Characterisation Of Novel Genetic Suppressor Elements Conferring Resistance To Cisplatin

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DECLARATION

I declare that the work of which this thesis is a record has been carried out by myself, unless otherwise stated and that the work has not previously been accepted for a higher degree.

Carolyn Louise Spraggon

ABSTRACT

Cisplatin is a chemotherapeutic agent which is thought to exert its cytotoxic effects by causing DNA damage in the form of crosslinks. The precise molecular mechanisms involved in the transduction of this damage to a cytotoxic outcome are as yet not fully characterised. Resistance to cisplatin is a major clinical problem making elucidation of its mechanisms of action and mechanisms of resistance to it an important area of investigation.

Genetic suppressor elements (GSE's) are small, randomly generated fragments of DNA which are isolated by conferring a selectable phenotype when expressed in target cells. They are powerful tools in the elucidation of recessive mechanisms of resistance to drug treatment. The work presented here describes the characterisation of GSE's generated by DNaseI digestion of equalised HeLa total cDNA library and isolated by Kirschling and Roninson (Chicago, USA) by cisplatin selection. Characterisation of GSE's generated from the random digestion of a p53 cDNA library are also included.

The GSE's isolated by Kirschling and Roninson have been transfected into the human ovarian adenocarcinoma cell line, A2780, and selected using cisplatin. Further examination of these cell lines revealed that the line generated by transfection of GSE 7.10 showed a high level of resistance to cisplatin (2-3.8 fold) and a high level of cross-resistance to an additional DNA damaging agent, ionising radiation. I have shown, by PCR amplification, that the GSE 7.10 construct is present in these transfectants and confirmed by DNA sequencing that it is 100% identical to a 188bp fragment of the human phosphoglycerate mutase-B gene. Analysis of the cell cycle checkpoints of these transfectants in response to DNA damaging agents has revealed that GSE 7.10 abrogates the G1/S checkpoint in response to ionising radiation, and suggests a reduction in the same checkpoint arrest in response to cisplatin. I have also purified and sequenced four of the p53 cisplatin GSE's and confirmed that they are fragments of the p53 gene.

Chapter 1

Chapter 1 Introduction

Chemotherapy has proved to be clinically successful in the treatment of many cancers. One of the main obstacles however to the ability to cure cancer is the resistance of many tumours to chemotherapeutic drugs and the lack of understanding of the mechanisms involved in this resistance. Understanding the precise mechanisms of action of chemotherapeutics should facilitate the circumvention of resistance and lead to the generation of novel, more selective agents.

One of the most successful groups of anti-cancer agents are the platinum co-ordination complexes, including *cis*-diamminedichloroplatinum(II) known as cisplatin (CDDP). This is an effective treatment for a variety of malignancies such as ovarian, testicular, bladder and head and neck tumours (1), and the main reason for treatment failure is believed to be the selective outgrowth of a drug resistant cell population.

The aim of work presented here was to characterise novel genetic suppressor elements which confer resistance, *in vitro*, to cisplatin in human tumour cell lines. In order to understand the approaches I have taken, it is important to understand the mechanisms involved in cisplatin toxicity and the cellular responses to cisplatin treatment. I have presented a description of what is known about the DNA damage which cisplatin induces and the pathway(s) leading from this damage to cell cycle arrests and / or apoptosis, processes which ensure that damaged DNA is not passed on to subsequent cellular generations. I have also described the genetic suppressor element approach in identifying novel recessive mechanisms of drug resistance.

1.1 Cisplatin Discovery

Cisplatin was discovered in 1965 by Rosenberg (2) as a consequence of administering an AC current to *E.coli* via platinum electrodes. This treatment prevented the bacteria from dividing and resulted in filamentous growth which is indicative of an SOS response to DNA damage. This was subsequently shown to be a result of a compound formed on the electrodes, i.e. *cis*$Pt(II)(NH_3)_2Cl_2$. Investigation in various mammalian tumour cells (3) (including carcinomas, sarcomas and leukaemias) revealed that this compound is active as a anti-tumour agent in the *cis* conformation, whereas the *trans* isomer is inactive. In 1979, cisplatin was approved as an anti-tumour drug.

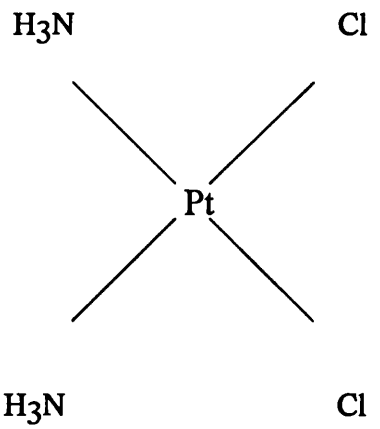


Figure 1.1.1 Chemical Structure of Cisplatin

Cisplatin is a neutral, square, planar co-ordination complex, which, when in the low intracellular chloride concentration environment, becomes an active, charged electrophile by substitution of the chloride groups by hydroxyls. As an electrophile it is capable of reacting with nucleophiles, such as nucleic acids.

1.2 Mode of Action of Cisplatin

1.2.1 DNA As The Target Of Cisplatin

The precise molecular mechanism of cisplatin cytotoxicity is uncertain, but there is strong evidence to suggest that the primary cytotoxic target of this drug is DNA. 1% of cellular cisplatin binds DNA and has been shown to induce various lesions within DNA, including monofunctional adducts, inter- and intra- strand crosslinks and DNA-protein crosslinks. The most common lesion (65%) is a 1,2 d(GpG) intrastrand crosslink, i.e. between two adjacent guanine bases, and studies suggest that this is the cytotoxic lesion resulting from cisplatin treatment (4, 5, 6). It has been observed that this type of crosslink is poorly repaired (7), possibly due to the binding of the HMG1 protein or proteins containing a HMG box (8). The *trans* isomer of the platinum co-ordination complex also induces lesions in DNA, but not the 1,2 d(GpG) intrastrand crosslink. The transplatin-induced lesions are more rapidly repaired than those caused by cisplatin and it is thought that it is the persistence of cisplatin-induced lesions which accounts for its higher cytotoxicity (9).

1.2.2 Responses to DNA Damage

Cell cycle arrest and apoptosis appear to be the two most important consequences of various types of DNA damage (10). Both of these processes ensure that the damage is eliminated from the cellular population, by allowing time for DNA repair prior to replication, or by destroying cells containing damaged DNA, respectively. Persistence of this damage could potentially lead to the generation of a population of cells with a transformed phenotype, so it is vital that the appropriate signal transduction pathways, downstream of DNA damage, are functioning correctly. One can envisage that interference in these pathways would be a possible mechanism of acquiring resistance to DNA damaging agents such as cisplatin.

1.3 Cell Cycle Control

1.3.1 Early Characterisation

In order to understand the consequences of cisplatin induced DNA damage, a description of the basic controls governing eukaryotic cell cycle progression is detailed in figure 1.3.1 and below.

The term 'cell cycle' incorporates all of the events which every cell undergoes from its release as one of the progeny of a parental cell, to its own division into two daughter cells. The cell cycle is divided into two main compartments, interphase and mitosis, both of which can be sub-compartmentalised. Essentially, interphase is the period during which the cell undergoes growth and replicates its sub-cellular components, including its DNA. Mitosis is the process of DNA segregation into two compartments which, as a result of cytokinesis, become two daughter cells.

In the early 1970's Rao and Johnson (11, 12) attempted to identify controls involved in the progression of the cell cycle. They employed cell fusion experiments using cells arrested in various phases of the cell cycle i.e. G1 + M, S + M and G2 + M. In all three cases, premature chromosome condensation occurred, indicating that there is a dominant factor in M-phase cells which drives cells towards cell division.

Much of the early investigation into the biochemical regulation of the cell cycle was carried out in the 1970's in the budding yeast *S.cerevisiae* and the fission yeast *S.pombe* (13, 14). This work yielded a great deal of information on the molecules controlling the cell cycle and their interactions. Many of these molecules exhibited a large degree of homology between these two yeast, suggesting that the mechanisms of control were evolutionarily conserved. This was confirmed by investigations in higher organisms including sea urchins, clams and *Xenopus* and also in mammalian systems (15).

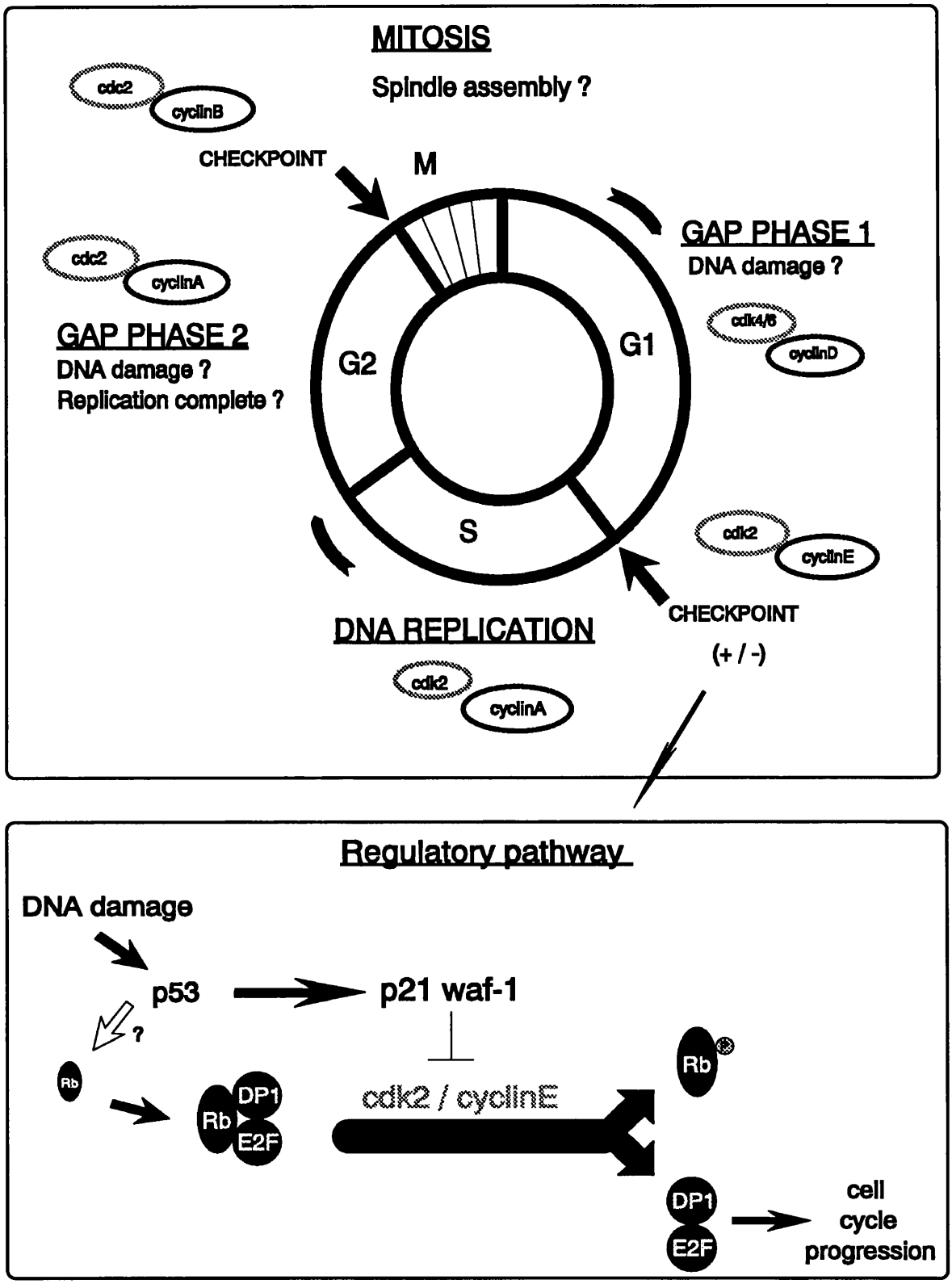


Figure 1.3.1 Summary of controls governing the eukaryotic cell cycle

The work in yeast revealed the presence of checkpoints in the cell cycle which were required to ensure that the cell division process was successful. In *S.cerevisiae*, the major checkpoint has been shown to be at the G1 to S phase boundary, the START site where a decision occurs as whether to mate, quiesce, or enter S phase and replicate DNA. Commitment to cell division beyond this point is irreversible and so facilitated the identification of cell division cycle (CDC) mutants, including the CDC28 and CLN1, 2, and 3 genes. These were subsequently shown to be homologous with the cyclin dependent kinases and cyclins, respectively, in mammals. Genes which are important in the S / G2 / M transitions were also identified, but *S.pombe* proved to be the more appropriate organism for this work, as its major checkpoint is at G2 / M.

In *S.pombe*, the G2/M checkpoint appears to be the most highly regulated and its transition is dependent upon cell size. Again, cell cycle mutants revealed important molecular components of the cell cycle machinery. A protein kinase homologue of the *S.cerevisiae* CDC28 protein was identified and named cdc2, this was found to function at both the G1/S and G2/M checkpoints. Homologues of the cyclins were also identified and were, in many cases, shown to substitute for the equivalent *S.cerevisiae* protein.

Work by Masui (16) on *Xenopus* oocytes, also in the early 1970's, revealed the importance of a maturation promotion factor in the development of the ovum to an egg i.e. the progression from G2 of meiosisI to metaphase of meiosisII. This progression is similar to the G2/M transition in mitosis. In the 1980's, MPF was purified by Maller (17) and was identified as a complex of a 34kDa protein and a 45kDa protein. The 34kDa component was shown to be homologous to the cdc2 protein of *S.pombe* and CDC28 of *S.cerevisiae* and the 45kDa protein to be a cyclin homologue.

Studies carried out by Tim Hunt in sea urchin eggs (18) identified 2 proteins with distinct patterns of expression during development i.e. reaching a maximum at mitosis and decreasing rapidly around metaphase. These have been identified as

cyclins A and B i.e. G2/M cyclins. The cyclin family has expanded rapidly in mammalian systems (A-H to date), all sharing sequence homology and usually a pattern of cyclical expression. Cyclins are found in complexes with specific partners, the homologues of the CDC28 and cdc2 proteins, termed cyclin-dependent kinases (cdk's) (19).

1.3.2 Cell Cycle Genes : Response To DNA Damage

The genes described above are essentially involved in the control of the normal cell cycle, but many genes involved in DNA damage detection and repair have also been identified in yeast. The RAD9 gene of *S.cerevisiae* was identified by Weinert and Hartwell (20) as being essential for the arrest of cell division following DNA damage. Irradiated *rad9* cells do not arrest at the G2 checkpoint as wild-type cells do, but continue to divide with damaged DNA and die after several rounds of replication. Mammalian cells in culture have been shown to arrest at the G2 checkpoint in response to cisplatin treatment (21), an arrest which is hypothesised to be dependent on the number of cisplatin adducts formed. The p53 tumour suppressor protein may play a role in this arrest but a comprehensive genetic explanation for this arrest has not as yet been presented. The response to cisplatin treatment has also been examined in *S.pombe* (20). Cells which are mutant for a protein kinase, wee1, which normally regulates cdc2 activity fail to arrest following treatment with cisplatin and those with an abrogated G2 arrest are hypersensitive to this drug. It appears therefore that cell cycle arrest is an important determinant of cisplatin sensitivity in yeast as well as mammalian cells. A whole family of RAD genes have been identified in *S.cerevisiae*, as well as homologous genes in *S.pombe*, suggesting a high level of evolutionary conservation in the response of cells to damaged DNA. Additional genes have been identified which are essential for G2 and S phase arrests (22) and whose protein products interact with various Cdc proteins, examples include the MEC genes and other RAD genes. It is

apparent that it is not only the genes involved in the regulation of the normal cell cycle which are evolutionarily conserved.

A human genetic disease, ataxia telangiectasia (AT), illustrates the conservation of the pathways which ensure the correct response to DNA damage. Individuals with AT display several disease-state characteristics, including an increased susceptibility to cancers of approximately 100-fold (for a recent review see (23) and references therein). This autosomal hereditary disease also correlates with an ability of cells to continue DNA replication in the presence of DNA damage, a feature indicative of a loss of the G1/S phase checkpoint arrest. These individuals have also been shown to be hypersensitive to X-rays and radio-mimetics known to cause DNA double strand breaks. Mutations in the *ATM* gene have been shown to be responsible for the AT phenotype. The gene has been cloned and sequenced and shows homology with the phosphatidylinositol 3-kinases from both mammals and yeast, suggesting that the gene may be involved in signal transduction. A number of recent papers have shown that there is also a high degree of homology with other yeast genes (*S.pombe* and *S.cerevisiae*) such as the TOR gene family, MEC1, TEL1 and *rad3*, mutations in which result in cell cycle defects in terms of arrest capability, chromosome structure and DNA repair (24, 25, 26, 27).

Studies in yeast have been vital in isolating genes involved in the eukaryotic cell cycle, both in the normal situation and in response to DNA damage. The degree of evolutionary conservation and number of genes involved in cell cycle controls illustrates the importance of this process and its importance in human disease states, particularly the development of a transformed phenotype.

1.3.3 Control Of CDK~Cyclin Activity

From the work in yeast and invertebrates, cdk's were shown to be responsible for cell cycle progression in concert with a variety of cyclin partners and control of these cdk~cyclin complexes is vital for the correct progression of the cell cycle. Cell cycle checkpoints help to ensure that a perfect copy of DNA is passed on to

daughter cells. The G1/S checkpoint ensures that DNA is intact and undamaged before replication proceeds and the G2/M checkpoint appears to ensure that DNA replication has been successfully completed before cell division occurs. It is the cdk~cyclin complexes which are the targets of the signal transduction pathway from DNA damage to cell cycle arrest (19).

Normal regulation of these complexes is governed by the activation and deactivation of the cdk component by dephosphorylation and phosphorylation respectively. This is controlled by kinases and phosphatases which also have homologues in *S.pombe* and *S.cerevisiae*. The mammalian homologue of *S.pombe* wee1 is responsible for phosphorylating cdc2 (the main G2/M cdk) on a tyrosine residue thus rendering it inactive and cdc25 for dephosphorylating the tyrosine residue and activating the protein. In turn, various kinases and phosphatases are responsible for activating and de-activating wee1 and cdc25 (28).

Recently, a number of mammalian cdk inhibitors have been identified which are induced in response to DNA damage and various growth signals (19). These function by directly binding to the cdk's rather than causing a subtle post-translational modification. Waf-1 (Cip1, p21, Sdi1), which is a universal cdk inhibitor, was identified by several groups as a protein being induced by wild type p53 (29, 30), as a protein which interacted with cdk's and a cyclin and as an inhibitor of DNA synthesis in senescent cells. Its control is described in more detail in section 1.3.4 but it is responsible for arrests at the G1/S checkpoint in response to DNA damage, and can also inhibit DNA synthesis (31).

1.3.4 The Role of p53 In The Signal Transduction Pathway From DNA Damage To Cell Cycle Arrests

The integrity of the DNA damage response pathway is crucial if DNA damaging agents such as cisplatin are to be cytotoxically effective. Modulation of various steps in the pathway may allow the sensitivity of cells to these agents to be

increased. A summary of the response pathway is shown in figure 1.3.2, indicating the points which are as yet uncharacterised.

In response to DNA damage, caused for example by cisplatin, it is hypothesised that the damage is detected and a signal transduction pathway is initiated. A DNA double strand break is known to induce p53-mediated cell cycle arrest (32), although the initial molecular DNA damage detector for cisplatin has not been identified. Several DNA damage recognition proteins (DRP's) have been characterised. Chu and Chang (33) and McLaughlin *et al.* (34) identified factors which recognised cisplatin-induced DNA damage and were elevated in cisplatin resistant cells, and Donahue *et al.* identified an additional factor (35) recognising the predominant cisplatin DNA lesion, d(GpG). Hughes *et al.* used a damaged DNA affinity precipitation technique to identify proteins which bound to cisplatin-induced DNA lesions, but not to undamaged DNA (36). The two predominant proteins were N-terminally sequenced and shown to be identical to the HMG-1 and HMG-2 proteins.

p53 is a nuclear phosphoprotein of 393 amino acids, the gene for which is mutated or lost in more than 50% of human tumours (37, 38). It has an N terminal acidic transactivation domain, a central region which contains very highly conserved sequences and has a sequence-specific DNA binding activity. The C-terminal domain contains the nuclear localisation sequences as well as regulatory phosphorylation sites, and is responsible for the oligomerisation of p53 dimers and tetramers. A schematic diagram of the functional regions of p53 is shown in fig. 1.3.3. The protein is highly regulated, and its main role appears to be that of a DNA sequence-specific transactivator.

