DEFECTIVE MISMATCH REPAIR AND CISPLATIN RESISTANCE IN OVARIAN CANCER CELLS

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For Lindsay

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To all my teachers;
past, present and future.

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ABBREVIATIONS

°C	Degrees centigrade
6TG	6-Thio-Guanine
A,T,G,C	Adenine, Thymine, Cytosine, Guanine
AAF	N-2-Acetyl-2-aminofluorene
ABC	Binding Cassette
atMDR	Atypical Multi-drug Resistance
ANLL	Acute non-lymphoblastic leukaemia
APRT	Adenosine phosphoribosyltransferase
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CNU	Chloro-nitosourea
DEPC	Diethyl polycarbonate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamminetetra-acetic acid
FACS	Fluoresence activated cell sorting
FITC	Fluorescein 5-isothiocyanate-conjugated
GTBP	G-T binding protein (hMSH6)
GSH	Glutathione
GST	Glutathione-S-Transferase
HRPL	Horseradishperoxidase-linked
HMG	High mobility group
HNPCC	Hereditary non-polyposis colorectal cancer
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
kDa	Kilodalton
LET	Linear energy transfer
LoH	Loss of heterozygosity
mA	milliamperes

M	Molar
m-AMSA	4'-(9-acridylamino)methanesulphon-m-aniside
MDR	Multi-drug resistance
MGMT	O^6-Methylguanine-DNA methyltransferase
MNNG	N-Methyl-N^1-nitro-N-nitrosoguanidine
MNU	N-Methyl-N-nitrosourea
mRNA	Messenger ribonucleic acid
MRP	Multi-drug resistance related protein
MT	Metallothionein
NER	Nucleotide excision repair
NSCLC	Non-small cell lung cancer
OD_{260}	Optical density at 260nm wavelength
O^6 MeG	O^6-Methylguanine
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PKC	Protein kinase C
PI	Propidium Iodide
RER	Replication error
SCLC	Small cell lung cancer
TGFβ	Transforming growth factor β
Topo I, II	Topoisomerase I or II
UV	Ultraviolet
V	Volts
XP	Xeroderma pigmentosum
XPA	Xeroderma pigmentosum complementation group A
XPG	Xeroderma pigmentosum complementation group G

ACKNOWLEDGEMENTS

ABSTRACT

The development of resistance to anticancer drugs poses one of the major obstacles to improving the survival from malignant disease. Most clinically effective anticancer agents act upon DNA. Resistance has been shown to arise by mechanisms that reduce the amount of drug reaching the DNA. However, drug resistance developing through alterations in the capacity of tumour cells to repair or respond to DNA damage, may be as important.

Microsatellite instability (RER+) is generally taken as an indication of defective DNA mismatch repair. Ovarian and breast carcinoma cell lines, selected for resistance to cisplatin and doxorubicin, were shown to display an RER+ phenotype as shown by multiple mutations at microsatellite loci in comparison to the parental line. The ovarian carcinoma cell line A2780 was shown to be RER– by microsatellite analysis of random sub-clones from the cell line. A2780/cp70, a cisplatin resistant cell line derived from A2780, displayed an RER+ phenotype when subject to the same analysis.

Ten independently derived, cisplatin resistant, A2780 cell lines were developed by selection with increasing concentrations of cisplatin to a final concentration of 15µM. These were shown to display stable resistance to cisplatin of between 4 fold (as determined by clonogenic assay) and 7 fold (as determined by MTT assay), when compared to the parental line. The RER+ phenotype was detected in all but one of these lines. An association between the RER+ phenotype and cisplatin resistance was further strengthened by the finding of microsatellite instability in resistant A2780 cell lines selected by a single exposure to 15µM cisplatin.

Mutations in genes involved in mismatch repair have been shown to be responsible for microsatellite instability. The biochemical basis of the RER+ phenotype in drug resistant A2780 derived cell lines was determined by *in vitro* repair assay. An inability to correct DNA mismatches, when repair was directed from 3' to the lesion, was observed, and hMutLα was identified as the defective component of mismatch repair by *in vitro* complementation. Further studies revealed the absence of hMLH1 and hPMS2 proteins, which constitute hMutLα, within the RER+ resistant cell lines. This arises from the failure of these cells to express hMLH1 mRNA. No defect in the expression of the other known mismatch repair genes was observed.

P53-dependent DNA damage responses were characterised in the RER+, drug resistant cell lines. A correlation was observed between RER+ status and loss of radiation induced G1 arrest, reduced expression of the CIP 1 gene and decreased levels of apoptosis after cisplatin exposure. This association between DNA mismatch repair and the DNA damage response pathway suggests that the mismatch repair system might act as a sensor for DNA damage. This supports the hypothesis that mismatch repair may be involved in determining the cellular sensitivity to cisplatin; defects in such repair leading to cisplatin resistance.

CHAPTER 1

GENERAL INTRODUCTION

1.1 CLINICAL DRUG RESISTANCE

The use of drugs to treat cancer has only been a reality over the past half-century since the introduction of the nitrogen mustards in the 1940's (Rhoads, 1946). During this period of time chemotherapeutic agents have brought about a number of notable success in the treatment of certain forms of cancer. Tumours such as malignant teratoma of the testis and childhood acute lymphoblastic leukaemia, which were once uniformly fatal, are now considered potentially curable in the majority of cases (Poplack et al. 1995). These few successes apart, and in spite of the enormous effort in terms of money and manpower to develop new forms of therapy, the majority of patients who develop a malignancy in the 1990's will eventually die from their disease.

There are many factors which underlie this failure to improve the prognosis associated with most forms of cancer. Poor public health awareness, inadequate screening tests and regional variations in treatment all help determine the likelihood of overall survival from cancer. However, one of the greatest obstacles to improving the outlook for patients with malignant disease is the inability of current therapies to permanently eradicate them of tumour. The innate response of tumours on exposure to chemotherapeutic drugs ranges from exquisite sensitivity to obstinate resistance. Unfortunately, tumours which initially show sensitivity to a particular therapeutic agent will often recur displaying resistance not only to that agent but often to a broader range of anti-cancer drugs. Understanding the molecular mechanisms that underlie acquisition of drug resistance and formulating new therapies to counteract its effects represents one of the fundamental challenges to oncologists.

1.2   TUMOUR HETEROGENEITY AND DRUG RESISTANCE.

There have been many different hypotheses advanced to account for the mechanism of treatment failure during cancer chemotherapy. These have included differences in the kinetics of cell growth between tumours (Shackney et al. 1978) and variations in the penetration of effective concentrations of chemotherapeutic agents in to tumours (Hanna et al. 1983). It is now widely accepted, however, that the ability of tumours to develop resistance to a wide variety of different anti-cancer agents derives from extensive phenotypic heterogeneity within the constituent cells (Dexter and Leith, 1986) This heterogeneity arises as a result of both the cellular genetic and phenotypic instability which characterise the growth of tumours (Nowell, 1976). It is assumed that within a tumour there exist many cells with different phenotypic characteristics which give a survival advantage under specific conditions. The selective pressures exerted by exposure to chemotherapeutic drugs, which are often mutagenic themselves, result in survival of cells which continue to develop further heterogeneity, and most likely resistance, as they grow during tumour progression. Using mathematical models it has been calculated that larger tumours display more heterogeneity, as do more slowly growing tumours, and are therefore more likely to display resistance to therapy (Goldie and Coldman, 1984) This is, generally, what is observed clinically.

Much of the work that has characterised drug resistance in tumours to date has revealed mechanisms of resistance whereby cells reduce the intracellular accumulation of active drug. It has therefore been assumed that by maximising the delivery of drug to its intracellular target the problem of drug resistance could be overcome (Hryniuk, 1995). This has formed the basis of numerous trials of high dose chemotherapy in the belief that increases in the extracellular dose of drug could overcome mechanisms which reduce intracellular accumulation. However, the results of these trials often show an initial benefit of high dose, over standard dose, treatment but with the eventual relapse of resistant tumour (Souhami, 1995). This lack of improvement in efficacy, despite better

drug delivery to the intracellular target, has suggested that mechanisms of drug resistance acting after the drug/target interaction may have a relatively greater clinical significance. It is generally assumed that DNA is the primary target for the action of cytotoxic anticancer agents. Advances in our understanding of DNA repair mechanisms has allowed determination of whether they play a role in the resistance to chemotherapeutic drugs. Of equal importance are the multiple pathways that interact after DNA damage to bring about death of the cell. It is becoming increasingly clear that there exist many points in these post-damage pathways where resistance to anti-cancer agents could arise.

Therefore one can broadly define three general areas in which cellular drug resistance can arise;

        (1).   Pre-target resistance.

        (2).   Drug/Target interactions and damage repair.

        (3).   Post-damage resistance.

The data presented in this thesis uses cell lines selected for resistance to the clinically successful anti-cancer drugs cis-diamminedichloroplatinum(II) (cisplatin) and doxorubicin. The following summary of the mechanisms by which cells develop resistance to anti-cancer drugs will, in general, focus on mechanisms relevant to these two agents.

1.3   MECHANISMS OF DRUG RESISTANCE IN TUMOUR CELLS OCCURRING PRIOR TO DRUG/TARGET INTERACTION.

1.3.1   Reduced intracellular accumulation of drug by decreased drug influx.

It would appear logical that if a tumour cell could reduce the amount of cytotoxic drug reaching the intracellular compartment for a given extracellular concentration of drug then it would display a greater degree of resistance. In the case of cisplatin it has been shown by various authors that *in vitro* tumour sub-lines, derived by exposure to increasing concentrations of drug, accumulate less cisplatin than the cells from which they have been derived (Andrews and Howell, 1990).

The mechanisms by which cisplatin enters the cell are still unclear. It would appear that cisplatin can enter the cell by a process of passive diffusion (Gale et al. 1973) but evidence has been presented that active transport of cisplatin can also occur. Other factors which may affect the active uptake of cisplatin in resistant cell lines include alterations in the membrane potential (Andrews and Albright, 1992) and altered signal transduction e.g. in the protein kinase A (Mann et al. 1991) or Ca^{2+}/Calmodulin pathways (Kikuchi et al. 1990). To tie all the data together a model has been proposed which suggests that the initial uptake of cisplatin into the cell occurs both by passive diffusion and by active transport through some form of gated channel (Gately and Howell, 1993). Characterisation of a number of gated channels suggests that flux through the channel can be regulated by alterations in membrane potential or by phosphorylation initiated by signal transduction pathways (Gately and Howell, 1993). Mutations arising in the protein could alter the rate of flux or specificity for substrate leading to a reduction in cisplatin uptake.

Evidence that gated channels may play some role in determining cisplatin sensitivity has come from comparing levels of specific membrane proteins in sensitive and resistant cell lines (Bernal et al. 1990). Sharp et al have shown overexpression of a 36kDa membrane protein in 41McisR6, a cisplatin resistant ovarian carcinoma cell line, this being associated with loss of sensitivity to inhibitors of active transport (Sharp et al. 1995). It has been shown that reduced cisplatin accumulation is probably one of the major determinants of resistance in cell lines such as 41McisR6 (Sharp et al. 1995).

However, the contribution that reduced accumulation plays in the development of cisplatin resistance *in vivo* remains unclear.

1.3.2 Reduced intracellular accumulation of drug by increased drug efflux.

Perhaps the most thoroughly characterised of all mechanisms of drug resistance to date have been those that increase the excretion of drug from the intracellular compartment. Clinical experience indicates that tumours which develop resistance after treatment with particular chemotherapeutic agents are also resistant to a range of agents to which they have never been exposed (Nooter and Herweijer, 1991). This phenomenon is referred to as multi-drug resistance (MDR). The anticancer drugs to which the MDR phenotype is observed share characteristics such as being lipophilic and of natural origin and include doxorubicin but not cisplatin. Cell lines which display an MDR phenotype can be derived, *in vitro*, by selection with drugs such as doxorubicin or vinblastine. It has been shown that such cells have a reduced ability to accumulate drugs, compared to the parent line, and that this is likely to be the main cause of the MDR phenotype (Dano, 1973). It has since been determined that altered expression of a specific energy dependent, unidirectional, drug efflux pump is responsible for the reduction in intracellular drug accumulation in these cells (Willingham et al. 1986). This is a 170kDa transmembrane glycoprotein named P-glycoprotein (Kartner et al. 1985).

That expression of P-glycoprotein can result in the development of multi-drug resistance *in vitro* has been proven by experiments in which the MDR1 gene was transfected into drug sensitive cells. This resulted in the development of an MDR phenotype in these cells without any prior exposure to anticancer drugs (Ueda et al. 1987). Despite such evidence the significance of the expression of P-glycoprotein in tumours is less clear. In general, tumours from tissues with high basal levels of MDR1

also display high expression of the gene. Such tumours (colon, pancreas, kidney etc.) are often intrinsically resistant to a wide range of anticancer drugs, showing a low response rate to chemotherapy. The increased expression of P-glycoprotein in resistant tumours can arise through enhanced gene transcription or by post-transcriptional stabilisation of MDR1 mRNA (Marino et al. 1990). Transcription can be influenced by genes such as p53 (Chin et al. 1992b).

One of the problems, however, in correlating the significance of P-glycoprotein expression and *in vivo* MDR is that it is unclear as to what level of P-glycoprotein expression, and in how many cells, is required to produce a resistant phenotype. A relationship has been shown *in vitro* between the expression of MDR1 and sensitivity to MDR related drugs for breast cancer biopsies (Keith et al. 1990). There has also been some recent evidence that in certain tumours such as neuroblastomas and soft tissue sarcomas high expression of MDR1 in the untreated tumour is associated with poor prognosis (Chan et al. 1990; Bourhis et al. 1989).

P-glycoprotein is not the only cell membrane associated protein involved in the development of multi-drug resistance by increased drug efflux. Another protein that belongs to the ATP binding cassette (ABC) transporter superfamily of proteins is MDR related protein (MRP). Gene amplification is commonly responsible for MRP over-expression in tumour cell lines selected for resistance by exposure to doxorubicin or etoposide (Cole et al. 1992). Transfection experiments using the MRP gene have shown that its expression *in vitro* can result in resistance to classical MDR type drugs such as doxorubicin and daunorubicin. There is little resistance to non-MDR drugs such as taxol or cisplatin (Zaman et al. 1994). Although the biochemistry of MRP action remains unclear it appears that not only may it pump drugs out of the cytoplasm but it may sequester drug by transportation into cytoplasmic vesicles (Cole et al. 1992). These vesicles can then be removed from the cell by exocytosis. As yet the role that MRP plays in the development of clinically relevant multi-drug resistance is unclear (Broxterman et al. 1995).

1.3.3. Altered intracellular drug metabolism.

Many anti-cancer drugs require biochemical modification within cells before becoming active. Intracellular enzymatic systems can also modify the drugs resulting in detoxification and excretion. Innate or acquired differences in the activities of such systems can result in the expression of a drug resistant phenotype.

The cytochrome P450 family of enzymes play a crucial role in the biotransformation of many drugs including anti-cancer drugs. Such biotransformation can either involve activation or inactivation of a drug. The levels of specific cytochromes P450 in an individual depend on factors such as sex, age, genetic polymorphism etc. Each of these factors could alter the specific metabolism of an anti-cancer agent resulting in relative resistance to the drug (Doehmer et al. 1993). Cyclophosphamide is an example of an anticancer agent that requires activation via the cytochrome P450 pathway (Clarke and Waxman, 1989).

Cells also possess a drug metabolism system based on the important intracellular thiol, Glutathione (GSH). GSH plays a role in decreasing the toxicity of certain drugs by combining to form glutathione-drug conjugates (catalysed by a family of Glutathione-S-transferase enzymes) which have reduced activity and are excreted in the urine or bile. GSH and other components of its metabolic pathway can also interact with and detoxify reactive oxygen species that arise spontaneously within the cell or as a result of the action of certain cytotoxic drugs. Such reactive oxygen molecules are highly toxic to the cell. There is evidence that the ability of a cell to maintain active GSH levels in the face of a toxic challenge may influence its ability to survive and hence its resistance (Meijer et al. 1990).

The clinical significance of the role of GSH in determining drug resistance is unclear. Elevated intracellular levels of GSH have been observed in many *in vitro* tumour cell lines with a good correlation to cisplatin resistance being observed in a few of these

(Mistry et al. 1990). In other cells it may be that levels of GSH within certain intracellular compartments, e.g. nuclear GSH, correlate more closely with cisplatin resistance (Meijer et al. 1990). Intranuclear GSH may be important in binding to monofunctional cisplatin/DNA adducts preventing formation of more toxic bifunctional forms. (Eastman, 1987a). *In vitro* studies have shown that intranuclear GSH levels do correlate with reductions in platinated DNA (Meijer et al. 1990).

Recently further evidence of an important role for GSH in the detoxification of cisplatin has arisen. It has been shown that GSH/cisplatin complexes within the cell can be actively transported across cell membranes by an ATP dependent glutathione S-conjugate export (GS-X) pump (Ishikawa and Aliosman, 1993). Doxorubicin and daunorubicin GSH conjugates may also be excreted by this pump (Ishikawa et al. 1995). It has been shown that over-expression of multidrug resistance-associated protein (MRP) results in an increased activity of the GS-X pump and that both proteins may in fact be the same (Muller et al. 1994)

The metallothioneins (MT) are a group of cysteine rich proteins which act as nucleophiles towards electrophilic agents such as cisplatin and may be involved in their detoxification. There is conflicting *in vitro* evidence as to the significance of MT in cisplatin resistance. Some studies show over-expression of MT mRNA and increased protein levels in a panel of human cell lines with acquired resistance to cisplatin (Kelley et al. 1988). In a panel of cisplatin resistant ovarian cancer cell lines, however, no correlation between MT mRNA over expression and cisplatin resistance was observed (Schilder et al. 1990). Therefore, although MT may be associated with induction of cisplatin resistance its causal role remains to be established.

1.3.4. Alterations in signal transduction pathways associated with drug resistance.

The intracellular signalling mechanisms which co-ordinate the vast array of cellular functions, although extensively investigated for their role in cell transformation, may also influence the sensitivity of cells to anticancer drugs. Cancer chemotherapeutic agents have been shown to disrupt these signal transduction pathways which may contribute to the evolution of drug resistant clones (Brunton and Workman, 1993).

It has been proposed that the substrate specificity of, and/or the rate of efflux through, P-glycoprotein can be controlled by phosphorylation of specific amino acids within the protein (Chin et al. 1992a; Chin et al. 1990a). Certain isoforms of protein kinase C (PKCα and β) have been shown to be elevated in cell lines with either intrinsic (Dolfini et al. 1993) or acquired resistance to doxorubicin and other drugs (Lee et al. 1992). A PKC mediated signal pathway has also been implicated in determining the sensitivity of cervical and ovarian carcinoma cells to cisplatin (Isonishi et al. 1990; Basu and Evans, 1994).

Transcriptional regulation of specific genes is the final step in many signal transduction pathways. It has been suggested that transcription of the MDR gene may be influenced by binding of the AP-1 complex, consisting of activated c-fos and c-jun (Volm, 1993; Ransone and Verma, 1990). There is evidence to show that cells with an MDR phenotype over-express c-fos and that cells transfected with and over-expressing c-fos display MDR. Also reduction of c-fos expression led to reversal of an MDR phenotype in actinomycin D resistant ovarian carcinoma cells (Scanlon et al. 1994). Other oncogenes which are involved in signal transduction pathways have also been shown to be altered in drug resistant cell lines *in vitro* (Kellen, 1994).

As yet the significance of differences in signal transduction pathways in the development of drug resistance in the clinical setting is completely unknown.

1.3.5. Drug resistance arising through alterations in target associated proteins.

The target of the majority of the clinically useful anticancer agents is DNA, although in some cases this interaction is mediated through DNA associated proteins. DNA topoisomerases (I & II) are enzymes involved in altering the tertiary structure of DNA to allow processes such as transcription and replication. The DNA topoisomerases act by generating breaks in the phosphodiester backbone of the DNA molecule (single strand for topo I and double strand for topo II) which allows passage of DNA strands through the gaps thus altering the topology of the DNA. The topoisomerases form transient covalent intermediates, called cleavable complexes, with the free ends of the DNA thus stabilising the strand break. The topoisomerases are the targets for a number of clinically useful anticancer agents. The main mechanism of action of these drugs is through binding to the topoisomerase protein, whilst covalently bound to the DNA, resulting in stabilisation of the cleavable complex. This halts the catalytic reaction prior to the re-ligation step. The stabilised topoisomerase/DNA complex presents a barrier to progression of the enzyme complexes involved in replication, transcription etc. The lesions become lethal to the cell when it tries to use the affected region of the genome as template (Liu, 1989).

The majority of the topoisomerase inhibitors are naturally derived drugs e.g. etoposide, doxorubicin etc. As such, many are substrates for P-glycoprotein. Tumour cells displaying the MDR phenotype will often display resistance to these drugs (Beck and Danks, 1991). It would appear intuitive that, as it is the presence of stabilised cleavable complexes that results in damage to the DNA, then the more complexes the greater the resultant damage. Atypical multidrug resistance (atMDR) (Danks et al. 1987), is observed as cross-resistance between topoisomerase inhibitors due to reductions in the levels of topoisomerase enzyme within a cell. Cells with reduced intracellular topo I are more resistant to the topoisomerase I inhibitors such as

camptothecin. Reduced levels of topoisomerase II has also been shown to correlate with resistance to the topo II inhibitors. In the P338/AMSA leukaemia cell line, resistant to m-AMSA, inactivation of one allele of topo IIα by hypermethylation results in a decreased level of protein in the cell (Johnson and Howard, 1982).

Atypical multidrug resistance has been shown to correlate with mutant forms of topoisomerase proteins as well as with differences in the relative amount of protein. Such mutant proteins may be less likely to form cleavable complexes or may have enhanced re-ligation of strand breaks thus altering the sensitivity of tumour cells to the effects of topoisomerase inhibitors.

1.4   DNA REPAIR AND DRUG RESISTANCE.

Despite the obvious importance of drug accumulation and metabolism in determining the sensitivity of a cell to a particular anti-cancer drug, many resistant cell lines have been characterised in which no difference can be detected in these parameters when compared to the parental line. In other cases the differences that are shown are not significant enough to account for the levels of resistance observed. This suggests that there may be alternative mechanisms of drug resistance which do not directly relate to the level of drug exposure.

Genomic and mitochondrial DNA are constantly subjected to endogenous and exogenous damaging stimuli which if uncorrected would be incompatible with life. Therefore all organisms have developed highly efficient enzyme systems that correct such damage before it becomes permanent and leads to cell death. Induction of DNA damage is the most common means by which anticancer drugs act. The ability of cells to repair this damage will obviously influence their sensitivity to such drugs.

1.4.1 DNA repair by direct reversal of damage.

In principle, the simplest biochemical mechanism by which damage to DNA can be repaired is one in which a single enzyme catalyses a single-step reaction which restores the structure of the genome to its normal state. There are several such single enzyme pathways known but only one has been linked to a drug resistance phenotype in human tumour cells.

Alkylating agents are electrophilic compounds with affinity for nucleophilic centres in organic macromolecules (Singer and Kusmierek, 1982). They include a wide variety of chemicals, many of which are proven, or suspected, carcinogens as well as several intermediates of normal cellular metabolism, such as S-adenosyl-methionine, (Lutz, 1990). The DNA molecule possesses numerous potential nucleophilic sites, suitable for alkylation. The N7 and O6 atoms of guanine and the N3 atom of adenine are the most reactive. O^6 alkylguanine has considerable biological importance in mammalian cells as it is implicated in mutagenesis. In general alkylating agents that produce little O^6-alkylguanine in DNA are weak carcinogens.

The alkylating agents can be either monofunctional or bifunctional. The former have a single reactive group and thus interact with a single nucleophilic centre in DNA. Bifunctional alkylating agents have two reactive groups, and each molecule is able, potentially, to react with two sites in the DNA. If the two sites are on opposite polynucleotide strands, interstrand cross-links will result. If the sites are situated on the same DNA strand then the reaction product is referred to as an intrastrand cross-link. Both types of cross-link are thought to represent an important class of chemical damage to DNA. As a result of this alkylating agents have found extensive clinical application in the treatment of a wide range of solid and haematological malignancies (Chabner and Myers, 1995).

The alkyltransferase enzymes are a group of highly conserved proteins that function in the direct repair of DNA damage brought about by alkylating agents. They act by removing the alkyl group from the nucleophilic centre on the DNA molecule to a conserved cysteine residue within the active site of the enzyme. The alkyl group is covalently, and irreversibly, linked to the protein and thus the alkyltransferase acts as a suicide enzyme (Lindahl et al. 1982). O^6-alkylguanine is the predominant substrate of the alkyltransferase detected in human cells, O^6 methyl-guanine methyltransferase (MGMT). Cells with effective levels of MGMT activity rapidly repair O^6-alkylguanine resulting in protection against cell killing, mutation, recombination and chromosomal aberrations induced by simple alkylating agents (Kaina et al. 1991). MGMT is also effective in removing chloroethyl monoadducts from the O^6 position of guanine induced by the chemotherapeutic chloroethylnitrosoureas (CNU's) (Gibson et al. 1985), thus preventing formation of potentially more cytotoxic DNA cross-links.

There is increasing evidence of a correlation between levels of MGMT within cells and resistance to monofunctional and bifunctional alkylating agents both *in vitro* and *in vivo*. Isogenic Chinese hamster and HeLa cell lines that varied only in the levels of MGMT, were used in a study by Preuss et al (Preuss et al. 1996). They showed that MGMT proficient cell lines were resistant to cell killing by the methylating drug streptozotocin, as well as a range of CNU's, whereas the same lines were not protected against chlorambucil, cisplatin or melphalan. Another study in human glioma cells has shown that the activity of alkyltransferase within cells does not correlate with sensitivity to cisplatin (Beith et al. 1997). The use of methylated viral DNA in a host cell reactivation (HCR) assay, in human melanoma cell lines, has confirmed the previously observed association between cellular resistance to methylating agents and the activity of MGMT (Maynard et al. 1989). Leukaemic myeloid cells from patients with acute non-lymphocytic leukaemia (ANLL) do not respond to the nitrosoureas. It has been shown that in myeloid cells freshly cultured from leukaemic patients the levels of MGMT are significantly higher than that found in bone marrow cells enriched for myeloid precursors

(Gerson and Trey, 1988). Selective inactivation of the alkyltransferase resulted in increased sensitivity of the leukaemic cells to nitrosoureas.

1.4.2 DNA repair by excision of altered or damaged bases.

The term excision repair covers a number of processes by which damaged or inappropriate bases are removed from the DNA strand and replaced by the normal nucleotide sequence and chemistry. It is of greater biological importance than repair by direct damage reversal due to the broader range of DNA damage that acts as substrate. Nucleotide excision repair (NER) is one such form of excision repair and has been shown to have an extremely broad specificity, being able to recognise a wide variety of chemical alterations to DNA (Sancar, 1994). Nucleotide excision repair is involved in the correction of bulky lesions that arise in DNA such as UV-induced photoproducts and adducts derived from exposure to agents such as cisplatin (Bohr, 1995). Defects in NER have been shown to be responsible for the rare, inherited, cancer prone syndrome Xeroderma pigmentosum (XP) (Kraemer et al. 1994). Analysis of the mutations which result in XP have helped identify many of the genes involved in nucleotide excision repair. The basic steps involved in NER, and the genes involved, are shown in figure 1.1. They include: (1) pre-incision recognition of DNA damage; (2) incision of the damaged DNA strand near the defect; (3) excision of the defective site with concomitant degradation of the excised strand; (4) replication to replace the excised strand with the opposite (intact) strand being used as a template; and (5) ligation of the correct strand into the DNA at the 3'end of the repair patch. The size of this "repair patch" in eukaryote species is about 30 nucleotides which is longer than that seen in the other types of excision repair (Freidman et al. 1995). The majority of genes involved in NER have now been cloned and recently a cell free system has been developed that allows analysis of the role of each gene product in the repair process (Aboussekhra et al. 1995).
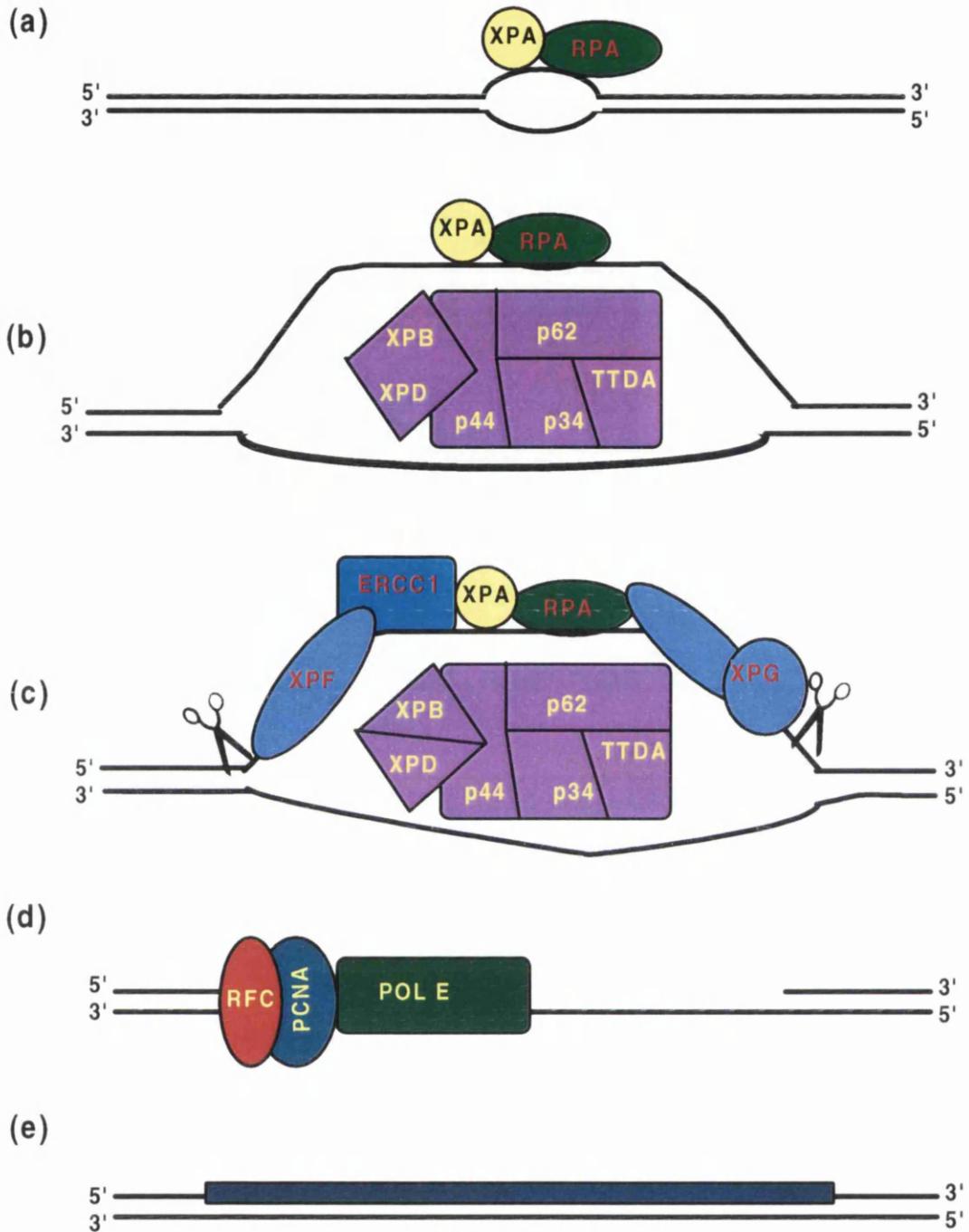
Figure 1.1 Model for nucleotide excision repair (NER).

(a). DNA damage is recognised by XPA protein in association with RPA
(b). Recruitment of TFIIH (six subunits) - helicase activity of of XPB and XPD
open out structure, enabling; (c). structure specific nucleases, ERCC1-XPF
and XPG, to cut DNA on either side of the damage.
(d). DNA polymerase epsilon (POL E) and accessory proteins, replication factor C
(RFC) and PCNA fill in the resulting gap, and (e) the new DNA is joined to the old
by DNA ligase.

Initial studies of the repair of bulky damage from DNA assumed that the efficiency of repair was uniform for the genome as a whole. Recently, however, there is evidence to suggest that preferential repair of certain active or essential genes is a general feature of DNA repair (Link et al. 1991). For example, in hamster cells, the dihydrofolate reductase gene is repaired more efficiently than the bulk of the genomic DNA (Bohr et al. 1987) and this may be related to differences in higher order chromatin structure around active genes. It has also been shown that, not only is damage repaired more efficiently from active genes but the actively transcribed strand is repaired preferentially to the non-transcribed strand (Mellon et al. 1987).Such repair has been classified as gene-specific or transcription coupled repair.

Chemotherapeutic drugs that target DNA often act through the formation of bulky adducts within the DNA molecule. It would therefore seem entirely reasonable that the ability of a cell to repair this damage might influence its susceptibility to the cytotoxic action of the drug. Cisplatin forms a variety of DNA adducts (Eastman, 1986), the most prevalent (>90%) of which are the N^7 d(GpG) and d(ApG) 1,2-intrastrand crosslink. Other platinum-DNA adducts include monofunctional, 1,3-d(GpNpG) and longer range intrastrand, interstrand and protein-DNA crosslinks. The trans-isomer of cisplatin is inactive in cell killing and is unable to form 1,2 intrastrand crosslinks which suggests that it is this lesion which is the most biologically active (Zamble and Lippard, 1995). It is still unclear, however, which of the cisplatin adducts has the most important role in cytotoxicity (Zamble and Lippard, 1995).

Evidence supporting the involvement of NER in the repair of platinated bases in DNA, comes from observations that cells deficient in such repair (both pro- and eukaryotic) are hypersensitive to cisplatin (Beck and Brubaker, 1973). For example cells deficient in the NER gene XPA show greater sensitivity to cisplatin than controls, as a function of the platinum bound to cellular DNA. They also display reduced repair of exogenous platinated DNA introduced into the cell (Chu, 1994). It has been proposed that both 1,2 and 1,3 intrastrand crosslinks can be removed by NER (Huang et al. 1994)

which suggests that the hypersensitivity in the XP cells may result from the inability to repair these lesions. The situation is confused, however, by evidence that 1,2 intrastrand crosslinks are not repaired, to any significant degree, using extracts from cells with functional NER (Szymkowski et al. 1992). This could indicate that the 1,2 crosslink is not the main cytotoxic lesion arising from cisplatin induced DNA damage.

Although the evidence presented above indicates that defects in NER result in increased sensitivity to cisplatin the connection between DNA repair and cisplatin resistance is by no means confirmed. Many of the initial studies were done on the mouse leukaemia cell line L1210 and showed that those lines selected for cisplatin resistance were much more effective in removing platinated DNA adducts from cellular DNA (Eastman and Schulte, 1988). It has recently been discovered, however, that the L1210 cell line has undergone a spontaneous mutation in the XPG gene at some point since its initial development, thus resulting in a DNA-repair defect (Vilpo et al. 1995). Therefore the parental line is actually hypersensitive to cisplatin and resistant lines probably have reverted to wild-type levels of repair. Ovarian cell lines have also been used in studies of cisplatin resistance and DNA repair. Several studies suggest that cisplatin resistant cell lines remove platinum adducts from the genomic DNA more rapidly than do sensitive lines (Lai et al. 1988; Parker et al. 1991).

