negative p53 mutant is introduced into the A2780/CP70 cell line (Brown *et al.*, 1993), which as discussed earlier has abrogated p53 function. Therefore, the PEP1-F peptide may have use in reversing cisplatin resistance in these specific genetic backgrounds. Furthermore, many tumour cells have inactivated p53 function yet retain wild-type TP53 gene sequence (Vogelstein and Kinzler, 1992). Using appropriate selection strategies, it may be possible to identify elements from a p53 retroviral expression vector library which can restore p53 function in such cells.

5.6 GENETIC INACTIVATION OF p53 IN MAMMALIAN CELLS

Gene targeting in murine embryonic stem cells has allowed the production of strains of mice which carry inactivating mutations in the TP53 gene (Clarke, 1995 and references therein). Such TP53 null mice have proved an invaluable resource in describing the biological roles of p53. Indeed, studies using either *in vivo* or cultured cells from TP53 null mice have indicated functional p53 to be essential for maintaining genomic integrity and to have a pivotal role in responding to DNA damage, by either inducing cell cycle arrest or apoptosis. Furthermore, the decision between these options appears heavily influenced by cell type (Clarke *et al.*, 1994; Midgely *et al.*, 1995). The majority of TP53 null mice survive to birth, but then rapidly develop tumours. However, the strong predisposition of TP53 null mice towards thymic lymphomas is a major limitation to using such models in the study of human cancer. To counter this, it has been demonstrated that the tumour spectrum may be altered by exposure to specific carcinogens (Kemp *et al.*, 1993). Another approach is to interbreed TP53 null mice with other mutant strains to alter the genetic background (Morgenbesser *et al.*, 1994; Clarke *et al.*, 1995). Inactivation of genes in a tissue specific manner may allow the analysis of the *in vivo* consequences of TP53 deficiency within chosen cell lineages.

A number of other studies have directly inactivated p53 within a variety of somatic cells of human origin by introduction of dominant negative p53 mutants (examples include McIlwrath *et al.*, 1994 and Fan *et al.*, 1995 among others). The advantage of these cultured systems is the ease with which they can be manipulated and

the cellular environment can be controlled. However, as previously discussed it may be difficult to relate observations from these contrived cell systems to the *in vivo* context. Recently, dominant negative p53 mutants have been used to separate the cell cycle arrest and apoptosis functions of p53 (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996; Rowan *et al.*, 1996). The major advantage of the GSE approach used in this thesis is the randomness of the selection of functional elements derived from p53. It may be possible using this technology to identify further portions or mutants of p53 which can function independently from its other biological roles. In conclusion, investigation into the biological roles of p53 in both tumour and normal cells and the possible manipulation of these may have important ramifications for the effective treatment of human malignancy.

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APPENDIX I

Sequence alignments of isolated p53 GSEs versus human TP53 cDNA. Comparisons were made using the FASTA program (contained within the GCG package). The human TP53 cDNA clone was obtained from the GenEMBL database (accession code: K03199). Coding sequences within this clone started from nucleotide 215. Predicted sites of translation initiation and termination for sense-oriented elements shown in red. Anticipated peptide sequences displayed in blue.

Antisense-oriented elements

GSE2: 100% identity in 102bp overlap

```
          699       689       679       669       659       649
HP53  GCTTGTAGATGGCCATGGCGCGGACGCGGGTGCCGGGCGGGGGTGTGGAATCAACCCACA
                                  ||||||||||||||||||||||||||||||
GSE2                              TGCCGGGCGGGGGTGTGGAATCAACCCACA
                                          10        20        30

          639       629       619       609       599       589
HP53  GCTGCACAGGGCAGGTCTTGGCCAGTTGGCAAAACATCTTGTTGAGGGCAGGGGAGTACG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GSE2  GCTGCACAGGGCAGGTCTTGGCCAGTTGGCAAAACATCTTGTTGAGGGCAGGGGAGTACG
           40        50        60        70        80        90

          579       569       559       549       539       529
HP53  TGCAAGTCACAGACTTGGCTGTCCCAGAATGCAAGAAGCCCAGACGGAAACCGTAGCTGC
      ||||||||||||
GSE2  TGCAAGTCACAG
           100
```