The p53 tumour suppressor protein has been shown to bind to a certain type of damaged DNA *in vitro* and form stable complexes (39). The damage was not however of the type induced by cisplatin but contained insertion deletion mismatches. Whether p53 has a direct role, *in vivo*, in the recognition of DNA damage remains unanswered, but evidence for the involvement of p53 in the

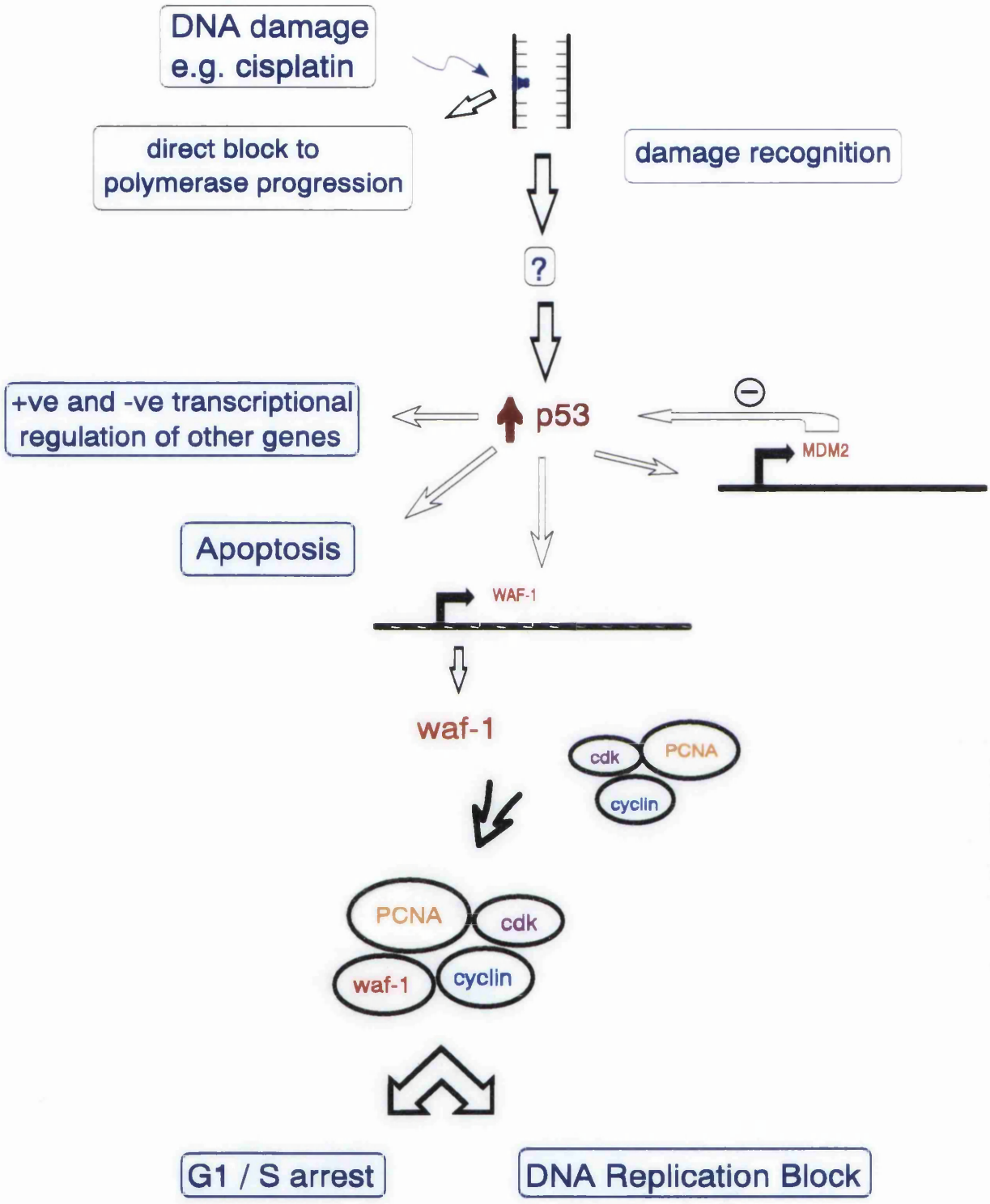


Figure 1.3.2 Molecular response to DNA damaging agents

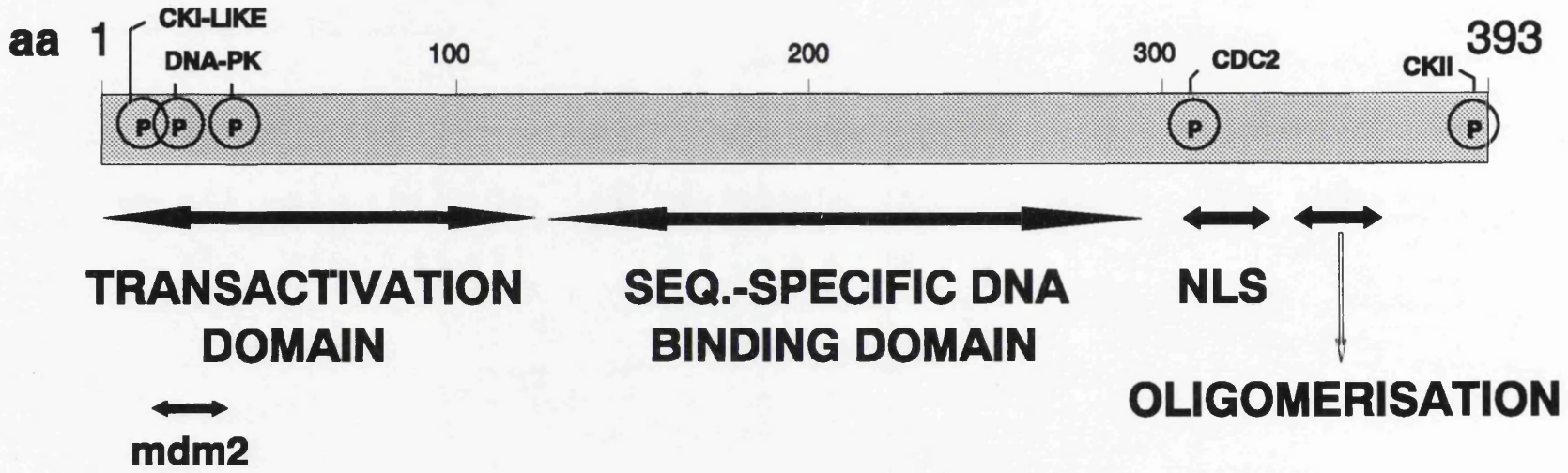


Figure 1.3.3 Outline of the functional domains of the p53 protein, indicating phosphorylation sites (Ⓟ), mdm2 binding site and the nuclear localisation sequence.

pathway leading from DNA damage to cell cycle arrests (and apoptosis) is very strong. p53 is involved in cell cycle progression and the normally unstable p53 protein is stabilised in response to DNA damage. It has been shown in p53 null transgenic mice however, that there is not an absolute requirement for p53 in the normal cell division cycle as these mice develop fully (40). These mice have a higher than normal incidence of tumours, suggesting that p53 plays a role in preventing abnormal or deregulated growth. This is supported by the observation that transfection of p53 into p53-deficient tumour lines restores G1 arrest (41) as does a shift to the permissive temperature of a ts^- mutant of p53 (42). Conversely dominant negative mutants of p53 have been shown to oligomerise with wild-type p53 and so abrogate its transactivating function (43), as well as abolishing their G1 arrest in response to DNA damage (44).

What involvement does p53 have in the response to DNA damage? The p53 response of cells following genotoxic insults such as UV, X and γ irradiation and cisplatin have been investigated. The kinetics of the p53 responses differ and appear to be cell type dependent (45, 46). Work on G1 arrest following treatment with DNA damaging agents suggests that p53 is involved in the G1/S checkpoint and possibly the G2/M checkpoint (47), but there appear to be alternative or additional mechanisms involved. p53-dependent G1 arrest in response to DNA damage can be abolished by viral proteins such as HPV E7 and E6 (48). The evolutionary function of these proteins is presumably to allow host cell replication to continue unhindered and so increase the amount of virus produced. Inhibition of the G1 arrest by the E6 protein occurs as it targets p53 for degradation. The E7 protein acts via an interaction with the retinoblastoma protein. The loss of p53-dependent G1 arrest can also be mediated by activated oncogenes e.g. H-ras (49).

How does p53 induce a G1/S arrest in response to DNA damage? p53 has a positive transcriptional activity and can interact with a number of proteins which are thought to be important in the cell cycle, some of which are transcription factors. It is thought that interaction with transcription factors reduces the

capability of the cell to undergo general transcription, while the transactivating activity of p53 results in gene-specific transcription. Several transcriptional targets of p53 have been identified, including WAF-1 which is an inhibitor of cdk's. Cyclin G was recently identified as being induced by p53 (50), but its role in the prevention of tumorigenesis is, as yet, uncharacterised. GADD45 (51), a protein involved in growth arrest following DNA damage is also known to be transcriptionally activated by the p53 protein. The mdm2 protein is thought to be involved in a negative feedback loop to terminate p53 activity (52). After induction by p53, mdm2 protein binds p53 near its N-terminal, in the acidic transactivation domain, thus blocking the transactivating function of the protein.

Waf-1 is an inhibitor of cdk's and can block the activity of several cdk~cyclin complexes. It functions by forming a quarternary complex with cdk / cyclin / PCNA (proliferating cell nuclear antigen) (53). As a result, the kinase activity of the cdk is deactivated and downstream targets, such as the retinoblastoma protein, whose phosphorylation is required for cell cycle progression, remain hypophosphorylated. PCNA is part of the DNA polymerase δ holoenzyme, which is active in DNA replication. This polymerase is primarily thought to be involved in DNA elongation, an activity which is curtailed when PCNA is bound in the quarternary complex of cdk / cyclin / PCNA / waf-1. This sequestration of PCNA does not however prevent DNA repair from occuring (54). This is logical when it is considered that the primary fuction of a G1/S arrest is for DNA repair to occur prior to replication. In transformed cells, formation of this quarternary complex has not been identified. This observation helps to explain the molecular reasons underlying the lack of G1 arrest in many transformed cells in response to DNA damaging agents and suggests that the inadequacy is upstream of waf-1 in the DNA damage response pathway. As such, waf-1 levels should provide an indication of whether it is the upstream p53 response which is being compromised in a cell incapable of damage-induced cell cycle arrests.

1.4 Apoptosis

Apoptosis is an active process of programmed cell death, distinct from necrosis, first identified by Kerr *et al.* (55, 56). It is characterised morphologically by cell swelling, nuclear chromatin condensation and changes in the cell surface. This is followed by release of apoptotic bodies which are removed by the phagocytic action of neighbouring cells. These morphological features are accompanied by non-random degradation of DNA into fragments of various defined sizes, those associated with chromatin domains i.e. approximately 50kb and internucleosomal fragments of 200bp. Fragments of 30kb have also been identified in response to cisplatin treatment (57).

The importance of apoptosis in preventing the development of transformed populations of cells is now largely undisputed. Not only are cells exposed to a large number of environmental toxins, but they are victim to their own inherent DNA replication and repair errors. There is the potential for two transformed cells to result from every cell division cycle, and viewed in this light, the rate of tumour development in each individual is surprisingly low. Apoptosis results in the removal of cells which have the potential to result in an expanded population of transformed cells. In this way, the survival of a single cell is forfeited in favour of the survival of the individual.

Many groups have helped to characterise the molecular signals which result in apoptosis, for example, Evan *et al.* showed that deregulated c-myc in serum starved rat fibroblasts leads to the apoptotic response (58). The p53 tumour suppressor protein has also been implicated in the apoptotic response in various lines in response to insult with various agents. Lowe *et al.* have shown distinct cases where apoptosis is dependent or independent of p53 function. Using thymocytes from transgenic p53 mice, glucocorticoid-induced apoptosis occured in p53 null lines, whereas radiation-induced apoptosis required the presence of p53 (59). Antisense p53 mRNA has been used to suppress growth factor withdrawal-

induced apoptosis in acute myoblastic leukaemia cells (60), thus emphasising the fact that p53 appears to play a pivotal role.

The purpose of chemotherapeutic treatment is to destroy the transformed cell population, whilst leaving normal tissue intact. Apoptotic cell death appears to be crucial to this process and a review by Dive and Hickman (61) details cellular responses to anticancer agents with respect to apoptosis. A linear relationship between cytotoxic agent dosage (including cisplatin) and apoptotic response has been recorded (62), and a CHO line shown to be deficient in DNA excision repair has been shown to be more sensitive to cisplatin treatment than the wild type CHO line. Exposure of the BM13674 cell line to cisplatin and other cytotoxic agents is known to result in apoptosis, yet it has recently been shown that okadaic acid, a tumour promoter and an inhibitor of protein phosphatases, prevents this apoptotic response (63). Blocking the apoptotic response of DNA-damaged cells provides a logical explanation for the importance of apoptosis in preventing tumour development. Cisplatin also induces apoptosis in an ovarian carcinoma cell line, CH1 (57), and has been shown to kill proliferating rather than quiescent rat thymocytes (64). As a common feature of transformed cells is their ability to undergo rapid cell division cycles, selective activity of anti-cancer drugs towards proliferating cells would prevent the killing of large numbers of normal cells.

The precise mechanisms of apoptotic cell death remain to be defined, but it is clear that a large number of factors are important, including extracellular growth signals, p53 status, proliferative state and the nature of the cellular insult.

1.5 Resistance To Anticancer Agents

There are many mechanisms which play a part in tumour-drug resistance, those which involve problems of delivery and other inherent and acquired mechanisms.

Delivery of drugs to a tumour site can be problematic as a result of alterations in the blood supply as well as other physiological and pharmacological factors. Different tumours exhibit different sensitivities to various drugs, for example, cell lines derived from bladder tumours are inherently more resistant to cisplatin treatment than testicular tumour-derived lines (65). There are a large number of possibilities as to why differential sensitivities arise, some of which are described in the following section.

1.5.1 Preventing Drug / Target Interactions

Acquired drug resistance results from a phenotypic change in the tumour in response to treatment, and can be imitated by selection of cells with drug *in vitro* (66, 67). A schematic diagram of various methods of acquired drug resistance are shown in Figure 1.5.1.

Changes in the accumulation of cytotoxic drugs can occur as a result of an increase in drug efflux and / or a decrease in drug influx. This has been characterised in the MDR (multi-drug resistant) phenotype, initially identified in the late 1960's (68, 69). Drug-selected cells were shown to have a lower level of drug accumulation than the parental cell line, a feature which was later identified as a result of overexpression of the MDR1 gene product (70, 71) due to amplification of the MDR1 gene (72). Increased expression of the MDR1 gene product, p-glycoprotein, has been identified in inherently resistant tumours and several relapsed tumours. P-glycoprotein is a integral membrane protein with an ATP binding domain and shows homology with transport proteins (73). It is therefore hypothesised that it acts as an energy-dependent efflux pump which can bind to various drugs and pump them out of the cell. This phenomenon however does not play a role in the clinic and in relapsed tumours in terms of cisplatin resistance (74, 75). An additional method of reduced drug accumulation is binding to extracellular proteins, such as serum proteins (76). Reduced accumulation of drug does not appear to be a physiologically relevant explanation

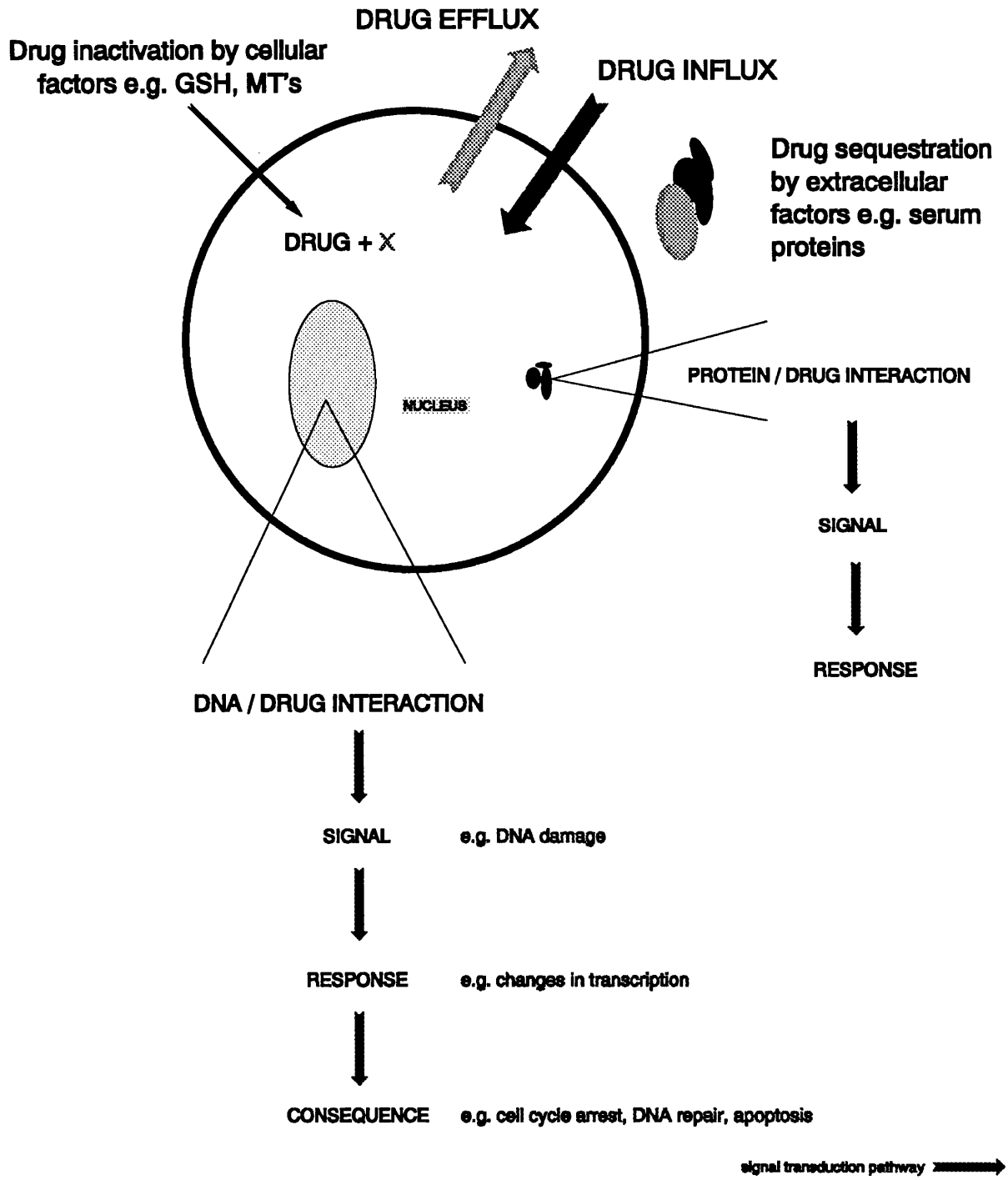


Figure 1.5.1 Schematic diagram of the mechanisms of drug resistance.

for cisplatin resistance although examples of cisplatin resistant lines with differential uptake levels have been observed (77, 78).

Intracellular molecules may also be responsible for 'sequestration' of cisplatin by reacting with it and so abolishing its reactivity. For example, nucleophiles such as the tripeptide glutathione (GSH) and the metallothionein (MT) proteins can react with cisplatin, so reducing its effective concentration and cytotoxicity. Although increases in GSH and MT intracellularly have been shown to correlate with increased resistance to cisplatin, this does not appear to be clinically important in the case of GSH and not a direct result of MT concentration (79).

1.5.2 DNA Repair and Adduct Tolerance

It can be envisaged that if a cell can rapidly repair DNA damage or tolerate greater levels of damage then it may display increased viability. It has been shown that cisplatin-DNA adducts can be repaired by nucleotide excision repair (80) although this may not be the only mechanism of repair of cisplatin-induced DNA damage. Several cisplatin-resistant cell lines have been shown to have increased DNA repair by ^{3}H-thymidine incorporation and loss of adducts (81, 82). This supports the hypothesis that it is the amount of damage which is the pertinent factor in the sensitivity of cells to cisplatin. In addition, a cisplatin-sensitive testicular line has reduced removal of intrastrand crosslinks as a result of cisplatin treatment compared with a cisplatin-resistant bladder line (83), strongly suggesting a role for DNA repair in determining the level of cisplatin resistance. In turn, this substantiates the evidence that the primary cytotoxic target of cisplatin is DNA.

1.5.3 Identification of Novel Resistance Mechanisms

As resistance to cisplatin has been shown to be clinically relevant, circumvention of this resistance should allow cisplatin treatment to be used to its most effective chemotherapeutic potential. As many tumours and cell lines exhibit a multi-drug resistance phenotype, independent of p-glycoprotein expression, identifying genes

involved in the resistance to cisplatin may elucidate general mechanisms of resistance for a large number of DNA-damaging agents. Identification of the most sensitive components of the DNA damage-induced cytotoxic pathway(s) should also aid the design of novel drugs and treatments.

Many of the genes which have been linked to drug resistance to date, have been identified as being elevated in the resistant phenotype. The relevance of the elevation of the MDR-1 gene product, p-glycoprotein, has been described previously (84) but appears to have little relevance in resistance to cisplatin. Other gene products which have been identified as elevated in resistant cell lines include the *bcl-2* protein. *Bcl-2* is a negative mediator of the apoptotic response (85), but there is a large amount of data detailing the importance of a family of *bcl-2*-related proteins which act in concert to control its activity (86). MDM2 is an oncogene and the protein has recently been shown to confer resistance to cisplatin-induced apoptosis in a human glioblastoma cell line when overexpressed (87). As *mdm2* is thought to act as a negative regulator of p53 function, it can be hypothesised that overexpression blocks the apoptotic function of p53 and so DNA-damaged cells survive. As mentioned in sections 1.3.4 and 1.5.2 respectively, damage recognition proteins and proteins involved in DNA repair have also been shown to be elevated in particular resistant tumours and cell lines (88, 67). Lu *et al.* (89) have recently identified a cisplatin-resistance locus (*crl1*), which, when overexpressed, confers cisplatin resistance. They employed a retroviral insertional mutagenesis strategy to identify this sequence i.e. proviral insertion resulted in an increase in expression from the retroviral promoter, and cells were selected for resistance to cisplatin treatment. The insertion site was then mapped. This system could of course have identified a locus, down-regulation of which resulted in resistance.

Recessively acting genes i.e. those genes in which a decrease in activity of gene product causes drug resistance, have been investigated less extensively. Loss of

heterozygosity and dominant negative mutations are often the causes of reductions in protein activity *in vivo* and may result in a resistant phenotype. Experimentally, dominant negative mutants of particular genes have been transfected into cells in culture to examine the role of reduction in protein activity. For example, transfection of the dominant negative mutant of the p53 gene, Val_{143} > Ala, into the A2780 cell line has been shown to correlate with a loss of the phenotypic characteristics associated with wild-type p53 function, such as cell cycle arrests (44). This line is also more resistant to cisplatin treatment than the parental cell line. Shaulian *et al.* (43) used a similar approach to examine the functional domains of the p53 protein. They generated C-terminal miniproteins of the p53 tumour suppressor protein and demonstrated that these fragments reduced p53 activity as determined by DNA binding and transformation assays i.e. these protein fragments or 'miniproteins' abrogated the activity of the endogenous, wild-type protein. An additional experimental method which has been extensively used to examine the effect of a reduction in the activity of an endogenous gene product is the introduction of antisense mRNA which can hybridise to the mRNA transcribed from the wild-type gene thus preventing its translation into protein. Antisense mRNA targetted at MDM2 was used to examine the result of a reduction in *mdm2* protein in a human glioblastoma cell line (87). The result was an increase in the sensitivity of this cell line to treatment with cisplatin, presumably because p53 activity was not downregulated by *mdm2* binding.

In an attempt to identify mechanisms of resistance resulting from a decrease in the activity of a gene product Gudkov *et al.* have taken an alternative approach. They have developed a technique using genetic suppresser elements (GSE's) in an attempt to identify recessive mechanisms of drug resistance (90).

1.6 Genetic Suppressor Elements

Genetic suppressor elements or GSE's are small gene fragments which, when transfected into a cell line, can act to suppress the level or activity of the protein synthesised from the full length wild-type gene. This gene fragment can act in antisense to decrease the amount of translated protein or in a sense orientation to decrease protein activity by a dominant negative mechanism. It is also possible that a GSE, which has sequence or conformational homology with the mRNA or protein encoded by a different gene, may reduce the level or activity of that protein. It can therefore be envisaged that this GSE approach has many potential applications, and could, theoretically, be used to identify genes involved in any biochemical pathway of interest. In the context of the work presented here, I have used GSE's to investigate genes involved in cisplatin resistance.

1.6.1 Generating Genetic Suppressor Elements

Figure 1.6.1 outlines the steps involved in the generation and isolation of genetic suppressor elements. The library is generated by random digestion of the cDNA of interest using, for example, DNaseI. The library is normalised to ensure that each digested fragment is equally represented. The fragments are ligated into a vector with the appropriate restriction sites and resistance markers for selection in bacteria and mammalian cell lines, and used to transform bacterial cells. The DNA is then isolated and used to transfect a packaging cell line. Packaging cell lines are capable of supporting the generation of infectious viral particles, and the supernatant from these cells can therefore be used to infect the cell line of interest. Infectants are selected using a dominant marker present in the vector and selected further for the phenotype of interest e.g. resistance to topo IIα inhibitors, cisplatin etc. Selected lines are therefore generated and the GSE insert can be isolated by PCR amplification (the nature of viral infection ensures that, in most cases, each cell will be expressing a single GSE). To confirm that the GSE which has been

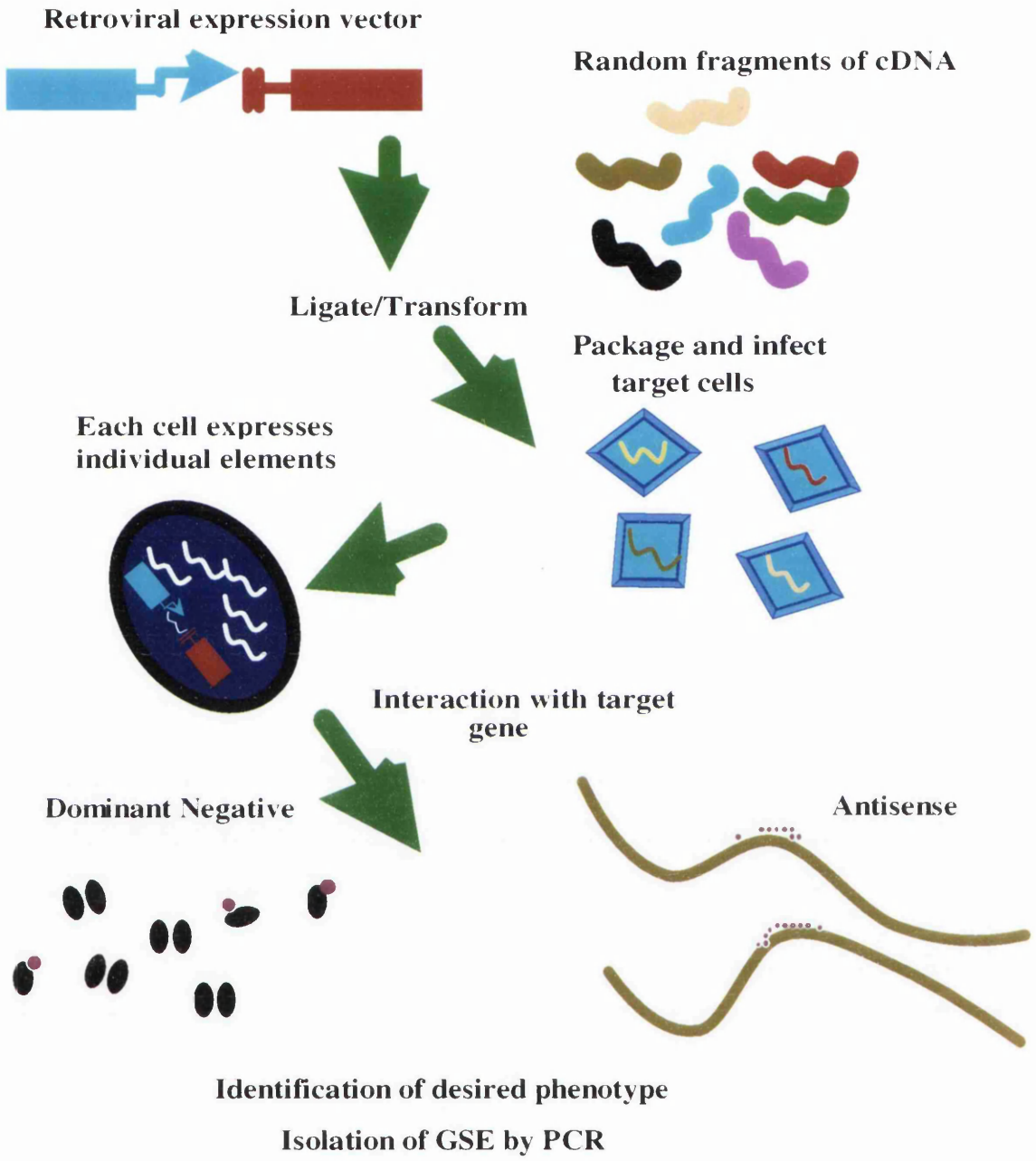


Figure 1.6.1 Generation and isolation of gene suppressor elements

isolated is responsible for the selected phenotype, it is then used to re-transfect the line of interest, and the selection procedure is repeated.