One of the difficulties that arises with the *in vitro* studies correlating cisplatin resistance and DNA repair are the different methods used to assess the repair of cisplatin adducts. Assays which expose cells to cisplatin then measure the removal of platinated adducts from the genomic DNA may be influenced by cisplatin induced alterations in cellular metabolism. In addition the effects of drug uptake and metabolism may influence the apparent differences in repair observed between cells. The host cell reactivation (HCR) assay is one way that such problems can be overcome. In such an assay plasmid or viral DNA containing some form of expressed reporter gene is treated with cisplatin exogenously then introduced in to the cells by transfection. The reporter gene will not be expressed if DNA damage persists and therefore the level of expression of the reporter

gene is a measure of the repair of the platinated DNA adducts. Conflicting results were obtained, as to the role of DNA repair in cisplatin resistance, when HCR assays were used in ovarian carcinoma cell lines (Parker et al. 1991; Moorehead et al. 1996).It has also been suggested that the difference between sensitive and resistant cells may not be related to the rate of repair of cisplatin damage in the genome as a whole. Zhen et al (Zhen et al. 1992) showed that the difference in repair between their resistant and sensitive lines occurred at the level of the gene specific repair of interstrand crosslinks.

Additional evidence has been produced from studies in lung cancer to suggest that differences in repair of cisplatin adducts might underlie the relative sensitivity of tumour cells to this drug. Non-small cell lung cancer (NSCLC) characteristically responds poorly to chemotherapy whereas small cell lung cancer (SCLC) tends to be responsive initially, although usually relapses with resistant disease. Using established tumour cell lines and primary cultures, set up from freshly excised tumours, HCR assays revealed that NSCLC lines displayed greater repair of transfected plasmid than SCLC lines (Nie et al. 1995). SCLC cells had greater repair than an excision repair deficient Chinese hamster ovary cell line which acted as control. Evidence from this, and other, studies have also suggested that alterations in signal transduction pathways, including $p185^{c-erbB-2}$ signalling, may influence the NER of cisplatin adducts (Arteaga et al. 1994).

Excision repair mechanisms are responsible for removal of the damage induced by chemotherapeutic agents other than cisplatin. However, less is known about the biochemical differences in repair between sensitive and resistant cell lines. Bifunctional alkylating agents, such as Melphalan, act by forming interstrand DNA crosslinks (Fox and Scott, 1980). Evidence from mouse and human *in vitro* tumour cell lines suggests that resistant lines have an enhanced capacity for removal of interstrand damage (Friedman et al. 1994). Evidence from human rhabdomyosarcoma xenograft studies suggests that elevations in the amount of DNA polymerase α and β may result in resistance to melphalan (Friedman et al. 1994) although further studies have failed to clarify their potential role in the resistant phenotype (Moynihan et al. 1996). Interestingly

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there has also been evidence presented suggesting that the levels or activity of DNA polymerase α, β and δ may influence sensitivity to cisplatin (Scanlon et al. 1989).

1.4.3   Damage recognition proteins and cisplatin resistance.

It may not only be the activity of the components of the NER pathway which influence the ability of cells to repair bulky DNA damage. A series of proteins have been identified which recognise specific alterations in the tertiary DNA structure caused by cisplatin adducts. Several of these proteins have also been found to be over-expressed in cisplatin resistant cells suggesting a possible relationship (Zamble and Lippard, 1995). A common feature of all these proteins is the presence of a stretch of about 80 amino acids known as the HMG domain, due to its initial identification in high mobility group proteins. The HMG proteins are abundant, non-histone, components of chromatin and may have a major structural role in the bending or looping of DNA (which may be important for transcription or recombination) (Lilley, 1992). It would appear that the HMG domain can recognise and bind to certain structural alterations in DNA.

The damage recognition proteins identified to date appear to recognise the structural alterations in DNA induced by cisplatin adducts but not those caused by transplatin . In addition the binding of HMG-domain proteins to cisplatin modified DNA is specific for the major 1,2-intrastrand adducts (Zamble and Lippard, 1995). There is, as yet, no direct evidence for such proteins being responsible for altered repair of platinated DNA. It has been proposed that these damage recognition proteins could either recruit other components of the NER complex to the site of damage or, in contradistinction, bind to and shield the damaged DNA from the repair complex resulting in persistence of the damage. Some support for this second model comes from studies in the yeast, S.cerevisiae. A damage recognition protein, IXR-1, which contains two tandemly repeated HMG domains, binds to cisplatin damaged DNA. Disruption of the IXR-1 gene resulted in cells which grew normally but displayed increased resistance to cisplatin, an

effect that has recently been linked to the NER pathway (McAnulty and Lippard, 1996; Brown et al. 1993)

A role for other forms of DNA repair, including base-excision and recombinational repair in removal of the damage induced by chemotherapeutic agents has been implied. For example, the E.coli recombination-repair deficient strain rec A13, is very sensitive to cisplatin (Beck and Brubaker, 1973). The incomplete characterisation of such repair pathways in mammalian cells limits the study of their significance or contribution to drug resistance. In conclusion, therefore, DNA repair has been shown to be an important mechanism by which cells restore the integrity of the DNA after damage introduced by exogenous agents. The role that these repair pathways play in the determination of innate or acquired resistance to DNA damaging chemotherapeutic agents *in vivo*, although hinted at, is as yet unclear.

1.5 DNA MISMATCH REPAIR AND ITS SIGNIFICANCE TO DRUG RESISTANCE.

1.5.1 Origin of Mismatch Errors.

One further, specific, DNA excision repair mechanism has become of increasing interest, in the fields of oncology and drug resistance, over the past few years. During replication, the fidelity with which DNA polymerases synthesise the new DNA strand is influenced by a number of factors. Most polymerases show a 200 - 400 fold discrimination against inserting an incorrect nucleotide into the nascent strand, although certain sequence contexts may be more prone to misincorporation errors. Frameshift mutations can also arise, due to misalignment between the primer and template strand resulting in addition or deletion of bases (Kunkel, 1992). Most polymerases possess a 3' - 5' proof-reading action which allows the enzyme to recognise, and remove, a misincorporated base, in most cases without dissociating from the DNA. This, on

average, appears to contribute about 100 fold to polymerase fidelity (Echols and Goodman, 1991) However, despite these features of DNA polymerases, mismatch errors can persist after replication. Such mismatches also arise during recombination between non-homologous stretches of DNA and from spontaneous or induced modifications of bases within double stranded DNA (Freidberg et al, 1995). It has been shown, therefore, that all cells possess additional enzyme pathways to correct such mismatches before they result in permanently fixed mutations within the genome.

1.5.2 Strand specificity of post-replication mismatch repair

To function properly, post-replication mismatch repair must discriminate the newly synthesised from the template DNA strand. Repair of the mismatch in the nascent strand results in correction of the error whilst repair of the template strand would simply compound the mistake by fixing the mutation. E.coli DNA is methylated on adenine residues within a specific GATC motif. Immediately after replication the newly synthesised strand is transiently undermethylated and mismatch correction is directed to lesions on this strand as a result of this characteristic (Radman et al. 1980) Other prokaryote species, such as S. pneumoniae are not methylated and the signal for strand discrimination is not known. This is also the situation in eukaryotes.

1.5.3 Long-patch mismatch repair in E.coli.

The mismatch repair system has been most fully characterised in prokaryotes, especially E.coli. The development of *in vitro* assays, which allowed determination of the mismatch repair activity in crude cell extracts, and complementation with purified mismatch repair proteins, facilitated this characterisation (Lahue et al. 1989). A similar *in vitro* mismatch repair assay has been developed for eukaryote mismatch repair and has been utilised in the work presented in this thesis (see Chapter 4 for description).

Recognition of the mismatched DNA represents the first step in the repair process, (figure 1.2(a)). The E.coli MutS protein has been shown to bind, in the form of a multimer, to DNA substrates containing mismatches (Su and Modrich, 1986). MutS binds to different mismatches with varying degrees of efficiency, thus G/T mismatches are bound strongly, whereas C/C mismatches are hardly recognised at all (Su et al. 1988). The binding of MutS to a mismatch, results in its interaction with a multimer of MutL protein to form a MutS/MutL complex. This is followed by further association of the MutS/MutL-mismatch complex with another protein, MutH. This protein, which has endonuclease activity, is found at hemimethylated GATC sites on the newly synthesised strand, which may be at a distance from the site of the mismatch. It is thought that the formation of the MutS/MutL/mismatch complex results in a conformational change in the DNA which approximates it to MutH. This activates the endonuclease function of MutH, resulting in the introduction of a single strand nick into the new strand (Au et al. 1992). It has been shown that the MutS/MutL complex will interact with the MutH/GATC site closest to it, irrespective of whether it is 5' or 3' to the mismatch. After introduction of the nick into the newly synthesised DNA strand, an exonuclease removes a section of DNA extending from the nick to up to 100 bases past the mismatch. Depending on the direction of repair this function may be performed by exonuclease I (3'-5' activity) or by exonuclease VII or RecJ (5'-3' activity) (Cooper et al. 1993). The process also requires the action of a helicase and a single strand binding protein. After removal of the segment containing the mismatch DNA polymerase III synthesises the correct strand which is then ligated into place by a DNA ligase.

1.5.4 Long-Patch mismatch repair in humans.

The mismatch repair system in eukaryotes has predominantly been characterised by the study of S.cereviseae and other yeasts. A high degree of genetic, biochemical and functional similarity has been observed between prokaryote and eukaryote systems. This

(a) Methyl-directed mismatch repair in E. coli

(b) Human Mismatch repair

1. Recognition

2. Excision

3. Resynthesis
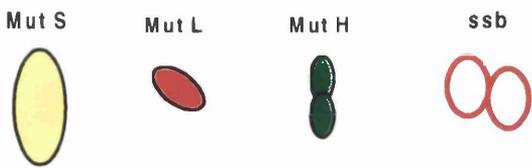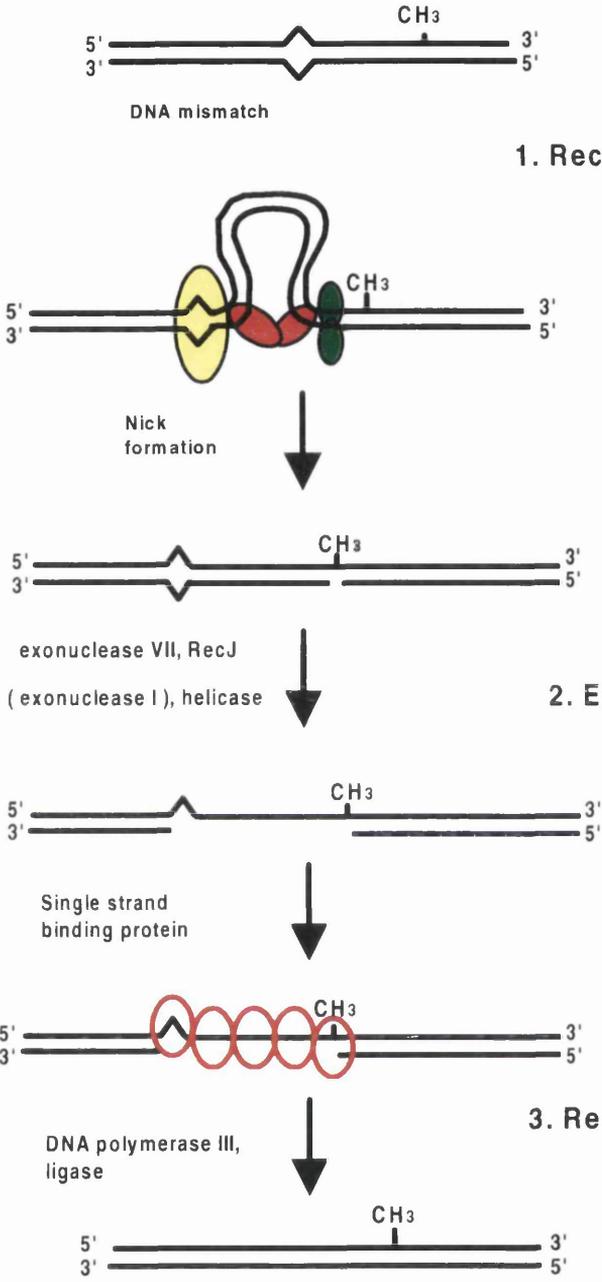
(ssb = single strand binding protein)
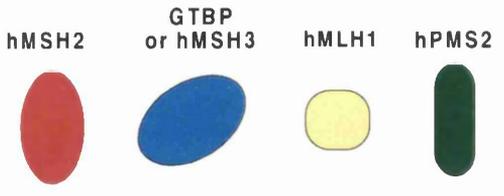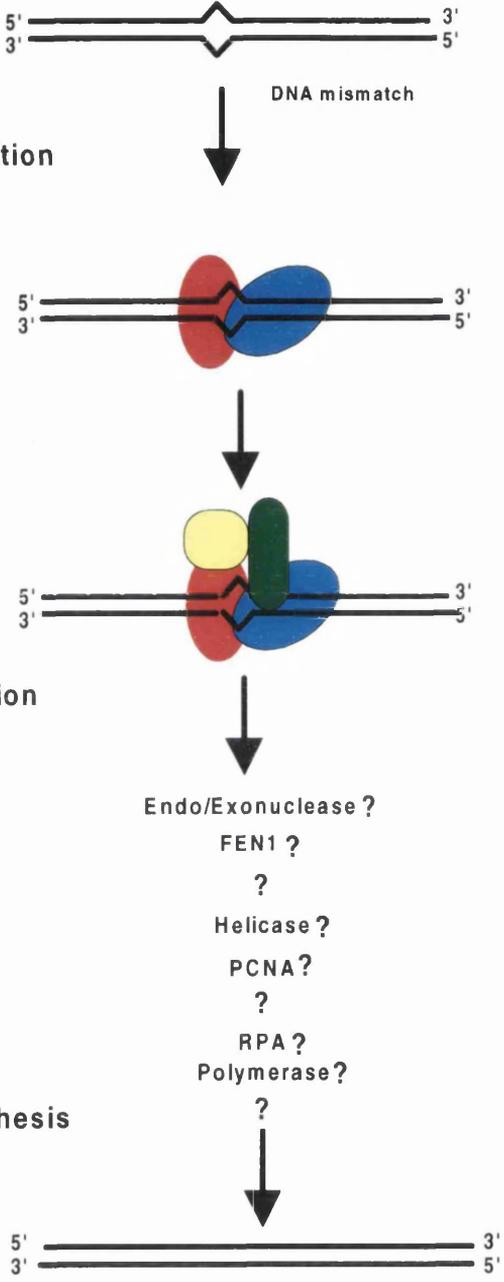
Figure 1.2 Long-patch DNA mismatch repair pathway (prokaryote & eukaryote)

Diagramatic representation of the pathways of DNA mismatch repair in prokaryotes (E.coli) and eukaryotes (humans). Question marks indicate postulated or unknown components of the system.

homology has also helped in the characterisation of the mismatch repair system in humans. The two striking differences between human mismatch repair and that seen in E.coli is the greater number of proteins involved in the former and the lack of GATC methylation as the signal for strand discrimination. It has been shown, *in vitro*, for both yeast and human mismatch repair, that introduction of a single strand nick into a mismatch containing construct directs repair to the nicked strand (Holmes et al. 1990). There has also been a suggestion that cytosine hemimethylation, in CpG islands, may direct mismatch correction (Hare and Taylor, 1985). However, the signal that determines repair strand specificity *in vivo* is unknown.

1.5.5 Human mismatch binding proteins.

As in prokaryotes the initial step in human mismatch repair is recognition of, and binding to, the mismatch (figure 1.2(b)). To date a number of mismatch binding proteins have been characterised in humans. Investigations into the genetic basis for one form of hereditary colon cancer, led to the discovery of a human mismatch binding protein which shared homology to E.coli MutS and S. cerevisiae MSH2 (Fishel et al. 1993a). This hMSH2 gene has been shown to bind to the full range of single base/base mismatches, including C/C mispairs not recognised by MutS, and also to insertion/deletion oligonucleotide heteroduplexes (Fishel et al. 1994). Cells with homozygous mutations in hMSH2 display a profound inability to correct any form of mismatch (Boyer et al. 1995).

It has subsequently been shown that hMSH2 functions as one subunit of a heterodimer, the other component of which is a 160kD MutS homologue called GTBP (G/T binding protein) (Drummond et al. 1995). *In vitro* data has shown that the hMSH2 and GTBP complex (known as hMutSα) form the major mismatch recognition activity in HeLa cells and that both proteins are required for binding to a G/T mismatched heteroduplex (Palombo et al. 1995). Analysis of cells mutated in GTBP reveals that the

mismatch binding specificity of both components of hMutSα are not identical. Mutants in GTBP are unable to repair all eight forms of base/base mismatch or single base insertion/deletion mismatches. They can, however, repair two, three or four nucleotide mismatches with up to 50% of the efficiency of wild-type cells (Drummond et al. 1995). The heteroduplex specificity of hMutSα has not been fully established, but the protein has been shown to bind with high specificity to G-T mispairs as well as 1- and 3-nucleotide insertion/deletion mismatches.

Two further human mismatch binding proteins, that are likely to be involved in mismatch repair, have been identified. The first eukaryotic MutS homologue to be identified, variously known as duc1, dug1 or MRP1 (Fujii and Shimada, 1989), was detected in the upstream control region of the DHFR gene. This gene, which has now been classified as hMSH3 due to its homology with MSH3 of S. cereviseae, has been sequenced. Using baculovirus synthesised human hMSH3 protein, many aspects of its function have also been characterised. It has been shown to bind with hMSH2 to form the, heterodimeric, hMutSβ complex. This would appear to have specificity for binding to mismatches in the form of insertion/deletion loops of two or more bases (Palombo et al. 1996). It is proposed that hMLH3 and hMLH6 display some level of redundancy, with the result that mutations in either protein do not lead to total loss of mismatch repair. hMSH2, on the other hand, appears to be vital for efficient mismatch binding and repair. Double mutants of the yeast homologues for hMSH3 and hMSH6 have been shown to have a repair defect as severe as that found for hMSH2 mutants (Marsischky et al. 1996). It has been suggested that the formation of such double mutants in human tumours is, statistically, a highly improbable event (Palombo et al. 1996). However, an endometrial carcinoma cell line, HUAA, has been shown to possess mutations in both hMSH6 and hMSH3, and displays loss of repair of both single base/base and insertion/deletion mismatches (Risinger et al. 1996). hMSH3 mutations have been observed in endometrial tumours (Risinger et al. 1996) and there is also a suggestion that

they may play a role in the development of certain haematological malignancies (Inokuchi et al. 1995).

A further protein that has a mismatch binding specificity different from that of hMSH2 or GTBP, has recently been identified. O'Regan et al (O'Regan et al. 1996) detected a dimer of proteins of 100kD and 140kD, different from hMutSα. This complex was found in cells lacking in hMSH2 and GTBP and has a binding preference for A/C and other pyrimidine/pyrimidine mismatches, as well as AT insertions or deletions in a poly(AT)$_n$ tract. The reason for multiple mismatch binding proteins in human mismatch repair is unclear although each protein may subserve a specific function.

1.5.6 Human MutL homologues.

Eukaryotic cells also display an increased complexity with regards to the component of mismatch repair homologous to MutL. To date three different MutL-like proteins have been identified in human cells. They have been named hMLH1, hPMS1 and hPMS2 in view of similarities to the MutL homologues found in S.cerevisiae. To further complicate matters, hPMS2 appears to be only one of a family of related genes located on chromosome 7, the functions of which are unknown (Nicolaides et al. 1995a). Tumour cells with mutations in hMLH1 or hPMS2, have been shown to be defective in repair of base/base and insertion/deletion mismatches, similar to the phenotype seen for hMSH2 mutants. Biochemical information is available only for hMLH1 and hPMS2 as yet, and this is limited. An HeLa cell activity that restores mismatch repair to extracts of hMLH1-deficient tumour cells has been isolated and shown to be a heterodimer of hMLH1 and hPMS2 (Li and Modrich, 1995). This has been designated hMutLα although the actual function of this complex, as with MutL itself, is unknown.

The other components of the mismatch repair system in humans are as yet uncharacterised. No human, or eukaryote, homologue of E.coli MutH protein has yet

been detected. Studies in yeast have suggested possible candidates for the exonucleases involved in removing the DNA strand containing the mismatch (Kolodner, 1996). There is also some evidence that the DNA polymerase responsible for repair synthesis may be one or more of α, ϵ or δ in view of the sensitivity of this process to the inhibitor aphidicolin (Thomas et al. 1991).

1.5.7 Mismatch repair mutations and cancer.

The significance of human mismatch repair has been reinforced over the past three years by the finding that defects in this repair pathway underlie the development of a variety of different types of tumour. Hereditary non-polyposis colorectal cancer (HNPCC) is one of the commonest of the hereditary cancer syndromes affecting as many as 1 in 200 individuals (Lynch et al. 1993). In its various forms it may be associated with an increased risk of endometrial, ovarian, renal tract and certain types of skin cancer as well as colonic adenocarcinoma. During an attempt to identify the genes responsible for this disorder using linkage analysis an increased frequency of mutation was observed in simple repetitive sequences known as microsatellite repeats (Aaltonen et al. 1993). It had been shown in yeast that such repetitive tracts of DNA displayed an elevated mutation rate in the presence of an abnormal mismatch repair pathway (Strand et al. 1993). The gene linked to the HNPCC phenotype on the short arm of chromosome 2 was shown to be homologous to the yeast MSH2 gene and is now known as hMSH2. Further characterisation of the genetic lesions underlying other HNPCC pedigrees led to the identification of the hMLH1, hPMS2 and hPMS1 genes. To date germ-line mutations in hMSH2 and hMLH1 have been shown to account for approximately 50% and 30% of cases of HNPCC although there are regional variations (Nystrom-Lahti et al. 1995). Mutations in hPMS2 and hPMS1 are much less commonly the inherited defect underlying HNPCC (Liu et al. 1996).

Mutations in mismatch repair genes have not only been detected in tumours arising in HNPCC kindreds. It has been shown that between 7 and 19% of sporadic colorectal tumours also show mutations in either hMSH2 or hMLH1. There is also an ever increasing literature which shows that mutations in components of the mismatch repair pathway may be responsible for a small but significant number of sporadic tumours from a wide range of tissues (Dams et al. 1995). Most commonly this has been through the identification of mutations in microsatellite repeat sequences in tumour tissue not found in normal tissue from the same patient.

Microsatellite sequences consist of short motifs of DNA, usually 1-6 bases long, repeated between 10 and 50 times in either perfect tandem or interrupted by other bases (Weber, 1990) Due to their repetitive nature frequent mispairing errors arise during replication(Schlotterer and Tautz, 1992), which are substrates for mismatch repair. Cells with defective mismatch repair show an increased mutational frequency at microsatellite repeats (microsatellite instability) and are said to have a replication error (RER+) phenotype. Of particular relevance to the work presented in this study is the observation of an RER+ phenotype in ovarian carcinomas. About 10-15% of sporadic ovarian carcinomas are RER+ (Han et al. 1993; King et al. 1995) and mutations in mismatch repair genes have been detected with increased frequency in particular histological subtypes (Fujita et al. 1995).

In RER+ sporadic tumours both alleles of the mismatch repair gene have to be inactivated. In some cases mutations have been identified in both alleles whereas in others, especially with hMLH1, loss of the non-mutated allele has been observed in the tumour (Hemminki et al. 1994). There has also been a suggestion that certain mutations may display a dominant negative phenotype hence negating the function of the normal allele (Parsons et al. 1995). In many sporadic tumours, which display evidence of a loss of mismatch repair, mutations of the four known mismatch repair genes are apparently absent (Katabuchi et al. 1995). This suggests that mutations in the other, as yet

undefined, components of mismatch repair may lead to malignant transformation and DNA polymerase δ has been implicated in one case (Dacosta et al. 1995).

1.5.8   Defective mismatch repair and tumorigenesis..

How do mutations in genes involved in mismatch repair result in the development of a wide range of different tumours? As mentioned above, loss of the mismatch repair pathway results in microsatellite instability throughout the genome. Bacteria and yeast with mismatch repair defects display a global increase in mutation rate, including non-repetitive sequences of DNA. Human tumour cell lines which display microsatellite instability have also been shown to have an increased rate of mutation at the HPRT gene (Bhattacharyya et al. 1994). One possibility is, therefore, that this increased global mutation rate results in a more rapid accumulation of mutations in oncogenes and tumour suppressor genes, resulting in tumour development. Such a " mutator phenotype" model for tumour development was proposed prior to the discoveries of defective tumour mismatch repair (Loeb, 1994).

Although an increase in the global mutation rate may affect any gene, it is intuitive that genes that possess microsatellite sequences, or other regions of repetitive DNA, have a greater risk of mutation. The gene for the androgen receptor has a trinucleotide CAG repeat within the first exon, and alteration in the length of this repeat has been reported in one case of prostatic cancer (Schoenberg et al. 1994). Perhaps of more relevance is that mutations in short stretches of repeated nucleotides have been found in the type II TGFβ receptor (TGFβ RII) (Markowitz et al. 1995). These frameshift mutations result in loss of TGFβ RII mRNA expression, and have been found in about 80% of RER+ colon tumours. TGFβ mediates growth inhibition in normal colonic epithelium, therefore the loss of the RII receptor would give a growth advantage to such cells.

Finally, it has been shown that repeats, consisting of 10-30 copies of the GT dinucleotide, are found in the non-translated regions and introns of a number of genes. These GT repeats have been shown to display weak transcriptional enhancer activity, which might be related to their ability to form the Z-DNA conformation (Hamada et al. 1984). It is possible that mutations arising in these GT repeat elements, as a result of defective mismatch repair, might alter the transcription of certain genes leading to tumour development.

1.5.9 Mismatch repair and Methylation tolerance.

The significance of DNA mismatch repair to drug resistance, and the impetus behind the studies presented in the following chapters, is the association between defects in this repair pathway and tolerance to specific DNA methylating agents. The concept of "tolerance" is one in which the damaged base is not removed from the DNA, but appears not to induce the cytotoxic effects that would normally arise from such a lesion (Karran and Bignami, 1994). As stated in section 1.4.1, agents which methylate DNA, such as MNNG and MNU, are mutagenic and cytotoxic due to the formation of O^6-methylguanine. Under normal circumstances this lesion is directly repaired by the enzyme MGMT. Functional loss of this enzyme, most commonly by epigenetic silencing, results in cellular hypersensitivity to killing and mutagenesis by such agents (Karran and Bignami, 1992).

It was through studies in bacteria, that the mismatch repair system was first implicated in the cytotoxicity of O^6-methylguanine lesions induced by methylating agents (Karran and Marinus, 1982). Mutation in the dam methylase responsible for methylation of d(GATC) sequences, in the newly replicated DNA strand, results in hypersensitivity to the cytotoxic actions of MNNG in E.coli. Additional mutations, in either MutS or MutL, result in double mutants which are no more sensitive to the killing action of MNNG than wild-type E.coli. This implicated these genes in the enhanced killing observed in

methylase deficient cells. Double mutant cells remain sensitive to the mutagenic effects of the alkylating agent which suggested the persistence of O^6-methylguanine lesions in the DNA despite loss of cytotoxicity. These observations led to the suggestion that O^6meG lesions in the helix can provoke a mismatch response (Karran and Marinus, 1982).

The concept of methylation tolerance in mammalian cells was first proposed by Goldmacher et al (Goldmacher et al. 1986). Exposure of the human lymphoblastoid cell line TK6, to a single high dose of MNNG, led to the isolation of the MT1 line. MT1 cells are a hundred times more resistant to the toxic effects of MNNG than TK6, but are slightly more sensitive to mutagenesis by this drug. It was shown that both cell lines have similar kinetics of MNNG adduct formation and removal, suggesting that MT1 cells tolerate adducts that are otherwise cytotoxic. The MT1 cell line was also shown to have an increased spontaneous mutation rate at the HPRT gene, thus indicating the likely presence of a mutator phenotype.

Subsequently, two further methyltransferase deficient, alkylation tolerant cell lines, one from CHO cells and one a Burkitt's lymphoma line were isolated (Branch et al. 1993). As well as showing increased resistance to MNU, both cell lines displayed an increased spontaneous mutation rate at HPRT and APRT loci and were subsequently shown to display microsatellite instability (Aquilina et al. 1994) They also both displayed loss of a specific GT mismatch binding function (Branch et al. 1993) resulting in loss of binding to O6meG·T and 6TG·T mismatches (Griffin et al. 1994). It has since been shown that MT1 cells are deficient in strand specific mismatch repair (Kat et al. 1993) with reduced MutSα activity (Drummond et al. 1995) due to mutation in both alleles of GTBP (Papadopoulos et al. 1995).

Tolerance to methylation damage has been further studied in colorectal adenocarcinoma cell lines (Branch et al. 1995). Several lines were shown to display defective G/T mismatch binding and were resistant to MNU. However these lines possessed abundant functional MGMT which, even after treatment with the inhibitor O^6-

benzylguanine, retained sufficient activity to efficiently remove the methylation damage. Thus the methylation tolerance of these lines could not be ascertained. Two lines, however, were shown to be lacking MGMT activity, yet one was sensitive to the effects of MNU and the other was resistant. The resistant line, SW48, was subsequently shown to be truly methylation tolerant and to display a mutator phenotype, both in a selectable gene and in microsatellite sequences(Branch et al. 1995). This cell line is proficient in G·T mismatch binding but has been shown to have lack of expression of hMLH1 mRNA (Liu et al. 1995) suggesting deficient MutLα activity.

The HCT116 colorectal adenocarcinoma cell line has also been shown to be deficient in hMLH1 and to be resistant to methylating agents(Koi et al. 1994). Introduction of chromosome 3 into these cells, via microcell fusion, resulted in restoration of mismatch repair function and increased sensitivity to MNNG. This was taken as proof that HCT116 cells were methylation tolerant. However, HCT116 cells possess functional MGMT and it has been shown that chromosome 3 may harbour other genes that interfere with MGMT action (Zunino et al. 1991). Therefore the introduction of an increased dose of these chromosome 3 genes may reduce the MGMT activity resulting in the increased sensitivity to MNNG. The methylation tolerance status of HCT116 therefore is not yet clear.

Although the association between defective mismatch repair and tolerance to certain methylating agents is clear, the mechanism underlying this phenomenon remains undefined. The tolerance phenotype is only observed with agents that introduce O^6MeG or 6-thioguanine (6TG) into the DNA. This suggests that structurally similar modifications at the 6-position of guanine are an important determinant in tolerance (Karran and Bignami, 1994). It has been proposed that these methylated bases are not a block to replication, but instead the DNA polymerase inserts the best fitting base into the nascent strand opposite the modified guanine. It has been shown that $O^6MeG·T$ introduces the least structural distortion to the DNA (Gaffney and Jones, 1989). In the absence of efficient methyltransferase function, the mismatched base pair is recognised by

the mismatch repair pathway as abnormal. Repair synthesis , which occurs in the strand opposite O^6MeG, is doomed to failure owing to the inability to find a good complementary match for the methylated base. It is proposed that repeated, futile, attempts to repair this mismatch eventually results in cell death, perhaps by the generation of strand breaks (Karran and Bignami, 1992). Tolerance would therefore arise when the mismatch repair system could no longer initiate these aborted attempts at repair.

There are several clinically active anticancer drugs, such as procarbazine and the methyltriazines, that act via introduction of O^6MeG into DNA (Hayward and Parsons, 1984). The clinical effectiveness of these drugs is compromised by the rapid appearance of tumour resistance. Our interest in the potential involvement of mismatch repair, in determining sensitivity or resistance to other classes of clinical anticancer agent, was fuelled by a number of observations. It has been shown that the mismatch repair system in bacteria can recognise lesions in DNA, other than those caused by methylating agents. A study by Feng et al (Feng et al. 1991) has shown that components of E.coli mismatch repair can recognise UV photoproducts in DNA. In addition dam mutant E.coli are sensitive to UV radiation, whereas dam, mutS double mutants are as resistant as wild type cells (McGraw and Marinus, 1980) This suggests that the mismatch repair system may have a function in the cellular response to UV damage in E.coli. The mismatch repair system may be also be involved in determining the cytotoxicity of cisplatin, since mutations in mutL reverse the cisplatin hypersensitivity of dam mutant E.coli (Fram et al. 1985a). Although there is no evidence to involve mismatch repair in human cisplatin sensitivity, studies in murine leukaemia and human ovarian carcinoma cell lines, have indicated that the ability of the replication apparatus to bypass cisplatin adducts on DNA is a component of cisplatin resistance (Mamenta et al. 1994). Conceptually this replicative bypass could be analogous to damage tolerance, although the mechanism is as yet undetermined. In view of the evidence that mismatch repair might be involved in the

recognition of a range of different DNA lesions, it has been suggested that it may act as a general sensor of DNA damage (Kat et al. 1993).

1.6   THE INFLUENCE OF POST-DNA DAMAGE RESPONSES ON RESISTANCE TO ANTICANCER DRUGS.

The importance of DNA repair, in the response of cells to DNA damaging cytotoxic agents, has already been stated. Until recently, the general consensus held that the tumour specific action of most anticancer agents was attributable to their genotoxic effects on actively proliferating cells. However, an increasing body of evidence suggests that cells possess a complex post-DNA damage response mechanism which determines the eventual fate of the cell.

When a cell suffers DNA damage, the defects must be detected, and signals sent to cellular control mechanisms in order to prevent the damage from being genetically fixed or propagated. One option is for the cell to pause in its replication cycle long enough for the damage to be repaired. Alternatively, the cell may respond to even low levels of damage by initiating an apoptotic response. Apoptosis has been described as "physiological" cell death, since it is a genetically determined cellular process, essential for normal development and maintenance of tissue homeostasis (Raff, 1992) Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction (Dive and Hickman, 1991) These results suggest that divergent types of cellular damage may lead to the generation of a common signal, or signals, that initiates the cell death programme. Accordingly, the ability of tumour cells to detect this damage, and activate the apoptotic response, may determine their ultimate sensitivity to cancer therapy. Evidence is accumulating from *in vitro* studies to support this concept (Lowe et al. 1993).

The components that constitute this post-DNA damage response pathway are gradually being identified. Central amongst them is the product of the tumour suppressor gene p53, which appears to act at a vital decision point in the pathway. Mutations in the p53 gene are found in about 50% of all human tumours, suggesting that its normal physiological function extends to the control of cell growth, as well as the response to DNA damage (Hollstein et al. 1991).

There are two major points in the cell cycle at which cells can temporarily arrest, in response to a variety of deleterious stimuli. The G1/S-phase arrest ensures that the cell repairs DNA damage prior to replication, whereas the G2 arrest allows repair before chromosomal segregation. P53 is central to the control of the G1/S checkpoint. Following DNA damage in normal cells there is a transient induction of the levels of p53 protein, occurring by a post-translational mechanism, which is temporally correlated with the G1 arrest (Kastan et al. 1991) The p53 gene has been shown to be regulate the transcription of a number of genes by binding to a specific consensus sequence (Farmer et al. 1992). One of these, p21 Waf1/Cip1, can bind to, and inactivate, cyclin dependent kinases, thus forming a link between the actions of p53 and arrest of the cell cycle (Harper et al. 1993). The p53 dependent induction of p21 Waf1/Cip1 also results in inhibition of proliferating cell nuclear antigen (PCNA), an essential DNA replication protein, which allows it to arrest DNA replication while permitting active DNA repair (Li et al. 1994).