GSE3: 78% identity in 121bp overlap

```
        919       909       899       889       879       869
HP53  ATGTAGTTGTAGTGGATGGTGGTACAGTCAGAGCCAACCTCAGGCGGCTCATAGGGCACC
                                  ||| || |||| || |||||||| ||  ||
GSE3                              GAGTCAGCCTCGGGTGGCTCATAAGGTTCC
                                          10        20        30

        859       849       839       829       819       809
HP53  ACCACACTATGTCGAAAAGTGTTTCTGTCATCCAAATACTCCACACGCAAATTTCCTTCC
      ||||| || ||  ||||||  || ||||| |||| ||||||   |  |||||||||| |
GSE3  ACCACGCTGTGGGGAAAAGGCTTCCTGTCTTCCAGATACTCGGGATACAAATTTCCTACA
           40        50        60        70        80        90

        799       789       779       769       759       749
HP53  ACTCGGATAAGATGCTGAGGAGGGGCCAGACCATCGCTATCTGAGCAGCGCTCATGGTGG
      || |||||||||||||| ||||| ||||| |
GSE3  ACCCGGATAAGATGCTGGGGAGGAGCCAGGC
           100       110       120
```

GSE4: 100% identity in 69bp overlap

```
     799        789        779        769        759        749        740
HP53   GATAAGATGCTGAGGAGGGGCCAGACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGCA
                                       ||||||||||||||||||||||||||||
GSE4                                   GCTATCTGAGCAGCGCTCATGGTGGGGGCA
                                                 10         20         30

     739        729        719        709        699        689        680
HP53   GCGCCTCACAACCTCCGTCATGTGCTGTGACTGCTTGTAGATGGCCATGGCGCGGACGCG
       |||||||||||||||||||||||||||||||||||||
GSE4   GCGCCTCACAACCTCCGTCATGTGCTGTGACTGCTTGTA
                40         50         60
```

GSE5: 79% identity in 53bp overlap

```
              839        829        819        809        799        789
HP53   AGTGTTTCTGTCATCCAAATACTCCACACGCAAATTTCCTTCCACTCGGATAAGATGCTG
                                       |||||||||||||||| |  ||||||||||||
GSE5                                CGGGATACAAATTTCCTTCCACCCTTATAAGATGCTG
                                          10         20         30

              779        769        759        749        739        729
HP53   AGGAGGGGCCAGACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGCAGCGCCTCACAAC
        |||||  |||||  |||
GSE5   GGGAGGAGCCAGGCCA
        40         50
```

Sense-oriented elements

GSE1: 100% identity in 33bp overlap

```
                                          M  D  V  P  P  G  S  T  K  R  A
                                             10         20         30
GSE1                                  ATGGATGGATGTGCCCCCAGGGAGCACTAAGCGAGCA
                                                 ||||||||||||||||||||||||||
HP53   AATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCCAGGGAGCACTAAGCGAGCA
        1080       1090       1100       1110       1120       1130

        L  P  N  M  S  E  *
        40         50
GSE1   CTGCCCAACATGAGTGAGTGA
       |||||||||
HP53   CTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAATAT
        1140       1150       1160       1170       1180       1190
```

GSE6: 90% identity in 87bp overlap

```
                                                M  G  M  N  X  L  X  I
                                               10         20         30
GSE6                                        ATGGATGGATGGGCATGAACCNCCTTNCCATC
                                                                :||| :|||||
HP53    ACTACATGTGTAACAGTTCCTGCATGGGCGGCATGAACCGGAGGCCCATCCTCACCATC
       920        930        940        950        960        970
```


I T L E D S X G N L L G R D N F E V R V
40 50 60 70 80 90
GSE6 ATCACACTGGAAGACTCCNNTGGGAACCTNCTGGGACGGGACAACTTTGAGGTTCGTGTT
HP53 ATCACACTGGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCATGTT
980 990 1000 1010 1020 1030
C A C P G *
100 110
GSE6 TGTGCCTGCCCTGGGTGAGTGAGTGA
HP53 TGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAG
1040 1050 1060 1070 1080 1090

LIST OF PUBLICATIONS ARISING FROM PhD THESIS

McIlwrath*, A, **Gallagher***, W. M., McGarry, L. and Brown, R. P53-dependent apoptosis and radio-resistance of human ovarian tumour cells, *manuscript submitted.* *The first two authors contributed equally to this work.

Gallagher, W. M., Cairney, M., Schott, B., Roninson, I. B. and Brown, R. Identification of p53 genetic suppressor elements which confer resistance to cisplatin. *Oncogene*, in press.

Anthoney, A., McIlwrath, A., **Gallagher**, W., Edlin, A. and Brown, R. (1996). Microsatellite instability, apoptosis and loss of p53 function in drug-resistant tumor cells. *Cancer Research*, **56**, 1374-1381.

The last two publications are included with this thesis for the reader's information