1.6.2 The Use of Genetic Suppressor Elements To Identify Recessive Mechanisms Of Resistance To Topoisomerase IIα Inhibitors

Gudkov *et al.* (91) identified GSE's which abrogate the resistance of cells to topoisomerase IIα inhibitors such as etoposide. Many inhibitors of topo IIα are used in cancer chemotherapy e.g. epipodophyllotoxins and anthracyclines, so understanding the reasons for resistance to these drugs is of clinical interest. Topoisomerase IIα is an enzyme involved in the negative and positive supercoiling of DNA and is therefore important in DNA replication and transcription. Topo IIα nicks and rejoins both strands of DNA, to achieve a two-fold change in DNA linkage number, by the formation of a cleavable complex with DNA. Topo IIα inhibitors act to stabilise this cleavable complex which contains the topo IIα protein bound to the cut ends of DNA. Prior to Gudkov's work it had been observed that cells which showed resistance to topo IIα inhibitors had decreased levels (or levels of activity) of topo IIα (105) suggesting that resistance was in some way due to a reduction in the number of cleavable complexes available for stabilisation.

Gudkov *et al.* attempted to 'artificially' reduce the level of topo IIα activity in HeLa cells by introducing DNA fragments derived by the random digestion of full length topo IIα cDNA, and examining their activity as topo IIα GSE's (91). This GSE approach has proved to be very successful and a number of topo IIα GSE's have been identified, including both sense and antisense gene fragments, which confer resistance to topo IIα inhibitors. The data also provides evidence for the functional importance of protein domains i.e. several of the sense GSE's derived from the topo IIα gene are fragments from the active site of the enzyme. The

fragments which are not derived from this region of the protein indicate the importance of regions with as yet unknown functions.

The same group have also randomly digested total poly(A)+ RNA-generated cDNA from NIH3T3 cells and used these fragments to look for resistance to topo IIα inhibitors (93). They isolated three GSE's, two from unknown genes and one which was derived from the kinesin gene, whose protein product is a microtubule-associated motor protein. Although kinesin has not previously been linked with responses to drug administration, the GSE was found to confer resistance to a number of drugs including etoposide and to a lesser extent cisplatin and increase sensitivity to microtubule poisons such as colchicine.

1.6.3 The Use of Genetic Suppressor Elements To Identify Recessive Mechanisms Of Resistance To Cisplatin

Within Roninson's group, Deborah Kirschling has generated GSE's which confer cisplatin resistance to human cells (94). These GSE's were derived by random digestion of normalised HeLa carcinoma cell cDNA. A retroviral library was generated from these fragments and infectious virus was generated in a packaging cell line. The viral supernatant was used to infect HeLa cells and HT1080 fibrosarcoma cells. Two rounds of selection for infectants with increased resistance to cisplatin were performed and the resulting fragments were tested for biological activity. To date, 15 cisplatin GSE's have been identified including both sense and antisense fragments from known and novel genes. None of these known genes has previously been specifically connected with drug resistance. The identity of the GSE's are shown in table 1.6.1. One can envisage however that reduction in the activity of a protein involved in DNA repair, apoptosis, pyrimidine biosynthesis or signal transduction may play a role in drug resistance. It is more difficult to generate a hypothesis for the involvement of a reduction in the activity or amount of an extracellular matrix protein or a glycolytic enzyme in this phenotype. As mentioned in section 1.6 however, it could be that the GSE's

are causing a reduction in the activity of a gene product other that from which they have been derived.

No.	Corresponding Gene	Orientation	Associated Function
Vector alone	-	-	-
1	XRCC1	sense	DNA repair
2	TRPM-2	sense	Apoptosis
3	Phosphoglycerate mutase B	sense	Glycolysis
4	CaM Kinase B	antisense	Signal transduction
5	Dihydrooratate dehydrogenase	antisense	Pyrimidine synthesis
6	Decorin	antisense	Extracellular matrix
7 - 15	Unknown	-	-

Table 1.6.1 Identity and function of the genes from which the 15 cisplatin GSE's were derived, and orientation of the GSE's.

1.6.4 p53 Genetic Suppressor Elements

The cisplatin GSE's which I have described were isolated from a cDNA library generated by digestion of total HeLa cell cDNA. An alternative approach, and one that Gudkov has previously employed in the case of topoisomerse IIα, is the generation of a gene cDNA library. Liam Gallagher (Dept. Medical Oncology, Glasgow University) has generated both human and murine p53 cDNA libraries, and using the methods previously described has isolated a number of p53-derived GSE's which confer resistance to cisplatin. Several of these GSE's have been isolated by PCR and DNA sequencing has revealed that they contain sequences of the p53 gene in preference over fragments from the vector in which the library was generated. This suggests that the GSE approach is a selective one which should be capable of identifying novel recessive mechanisms of drug resistance.

1.7 Tetracycline Inducible Expression System

To confirm any phenotype which correlates with tranfection of a GSE, it would be interesting to controllably induce expression of that GSE. Myself and Margaret Cairney (Dept. of Medical Oncology, University of Glasgow) are attempting to generate an inducible gene expression system based on tetracycline responsive elements, as described by Bujard *et al.* (95), in various cell lines of interest. This system is being used in preference to other inducible systems due to its efficiency as a very clean on/off switch. Many other inducible systems can 'leak' as the element or molecule which is used as the switch is endogenous to the cell line in use. For example, the metallothionein and dexamethasone systems both use inducer molecules which are often present in cells at a low concentration.

The tetracycline system requires the use of two plasmids, one incorporating the transactivator construct, the other carries the tetracycline operator sequence. The transactivator consists of the tet repressor sequence from *E.coli* fused to VP16 the herpes virus transactivator. This construct acts as a transcription factor, the target of which is a minimal eukaryotic promotor with a series of tet operator sequences and the gene of interest. In the presence of tetracycline, the transactivator binds to tetracycline and so is prevented from binding to the operator sequences. As a result, the gene of interest is not transcribed. When tetracycline is removed, the transactivator is free to bind the operator sequence and transcribe the gene (or GSE) of interest. As tetracycline is not an endogenous molecule of mammalian cells, expression of the GSE should be high when tetracycline is absent, and the concentration of tetracycline required to block expression can be determined using a cell line containing a luciferase reporter system, that is, SM161.

1.8 AIMS

The aim of the work presented here has been the further characterisation of the cisplatin GSE's isolated by Kirschling *et al.* (94). I have specifically concentrated on GSE 7.10, that derived from the human phosphoglycerate mutase-B gene for reasons which will be described later. I have been able to confirm that this GSE does confer resistance to cisplatin in an additional cell line to those described by Kirschling *et al.*, that is the human ovarian adenocarcinoma cell line, A2780. I have further investigated their cross-resistance to another DNA-damaging agent, ionising radiation and examined their ability to initiate cell cycle arrests after exposure to both cisplatin and ionising radiation. I have also carried out some initial preparations for developing the tetracycline-dependent inducible expression system and examined some of the p53 cisplatin GSE's isolated by Liam Gallagher.

Chapter 2

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Manufacturers

Chemicals	Sigma, BDH, Pharmacia, Fluka, Fisons
Restriction Enzymes	Gibco BRL, Pharmacia
Cisplatin	Sigma
Radiochemicals	Amersham International
Oligonucleotide Primers	Oswell DNA Service
Molecular Weight Markers	Gibco BRL
Antibodies	Dakopatts, Sigma
PCR Reagents	Boehringer Mannheim
Wizard PCR Clean-Up Kit	Promega
Wizard DNA Clean-Up Kit	Promega
Prime-It II Kit	Stratagene
Nucleon 1 DNA Extraction Kit	Scotlab
TRIzol™ RNA Extraction Reagent	Gibco BRL
Competent Cells	Stratagene (Epicurian Coli SURE)
	Gibco BRL (DH5-α Sub-cloning Efficiency)
Plasmid Purification Kits	Qiagen, Flowgen
Nick Columns	Pharmacia
Transfer Membrane	Amersham International
Autoradiography Film	Kodak
Plastics	Sterilin, Falcon
Aerosol Resistant Tips	Molecular Bioproducts
Tissue Culture Media	Gibco BRL, Globepharm
Supplements for Media	Gibco BRL
Calcium Phosphate Transfection Kit	Gibco BRL

Tissue culture plastics	Nunclon, Falcon
24 Microwell Plates	Nunclon, Corning
Centrifuges	Sorvall
FACS Machine	Beckton Dickinson
PCR Machines	Hybaid, Perkin Elmer
Sequencing	Applied Biosystems Inc. using Dye Deoxy
Sequence Analysis	GCG Package
Gel Tanks	Pharmacia
X-Ray Developer	Kodak
UV Crosslinker	Stratagene

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

2.1.2 Radiation

All performed using γ rays from a ^{60}Co source.

2.1.3 Oligonucleotide Primers

H2693 / NO853 / P1785	(Reverse)	AGCTACGTTGCCAAACCTA
H2694 / NO854 / P1786	(Forward)	CCTGGAGACGCCATCCAC

2.1.4 Plasmids

Common Features of pUH Tetracycline Plasmids

From H.Bujard (95).

pBR322 derived plasmid, with a hCMV promoter/enhancer.

β-lactamase resistance gene.

Specific Features of pUH Tetracycline Plasmids

pUHD 15-1	Contains the tTA gene as a VP16 fusion.
pUHD 151-1	Contains the tTAs gene.
pUHD 10-3	Contains heptamerised upstream tet-operators.
	Multiple cloning site (MCS).
pUHC 13-3	Contains heptamerised upstream tet-operators in two different orientations.
	Luciferase gene.
pUHC 13-4	Contains heptamerised upstream tet-operators in two different orientations.
	Luciferase gene.

Other Plasmids

pLNCX7.10	pLNCX Accession no. M28247 (96)
	Generated by HindIII / ClaI digestion of both pLNCX and GSE 7.10, followed by ligation of the plasmid backbone with the GSE fragment
	Ampicillin resistance gene.
pIC19R	Accession no. VB0092 (97)
	Ampicillin resistance gene.
	MCS.
	Lac Z selection
pSVneo	(98)
	Ampicillin resistance gene.
	Neomycin resistance gene.
pCEP-WAF-1-S	(30)

2.1.5 Solutions

Phosphate Buffered Saline (PBS)

0.8% NaCl

0.115% Na_2HPO_4

0.02% KCl

0.02% KH_2PO_4

PBT

PBS

0.1% Tween-20

0.5% BSA fraction V (w/v)

TBE(1x) pH8

89mM Tris borate

89mM Boric acid

2.5mM EDTA

TE pH8

10mM Tris

1mM EDTA

20x SSPE

3.6M NaCl

0.2M NaH_2PO_4

0.02M EDTA pH7.7

made up with DEPC treated water

0.1% DEPC Treated Water

999ml double distilled H_2O

1ml DEPC

Autoclave solution

10x MOPS RNA Running Buffer

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

0.05M Na acetate pH7.0

0.01M Na_2EDTA

made up with DEPC treated water

100x Denhardt's Solution

2% (w/v) BSA fraction V

2% (w/v) Ficoll™

2% (w/v) PVP

made up with DEPC treated water

Prehybridisation Buffer

5x SSPE

5x Denhardt's solution

0.5% SDS (w/v)

50% formamide (v/v)

made up with DEPC treated water

Formaldehyde Gel Loading Buffer (for Northern blotting)

50% glycerol

1mM EDTA (pH 8)

0.25% bromophenol blue

0.25% xylene cyanol FF

DNA Dye Mixture

30% v/v Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase)

Prepared in dimethyl formamide.

CIP Buffer

10mM $ZnCl_2$

10mM $MgCl_2$

0.1M Tris·HCl (pH8.3)

Equilibrated Phenol

To phenol (Rathburn) 0.1% (w/v) 8-hydroxyquinoline was added. An equal volume of 1M Tris base pH8 was added and, after settling, the upper phase discarded. This was repeated. An equal volume of 0.1M Tris base pH8 and 0.2% (v/v) β-mercaptoethanol was added and mixed well. This was left to settle and the upper phase discarded. Repeat this step. This was stored at 4°C away from light with a small volume of Tris covering the surface.

2.2 Tissue Culture Techniques

2.2.1 General Techniques

Aseptic manipulations were performed using sterilised glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown at 37°C as monolayers in supplemented RPMI (Rosswell Park Memorial Institute; see below for supplements) medium in the presence of 5% CO_2. They were stored by

freezing in RPMI with 10% di-methyl sulphoxide (DMSO) in 1ml cryotubes at -70°C and were then maintained in liquid nitrogen. GSE transfected cells were regularly exposed to 600μgml^{-1} G418 to confirm the presence of the co-transfected G418 resistance plasmid.

2.2.2 Tissue Culture Media

RPMI

88 ml RPMI 1640 (10x)

800ml sterile distilled water

26.6ml 7.5% $Na(CO_3)_2$

10ml 100mM Na pyruvate

10ml 200mM L-glutamine

1ml 1M NaOH

100ml foetal calf serum

5ml penicillin / streptomycin (50mg)

Special Liquid Medium

500ml Special Liquid Medium

50ml 200mM L-glutamine

50ml foetal calf serum

2.5ml penicillin/streptomycin (25mg)

Trypsin

20ml 10x PE

25ml trypsin (2.5% stock)

205ml sterile distilled water

2.2.3 Cell Lines

A2780	A human, ovarian adenocarcinoma cell line obtained from R.F. Ozols and T.C. Hamilton, Fox Chase Cancer Center, Philadelphia.
A2780cp70	A cisplatin resistant derivative of A2780. (For a more complete description of A2780 and A2780cp70 see Behrens *et al.*, 1987 (66).)
MCF-7	A human, breast adenocarcinoma cell line (92).
SM161	An established murine epithelial cell line transfected with a luciferase construct driven by the 6kB promoter of the e-cadherin receptor.

2.2.4 Subcloning of A2.14

Cells were seeded at $1x10^3$ per 90mm diameter dish and incubated for 8 - 12 days. Colonies were picked into $25cm^2$ flasks using sterile pipette tips.

2.2.5 Clonogenic Assay

Cells were seeded at $1x10^3$ per 90mm diameter dish on day 1. Cells were treated with either radiation or cisplatin on day 2 after medium replenishment. Colonies were grown for 10-14 days then stained with 1x Giemsa stain for 10 minutes and rinsed.

2.2.6 Cell Cycle Assay

Tissue Culture

Cells were seeded at $2x10^5$ cells per 90mm tissue dish in supplemented RPMI on day 1. On day 4, the medium was replenished prior to treatment with radiation or cisplatin. 6, 24 and 48 hours after treatment the medium was replenished with supplemented RPMI containing bromo-deoxyuridine (BrdU) at a final concentration of 10μM. After a 4 hour incubation in BrdU, the medium was removed and retained, and the cells were lifted by trypsinisation. The combined medium and cells were centrifuged at 500xg for 5 minutes and the supernatant was discarded. The pellets were resuspended in 5ml of cold 70% ethanol and stored at 4°C for up to two weeks.

Cell Staining

Cells were pelleted by centrifugation at 500xg for 5 minutes and the supernatant was discarded. The pellets were resuspended in 1ml cold PBS and transferred to 1.5ml microcentrifuge tubes prior to centrifugation at 300xg for 5 minutes at 4°C. The cells were again washed in 1ml cold PBS, pelleted as previously and permeabilised with 1ml 2M HCl for approximately 30 minutes. Cells were pelleted by centrifugation at 2000xg for 5 minutes at 4°C (these conditions used for all subsequent centrifugations unless indicated) and washed twice in 1ml PBS. The

cells were then resuspended in 1ml PBT as a blocking agent, pelleted and incubated with 100µl of a 1:40 dilution of mouse α-BrdU MAb in PBT for at least 1 hour at room temperature. Pelleting was followed by two washes in PBT and then incubation with secondary antibody, 1:40 dilution in PBT of FITC-conjugated α-mouse MAb for at least 30 minutes. The cells were washed once in PBT and once in PBS prior to incubation with $1mgml^{-1}$ propidium iodide for at least 20 minutes at room temperature or overnight at 4°C. The cells were pelleted and resuspended in an appropriate volume of PBS in preparation for FACS analysis.
Cell cycle distribution was determined by FACS analysis counting in excess of $2x10^4$ events unless otherwise stated.

2.2.7 Transfection Of Plasmid DNA Into Cell Lines

$1x10^6$ cells were seeded in 15ml supplemented Special Liquid Medium per 90mm dish on day 1. Medium was replenished on day 2, approximately 3 hours prior to transfection. A calcium phosphate transfection protocol was followed to co-transfect the tetracycline operator plasmid DNA and neomycin resistance marker plasmid DNA.
Control transfections were performed using supplied salmon sperm DNA in combination with the resistance marker plasmid, a transfection procedure in the absence of DNA and also untreated cells. The cells were incubated in the transfection mixture for 12 - 16 hours before medium was replenished on day 3. On day 4, G418 (geneticin) was added to the cells in fresh supplemented SLM as selection. After 8 - 14 days incubation, colonies were picked with sterile Gilson pipette tips into individual wells of 24 microwell plates containing approximately 2.5ml supplemented RPMI. Tetracycline was added where required.

2.3 Nucleic Acid Extractions

All work with RNA & DNA was carried out using autoclaved solutions, where appropriate. Disposable gloves and plasticware, autoclaved glass pipettes and aerosol resistant tips were used throughout. DNA & RNA quantitation was performed using a combination of visual assessment on agarose gels in 0.5x TBE, 0.5μgml^{-1} ethidium bromide when run against known standards, and spectrophotometrically using O.D.260.

Gel apparatus to be used in RNA analysis was washed (99) to destroy contaminating RNases : apparatus was washed thoroughly in diluted Decon and rinsed. This was followed by a rinse in ethanol, and a 15 minute soak in 3% H_2O_2. The apparatus was rinsed in DEPC treated water prior to use. DEPC treated solutions were also used where appropriate (see text for details).

2.3.1 DNA Extraction

DNA was extracted from cell lines using the Nucleon 1 kit, following the supplied protocol.

2.3.2 Routine Extraction Of DNA

Routine concentration of DNA samples was carried out as detailed. To the DNA sample, 0.1 volumes 3M sodium acetate pH8, 2.5 volumes 100% ethanol and 1% glycogen was added. The DNA was left on dry ice for approximately 15 minutes and then centrifuged at 12000xg at 4°C for 15 minutes. The pellet was washed in 70% ethanol and DNA was re-pelleted at 12000xg for 10 minutes at 4°C. The pellet was air-dried and resuspended in TE pH8.

2.3.3 RNA Extraction

Medium was poured off monolayer cultures of near-confluent cells grown in 125cm^2 flasks. 10ml of TRIzol reagent was added to lyse the cells which were

then transfered into sterile 15ml tubes and left at room temperature for approximately 5 minutes. 2ml of chloroform:amyl alcohol (24:1) was added to each tube. After shaking the tubes for approximately 15 seconds and a 2-3 minute incubation at room temperature, the contents were centrifuged at 1600xg at 4°C for 20 minutes. The upper aqueous phase was removed to a fresh 15ml tube to which 5ml isopropanol was added and left to incubate at room temperature for 10 minutes. After centrifugation at 1600xg at 4°C for 20 minutes, the supernatant was discarded and 10ml 75% ethanol (DEPC treated) was added and the pellet was partially resuspended by vigorous vortexing. The pellets were air dried at room temperature after a 15 minute centrifugation at 4°C at 1600xg. The pellets were resuspended in 50-200µl DEPC treated water in autoclaved 0.5ml eppendorf tubes and warmed to 55°C for 5 minutes to aid dissolution. Samples were stored at -70°C.

2.4 Polymerase Chain Reaction

Pre and post PCR reagents, reactions and equipment were kept separate in order to prevent cross contamination. In addition, aerosol resistant tips were used throughout.

2.4.1 PCR Amplification of DNA

To 10µl of DNA, 90µl of 'master mix' was added. Master mix was prepared as follows (per sample)

Taq buffer	10µl	
dNTP mix	2µl	(from 40mM total concentration stock, each dNTP = 10mM)
primers	1µg of each	
Taq enzyme	0.4µl	
sterile water	to total volume of 90µl	

75µl of paraffin oil was added to each sample to prevent evaporation.

The GATC base content of each primer was used to estimate the optimum annealing temperature, and the average of the two values was used in the reaction.

$$2(A+T) + 4(G+C) = \text{annealing temperature}$$

The reaction was carried out in a Omnigene thermal cycler under the following conditions

94°C	3 minutes	
94°C	1 minute	
56°C	1 minute	30 CYCLES
72°C	1 minute	
72°C	7 minutes	

The PCR products were purified using a Wizard DNA Clean-Up kit and protocol, and the products analysed by gel electrophoresis.

2.5 DNA Sequencing

Purified PCR products were amplified prior to sequencing as follows :

PCR product	5µl
primers	3.2pmoles of each
sterile water	to final volume of 10.5µl
DyeDeoxy Termination mix	9.5µl

PCR was performed using the following protocol

Preheat to 96°C	
96°C for 15 secs	
50°C for 1 sec	25 CYCLES
60°C for 4 mins	
Soak 4°C	

80µl of sterile water was then added to the reactions and DNA was extracted with 100µl of phenol / chloroform / water at a ratio of 68:18:14 by centrifugation at 5000xg for 5 minutes. DNA was extracted as detailed (section 2.3.2) The DNA was left to air-dry, then sequenced and analysed. Sequencing was performed using the dideoxy chain termination technique of Sanger *et al.* (100) as detailed in the protocol which accompanies the DyeDeoxy kit (ABI).

2.6 RNA Analysis

2.6.1 RNA Integrity

To verify the integrity of the extracted RNA, it was separated electrophoretically. RNA samples were prepared as follows : to approximately 10µg of RNA, TE pH8 was added to a total volume of 4µl. The RNA was incubated at 65°C for 10 minutes then chilled on ice prior to the addition of 1µl of DNA running dye. The samples were loaded onto a 1.5% agarose gel containing 0.5µgml^{-1} ethidium bromide, and run in 300ml 0.5x TBE containing the same concentration of ethidium bromide. The gel was run at 7.5Vcm^{-1} for approximately 1 hour. RNA was visualised on a UV transilluminator.