Not only can p53 bring about a G1 arrest in response to DNA damage, but it is also involved in the determination of an apoptotic response to damage in particular types of cell. In certain cells, ionizing radiation initiates death through apoptosis, and this response has been shown to depend on the presence of wild-type p53 (Clarke et al. 1993). In addition, p53 mediates the effects of some chemotherapeutic agents in initiating apoptotic death (Lowe et al. 1993).That p53 plays this central role in determining the response of a cell to DNA damage, has been further reinforced by the study of cells lacking, or possessing mutant, p53. Thus, it has been shown that in a series

of 8 different tumour cell lines, the sensitivity to ionising radiation correlated closely with the constitutive levels of p53, the level of p53 induction after radiation and the presence of a G1 arrest (Siles et al. 1996).

Lowe et al, have shown in mouse embryonic fibroblasts that p53 is required for an efficient apoptotic response to ionising radiation and drugs such as etoposide and doxorubicin (Lowe et al. 1993). They have confirmed the requirement for functional p53 in apoptosis, arising from radiation and doxorubicin induced damage, in transformed embryonic fibroblast xenografts transplanted into nude mice (Lowe et al. 1994). Those xenografts which were resistant to these damaging agents harboured mutations in the p53 gene and displayed less apoptosis. The authors suggested that these results could explain the association, observed clinically, between p53 mutation and poor patient prognosis seen in certain tumours. Transfection of a dominant negative mutant p53, into a wild-type p53, radiation sensitive, ovarian cancer cell line (A2780) led to loss of the G1 arrest and increased resistance to ionising radiation (McIlwrath et al. 1994). This, along with a correlation between G1 arrest and radiation sensitivity across a range of tumour cell lines, further suggests that p53 and its role in mediating a G1 arrest are important in sensitivity to radiation.

Unfortunately nature is never straightforward and an increasing number of reports show that the response to DNA damage need not depend on p53 at all, or is influenced by factors which act after p53 in the pathway. Using isogenic colorectal carcinoma cell lines, that differed only in their p53 status, Slichenmyer et al (Slichenmyer et al. 1993) showed that loss of the G1 checkpoint was not associated with altered sensitivity to ionising radiation, or the topoI inhibitor camptothecin. In these cells it appeared that the G2 checkpoint was more important in determining radiosensitivity. It has been suggested that arrest in G2 is in fact a prerequisite for cell death, and that essential events occur during G2 which are required for death to occur by apoptosis (Eastman, 1990). Additional studies have shown that in certain cell types, sensitivity or resistance to different DNA damaging agents cannot be directly correlated with the

functional state of p53. Wu and El-Deiry have shown that the sensitivity of a variety of haemopoietic and solid tumour cell lines to various anti-cancer drugs was not dependent on the p53 status (Wu and Eldeiry, 1996).

How can these conflicting results, concerning the importance of p53 functions in determining the sensitivity of cells to DNA damage, be reconciled? It is proposed that, although p53 does play an important role in determining whether a cell enters a cell-cycle arrest or the apoptotic pathway after DNA damage, the final outcome of such a decision depends upon the genetic context in which it is made. Factors which influence the apoptotic pathway downstream of p53, such as members of the bcl2 and bax gene families, may determine the ability of a cell to undergo apoptosis irrespective of the p53 status. In certain cells, or in response to certain apoptotic stimuli, it has been shown that a p53 independent apoptotic response can occur which is also influenced by bcl2 (Strasser et al. 1994). It has also been shown that in cells in which G1 arrest and apoptosis depend upon normal p53 function, the response to ionising radiation depends upon the presence of exogenous growth factors (Canman et al. 1995). It may be the balance between death and growth factors, within a particular cellular environment, which determines the sensitivity to damage.

As already indicated, numerous studies have investigated the importance of p53 status, cell cycle checkpoints and apoptosis in the response to various forms of DNA damage. It is hoped that the further characterisation of these damage response mechanisms will identify the particular determinants which influence sensitivity or resistance. It can already be seen, however, that post-DNA damage responses are likely to play an important role in determining clinical responses to treatment. Tumours derived from cells which undergo apoptosis rapidly after DNA damage, in a p53 dependent manner, might be expected to be more responsive to therapy. Most of the tumours that do respond well to radiation and systemic chemotherapy (childhood ALL, lymphomas, germ cell tumours, etc.) are derived from cells in which the DNA damage induced p53 response is most likely apoptotic (Kastan et al. 1995a). Tumours in which the p53

mediated response to damage is one in which G1 arrest predominates, might be likely to survive following exposure to DNA damaging agents. Accordingly, it has been proposed that many of the inherently resistant solid tumours, derive from cell types which do not readily undergo p53 dependent apoptosis after DNA damage (Kastan et al. 1995a).

Finally, one component of the post-DNA damage pathway which is of increasing interest are the signals produced by DNA damage which activate the damage response pathways. It has been shown that double-strand DNA breaks are sufficient to initiate induction of p53 in tumour cells (Nelson and Kastan, 1994). However, patients with the inherited cancer prone condition ataxia-telangiectasia (AT), have a defect in the induction of p53 in response to ionising radiation (Canman et al. 1994). This suggests a defect in the signal transduction pathway from DNA damage to p53. Recently the genetic defect underlying AT has been shown to reside in a gene commonly involved in signal transduction (Lehmann and Carr, 1994).

The suggestion that the mismatch repair system might act as a general sensor of DNA damage (Kat et al. 1993) would place it within the pre-p53 signal transduction path. In determining whether alterations in mismatch repair might be involved in the signalling of DNA damage to the effectors of apoptosis, or cycle arrest, a system in which the cellular responses to damage are well characterised is essential. Our group have previously characterised the p53, cell-cycle and apoptotic responses of the ovarian carcinoma cell line A2780, to ionising radiation and a variety of chemotherapeutic agents (McIlwrath et al. 1994; Brown et al. 1993). The post-DNA damage responses of a series of cisplatin resistant, RER+, derivatives of this cell line have been characterised in the work set out in this thesis.

1.7 THESIS AIMS.

DNA is the principle target for the majority of successful anticancer drugs. Evidence from the literature has been presented, to support the concept that cellular processes that occur after DNA damage by anticancer drugs, may have more clinical significance than those which influence the initial levels of damage. The involvement of different mechanisms of DNA repair in cytotoxic drug resistance has been discussed. Specific reference has also been made to the evidence that mutations in DNA mismatch repair, in human tumour cell lines, can result in the development of resistance to certain DNA alkylating agents. The possibility that mismatch repair may also be involved in determining the sensitivity of cells to other cytotoxic lesions introduced into DNA has been raised. A brief review of the interaction between DNA damage, DNA damage repair and post-DNA damage response mechanisms has also been presented.

The aim of this thesis is to determine whether resistance to clinically important anticancer drugs, such as cisplatin and doxorubcin, could be determined by defects in the mismatch repair pathway in human tumour cells. Characterisation of the mismatch repair system is performed on a series of tumour cell lines selected for resistance to cisplatin and doxorubicin. To further investigate whether mismatch repair is involved in the development of cisplatin resistance, and possible interactions with determinants of post-DNA damage response, a series of cisplatin resistant ovarian carcinoma cell lines are developed.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The following section lists routinely used materials . Those used less frequently are described in the appropriate methods section.

2.1.1 Chemicals

All chemicals were of the highest available quality and were obtained from Gibco BRL, BDH Chemicals, Pharmacia LKB, Severn Biotech, Rathburn or Sigma Chemicals.

2.1.2 Radiochemicals

$(\alpha^{32}P)DTP$, used for labelling DNA probes and $(\alpha^{35}S)dATP$ for labelling M13 sequencing ladder were obtained from Amersham International.

2.1.3 Equipment

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

Automated DNA sequencer   Applied Biosystems,

Autoradiography film   Kodak, Cambridge. UK
                                   Fuji, Tokyo. Japan.

Cell Counter   Coulter Electronics. Luton, Beds. UK.

Confocal Microscope   Biorad. Hemel Hempstead. UK.

Densitometric Analysis
   Laser Densitometer   PDI, New York. USA.

Quantitation Software   PDI, New York. USA.

Electrophoresis Tank   IBI Ltd., UK.

                                 Flowgen. Lichfield. UK.

                                 Life Technologies. Paisley. UK.

Electroblotting System   Millipore, Watford, UK.

                                 Biorad. Hemel Hempsted. UK.

FACS Machine and Software   Becton Dickenson, San Jose, CA

Film Processor   Kodak, Cambridge, UK.

Gel Drier   Biorad. Hemel Hempstead. UK.

Hybridisation Membranes

    Hybond-N   Amersham International, Amersham UK.

    Immobilon-P   Millipore, Bedford, UK.

Hybridisation Oven and Bottles Hybaid Ltd., Middlesex, UK.

Liquid scintillation analyzer   Canberra Packard, Pangborne, Berks.

                                 UK.

Microplate reader   Molecular Devices. Alpha Labs,

                                 Eastleigh. UK.

PCR Thermal cycler   Hybaid, Teddington. UK.

                                 Perkin Elmer Cetus, Conn. USA.

LEP Scientific, Milton Keynes. UK.

UV Crosslinker   Stratagene, Cambridge. UK.

UV Transilluminator/imager   Appligene, Chester-le-Street. UK.

2.1.4 Restriction endonucleases and other enzymes

Restriction endonucleases   Gibco BRL

Taq polymerase   Boehringer Mannheim

RNAse A   Pharmacia LKB

Proteinase K   Boehringer Mannheim

2.1.5 Size markers

DNA Size Markers
Phage λ (HindIII)   Gibco BRL
123bp DNA ladder   Gibco BRL

RNA Size Markers
0.24-9.5Kbp RNA ladder   Gibco BRL

Protein Size Markers
14.3-200.0KDa prestained   Gibco BRL
6.9-202 KDa prestained   Biorad

2.1.6 Buffers, solutions and media

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

<u>Blocking Solution</u>

5% Marvel® non-fat milk powder

50mM Tris HCl pH 7.5

50mM NaCl

1mM EDTA

0.01% Tween-20

1mM DTT

<u>Buffer A</u>

25mM Hepes/KOH, pH8

1mM EDTA

1mM benzamidine

2mM 2-mercaptoethanol

0.5mM PMSF

0.5mM spermidine

0.1mM spermine

<u>0.1% DEPC Treated Water</u>

999ml double distilled H_2O

1ml DEPC

Leave at 37°C for 24h

Autoclaved solution

<u>Denaturing Solution</u>

1.5M NaCl

0.5N NaOH

DNA Loading Dye Mixture

30% v/v Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol

Formaldehyde Gel Loading Buffer (for Northern blotting)

50% glycerol

1mM EDTA (pH 8)

0.25% bromophenol blue

0.25% xylene cyanol FF

Genescreen

0.5M Na_2HPO_4

0.5M NaH_2PO_4

Glycine Buffer

0.1M NaCl

0.1M Glycine

pH to 10.5 with NaOH

High Salt Lysis Buffer

500mM NaCl

1% NP-40

50mM Tris pH 7.5

Protease Inhibitors (1×)

Hybridisation Buffer

50mM PIPES

50mM NaH_2PO_4

50mM Na_2HPO_4

100mM NaCl

1μM EDTA

5% SDS

(100μg/ml sonicated salmon sperm DNA. Southern blot only)

Made with DEPC treated water

10x MOPS RNA Running Buffer

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

0.05M Na acetate pH7.0

0.01M Na_2EDTA

Made with DEPC treated water

pH to 7 with glacial acetic acid

Autoclaved solution

Neutralization solution

1M Tris (pH 7.4)

1.5M NaCl

NP-40 Lysis buffer

150mM NaCl

1.0% NP-40

50mM Tris (pH8.0)

PBT

PBS

0.1% Tween-20

1.0% BSA fraction V

PBST

PBS

0.1% Tween-20

Phosphate Buffered Saline (PBS)

0.8% NaCl

0.115% Na_2HPO_4

0.02% KCl

0.02% KH_2PO_4

Protease inhibitors (100x)

0.1mg/ml Aprotinin

0.1mg/ml Pepstatin

0.1mg/ml Chymostatin

0.05M Benzamidine

0.05M PMSF

0.1mg/ml Leupeptin

Stored at -70°C

RNA Loading Dye

95% Formamide

20mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol FF

0.1 Volume of 50% glycerol

RPMI medium.

88 ml RPMI 1640 (10×)

800ml sterile distilled water

26.6ml 7.5% $Na(CO_3)_2$

10ml 100mM Na pyruvate

10ml 200mM L-glutamine

3.5ml 1M NaOH

100ml Foetal calf serum

5ml Penicillin/Streptomycin (50mg)

Resolving gel (6%)

21.5ml Water

10ml 30% Acrylamide, 0.8% bis-acrylamide

12.5 ml 1.5M Tris (pH 8.8)

0.5ml 10% SDS

0.5ml 10% Ammonium persulphate (freshly made)

5ml 1% polyacrylamide

40μl TEMED

Stacking gel (5%)

6.8ml Water

1.7ml 30% Acrylamide, 0.8% bis-acrylamide

1.25ml 1.0M Tris (pH 6.8)

0.1ml 10% SDS

0.1ml 10% ammonium persulphate

10μl TEMED

SSC (20×)

3M NaCl

0.3M Tri-sodium citrate

20x SSPE

3.6M NaCl

0.2M NaH_2PO_4

0.02M EDTA pH7.7

made up with DEPC treated water

Tank buffer (5×)

0.25 Tris base

0.5M Glycine

0.5% SDS

TAE(1×) pH8

242g Tris borate

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8)

Make to 1L with H_2O

TBE(1x) pH8

89mM Tris borate

89mM Boric acid

2.5mM EDTA

TBST

100mM Tris pH8

150mM NaCl

0.05% Tween 20

TE pH8

10mM Tris

1mM EDTA

Transfer buffer

48mM Tris base

39mM Glycine

0.038% SDS

20% Methanol

Trypsin

360ml Sterile distilled water

40ml 10×PE

40ml Trypsin (2.5% stock)

Western loading dye

2.5ml Tris

1g SDS

200µl 0.5M EDTA

5ml Glycerol

2.5% Bromophenol blue

2.5ml ddH$_2$O

2.1.7 Cell lines

A2780

Human ovarian adenocarcinoma cell line derived from omental metastasis from an untreated patient (Eva et al. 1982). Received from R.F. Ozols and T.C. Hamilton, Fox Chase Cancer Centre, Philadelphia.

A2780/cp70

Cisplatin resistant derivative of A2780 selected for resistance by exposure to increasing concentrations of cisplatin to maximum of 70μM (Behrens et al. 1987)

A2780/AD

Doxorubicin resistant derivative of A2780, isolated by repeated exposures to doxorubicin (Rogan et al. 1984). Expresses high levels of p-glycoprotein (Sugawara et al. 1988)

MCF7

Human breast adenocarcinoma cell line established from the pleural effusion of a 69 year old female (Soule et al. 1973).

MCF7/AD

Doxorubicin resistant derivative of MCF7 isolated by selection against increasing concentrations of drug (Sinah et al. 1986)

OV1/p

Ovarian carcinoma cell line (originally named IGROV1) (Benard et al. 1985). Received from Dr. J. Benard. Institut Gustave Roussy, Villejuif, France.

OV1/DDP

Cisplatin resistant derivative of OV1/p selected for resistance by (Teyssier et al. 1989)

2.1.8   Antibodies and immunological agents

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

Antigen	Antibody	Isotype	Company
p53	DO-1	Mouse IgG$_{2a}$	Oncogene Science
hMLH1	G168-15	Mouse IgG$_1$	Pharmingen
hPMS2	Ab-1(Clone 9)	Mouse IgG$_1$	Calbiochem
hMSH2	Polyclonal	Rabbit	Dr.J.Jiricny
GTBP	Polyclonal	Rabbit	Dr.J.Jiricny
Vinculin	Vin-11-1	Mouse IgG$_1$	Sigma
BrdUrd	Bu20a	Mouse IgG$_1$	Dako

Anti-mouse IgG horseradish peroxidase-linked (HRPL) rabbit antibody (Amersham) and anti-rabbit IgG (HRPL) donkey antibody (Amersham) were used as secondary detection agents in Western immunoblot studies. Anti-mouse IgG fluorescein 5-isothiocyanate-conjugated (FITC) goat antibody (Sigma) was used in immunohistochemistry and cell cycle analyses.

2.1.9 Plasmids and molecular probes

The following plasmids were used in the formation of probes for Northern blotting. The pBSK-MSH2 plasmid was provided by Prof. B. Vogelstein, Johns Hopkins University, Baltimore, Maryland, USA. The probes were derived from DNA fragments obtained using the restriction enzymes indicated.

Plasmid	cDNA	Probe fragment	Reference
pCRII-GAPDH	Human GAPDH	EcoRI (720bp)	(Tso et al. 1985)
pBSK-MSH2	Human hMSH2	ClaI/XbaI (3.1kb)	unpublished

2.1.10 PCR primers

The primers used for PCR of microsatellite repeat sequences, along with the type of repeat, chromosomal location and locus heterozygosity (PIC) are shown in Table 2.1 and 2.2. The majority of these primer pairs were obtained from Research Genetics, Huntsville, AL. USA. The others, where indicated, were a gift from Dr.D.Black (BICR, Glasgow).

Primers for PCR synthesis of oligonucleotide probes for Northern and Southern blotting are shown below. The sequences for these were determined using the PRIME function of the GCG software package and were synthesised either by Oswel DNA Service (Southampton, UK.) or in-house using an Applied Biosystems DNA synthesiser.

hMLH1

F   5'- ACG TTT CCT TGG CTC TTC -3'

R   5'- CCT CAC ATC CAA TTT CTA TC -3'

Product length - 730bp

hPMS2

F   5'- TCGAGT ACA GAA CCT GCT AAG -3'

R   5'- GTG AAA TGA AAC CTG AGA GT -3'

Product length - 804bp

PCR primers for use in RT-PCR analysis were derived using the GCG PRIME programme and were synthesised on an Applied Biosystems DNA synthesiser. Primer sequences are given below.

hMSH2

S5   5'- CAC CTG TTC CAT ATG TAC -3'

S6   5'- AAA ATG GGT TGC AAA CAT GC -3'

Locus	Location	Repeat Type	Sequence of Primers	Heterozygosity	Reference
CHRNB1	17pter-p12	CA	CTC GAG CCC CCG CAT TCA AGA A AAC TTT ACT ACA GGA GTT ACA CCC	0.88	Guzzetta,V. et al. 1992
D17S261	17p12-p11.2	CA	CAG GTT CTG TCA TAG GAC TA TTC TGG AAA CCT ACT CCT GA	0.5	Weber,JL 1990
D17S351	17q12-q21	CA	TTG ACC GGG GTA GAG AAC TC TCT CAG TAC TTC CCG TGA CC	0.75	Accessed through Genome database.
D17S579	17q12-q21	CA	AGT CCT GTA GAC AAA ACC TG CAG TTT CAT ACC AAG TTC CT	N/A	Hall,J.et al. 1992
D17S183	17q12-q21	CA	GTA CAT AGC ATG GGT GCA GCT ACA AAC TGA TGT GGG CTC TAG	0.4	Yagle, M. et al. 1989
D17S855	17q12-q21	CA	GGA TGG CCT TTT AGA AAG TGG ACA CAG ACT TGT CCT ACT GCC	0.82	Anderson, L.A. et al. 1993
D17S856	17q12-q21	AAAG	AAG GCA AGA CTT CGT CGA GA CAT TCC CTG GTC CTG TGC	0.4	Anderson, L.A. et al 1993
D17S796	17p13	CA	CAA TGG AAC CAA ATG TGG TC AGT CCG ATA ATG CCA GGA TG	0.8	Gyapay,G. et al. 1994
D17S702	data unobtainable	CA	AGC AAC ACA TAT CAG GGG C TGT AGG TTG ACC TTA AGG C	N/A	Unpublished.Accessed via Genome Database.
D17S793	data unobtainable	CA	TCT TTG ACC CAG ACC TCT AA TGT TGG AGT TAA TGT GCC AT	0.7	Unpublished.Accessed via Genome Database.
D17S801	data unobtainable	CA	CCT CAA ACC GGA CAA CTA TTT CAG AGA GCA AGA TCC TAC CTC	0.85	Gyapay,G. et al. 1994
THRA-1	17q12-21	CA	GGG CAA AAA TGT CTT AAG C CAG CAT AGC ATT GCC TTC	0.81	Bowcock,A.M. et al. 1993

Table 2.1 Chromosome 17 microsatellite repeat sequences.

Locus	Chromosomal location	Sequence of PCR primers	Heterozygosity
D2S111	2q	TTTTCT TTT TTG CAG TTT ATC C CAC TTC AGT GCC TTC TTG AGA	0.81
D2S172	2q	GCA AAG GCA CAA CTG CTT A CAT CCA GGT TGC TGT GAA T	0.92
D2S121	2p	GCT GAT ATT CTG GTG GGA AA GGC AAG AGC AAA ACT CTG TC	0.81
D2S168	2p	CCG GAC AAC AGA GCG A AGC TCA TTG CTA TGG GAG TA	0.82
D2S123	2p	AAA CAG GAT GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	0.76
D6S274	6p	CTC ATC TGT TGA ATG GGG AT CTT AAA TGC TAT GCC TTC CG	0.81
D6S311	6q	ATG TCC TCA TTG GTG TTG TG GAT TCA GAG CCC AGG AAG AT	0.91
D6S294	6p	TGC ATT GTT GTC ATG CCT TAA AGT CTC CAT CTT CGA TTG T	0.81
D6S261	6q	GTG AAA CCC TGT CTC ACT GC GGA TTT ATA GGA CCA TGC CA	0.82
D3S1298	3p	AGC TCT CAG TGC CAC CCC GAA AAA TCC CCT GTG AAG CG	0.87

Table 2.2 Microsatellite loci on chromosomes 2, 3 and 6.

Legend: Locus symbol, chromosomal location, heterozygosity and sequence of PCR primers for the microsatellite repeat sequences on chromosomes 2, 3 and 6, used in the studies on microsatellite instability. All information obtained from Gyapay,G. et al, 1994.

Product length - 508bp

hMLH1

F   5'- ACG TTT CCT TGG CTC TTC -3'

R   5'- GGG CTT TCA GTT TTC CAT C -3'

Product length - 440bp

hPMS2

F   5'- TCG AGT ACA GAA CCT GCT AAG -3'

R   5'- GTG AAA TGA AAC CTG AGA GT -3'

Product length - 804bp

2.2   EXTRACTION OF NUCLEIC ACIDS AND PROTEINS

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

2.2.1 RNA Extraction

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

2.2.2   DNA Extraction

Genomic DNA was extracted from monolayer cultures using the Nucleon I DNA extraction kit (Scotlab) following manufacturers protocol. For extraction of plasmid DNA from large scale (50 100ml) bacterial culture the Quiagen plasmid Maxi-kit was used.

2.2.3   Protein preparation

Cells, grown in monolayer culture, were washed with PBS then lysed for 5 minutes using high salt lysis buffer supplemented with 1× protease inhibitors. Cell supernatant containing extracted proteins was collected by centrifugation of lysate in cooled Eppendorf tubes at 4°C (15000rpm/ 15min). Supernatant stored at -70°C.

For total cell, or direct cell, lysis 10 volumes of sample buffer were added to the harvested cells and the suspension mixed vigorously. The sample was then boiled for 5 min. Chromosomal DNA was sheared by, firstly, passing the sample repeatedly through a 20-guage hypodermic needle and then through a 26-gauge needle. The sample was then spun at 10,000g for 10 min. The supernatant was collected.

Estimation of protein concentration performed using Bio-Rad Protein assay with standard curve constructed using Bovine serum albumin.

2.3 PREPARATION OF ^{32}P RADIOLABELLED PROBES

2.3.1   Purification of DNA for probe synthesis

DNA synthesised from PCR amplification was purified from excess primers and unincorporated nucleotides using the Wizard PCR clean kit (Promega). DNA excised from plasmid vector using appropriate restriction endonucleases was purified from vector DNA by electrophoresis on a 0.8% low melting point agarose, 1× TBE, 0.25µg/ml

ethidium bromide, gel. DNA was visualised by transillumination with UV light and an agarose plug containing the desired DNA fragment was excised from gel using a sterile scalpel blade. Agarose plug dissolved by heating to 65°C for 5 minutes in 5 volumes 20mM Tris.Cl (pH 8.0) and 1mM EDTA. Solution cooled to room temperature and equal volume of phenol added. Aqueous phase recovered by centrifugation and re-extracted with phenol/chloroform and then chloroform. DNA recovered by ethanol precipitation and dissolved in water.

2.3.2   Random priming of double stranded DNA

(α^{32}P) dCTP labelled dsDNA probes (50 - 100ng) were produced using the Prime-it kit (Stratagene) following manufacturers protocol.

2.3.3   Nick column purification

Radiolabelled probes were separated from unincorporated ^{32}P-labelled nucleotides using disposable Nick columns (Pharmacia). Briefly, Nick column washed with 800µl water, then products of random priming reaction loaded onto column. 400µl water added to column and eluate collected. 400µl elution repeated.

2.3.4   Determination of specific activity of probe.

The specific activity of radiolabelled probes was determined by taking a 5µl sample of the second 400µl eluate from the nick column purification and measuring the number of disintegrations per minute (dpm) using a liquid scintillation counter. A specific activity of > 10^7 dpm was required for the probe to be used in hybridisation.

2.3.5 Denaturation of probes

Probes were denatured before hybridisation by adding 0.1 volume of 3M NaOH and leaving for 5 minutes at room temperature. 0.05 volumes of 1M tris HCl (pH 7.5) were then added and left to incubate for 5 minutes on ice followed by the addition of 0.1 volume of 3M HCl and a further 5 minute incubation at 0°C.

2.4 SEPARATION AND HYBRIDISATION OF RNA

2.4.1 Separation and Northern transfer of RNA

Methodology for separation and transfer of RNA emulated instructions detailed by Sambrook et al, (Sambrook et al. 1989). 1.2% (w/v) agarose gels were prepared by dissolving 2.4g of agarose in 150ml of water then cooling to 60°C. 20μl of 0.5mg/ml ethidium bromide, 19.5ml of 10× MOPS RNA running buffer and 36ml of formaldehyde were added and the gel poured immediately. To 20μg of total RNA in 12μl, 25μl formamide, 8μl formaldehyde and 5μl 10× MOPS RNA running buffer were added. The samples were heated to 65°C for 15 minutes and chilled on ice before addition of 5μl RNA gel loading buffer. After electrophoresis for 4h at 56V, the gels were imaged using UV transillumination and photographed. Gel subsequently soaked in 50mM NaOH for 20min, rinsed in D.E.P.C. treated water and soaked in 20× SSC for 45min. RNA was transferred onto Hybond-N membranes by capillary action using 20× SSC as transfer buffer. Membranes were rinsed in water then exposed to UV for 6min, in Stratalinker (Stratagene), to allow fixation.

2.4.2 Hybridisation of blots

Pre-hybridisation of blots was carried out using hybridisation buffer at 65°C for at least 1hr. Blot subsequently incubated with denatured, radiolabelled probe in 10ml hybridisation buffer at 65°C overnight.

2.4.3 Washing

Following hybridisation blots were rinsed 3× in 10ml wash buffer for 2min, followed by wash in 10ml wash buffer at 65°C, with change of buffer every 30min, until background level of radioactivity reduced sufficiently to allow specific signal to be detected. Wash buffer consisted of either 1×, 5× or 20× SSC depending on stringency required to reduce background radioactivity.

2.4.4 Autoradiography

Following washing, blots were blotted dry, wrapped in plastic film and exposed to Kodak XAR film in film cassette with tungstate intensifying screens. Loaded film cassettes were held at -70°C until developed.

2.5 SEPARATION AND HYBRIDISATION OF DNA

2.5.1 Restriction digestion of genomic DNA

40µg genomic DNA (50µl) added to 25µl H_2O and 10µl 10× restriction buffer. Incubated for 3hr at 4°C with occasional mixing using capillary tube. 50units restriction enzyme added and stirred for 3min before incubating at 37°C for 3hr. Further 50 units restriction enzyme added and incubation continued for 6hr. Digested DNA was ethanol precipitated and resuspended in 25µl H_2O.

2.5.2 Separation and Southern transfer of DNA

Methodology for separation and transfer of DNA emulated instructions detailed by Sambrook et al, (Sambrook et al. 1989). 2.4g agarose dissolved in 300ml 1× TAE, 6µl 0.5mg/ml ethidium bromide and poured. DNA sample heated to 56°C for 3min then mixed with 5µl DNA loading dye and loaded onto gel. Gel was run at 20V, for 18hr, using 1× TAE as electrophoresis buffer which was recirculated using a mini-peristaltic pump. After electrophoresis, gel photographed under UV transillumination then soaked in denaturing solution for 45min. Gel rinsed in H_2O then soaked in neutralization solution, 30min, with gentle agitation. Neutralization solution changed and gel soaked for further 15min then placed in 1× Genescreen to equilibrate. DNA fragments transferred to Hybond-N filter by capillary action, using 1× Genescreen as transfer buffer. Transfer performed over 18hr. Membranes were rinsed in water then exposed to UV for 6min, in Stratalinker (Stratagene), to allow fixation.

2.5.3 Hybridisation of blots

Prehybridisation of the blot was performed using 30ml hybridisation solution, containing 100µg/ml sonicated salmon sperm DNA as in 2.4.2. Radiolabelled probe was added to 10ml of hybridisation solution, containing 50µg/ml salmon sperm DNA, and incubated with the blot for 18hr at 65°C.

Washing and autoradiography of blots performed in identical manner to that detailed in 2.4.3 and 2.4.4.

2.6 GEL SEPARATION AND IMMUNODETECTION OF PROTEINS

2.6.1 Sample preparation

Protein extracts were prepared and quantified as detailed in section 2.2.3. 75µg protein was mixed with 1/5 volume Western loading dye and boiled for 3min to facilitate protein denaturation.

2.6.2 Separation of proteins by SDS-gel electrophoresis

A resolving gel was cast (the percentage gel depending upon size of protein to be detected) and allowed to set for 1hr before overlaying with stacking gel. Samples and protein size markers were then added and proteins electrophoresed at 200V/ 40mA until dye front had migrated through gel. 1×Tank buffer used as electrophoresis medium.

2.6.3 Western transfer of proteins by electroblotting

Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore.) using a semi-dry electroblotter. The membrane was wetted in methanol then Transfer buffer. Six sheets of 3mm chromatography paper (Whatman, Maidstone, UK.) wetted in Transfer buffer were laid on anode followed by PVDF membrane, gel and another six sheets pre-wetted paper with cathode placed on top. The electroblotter was run at 200mA for 1hr. To assess evenness of protein transfer the gel was subsequently stained in Coomassie stain (0.2% Coomassie Brilliant blue R250 in a 50:50:7 v/v ratio of methanol: H_2O:galcial acetic acid) for 4hr, then destained using 25:68:7 v/v ratio of methanol: H_2O:galcial acetic acid.

2.6.4 Immunodetection of proteins on Western blot

Western membranes were incubated with Blocking solution at 4°C for 4hr with constant gentle agitation. The membrane was then incubated overnight, at 4°C, with primary antibody, diluted in Blocking solution, and subsequently washed in PBST (3× 5min). The membrane was then incubated with Blocking solution, containing secondary antibody, for 2hr then washed with PBST. The membrane was then incubated with a chemiluminescence substrate (Enhanced Chemiluminescence kit, Amersham. UK) for 1 minute, and exposed to radiographic film to enable bound complexes to be visualised.

2.7 AMPLIFICATION AND DETECTION OF DNA AND RNA

2.7.1 Standard PCR

All PCR manipulations were carried out using dedicated micro-pippetors, aerosol resistant tips and plasticware that had been sterilised by brief exposure to UV irradiation. Water and other non-DNA containing reagents were also exposed to UV irradiation in an attempt to reduce contamination of samples with extraneous DNA. PCR was carried out in a total reaction volume of 25µl in 0.5ml Eppendorf tubes or thin-walled 96 well plates (Hybaid, UK.) Template DNA was used in concentrations ranging from 50 -100ng. PCR primers obtained from Research Genetics (Huntsville, USA.) were used at a final concentration of 160nM, whereas with other primers the final concentration ranged from 0.5 to 1.0 mM. 2.5µl of 10× PCR buffer, 0.8µl dNTP mix and 0.2µl Taq polymerase (all Boehringer Mannheim) as well as 0.1µl $\alpha^{32}P$ dCTP were added to template DNA and primers and the reaction volume attained using sterile H_2O. 1 drop sterile mineral oil was placed on top of reaction mixture prior to amplification in thermal cycler.

Thermal cycler perameters were individualised for each primer / template combination. Annealing temperatures were obtained, either from the Prime programme of the GCG software package or were calculated from the equation;

$$\text{Annealing temperature } (\, ^\circ\text{C} \,) = 2 \times (\, \text{A+T}) + 4 \times (\, \text{G+C} \,)$$

where G,C,A,T are the number of each nucleotide in the primer. Standard melting and extension temperatures of 94°C and 72°C, respectively, were used in each reaction unless otherwise stated. The number of cycles of amplification was generally either 30 or 35.

A technique of "Touchdown" PCR was used for many of the reactions involving amplification of microsatellite repeats. A fuller description of the theory behind this technique is given in Appendix B and Don et al, (Don et al. 1991).

2.7.2 RT-PCR

cDNA was formed from 2μg total RNA using the Superscript II Pre-amplification kit (Gibco.BRL.) as per manufacturers protocol. Oligo(dT)$_{12\text{-}18}$ primers were used to prime the reverse transcription reaction. A control in which no reverse transcriptase was added was used to detect contamination with genomic DNA. Once formed the cDNA was treated with RNAaseH to remove residual RNA. 1-2μl of the reaction product was used as template in standard or Touchdown PCR reaction using gene specific DNA primers.

2.7.3 Synthesis of DNA size ladder

To allow accurate calculation of the size of PCR amplified microsatellite repeat sequences on polyacrylamide gels a DNA sequence ladder was synthesised using the M13mp18 bacteriophage as template. The ladder was synthesised using the Sequenase version 2.0 DNA sequencing kit (USB.) following the manufacturers protocol and labelling the products by addition of S^{35} dATP to the reaction.

2.7.4 Agarose gel electrophoresis

0.8 - 2.0g agarose was dissolved in 100ml 1× TBE and 2μl 0.5mg/ml ethidium bromide added prior to casting. DNA samples were mixed with 1/5 volume DNA loading dye prior to loading on gel. Gels were electrophoresed in 1× TBE buffer at 200V.

2.7.5 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels (the percentage polyacrylamide dependent on the size of DNA fragments requiring resolution) were used to resolve individual microsatellite repeats. Gels were cast between pre-cleaned glass plates one of which was coated with a silicone solution (Repelcote, Sigma. UK.). The glass plates were sealed to form a mould for the gel using adhesive tape.