2.6.2 Denaturing Formaldehyde Gel For Northern Analysis

RNA samples were prepared according to the protocol detailed in the Amersham Hybond N booklet. To approximately 20μg RNA (final volume made up to 6μl with DEPC treated water) 12.5μl formamide, 4μl formaldehyde and 2.5μl 10x MOPS were added. These samples were then incubated at 65°C for approximately 10 minutes and chilled on ice prior to the addition of 2.5μl of RNA running dye. 6 μg of 0.24 - 9.6kb RNA ladder was prepared in the same way.

The samples were loaded onto a pre-run (5 minutes at $5Vcm^{-1}$) 1% agarose denaturing gel prepared as follows : 4g agarose was added to 292ml DEPC treated water and 40ml 10xMOPS. The agarose was melted and left to cool to approximately 50°C prior to the addition of 68ml formaldehyde and $0.5\mu gml^{-1}$ ethidium bromide. The gel was cast and left to set in a fume hood. The gel was run at $3.5Vcm^{-1}$ for approximately 5 hours and at $1Vcm^{-1}$ overnight. The gel was then photographed under UV before transfer.

2.6.3 Northern Transfer of RNA

The denaturing gel was cut to size and the RNA transfered, over approximately 20 hours, onto Hybond-N membrane using 20x SSC as the transfer buffer. The membranes were left to air dry for approximately 10 minutes prior to UV crosslinking in a Stratalinker oven.

2.6.4 Hybridisation Of Northern Blots

Blots were pre-hybridised overnight in 25ml of pre-hybridisation buffer at 42°C using a Hybaid oven and Hybaid roller bottle system (bottles were rinsed in DEPC treated water before use). Labelled probe was then added to the pre-hybridisation buffer and incubated at 42°C overnight.

2.6.5 Molecular Probes

Probes were generated by random priming using α-^{32}P-dCTP.

WAF-1 : This 12.4 kb cDNA fragment was used for Northern blotting and was derived from the plasmid, pCEP-WAF-1-S (30). The template was obtained from Margaret Cairney and had been prepared by digestion of the plasmid, using the appropriate restriction enzyme i.e. *Not*I.

GAPDH : Probe was generated by digestion with *Eco*RI and obtained from Liam Gallagher.

2.6.6 Washing Membranes

Following formamide hybridisation, membranes were washed at room temperature for 15 miutes in 1xSSPE and 0.5% SDS, followed by a 1 hour wash in 0.5xSSPE / 0.5% SDS at 65°C. The final wash in 0.1xSSPE / 0.5% SDS was carried out for approximately 30 minutes at 65°C.

2.6.7 Autoradiography

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

2.7 Microbiological Techniques

2.7.1 Bacterial Culture

Bacteria were maintained on Luria agar (1.5% agar in L-broth) plates at 4°C. Ligations were maintained as described, with the addition of 40µl X-gal (20mgml^{-1}) and 8µl IPTG (100mgml^{-1}). Bacteria were stored for long periods in 15% glycerol in L-broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at -70°C.

2.7.2 Transformation Of Bacterial Cultures With Plasmid DNA

50µl of competent bacteria were added to 1-10ng of transforming DNA. The protocols provided with each cell strain were followed. Cells were grown overnight at 37°C on appropriate selection media and picked, using sterile Gilson pipette tips, into 10ml or 5ml of LB medium containing 100mgml^{-1} ampicillin for plasmid retrieval using the Qiagen Maxi Preparation kit and the Flowgen SpinBind procedure respectively.

2.7.3 Recovery Of Plasmid DNA From Bacterial Cultures

5ml of a 10ml culture was used to innoculate 500ml of L-broth containing 100mgml^{-1} ampicillin (for selection) and this culture was grown to stationary phase overnight. The Qiagen Maxi Preparation kit was used according to manufacturers recommendations to obtain plasmid DNA. The second centrifugation (step 5) was replaced by filtration through Whatman 3MM paper. Alternatively, plasmids were purified from 4.5ml of a 5ml culture using the Flowgen SpinBind protocol.

2.8 Plasmid Manipulations

2.8.1 Restriction Digests

Restriction digests were carried out according to manufacturer's instructions, with the total enzyme concentration in the reaction not exceeding 10%. Double digests were performed using *Hin*dIII and *Cla*I in the same reaction using REactI as the buffer. To compensate for the low activity of *Hin*dIII in this buffer i.e. ~30%, three times the number of recommended units of *Hin*dIII were used. Reactions were carried out at 37°C for 2-6 hours.

2.8.2 De-Phosphorylation Reactions

De-phosphorylation of restriction digested plasmids were performed with CIP (calf intestinal phosphatase). The protocol detailed in Sambrook *et al.* (99) suggests the use of 1 unit of CIP per 100pmoles of DNA, however CIP was used in excess of this concentration. A standard phenol chloroform DNA extraction was performed to isolate digested plasmid DNA. DNA was precipitated with 2.5 volumes of ethanol and resuspended in 90µl of 10mM Tris·HCl pH8.3. 10x CIP buffer was added to a final concentration of 1x, and the appropriate volume of CIP enzyme was added. After an incubation at 37°C for at least 30 minutes, SDS and EDTA pH8 were added to final concentrations of 0.5% and 5mM respectively. Incubation in this mixture at 56°C for 30 minutes is sufficient to inactivate the phosphatase. Following cooling to room temperature, DNA was extracted once with phenol at room temperature and once with phenol / chloroform at 4°C. DNA was precipitated as detailed in 2.3.2 and the pellet was resuspended in TE and stored at -20°C.

2.8.3 Ligation Reactions

Ligation reactions were carried out in a final volume of no greater than 10µl so as to maintain a high concentration of 5' and 3' DNA ends. The reactions were performed using an excess of the insert (~10x) in 1x ligase buffer in the presence of $10\mu g\mu l^{-1}$ BSA and incubated overnight at room temperature. The ligated products were used to transform DH5α sub-cloning efficiency competent cells as described in manufacturer's instructions. The transformants were selected on Luria agar plates using ampicillin, X-gal and IPTG (see section 2.7.1) incubated overnight at 37°C. The plates were left at 4°C for approximately 4 hours to allow the blue colour in the colonies to develop, then white colonies were picked, cultured and plasmid DNA purified as described in section 2.7.3. Blue colonies were treated in the same way as a comparison.

Chapter 3

Chapter 3 : Results

3.1 Introduction

Fifteen putative cisplatin genetic suppresser elements (GSE's) had previously been isolated by Kirschling *et al.* (94) from a pool of cDNA fragments derived from a DNaseI digestion of a normalised HeLa cDNA library. In order to confirm that these GSE's confer cisplatin resistance we have transfected them, expressed in the pLNCX vector, into the A2780 human ovarian adenocarcinoma cell line and examined the transfectants by clonogenic assay. The experimental results detailed here confirm that resistance to cisplatin is conferred by several of these GSE's.

Cisplatin is thought to exert its cytotoxic effects as a consequence of its DNA-damaging activity. I was interested to determine whether GSE-induced cisplatin resistance was a result of interference within a DNA-damage response pathway common to more than one DNA damage-inducing agent. To investigate the possibility of a multi-agent resistant phenotype, resistance to γ radiation was assessed again by clonogenic assay.

PCR amplification of GSE DNA and DNA sequencing were used to verify the presence of the GSE DNA and so validate the results of the resistance analyses, as well as confirming the genomic origin of the DNA.

Many agents which induce DNA damage have been shown to result in cell cycle arrest checkpoints (10). I was interested to examine the checkpoint phenotypes, in response to DNA damage in the GSE transfectants and so carried out an analysis of G1/S and G2/M checkpoints in response to ionising radiation and cisplatin

One pathway which is thought to be important in the response to DNA damage is the p53 / waf-1 mechanism (fig. 1.3.2). I was interested to determine whether it is this pathway which the GSE's are interfering in and so analysed waf-1 RNA levels using a Northern analysis approach.

3.2 Resistance to Cisplatin

The GSE's which were used to transfect the A2780 cell line were all originally isolated as a consequence of conferring cisplatin resistance to HeLa and / or HT1080 cell lines. The lines generated by transfection of GSE 7.10 into the A2780 cell line are shown in table 3.2.1 along with descriptions of other cell lines used. Unless otherwise noted, the transfected lines represent geneticin resistant colonies pooled after DNA transfection and selection for the GSE shown.

CELL LINE	DESCRIPTION
A2780	Parental cell line
A2.9	LNCX vector alone
A2.14	GSE 7.10
A2.14.5[3]	GSE 7.10
A2.14.9[3]	GSE 7.10
A2.14.10[3]	GSE 7.10
A2.22	GSE 7.10
A2.15	GSE 7.1
A2.23	GSE 7.1
A2.16	GSE E4
A2.24	GSE E4
53.1.6	V_{143}→A dominant negative p53 mutant

3 denotes a subclone of the A2.14 cell line

Table 3.2.1 Description of the cell lines examined in the resistance (and other) experiments.

The resistance to cisplatin of the GSE transfectants compared with the parental A2780 line and the pLNCX transfected control line A2.9, was examined by clonogenic survival assay. The resistance values were calculated by dividing the surviving fraction (s.f.) after cisplatin treatment of the transfectants with either

A2780 s.f. values or the vector alone transfectants, A2.9. The plating efficiency of each line is also recorded. The results of a 24 hour exposure to 1μM cisplatin are detailed in table 3.2.2.

CELL LINE	GSE	PLATING EFFICIENCY	MEAN SURVIVING FRACTION (a)	STANDARD ERROR	MEAN RESISTANCE
A2780	none	30%	0.15 (12)	0.007	1
A2.9	pLNCX	54%	0.15 (2)	ND	1
A2.22	7.10	34%	0.43 (8)	0.025	2.9
A2.15	7.1	38%	0.25 (6)	0.021	1.7
A2.23	7.1	12%	0.22 (8)	0.042	1.5
A2.16	E4	11%	0.25 (8)	0.03	1.7

(a) Surviving fraction in relation to untreated controls. Numbers in parenthesis represent the number of experiments, plating 10^3 cells per experiment.

Table 3.2.2 Results of clonogenic survival assays after a 24 hour exposure to 1μM cisplatin.

All of the lines examined show a degree of resistance to cisplatin treatment with the A2.22 cell line showing the highest levels i.e. 2-3.8 fold resistance compared with the A2780 and A2.9 cell lines.

The sensitivities of the GSE transfectants to a one hour exposure to 20μM cisplatin was examined and the results shown below.

CELL LINE	GSE	PLATING EFFICIENCY	MEAN SURVIVING FRACTION (a)	STANDARD ERROR	MEAN RESISTANCE COMPARED WITH A2.9
A2.9	pLNCX	29%	0.012 (12)	0.004	1
53.1.6	none	32%	0.03 (4)	0.003	2.5
A2.22	7.10	50%	0.071 (12)	0.015	5.9
A2.14	"	17%	0.024 (12)	0.009	2
A2.14.10	"	21%	0.093 (4)	0.01	7.8

(a) Surviving fraction in relation to untreated controls. Numbers in parenthesis represent the number of experiments, plating 10^3 cells per experiment.

Table 3.2.3 Results of clonogenic survival assays after a one hour exposure to 20μM cisplatin. The response of a line transfected with a dominant negative mutation of p53, 53.1.6, is also shown for comparison.

These clonogenic assays show that the lines generated by transfection of GSE 7.10 into the A2780 cell line exhibit an increased resistance to a 1 hour exposure to 20μM cisplatin as well as the resistance observed in response to a 24 hour exposure to 1μM cisplatin. It is interesting to note that the subclone of A2.14, A2.14.10, has a resistance factor higher than that of both A2.14 and A2.22. This may reflect a highly resistant sub-population of transfectants from the population of cisplatin selected A2.14 cells. For instance, this may represent a sub-population of cells which highly express the GSE and will uniformly express the GSE in clonal isolates.

3.3 Resistance to Ionising Radiation

Clonogenic assays were used to determine the levels of resistance of the GSE transfectants to 2 Grays ionising radiation compared with the pLNCX transfected control line A2.9. The resistance values were calculated by expressing the surviving fraction (s.f.) of the A2.9 cell line at 2 Gy as 1.0 and comparing the s.f. values of the other lines to this. The plating efficiencies have also been determined, and are comparable between the transfected lines. The results of the clonogenic assays are summarised in table 3.3.1.

The results of these assays suggest that several of the GSE 7.10 transfectants have an increased resistance to ionising radiation compared with the control cell line generated by transfection of the pLNCX vector alone. As in the previous experiments using cisplatin, the A2.22 cell line appears to confer the most significant level of resistance to treatment with a DNA damaging agent. For this reason I have concentrated on the analysis of the cell lines generated by transfection of GSE 7.10 into the A2780 cell line i.e. A2.22 and A2.14 and its subclones.

The resistance of the GSE transfectants was also compared with the A2780 parental cell line following treatment with ionising radiation. The results of these assays were ambiguous as a result of abnormally high levels of resistance in this batch of cells. Other procedures in the laboratory gave anomolous results with this stock of A2780 cells which have subsequently been discarded. Consequently, confirmation of the radio-resistance results compared with the A2780 cell line will be required.

CELL LINE	GSE	PLATING EFFICIENCY	MEAN SURVIVING FRACTION (a)	STANDARD ERROR	MEAN RESISTANCE COMPARED WITH A2.9
A2.9	pLNCX	27%	0.034 (4)	0.014	1
A2.14	7.10	13%	0.11 (4)	0.025	3.2
A2.14.9	"	20%	0.083 (3)	0.033	2.4
A2.14.10	"	20%	0.177 (4)	0.018	5.2
A2.22	"	35%	0.243 (4)	0.026	7.1

(a) Surviving fraction in relation to untreated controls. Numbers in parenthesis represent the number of experiments, plating 10^3 cells per experiment.

Table 3.3.1 Results of clonogenic assays after exposure to 2 Gy ionising radiation.

3.4 PCR Amplification Of GSE 7.10 From A2.14 And A2.22

PCR amplification was employed to verify that the GSE DNA was still present within the GSE 7.10 transfectants. To carry out molecular analyses, DNA was extracted from the cell lines transfected with GSE 7.10 i.e. A2.14 and A2.22 as well as from vector alone transfectants, A2.9 and the parental cell line, A2780. The presence of DNA was confirmed by gel electrophoresis and the concentration determined by spectrophotometry.

Details of the primer binding sites are shown in fig. 3.4.1. The primers are complimentary to sequences within the pLNCX vector which flank the GSE insertion site, so amplification of the insert and flanking vector sequences would be expected. To ensure that the amplification process was specific, DNA from the A2780 parental cell line was treated in the same way as the transfectants. The

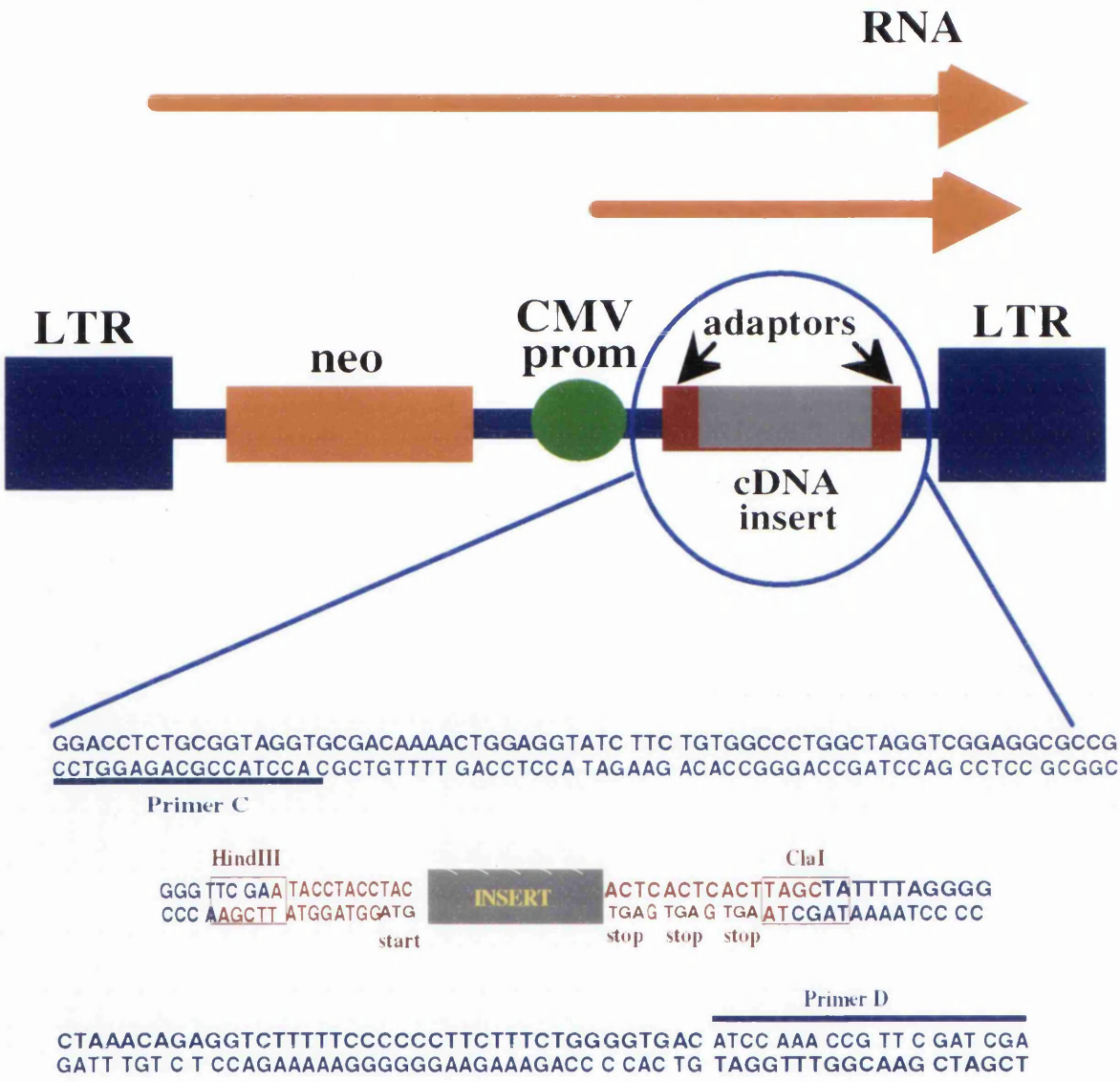


Figure 3.4.1 Diagram of the pLNCX vector detailing relevent restriction sites and primer binding sites

pLNCX$^{7.10}$ plasmid was also amplified to act as a marker in the agarose gel analysis.

The photograph in fig. 3.4.2 shows the electrophoretic separation of the PCR products obtained. Amplification of the two negative controls i.e. A2780 DNA template and water, did not result in any product being generated, so amplification of the other samples can be presumed to be specific. The pLNCX$^{7.10}$ plasmid amplified to give a product of approximately 300bp as did the DNA extracted from the GSE 7.10 transfectants, A2.14 and A2.22. This suggests that the same fragment of DNA has been amplified in all three cases, indicating that the pLNCX$^{7.10}$ plasmid is still present in the transfectants. DNA from the A2.9 line, which has been transfected with the pLNCX plasmid without a GSE insert, yielded a product in the region of 100bp, suggesting that GSE7.10 is approximately 200bp. The intensely stained smears at the foot of the gel are the result of primer dimerisation.

3.5 Sequencing To Confirm Identity of GSE 7.10

GSE 7.10 had been identified by Kirschling *et al.* (94) to be a fragment of the human phosphoglycerate mutase B gene (PGAM-B), acting in a sense orientation. In order to confirm the identity of the PCR amplified DNA, the PCR products obtained from the A2.14 and A2.22 cell lines were purified and prepared for DNA sequence analysis. The sequences obtained were entered into the GenBank™ /EMBL DNA sequence database and the search confirmed that the PCR products are 100% identical to a 188 base pair fragment of the human phosphoglycerate mutase-B (PGAM-B) gene (fig. 3.5.1) (101).

PGAM-B is a glycolytic enzyme which catalyses the conversion of 3-phosphoglycerate to 2-phosphoglycerate using 2,3 bisphosphoglycerate as a cofactor. It is highly conserved from yeast through to man and a homologous

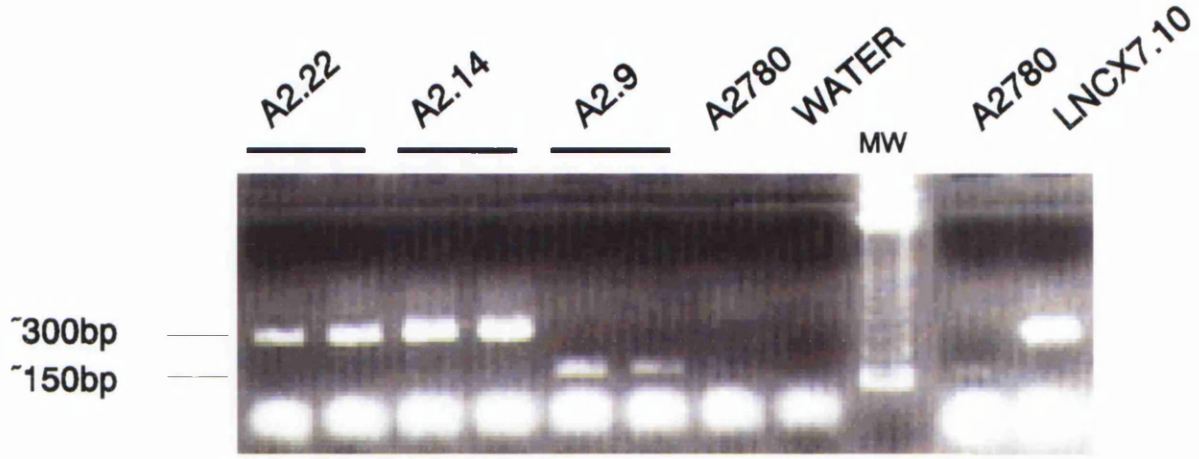


Figure 3.4.2 Gel showing the results of the PCR amplification of total DNA isolated from A2.22, A2.14, A2.9 and A2780 using primers complementary to sequences of the LNCX plasmid vector. Controls are water and the LNCX vector incorporating GSE 7.10. (MW = 123bp DNA ladder)

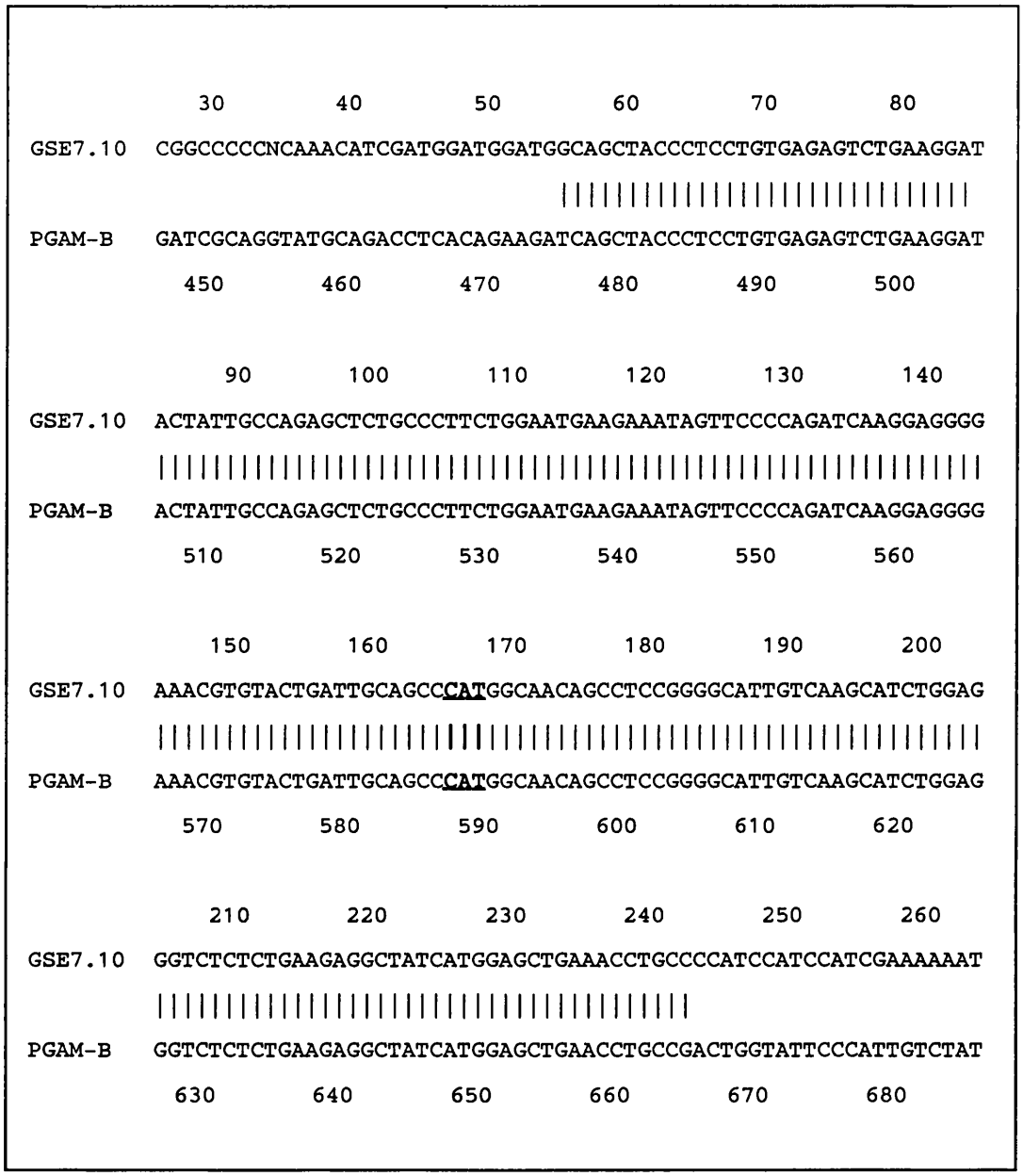


Figure 3.5.1 DNA sequence alignment of GSE 7.10 with human phosphoglycerate mutase B (accession no. J04173). The GSE was isolated by PCR from both the A2.14 and A2.22 cell lines (see section 3.4 and 3.5). The highlighted bases represent the codon for the histidine residue (His181) of the active site of the enzyme.

enzyme has also been identified in bacteria. At least two isoforms of the enzyme exist in man, one is muscle-specific, the M isoform, and another is non-muscle-specific, the B isoform. The enzyme is thought to act as a dimer and has not previously been linked to any form of drug resistance.