Gels were formed using pre-mixed 30% Acrylamide / 0.85% bis-Acrylamide / 7M Urea (Easigel). For an 8% polyacrylamide gel the following recipe was used;

> 40ml   Acrylamide / bis-Acrylamide solution
>
> 280μl   10% Ammonium persulphate solution (made
>
> freshly)
>
> 14μl   TEMED

Once the gels were poured wells were formed using sharkstooth combs. Once set the gels were pre-run for 30 minutes at 35mA/ ≈1800V. After the pre-run the wells were cleared of unpolymerised polyacrylamide and urea using 1× TBE buffer. DNA samples for electrophoresis were mixed with 2/3 volume Fromamide loading dye and heated to 75°C for 5 minutes prior to loading on gel. The gel was run at 35mA in 1× TBE buffer until the xylene cyanol FF band was at the bottom of gel, then blotted onto a sheet of 3mm chromatography paper (Whatmann, UK) and dried under vacuum at 80°C for 1 hour. It was then exposed to radiographic film in a cassette with tungstate intensifying screens at -70°C.

2.8 TISSUE CULTURE TECHNIQUES

2.8.1 General cell culture methods

Aseptic manipulations were performed using sterile glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown at 37°C as monolayers in supplemented RPMI medium in the presence of 5% CO_2. All cell lines were regularly analysed for mycoplasma infection. Cell stocks were formed by freezing 10^6 cells, in 1ml RPMI with 10% di-methyl sulphoxide (DMSO), at -70°C in cryotubes (Nunc, UK). After 24 hours, samples were transferred to liquid nitrogen. To reduce the chances of genetic drift cell lines were replaced from frozen stocks regularly.

2.8.2 Selection of cisplatin resistant lines

1×10^6 A2780 cells were grown in F75 tissue culture flasks (Bibby Sterlin, Aberbargoed. UK) for 36 hours to attain log growth phase. A 10mM stock solution of cisplatin was made freshly for each selection. Stock cisplatin solution was added to RPMI medium to obtain the desired final concentration. Cells were then exposed to the cisplatin containing medium for 24 hours at which point it was replaced by fresh medium. Cells were allowed to grow for approximately 7 days , with changes of medium as required. Cells surviving after this period were harvested by trypsinisation and 1×10^6 cells were placed in a new F75 flask for the next round of selection. The concentrations of cisplatin used in the selection process were 1μM, 2μM, 4μM, 6μM, 8μM, and 15μM.

2.8.3 Clonogenic assay

1×10^3 cells were plated in 10cm tissue culture dishes, with 5 replicates for controls and for each drug concentration, per cell line. Cells were allowed to grow for 24 hours, at which point medium was removed and replaced with fresh medium containing the

required concentration of cisplatin. After the desired period of exposure this medium was replaced by fresh, drug free, medium and the cells allowed to grow for 10 days with changes of medium as required. 1mL Giemsa's stain was added to the medium in each dish and left for 10 minutes to allow cell staining. Medium was then removed and dishes washed gently in water. Colonies were counted manually with an attempt made to standardise the size of colonies counted between dishes.

2.8.4 MTT assay

Using a cell suspension of density 2.5×10^3 cells/ml medium, cells were plated out in volumes of 200µl into microwell plates and incubated for 3 days. On day 4 the plates were exposed to medium containing cisplatin for 1 hour, then replaced in the incubator with fresh drug-free medium. On days 5-7 the medium was refreshed and the plates re-incubated. On day 8 the medium was removed and the MTT solution added (200µl RPMI, 1/100 Hepes and 50µl of 5mg/ml MTT dissolved in PBS and filter sterilised). The plates were wrapped in silver foil, incubated for 4 hours at 37°C, then the MTT solution removed and replaced with 200µl DMSO and 25µl glycine buffer. The optical density at 570nm of each well was then determined using a microplate reader.

2.9 IMMUNOCYTOCHEMISTRY

2.9.1 Immunohistochemistry of cultured cells

To the wells of a chamber slide (Nunc, Naperville, Il. USA) 5×10^4 cells of each cell line, in 250µl of RPMI medium, were added and grown for 48 hours. After removal of the medium the cells were washed with PBS, the plastic gasket removed and the slide dried in air. Slides could be wrapped in parafilm at this point and stored at -70°C. Dried slides were placed in methanol at -20°C for 20 minutes to allow fixation of cells and then air dried for a further 40 minutes. Normal horse serum, diluted 1:10 with 0.1% BSA in PBS,

was added to the cells as a blocking agent and left to incubate for 20 minutes at room temperature in a humid atmosphere. Excess liquid was removed and 50μl primary antibody, diluted in 0.1% BSA in PBS, added and left to incubate as before for 2 hours. Slides were subsequently immersed in 0.1% Tween in PBS for 2× 10 minutes before incubation for 30 minutes at room temperature with secondary antibody, again diluted in 0.1% BSA in PBS. Two further 10 minute washes in 0.1% Tween in PBS followed. The slides were then mounted using Vectashield (Vector Labs, Burlingame. Ca. USA) and the edges of the coverslip sealed with nail varnish. Slides were then analysed using confocal microscopy.

2.9.2 Cell cycle analysis

Determination of the proportion of cells, within a given population, in different stages of the cell cycle was assessed using the method previously described by Kastan et al (Kastan et al. 1991a). Exponentially growing cells were plated at 5×10^5/10cm plate in RPMI medium and incubated at 37°C for 3-4 days. At this point the cells were irradiated with 2Gy ionising radiation in the form of γ-rays from a 60Co source. The cells were incubated for a further 24 hours at which point the medium was replaced with RPMI medium containing bromo-deoxyuridine (BrdUrd) at a final concentration of 10μM. After incubation for a further 4 hours at 37°C, the medium was removed and cells removed from the plates by trypsinisation. Cell pellets were obtained by centrifugation of the trypsin/cell suspension at 500G for 5 minutes and removal of the supernatant. The pellets were resuspended in 5ml, ice-cold, 70% ethanol and could be stored for up to 2 weeks at 4°C.

For analysis cell pellets were resuspended in 1ml cold PBS, transferred to 1.5ml microcentrifuge tubes, pelleted by centrifugation at 300G for 5 minutes at 4°C (these conditions were used for all subsequent centrifugations) and the PBS wash repeated one further time. Cells were then permeabilised by incubation in 2M HCl for approximately 30 minutes, at room temperature. Cells were collected by centrifugation and washed twice in

1ml PBS, then in 1ml PBT which acts as a blocking agent. The pelleted cells were incubated with 100µl of 1:40 mouse anti-BrdUrd monoclonal Ab, in PBT, for 1 hour at room temperature, washed twice in PBT then incubated with a 1:40 dilution of FITC conjugated goat anti-mouse 2°Ab, in PBT, for 30 minutes. The cells were washed once in PBT and once in PBS prior to incubation with 1mg/ml propidium iodide for at least 20 minutes at room temperature or overnight at 4°C. Cells were pelleted and resuspended in PBS to allow analysis using the FACScan flow cytometer. The red (propidium iodide) and green (FITC) emissions from each cell were separated using the standard optics of the FACScan, and cell cycle distribution was determined by counting in excess of 2×10^4 events.

2.10   MISMATCH REPAIR ASSAYS

2.10.1 *In vitro* mismatch binding assay.

Cell extracts were prepared by a modification of the method described by Jiricny (Jiricny et al. 1988). Briefly, cells were harvested and suspended in three packed cell volumes of buffer A, followed by homogenisation in a Dounce homogeniser. One volume of glycerol was added, followed by 1/10 volume saturated, neutralised ammonium sulphate solution. This was incubated for 30 minutes then centrifuged at 90,000 rpm for 60 minutes. All procedures were performed at 4°C.

Gel retardation assays were performed at 20°C. 5µl of cell extract was added to 20µl of the following reaction mix: 25mM Hepes·KOH, pH 8.0, 0.5mM EDTA, 0.1mM $ZnCl_2$, 0.5mM dithiothreitol, 10% glycerol, 50µg/ml poly (dI·dC)$_2$ (Sigma), 0.4µl of 'cold competitor', a 34mer ds complementary oligonucleotide, was added to each sample. This was incubated at room temperature for 5 minutes. Labelled heteroduplex oligonucleotide was added and a further 20 minutes incubation performed. 2µl of loading buffer (Bromophenol blue in 50% glycerol) was added and half of the sample loaded onto a 6%

polyacrylamide: TBE gel. Electrophoresis was performed at 70V in TBE until the dye front had just migrated out of the gel.

2.10.2 *In vitro* mismatch repair assay

Nuclear extracts were prepared according to the method of Challberg and Kelly (Challberg and Kelly, 1979), except that all buffers contained 0.1% PMSF. Nuclear pellets were resuspended in 0.05M Hepes, pH 7.5; 0.5mM dithiothreitol; 0.1% PMSF; 10% sucrose. After addition of 0.031 volumes 5M NaCl, the nuclear suspension was mixed for 1 hour. Nuclei were centrifuged at 15,000g for 20 minutes, and the resulting supernatant concentrated by ammonium sulphate precipitation. The ammonium sulphate pellet was dissolved and dialysed against buffer containing 0.1% PMSF. The resulting dialysate was clarified by centrifugation at 14,000g for 15 minutes, the protein concentration determined and small aliquots stored at -70°C.

100ng of mismatched f1MR construct was added to 50µg of nuclear extract and incubated at 37°C for 15 minutes. After incubation reactions were stopped by addition of 30µl of 25mM EDTA, 0.67% SDS followed by treatment with proteinase K. DNA was purified by phenol extraction and the extent of mismatch correction quantitated by determined of sensitivity to the appropriate restriction enzymes.

CHAPTER 3

MICROSATELLITE INSTABILITY AND DRUG RESISTANCE

3.1 INTRODUCTION

Cells from a significant number of sporadic tumours, of different tissue origin, have been shown to display microsatellite instability. This has also been shown to be the case for *in vitro* cell lines derived from human tumours of various types (Boyer et al. 1995; Orth et al. 1994). An association between defective mismatch repair, characterised by microsatellite instability, and resistance to certain types of alkyalting agents has been proven conclusively in a number of different *in vitro* cell systems (Karran and Bignami, 1994). The alkylating agents for which this specific type of resistance has been shown are of limited clinical significance. It is therefore important to determine whether there is an association between microsatellite instability in tumour cells and resistance to those anticancer agents of clinical importance.

The ovarian epithelial adenocarcinoma cell line A2780 was originally derived from a patient with no prior exposure to chemotherapy (Eva et al. 1982). This line, and derivatives selected for resistance to anti-cancer drugs such as cisplatin, have been used extensively in the study of the molecular determinants of drug resistance. As a result of this many of the differences between A2780 and its drug resistant derivatives are characterised at the molecular level (Rogan et al. 1984; Brown et al. 1993; Behrens et al. 1987; Andrews and Howell, 1990). In view of this substantial molecular characterisation these cell lines, and several others, were used in the analysis of microsatellite instability.

To detect the presence of microsatellite instability comparison is made of the length of microsatellite repeat sequences at identical loci between cell lines. Although there is some debate as to whether the likelihood of detecting microsatellite instability depends on the locus studied (Tautz, 1990), microsatellite repeats on chromosome 17 were used primarily. Chromosome 17 is known to harbour genes involved in the development of drug resistance and the malignant phenotype (Fry et al. 1991; Brown et al. 1993). Recessive mutations that arise in such genes may be important in the

development of an altered cell phenotype, such as the acquisition of drug resistance. One way of uncovering recessively acting mutations in genes is by looking for the development of loss of heterozygosity (LoH) between the normal or parent cell and that with the altered phenotype. Microsatellite repeats have been used as chromosomal markers in LoH studies due to their spread throughout the genome and their high level of polymorphism (Gruis et al. 1993). It was hoped that the microsatellites used in this study would provide not only information about microsatellite instability but perhaps also evidence of chromosome 17 specific LoH in the resistant cell lines.

The analysis of microsatellite repeat sequences was performed using the polymerase chain reaction (PCR). Certain parameters influence the success and accuracy of amplification of microsatellite DNA . In each cycle of amplification the DNA template and primer sequences go through steps of denaturation, annealing and elongation. The temperature at which the primers anneal to the template strand is critical and is a function of the length and sequence context of the primers themselves. If the temperature of the annealing step is above the optimal annealing temperature for the specific primers then there will be a much reduced efficiency of amplification. If the annealing temperature is below optimal then the primers become more likely to anneal not only to the specific template sequences but also to non-specific sequences leading to an increase in non-specific amplified products. In the analysis of microsatellite repeat sequences reported here a technique of "Touchdown" PCR was used (Don et al. 1991) to improve the efficiency of DNA amplification. It also permitted the use of a single set of reaction conditions for a number of different PCR primer pairs without significant loss of amplification specificity. This technique is more fully discussed in Appendix B.

The sections that follow present the results of the search for microsatellite instability in drug resistant tumour cell lines. It subsequently proceeds to study further parameters of the drug resistant phenotype in cisplatin resistant cell lines derived from A2780. Finally it assesses whether certain specific features of the post DNA-damage

response pathway are altered in the drug resistant cell lines as compared to the lines from which they are derived.

3.2 MICROSATELLITE ANALYSIS OF DRUG RESISTANT TUMOUR CELL LINES

3.2 1 Comparison of chromosome 17 specific, microsatellite repeat sequences in A2780 and drug resistant cell-line derivatives.

Microsatellite repeat sequences from different chromosome 17 loci were amplified using PCR in DNA from A2780, A2780/cp70 and A2780/AD. Figure 3.1 shows the autoradiographic images obtained from the radiolabelled PCR products, after electrophoresis on denaturing polyacrylamide gels. When the PCR amplified products, from each of the chromosome 17 microsatellite repeats in A2780/cp70 and A2780/AD, were compared to those from A2780 differences were observed in the size of alleles at certain loci. In Figure 3.1(a) the microsatellite loci D17S579 and D17S183 are shown for each of the three cell lines. D17S183 is a heterozygous repeat locus with the dominant alleles in A2780 migrating at 93bp and 99bp. The allelic pattern in A2780/AD is exactly identical to that of the parent line whilst that in A2780/cp70 shows a reduction in the size of the longer allele by 2bp (one repeat length). It can be observed that the allelic pattern seen usually consists of a series of bands with one often being more prominent than the others. The fainter bands, often known as "stutter bands", represent artefacts that arise during PCR by several mechanisms (Tautz, 1990).

D17S579 is homozygous with the predominant band in A2780 migrating at a size of 108bp. The same allele in both A2780/cp70 and A2780/AD has changed in size. In A2780/AD the dominant band would appear to be one repeat length larger (110bp) whilst in A2780/cp70 the strongest band on the autoradiograph is two repeat lengths shorter (104bp). Other examples of the changes in microsatellite repeat sequence seen in
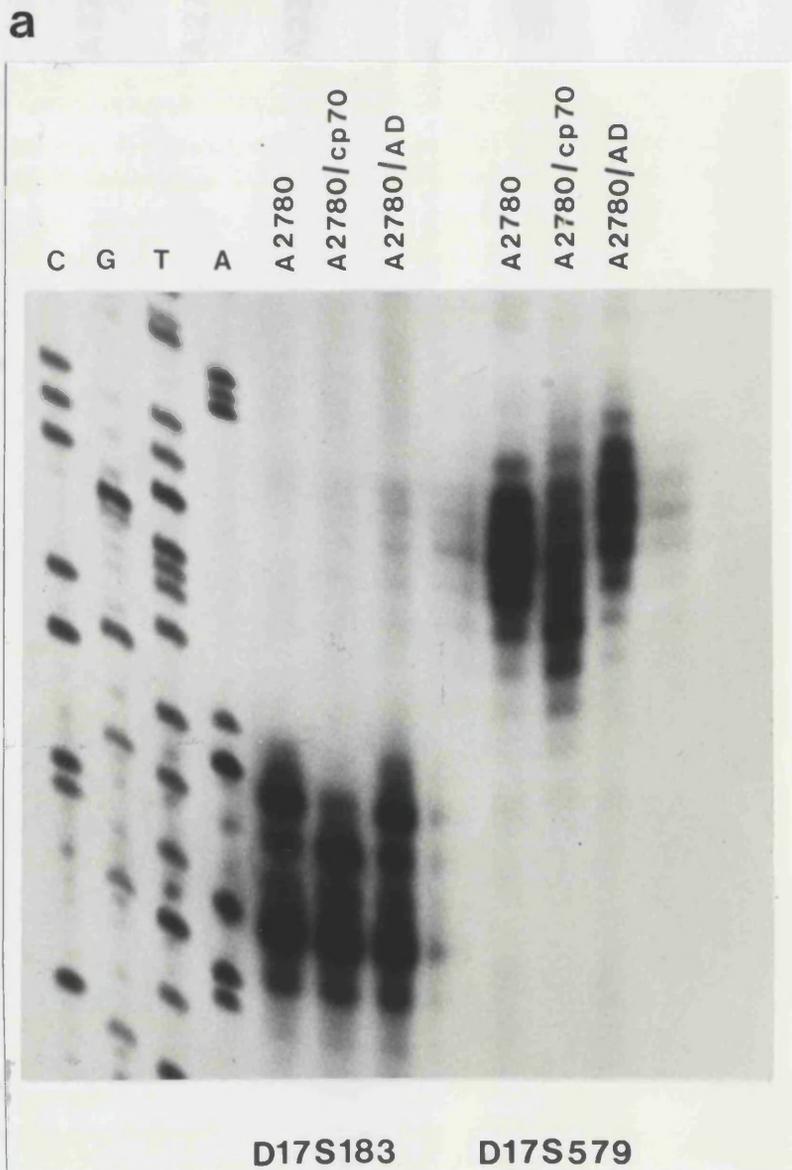
Figure 3.1(a) Mutations of microsatellite loci in drug resistant A2780 cell lines.

PCR amplification of the microsatellite loci D17S183 and D17S579 in DNA from A2780, A2780/cp70 and A2780/AD. The pattern of microsatellite alleles in A2780/cp70 and A2780/AD can be seen to differ from that in A2780 in D17S579 whilst only A2780/cp70 differs from A2780 in D17S183. These differences represent microsatellite mutations. An M13 phage DNA sequencing ladder (C,G,T,A,) is included to allow determination of allele size.

Figure 3.1 (b), (c), (d) & (e) Mutation in microsatellite loci on chromosome 17 and 2 in drug resistant, A2780 derived, cell lines.

PCR amplification of microsatellite loci D17S351, D17S261, D17S702 and D2S172 in DNA from A2780, A2780/cp70 and A2780/AD. A number of different types of microsatellite mutations are observed in the different loci from chromosome 17 and 2. Sizes (in base pairs) are represented by arrows and were calculated from an M13 phage sequence ladder. This allows an estimation of the length of the microsatellite alleles.

b

A2780   A2780/cp70   A2780/AD

85 bp →

79bp →

D17S351

c

A2780   A2780/cp70   A2780/AD

140bp →

D17S261

d

A2780   A2780/cp70

310 bp →

270 bp →

D17S702

e

A2780   A2780/cp70   A2780/AD

260bp →

D2S172
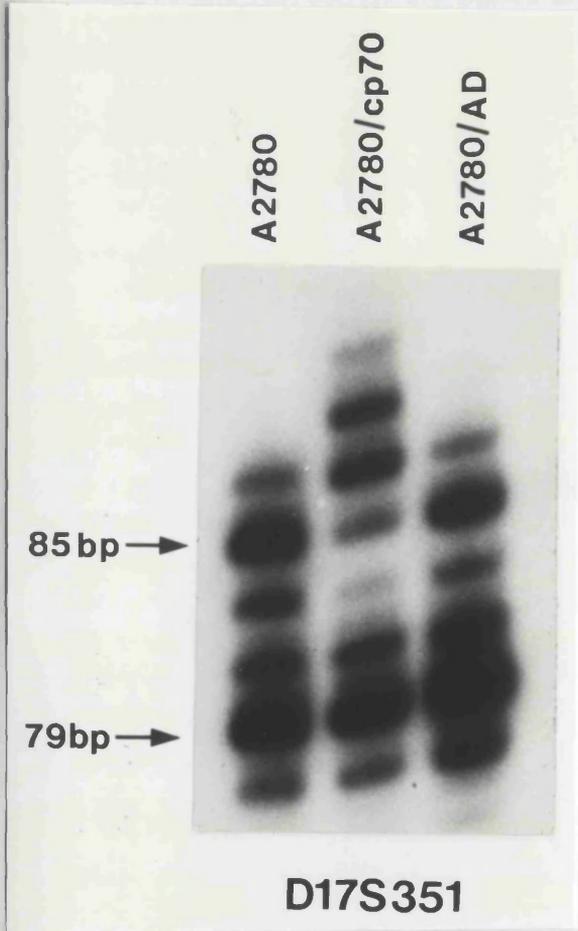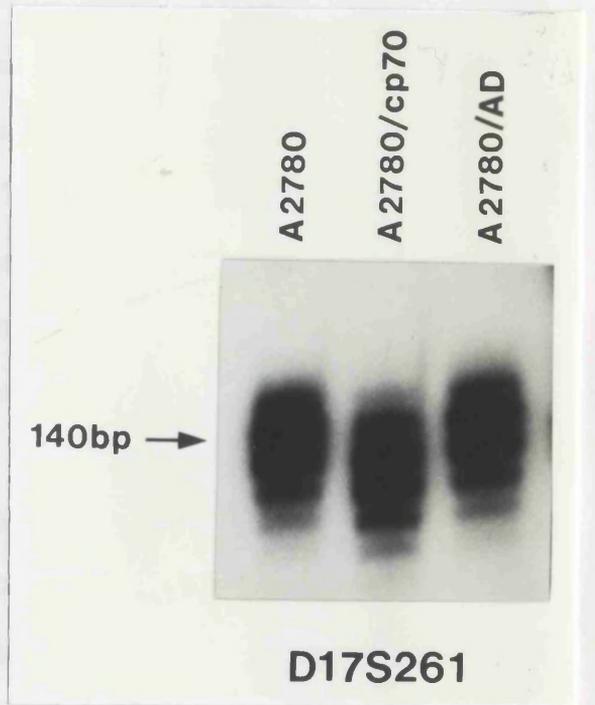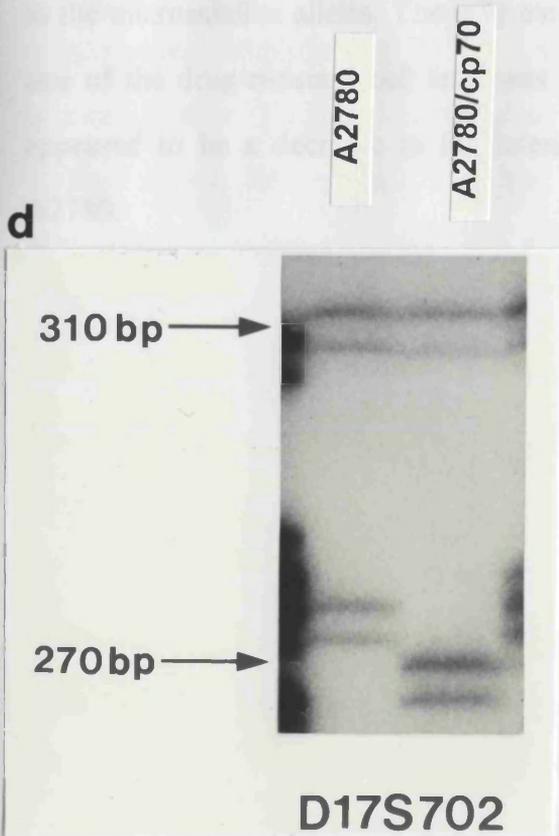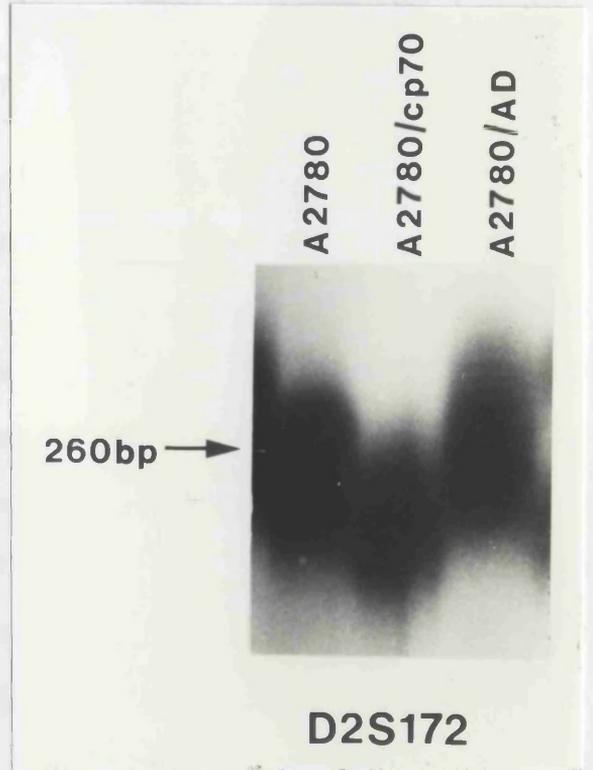
A2780/cp70 and A2780/AD are shown in Figure 3.1(b), (c) and (d). The microsatellite D17S351 (Figure 3.1(b)) shows the opposite picture to that of D17S183 with A2780/cp70 showing a gain of one repeat unit in the size of the larger allele as compared to both A2780/AD and the parental line.

The presence of changes in the repeat number is not confined to microsatellite repeats residing on the long arm of chromosome 17, of which the three previous loci are examples. D17S261 is on chromosome 17p and shows a smaller sized repeat in A2780/cp70 than in the parental line, (Figure 3.1(c)). Microsatellite mutation was also seen with the, theoretically, more stable tetranucleotide repeats as can be seen at locus D17S702 in A2780/cp70. Here the smaller of the two alleles has lost 3 repeat lengths (12bp) compared to that in the parental line, (Figure 3.1(d)). Not all the microsatellite loci studied on chromosome 17 showed differences when the three cell lines were compared. Table 3.1 reveals that A2780/AD only showed evidence of a microsatellite mutation at D17S579 with all other loci being identical to A2780. In the case of A2780/cp70 however six out of the ten loci studied showed evidence of length changes in the microsatellite alleles. The only evidence of LoH occurring at any of these loci in one of the drug-resistant cell lines was at locus D17S801 in A2780/AD where there appeared to be a decrease in the intensity of the shorter allele compared to that in A2780.

The microsatellite mutations detected at loci on chromosome 17 could potentially have arisen due to some specific feature of that chromosome and not represent an increased propensity for mutation throughout the genome as a whole. To determine whether the changes observed in chromosome 17 microsatellites were detected elsewhere, four microsatellite repeat sequences from chromosome 2 were subjected to the same type of analysis in the three cell lines. Previous studies on LoH in ovarian carcinoma samples have shown that chromosome 2 is often affected. Therefore supplementary information would potentially be available by using microsatellite repeats from this chromosome in the analysis of microsatellite mutation. Two of the

microsatellite repeats were chosen from the short arm of the chromosome and two from the long arm. As can be seen in figure 3.1(e) microsatellite mutations also arise on chromosome 2 . In this case the homozygous microsatellite repeat at D2S172 shows a reduction in the size of the repeat in A2780/cp70 but an increase in the allelic length in A2780/AD. Evidence for a mutation in the size of the microsatellite repeat at D2S123, which is on the opposite arm of chromosome 2 to D2S172, was also observed in A2780/cp70 (Table 3.1). The same repeat in A2780/AD was very difficult to amplify and no reproducible data was obtained. Therefore it would appear that the increased frequency of mutation at microsatellite repeat loci observed in A2780/cp70 and A2780/AD when compared to A2780 is not limited to chromosome 17 suggesting that it may be a feature of the genome as a whole.

3.2.2 Characterisation of microsatellite repeat loci in further tumour cell lines and drug resistant derivatives.

Although an increased frequency of microsatellite mutation was observed in the drug resistant derivatives of A2780 it was possible that this was an isolated feature of this cell lineage. To test this, the same range of microsatellite repeat sequences were studied in two different cell lines and sub-lines derived from them by selection for resistance to anti-cancer drugs. The cell line OV1/p (also known as IGROV1) was derived from tumour tissue taken from a previously untreated patient with a stage III ovarian carcinoma (Benard et al. 1985). OV1/DDP, a cisplatin resistant derivative of OV1/p, was derived by repeated exposure to increasing concentrations of cisplatin (Teyssier et al. 1989). The MCF7 human breast carcinoma cell line was derived from a metastatic breast cancer (Soule et al. 1973). The doxorubicin resistant line MCF7/AD was derived by selection with increasing concentrations of doxorubicin (Sinah et al. 1986).

The evidence that there are changes in the microsatellite repeats between the parental and drug-resistant derivatives for both these cell lines is shown in figures 3.2 and 3.3. In MCF7 and MCF7/AD the chromosome 17 microsatellite loci displayed are all homozygous, which was also true for all but one of the loci studied. The changes observed are predominantly due to increases in the size of the microsatellite repeat in the drug-resistant derivative line and tend to take one of two forms. This is either a shift in the size of the microsatellite allele by one repeat unit length or a more profound shift of two or greater repeat lengths. Such changes in microsatellite repeats have been termed Type II and Type I mutations respectively (Thibodeau et al. 1993). The mutations observed at loci D17S796 and D17S261 in MCF7/AD (Figure 3.2) are therefore of Type II whereas at CHRNB1 it is a Type I mutation. As with the A2780 derived cell lines the presence of microsatellite mutation is not confined to loci on chromosome 17. The homozygous microsatellite repeat locus at D2S121 shows a Type I mutation in the MCF7/AD cell line compared to the parental line.

As with the multi-drug resistant MCF7/AD, the cisplatin resistant cell line OV1/DDP also exhibits evidence of mutations at microsatellite loci when they are compared to the parental line OV1/p. Mutations are observed at microsatellite loci on chromosomes 2,3 and 17 which again suggests a global predisposition to microsatellite mutation. Type I and II microsatellite mutations are both observed in this cell line. Characterisation of the MCF7 and OV1/p derived cell lines with the complete set of microsatellite repeat sequences on chromosomes 2,3 and 17 used for analysis in A2780 lines shows frequent mutations (table 3.1). Six out of the twelve loci for which data is available in MCF7/AD show mutation whereas for OV1/DDP it is five out of fourteen. LoH is observed in both sets of drug resistant cell lines when compared to the parental lines. In MCF7/AD there is an obvious loss of an allele with the microsatellite repeat D2S172 which is on the long arm of chromosome two. This was the only one of all the microsatellite repeats used in the MCF7 lines to be heterozygous. OV1/DDP shows LoH at D2S121 on the short arm of the chromosome with the remaining allele also
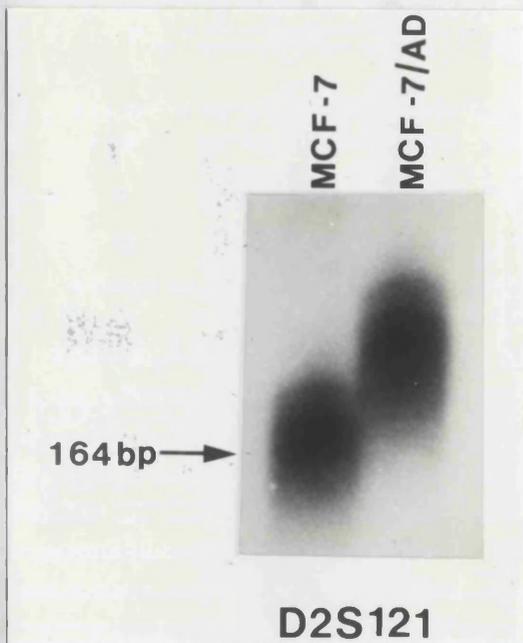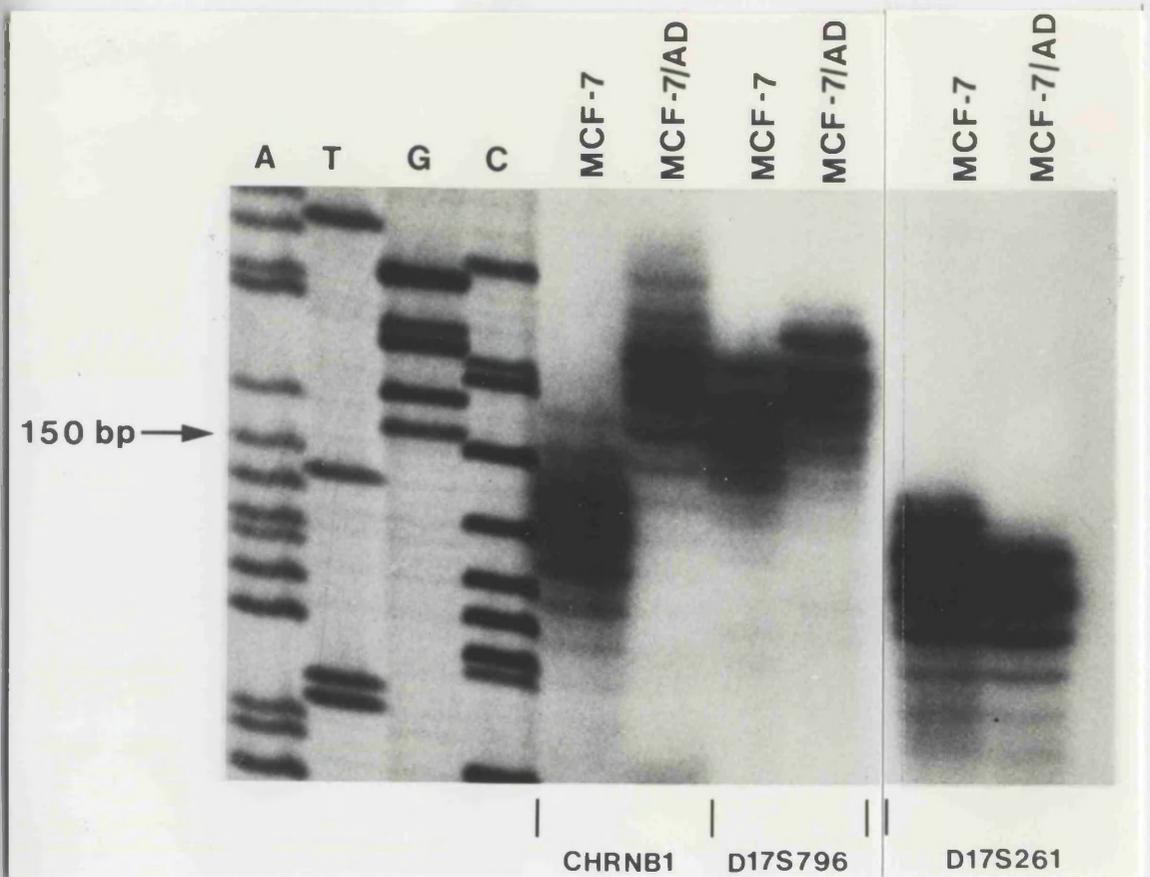
Figure 3.2 Mutations at microsatellite loci in the MCF7/AD cell line.