3.6 Subcloning of A2.14

The ionising radiation and cisplatin resistance experiments described previously suggest that one of the two independently GSE 7.10-transfected cell lines, A2.22, has a greater level of resistance than the other i.e. A2.14. When viewed by light microscopy, the gross morphology of these two transfected lines differed significantly. The A2.22 cells are homogeneously small and rounded, whereas the A2.14 cell line is more heterogeneous, in that both small rounded cells and larger, more flattened cells are present within the same population. I was interested to investigate whether the two morphologically different populations of the A2.14 displayed different characteristics in terms of their resistance to DNA damaging agents, and whether this resulted in the population as a whole, having a less dramatic phenotype than the A2.22 cell line.

In an attempt to isolate these two morphological populations within the A2.14 line, cells were grown and colonies were picked and expanded. All of the colonies however contained cells of both morphologies and so it proved impossible to segregate the cells on this basis. Three clones were maintained for further investigations and were designated A2.14.5, A2.14.9 and A2.14.10.

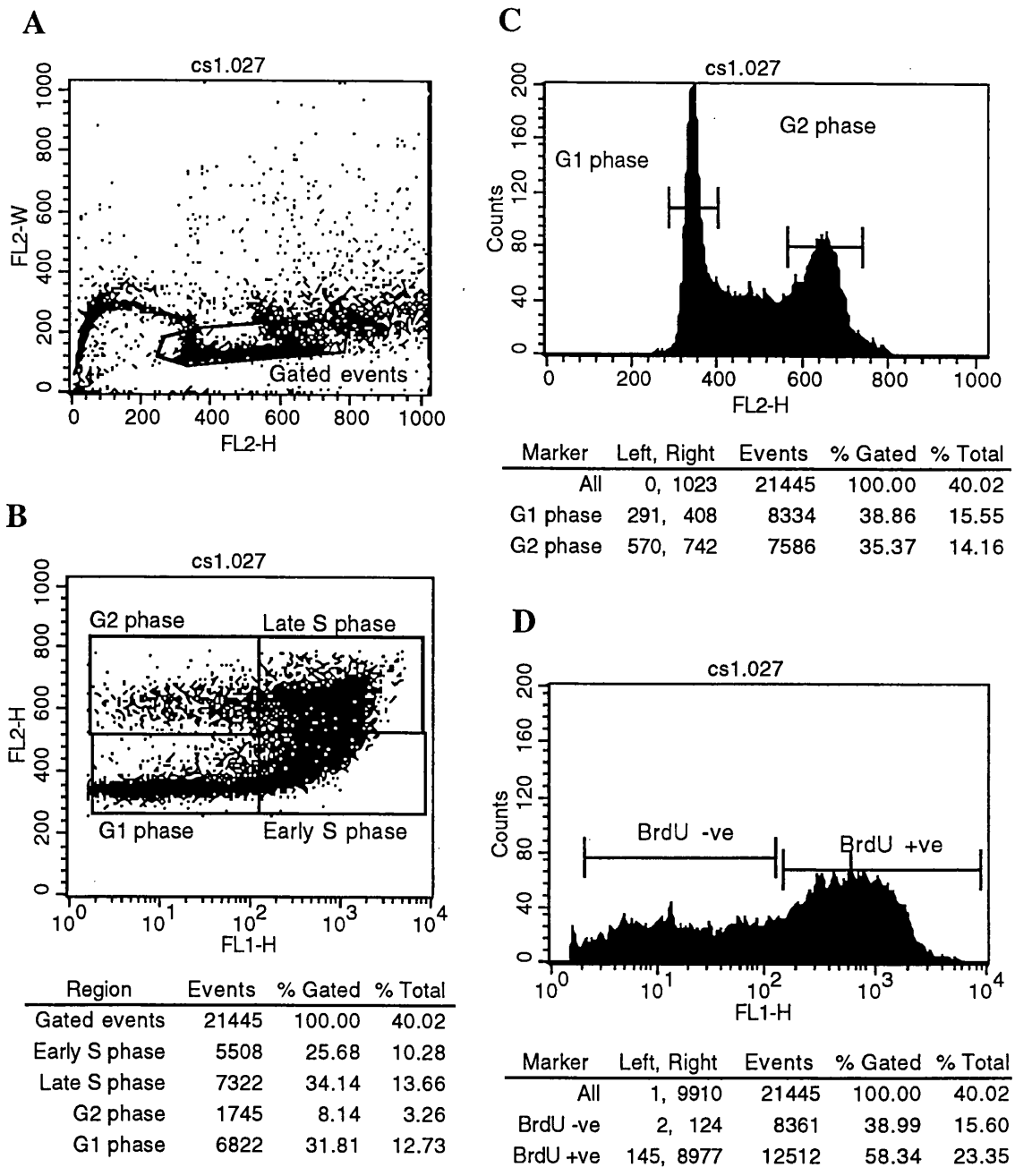
3.7 Cell Cycle Arrest Assay

Many DNA damaging agents have been shown to induce cell cycle checkpoint arrests. In order to assess any effect on cell cycle checkpoints which the GSE's were exerting, an assay to determine the percentage of cells in each phase of the cell cycle in response to cisplatin and ionising radiation treatment was carried out. Both agents cause DNA damage correlating with an arrest at the G1/S and G2/M checkpoints (102, 21, 44). This assay therefore assesses the ability of the GSE transfectants to block DNA replication and mitosis in response to DNA damaging agents. Wild-type cell cycle distribution is determined by analysing untreated cells, and this data is used as a comparison.

The cell cycle assay relies on the detection of a fluorescent antibody which binds the DNA replication-positive marker, 5-bromodeoxyuridine (BrdU) and the fluorescent activity of propidium iodide (PDI), a DNA intercalating agent. The staining achieved is therefore an accurate indicator of DNA replication and DNA content i.e. 2n and 4n. Table 3.7.1 describes the BrdU status and DNA content expected for each cell cycle phase.

The assay is performed on asynchronous cells grown in culture, and the results assessed by FACS (Fluorescence Activated Cell Sorting) analysis. The cells are sorted into separate populations as a result of differences in fluorescent staining and counted. Typical examples of the data produced are shown in figs. 3.7.1 and 3.7.2.

The numerical data generated by histogram B is used to calculate the percentage of treated cells in the G1, S and G2 phases of the cell cycle compared with the percentage of untreated cells in each phase. For example, if examining the ability of the cells to block DNA replication in response to DNA damage, the percentage of cells in S-phase is calculated with respect to the percentage of untreated cells in S-phase. The result is therefore a measurement of the incorporation of BrdU into DNA. In theory, for cells which are capable of blocking



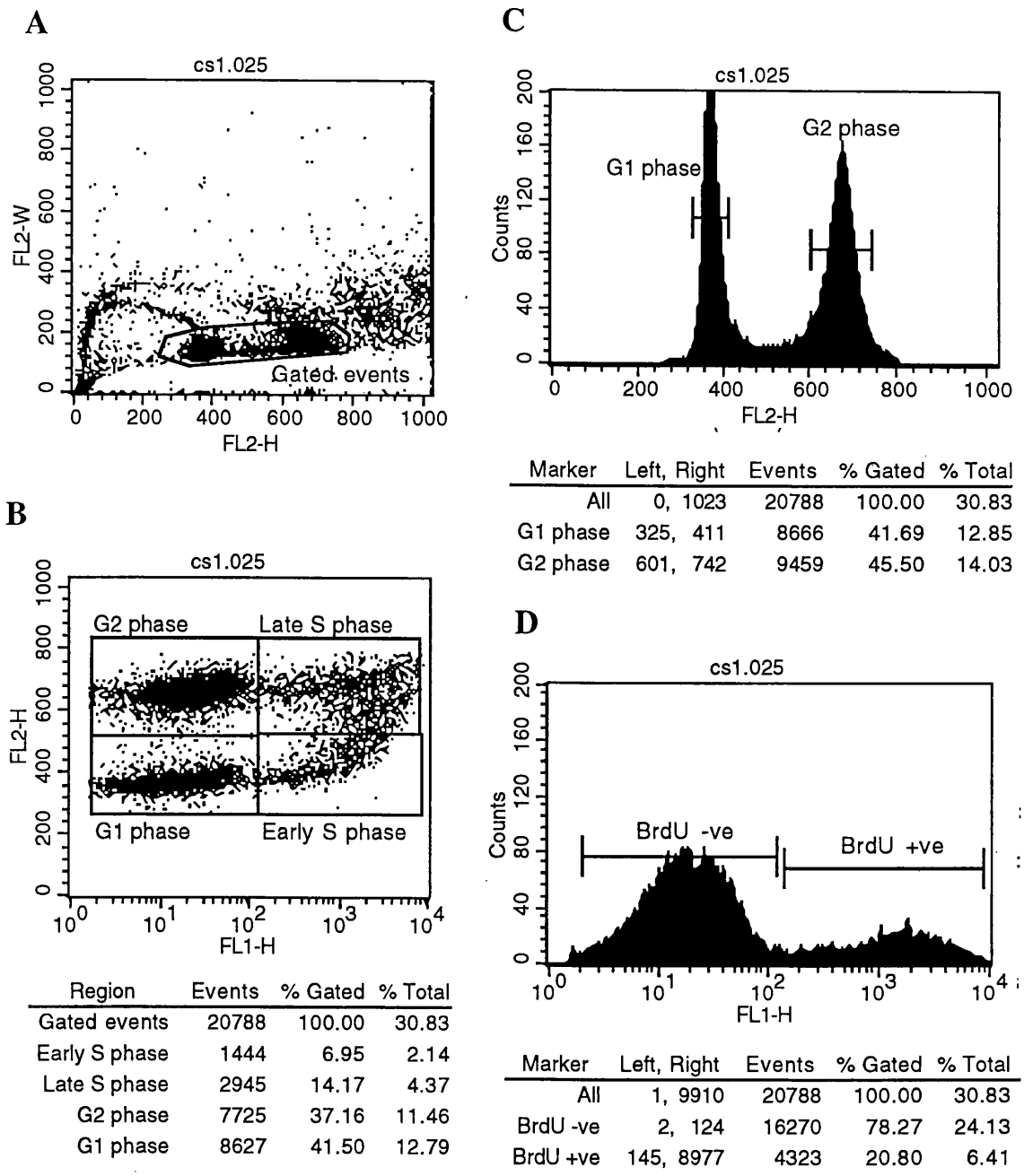
Region	Events	% Gated	% Total
Gated events	21445	100.00	40.02
Early S phase	5508	25.68	10.28
Late S phase	7322	34.14	13.66
G2 phase	1745	8.14	3.26
G1 phase	6822	31.81	12.73

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	21445	100.00	40.02
G1 phase	291, 408	8334	38.86	15.55
G2 phase	570, 742	7586	35.37	14.16

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	21445	100.00	40.02
BrdU -ve	2, 124	8361	38.99	15.60
BrdU +ve	145, 8977	12512	58.34	23.35

Figure 3.7.1 Example of results of cell cycle FACS analysis. The histograms represent a population of untreated cells which have been pulsed with BrdU and stained with propidium iodide (PDI) prior to analysis.

FL1-H : Represents BrdU staining e.g. histogram D represents the number of cells (y-axis) emitting any particular intensity of fluorescence generated by binding of the FITC conjugated antibody to BrdU. **FL2-H** : Represents PDI staining e.g.histogram B classifies each cell in terms of its BrdU (x-axis) and PDI (y-axis) content. **FL2-W** : Represents a measurement of cell size. This reading allows the exclusion from the analysis of cell aggregates (see histogram A).



Region	Events	% Gated	% Total
Gated events	20788	100.00	30.83
Early S phase	1444	6.95	2.14
Late S phase	2945	14.17	4.37
G2 phase	7725	37.16	11.46
G1 phase	8627	41.50	12.79

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	20788	100.00	30.83
G1 phase	325, 411	8666	41.69	12.85
G2 phase	601, 742	9459	45.50	14.03

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	20788	100.00	30.83
BrdU -ve	2, 124	16270	78.27	24.13
BrdU +ve	145, 8977	4323	20.80	6.41

Figure 3.7.2 Example of results of cell cycle FACS analysis. The histograms represent a population of cells which were pulsed with BrdU and stained with PDI following a one hour incubation with 20μM cisplatin.

When the histograms for these treated cells are compared with those for the untreated cells, then it is clear that there has been a large reduction in the proportion of cells in S phase following cisplatin treatment. This is seen in both histograms B and D, where the shift from BrdU+ cells to BrdU- cells is clear. One can conclude therefore that the cisplatin treated cells have arrested at the G1 checkpoint, thus preventing progression to the S phase of the cell cycle where DNA replication would occur.

CELL CYCLE PHASE	BrdU STATUS	DNA CONTENT (propidium iodide staining)
G1	–	2n
S	+	2n → 4n
G2	+	4n
M	+	4n → 2n

Table 3.7.1 BrdU status and DNA content of cell cycle phases.

entry into S-phase and so blocking DNA replication, the resultant value would approach zero, whereas those cells unable to arrest at this checkpoint would have a value for BrdU incorporation approaching 100%. In reality, in the A2780 cell line, a value of 20 - 40% is consistent with a population of cells with a functional G1 / S arrest, and so a value in excess of this indicates continuation of DNA replication following genotoxic insult.

Staining is carried out at 3 time points after the DNA damaging treatment, 6 hours to examine the G2 checkpoint, 24 hours for the G1 checkpoint and also at 48 hours.

The controls used in these assays were the parental line, A2780, and / or the vector alone transfectant, A2.9. The 53.1.6 line was also assessed. This line has been transfected with a dominant negative mutant of p53 (codon143 val->ala) and has been extensively shown, in this laboratory, to have lost its capability to arrest at the G1 to S-phase checkpoint in response to ionising radiation and cisplatin (44). Unpublished data also suggests that this line is incapable of arresting at the G2 to M checkpoint in response to cisplatin treatment.

3.7.1 Ionising Radiation Induced Cell Cycle Arrests

Six of the GSE transfected cell lines were examined in terms of their response to ionising γ radiation. The results of these assays are shown in fig.3.7.3 and 3.7.4 and usually represent 3 samples of treated and untreated cell populations.

The controls in the experiment shown in fig.3.7.3 were the A2780 parental line (▲) and the A2.9 vector alone transfectant line (■). Both of these lines were expected to exhibit a functional G1/S phase checkpoint arrest in response to ionising radiation. The data shows that this is the case for the A2780 and A2.9 cell lines i.e. at the 24 hour time-point, the percentage of these cells able to incorporate BrdU into their DNA has decreased by 50-60% compared with untreated cells. The other control line is 53.1.6 (•). This has been transfected with a dominant negative mutant of p53 and as a result, the cell cycle arrest potential of this line is severely compromised (44). The results confirm that this is the case as there is a reduction of only 35% in the number of actively cycling cells post-radiation. As expected, the maximal reduction in the percentage of cells in S phase is seen 24 hours after radiation. This is consistent with previous data (44). Of the GSE transfectants analysed here, at least two show a severely compromised ability to arrest at the G1/S checkpoint in response to ionising radiation as measured by a reduction in BrdU incorporation.

The A2.22 line (□), which has been transfected with GSE 7.10 exhibits the least reduction in the percentage of $BrdU^+$ cells following exposure to ionising radiation, and is striking in that the percentage of actively cycling cells at the G1/S transition 24 hours after treatment is greater than the 53.1.6 (mutant p53) line. The line generated by an independent transfection of GSE 7.10, A2.14 (Δ), shows less of a reduction in $BrdU^+$ cells than the vector alone control line.

The A2.23 line (o), which has been transfected with GSE 7.1 has a reduction in G1 arrest in response to γ radiation. At the 24 hour time point, this line has a reduction of approximately 35% in the number of actively cycling cells, a value similar to that for the 53.1.6 cell line. The other line examined in this experiment

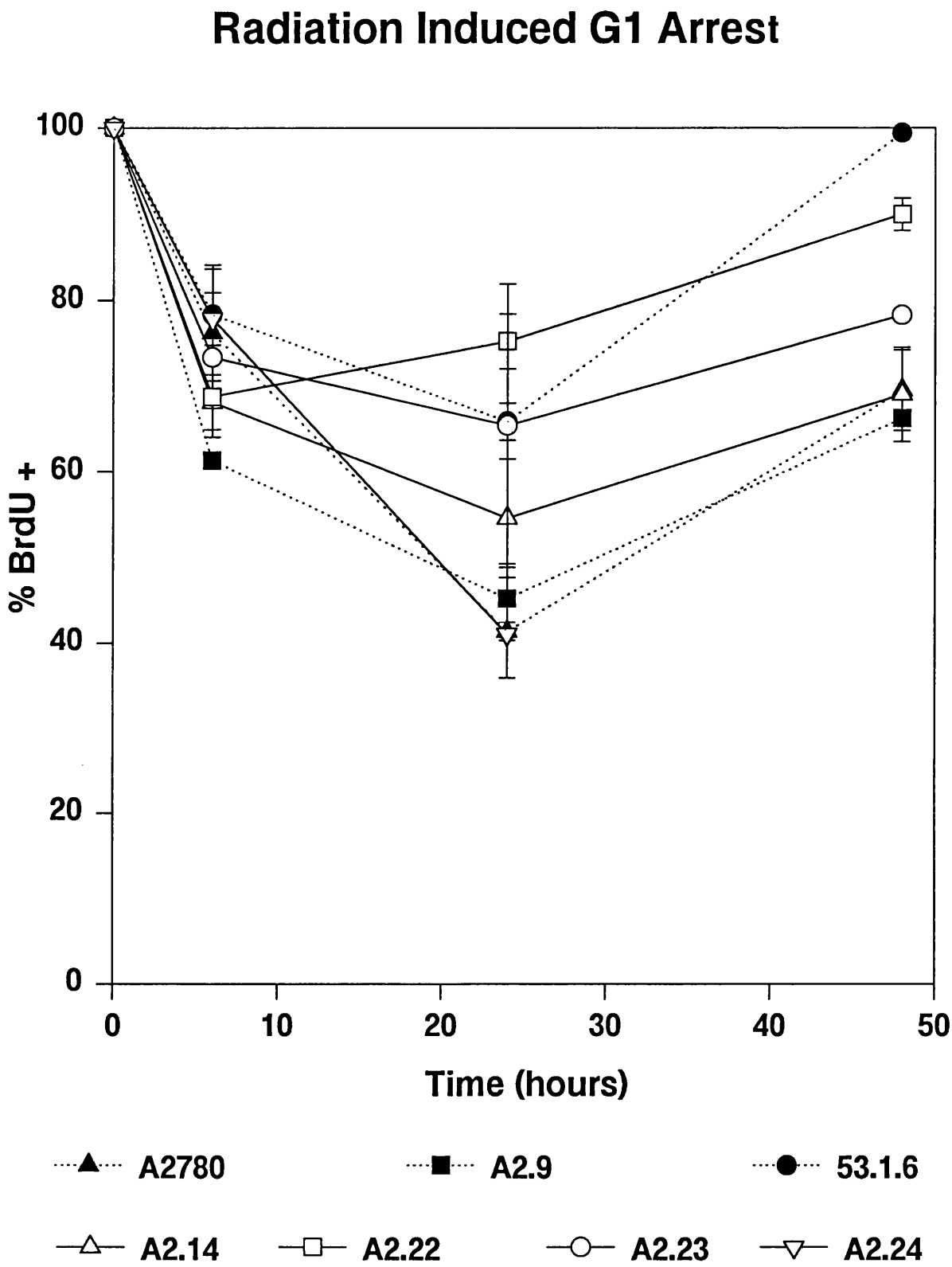


Figure 3.7.3 Graph details the results of FACS analysis, showing the percentage of BrdU positive GSE transfectants following treatment with 2Gy ionising radiation

is the A2.24 line, which has been transfected with GSE E4. This line appears to have a functional arrest, in that approximately 60% of cells are arrested at 24 hours post-treatment, a value similar to that for the A2.9 and A2780 control lines.

To confirm the results described above, i.e. that the GSE 7.10 transfectants, A2.14 and A2.22 have a reduced capability to undergo G1 arrest in response to ionising radiation, the experiment was repeated. The values recorded at the 24 hour time point are shown below.

CELL LINE	A2780	A2.9	53.1.6	A2.14	A2.22
MEAN % $BrdU^+$ CELLS	38.6	49.3	69.7	47.5	75.9
STANDARD ERROR	0.96	5.80	10.68	0.55	0.95
SAMPLE NUMBER	3	3	3	3	4

Table 3.7.2 Mean % values of $BrdU^+$ cells 24 hours after exposure to 2Gy ionising radiation. This describes the percentage of treated cells which are still actively cycling through the G1/S checkpoint.

As in the previous experiments, the A2780 and A2.9 lines show a decrease in actively cycling, $BrdU^+$ cells of greater than 50%, whereas the 53.1.6 mutant p53 transfectants show only a limited reduction in the percentage of $BrdU^+$ cells after treatment at 2Gy. The A2.22 line shows a very limited reduction in $BrdU^+$ cells, that is, only 24%. This supports the initial data that the transfected GSE is exerting a cell cycle effect i.e. a reduction in the capability to arrest at the G1 checkpoint after exposure to ionising radiation. The A2.14 cell line appears to act in a wild-type manner with respect to a G1 arrest in this experiment, having a greater than 50% reduction in cells actively synthesising DNA.

Fig. 3.7.4 shows the results of an additional experiment, examining the cell cycle of the A2.15 and A2.16 lines in response to ionising radiation. These lines have been transfected with GSE 7.1 and E4 respectively. In this experiment, the control lines do not exhibit the extreme differences which would be expected. The 53.1.6 mutant p53 transfectant has 70 % of cells able to incorporate BrdU into their DNA in response to ionising radiation, indicating a loss of G1 arrest. The A2.9 line however also has a large percentage of cycling cells after treatment, approximately 60%. The low level of reduction in the percentage of $BrdU^+$ cells in the A2.9 line makes the data generated from the GSE transfectants difficult to interpret. Both transfectants have a greater reduction in the percentage of $BrdU^+$ cells than either the A2.9 or 53.1.6 cell lines. The indication is therefore that the two GSE transfected lines have a functional G1 arrest in response to ionising radiation.

The percentage of cells with a 4n DNA content was also measured to determine the G2 arrest phenotypes. The data is not shown as all lines exhibited a strong G2/M checkpoint arrest in response to ionising radiation.

In conclusion, these experiments show that transfection of at least two of the GSE's into the A2780 cell line i.e. GSE 7.10 and GSE 7.1 results in an abnormal response to ionising radiation in terms of the ability of the cells to incorporate BrdU into their DNA. This suggests that these transfectants are able to continue replicating their DNA following damage, that is, they have a reduced capacity to arrest at the G1 / S checkpoint. GSE 7.10 is of particular interest as A2.22 (a line generated by transfection of GSE 7.10) shows the most extreme loss of G1 arrest, comparable to that of a line containing functionally mutant p53, and the A2.14 line which was generated by an independent transfection of the same GSE also exhibits a compromised G1 arrest phenotype.

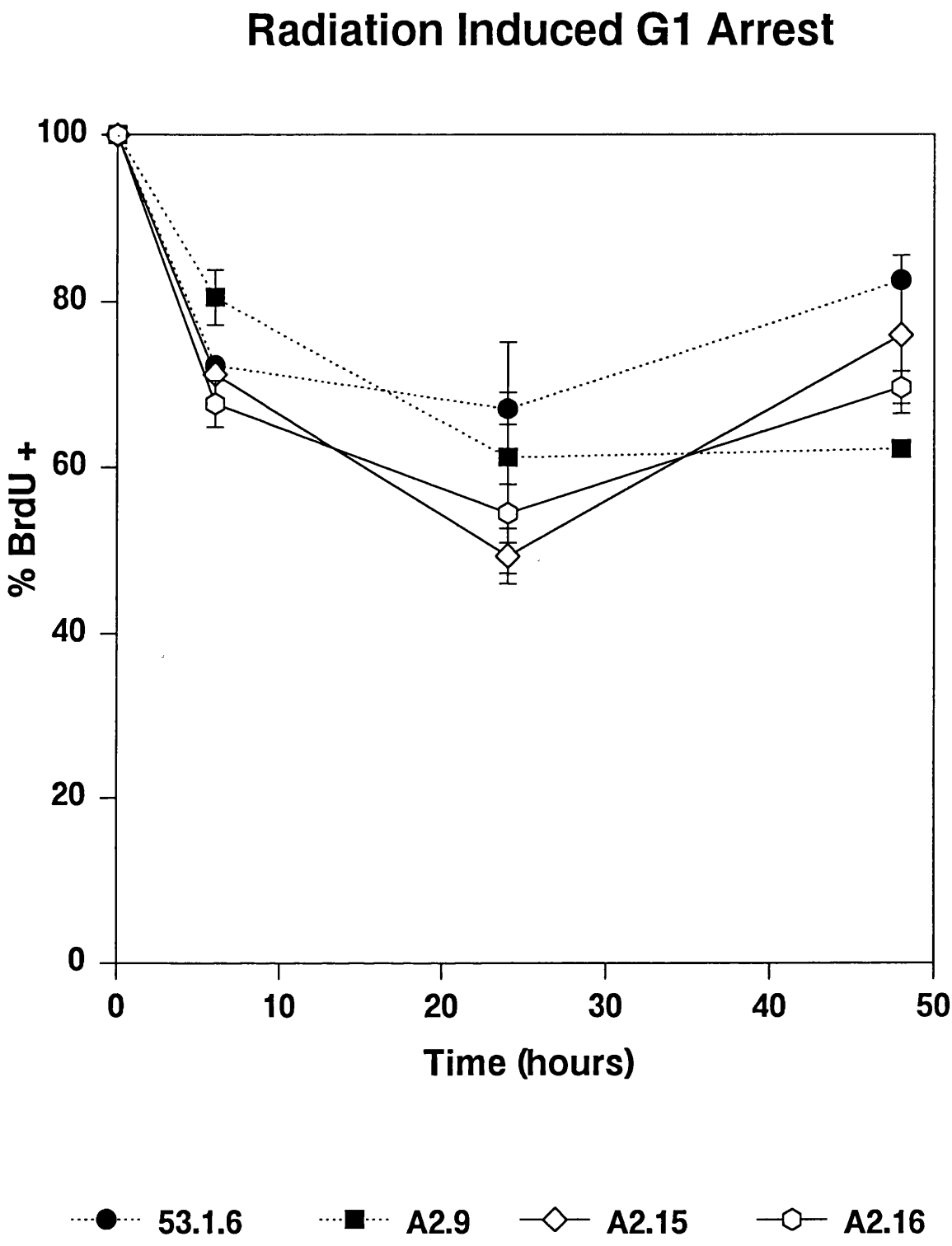


Figure 3.7.4 Graph details the results of FACS analysis, showing the percentage of BrdU positive GSE transfectants following treatment with 2Gy ionising radiation

3.7.2 Cisplatin Induced Cell Cycle Arrests

G1 Arrest

Figure 3.7.5 shows the G1 arrest data in response to a 20μM cisplatin exposure over a one hour time period. The results usually represent 3 samples of treated and untreated cell populations.

The A2.9 vector alone control line (■) shows a reduction in the percentage of $BrdU^+$ cells at 24 hours of less than 20%. This is a surprising result as this line should exhibit an arrest at the G1 / S checkpoint in response to cisplatin treatment. The reduction of $BrdU^+$ cells at 48 hours is significantly greater, i.e. approximately 80%. The cell line generated by transfection of the A2780 cell line with a dominant negative mutant of p53, 53.1.6, (•) has previously been shown to have lost its ability to arrest at the G1 checkpoint in response to DNA damaging agents. Consistent with these observations, the 53.1.6 cell line has a reduction in $BrdU^+$ cells of 10% at 24 hours. This reduction does continue to the 48 hour time point, but the reduction is less than for the A2.9 control line.

Of the three GSE transfected lines (all with GSE 7.10) two, A2.14 (Δ) and A2.22 (□), have no reduction in the percentage of $BrdU^+$ cells at 24 hours. This suggests that both of these lines are capable of continuing DNA replication after cisplatin treatment, that is, they do not arrest at the G1 / S checkpoint. The sub-clone of A2.14, A2.14.5 (o), showed a wild-type arrest phenotype. The phenotypes at 48 hours were unusual when compared to previous experiments in this group as the percentage of BrdU+ cells would be expected to rise. The absence of this may be due to nutrient depletion of the culture medium.

From this data, it appears that the GSE 7.10 transfectants, A2.14 and A2.22 have a decreased ability to arrest at the G1 checkpoint in response to treatment with the DNA damaging agent cisplatin. The sub-clone of A2.14, A2.14.5, appears to have a wild-type arrest in these conditions. It should be noted that this clonal population has not had the presence of GSE 7.10 confirmed by PCR amplification.

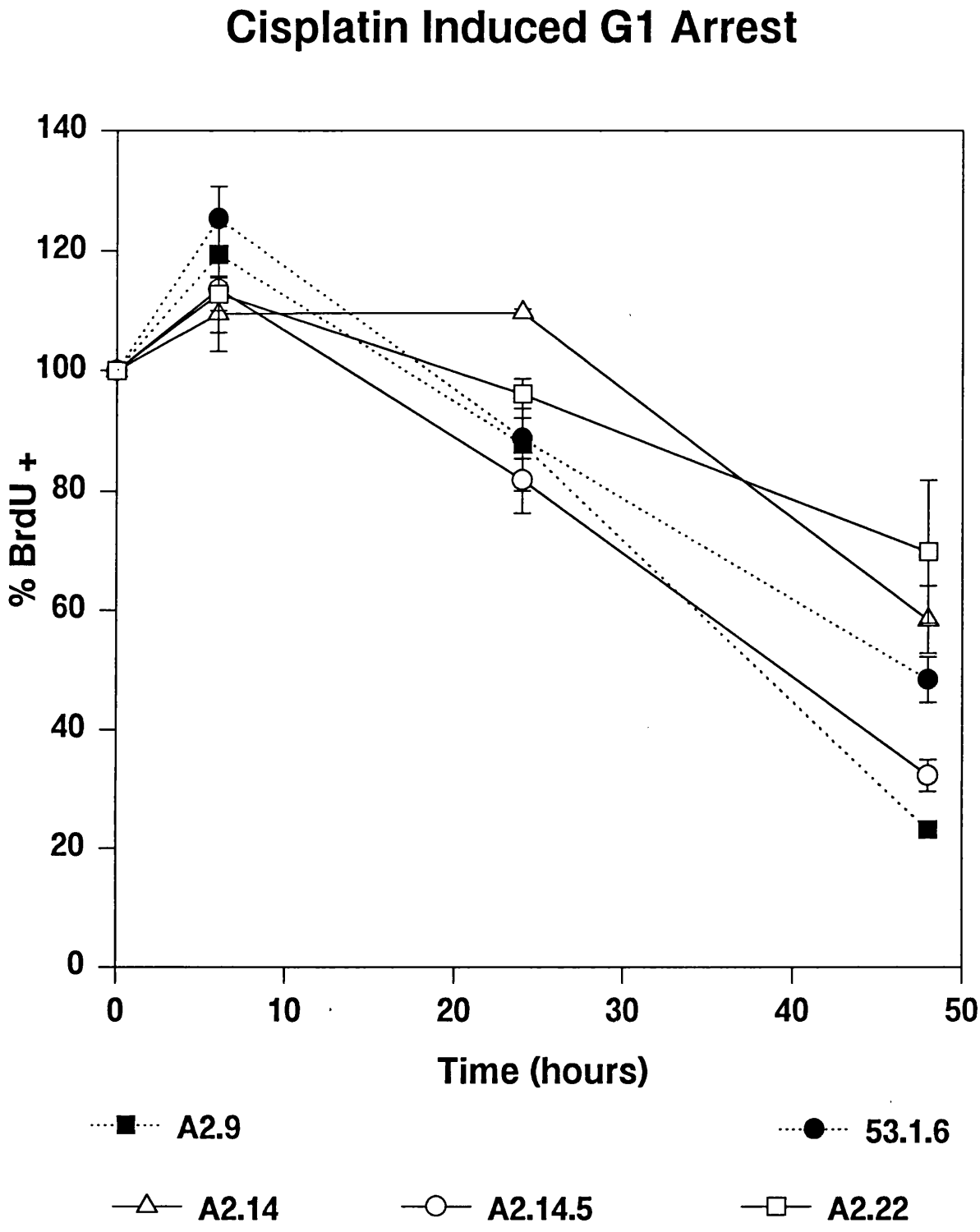


Figure 3.7.5 Graph details the results of FACS analysis, showing the percentage of BrdU positive GSE transfectants following treatment with 20uM cisplatin

The A2.9 vector alone control line used in this assay does not however conform to the expected wild-type arrest phenotype expected. The results of this assay therefore require confirmation.

G2 Arrest

Cisplatin has previously been shown to induce cell cycle arrest at the G2/M checkpoint as well as at G1/S (102, 21). Figure 3.7.6 shows the data relating to G2 arrest as determined by the percentage of cells with a 4n DNA content. The results usually represent 3 samples of treated and untreated cell populations.

The ability to arrest at the G2 checkpoint is assessed at the 6 hour time point. At the 6 hour time point, the GSE transfectants showed moderate changes in the percentage of cells containing 4n DNA. As can be seen in fig. 3.7.6, the more dramatic changes in the 4n DNA population occurred at 24 hours. At this stage, the vector alone control line, A2.9 (■), showed a large increase in the percentage of 4n cells, approximately 10-fold greater than at time = 0. In comparison, the 53.1.6 mutant p53 cell line (•) showed only a 4-fold increase in the percentage of cells with a 4n DNA content by 24 hours. This value decreased rapidly from 24 to 48 hours, whereas the A2.9 line maintained its arrest towards the 48 hour time point.

The GSE transfected cell lines displayed a phenotype intermediate between these two extremes. The A2.22 line (□) had approximately a 6-fold increase in 4n cells by 24 hours which fell off slightly by 48 hours. A2.14.5 (o) had a 4-fold increase in 4n cells by 24 hours, and this value continued to rise towards 48 hours. A2.14 (Δ) had only a 2-fold increase in 4n cells by 24 hours and a 5-fold increase at 48 hours. These results suggest that the GSE 7.10 transfectants show a reduction in their G2/M arrest capability when compared to a pLNCX transfected vector alone control line. This reduction in cells with a 4n DNA content occurs at the 24 hour time-point rather than at 6 hours after treatment with cisplatin and does not represent a reduction to the level seen in the dominant negative mutant p53

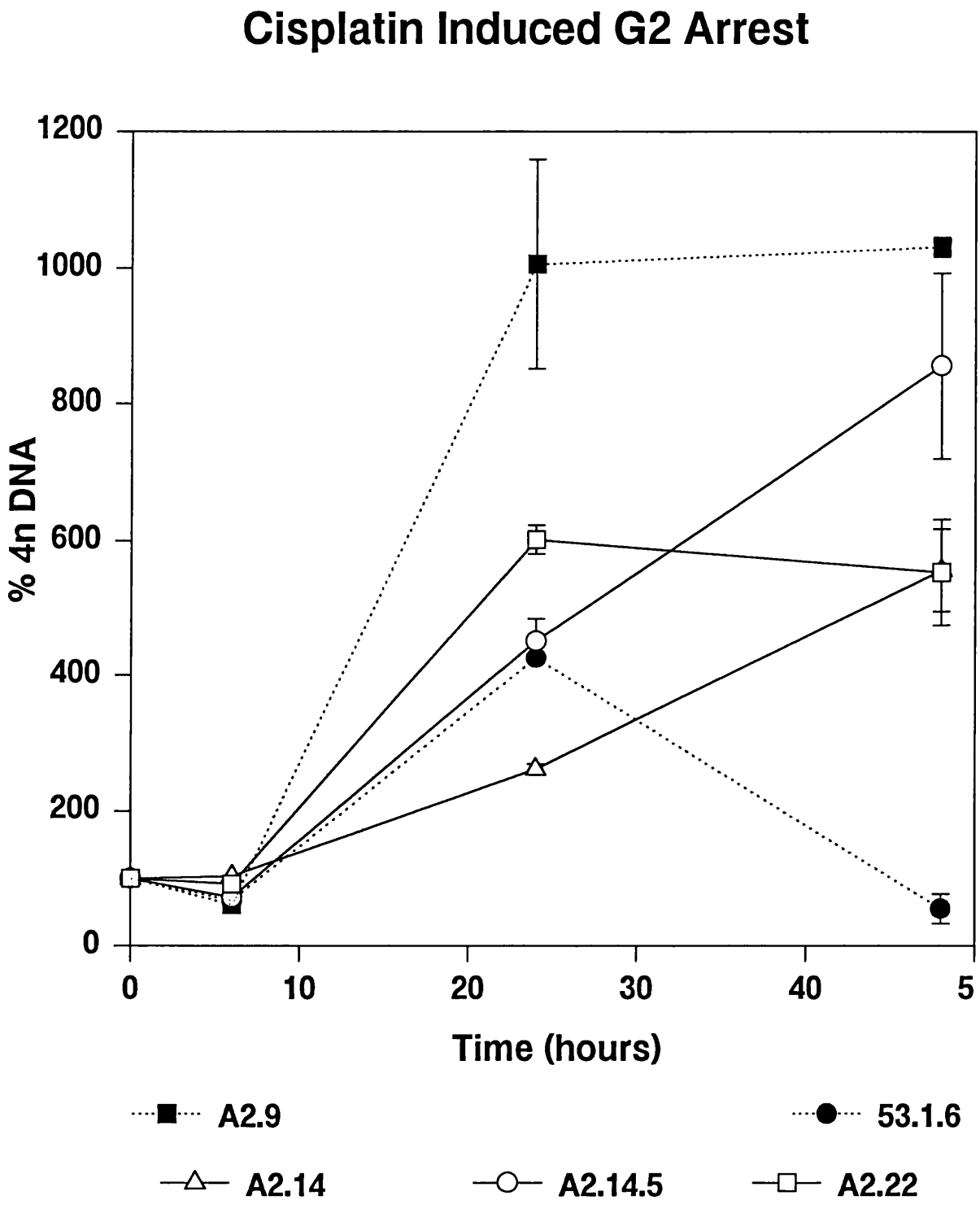


Figure 3.7.6 **Graph details the results of FACS analysis, showing the percentage of GSE transfectants with a 4n DNA content following treatment with 20uM cisplatin**

transfectants. Consequently, this data requires clarification in order to determine whether the reduction in the percentage of cells with a 4n DNA content is significant.

The doubling time of the cell lines examined in the cell cycle arrest assays should also be determined. This will confirm that the presence or absence of cell cycle arrests is not an artefact due to a change in doubling time resulting from the transfection procedure.

3.8 Northern Blotting To Examine waf-1 RNA Levels

Waf-1 expression is induced by elevated levels of p53 protein in response to DNA damage (30), therefore the level of waf-1 RNA is an indication of p53 transactivation activity. In order to determine whether the p53/waf-1 pathway is being interfered with by transfection of GSE 7.10, waf-1 RNA levels have been estimated in the GSE 7.10 transfectants by Northern blot analysis. Figure 3.8.1a shows the waf-1 RNA levels in the GSE 7.10 transfectants as well as the parental cell line, A2780, and the vector alone transfectant, A2.9. Also examined was a line sub-cloned from A2.14, A2.14.5, and A2780cp70, a cisplatin resistant derivative of the parental A2780 cell line, which exhibits a marked reduction in p53 activity and would therefore be expected to have reduced levels of waf-1 RNA. The lower autoradiograph shows the hybridisation with a GAPDH probe, used in order to take account of the initial loading amount of RNA.

The graph in figure 3.8.1b shows the relative densities of the waf-1 hybridisation signals in the various lines tested. The RNA loading levels were corrected using the GAPDH signals in the same lines using the autoradiographs shown in fig. 3.8.1a. The maximum fold difference between the lines is low enough to indicate that there is no significant difference in waf-1 RNA levels between the samples. It would be expected that the A2780cp70 cell line would have a reduction in the levels of waf-1 RNA as it lacks functional p53 and has been confirmed previously.

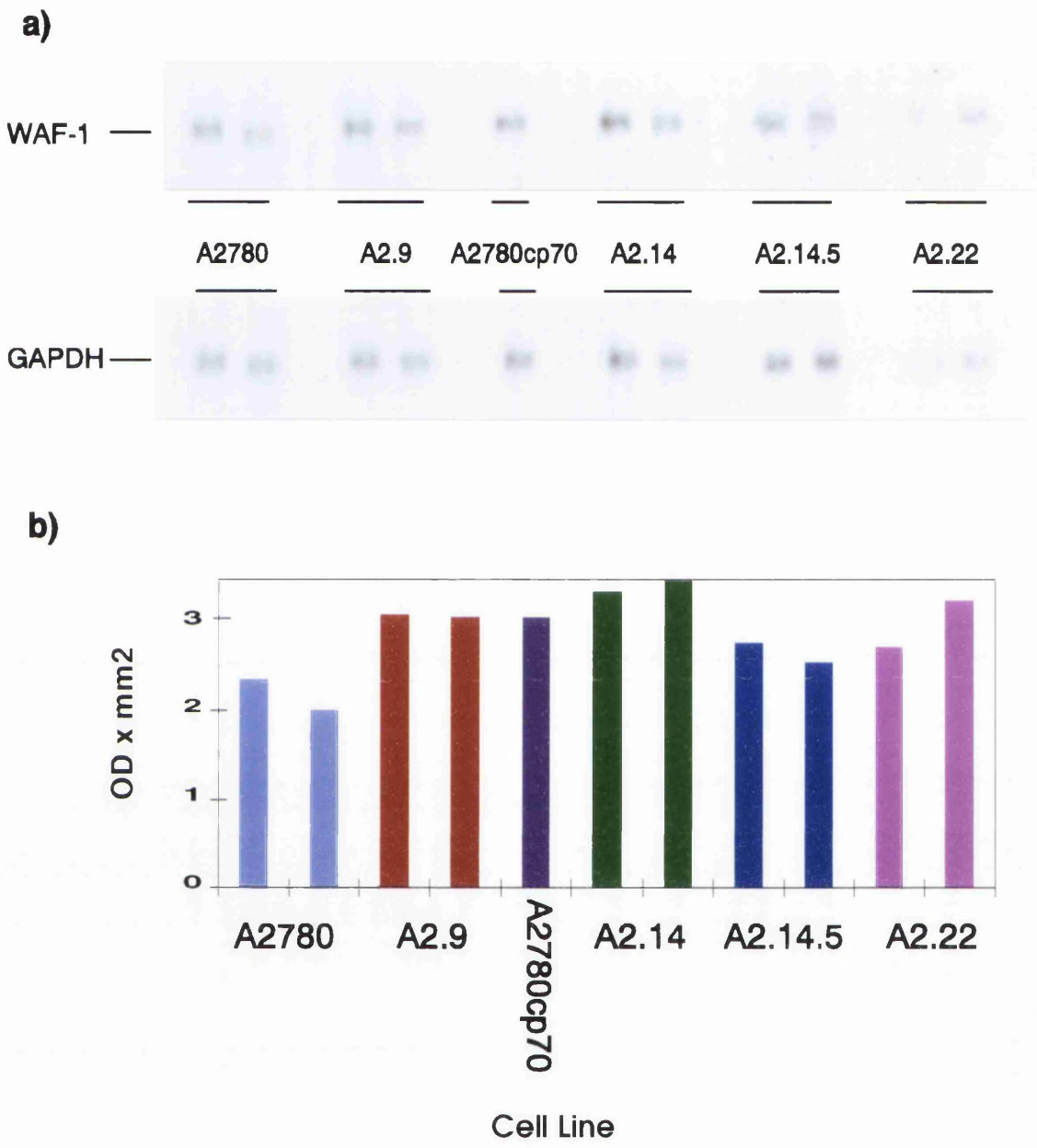


Figure 3.8.1 Comparison of waf-1 and gapdh mRNA levels from a Northern blot using a WAF-1 probe.

a) autoradiograph of blots

b) density of WAF-1 bands normalised for loading with GAPDH band density

3.9 Tetracycline Inducible System

In order to confirm that the transfection of GSE 7.10 is responsible for the phenotypic effects described, an inducible gene expression system based on tetracycline responsive elements (95) is being developed. This system is used in preference to other inducible systems due to its reported efficiency as a very clean on/off switch. Many other inducible systems can 'leak', that is, a low level of constitutive expression occurs as the element or molecule which is used as the switch is endogenous to the cell line in use, for example, the metallothionein and dexamethasone systems.

The tetracycline system requires the use of two plasmids, one which incorporates the transactivator construct, the other carries the tetracycline operator sequence. The transactivator consists of the tet-repressor sequence from *E.coli* fused to VP16 the herpes virus transactivator. This construct acts as a transcription factor, the target of which is a minimal eukaryotic promoter with a series of tet-operator sequences and the gene of interest. In the presence of tetracycline, the transactivator binds to tetracycline and so is prevented from binding to the operator sequences. As a result, the gene of interest is not transcribed. When tetracycline is removed, the transactivator is free to bind the operator sequence and transcription of the gene (or GSE) of interest results.

The work detailed here describes only the initial steps carried out in the generation of this system.

3.9.1 pUH Plasmid Purification

The plasmid constructs for use in the tetracycline inducible system are described in section 2.1.4 and were obtained from H. Bujard (95). The plasmids were rehydrated as instructed and used to transform Epicurian Coli SURE competent cells according to the manufacturer's instructions. Plasmid DNA was purified

using the Qiagen maxi prep. protocol. Diagnostic restriction digests were performed to ensure that the constructs were intact as expected.

3.9.2 Transfection

Transfection of the tetracycline transactivation complex tTA was achieved using the calcium phosphate technique. The pUHD15-1 plasmid was co-transfected into a resistant derivative of the A2780 cell line, A2780cp70 and also into the MCF-7 cell line with a neomycin resistance marker plasmid as detailed:-

- 20μg / 10μg plasmid DNA, pUHD15-1 along with 4μg / 2μg G418 resistance marker plasmid ZIPneoSV(X)1 or pSVneo.