PCR amplification of microsatellite loci CHRNB1, D17S796, D17S261 D2S121 and D2S172, in DNA from MCF7 and MCF7/AD cell lines, shows evidence of mutation in the latter cell line. Type I (CHRNB1, D2S121) and II (D17S796, D17S261) microsatellite mutations are observed. Loss of heterozygosity is seen in D2S172 with no upper allele detected in MCF7/AD.
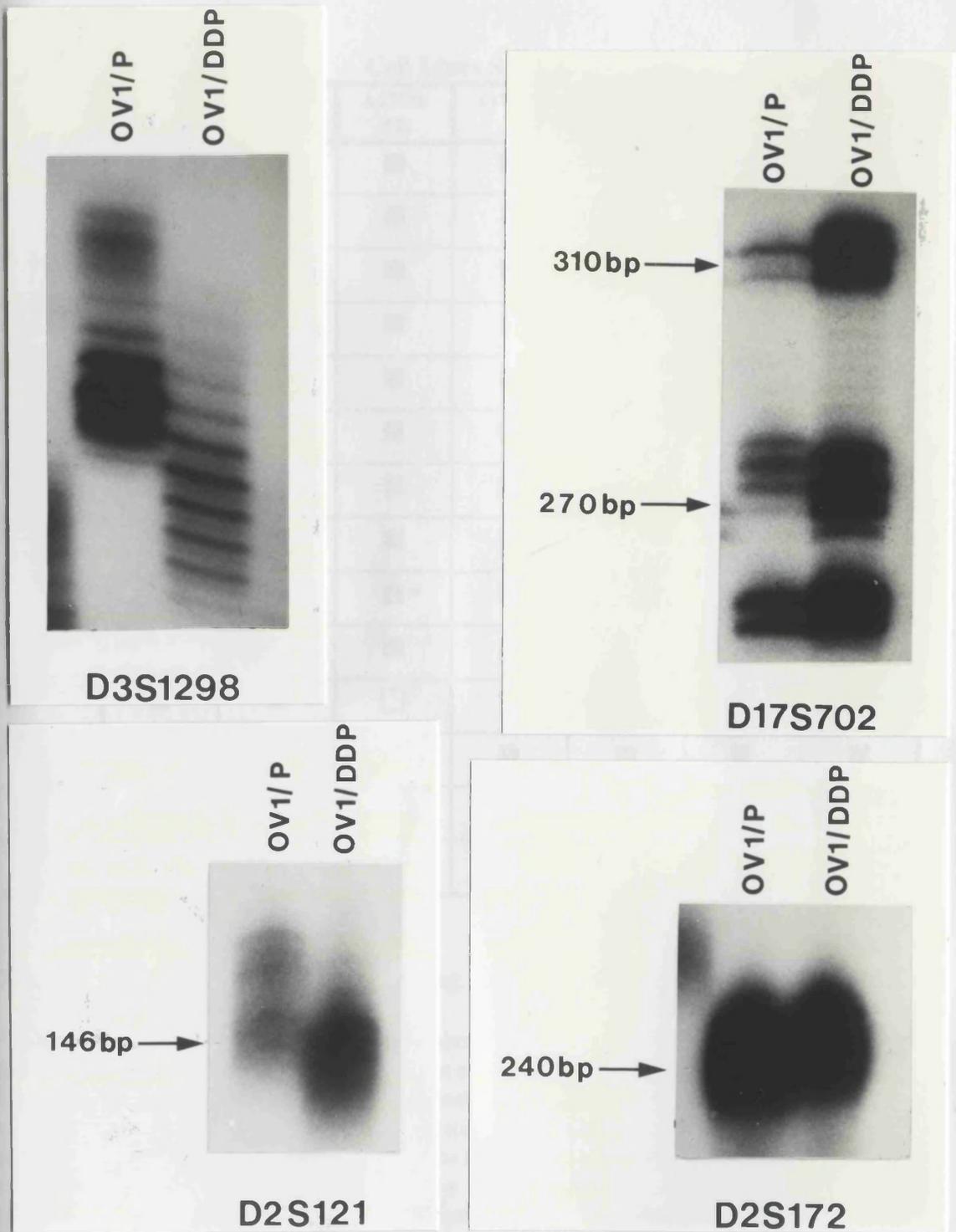
Figure 3.3 Mutations at microsatellite loci in the OV1/DDP cell line.

PCR amplification of microsatellite loci D3S1298, D17S702, D2S121 and D2S172 in DNA from OV1/p and OV1/DDP shows evidence of mutations in the latter cell line. As with MCF7/AD, type I (D3S1298) and type II (D17S702, D2S172) microsatellite mutations are observed. Loss of heterozygosity can be detected in D2S121 from the disappearance of the upper allele in OV1/DDP. The lower allele shows a shift in size compared to OV1/p indicating microsatellite mutation.

Cell Lines Studied

	A2780	A2780/cp70	A2780/AD	OV1/p	OV1/DDP	MCF-7	MCF-7/AD
CHRNB-1	WT ✓	M	WT	WT	WT	WT	M
D17S796	WT ✓	WT	WT	WT	WT	WT	M
D17S261	WT	M	WT	WT	M	WT	WT
D17S856	WT ✓	WT	WT	WT	WT	N/A	N/A
D17S855	WT ✓	WT	WT	WT	M	N/A	N/A
D17S183	WT ✓	M	WT	WT	WT	WT	M
D17S579	WT	M	M	WT	WT	WT	WT
D17S351	WT ✓	M	WT	WT	WT	WT	WT
D17S801	WT ✓	M	WT *	WT	WT	WT	M
D17S193	WT ✓	WT	WT	WT	WT	WT	M
D2S123	WT	M	N/A	WT	WT	WT	WT
D2S172	WT	M	M	WT	M	WT	WT
D2S121	WT	N/A	N/A	WT ✓	M *	WT ✓	M *
D3S1298	WT	N/A	N/A	WT	M	WT	WT

Table 3.1 Microsatellite mutations in drug resistant cell-lines.

The microsatellite repeats at each of the loci above were amplified using the PCR primers detailed in Table 2.1 & 2.2, as discussed in the methods section. Evidence for microsatellite mutation was taken as a shift in the size of one or both alleles compared to those of the parental line after electrophoresis on a denaturing polyacrylamide gel. Loss or reduction in intensity of one allele in a heterozygous repeat was taken to represent a loss of heterozygosity. The majority of experiments were repeated to ensure that any variation between alleles was not an artefact of the PCR process. Subtle changes arising between alleles were independently assessed before final classification.

Legend:

- ■ (blue) = Wild type microsatellite allele
- ■ (red) = Mutant microsatellite allele present as compared to wild type
- □ = Data unavailable
- ✓ = Represents heterozygous locus potentially informative for LoH. All other loci were homozygous and thus uninformative.
- * = Represents LoH at locus indicated.

displaying a decrease in the size of the repeats suggesting microsatellite mutation. About half of the microsatellite repeat loci in OV1/p were heterozygous and thus potentially informative for LoH.

3.2.3 Determination of the microsatellite instability phenotypes in A2780 and A2780/cp70.

As seen in table 3.1, the cisplatin resistant cell line A2780/cp70 shows mutations in eight of the twelve microsatellite repeat loci studied, when compared to A2780. This was a greater percentage of mutated microsatellite loci than found in any of the other drug-resistant cell lines, when compared to the parental lines. In view of this, and the fact that a great deal of experience had been gained with the A2780 and A2780/cp70 cell lines in our group, it was decided to use these lines for further characterisation of the microsatellite mutation phenotype.

An increased frequency of microsatellite mutation is termed microsatellite instability and several primary ovarian carcinoma cell lines have been shown to display this feature (Orth et al. 1994). The detection of instability in A2780/cp70, upon direct comparison of microsatellite repeats with A2780, does not eliminate the possibility that A2780/cp70 is displaying a phenotype of instability already present in the parental line. Therefore to determine whether the presence of microsatellite instability was a feature of the A2780 cell line, or had potentially been acquired during the development of the cisplatin resistant A2780/cp70, use was made of a sub-clone analysis. Sub-clones of a cell line with a phenotype of microsatellite instability are likely, with further growth, to continue to develop microsatellite mutations at a detectable frequency. Sub-clones from a cell line without this phenotype should not.

Fifteen sub-clones of A2780 and 20 sub-clones of A2780/cp70 were derived by isolating individual clones, at random, from sub-confluent cultures of either cell line, as described in chapter 2. The DNA from the different sub-clones was analysed for

evidence of microsatellite mutation by PCR amplification at four microsatellite repeats on chromosome 17, four on chromosome 2, two on chromosome 6 and one on chromosome 3. The size of the resultant microsatellite alleles were then compared to those from the cell line from which the sub-clones had originated. The microsatellite repeat loci on chromosomes 17,2 and 3 were those used previously in the analysis of the drug resistant cell lines. The four loci from chromosome 17 were all (CA)n repeats and were chosen by the fact that the had consistently amplified well by PCR. Three of the repeats (CHRNB1, D17S183 and D17S351) had all shown microsatellite mutations in A2780/cp70 as compared to the A2780 locus. D17S796 had shown the same sized repeat in both cell lines. Figure 3.4 shows the microsatellite repeats D17S796 and D17S183 at the fifteen A2780 sub-clones, as well as in the original A2780 clone from which they were derived. The sub-clones are marked as A2780 p1 to p15. In D17S796 the heterozygous nature of the repeat is observed in all the lanes, with fainter stutter bands observed around a distinct band representing the microsatellite allele. A similar picture is observed at microsatellite D17S183. Taking into account minor artefactual variations there is no evidence of any microsatellite mutation having occurred in any of the A2780 sub-clones as compared to the original clone. Analysis of all eleven microsatellite repeats in the A2780 sub-clones did not detect any alterations in the size of the repeats as compared to the original A2780 clone (table 3.2).

Analysis of A2780/cp70 and the twenty sub-clones isolated from it was performed in the same manner using the panel of eleven microsatellite repeat sequences. The results of amplifying the A2780/cp70 sub-clones at the microsatellite loci D17S183 and D17S796 are demonstrated in Figures 3.5. With D17S183 all but two of the sub-clones have produced products after PCR. The size of the microsatellite alleles in this heterozygous locus are identical to that of the original A2780/cp70 line (as indicated by the arrows) in all but two of the sub-clones; numbers 6 and 20. In these two lines there is an identical reduction in the size of the smaller allele of the repeat by one repeat unit length. More typical of the results seen with the A2780/cp70 sub-clones, locus D17S796
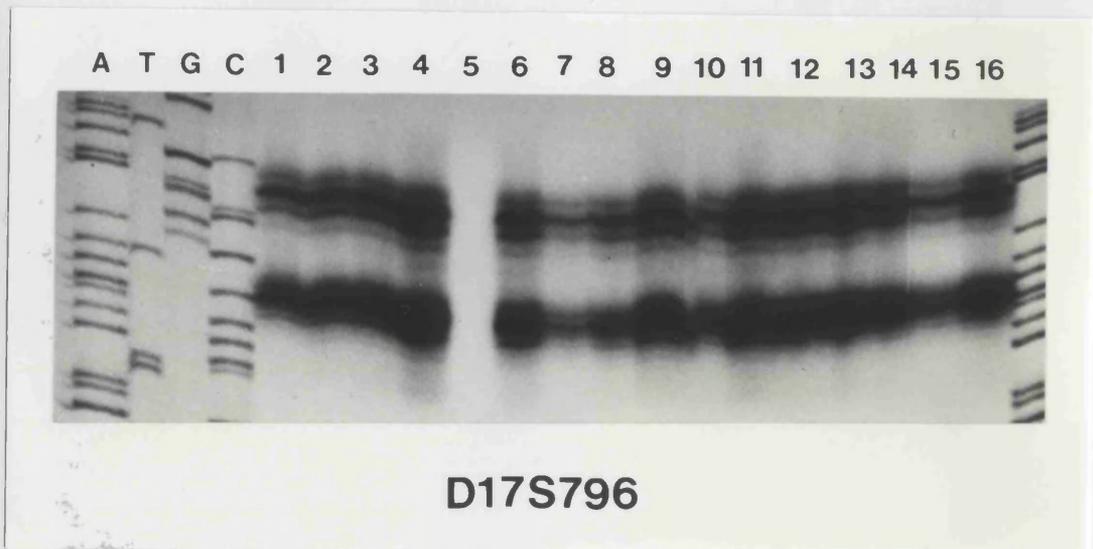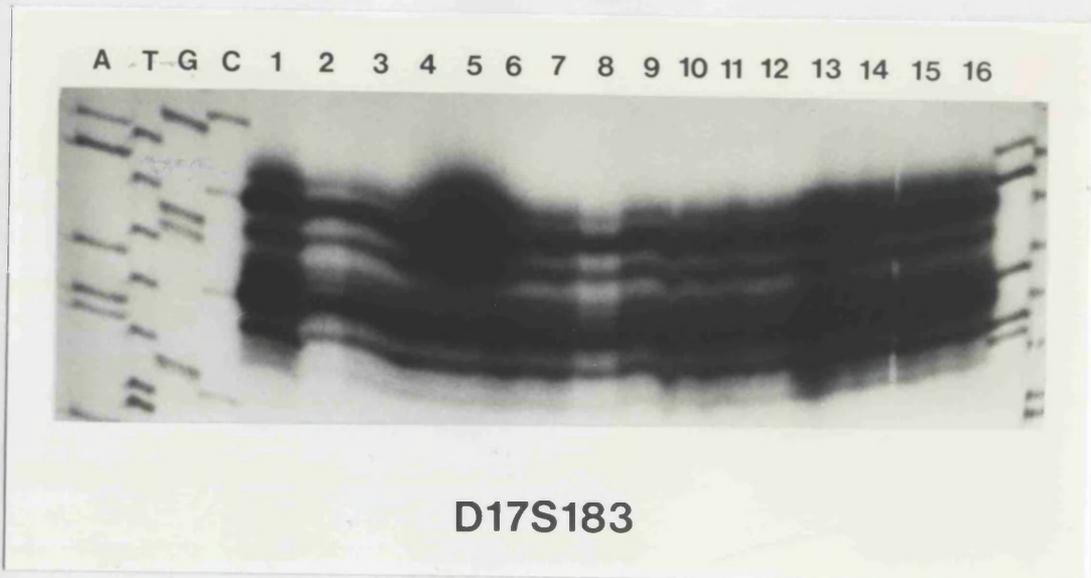
D17S183

D17S796

Figure 3.4 Analysis of random A2780 sub-clones for microsatellite mutation.

PCR amplification of D17S183 and D17S796 microsatellite loci in DNA from fifteen randomly isolated sub-clones of A2780. No difference is observed in the size of the microsatellite alleles between any of the sub-clones or with parental A2780 DNA. Sub-clone 5 in D17S796 did not amplify.

The M13 phage sequence ladder allows accurate comparison of allele sizes.

Legend	A,T,G,C,	M13 phage sequence ladder
	Lane 1 - 15	Sub-clones of A2780
	Lane 16	parental A2780
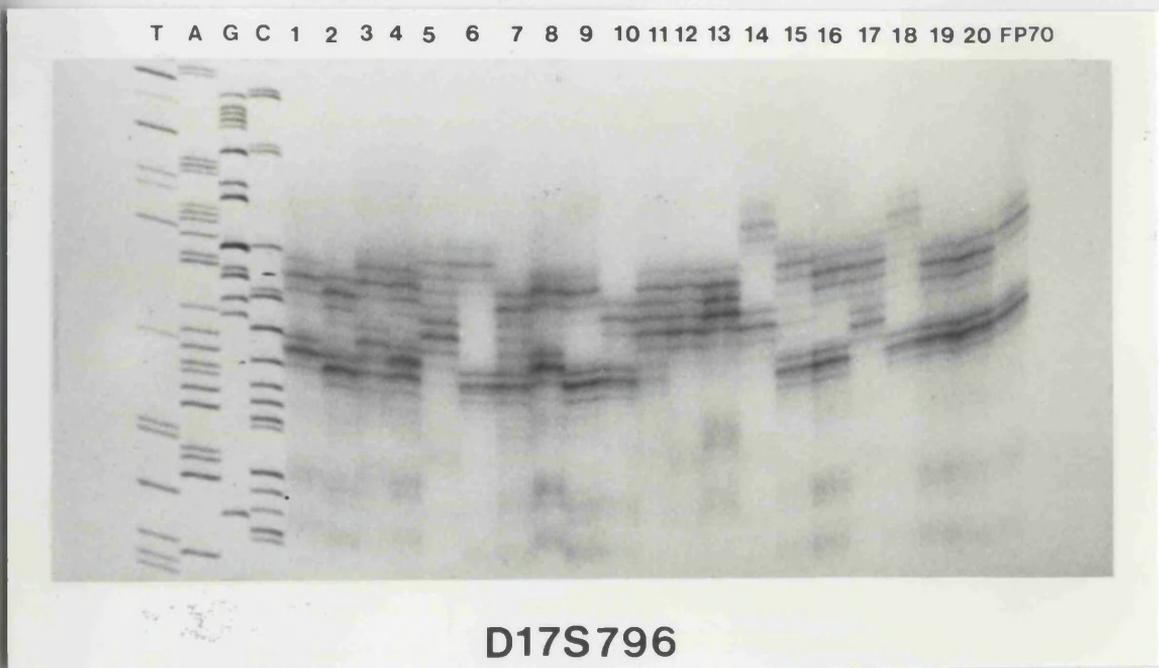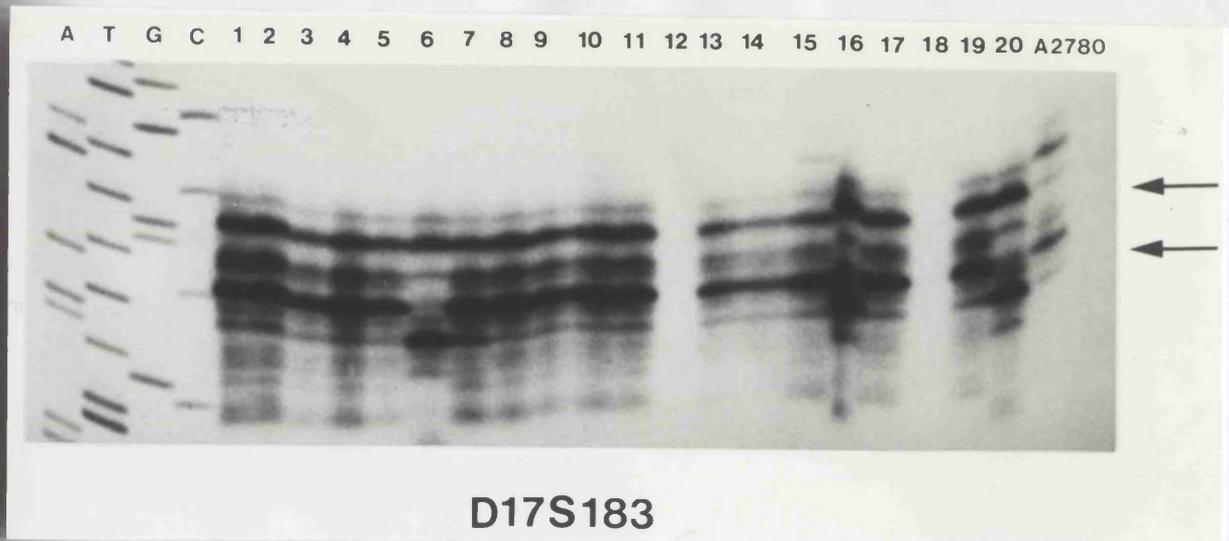
D17S183

D17S796

Figure 3.5 Analysis of random A2780/cp70 sub-clones for microsatellite mutation

PCR amplification of D17S183 and D17S796 microsatellite loci in DNA from twenty randomly isolated sub-clones of A2780/cp70. Evidence of mutation can be observed in sub-clones by comparison with the size of the microsatellite alleles in parental A2780/cp70, (A2780/cp70 allele size, in D17S183, is represented by arrows). Sub-clones 12 & 18 in D17S183 did not amplify with PCR.

Legend   A,T,G,C,   M13 phage sequence ladder.

Lanes 1 - 20   Sub-clones of A2780/cp70

A2780 sub-clones.

CHRNB-1	D17S351	D17S183	D17S796	D2S172	D2S111
0/15	0/15	0/15	0/15	0/15	0/15

D2S123	D2S121	D6S311	D6S274	D3S1296
0/15	0/15	0/15	0/15	0/15

A2780/cp70 sub-clones.

CHRNB-1	D17S351	D17S183	D17S796	D2S172	D2S111
15/20	15/19	2/18	15/20	N/D	11/11

D2S123	D2S121	D6S311	D6S274	D3S1296
17/18	ND	17/18	ND	19/19

Table 3.2.   Incidence of microsatellite mutation in independent, non-selected, sub-clones of A2780 and A2780/cp70.

DNA from 15 random sub-clones of A2780 and 20 random sub-clones of A2780/cp70 was amplified, by PCR, using primers specific for the microsatellite repeat sequences indicated. The numerator for each microsatellite locus indicates the number of sub-clones displaying mutation in the size of the microsatellite repeat. This is by comparison to the microsatellite in the parental cell line. The denominator represents the number of sub-clones for which assessable data was obtained.

ND = data not obtained for this microsatellite locus.

reveals an extremely high degree of microsatellite mutation as compared to the original line. Five of the sub-clones retain the same allele sizes as A2780/cp70 whereas the remaining fifteen show type I (e.g. sub-clone 18) or type II (e.g. sub-clone 6) mutations. With most of the sub-clones the mutation only occurs in one allele and this is most commonly in the larger allele. This phenomenon of more frequent mutation occurring in the larger allele of a heterozygous locus was also observed by Orth et al. (Orth et al. 1994) when looking at sub-clones of an ovarian carcinoma cell line with a chromosome 17 specific microsatellite. Sub-clone 14 shows an alteration in the size of both microsatellite alleles, both being increased in size, whereas in all other cases the mutation occurs in only one allele. A high frequency of alterations in the size of the microsatellite alleles in the A2780/cp70 sub-clones was observed at all the other loci studied, (Table 3.2).

Therefore, to summarise the results obtained from sub-clone analysis from A2780 and A2780/cp70; no size alterations in the microsatellite repeats was observed in the 165 loci studied in A2780 sub-clones. With A2780/cp70 sub-clones, however, over 70% of the microsatellite loci analysed showed evidence of mutation. It would therefore appear that A2780 does not possess a phenotype of microsatellite instability which would suggest from the results with A2780/cp70 and A2780/AD (Table 3.1) that the instability seen in these lines has arisen at some point in their derivation from the parent line. The high frequency of mutation seen with A2780/cp70 sub-clones would tend to confirm the presence of a microsatellite instability phenotype associated with this cell line.

3.2.4   Characterisation of the role of p53 in development of microsatellite instability in A2780 derived cell lines.

The demonstration that the phenotype of microsatellite instability, also classified as a replication error (RER+) phenotype, was acquired by the cisplatin and doxorubicin

resistant derivatives of A2780 during their development, raised the question of how such a change in genomic stability had arisen. The p53 tumour suppressor gene is known to be involved in maintaining the integrity of genomic DNA prior to entry into S-phase or mitosis . Mutations in the p53 gene are associated with an increase in genomic instability usually in the form of aneuploidy, damaged chromosomes or gene amplification (Tainsky et al. 1995). A2780 has been shown to possess functionally normal p53 with wild type sequence whereas, although A2780/cp70 has wild type sequence it displays abnormal p53 function (Brown et al. 1993). The possibility arose, therefore, that the RER+ phenotype observed in A2780/cp70 was a variant of genomic instability consequent on the abnormal p53 function.

The dominant negative effect displayed by mutants of p53 on wild-type protein function, was used to determine whether p53 mutation could play a role in the development of an RER+ phenotype in A2780/cp70. p53 cDNA under the control of a CMV promoter, and containing a mutation at codon 143 resulting in the substitution of alanine for valine (Baker et al. 1990), has previously been stably transfected into A2780 cells by calcium phosphate precipitation (Brown et al. 1993). Transfection of this particular mutant p53 into cells containing wild-type p53, has been shown to abolish the G1 arrest occurring after ionising radiation and has been suggested to act in a dominant negative manner to inactivate the wild-type p53 function (Kuerbitz et al. 1992). It was hypothesised that, if loss of p53 function was in some way responsible for the development of an RER+ phenotype, then these mutant p53 transfectants should display evidence of microsatellite instability when compared to wild-type A2780. The RER+ status of the mutant p53 transfected A2780 was determined by analysis of seven microsatellite repeat sequences, in fourteen random non-selected sub-clones of the original transfected line. These sub-clones have been shown to express mutant p53 by ELISA and to have defective p53 function by loss of inhibition of DNA synthesis after ionising radiation (McIlwrath et al. 1994a). The results from two of the microsatellite repeat loci, D17S796 and D17S351, are displayed in Figure 3.6. There is no evidence of

D17351
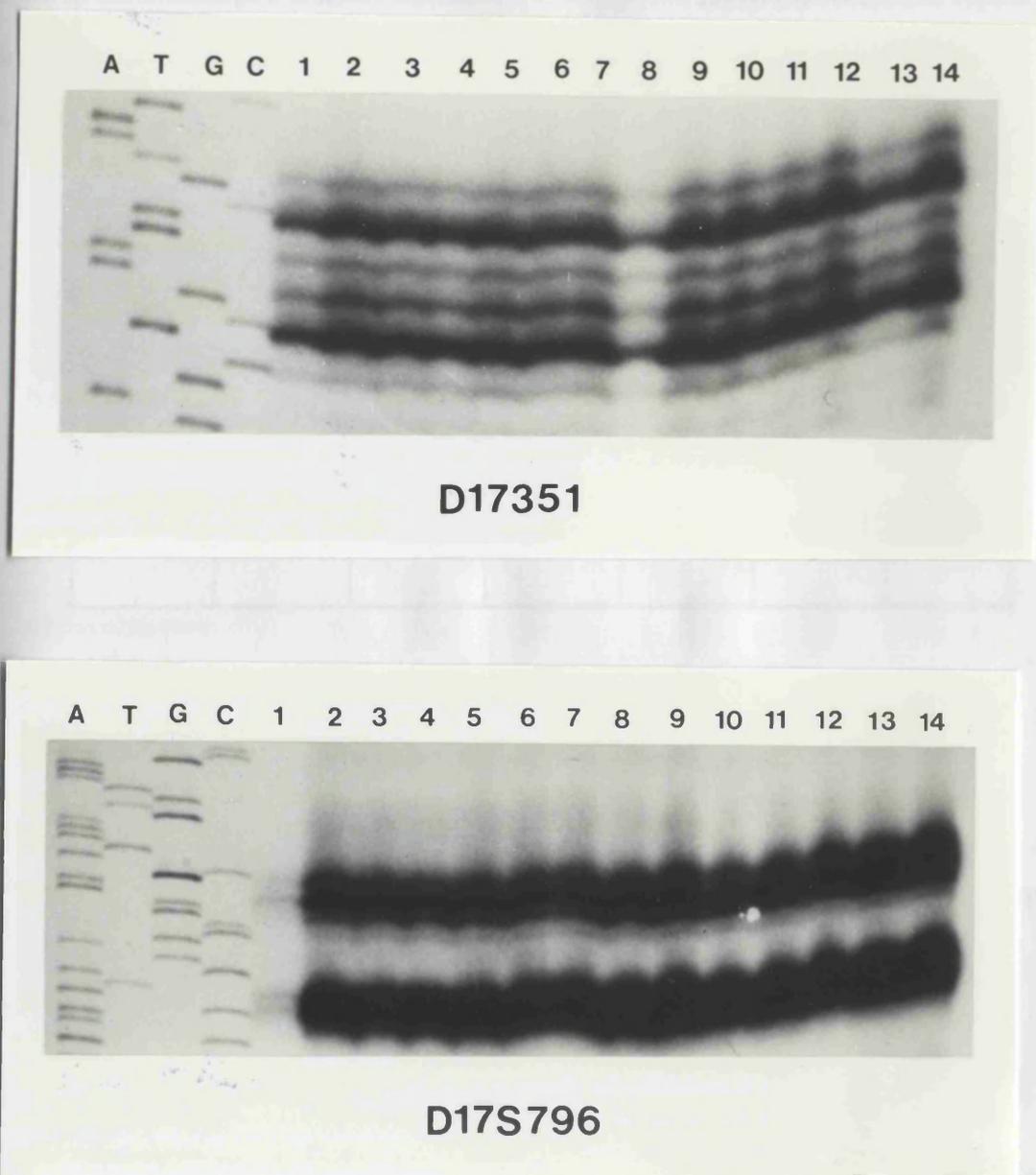
D17S796

Figure 3.6 Analysis of the effect of mutant p53 on microsatellite stability in A2780.

PCR amplification of microsatellite loci at D17S351 and D17S796, in DNA from 14 mutant p53 transfectants of A2780. Each of the transfectants has been shown to express mutant p53 and have defective p53 function. No evidence of microsatellite mutation is observed between the transfectants, all of which have identical microsatellite alleles to parental A2780.

Legend   A,T,G,C,   M13 phage sequence ladder.

Lanes 1 -14   Mutant p53 transfectants of A2780.

alteration in the size of the microsatellite repeat alleles in either of these loci, when compared to the wild-type A2780. Table 3.3 shows that no evidence of mutation was found in the mutant p53 transfectant clones at any of the seven microsatellite repeat loci. The lack of microsatellite instability in cells with no functioning p53 suggests that it is not responsible for this form of genomic instability.

Microsatellite loci

CHRNB1	D17S181	D17S183	D17S796	D6S311	D2S172	D2S121
0/13	0/14	0/14	0/14	0/13	0/8	0/10

Table 3.3   Incidence of microsatellite mutation in clones of A2780 transfected with mutant p53.

Numerator in each lane represents number of mutant microsatellite alleles detected compared to wild type A2780. Denominator represents number of clones for which results obtained.

3.2.5   Incidence of microsatellite mutation in A2780 cell lines selected for resistance by single exposure to cisplatin.

A2780/cp70 cells have been repeatedly grown in culture since their initial derivation. It is possible that the development of the RER+ phenotype arose during prolonged growth in culture and was not directly related to the selection for resistance to cisplatin. To examine whether a relationship existed between acquisition of an RER+ phenotype and selection for resistance to cisplatin in A2780 cells, cells selected for cisplatin resistance by a single drug exposure were used. It has been shown previously that single step selection protocols reliably and quantitatively select for resistant cells in

a population (Thacker and Stretch, 1985). Clonogenic analysis of A2780 cells, given a single 24 hour exposure to cisplatin concentrations of between 15 μM and 30μM, has suggested the existence of a sub-population of resistant cells which appear as a tail on the survival curve (McLaughlin et al. 1991). It has subsequently been shown that these cells are, most probably, resistant due to the development of stable genetic mutations and that the resistant phenotype was maintained after growth in the absence of drug selection (McLaughlin et al. 1991). The advantage of using such single-step selected resistant clones is that, due to the short selection process, they have not had the opportunity to diverge genetically any more than, for example, the non-selectively isolated sub-clones of A2780 used in section 3.2. Therefore the identification of phenotypic differences in the resistant cells, as compared to parental A2780, are likely to correlate more strongly to the development of cisplatin resistance and not represent an epiphenomenon.

DNA was obtained from eleven previously derived single step selected, cisplatin resistant, A2780 clones (A2780/SCP1 to SCP11) (McLaughlin et al. 1991). These had been selected by a single exposure to 15μM cisplatin for 24 hours. PCR amplification was then performed using primers for ten microsatellite loci, four on chromosome 17, three on chromosome 2 and three on chromosome 6. During the initial investigation of the single-step selected clones it became clear that A2780/SCP11 was in fact the same line as A2780/SCP5 but was included in subsequent analyses to control for reproducibility.

Of the eleven A2780/SCP clones only three displayed microsatellite mutation. Two of the clones, A2780/SCP2 and SCP5, showed an alteration in the size of the CHRNB1 microsatellite repeat on chromosome 17 as shown in figure 3.7(a). The increase in the size of the larger allele observed in A2780/SCP5 is also seen with SCP11 (not shown). Multiple microsatellite mutations at loci on all three chromosomes studied were observed with A2780/SCP6. Figure 3.7 (a-d) shows the alterations seen at CHRNB1, D17S796, D2S172 and D6S261 when comparing A2780/SCP6 with A2780.

Figure 3.7 Mutations at microsatellite loci in A2780/SCP cell lines.

PCR amplification of microsatellite loci D17S796, CHRNB1, D2S172 and D6S261 in cisplatin resistant A2780/SCP cell lines. These lines were derived from A2780 by a single exposure to 15μM cisplatin for 24 hours. Alterations in the size of microsatellite alleles compared to those in A2780 are indicative of mutation.

Legend

D17S796 & CHRNB1:- Lanes 1 & 5:- A2780; Lane 2:- A2780/SCP2
        Lane 3:- A2780/SCP5; Lane 4:- A2780/SCP6.

D2S172: Lane:- (1) A2780/SCP1; (2) A2780/SCP2; (3) A2780/SCP3; (4) A2780/SCP4;
      (5) A2780/SCP2; (6) A2780/SCP5; (7) A2780/SCP6; (8) A2780/SCP7;
      (6) A2780/SCP8; (10) A2780/SCP9; (11) A2780/SCP10; (12) A2780/SCP6*
      (13) A2780

D6S261: Lane:- (1) A2780/SCP ; (2) A2780/SCP ; (3) A2780/SCP6; (4) A2780/SCP6*
      (5) A2780/SCP ; (6) A2780/SCP ; (7) A2780/SCP ; (8) A2780/SCP ;
      (9)A2780/SCP ; (10) A2780/SCP ; (11) A2780/SCP ; (12) A2780 .

A2780/SCP6* represents DNA extracted from a later passage of the A2780/SCP6 cell line.
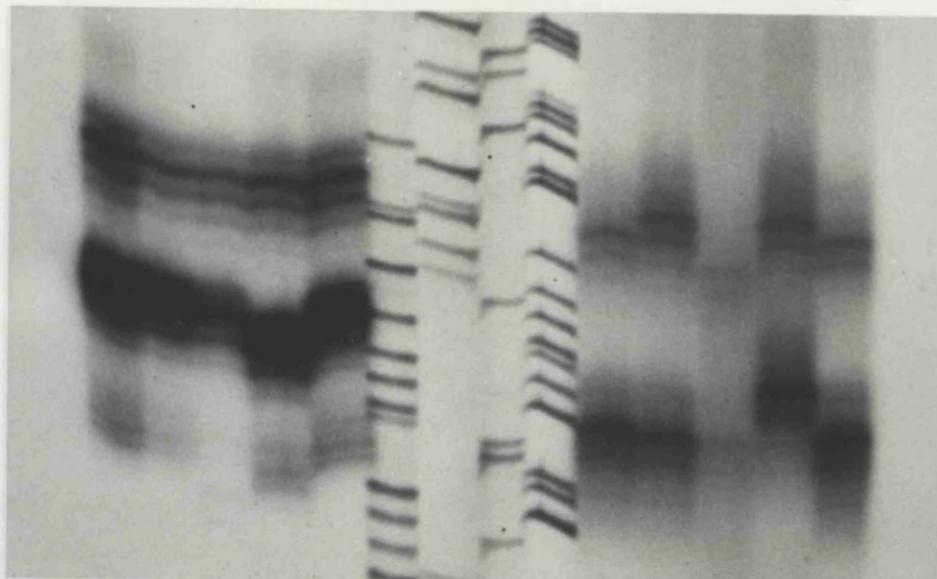
D17S796   CHRNB1
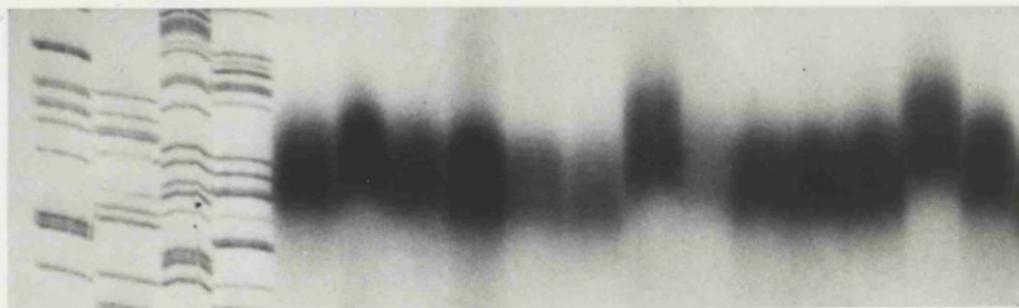
D2S172

D6S261

In amplification of the microsatellite loci on chromosome 2 and 6 a second, independently extracted, sample of A2780/SCP6 DNA was amplified to ensure the reproducibility of the observed mutations. The results of all the microsatellite loci amplified in the A2780/SCP clones is presented in table 3.4, although the data from the repeat sample of A2780/SCP6 and from A2780/SCP11 is left out of the final analysis. Of the ten single-step selected clones studied, seven showed no change in the size of microsatellite repeat length at any of the ten microsatellite loci when compared to the parental A2780. A2780/SCP2 and A2780/SCP5 both display a microsatellite mutation at just one locus. An alteration in the size of a microsatellite repeat allele is observed in six of the ten loci studied with the A2780/SCP6 cell-line. This high frequency of microsatellite mutation is consistent with A2780/SCP6 displaying an RER+ phenotype as classified by accepted criteria (Thibodeau et al. 1993). Although showing microsatellite mutation at one locus, A2780/SCP2 and SCP5 do not, with respect to the information obtained, fulfil the RER+ criteria. The data presented in this section, therefore, indicates that an RER+ phenotype can be selected for, along with that of cisplatin resistance, by a single exposure to a high concentration of the drug.