The colonies were selected with G418 and picked into 24-well plates and expanded into tissue culture flasks. Resistant colonies have been frozen for storage at -70^{o}C.

Approximately 50% of the A2780cp70 transfectants (pSVneo + pUHD15-1) which were picked into the 24-well plate were viable. None of the MCF-7 colonies grew once transferred into the 24-well plates. These observations appear to be independent of original size of colony, time at which they were picked and neomycin resistance marker plasmid used. Control cells (MCF-7 and A2780cp70) were transfected with salmon sperm DNA and resistant colonies developed on the master plates. However, these were not picked into 24-well plates so the explanation for the low / zero viability of the pUHD15-1 transfectants is unknown. Both of these cell lines have previously been transfected successfully, so an inherent cellular problem is unlikely.

In the absence of tetracycline, this inducible system is switched on. It is possible in these transfection conditions that the pUHD15-1 plasmid is toxic at its site of integration. As a result, the transfections were repeated in the presence of tetracycline as well as G418, the rationale being that the presence of tetracycline prevents transcriptional activation. In both the presence and absence of tetracycline, resistant colonies of A2780cp70 and MCF-7 transfectants appeared

and were large enough to be picked into the 24-well plates. At least 50% of the A2780cp70 transfectants were viable and were expanded into tissue culture flasks and frozen stocks were made. Tetracycline did not appear to play a role in the viability of the resistant A2780cp70 colonies, nor the MCF-7 transfectants. Colonies of G418-resistant MCF-7 transfectants appeared on the master plates in the presence and absence of tetracycline. These were picked into 24-well plates in the presence and absence of tetracycline and incubated as normal. 100% of these selected colonies proved to be inviable.

3.9.3 Preparation of GSE 7.10 For Use In The Tetracycline Inducible Expression System

In order to induce expression of GSE7.10 in this system, it was necessary to sub-clone the GSE into pUHD10-3, downstream of the heptamerised tet operator sequences. GSE 7.10 was cloned into the pLNCX vector as a *Hae*II / *Cla*I fragment, however, this could not be easily cloned into the pUHD10-3 vector. Consequently, an intermediate vector had to be used in the sub-cloning process (see fig 3.9.1), pIC19R (97). The GSE was cut out of the pLNCX vector as a *Hin*dIII / *Cla*I fragment and the pIC19R vector was also digested with *Hind*III / *Cla*I. These digestions resulted in complementary 'sticky ends' allowing the GSE to ligate in a known orientation. In order to increase the percentage of specific ligations and reduce the number of religations and doublet formation, the reaction was also performed using dephosphorylated pIC19R. The pIC19R and GSE7.10 *Hin*dIII / *Cla*I fragments were ligated as detailed in section 2.8.3, employing X-gal and ampicillin as selection procedures. The use of selection by α-complementation relies on the expression of β-lactamase from the lacZ gene on the pIC19R plasmid. When this gene is expressed, blue colonies form in the presence of X-gal and the inducer IPTG. The lacZ gene spans the multi-cloning site of pIC19R, therefore, if the GSE fragment were to successfully ligate into the vector, the lacZ gene would be disrupted and white colonies would result.

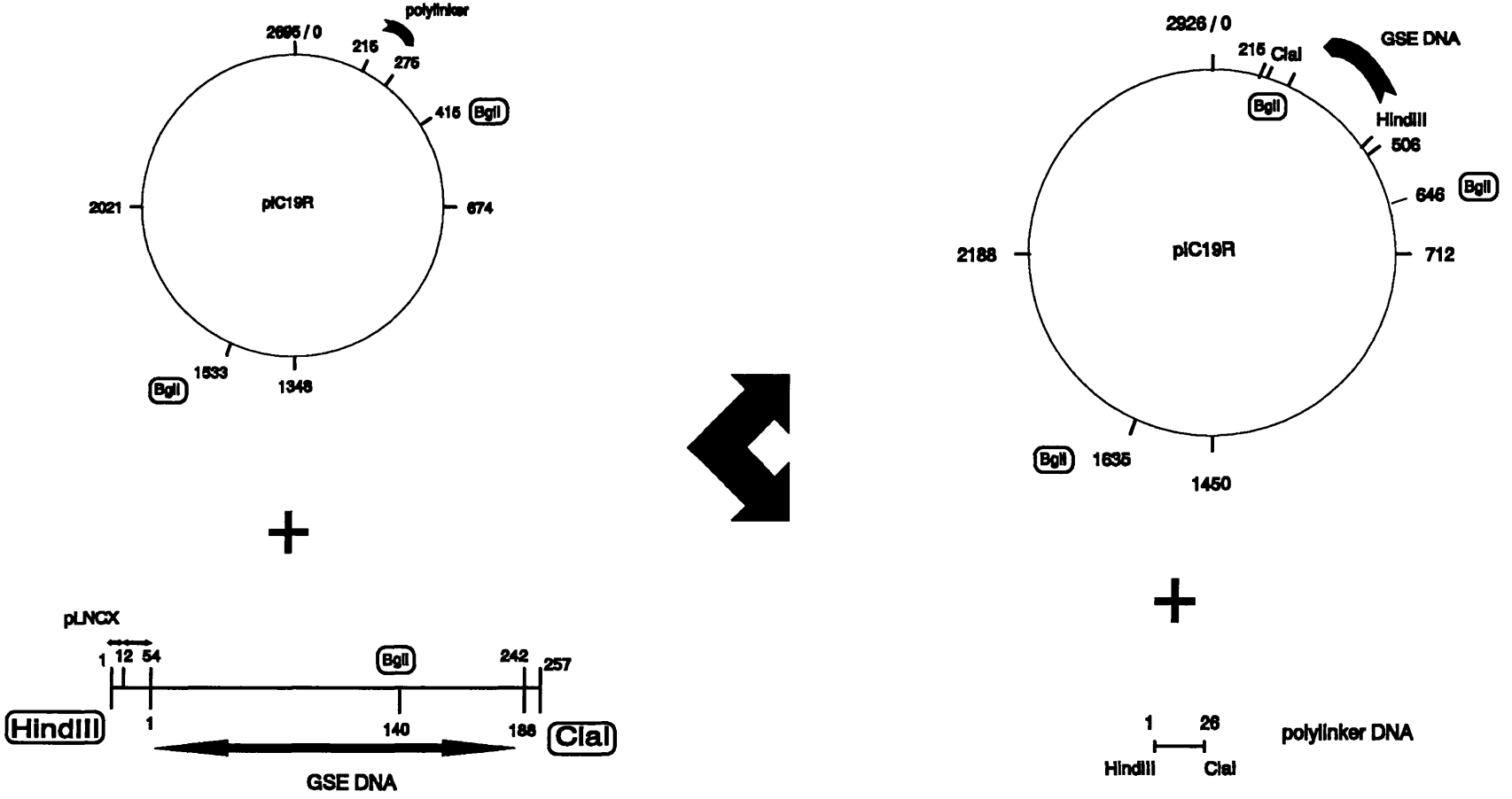


Figure 3.9.1 Schematic diagram of the pIC19R plasmid and the GSE 7.10 construct. The GSE7.10 construct was derived from the pLNCX plasmid by HindIII/ClaI digestion. The pIC19R plasmid was also digested with these enzymes. Restriction sites for the diagnostic digestions i.e. BglI and HindIII/ClaI are shown.

Problems can arise if the insert DNA is a small enough fragment that does not prevent the lacZ gene from being expressed, in this case, blue colonies which would usually be ignored may contain the plasmid and ligated insert.

The ligation reactions were used to transform bacteria, and plasmid DNA from in excess of thirty white colonies was purified. Subsequent restriction digests of these DNA samples to confirm identity and the presence of accurate ligation products all resulted in DNA fragments whose size suggested that pIC19R doublets had formed. Plasmid DNA was then extracted from three blue colonies and digested in the same manner as the DNA extracted from the white colonies. All three samples revealed DNA fragments of a size consistent with successful ligation of GSE 7.10 into pIC19R by both *Hin*dIII / *Cla*I and *Bgl*I digestions (fig.3.9.2) It must therefore be assumed that the GSE is of such a size that read-through is possible.

3.10 p53 GSE's

The results described so far detail the analysis of cisplatin GSE's which were generated by digestion of total HeLa cell cDNA. As previously mentioned however (section 1.6.2) it is possible to isolate GSE's derived from digestion of a single gene cDNA. As there is a large body of evidence to suggest that p53 plays a pivotal role in the response to DNA damaging agents, including cisplatin, Liam Gallagher (CRC Dept. of Medical Oncology, University of Glasgow) has attempted to isolate genetic suppressor elements derived from both the human and murine p53 genes which confer resistance to cisplatin.

3.10.1 PCR Amplification of Cisplatin Selected GSE's

A2780 cells which had previously been infected with vector containing both murine and human p53 GSE's were selected for resistance to cisplatin. The GSE's

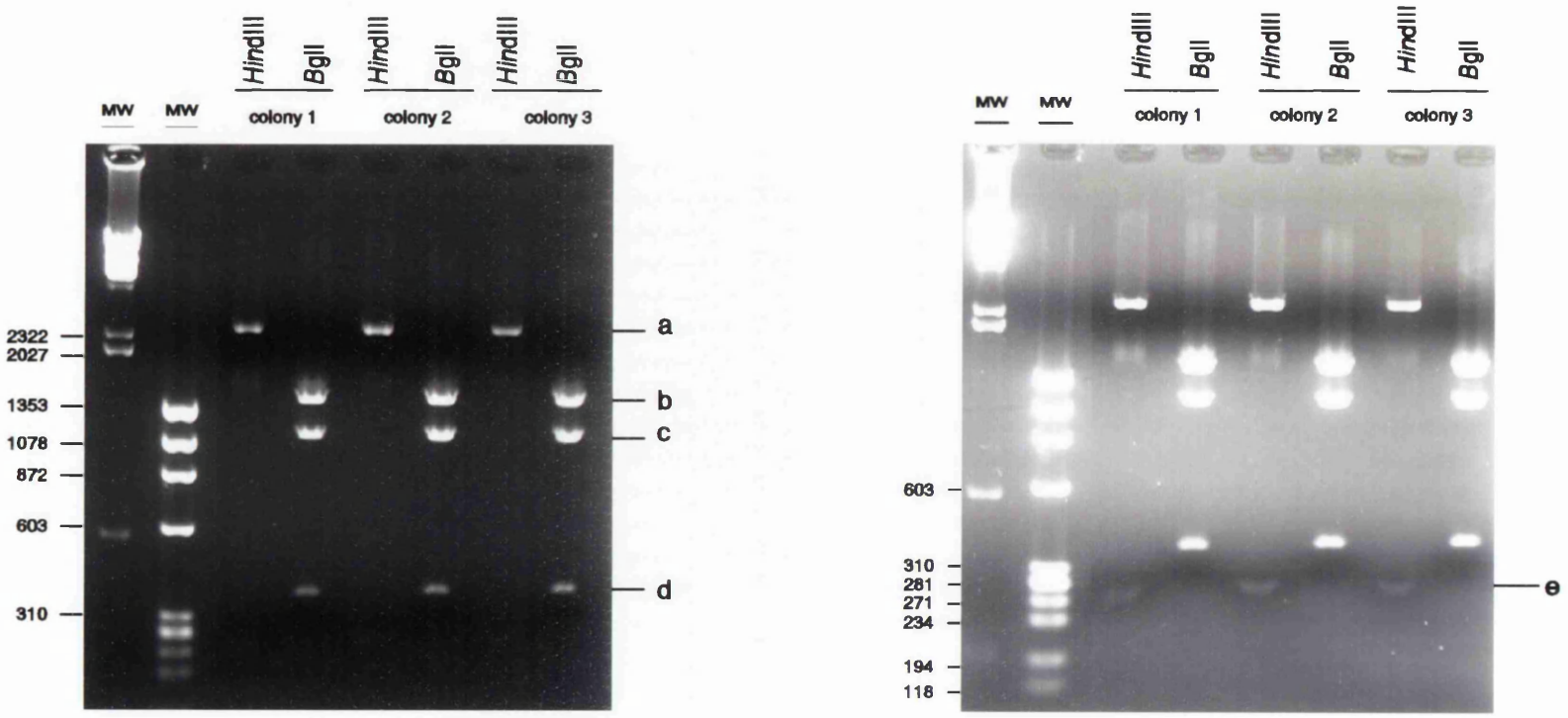


Figure 3.9.2 Electrophoretically separated restriction digests of three blue colonies generated by transformation of *E.coli* cells with a plasmid generated by ligation of the HindIII / ClaI digested pIC19R plasmid and the GSE 7.10 HindIII / ClaI fragment. Two different exposures of the same gel are presented to show the full range of restriction products generated (a - e).

SUMMARY

colonies 1-3 *Hin*dIII / *Cla*I : 2.5kb, 280bp
colonies 1-3 *Bgl*I : 1.5kb, 1.1kb, 400bp

present in 32 of the resistant cell lines were isolated by PCR amplification and purified using the Wizard PCR Clean-Up protocol. Master mix was prepared as detailed (section 2.4.1), using 1μg of primers P1785 and P1786, complimentary to pLNCX flanking DNA sequences. (see fig.3.4.1 for more detail). Figure 3.10.1 shows the electrophoretic separation of these amplified sequences. It is clear from this data that increasing the stringency of cisplatin selection results in the isolation of cell populations which contain a single GSE i.e. in the less stringently selected samples, there are a number of products, whereas increased cisplatin concentrations result in a reduction in the number of products isolated. Four of the cell populations which appeared to have a strong band and little smearing (indicating a single amplified product) were sequenced and analysed in comparison with human p53 (Accession no. K03199). The results of this analysis are shown in the appendix and summarised in table 3.10.1.

It is clear from this and other data in the group that the GSE technique has great potential in terms of isolating p53 fragments which confer cisplatin resistance. If the system was of low selectivity, then the appearance of significant numbers of GSE's derived from the digested vector would be expected. This has not occurred.

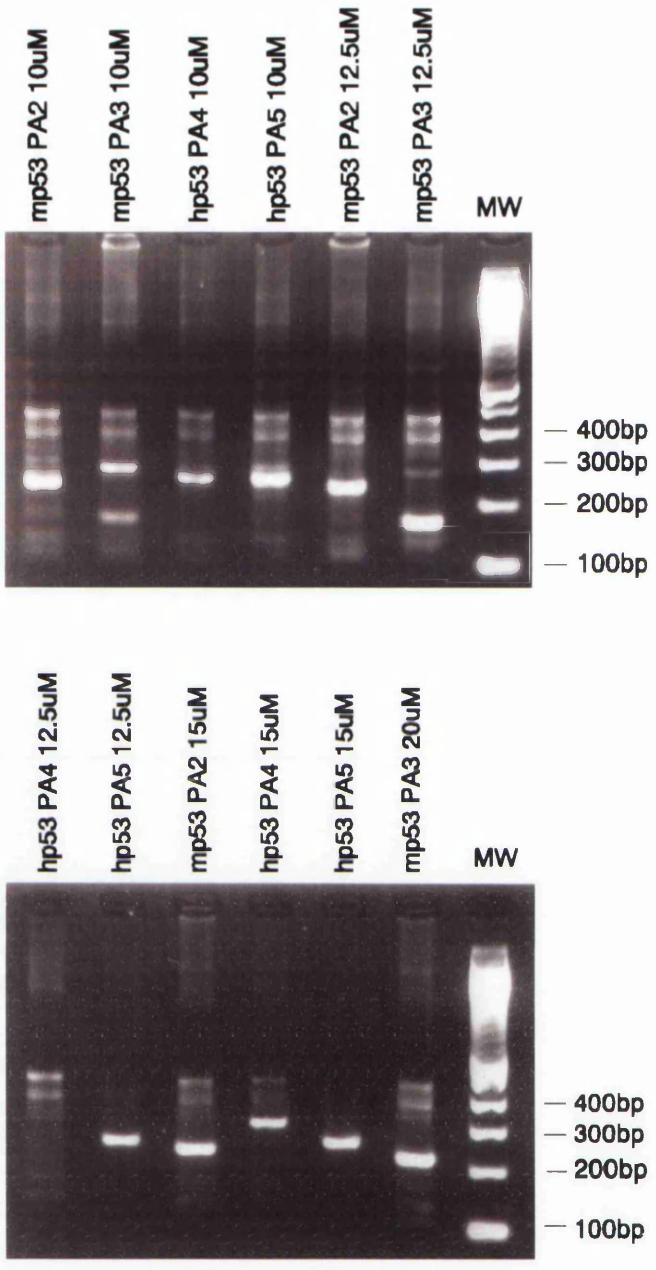


Figure 3.10.1 2% agarose gel separation of purified p53 GSE PCR products

MW	100bp ladder
mp53	GSE derived from murine p53
hp53	GSE derived from human p53
PAx	designated name
xuM	micromolar concentration of cisplatin selection carried out over 24 hours

Table 3.10.1

Pages 84-87 describe the homology determined by sequence alignment for the p53 GSE's described. Full details of the alignments are shown in the appendix.

Designation	Cisplatin Selection	Primer	Orientation	Identity to hump53t	Band Size On Agarose Gel (bp)	Overlap with p53 (bp)	Overlap with p53 (amino acids)	p53 Region
human p53 PA5 B1	15μM / 24hrs	H2693	antisense	72.7% in 22bp	280	983 - 1005	256 - 263	Conserved DNA binding domain. Covers LFS site
			sense	66.7% in 48bp		297 - 339	27 - 41	Covers MDM2 site
		H2694	antisense	77.3% in 22bp		982 - 1004	256 - 263	Conserved DNA binding domain. Covers LFS site.
			sense	60% in 45bp		600 - 644	128 - 143	Conserved DNA binding domain.

Designation	Cisplatin Selection	Primer	Orientation	Identity to hump53t	Band Size On Agarose Gel (bp)	Overlap with p53 (bp)	Overlap with p53 (amino acids)	p53 Region
human p53 PA4 B1	15μM / 24hrs	H2693	sense	93.4% in 121bp	320	1407 - 1528	downstream of translated region	NA
			antisense	78.3% in 23bp		1045 - 1068	277 - 285	C terminal end of conserved DNA binding domain
		H2694	sense	94.2% in 120bp		1408 - 1528	downstream of translated region	NA
			antisense	100% in 9bp		749 - 758	178 - 181	Conserved DNA binding domain, covers SV40 large T-Ag site

Designation	Cisplatin Selection	Primer	Orientation	Identity to hump53t	Band Size On Agarose Gel (bp)	Overlap with p53 (bp)	Overlap with p53 (amino acids)	p53 Region
murine p53 PA3 B1	20μM / 24hrs	H2693	antisense	59.4% in 32bp	230bp	1426 - 1456	downstream of translated region	NA
			sense	100% in 9bp		1240 - 1249	345 - 347	oligomerisation domain
		H2694	antisense	70.8% in 24bp		1235 - 1259	340 - 348	oligomerisation domain
			sense	76.9% in 13bp		448 - 461	78 - 82	transactivation domain

Designation	Cisplatin Selection	Primer	Orientation	Homology to hump53t	Band Size On Agarose Gel (bp)	Overlap with p53 (bp)	Overlap with p53 (amino acids)	p53 Region
murine p53 PA2B1	15μM / 24hrs	H2693	antisense	81.2% in 48bp	250	775 - 818	186 - 201	Conserved DNA binding domain
			sense	63.3% in 49bp		1617 - 1665	downstream of translated region	NA
		H2694	antisense	89.6% in 48bp		772 - 818	185 - 201	Conserved DNA binding domain
			sense	59.1% in 66bp		8 - 69	upstream of translated region	NA

Chapter 4

Chapter 4 Discussion

Although DNA damage is generally accepted as the chemotherapeutic target for cisplatin, the precise mechanisms by which cisplatin exerts its cytotoxic effects are not well characterised. As a result, it is difficult to ascertain all of the molecular mechanisms which can confer resistance to cisplatin although some have been identified (103). Many of the mechanisms so far identified are predicted to act in a dominant manner, that is, increased protein levels or activity results in increased drug resistance. The use of genetic suppressor elements offers a method with the potential to identify recessive mechanisms of drug resistance and may therefore elucidate novel mechanisms of resistance and help understand the exact mechanism of action of various drugs. Described in this study are two groups of GSE's, those generated by DNaseI digestion of an equalised total HeLa cDNA library i.e. the isolation of the cisplatin resistance GSE's and those generated by DNaseI digestion of a specific gene i.e. p53. Both groups have been isolated by cisplatin selection following introduction and retroviral expression in human cell lines.

The work presented here confirms the identity of a genetic suppressor element, designated GSE 7.10, as a 188bp fragment of the B-type isozyme of human phosphoglycerate mutase, an enzyme which is involved in the glycolytic pathway. This GSE was originally isolated as a consequence of selection for cisplatin resistance in HeLa cells and HT1080 cells after infection with the pLNCX~GSE 7.10 construct (94). GSE 7.10 has been shown, in this study, to confer resistance

to cisplatin in an additional cell line, the ovarian adenocarcinoma derived cell line, A2780. In addition, the response of the GSE 7.10 transfected A2780 lines to ionising radiation, another agent capable of causing DNA damage, has been investigated. The results of this aspect of the study suggest that GSE 7.10 can confer resistance to ionising radiation in the A2780 cell line. Assays designed to analyse the cell cycle characteristics of the GSE 7.10 transfectants in response to ionising radiation and cisplatin have also been carried out. They revealed the absence of a G1/S phase checkpoint in cells transfected with GSE 7.10 in response to ionising radiation in one line of transfectants (A2.22), whereas another line transfected with the same GSE (A2.14) displayed a more modest reduction in arrest capability. The response of the GSE 7.10 transfectants to cisplatin mirrored those of the response to ionising radiation, in that the A2.22 line showed a significant reduction in the ability to arrest at the G1/S checkpoint, whereas the A2.14 line appeared to have a fully functional arrest. The G2/M arrest was also examined in these assays. This arrest appears to be intact in response to ionising radiation, but the response to cisplatin treatment is less clear, as arrest appears to have been lost at the 6 hour time-point, but functional at 24 hours. These results require further confirmation.

Cisplatin and ionising radiation both result in DNA damage and this is thought to be the mechanism by which they exert their cytotoxic effects. One of the current models (see section 1.3.4) for the pathway which is initiated by DNA damage involves the elevation of p53 protein levels, in turn transactivating the WAF-1 gene, leading to cell cycle arrest. As the presence of GSE 7.10 results in a loss (or at least a reduction) of G1/S arrest capability, waf-1 RNA analysis is an important

experiment in attempting to identify a possible pathway by which GSE 7.10 may function. The waf-1 RNA levels seen in the preliminary Northern experiment presented do not confirm or rule out the involvement of the p53 pathway in the activity of the GSE, as a control cell line known to have reduced levels of waf-1 RNA, A2780cp70, showed no significant difference from the waf-1^+ line, A2780, from which it was derived. This type of analysis would also be interesting to carry out on RNA isolated from the GSE transfectants following treatment with cisplatin, when waf-1 would be transcriptionally induced if the p53 pathway were intact.

There is much evidence from Gudkov's work that the GSE approach is a powerful way of identifying recessive mechanisms of drug resistance as well as identifying important functional areas of proteins. It is anticipated that the isolation of cisplatin GSE's derived from the p53 gene will not only be of use in manipulating the response to cisplatin but also provide further insight into the functional domains of the p53 protein. The early data suggests that this approach works well as the great majority of active GSE's are derived from the p53 gene and not from randomly digested sections of the pLNCX vector. The four p53 GSE's sequenced here show various levels of homology with the human p53 protein, and further sub-cloning and selection of these GSE's should result in a clearer gel band i.e. indicating the presence of a single GSE and more accurate sequencing data.

To unequivocally confirm many of the results presented here, the use of an inducible expression system will be important. Many of the older inducible systems lack the tight on/off control of the tetracycline system which has been developed by Bujard and Gossen (95), hence the reason for favouring this system.