3.3 DEVELOPMENT OF CISPLATIN RESISTANT A2780 CELL-LINES BY SELECTION AGAINST INCREASING CONCENTRATIONS OF CISPLATIN.

The data presented in the previous section suggests an association between cisplatin resistance in A2780 cells and the development of an RER+ phenotype. To enable further analysis of this proposed association a new set of cisplatin resistant derivatives were developed from a clonal population of A2780 cells (for detailed method see chapter 2.8.2). Starting with a clonal population of A2780 should reduce the influence that genetic differences between the cells have on the development of a resistant phenotype. Variations in the drug induced selection pressure on each cell line were kept to a minimum by running each drug selection in parallel. The concentration of

Microsatellite locus

Legend symbols used below: ■ = microsatellite alleles identical to A2780 wild type; ■(mut) = mutant microsatellite alleles; □ = data unavailable.

	CHRNB1	D17S351	D17S183	D17S796	D2S111	D2S172	D2S121	D6S311	D6S261	D6S274
A2780/ SCP1	■	□	■	■	■	■	□	■	■	■
A2780/ SCP2	■(mut)	□	■	■	■	■	□	■	■	■
A2780/ SCP3	■	■	■	■	■	■	■	■	■	■
A2780/ SCP4	■	■	■	■	■	■	■	■	■	■
A2780/ SCP5	■(mut)	■	■	■	■	■	■	■	■	■
A2780/ SCP6	■(mut)	■	■	■(mut)	■(mut)	■(mut)	■(mut)	■	■(mut)	■
A2780/ SCP7	■	■	■	■	■	■	■	■	■	■
A2780/ SCP8	■	■	□	■	■	■	■	■	■	■
A2780 SCP9	□	■	□	■	■	■	■	■	■	■
A2780 SCP10	■	■	■	■	■	■	■	■	■	■

Table 3.4. Microsatellite mutation occurring in clones of A2780 selected for resistance by single exposure to 15μM cisplatin for 24hr.

This table displays the incidence of microsatellite repeat sequence mutation, at the loci indicated, in independent cell lines derived from A2780 by selection with a single exposure to 15μM cisplatin for 24hr. Mutations were identified by a shift in the size of PCR amplified microsatellite DNA from the cell line as compared to that from parental A2780.

Legend:

■ = Microsatellite alleles identical to A2780 wild type.

■ = Mutant microsatellite alleles, as compared to A2780 wild type.

□ = Data unavailable.

cisplatin used for the initial drug selection was that which had been shown previously to give a surviving fraction of between 20% and 30% for A2780 (McLaughlin et al. 1991). This was a drug exposure of 1μM cisplatin for 24 hours. Further steps of selection against increasing doses of cisplatin were performed to a final exposure of 15μM cisplatin for 24 hours. At this stage the surviving cells in each of the nine derived lines (A2780/MCP1-9) were maintained in cisplatin free medium, with frozen stocks being suspended in medium containing 10% DMSO.

3.4   MEASUREMENT OF LEVEL OF RESISTANCE TO CISPLATIN IN A2780 DERIVED CELL-LINES.

Correlation of the development of an RER+ phenotype with selection for drug resistance in A2780 requires not only evidence of microsatellite instability but also proof of stable resistance to cisplatin in the derived cell lines. To this end the resistance to cisplatin of the A2780 derived lines; A2780/cp70, A2780/MCP1 to MCP9 and A2780/SCP5, 6 and 3 were determined and compared to the parental line. Two different techniques were used in analysis of the cisplatin resistance of the A2780 derived lines.

3.4.1 Clonogenic cell survival assay.

One method of determining the level of cisplatin resistance of the derived A2780 cell-lines is to assay cell survival after exposure to cisplatin. This assay depends on the demonstration of the reproductive integrity of the surviving cells, as evidenced by clonogenicity. To increase the information on cisplatin resistance obtained by this analysis two different cisplatin exposures were used in the clonogenic assay. One set of data were obtained using exposure to a high concentration of cisplatin over a short period of time (20μM for 1 hour). There is some evidence that this may be similar to the drug exposure experienced by tumour cells *in vivo* after intra-venous administration

(Wilson, 1992). The other data was obtained using exposure to a lower concentration of cisplatin for a longer period of time (1μM for 24 hours). Three separate clonogenic assays were performed using the lower cisplatin exposure and two assays at the higher exposure. Determination of the stability of any cisplatin resistance observed, under growth in the absence of a selection pressure, was also attempted. To this end, for a particular level of cisplatin exposure, each successive clonogenic assay performed on an individual cell-line used cells that had undergone further growth in the absence of cisplatin.

The cumulative data on all clonogenic survival assays is presented in Figure 3.8 and in Table 3.5. Figure 3.8(a) displays, in diagrammatic form, the fraction of cells surviving exposure to 1μM cisplatin / 24 hours compared to non-treated cells for cell lines A2780, A2780/cp70 and A2780/MCP1 to MCP9. Each column represents the mean of three experiments, consisting of at least 4 plates per cell line. A2780 shows the smallest surviving fraction indicating that it is more sensitive to the cytotoxic effects of this concentration of cisplatin than are the other lines. A2780/cp70 cells exposed to cisplatin show no significant difference in survival as compared to no drug controls. It also displays the highest surviving fraction of any of the cell lines suggesting that it possesses the greatest resistance to this level of cisplatin exposure. The difference in survival between A2780/cp70 and A2780 cells at 1μM cisplatin / 24 hours is highly significant using paired t-test analysis (p = 0.001). The A2780/MCP cell lines have surviving fractions intermediate between A2780 and A2780/cp70 indicating that they have greater resistance to this level of cisplatin exposure than A2780. A2780/MCP6 displays the greatest surviving fraction whilst A2780/MCP9 displays the least, although this is still approximately 50% greater than that for A2780. Using the paired t-test method the difference in surviving fraction between each of the A2780/MCP cell lines and A2780 is highly significant (p values all < 0.001).

The mean surviving fractions of the cell lines after exposure to 20μM cisplatin / 1 hour is displayed in figure 3.8(b). A2780 is again the most sensitive cell-line to this
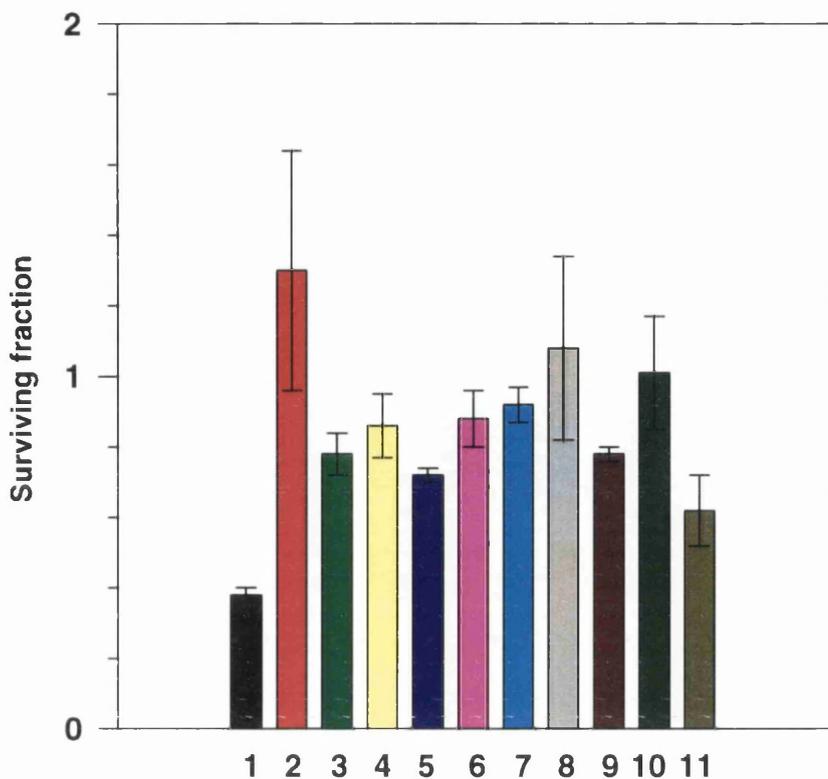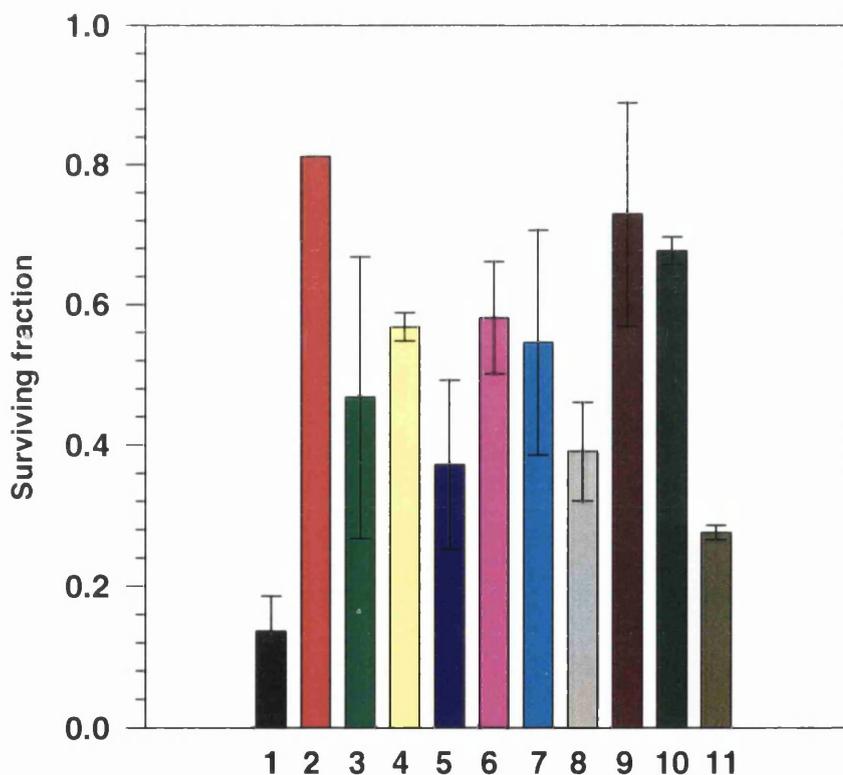
Figure 3.8 Clonogenic determination of cisplatin resistance in A2780 and derived cell lines.

For each cell line the mean survival fraction was determined by comparing the number of colonies formed in replicates of cisplatin treated cells compared to non-treated controls. Error bars represent one standard error of the mean.

level of cisplatin although with about three times less cells surviving than for the 1μM cisplatin exposure. The mean surviving fraction to 20μM cisplatin / 1 hour is reduced for all of the cell-lines, suggesting that this cisplatin exposure was more cytotoxic to the A2780 derived cells than 1μM over 24 hours. As before, A2780/cp70 shows the greatest surviving fraction whilst the A2780/MCP cell lines display surviving fractions intermediate between that of A2780 and A2780/cp70. Therefore, at this different level of cisplatin exposure the A2780/MCP cell lines display greater resistance than A2780. The surviving fraction of each replicate, of each cell line exposed to 20μM cisplatin / 1 hour, was compared by paired t-test analysis to that of the replicates of A2780. The differences in surviving fraction observed were all highly significant (p < 0.001).

The relative resistance to cisplatin for each of the cell-lines when compared to A2780 is shown in Table 3.5. This, as would be expected, confirms the data in figure 3.8 and shows that A2780/cp70 has the highest degree of resistance to cisplatin at both levels of exposure. The differences in relative resistance between the A2780/MCP cell-lines tend to be similar at both levels of cisplatin exposure. Cell-lines with low fold resistance to 1μM cisplatin also tend to be amongst those with the lowest fold resistance to 20μM cisplatin, e.g. A2780/MCP3. Those with the highest fold resistance at 1μM cisplatin also tend to be among the highest at 20μM cisplatin, e.g. A2780/MCP8. The mean plating efficiency was also calculated for the no-drug controls of each cell-line. Although there was a degree of variation, for most cell-lines it was in the range of 25 - 45%. In view of this there was no evidence that the results were influenced by the poor plating efficiency of cells in any particular cell-line.

3.4.2 MTT cell viability assay.

The results presented in the previous section indicate that the A2780/MCP cell lines are more resistant to cisplatin than the parental cell line, though not to the same degree as A2780/cp70. However, this data has been obtained using only two different

Cell Lines	Relative Resistance 1uM Cisplatin/24hr	Relative Resistance 20uM Cisplatin/1hr	Plating Efficiency
A2780	1	1	30.3%
A2780/cp70	3.3 +/- 0.6	8.7 +/- 0.0	43.4%
A2780MCP1	2.0 +/- 0.3	4.4 +/- 3.2	39.3%
A2780MCP2	2.2 +/- 0.3	4.5 +/- 1.4	44.4%
A2780MCP3	1.9 +/- 0.2	3.2 +/- 1.3	46.7%
A2780MCP4	2.3 +/- 0.1	4.6 +/- 1.3	48.2%
A2780MCP5	2.4 +/- 0.3	4.8 +/- 2.8	46.2%
A2780MCP6	2.7 +/- 0.6	3.0 +/- 0.5	36.8%
A2780MCP7	2.1 +/- 0.1	6.3 +/- 3.2	41.0%
A2780MCP8	2.5 +/- 0.4	5.5 +/- 2.0	44.6%
A2780MCP9	1.6 +/- 0.3	2.2 +/- 0.6	38.7%

Table 3.5 Relative cisplatin resistance of A2780 derived cell lines as compared to parental A2780.

The relative resistance to cisplatin of A2780/cp70 and A2780/MCP cell lines, as compared to A2780, was determined by clonogenic survival assay. Two different cisplatin exposures were used in the assays. The results displayed are the mean of at least two independent experiments (+/- 1 S.E.). To reduce error 4 or 5 repeats were performed per experiment, for both control and drug exposed cells. The plating efficiencies displayed represent an example of the values obtained throughout the clonogenic assays.

levels of cisplatin exposure. To further compare the differences in cisplatin resistance between the A2780/MCP cell lines and A2780, the sensitivity of cells from each line to a wide range of concentrations of cisplatin was measured. The eight different concentrations of cisplatin used consisted of serial 5-fold dilutions starting from 10^{-4} M. From the data obtained in each of these assays a graph of dose versus response to cisplatin can be plotted.

To characterise the curves of dose versus response for each of the cell-lines a non-clonogenic method, the MTT assay, was utilised. The reasons for this were essentially practical. To calculate dose/response curves using clonogenic assays for multiple cell-lines is logistically difficult, with regards to the culture of multiple replicates of each line for each drug concentration. The common answer to this problem is to perform the assay in batches of cell-lines. This can lead to differences in the exact amount of drug that the cells are exposed to due to batch to batch variation in stock solutions of the drug. This could be a particular problem with cisplatin as it has a low solubility in water at room temperature. Thus the ability to expose all replicates of the different cell-lines to the same batch of drug, at the different concentrations required, would remove this source of variation. The MTT assay makes this approach feasible. One theoretical drawback of non-clonogenic assays of drug resistance is that they cannot differentiate between cytostatic and cytotoxic effects of the drug. A clonogenic assay is dependent on the survival of cells with the ability to grow and divide in a clonal manner. Non-clonogenic assays often determine the viability of a cell, after exposure to a cytotoxin, by using a metabolic end-point. Cytotoxic drugs may slow the metabolic processes in a cell without killing it and hence lead to an overestimation of the resistance of a cell-line. This may be less of a worry when using cisplatin as the effects on tumour cells produced tend to be mainly cytotoxic.

The M.T.T. assay was used to determine the viability of the different cell-lines after exposure, for 24 hours, to cisplatin concentrations ranging from 10^{-4}M to 10^{-9}M. Only metabolically viable cells can reduce the yellow M.T.T. dye to a purple Formazan

product, whose concentration can be measured spectroscopically. For each cell-line, control cells to which no drug was added were included to allow calculation of the relative survival of viable cells. Figure 3.9 shows the dose/response curves for A2780, A2780/cp70 and all of the A2780/MCP cell-lines. Each curve represents the mean values obtained from one experiment with eight repeats per drug concentration. At very low concentrations of cisplatin there is a high level of cell survival with wide overlap between different lines. Very high concentrations of cisplatin (e.g. 0.1mM) are uniformly lethal for all the cell-lines. However, in the range of exposure from $0.16\mu M$ to $20\mu M$ cisplatin for 24 hours it can be seen clearly that the curves for A2780 and A2780/cp70 are separate and distinct from those of the A2780/MCP lines. A2780 shows the lowest cell survival across this range of cisplatin concentrations and there is no crossing-over with the plots of the other cell-lines. A similar situation is observed for A2780/cp70 except that it shows the highest levels of cell survival. The curves for the A2780/MCP cell-lines show levels of cell survival intermediate between that for the other two lines, confirming that they have developed resistance to cisplatin by the selection protocol although not to the level seen with A2780/cp70. There is a reasonable degree of overlap between the curves for the A2780/MCP cell-lines at these cisplatin concentrations. The standard errors have been calculated for the mean cell survival at each drug concentration in each of the cell lines. They suggest that the differences in cell survival, i.e. the resistance, seen between A2780 and A2780/cp70 is significant as there is no overlap between the error bars representing the standard error. This can also be seen for A2780 and the A2780/MCP cell lines. To confirm that the differences in resistance observed between the cell lines is statistically significant data from one particular drug dose point ($1.6 \times 10^{-7}M$)was chosen. The relative cell survival as compared to controls was calculated for each replicate in each of the cell lines. A one-sided, paired t-test was then used to compare the data from each of the cell lines as compared to A2780. The results from the A2780/cp70 and A2780/MCP cell lines all displayed highly significant results ($p < 0.01$) suggesting that the differences in cell survival observed are real and not arising by chance.
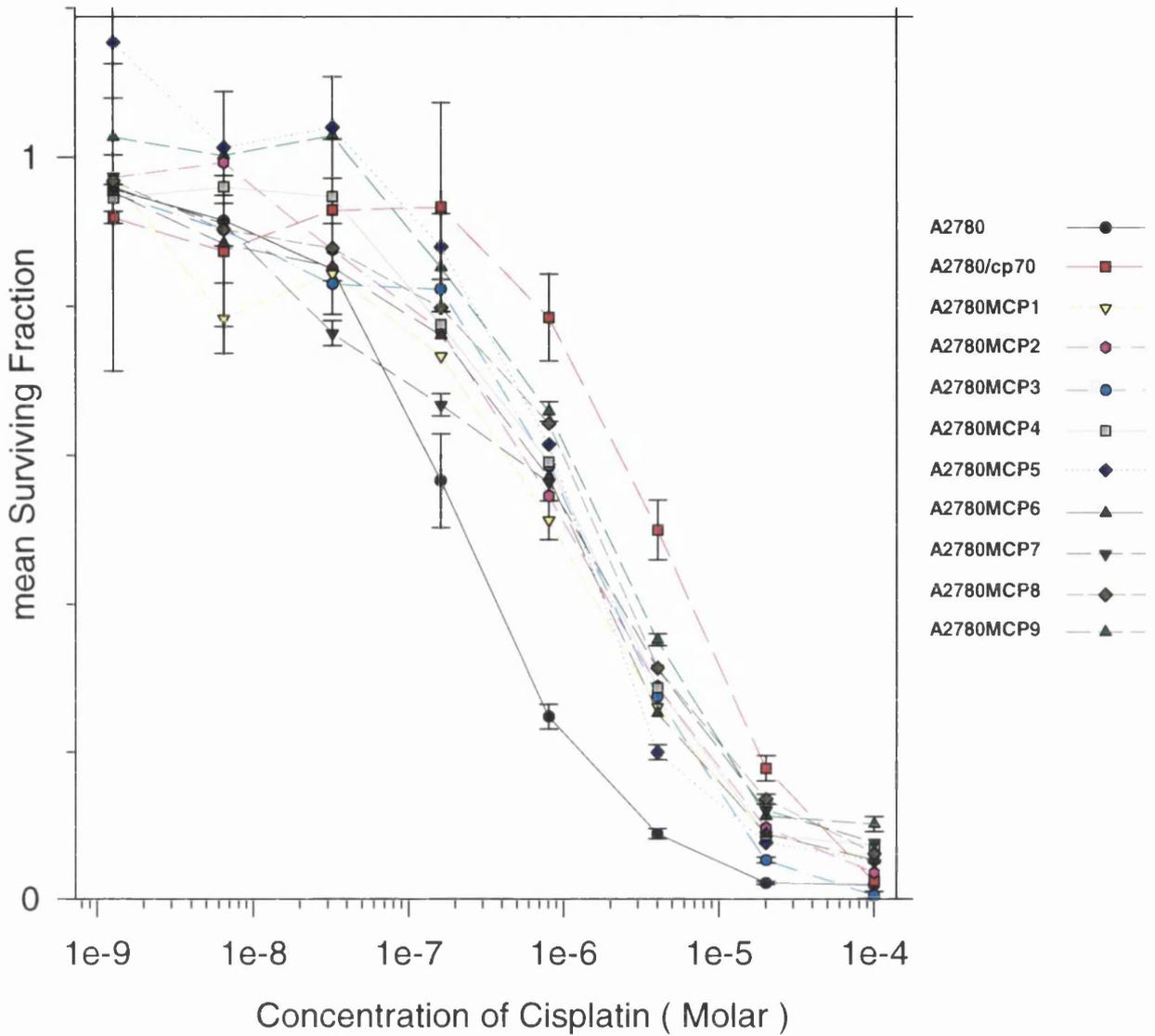
Figure 3.9 Cisplatin dose / response curves for A2780, A2780/cp70 and A2780/MCP cell lines.

Cisplatin dose/response curves for A2780, A2780/cp70 and A2780MCP lines derived using MTT viability assays. Mean survival fraction calculated by dividing mean value of 8 repeat assays at each drug concentration by that for no drug control.

Error bars represent +/- one standard error of mean.

One commonly used method of comparing the data on cellular drug sensitivity from dose/response curves in a numerical form is by calculating ID_{50} values. This represents the concentration of cisplatin required to inhibit cell viability by 50% and is calculated by determining the drug concentration that reduced cell survival to 50% of that seen in controls. The ID_{50} values for the set of dose/response curves from one cell viability assay are displayed in Table 3.6 along with the relative resistance compared to A2780. The mean relative resistance calculated from the results of all the M.T.T. assays is also included. It can be seen that A2780/cp70 displays a much higher relative resistance (approximately 20 fold) using this type of assay compared with the clonogenic assay. However this level of resistance is characteristic of A2780/cp70 under these assay conditions (Plumb et al. 1993). The mean of the relative resistance for the A2780/MCP lines ranges from 4.1 to 7.3. This data again demonstrates the development of a stable cisplatin resistant phenotype by the A2780/MCP cell-lines as compared to the cell of origin, A2780.

Concurrent with the determination of the dose/response curves to cisplatin for the A2780/MCP cell-lines those of three of the A2780/SCP lines were also derived. Two of the lines that had displayed evidence of microsatellite mutation, A2780/SCP5 and SCP6, were studied along with A2780/SCP3 which had shown no microsatellite changes. The cisplatin resistance of these cell-lines had been characterised previously (McLaughlin, 1991) and all of the lines had displayed a low fold resistance relative to A2780. However, it was possible that the phenotype of resistance could have been altered over the period since the lines were originally derived. In view of the alteration in phenotype observed in A2780/SCP5, SCP6 and SCP2, displayed by microsatellite mutation, it was felt to be prudent to confirm that the SCP lines retained their resistance to cisplatin. Figure 3.10 represents the dose/response curves for A2780, A2780/cp70 and A2780/SCP3, SCP5 and SCP6. The three A2780/SCP lines have curves that lie intermediate between those for A2780 and A2780/cp70, although closer to that of the former but with no overlap. As can be seen in Table 3.6 the A2780/SCP cell lines show
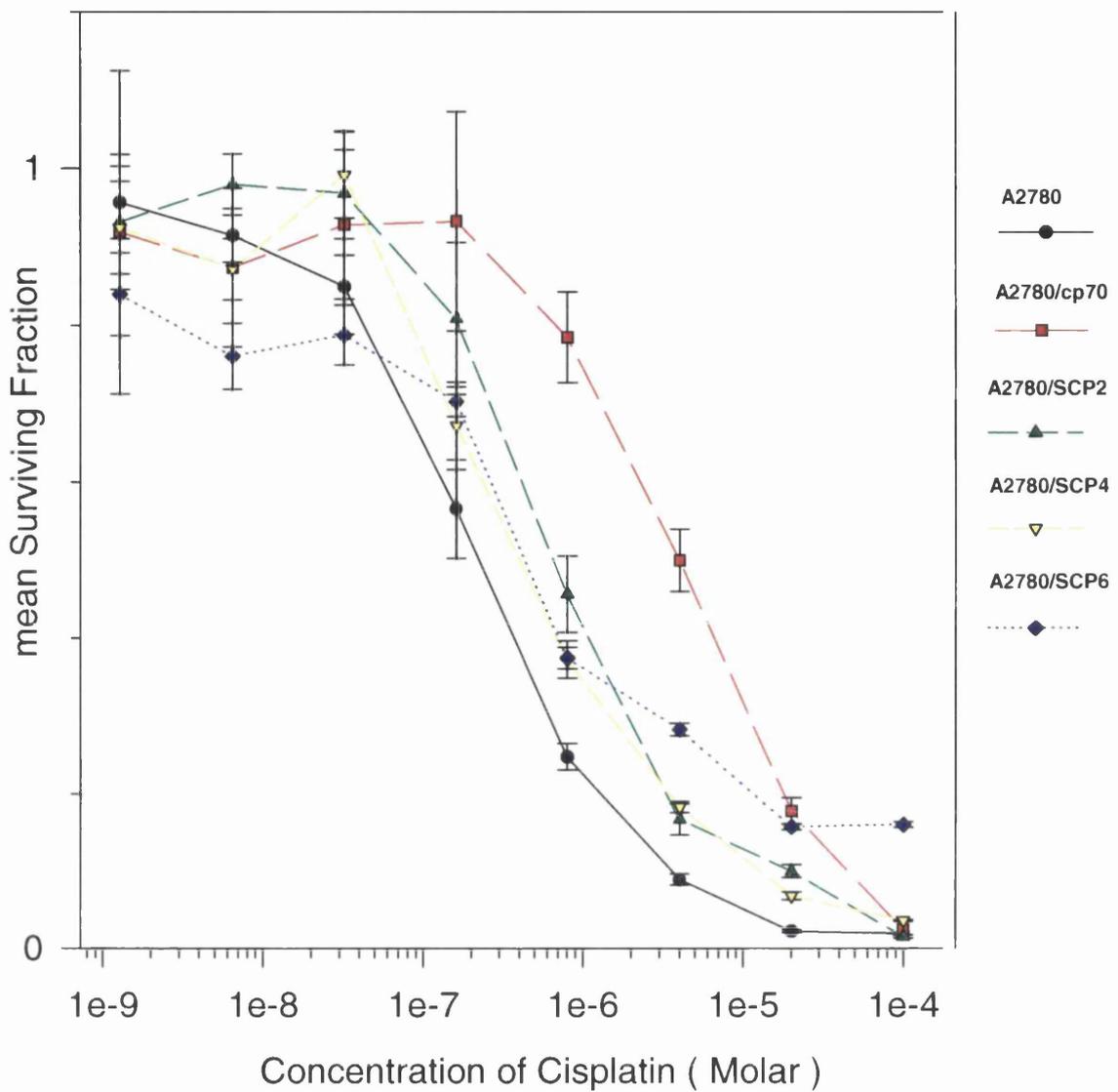
Figure 3.10 Cisplatin dose / response curves for A2780, A2780/cp70 and A2780/SCP cell lines derived using MTT cell viability assay.

Comparison of dose/response curves for A2780, A2780/cp70 and A2780/SCP cell lines derived using an MTT viability assay. The mean survival fraction was calculated by dividing the mean value of 8 repeats for each drug concentration by that for the no drug control. Error bars represent +/- one standard error of the mean.

Cell Line	Lethal Dose (LD)$_{50}$ - µM	Relative Resistance	Mean relative resistance +/- SE
A2780	2.214×10^{-7}	1	1
A2780/cp70	4.093×10^{-6}	18.5	19.6 +/- 1.2
A2780MCP1	8.182×10^{-7}	3.7	4.3 +/- 0.4
A2780MCP2	1.015×10^{-6}	4.6	4.6 +/- 0.04
A2780MCP3	1.248×10^{-6}	5.6	7.3 +/- 2.1
A2780MCP4	1.151×10^{-6}	5.2	7.1 +/- 1.9
A2780MCP5	1.102×10^{-6}	5.0	5.2 +/- 0.2
A2780MCP6	1.032×10^{-6}	4.7	4.1 +/- 0.6
A2780MCP7	8.476×10^{-7}	3.8	4.3 +/- 0.4
A2780MCP8	1.536×10^{-6}	6.9	5.3 +/- 0.8
A2780MCP9	1.683×10^{-6}	7.6	5.6 +/- 2.0
A2780SCP3	4.575×10^{-7}	2.1	3.5 +/- 0.3
A2780SCP6	4.862×10^{-7}	2.2	1.7 +/- 0.4
A2780SCP5	7.085×10^{-7}	3.2	1.4 +/- 0.7

Table 3.6 Relative resistance to cisplatin of A2780 derived cell lines compared to parental A2780, as determined by MTT assay

MTT cell viability assays were used to determine the resistance, to a range of cisplatin concentrations, of A2780 and derived cell lines. The LD50 was calculated as the concentration of cisplatin required to decrease viability of plated cells by 50%, as determined from the dose/response curve. The relative resistance, compared to A2780, was calculated for all cell lines by comparison of LD50 values. The LD50 values and relative resistance displayed are the results of one MTT assay. The mean relative resistance (+/- S.E.) was determined for each cell line, using data from two to three separate MTT assays.

2 to 3 fold resistance to cisplatin over A2780 by comparison of ID_{50} values for this set of dose/response curves. The mean relative resistance from two MTT assays confirms this low fold resistance to cisplatin. Use of the data for each of these cell lines at the cisplatin dose of $1.6 \times 10^{-7}M$ allows calculation of the relative cell survival as before. Using this in a paired t-test it can be shown that, when compared to A2780, the increased levels of cell survival for A2780/SCP3 and SCP6 are significant ($0.001 < p < 0.05$). In the case of A2780/SCP5 the difference approaches statistical significance ($0.1 > p > 0.05$).

3.5 DETERMINATION OF THE FREQUENCY OF MICROSATELLITE MUTATION IN CISPLATIN RESISTANT A2780/MCP CELL-LINES.

Having established that the A2780/MCP cell-lines displayed a stable cisplatin resistant phenotype it was important to determine whether they had also acquired an RER+ phenotype as characterised by microsatellite instability. DNA was isolated from all of the A2780/MCP cell-lines. Using a similar set of PCR primers to those used for characterisation of the A2780/SCP cell-lines, each line was investigated for alterations in the length of microsatellite repeats on chromosomes 17, 2 and 6. Examples of the resulting autoradiographs can be seen in Figure 3.11. At locus D2S111, the size of the microsatellite alleles observed in wild-type A2780 are represented by the two arrows. The alleles in A2780/MCP6, MCP7 and MCP8 are all identical to that of the parental cell-line. In A2780/MCP1, MCP2, MCP4, MCP5 and MCP9 there has been an increase in the size of the larger allele by 4bp (2 repeat units) whilst in A2780/MCP3 this allele has decreased in size by the same amount. There is no alteration in the size of the smaller allele. The microsatellite repeat at locus D2S172 is homozygous but shows a similar pattern of mutation to that found at D2S111. Again the size of the allele found in parental A2780 is represented by the arrow. It would appear that A2780/MCP6 to MCP8 all have microsatellite repeats of identical length to that in A2780. The other

A2780/MCP cell-lines all have an increase in the size of this repeat length except for MCP5 and MCP3 which show decreases in size.

When analysis of the microsatellite repeats at all eleven loci studied was performed it was found that all but one of the A2780/MCP cell-lines displayed evidence for microsatellite mutation (Table 3.7) A2780/MCP7 was the line with no obvious evidence of microsatellite mutation. In the other lines the number of mutations varied from a single altered locus (MCP8) to four out of eleven loci showing mutation (MCP2 and 3) An interesting feature of these results is that the great majority of the mutations arise in microsatellite loci on chromosome 2, with only two mutated microsatellite repeats on chromosome 17 and none on chromosome 6. There was no particular reason why chromosome 2 microsatellites should be more prone to mutation in these cell-lines. As stated previously there are certain characteristics of microsatellite repeats that make it more likely that replication errors will occur. However, why such a clustering of mutations should occur is unclear.

These results indicate that use of a multiple-step selection protocol for isolating cisplatin resistant cell-lines is also associated with the selection for a phenotype of microsatellite instability. This occurs more frequently with selection by multiple drug exposures than with the single-step selected resistant cell-lines.

3.6 CHARACTERISATION OF P53 FUNCTION IN CISPLATIN RESISTANT A2780 CELL LINES.

Of all the cisplatin resistant A2780 cell lines studied, A2780/cp70 displays the greatest degree of cisplatin resistance and the highest frequency of microsatellite instability. A2780/cp70 possesses a phenotype of resistance to a number of different anticancer drugs despite having been selected only for resistance to cisplatin (Vasey et al. 1995; Hamaguchi et al. 1993). The mechanism for this multi-drug resistance is not clear. It would appear not to be due to an increased expression of P-glycoprotein as
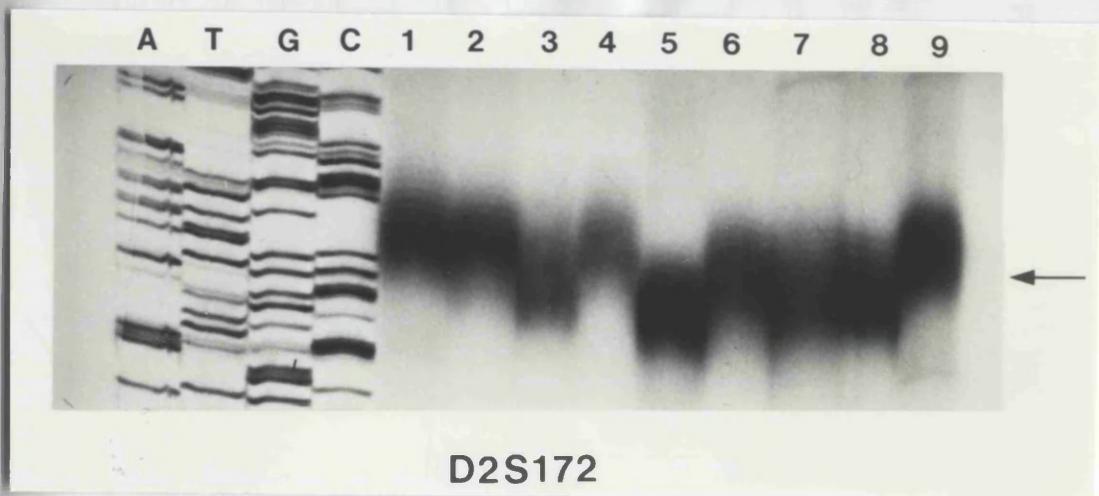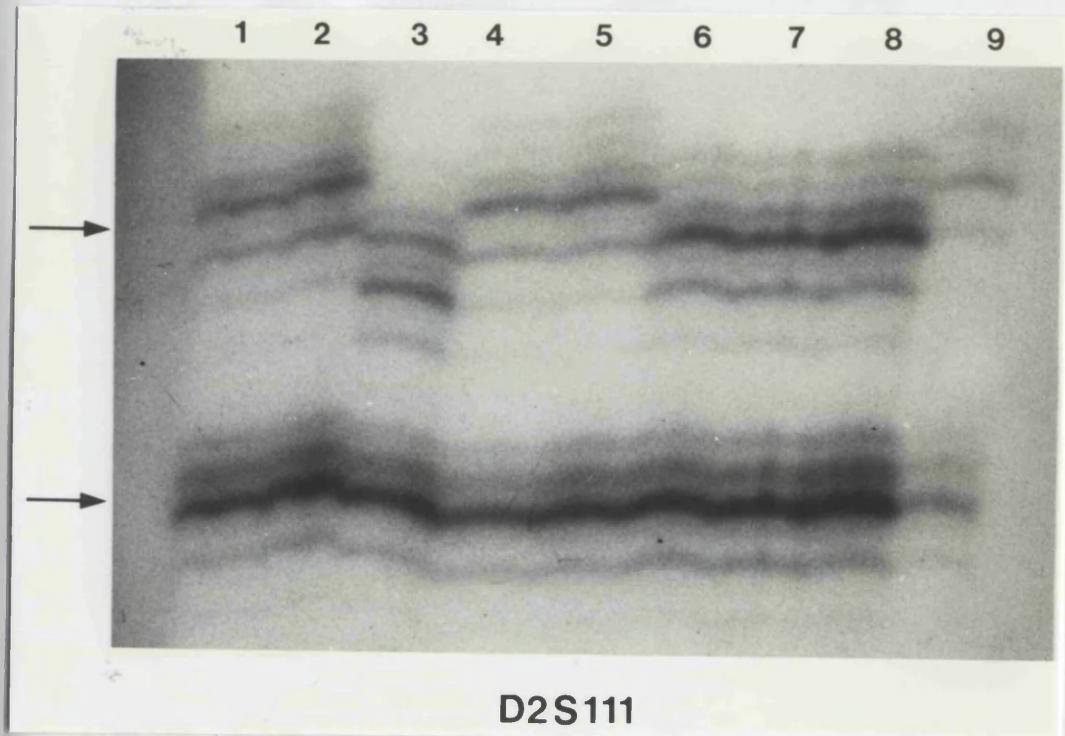
D2S111

D2S172

Figure 3.11 Mutation at microsatellite loci in the cisplatin resistant A2780/MCP
cell lines.

PCR amplification of microsatellite loci (a) D2S111 and (b) D2S172 in A2780/MCP cell lines.
These were derived from A2780 by selecting for resistance to increasing concentrations of
cisplatin. The size of the microsatellite alleles observed in parental A2780 is indicated by
arrows.

Legend A,T,G,C, M13 phage sequence ladder

Lanes 1-9 A2780/MCP1 - A2780/MCP9

Microsatellite loci

	A2780/ MCP1	A2780/ MCP2	A2780/ MCP3	A2780/ MCP4	A2780 MCP5	A2780/ MCP6	A2780/ MCP7	A2780/ MCP8	A2780/ MCP9
CHRNB1	■	■	■	■	■	■	■	■	■
D17S796	■	■	■	■	■	■	■	■	■
D17S183	■	■	■	■	■	■	■	■	■
D17S351	■	■	■	■	■	■	■	■	■
D2S172	■	■	■	■	■	■	■	■	■
D2S121	■	■	■	■	■	■	■	■	■
D2S111	■	■	■	■	■	■	■	■	■
D2S123	■	■	■	■	■	■	■	■	■
D6S261	□	■	■	■	■	■	■	□	□
D6S311	■	■	■	■	■	■	■	■	□
D6S274	■	■	■	■	■	■	■	■	■

Table 3.7   Incidence of microsatellite mutation in multiple step selected, cisplatin resistant, A2780 cell lines.

DNA from cisplatin resistant A2780 cell lines, derived by selection with increasing concentrations of cisplatin, was amplified by PCR using microsatellite specific primers. Mutation was characterised by a shift in the size of microsatellite DNA compared to that from parental A2780.

Legend:

■ = Microsatellite alleles identical to A2780 wild type.

■ = Mutant microsatellite allele detected as compared to A2780 wild type.

□ = Data unavailable.

there is no evidence for amplification of the Mdr gene. Of the other RER+ cell lines, A2780/AD (Vasey, 1996) and MCF7/AD have also been shown to display a phenotype of multi-drug resistance.

As previously indicated A2780/cp70 cells possess wild-type copies of the gene for the p53 tumour suppressor protein (TP53). However, it has been shown that the cells display decreased levels of p53 function after a DNA damaging stimulus (Brown et al. 1993). A number of recent studies have revealed a relationship between functional integrity of p53 in tumour cells and their sensitivity to anti-cancer agents (Lowe et al. 1994; Lowe et al. 1993). Defects in the function of p53 in certain cell types would appear to increase the resistance of these cells to a range of anti -cancer agents that act by damaging DNA. A2780/cp70 would appear to represent one cell line in which abnormal p53 function could be associated with the development of multi-drug resistance.

The expression and function of TP53 in the A2780/MCP cell lines was determined in order to ascertain whether they displayed TP53 defects that could be involved in development of cisplatin resistance.

3.6.1   Analysis of p53 protein levels expressed in A2780 and drug resistant derivatives.

The basal levels of TP53 in A2780, A2780/cp70 and the A2780/MCP cell lines were compared to determine whether there were obvious differences between the cell lines. In general, cells which have not been exposed to a p53 inducing stimulus express low basal levels of p53 protein (Reich and Levine, 1984). This is due to the very short half-life of p53, about 20 minutes in most cell types (Levine, 1992), caused by rapid degradation of the protein by the ubiqitin system. Specific mutations in conserved domains of the p53 gene sequence can increase basal p53 levels in tumour cells (Bartek et al. 1990). This arises because the mutant protein has a much longer half-life in the

cell, usually in the order of hours rather than minutes (Finlay et al. 1988). A range of monoclonal and polyclonal antibodies are available that react with particular epitopes of the p53 protein. The detection of raised levels of p53 in cells by immunohistochemistry, most commonly correlates with the presence of mutant p53 protein. Several studies have shown, however, that elevated intracellular p53 can arise from an apparently wild-type gene (Righetti et al. 1996a). This suggests that there may be other means, apart from mutation, by which p53 can be stabilised in the cell leading to abnormal function (Vogelstein and Kinzler, 1992). A2780/cp70 which is known to possess wild-type p53 gene sequence has been shown to have accumulated p53 by immunoblot analysis when compared to A2780 (Brown et al. 1993).

In order to assess the relative basal levels of p53 in the A2780/MCP and A2780/cp70 cell-lines compared to A2780, a Western immunoblot assay was employed (for method see Chapter 2.6). The DO-1 antibody (p53Ab6. Oncogene Science. Manhasset, N.Y.) was used for detection of TP53 proteins on the Western membrane. This is a monoclonal antibody that cross-reacts with both normal and mutant forms of p53 at an epitope near the amino terminus of the protein (Vojtesek et al. 1992). Several repeats of the p53 western immunoblot were performed using newly derived extracts of all the cell-lines under study. As can be seen from figure 3.12, TP53 protein was detectable in all of the cell-lines. In agreement with previous studies A2780/cp70 shows a higher TP53 level than does A2780 (Brown et al. 1993). The levels of TP53 in the A2780/MCP cell-lines vary with regards to each other and to A2780. Laser densitometry of the autoradiographs, produced from each Western immunoblot, was performed to allow semi-quantitative analysis of the differences in basal TP53 levels between the cell lines. Each autoradiograph was scanned using the laser densitometer and the average optical density (O.D.) of each spot on the autoradiograph calculated using associated software (Chapter 2.6, materials and methods). The data from laser densitometry on five, independently performed Western immunoblots, permitted calculation of a mean level of basal TP53 expression. This mean value could then be
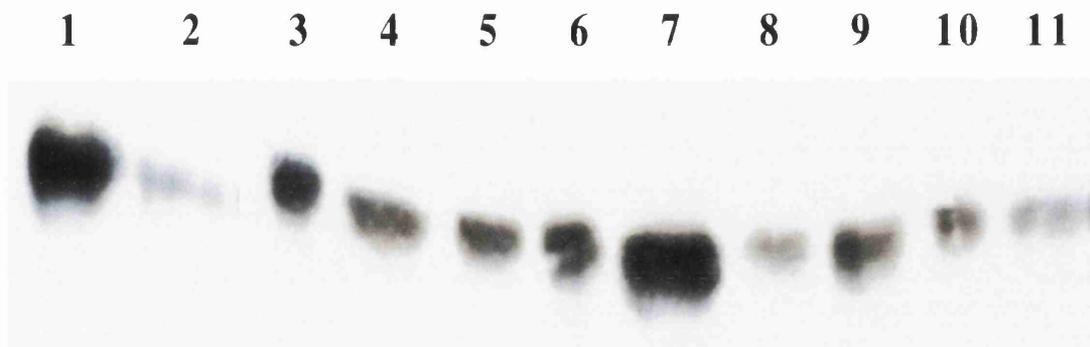
Figure 3.12. p53 protein levels in A2780 and derived cisplatin resistant cell-lines

Equal quantities of cell extract from the cell lines indicated were subjected to electrophoresis through an SDS-polyacrylamide gel. After transfer to a nylon membrane p53 protein detection was performed using MAb DO1. Bound antibody was detected using chemiluminescence.

The figure shows immunodetection of p53 protein in each of the cell extracts indicated. Repeats of the Western immunoblot resulted in some variation in the levels of p53 seen within a cell line. This was most probably a result of variability in transfer of protein to the nylon membrane.

Legend:

(1) A2780/cp70   (2) A2780   (3) A2780/MCP1   (4) A2780/MCP2

(5) A2780/MCP3   (6) A2780/MCP4   (7) A2780/MCP5   (8) A2780/MCP6

(9) A2780/MCP7   (10) A2780/MCP8   (11) A2780/MCP9

compared to that obtained for the A2780 cell line to give a relative level of basal TP53. This data is presented in Table 3.8. It confirms that A2780/cp70 possesses the highest levels of basal TP53 as compared to A2780. The variation in the basal TP53 levels in the A2780/MCP cell lines is also observed. However, none of the A2780/MCP cell lines show a statistically significant difference in the basal expression of TP53 when compared to A2780 (p>0.5).

3.6.2 Flow cytometric analysis of cell cycle changes in cisplatin resistant A2780 derived cell-lines after ionising radiation.

The p53 protein has a central role in two of the main responses to cellular insult, especially those arising through DNA damage. It is involved in the maintenance of cell-cycle checkpoints at the G_1/S and G_2/M boundaries. The G_1/S arrest prevents cells from replicating damaged DNA by stopping progression through the cell-cycle prior to S-phase and providing more time for DNA repair (Kastan et al. 1995). This cell-cycle arrest comes about through the transcriptional activation of specific genes, such as Waf1/Cip1, by p53 (El-Deiry et al. 1994). In many cells it would also appear to play a central role in the apoptotic response after DNA damage. To study the function of p53 in the cisplatin resistant A2780 cell-lines, the ability of cells to delay replicative DNA synthesis after a damaging stimulus was determined. The DNA damaging stimulus used in these studies was ionising radiation from a Cobalt source. The use of anti-cancer agents such as cisplatin as the source of DNA damage would have introduced potential sources of variability between the cell-lines. For example, although cells would be exposed to the same concentration of cisplatin, variation in drug uptake and metabolism could result in different amounts of DNA damage being induced in each case. Without directly measuring the level of platinated DNA in each cell-line we would not be able to determine whether changes observed in the p53 function were due to differences in the levels of DNA damage. With ionising radiation the problems of uptake and metabolism

Cell line	Relative p53 protein level.[a]	Percentage of apoptotic cells.[b]	Relative CIP1 mRNA levels.[c]
A2780	1	22	1
A2780/cp70	1.66 (+/- 0.5)	1	0.29
A2780/MCP1	1.36 (+/- 0.5)	4	0.52
A2780/MCP2	0.81 (+/- 0.35)	2	0.55
A2780/MCP3	1.03 (+/- 0.3)	1	0.32
A2780/MCP4	1.01 (+/- 0.45)	1	0.58
A2780/MCP5	1.35 (+/- 0.6)	8	0.33
A2780/MCP6	0.72 (+/- 0.2)	7	0.49
A2780/MCP7	1.03 (+/- 0.3)	20	0.95
A2780/MCP8	0.92 (+/- 0.25)	1	0.66
A2780/MCP9	0.64 (+/- 0.25)	1	0.56

Table 3.8   Analysis of p53 dependent variables in A2780 and cisplatin resistant derived cell lines.

Comparison of basal TP53 protein expression and two indicators of basal p53 function in parental A2780, A2780/cp70 and A2780/MCP1 - MCP9.

Legend

a. p53 protein level, relative to A2780, as determined by laser densitometry of autoradiographic images. Protein detected by Western immunoblot using DO1 antibody. Mean of 5 experiments (+/- one standard error).

b The percentage of total cells undergoing apoptosis 72hrs after treatment with 40μM cisplatin as detected by flow cytometry of fluorescently labelled DNA strand breaks. Values show means of duplicate experiments with 15,000 cells counted per experiment.

Data supplied by A. McIlwrath. Dept Medical Oncology. Glasgow University.

c. Relative basal levels of Cip1 mRNA determined by Northern blot analysis by hybridisation to Cip1 cDNA probe. Standardisation was by rehybridisation against G3PDH probe.

Data supplied by L. Gallagher. Dept. Medical Oncology. Glasgow University.

do not apply and it is assumed that each cell receives an equivalent level of DNA damage.

To evaluate the changes in cell cycle progression after ionising radiation a simultaneous flow cytometric analysis of DNA content and DNA synthesis (determined by propidium iodide staining and BrdUrd incorporation respectively) was made. The use of pulses of BrdUrd and the ability of flow cytometry to analyse single cells provides a dynamic assessment of the cell cycle. Using this method the fraction of cells in G1, S, and G2/M phases can be easily measured and distinctions can be made between a G1 arrest and an S-phase arrest (Kastan et al. 1991a). In the protocol used (see Chapter 2.9.2) 5×10^5 cells, of each cell-line, were plated in duplicate for irradiated and control end-points. Flow cytometry was carried out on a fluorescence activated cell sorter (FACS). For each sample at least 20,000 cells were counted and a histogram of total DNA content versus replicated DNA plotted using appropriate software. The different phases of the cell cycle (G1, S, G2) can be identified on the histogram and the effects of exposure to ionising radiation, on progression through the cell cycle, can also be visualised. In the A2780 control cells the progression from G1, through S-phase to G2 can be clearly seen (figure 3.13(a)). After ionising radiation there is a decrease in the number of cells in S-phase in A2780 (figure 3.13(b)) and an accumulation of cells in G2. This is consistent with the possession of functional p53 which acts to prevent cells entering S-phase. The A2780/cp70 controls show similar progress through the cell cycle to that seen with A2780 (figure 3.13(c)). However, after irradiation a much higher proportion of cells continue to progress into S-phase, (figure 3.13(d)). As an example of an A2780/MCP cell-line, A2780/MCP9 cells also show evidence of a reduction in new DNA synthesis after irradiation and an accumulation of cells in G1 and G2 phases of the cycle, (figures 3.13(e) & (f)).

The percentage of cells in each phase of the cell cycle for the cell-lines displayed is shown in the table in figure 3.13. This confirms that;

Figure 3.13   Changes in cell cycle dynamics in A2780 and derived cell-lines after
irradiation

Cell-cycle histograms are displayed for control and post-irradiation cells in each of

A2780, A2780/cp70 and A2780/MCP9 lines. Cell cycle analysis was accomplished by

flow cytometric measurement of cells after bromodeoxyuridine (BrdUrd) incorporation,

during a 4h pulse, and propidium iodide (PI) staining. The different components of the

cell cycle can be differentiated on the histogram as indicated in (a). The percentage of

cells in each part of the cycle can then be determined.

After irradiation there is a decrease in the number of cells entering S-phase in A2780

whereas in A2780/cp70 there is no significant difference over control. A2780/MCP9

shows some degree of decreased entry into S-phase.

Figure 3.13

(a) A2780 control

(b) A2780 2Gy

(c) A2780/cp70 control

(d) A2780/cp70 2Gy

(e) A2780/MCP9 control
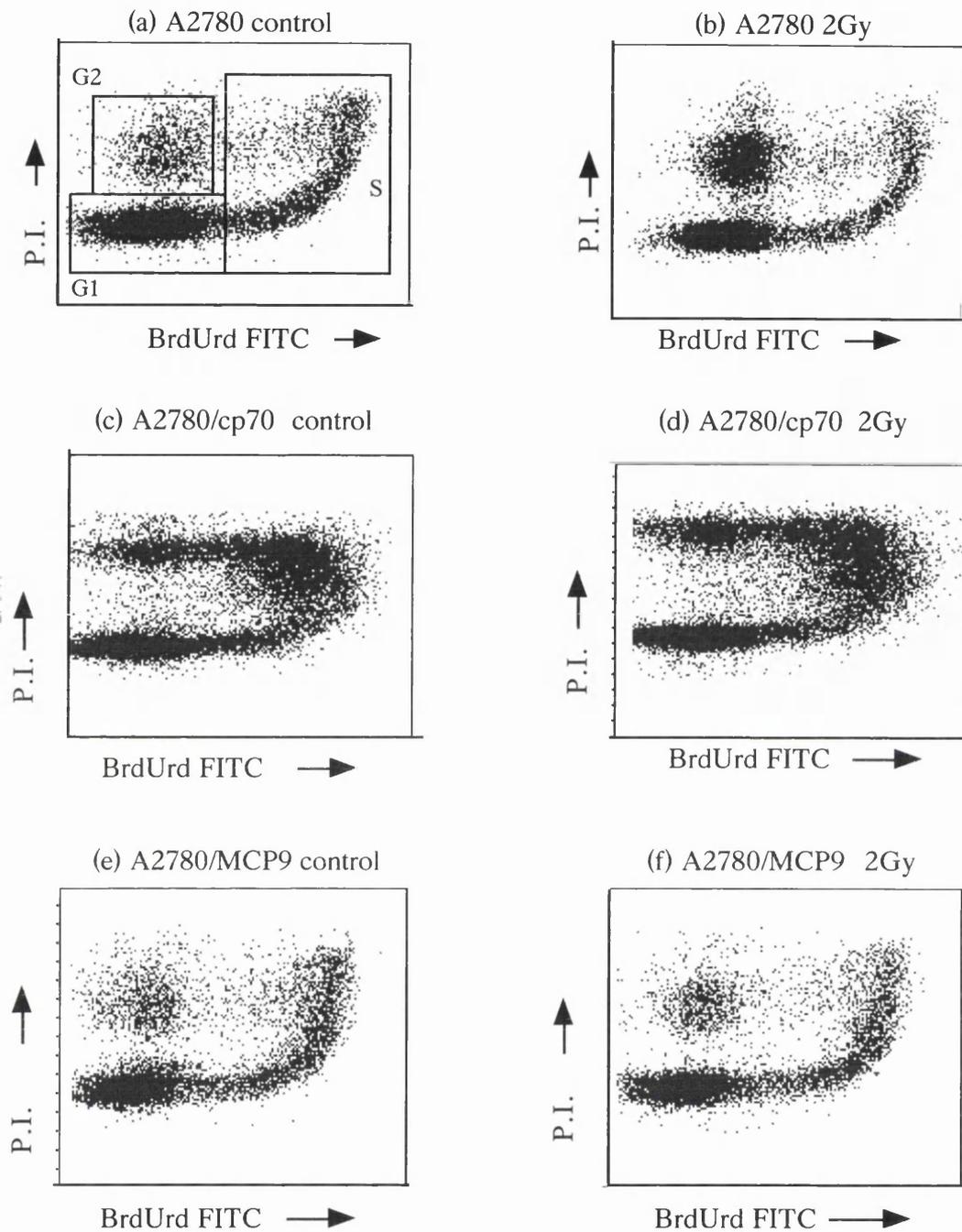
(f) A2780/MCP9 2Gy

	Controls			Irradiated 2Gy		
	A2780	A2780/ cp70	A2780/ MCP9	A2780	A2780/ cp70	A2780/ MCP9
%G1	58.7	52.5	45.8	65.6	48.3	56.3
%S	32.9	35.4	48.8	20.7	37.3	39.6
%G2	7.3	9.1	1.0	12.6	11.5	5.0

(a) in A2780 there is accumulation of cells in G1 and G2 and a fall in the number in S-phase after irradiation,

(b) A2780/cp70 displays no significant change in the percentage of cells in each phase of the cycle after irradiation.

These results are consistent with previous observations on these cell-lines, (Brown et al. 1993). In the case of the A2780/MCP9 cell line there is a reduction in the number of cells entering S-phase after irradiation, but this is less than that observed in A2780. This suggests some degree of loss of p53 function in this cell line.

Three separate analyses of the differences in cell-cycle progression after irradiation, were performed on A2780, A2780/cp70 and the A2780/MCP cell lines. In order to compare the differences between the cisplatin resistant cell lines and A2780, the cumulative data from all three independent cell cycle experiments was analysed, (this data can be found in appendix A). The mean of the fraction of S-phase cells present in each cell line after 2Gy irradiation was calculated as well as the standard error of the mean (Table 3.9). The results show that over the three experiments approximately 50% more cells enter S-phase after irradiation of A2780/cp70 than with A2780. A similar result is observed for all but one of the A2780/MCP cell-lines with a proportionally larger number of cells entering S-phase after radiation than in A2780. It would appear, therefore, that there has been a reduction in the ability of cells from these lines to inhibit DNA replication after a DNA damaging stimulus.

As the cell-cycle data is not likely to conform to a normal distribution, a non-parametric method was used to asses the significance of the differences in relative mean fraction of cells in S-phase after irradiation, between cell-lines. Using a Wilcoxon rank sum test the comparison between A2780 and each of the cisplatin resistant derivatives was significant (0.05>p>0.01) in all but two cell lines. A2780/MCP7 showed no significant difference in its response to radiation when compared to A2780. With A2780/MCP9 more cells entered S-phase after irradiation than in the case of A2780 in

Cell Lines	Mean fraction of cells in S-phase 24hr after 2Gy radiation (+/- S.E.)	Relative change in % S-phase cells after 2Gy radiation compared to A2780
A2780	0.60 (0.07)	1.0
A2780/cp70	0.90 (0.16)	1.5
A2780MCP1	0.81 (0.07)	1.35
A2780MCP2	0.76 (0.17)	1.27
A2780MCP3	0.79 (0.1)	1.32
A2780MCP4	1.06 (0.25)	1.77
A2780MCP5	0.80 (0.17)	1.33
A2780MCP6	0.77 (0.24)	1.28
A2780MCP7	0.60 (0.09)	1.0
A2780MCP8	0.76 (0.05)	1.27
A2780MCP9	0.64 (0.1)	1.07

Table 3.9   Effects of ionising radiation on entry into S-phase in cells from A2780, A2780/cp70 and A2780/MCP cell lines.

The mean of the fraction of cells entering S-phase after 2Gy ionising radiation was calculated from duplicates of each cell line compared to non-irradiated controls. This was accomplished using FACS analysis with at least 20,000 cells counted per cell line,. The analysis was performed in each of 3 separate cell-cycle assays and the mean fraction of cells in S-phase, 24hr after irradiation, was calculated. This was then compared to the mean fraction of S-phase cells after irradiation in A2780 to obtain the relative change in S-phase fraction.

two out of the three experiments. In the final experiment no evidence of a loss of cell-cycle arrest at G_1 was observed. Over the three experiments the loss of G_1 arrest in this cell-line approached but did not reach statistical significance (p> 0.05).

Analysis of the data for the cisplatin resistant cell-line A2780/MCP5 reveals a change in the status of the G_1/S checkpoint over the temporal course of the three experiments. A period of five months lapsed between the first and second cell cycle experiments and, although the cells were not continuously grown over this period of time, a significant number of cell doublings took place. The second and third repetitions of the cell cycle experiment were approximately one month apart during which time the cells had been grown continuously in culture. This represents approximately 20 additional cell doublings for each line between these two time points. For A2780/cp70 and most of the A2780/MCP cell lines a relative increase in the number of cells progressing into S-phase after irradiation is observed in the first cell cycle assay and is reproduced in the subsequent experiments. As shown in Table 3.10, A2780 and A2780/MCP7 display an obvious decrease in the relative number of post-radiation S-phase cells in all three experiments. A2780/MCP5 shows very little difference to A2780 in the first two cell cycle assays. However, with the further period of culturing of the cells between the second and third experiments this cell-line developed an obvious increase in the number of cells progressing into S-phase after radiation. This suggests that the further culture of this cell-line has brought about an decrease in the ability to arrest in G1 after irradiation.

3.6.3   Characterisation of apoptosis in cisplatin resistant A2780 cells as a measure of p53 function.

To further determine if cisplatin resistant derivatives of A2780 displayed differences in p53 function as compared to the parental cell-line, the ability of cells to undergo apoptosis after exposure to cisplatin was measured. This work was carried out

	Fraction of S-phase cells 24h after 2Gy ionising radiation compared to non-irradiated controls.		
Cell Line	Passage I Dec 1994	Passage II May 1995	Passage III June 1995
A2780	0.51	0.73	0.56
A2780/MCP7	0.42	0.69	0.68
A2780/MCP5	0.58	0.67	1.15

Table 3.10   Changes in post-irradiation cell-cycle characteristics of A2780/MCP5 with prolonged growth in culture

The table shows the fraction of cells in each line entering S-phase after 2Gy of ionising radiation as compared to non-irradiated control cells. Three separate experiments were performed, with a substantial period of continuous cell culture occurring between experiments. For A2780 and A2780/MCP7 it can be seen that there is a significant reduction in the number of cells entering S-phase after irradiation which remains relatively constant between experiments. With A2780/MCP5, however, there is no change in the fraction of cells entering S-phase post-irradiation between the first two experiments but a significant increase in the fraction of cells entering S-phase in the third. This suggests some change occurring in A2780/MCP5 resulting in a reduction in the post-irradiation delay of cell entry into S-phase.

in collaboration with A.McIlwrath. It has been shown previously that the apoptotic response to cisplatin induced DNA damage in A2780 cells is dependent on functional p53 (A. McIlwrath. Pers. Comm.). A widely used biochemical marker for apoptosis is the non-random fragmentation of DNA. This is normally detected by the initial appearance of 300-kbp and 50-kbp fragments of DNA followed subsequently, in most but not all cases, by the internucleosomal cleavage to 180-200 bp integers (the DNA ladder) (Oberhammer et al. 1993). Using field-inversion gel electrophoresis to separate these fragments it was shown that between 48 and 96 hours after exposure to 40µM cisplatin A2780 cells displayed the appearance of 300-kbp and 50-kbp fragments. The A2780/cp70 cells displayed markedly less DNA fragmentation after the same cisplatin exposure. No nucleosomal sized DNA fragments were observed in either cell type. To allow quantification of this apparent reduction in apoptosis a flow cytometric assay was used. This technique, known as a TUNEL assay (Gorczyca et al. 1993) relies on the principle of end-labelling of DNA fragments with a fluorescent tag (FITC) using terminal deoxytransferase (TdT) which can then be detected in the flow cytometer. Labelling of total DNA with propidium iodide allows analysis of the number of cells with fragmented DNA in the cell population as a whole and displays a characteristic pattern on the cell histogram. Seventy-two hours after exposure to equitoxic concentrations of cisplatin it was shown that there was little increase in the number of apoptotic A2780/cp70 cells compared to controls. With A2780 on the other hand a significant proportion of apoptotic cells were observed at this time point. Similar measurements were made for the A2780/MCP cell-lines the results of which are shown in Table 3.8. There would appear to be a reduction in the number of cells engaging apoptosis after exposure to cisplatin for all the A2780/MCP cell-lines except MCP7. In the A2780/MCP cell-lines no measurements have been made of the actual levels of DNA damage induced by exposure to a specific concentration of cisplatin. Therefore, the diminished apoptotic response observed in these cells when compared to A2780 may be as a result of differences in the uptake or metabolism of cisplatin or in the signal that engages an apoptotic response.

3.6.4 Determination of basal and induced Cip1/Waf1 mRNA levels in cisplatin resistant A2780 cell-lines.

TP53 is involved in the transcriptional regulation of the Cip1/Waf1 gene and is necessary for radiation induced G1 arrest (El-Deiry et al. 1994). To determine the basal levels of expression of Cip1/Waf1 in A2780 and derived cell-lines, detection of mRNA was performed using a Northern blot technique. This work was carried out in collaboration with L. Gallagher. A plasmid containing the Cip1/Waf1 cDNA (pCEP-WAF1-S) was used as the probe for detection of Cip1/Waf1 mRNA. It had been shown that A2780/cp70 cells possessed a reduced basal level of Cip1/Waf1 mRNA as compared to A2780 (Gallagher, 1996). A2780/cp70 cells also displayed a much smaller increase in the rise in the level of this specific mRNA after 2Gy of γ-radiation or exposure to 1μM cisplatin for 1 hour when compared to A2780 (Gallagher, 1996). When the basal levels of Cip1/Waf1 mRNA were determined by Northern blotting in the A2780/MCP cell-lines a reduced expression of this gene was observed in all the lines compared to A2780. However, the expression of Cip1/Waf1 mRNA was greatest in the A2780/MCP7 cell-line. (Table 3.8).

3.7. CONCLUSIONS.

The data presented in this chapter reveals, for the first time, the possibility of an association between microsatellite mutation and resistance to cisplatin and doxorubicin in tumour cell lines. However the detection and analysis of microsatellite mutation is fraught with potential sources of error. Could it be that the microsatellite mutation detected is artefact and that microsatellite instability does not exist in these cell lines?

Two areas of significant controversy exist in the analysis of microsatellite mutation;

(a); How does one define microsatellite mutation and microsatellite instability and identify them practically?

(b); How does one differentiate between the appearance of a mutation in a microsatellite repeat representing a random event and that associated with defective mismatch repair?

One of the practical problems that arises in interpretation of microsatellite mutations is differentiating true mutations from artefacts that can arise during the process of analysis. "Stutter" bands, caused by slippage of Taq polymerase during PCR, and aberrant migration of amplified DNA through polyacrylamide gels are two potential sources of artefact. Practical techniques employed to reduce such sources of error have included using high fidelity polymerases, fluorescently labelled PCR primers and automated DNA sequencers. In this work the reproducibility of changes in microsatellite size was used as the best indicator that such a change had not arisen artefactually during analysis. In cases in which interpretation of the autoradiograph was not clear assessment by an independent observer was obtained.

The determination of microsatellite instability depends on showing that the frequency of mutation detected at microsatellite repeat sequences, in a particular cell line, is greater than that expected in a normal cell. This can be calculated by finding the percentage of microsatellite loci that show mutations out of the total studied and comparing to the frequency expected in normal cells. However the question arises as to how many microsatellite loci have to be studied in order for instability to be determined? For example does the detection of a microsatellite mutation at one of two loci studied constitute microsatellite instability? Recent evidence suggests that the mutation rate of microsatellite sequences in normal tissues, such as peripheral T-lymphocytes and fibroblasts, may be as high as 3×10^{-3} per allele, which is an order of magnitude higher than previously quoted values (Hearne et al. 1992; Shibata et al. 1994; Hackman et al. 1995). This frequency of microsatellite mutation is similar to that observed in some sporadic tumours previously defined as displaying an RER+

phenotype (Hackman et al. 1995). Thus the detection of a single microsatellite mutation is not sufficient to allow differentiation between cells with functional and non-functional mismatch repair. In the literature to date the term microsatellite instability has been applied to cell lines or tumours in which as few as two microsatellite loci have been studied. It has been suggested that the minimum criteria for determining microsatellite instability should be the detection of mutations occurring in at least two out of five microsatellite repeat sequences (Thibodeau et al. 1993).

The drug resistant derivatives of A2780, OV1/p and MCF7 display mutations in at least two microsatellite loci out of, up to, fourteen studied. The cisplatin resistant lines A2780/SCP6 and A2780/MCP1-6 and 9 also show mutation in at least two out of the ten or eleven loci studied. These lines, therefore, all fulfil the criteria for displaying true microsatellite instability.

A problem that frequently arises when studying microsatellite instability in cell lines is that of polyclonality. Cells from a line cultured over a prolonged period are likely to develop some genetic differences from each other due to mutation, hence becoming a polyclonal population. In the case of microsatellite sequences, even when the cell line possesses normal mismatch repair, with time mutation will result in sub-populations within the line which display differences in the length of microsatellite repeats. One result of this is that studies between derivatives of a cell line could detect differences in the microsatellites arising as a result of its polyclonal nature but interpret them as being due to instability. This problem can be resolved by analysing the microsatellite repeats in a series of sub-clones derived from the line (Shibata et al. 1994). If the sub-clones are derived from a single clone of the original cell-line then the detection of multiple microsatellite mutations not present in the parent clone represents further replication errors occurring during growth of the sub-clones (Shibata et al. 1994). The results from the sub-cloning of the A2780 and A2780/cp70 cell-lines strongly suggests that the latter has developed an RER+ phenotype not seen in the parent line. Counting the number of mutations arising in the 165 microsatellite alleles

studied in A2780 sub-clones, allows calculation of a spontaneous mutation frequency for microsatellites in this line of less than 6×10^{-3}/ allele. A2780/cp70 on the other hand shows a mutational frequency two orders of magnitude higher than this. Sub-clone analysis has not been done for the line A2780/AD. However, the observation of mutations at two of fourteen microsatellite loci in the resistant cell-line combined with the low rate of spontaneous mutation in A2780 suggests (but does not prove) that this line also displays acquisition of an RER+ state. A similar argument can be advanced for the A2780/MCP and SCP cell lines showing microsatellite mutations. Without further determination of the spontaneous frequency of microsatellite mutation in the OV1/p and MCF7 cell-lines the significance of the frequent mutations observed in their drug resistant derivatives remains uncertain.