The work described here therefore confirms that GSE 7.10 confers resistance to cisplatin and ionising radiation and can result in the abrogation of cell cycle arrests induced by these agents. It does not however indicate the mechanism by which the GSE exerts these functions. The GSE acts in a sense orientation i.e. at the protein level, and is derived from the phosphoglycerate mutase-B gene. If the GSE were to be inhibiting the parental gene product i.e. the glycolytic enzyme, how could this produce changes in the response to DNA damage? It is widely acknowledged that diet can play an important role in the development of malignant disease. The role of glycolysis is to generate energy in the form of ATP by the conversion of glucose to pyruvate, as well as generating substrates for other biochemical processes. An important product of the respiratory pathway is NADH. A recent paper (104) describes a link between NAD and p53 expression. NAD is the main substrate of the enzyme PARP (polyADP ribose polymerase), and Whitacre *et al.* have demonstrated that a reduction in the available NAD pool or a reduction in PARP activity correlate with a reduction in p53 expression in response to DNA damage. Both p53 and PARP activities are known to increase in response to DNA damage, with the latter being induced within minutes of the damage occurring. This group have therefore hypothesised that PARP activity is in some way instrumental in the induction of p53 expression. If GSE 7.10 were blocking the activity of phosphoglycerate mutase-B, then the glycolytic pathway would be slowed so the availability of NAD may become a limiting factor in the induction of the p53 response. Examination of levels of NAD and polyADP ribose in the GSE transfectants would indicate whether it is altering the respiratory pathway.

As the GSE is acting at the protein level, it is possible that its inhibitory activity is promiscuous in that it can compromise the activity of another enzyme or protein. If the protein fragment were to fold in such a way that it mimicked the phosphorylation site of another protein, then it could compete for the action of the relevant kinase. Alternatively, if its conformation is such that it is able to bind the active site of an enzyme then it could alter activity in this way. There are, therefore a large number of possible mechanisms of action of the GSE which remain to be defined.

The use of the tetracycline inducible system will be important in confirming the results presented here particularly for the cell cycle assays. This system will also facilitate additional characterisation to be carried out on the GSE 7.10 transfectants. This GSE appears to confer the ability for cells to continue through the normal G1/S checkpoint following treatment with DNA damaging agents which normally induce this arrest. Apoptosis is an additional (or subsequent) event which has been shown to occur in response to DNA damage. FACS analysis of the GSE transfectants in response to cisplatin and ionising radiation treatment is a crucial experiment to carry out in order to examine the extent of the activity of the GSE in response to DNA damage. If the GSE is also capable of blocking the apoptotic response to DNA damage, it would be implicated in both of the central response pathways to DNA damage. The resistance, cell cycle and apoptosis assays would also be interesting to perform with cytotoxic agents which do not act via a DNA damage pathway e.g. taxol.

As the GSE is acting in a sense orientation i.e. at the protein level, it will be interesting to determine the proteins with which the GSE peptide interacts. There are a number of methods which could be used to identify such proteins, for example, immunoprecipitation assays potentially using the GSE as a GST fusion protein, the yeast two hybrid system and ELISA. Analysis of the primary sequence of the GSE may offer an insight into possible interactions as could a model of the putative secondary structure, prior to the interaction assays. Sequence motifs could reveal consensus sequences of potential binding sites for proteins involved in key cell cycle events or other biochemical processes. If the GSE were blocking the activity of the parental gene product, i.e. phosphoglycerate mutase-B, then overexpression of the parental gene may expose an increased sensitivity to cytotoxic agents.

The GSE method has been shown to be a powerful technique for identifying genes and regions of proteins important in a drug resistant phenotype. The identification of a fragment of the human phosphoglycerate mutase-B gene as a cisplatin GSE which also results in cell cycle peturbations in response to DNA damage potentially exposes a novel mechanism (or a new molecule) involved in the resistance to DNA damaging agents.

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Appendix : Sequence Alignment of p53-Derived Cisplatin GSE's

Sequence Alignment of Forward (primer h2693) and Reverse (primer H2694) Primed Strands of hp53 PA5 B1

a) 82.0% identity in 189 bp overlap

```
       219        209        199        189        179        169
H2693 ATCCACGCTGTTTTGACCTCCATAGAAGACAACGGGACCGATCCAGCCTCCGCGGCCCCA
                                          ||  ||  |||||||| ||||| |||||||
H2694 CCTTTGNGNTATACACGGCCCNGGCCCCCAAAAAGGTTCGATCCAGTCTCCGTGGCCCCA
               10         20         30         40         50         60

       159        149        139        129        119        109
H2693 AGCTTATGGATGGATGAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGT
       |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
H2694 CGCTTATGGATGGATGAGCACCGGCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGG
               70         80         90        100        110        120

         99         89         79         69         59         49
H2693 CTGCTGCCAGTGGCGATAAGTCGTGTCTGAGTGAGTGAATCGATCGGGGCCCAGATTTTA
       |||||||||||||||||||||| ||||||||||||||||||                 ||||||||
H2694 CTGCTGCCAGTGGCGATAAGTCGGGTCTGAGTGAGTGAATCGATAAAATAAAAGATTTTA
              130        140        150        160        170        180

         39         29         19          9
H2693 TTTAGTCTCCANATTTTGGGGCTGCTGAAAGCNNNNNNCNCA
       ||||||||||:|    ||||    |||||| ::::::|:
H2694 TTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCCACCTGTAGGTTTGGCAAGCTGCTA
              190        200        210        220        230        240
```

b) 100.0% identity in 8 bp overlap

```
                  10         20         30         40         50
H2693      TGNGNNNNNNGCTTTCAGCAGCCCCAAAATNTGGAGACTAAATAAAATCTGGGCCC
                                    ||||||||
H2694 CCTTTGNGNTATACACGGCCCNGGCCCCCAAAAAGGTTCGATCCAGTCTCCGTGGCCCCA
               10         20         30         40         50         60
```

Sequence Alignment of hp53 PA5 B1 (H2693) With Human p53t, Accession no. K03199

a) 72.7% identity in 22 bp overlap

```
              70         80         90        100        110        120
H2693 CGATTCACTCACTCAGACACGACTTATCGCCACTGGCAGCAGACACTGGTAACAGGATTA
                                       |||||| ||    || ||||||
hump53 TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGGTAATCTACTGG
              960        970        980        990       1000       1010
```

b) 66.7% identity in 48 bp overlap

```
             219        209        199        189        179        169
 H2693 GACGCCATCCACGCTGTTTTGACCTCCATAGAAGACAACGGGACCGATCCAGCCTCCGCG
                                          ||| |||||  |  |  |||  ||    ||
hump53 ACATTTTCAGACCTATGGAAACTACTTCCTGAAAACAAC--GTTCTGTCC--CCCTTGCC
         270        280        290        300          310          320

             159        149        139        129        119        109
 H2693 GCCCCAAGCTTATGGATGGATGAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTAC
       | |||||||  |||||||
hump53 GTCCCAAGC-AATGGATGATTTGATGCTGTCCCCGGACGATATTGAACAATGGTTCACTG
             330        340        350        360        370        380
```

Sequence Alignment of hp53 PA5 B1 (H2694) With Human p53t, Accession no. K03199

a) 77.3% identity in 22 bp overlap

```
        159        149        139        129        119        109
 H2694 CGATTCACTCACTCAGACCCGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTA
                                         ||||||  ||    |||  ||||||
hump53 TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGGTAATCTACTGG
             960        970        980        990       1000       1010
```

b) 60.0% identity in 45 bp overlap

```
             190        200        210        220        230
 H2694 TTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCCACCTGTAGGTTTGGCAAGCTG--C
                                        | ||  ||  |    |  ||||  ||  |  |||   |
hump53 GCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCC
       570        580        590        600        610        620

       240        250       259
 H2694 TAAACCCACCCNGGGANCTNG
        | |||  |||:| |
hump53 AAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGC
       630        640        650        660        670        680
```

Sequence Alignment of Forward (primer h2693) and Reverse (primer H2694) Primed Strands of hp53 PA4 B1

a) 82.7% identity in 220 bp overlap

```
     269       259       249       239       229       219
H2694 TGCCAAACCTACAGGTGGGGTCTTTCATTCCCCCCTTTTTCTGGAGACTAAATAAAATCT
                                        |||||  | | |:|||||||||||||| |||
H2963      AANAGGNCNGNNAATAANTNCCAGGGCCCCCAATATTTNGAGACTAAATAAATTCT
                  10        20        30        40        50

     209       199       189       179       169       159
H2694 TTTATTTTATCGATTCACTCANTCATGGNAGTCCTGGGTGCTTCTGACCCACACCTATTG
        :|||||||||||||||||:|||||||:|||||||||||| |||||||| ||||||||||||
H2963 GGCCNTTTATCGATTCACTCACTCATGGAAGTCCTGGGTGATTCTGACGCACACCTATTG
        60        70        80        90        100       110

     149       139       129       119       109       99
H2964 CAANCAAGGGTTCAAAGACCCAAAATCCATAATGCCAGGGGAGGGAGAGATGGGGGTGGG
      |||:|||||||||||||||||||| | |||  ||| ||||||||||||||||| |||||||
H2963 CAAGCAAGGGTTCAAAGACCCAAGACCCAATATGGCAGGGGAGGGAGAGATTGGGGTGGC
       120       130       140       150       160       170

      89        79        69        59        49        39
H2964 AGACTGTCAGTGGGGAACAAGAAGCATCTATCCATGAGCGTGGGACCACGGAGGCTGAAT
      || ||| |||||||||||||  ||| ||| |||||| ||| |||| || |||||||||| ||
H2963 AGGCTGCCAGTGGGGAACAGTAAGTATCCATCCATAAGCTTGGGGCCGCGGAGGCTGGAT
       180       190       200       210       220       230

      29        19        9
H2964 CGAAANCGGTCGGGGGCCCTTAAGNCNNTT
      ||   :||||
H2963 CGGTCCCGGTGTCTTCTATGGAGGTCAAAACAGCGTGGATGGCGTCTCAAGGG
       240       250       260       270       280
```

b) 63.4% identity in 41 bp overlap

```
         20        30        40        50        60        70
H2964 CCCCGACCGNTTTCGATTCAGCCTCCGTGGTCCCACGCTCATGGATAGATGCTTCTTGTT
                                        || | | |||||||| || | ||    || |
H2963 AGACTAAATAAATTCTGGCCNTTTATCGATTCACTCACTCATGGA-AG-TCCTGGGTGAT
               50        60        70        80        90

         80        90        100       110       120       130
H2694 CCCCACTGACAGTCTCCCACCCCCATCTCTCCCTCCCCTGGCATTATGGATTTTGGGTCT
       |  ||  |||
H2693 TCTGACGCACACCTATTGCAAGCAAGGGTTCAAAGACCCAAGACCCAATATGGCAGGGGA
      100       110       120       130       140       150
```

Sequence Alignment of hp53 PA4 B1 (H2693) With Human p53t, Accession no. K03199

a) 93.4% identity in 121 bp overlap

```
        229         219         209         199         189         179
H2693   GCCTCCGCGGCCCCAAGCTTATGGATGGATACTTACTGTTCCCCACTGGCAGCCTGCCAC
                                            ||||  ||||||||||||  ||||||  ||||
hump53  AGAAGGGCCTGACTCAGACTGACATTCTCCACTTCTTGTTCCCCACTGACAGCCTCCCAC
          1380        1390        1400        1410        1420        1430

        169         159         149         139         129         119
H2693   CCCAATCTCTCCCTCCCCTGCCATATTGGGTCTTGGGTCTTTGAACCCTTGCTTGCAATA
        |||  ||||||||||||||||||||  ||||||  ||||||||||||||||||||||||||||
hump53  CCCCATCTCTCCCTCCCCTGCCATTTTGGGTTTTGGGTCTTTGAACCCTTGCTTGCAATA
          1440        1450        1460        1470        1480        1490

        109         99          89          79          69          59
H2693   GGTGTGCGTCAGAATCACCCAGGACTTCCATGAGTGAGTGAATCGATAAANGGCCAGAAT
        ||||||||||||||  ||||||||||||||||
hump53  GGTGTGCGTCAGAAGCACCCAGGACTTCCATTTGCTTTGTCCCGGGGCTCCACTGAACAA
          1500        1510        1520        1530        1540        1550
```

b) 78.3% identity in 23 bp overlap

```
          60          70          80          90          100         110
H2693   GGCCNTTTATCGATTCACTCACTCATGGAAGTCCTGGGTGATTCTGACGCACACCTATTG
                                            ||||||||  ||  |  |  ||||||
hump53  GGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAG
          1020        1030        1040        1050        1060        1070
```

Sequence Alignment of hp53 PA4 B1 (H2694) With Human p53t, Accession no. K03199

a) 94.2% identity in 120 bp overlap

```
          40          50          60          70          80          90
H2694   CCTCCGTGGTCCCACGCTCATGGATAGATGCTTCTTGTTCCCCACTGACAGTCTCCCACC
                                            ||||||||||||||||||||||  ||||||||
hump53  GAAGGGCCTGACTCAGACTGACATTCTCCACTTCTTGTTCCCCACTGACAGCCTCCCACC
          1380        1390        1400        1410        1420        1430

          100         110         120         130         140         150
H2694   CCCATCTCTCCCTCCCCTGGCATTATGGATTTTGGGTCTTTGAACCCTTGNTTGCAATAG
        ||||||||||||||||||  ||||  |||  ||||||||||||||||||||:|||||||||
hump53  CCCATCTCTCCCTCCCCTGCCATTTTGGGTTTTGGGTCTTTGAACCCTTGCTTGCAATAG
          1440        1450        1460        1470        1480        1490

          160         170         180         190         200         210
H2694   GTGTGGGTCAGAAGCACCCAGGACTNCCATGANTGAGTGAATCGATAAAATAAAAGATTT
        |||||  ||||||||||||||||||:||||
hump53  GTGTGCGTCAGAAGCACCCAGGACTTCCATTTGCTTTGTCCCGGGGCTCCACTGAACAAG
          1500        1510        1520        1530        1540        1550
```

b) 100.0% identity in 9 bp overlap

```
      89        79        69        59        49        39        30
H2694 GACTGTCAGTGGGGAACAAGAAGCATCTATCCATGAGCGTGGGACCACGGAGGCTGAATC
                                          |||||||||
hump53 CATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCT
       720       730       740       750       760       770

      29        19         9
H2694 GAAANCGGTCGGGGGCCCTTAAGNCNNTT

hump53 GGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGA
       780       790       800       810       820       830
```

Sequence Alignment of Forward (primer h2693) and Reverse (primer H2694) Primed Strands of mp53 PA3 B1

a) 69.5% identity in 141 bp overlap

```
    199       189       179       169       159       149       140
H2693 CACCAACAACCCTGTTTTGACCTCCATAAAACACCGGGGCCGATCCAACCTCCTCGCCTC
                                        ||| | : :  ||||||  |||||  |   |
H2694              GAAAGANCTTCATTGCGNCCACAACANTNTTGATCCAGTCTCCTTGGTCC
                          10        20        30        40        50

    139       129       119       109        99        89        80
H2693 CAAGCTTATGGATGGATGCATTCAGCTCCGGTTCCCACCGATCAAGGCGAGTTACATGAT
      ||  |||||||||||||||:|||||||||||||| :|||| |||||||||  ||||||||:|
H2694 CACGCTTATGGATGGATNCATTCAGCTCCGGCNCCCAACGATCAAGGGGAGTTACATNAG
                60        70        80        90       100       110

     79        69        59        49        39        29        20
H2693 TGATTGATTCGATCAGGGTCCAGGACCCTAATTATTCTCCAANNTTAAGGGNNCGGGNNC
      |||  |||  |||  |  |   | :|  ||   |  |   ||  ||||||  ::   |  |||:
H2694 TGAGTGAATCGCT-AACATANAAGATTCGATCTAGTCTCCAGAAAAAGGGGGGAATGAAA
              120       130       140       150       160
```

75.0% identity in 16 bp overlap

```
          90        100       110       120       130       140
H2693 TAACTCGCCTTGATCGGTGGGAACCGGAGCTGAATGCATCCATCCATAAGCTTGGAGGCG
                                          ||  |||  ||:|||  ||
H2694 NTTGATCCAGTCTCCTTGGTCCCACGCTTATGGATGGATNCATTCAGCTCCGGCNCCCAA
      30        40        50        60        70        80
```

Sequence Alignment of mp53 PA3 B1 (H2693) With Human p53t, Accession no. K03199

a) 59.4% identity in 32 bp overlap

```
      280        290        300        310        320        330
H2693 CNTCGTTNTTNCTGNAAACAAAATNAGNTCCTGCATNCNANCNNCA-CNCCCCCACCCCC
                                          | ||  |:|:|:|::||  |:|  |||  ||||
hump53 TGACATTCTCCACTTCTTGTTCCCCACTGACAGCCTCCCACCCCCATCTCTCCCTCCCCT
          1400       1410       1420       1430       1440       1450

       340
H2693 NCAGCTCTCG
       :|
hump53 GCCATTTTGGGTTTTGGGTCTTTGAACCCTTGCTTGCAATAGGTGTGCGTCAGAAGCACC
          1460       1470       1480       1490       1500       1510
```

b) 100.0% identity in 9 bp overlap

```
                                    349        339        329        320
H2693                                 CGAGAGCTGNGGGGGTGGGGGNGTGNNGNT
                                      |||||||||:
hump53 ATCCGTGGGCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAG
       1210       1220       1230       1240       1250       1260
```

Sequence Alignment of mp53 PA3 B1 (H2694) With Human p53t, Accession no. K03199

a) 70.8% identity in 24 bp overlap

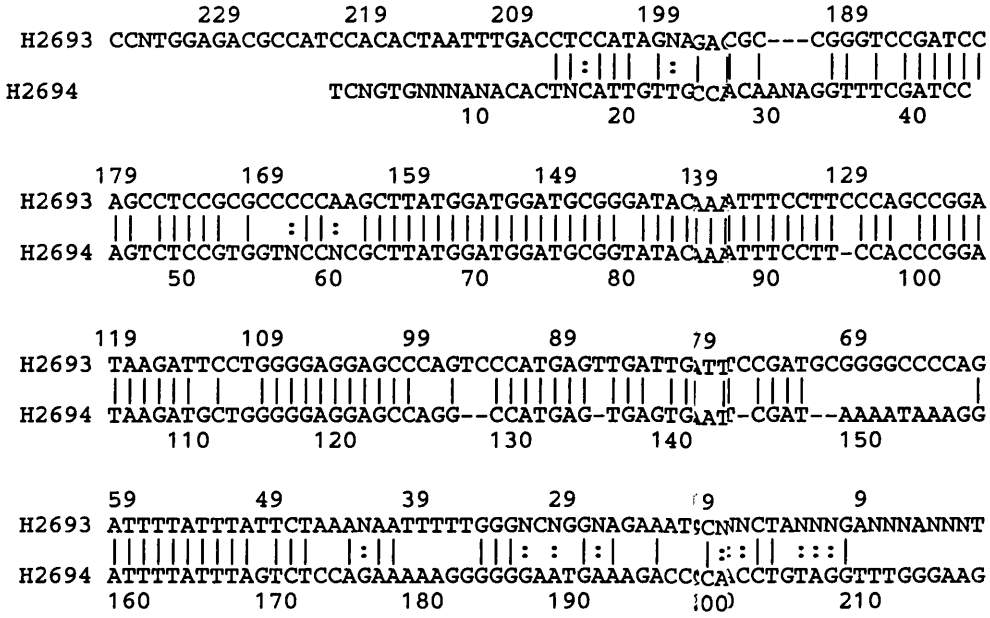
```
          109        99         89         79         69         59
H2694 CTCACTNATGTAACTCCCCTTGATCGTTGGGNGCCG-GAGCTGAATGNATCCATCCATAA
                                        |: ||| ||||||||||:  || |
hump53 TCAGATCCGTGGGCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACT
          1210       1220       1230       1240       1250       1260
```

b) 76.9% identity in 13 bp overlap

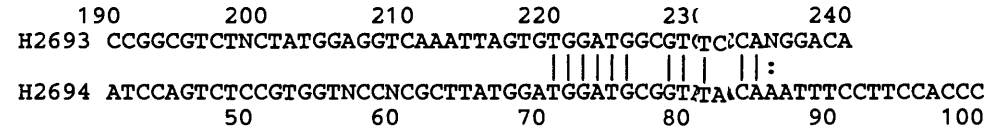
```
            50         60         70         80         90         100
H2694 CTTGGTCCCACGCTTATGGATGGATNCATTCAGCTCCGGCNCCCAACGATCAAGGGGAGT
                                      |||||||  |:||
hump53 AGGCTGCTCCCCCCGTGGCCCCTGCACCAGCAGCTCCTACACCGGCGGCCCCTGCACCAG
        420        430        440        450        460        470
```

Sequence Alignment of Forward (primer h2693) and Reverse (primer H2694) Primed Strands of mp53 PA2 B1

a) 65.7% identity in 201 bp overlap

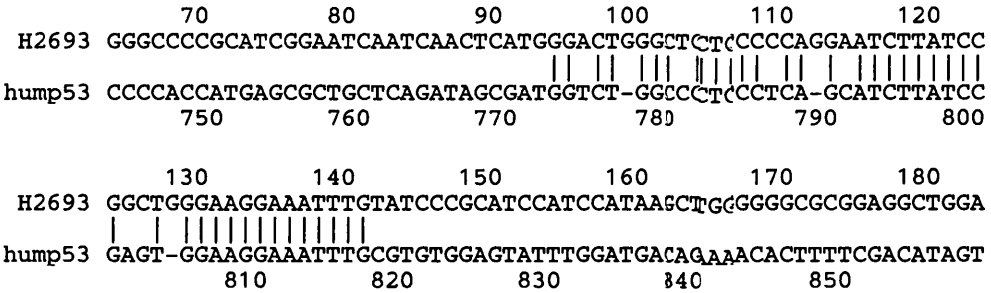


b) 73.3% identity in 15 bp overlap



Sequence Alignment of mp53 PA2 B1 (H2693) With Human p53t, Accession no. K03199

a) 81.2% identity in 48 bp overlap



b) 63.3% identity in 49 bp overlap

```
         89        79        69        59              49        39
 H2693 ATGAGTTGATTGATTCCGATGCGGGGCCCCAGATTTTA-----TTTATTCTAAANAATTT
                                        ||||||||     |||| | | | : || |
hump53 AGGAGGATGGGGAGTAGGACATACCAGCTTAGATTTTAAGGTTTTTACTGTGAGGGATGT
         1590      1600      1610      1620      1630      1640

                29        19        9
 H2693 TTGGG-NCNGGNAGAAATGCNNCTANNNGANNNANNNT
       ||||| : :| :|||||||
hump53 TTGGGAGATGTAAGAAATGTTCTTGCAGTTAAGGGTTAGTTTACAATCAGCCACATTCTA
         1650      1660      1670      1680      1690      1700
```

Sequence Alignment of mp53 PA2 B1 (H2694) With Human p53t, Accession no. K03199

a) 89.6% identity in 48 bp overlap

```
       159       149       139       129       119       109
 H2694 AAATCCTTTATTTTATCGATTCACTCACTCATGGCCTGGCTCCTCCCCCAGCATCTTATC
                                        |||| ||||| ||| || ||||||||||||
hump53 GCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGGCCCCT-CCTCAGCATCTTATC
              750       760       770       780       790

        99        89        79        69        59        49
 H2694 CGGGTGGAAGGAAATTTGTATACCGCATCCATCCATAAGCGNGGNACCACGGAGACTGGA
       || ||||||||||||||||
hump53 CGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGT
     800       810       820       830       840       850
```

b) 59.1% identity in 66 bp overlap

```
              70        80        90       100       110       120
 H2694 CGCTTATGGATGGATGCGGTATACAAATTTCCTTCCACCCGGATAAGATGCTGGGGGAGG
                                  | ||||||||    |||||    ||  |
hump53                          GTCGACCCTTTCCACCCCTGGAAGAT----GGAAATA
                                      10        20            30

             130       140       150       160       170       179
 H2694 AGCCAGGCCATGAGT-GAGTGAATCGATAAAATAAAGGATTTTATTTAGTCTCCAGAAAA
       | || |     || || |||||     || |||| |||
hump53 AACCTGCGTGTGGGTGGAGTGTTAGGACAAAAAAAAAAAAAAAAAAGTCTAGAGCCACCG
            40        50        60        70        80        90
```