One of the reasons behind choosing highly polymorphic microsatellite markers, on four different chromosomes, for this study was to maximise detection of loss of heterozygosity (LoH). Despite this only three cases of LoH were observed in all the loci studied throughout the four drug resistant tumour cell lines. Loss of heterozygosity has been reported frequently on chromosomes 2,3,6 and 17 in ovarian tumours in other studies (Jacobs and Lancaster, 1996). Thus, the low frequency of LoH in these cell lines is not likely to be the result of studying loci in which LoH does not result in a specific growth advantage to the cell. It has been hypothesised, that cell lines displaying microsatellite instability can show apparent LoH, if the change in the size of one allele of a heterozygous pair results in both alleles ending up the same length, i.e. apparently homozygous (Contegiacomo et al. 1995). This may be what is observed with OV1/DDP in which the autoradiographic signal from the single remaining allele of D2S121 appears denser than does the corresponding allele in OV1/p.

During analysis of the drug resistant cell-lines the chromosomal locations of two of the mismatch repair genes, hMSH2 and hMLH1, were resolved (Bronner et al. 1994; Fishel et al. 1993). hMSH2 is located on chromosome 2p22-21 whereas hMLH1 if found at 3p21-23. It has been shown that mutations in hMSH2 and hMLH1 are

responsible for >80% of tumours in HNPCC (Han et al. 1995; Liu et al. 1994). Certain microsatellite markers were shown to be closely linked to these genes, e.g. D2S123 with hMSH2 and D3S1298 with hMLH1 (Papadopoulos et al. 1994; Peltomaki et al. 1993). These microsatellites were therefore included in the characterisation of the drug resistant cell-lines. The aim was to determine whether LoH could be detected at either of these loci and thus suggest the possible involvement of either of these genes in the phenotypes observed. Unfortunately both loci were homozygous in all of the cell-lines and therefore uninformative for LoH although microsatellite mutation was observed. It has been shown that LoH of linked markers is not a feature of either the 2p or 3p forms of HNPCC (Aaltonen et al. 1993; Lindblom et al. 1993) although LoH at 3p21.3-23 has been observed in sporadic non-small cell lung cancer (Neville et al. 1996), oral cancer and oesophageal cancer. One report shows microsatellite instability associated with LoH at 3p21 in non-small cell lung cancer (Wieland et al. 1996).

It would also appear that in general cells which display microsatellite instability do not accumulate the chromosomal rearrangements and deletions associated with frequent loss of heterozygosity (Yee et al. 1994; Thibodeau et al. 1993). The low frequency of LoH observed in the RER+ drug resistant cell lines studied here would tend to confirm this observation.

The high frequency of microsatellite instability observed in the A2780/MCP cell lines is very striking. This data along with that from the other drug resistant derivatives of A2780, MCF7 and OV1/p suggests a possible association between selection for drug resistance and selection for an RER+ phenotype. It should be noted however that not all cell lines selected for drug resistance show microsatellite instability. In a recent analysis of different ovarian, lung and testicular cancer cell lines with their cisplatin resistant derivatives, none of the resistant lines displayed any evidence of microsatellite mutation (G. Hirst. Pers comm.).

The data from the clonogenic and MTT assays prove, conclusively, that the A2780 cell lines selected using exposure to cisplatin show greater resistance to the cytotoxic effects of this drug than A2780. As stated , the repeats of the clonogenic and MTT assays were carried out several months apart each time. The cell lines had undergone further rounds of cell division and growth between each of the assay repeats. During these assays all cells were cultured using cisplatin free medium. The data from each of the clonogenic assays and the MTT dose/response curves can be seen in Appendix A. The results indicate that not only do the cell lines show greater resistance to cisplatin but that this is a stable phenotype that persists despite growth in the absence of the drug. It is important to show this as it suggests that the resistant phenotype has arisen through stable genetic change.

The other significant association to arise from these results is that observed between the presence of an RER+ phenotype and a reduction, or loss, of certain p53 dependent functions. In the cisplatin resistant A2780 cell-lines analysed, which displayed evidence of microsatellite mutation or instability, there was a reduction in p53 dependent apoptosis and cell-cycle arrest after DNA damage. It is important to note that not all apoptosis is p53 dependent (Clarke et al. 1993). However, the loss of an apoptotic response to DNA damage in A2780 cells transfected with dominant negative p53 mutants, indicates that apoptosis is p53 dependent in these cells (Vasey et al. 1996). Among all the cisplatin resistant A2780 cell-lines selected by multiple drug exposure, A2780/MCP7 was the only line not to display microsatellite mutation. It appeared to possess levels of p53 activity, using all three measures of p53 function analysed, similar to that of parental A2780 cells. This implies that this cell-line may have acquired its resistance to cisplatin by mechanisms different to those seen in RER+ cisplatin resistant A2780 cell-lines.

The A2780/MCP cell lines, other than MCP7, all show loss of DNA damage induced p53 function as characterised by the three assays employed. A2780/cp70 tended to show, in general, significant loss of p53 activity in all three assays. Other lines

displayed variable degrees of loss of p53 activity, depending on the assay used. For example, A2780/MCP9 shows very little loss of G_1 arrest after ionising radiation but marked reduction in the basal expression of Cip1 and the ability to induce apoptosis after cisplatin damage. A2780/MCP5 shows a markedly reduced level of basal Cip1 mRNA expression (table 3.9). At the same time after development of this cell line that the Cip1 mRNA was extracted it also displayed a level of post-irradiation G_1 arrest similar to that of A2780. Further expansion of this cell-line, by growth in culture, was associated with loss of radiation induced inhibition of DNA synthesis and reduced apoptosis by cisplatin. Thus the further growth in culture appeared to be associated with loss of these p53 mediated responses. With the obvious exception of A2780/MCP7, it would appear that all the A2780/MCP lines except MCP5 developed phenotypes of microsatellite instability, cisplatin resistance and loss of p53 dependent functions during the original selection with cisplatin. A2780/MCP5 on the other hand has only developed the full phenotype of lost p53 function after further expansion of the RER+ cell-line.

As stated in this chapter the cell-cycle characteristics of the cisplatin resistant cell-lines were analysed using 2Gy ionising radiation as the source of DNA damage. The reasons for this have been described. However, although the A2780/MCP cell-lines all showed resistance to cisplatin compared to parental A2780 cells it is unknown as to whether they show greater resistance to ionising radiation. A2780/cp70 and other cell-lines selected for resistance to cisplatin do demonstrate cross-resistance to multiple cytotoxic agents including ionizing radiation, melphalan, doxorubicin, etoposide and mitoxantrone (Vasey et al. 1995; Hamaguchi et al. 1993). This implies that the A2780/MCP cell-lines could well display resistance to ionizing radiation, although this remains to be tested formally. Assessing the cell-cycle parameters of each cell line treated with equitoxic doses of cisplatin would be another experiment that would help clarify the p53 dependent functions of these cells.

There is, however, no indication as to how the development of an RER+ phenotype and abnormal p53 function are associated. It has already been stated that at a

cDNA level the sequence of A2780/cp70 is identical to that of A2780 (Brown et al. 1993). However, TP53 accumulates in the nuclei of the former cell-line suggesting stabilisation of the protein. This accumulation of TP53 in A2780/cp70 is confirmed by the results presented here; however there would appear to be no consistent correlation between basal p53 protein levels and p53 function in the A2780/MCP cell lines. One possibility is that, in some way, development of an RER+ phenotype increases the likelihood that a p53 defective clone will be selected for during exposure to cisplatin. Although the p53 gene has not been sequenced in the A2780/MCP cell lines it may be that defective p53 function in these lines is not a result of mutation in the p53 protein. There is some evidence to suggest that in certain tumour types there is an inverse correlation between the presence of an RER+ phenotype and mutations in p53 (Uchida et al. 1994; Strickler et al. 1994). It may be that the presence of the RER+ phenotype results in inactivation of the p53 gene or protein by other mechanisms thus removing the necessity for selection of mutants in these cell lines.

The A2780/cp70 cell line has been grown over many years in culture and displays the most prominent RER+ phenotype of all the cisplatin resistant A2780 cell lines studied. It would appear likely that this has arisen due to a growth advantage for the RER+ cells during culture, with the result that they now constitute the whole population of A2780/cp70 cells. In the A2780/MCP cell lines, having undergone less expansion in culture since initial selection, RER+ cells have had less time to exert a growth advantage. These lines may therefore be a mixture of RER+ and RER− cells. This could explain the intermediate levels of resistance to cisplatin and the reduced loss of p53 dependent functions when compared to A2780/cp70. Further growth in culture might increase the proportion of RER+ cells in the population. This could be an explanation for the apparent loss of p53 functions with further culturing of the A2780/MCP5 cell line. The existence of mechanisms of cisplatin resistance inA2780 cell lines that do not depend on the acquisition of an RER+ phenotype or the loss of p53 functions is indicated by A2780/MCP7.

Addendum to Chapter 3

The cisplatin resistance (as measured by clonogenic assay) and p53 dependent DNA damage responses of A2780, A2780/cp70 and the A2780/MCP cell lines has previously been published (Table 4. Anthoney et al. 1996). The published data on fold resistance to cisplatin, relative p53 protein level and S-phase fraction of cells after 2Gy ionising radiation differs quantitatively from that presented in this chapter (tables 3.5, 3.8, 3.9). The published results represent an interim analysis of data obtained from a smaller number of experiments than those presented in the thesis. Further experiments were performed between publication of the paper and writing of the thesis.

Although there is a difference in the numerical values presented between the two sets of data there is no significant change in the conclusions that can be drawn. Thus all the A2780/MCP cell lines show increased resistance to cisplatin compared to A2780, with A2780/MCP1 having a greater fold resistance in the final analysis as compared to the interim data. No consistent difference in the levels of p53 in the A2780/MCP cell lines as compared to A2780 were observed in either set of data. Finally, each of the A2780/MCP cell lines showed evidence of a loss of inhibition of entry into S-phase after 2Gy which was similar in both sets of data. As discussed in the paper by Anthoney et al, one possible explanation for differences in the data sets is that the A2780/MCP cell lines appear to evolve, with regards to loss of p53 function, with prolonged growth in culture.

CHAPTER 4

*CHARATERISATION OF MISMATCH REPAIR DEFECT IN CISPLATIN
RESISTANT OVARIAN CARCINOMA CELL LINES*

4.1 INTRODUCTION.

A significant association has been identified between the phenotype of resistance, or tolerance, to certain types of cytotoxic anti-cancer agents and the replication error (RER+) phenotype, characterised by an increased mutation rate at microsatellite repeat sequences. Evidence for this comes from tumour cell lines resistant to alkylating agents (Branch et al. 1995), as well as from the cisplatin and doxorubicin resistant lines presented in the previous chapter. The RER+ phenotype has since been shown to arise from defects in the process of mismatch repair, characterised by the finding of mutations in genes homologous to those known to be involved in prokaryotic mismatch repair (Modrich and Lahue, 1996). Tumour cell lines with alkylation tolerance have been shown to harbour mutations in mismatch repair genes. Confirmation that mutations in these genes actually result in defective mismatch repair comes from the use of *in vitro* repair assays (Boyer et al. 1995). The whole story is not cut and dried however. A significant percentage of HNPCC tumours expressing an RER+ phenotype do not display mutations in any of the known mismatch repair genes (Rhyu, 1996). This suggests that there are components of the mismatch repair pathway as yet uncharacterised, mutations of which may play a role in the development of the malignant phenotype and perhaps anti-cancer drug tolerance. Therefore it is important to attempt to characterise the underlying genetic and biochemical defects in mismatch repair in the RER+ cisplatin resistant A2780 cell lines.

A variety of different biochemical and genetic methods have been employed to characterise the involvement of mismatch repair gene mutations in certain forms of cancer. Genetic linkage analysis in HNPCC kindreds, and PCR utilising degenerate primers was involved in the identification of hMSH2 and hMLH1 (Lindblom et al. 1993; Peltomaki et al. 1993). Degenerate PCR, with primers targeted for conserved regions of known bacterial and yeast mismatch repair genes, allowed the identification of the coding sequences for the genes (Bronner et al. 1994; Fishel et al. 1993). Subsequent confirmation that germ-line and somatic mutations in these genes were

found in individuals affected with HNPCC came from sequencing genomic or cDNA from normal and tumour tissues (Leach et al. 1993). The hPMS1 and hPMS2 genes were identified during a search for homology with bacterial and yeast mismatch repair genes in a human gene database (Papadopoulos et al. 1994).

Identification of homologues of the E.coli. MutS protein have made use of the ability of such proteins to bind to mismatches in DNA. In these experiments, binding of a protein to an oligonucleotide DNA-duplex containing a mismatch results in retardation of the electrophoretic migration of the duplex compared to unbound oligonucleotide. Using protein purification and HPLC techniques, followed by gel retardation assays with mismatched oligonucleotides, GTBP (hMSH6) (Palombo et al. 1995) and an, as yet, uncharacterised A-T mismatch binding protein (Stephenson and Karran, 1989) have been isolated.

Biochemical assays of mismatch repair have been used to determine whether RER+ cell lines display defective repair. Complementation studies with cell lines having defined mismatch repair mutations, allows isolation of the defective repair components in any RER+ line. The mismatch repair assays use a variety of DNA templates with single base-base or more complex insertion/deletion mismatches engineered into them. Addition of such templates to nuclear extracts from the cell line under study allows measurement of the rate of mismatch repair. To detect that repair has occurred the mismatch is engineered into a "reporter" region that allows differentiation between repaired and unrepaired DNA. In one assay the mismatch is constructed in a β-galactosidase reporter gene, in either a mammalian shuttle vector (Parsons et al. 1993) or M13 phage (Umar et al. 1994). After repair in the nuclear extract, the DNA construct is introduced into mismatch repair deficient E.coli and the difference in the ratio of blue to white colonies or plaques counted. This ratio is determined by the restoration of β-galactosidase function on removal of the mismatch. Other types of reporter constructs have been used in assays of mismatch repair.

Development of an RER+ phenotype is a very frequent event in the selection of cisplatin resistant clones of the A2780 cell line, as demonstrated in chapter 3. This RER+ status is determined only by the presence of microsatellite instability. It is therefore necessary to characterise the mismatch repair function of these cisplatin resistant A2780 derived cell lines. The following sections present data on the biochemical and molecular analysis of mismatch repair in these cell lines.

4.2   CHARACTERISATION OF MISMATCH BINDING COMPONENTS OF A2780,A2780/CP70 AND A2780/MCP CELL LINES.

4.2.1   Immunoblot analysis of mismatch binding proteins.

Immunological detection of mismatch repair proteins has been used to characterise the mismatch repair system in tumour cell lines (Palombo et al. 1995). This allows a gross comparison of the cellular protein levels but does not provide information on the functional state of such proteins in the cell.

To determine whether differences exist in the detectable levels of the mismatch binding proteins hMSH2 and hMSH6 (GTBP), in A2780 and its cisplatin resistant derivatives, an immunoblot analysis was performed. Protein extracts were prepared from confluent cultures of each cell line as detailed in chapter 2, section 2.3. The concentration of protein in each extract was determined using a modified Bradford assay (Bradford, 1976). An equal quantity of total cell extract was then subjected to gel electrophoresis and Western blot analysis.

A polyclonal antibody, prepared from rabbits injected with a GST fusion polypeptide corresponding to amino acid residues 27 - 158 of hMSH2 (Palombo et al. 1995),was used in the detection of hMSH2 protein. This was kindly supplied by Dr. J. Jiricny (Istituto di Ricerche di Biologia Molecolare. Pomezia, Italy.). When this antibody was used to probe the Western immunoblot membrane a specific signal was

detected with each of the cell lines. A doublet band running at about 100kDa was observed. This is the predicted size of the hMSH2 protein although in previous experiments using nuclear extracts a single band of this size has been observed in HeLa cells (Palombo et al. 1995). As can be seen in figure 4.1 hMSH2 is detected in each of the cell lines analysed although the level appears to vary between the lines. A relatively strong signal for hMSH2 is observed for A2780, A2780/cp70 and A2780/MCP1 & MCP3 to 6. The other A2780/MCP lines show apparently reduced levels of hMSH2 protein. However, when the same membrane was probed with an antibody against vinculin, the same variation in protein levels between the cell lines was observed.

The level of vinculin expression in cells is consistent and it acts as a control for protein loading and transfer in the experiment. The A2780/MCP cell lines which appeared to express low levels of hMSH2 also displayed low signals with vinculin antibody. This suggests that the differences in the hMSH2 levels between the A2780 cell lines arises from unequal transfer of protein to the Western membrane and not from true differences in hMSH2 levels. The levels of hMSH2 detected in A2780 and A2780/cp70 were similar. It is therefore unlikely that differences in the RER status of these lines arises from altered levels of hMSH2.

The hMSH2 protein is known to form a heterodimeric complex, known as hMutSα (Drummond et al. 1995), with another protein hMSH6 (GTBP). Western immunoblot analysis of the levels of hMSH6 in the cisplatin resistant A2780 cell lines was performed using a polyclonal antibody raised against amino acids 750-928 of the hMSH6 protein (supplied by Dr. J. Jiricny). As can be seen from figure 4.1(b) hMSH6 also appears to be present in all of the cell lines. The same pattern of variation in the level of protein between the cell lines is seen as for hMSH2. The signal is weakest in those lanes which show the lowest vinculin levels (A2780/MCP2,7,8 and 9) suggesting that the difference between hMSH6 levels in the cells arises due to unequal protein transfer and does not represent true variation between the cells. A2780 and A2780/cp70

(a) hMSH2
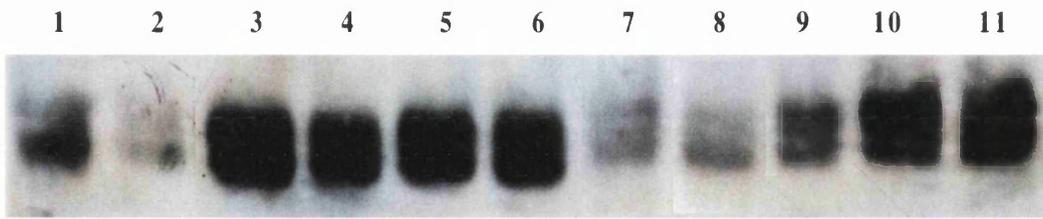
(b) hMSH6 (GTBP)
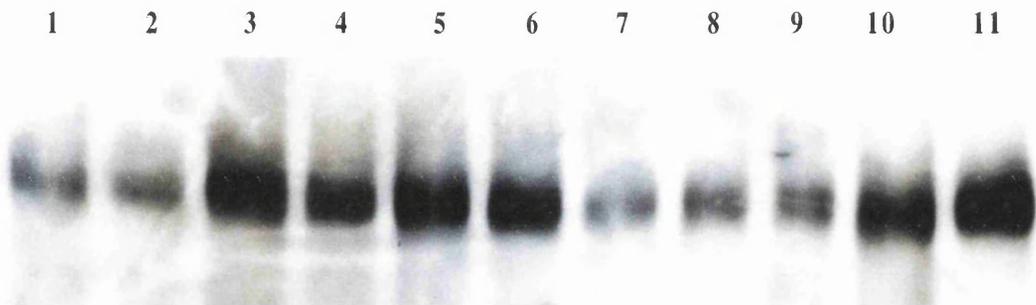
(c) Vinculin
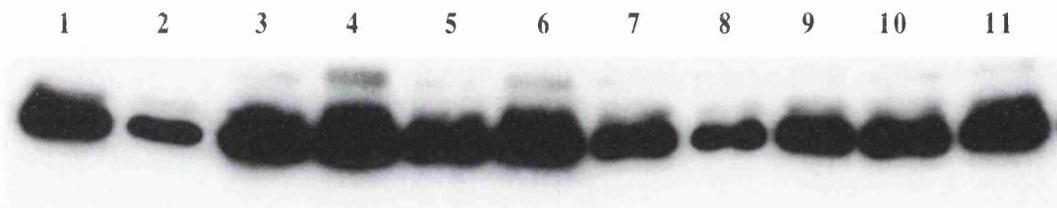
Figure 4.1 Basal levels of hMSH2 and hMSH6 protein in A2780 and cisplatin resistant derived cell-lines.

Western immunoblot representing basal levels of, (a) hMSH2, (b) hMSH6 and, (c) vinculin proteins in the cell lines indicated. Equal amounts of cellular total protein extract were analysed per cell-line. Immunodetection of vinculin was used to control for equivalence of total protein on the Western blot.

Legend.

(1) A2780/MCP1   (2) A2780/MCP2   (3) A2780/MCP3   (4) A2780/MCP4
(5) A2780/MCP5   (6) A2780/MCP6   (7) A2780/MCP7   (8) A2780/MCP8
(9) A2780/MCP9   (10) A2780/cp70   (11) A2780

show similar protein levels, again suggesting that the RER+ phenotype is not due to differences in the level of hMSH6.

To determine whether alterations in the levels of hMSH2 or hMSH6 proteins were associated with the RER+ phenotype observed in the A2780/AD cell line immunoblot analysis was performed using the same antibodies. A2780, A2780/cp70, A2780/MCP1 and 5 were also run for comparison (figure 4.2). Differences in the signals obtained with hMSH2 and hMSH6 are seen between the cell lines with A2780/AD showing a reduced signal with hMSH6. However, when the signal to vinculin protein is analysed it would appear that there is a reduced level of A2780/AD protein on the membrane compared to the other cell lines. Again, therefore, it can be assumed that the differences in hMSH6 and hMSH2 observed between A2780/AD and the other lines arises from unequal protein transfer and does not represent a true variation.

The results from the hMSH6 and hMSH2 immunoblot assays would suggest, therefore, that differences in the levels of these proteins between A2780 and cisplatin or doxorubicin resistant derivatives are not responsible for the RER+ phenotype observed.

4.2.2 Mismatch binding activity in cisplatin resistant A2780 cell lines.

The results of the preceding section suggest that no significant differences exist in the levels of hMSH2 or hMSH6 between the various cisplatin resistant A2780 derived cell lines. They do not, however, give any indication as to the ability of these proteins to bind to mismatches occurring in DNA. Therefore a gel-retardation assay was employed to identify whether components of cell extracts from A2780 and derived cells could bind to specific DNA mismatches. This work was performed in collaboration with Dr. R. Brown and A. McIlwrath, with advice and assistance from the laboratory of Dr. P. Karran.
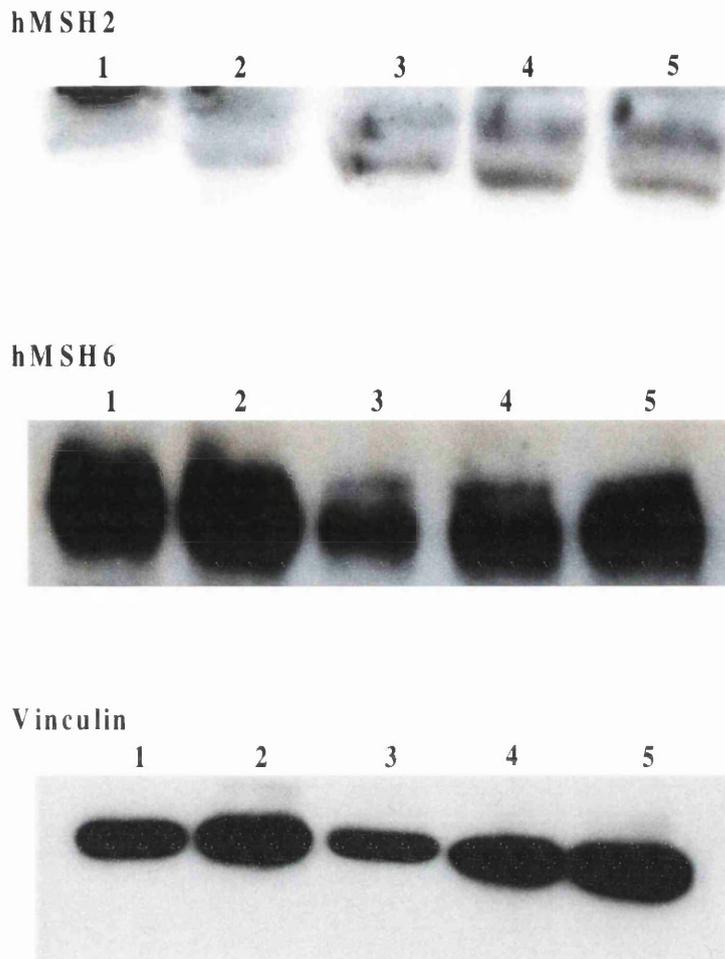
Figure 4.2 Basal levels of hMSH2 and hMSH6 proteins in A2780/AD
A2780 and, cisplatin resistant, derived cell-lines

Western immunoblot representing basal levels of hMSH2, hMSH6 and vinculin proteins in the cell lines indicated. Equal amounts of cellular total protein extract were used for each line. Each image represents the same blot probed with antibody to the specific protein indicated. Immunodetection of vinculin was used to control for differences in the amount of total protein on the blot.

Legend.   (1) A2780, (2) A2780/cp70, (3) A2780/AD,
(4) A2780/MCP1, (5) A2780/MCP5.

Gel-retardation assays have been used previously to identify proteins that can bind to specific mismatches in synthetic oligonucleotides. Jiricny et al. (Jiricny et al. 1988) used this method to identify a 200kDa complex that bound specifically to G/T mismatches and later identified this as GTBP (Hughes and Jiricny, 1992). Stephenson and Karran (Stephenson and Karran, 1989) distinguished both G/T and A/C mismatch binding proteins in extracts from a human Burkitt's lymphoma cell line by a similar procedure.

In the present assay a synthetic 34-mer oligonucleotide was synthesised that contained either a complementary G/C pairing at nucleotide number 16 (homoduplex) or a mismatch, either G/T or A/C, at the same position (heteroduplex). End-labelled oligonucleotide was added to cell extracts that had been previously exposed to poly(dI· dC) and 40fmol of unlabelled nonmismatched oligonucleotide to remove non-specific DNA binding proteins. The reaction was left for 20 minutes at room temperature and the products electrophoresed on non-denaturing polyacrylamide gels prior to detection by autoradiography.

Oligonucleotide that is not bound by protein migrates quickly through the gel and can be observed at the bottom of the autoradiograph (figure 4.3). Addition of labelled heteroduplex oligonucleotide (A/C mismatch) resulted in the formation of a specific retardation complex which was observed in lane 1 of all three cell lines. If, however, a labelled heteroduplex oligonucleotide (G/T mismatch) was added to the cell extract a different retardation complex, consisting of a more slowly migrating band, was observed (A2780 lane 2). If the A2780 cell extract was pre-incubated with a 100-fold excess of unlabelled G/T mismatched oligonucleotide prior to addition of the labelled heteroduplex, then the mismatch specific retardation complex was not observed (A2780 lane3) showing that this complex was specific for G/T mismatches. The A2780/AD cell line also showed formation of this G/T mismatch specific retardation complex at a level equivalent to that seen with A2780 (A2780/AD lane 2). A2780/cp70, however, showed a greatly reduced level of this retardation complex (
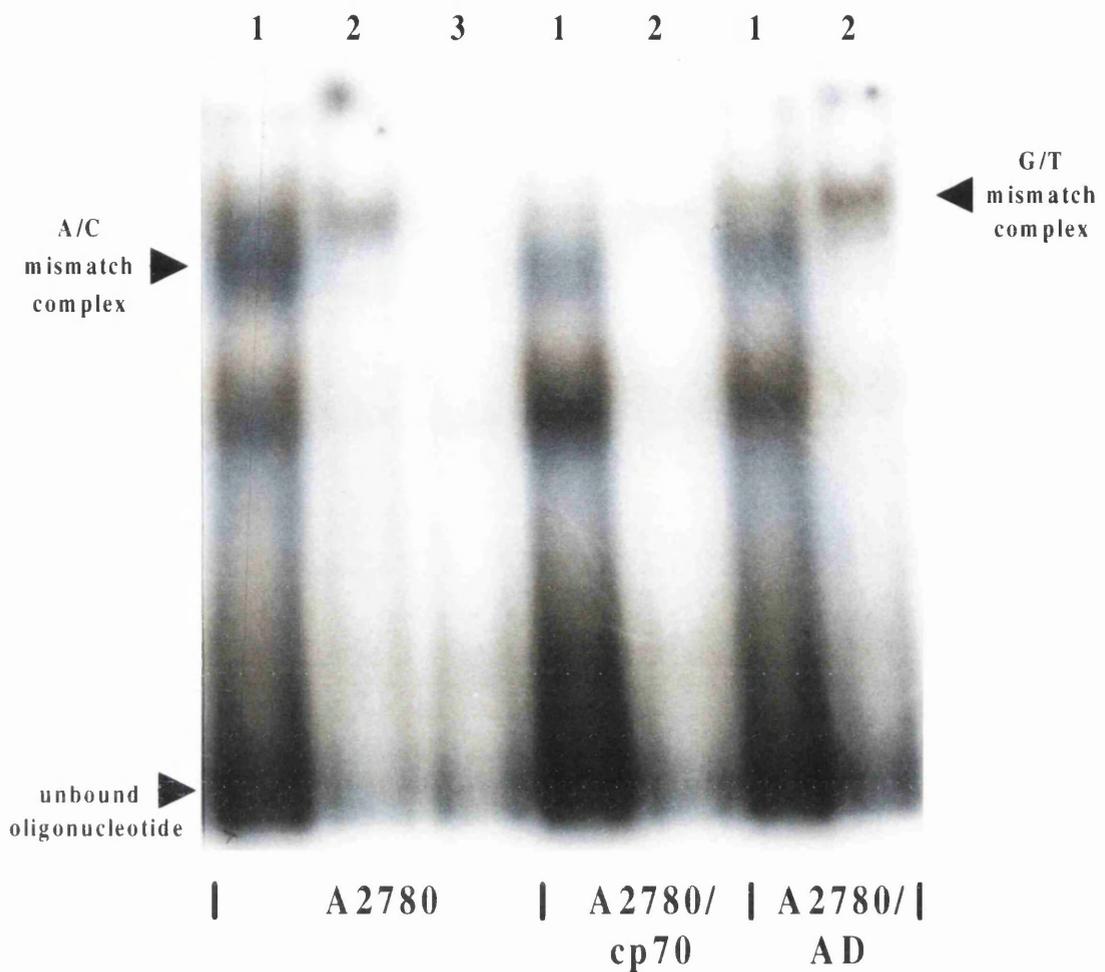
Figure 4.3 Detection of mismatch binding proteins in cell extracts from A2780, A2780/cp70 and A2780/AD by gel retardation.

Cell extracts from the cell lines indicated were incubated with end-labelled oligonucleotides containing either A/C or G/T mismatches. Binding of proteins to the mismatch was indicated by retardation of the oligonucleotide on the gel as compared to unbound oligonucleotide. Specificity of binding to a particular mismatch was determined by pre-incubation of the cell extract with unlabelled oligonucleotide containing the same mismatch.

Although not shown, no specific retardation complexes were observed using completely matched, labelled, homoduplex oligonucleotides.

Legend   Lane1. A/C mismatched oligonucleotide

                     Lane2. G/T mismatched oligonucleotide

                     Lane3. G/T mismatched oligonucleotide (pre-incubation with unlabelled mismatched oligonucleotide)

A2780/cp70 lane 2). This suggests that the A2780/cp70 cells are defective in a G/T mismatch binding function that is present in A2780 whilst still retaining separate A/C mismatch recognition. This result was reproducible using independently isolated cell extracts from each cell line, suggesting that degradation of proteins during extraction were unlikely to be a cause of the lack of A2780/cp70 G/T mismatch binding.

4.3 ASSESSMENT OF MISMATCH REPAIR ACTIVITY IN A2780 DERIVED CELL LINES USING AN IN VITRO REPAIR ASSAY.

The RER+ phenotype is evidence of a defect in the mismatch repair system within a cell. The ability to detect the presence of mismatch repair proteins, such as hMSH2, by immunological assays indicates that a cell is able to transcribe and translate the appropriate gene. It does not determine, however, the functional state of the resultant protein. To assess the function of the mismatch repair system in A2780 and its drug resistant derivatives a biochemical assay of mismatch repair was used. The *in vitro* mismatch repair assay was performed in collaboration with Dr. James Drummond and Dr. Paul Modrich (Duke University, Durham, N.Carolina, USA.) on cells provided from this laboratory.

The basis of the *in vitro* assay is measurement of the rate of repair of a synthetic mismatched template by extracts of the cell line under study. The template used in this assay was based on the phage f1MR (Su et al. 1988). A 6440 base-pair heteroduplex was formed from f1MR containing a strand specific break at position 6440/0 (figure 4.4). It has been shown previously that in mammalian cells a strand break is sufficient to determine the directionality of mismatch repair (Holmes et al. 1990) Depending upon which strand of the heteroduplex the break resided determined whether it was 3' or 5' to the mismatched bases (figure 4.4). At a position in the heteroduplex 808 bases from the strand break a variety of mismatched bases could be engineered. These included single base-base mismatches and di, tri and tetranucleotide insertion/deletion
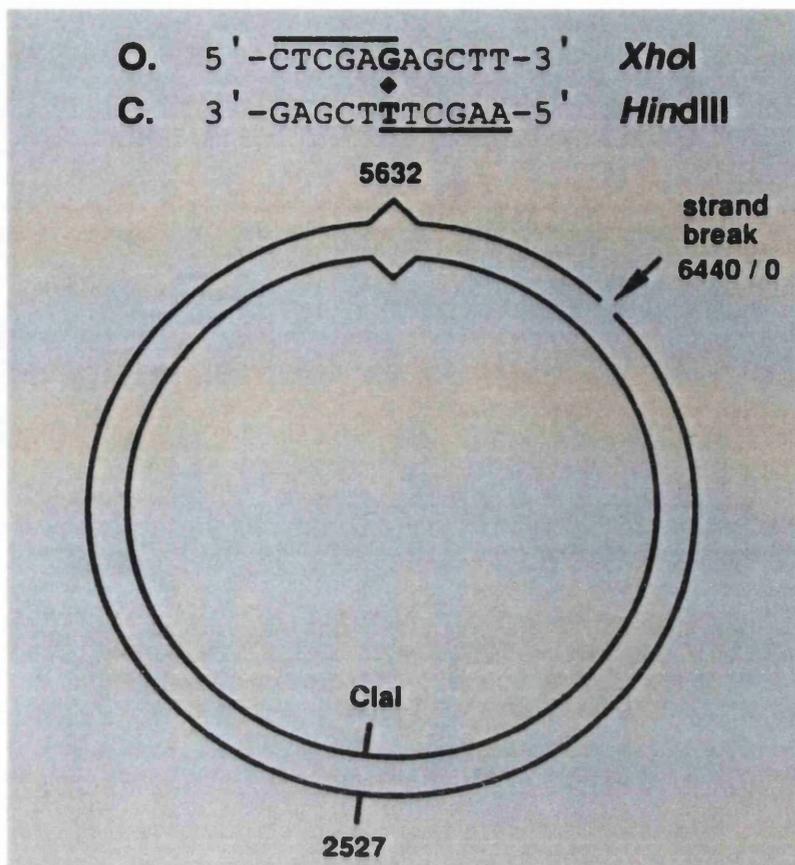
Figure 4.4 Heteroduplex substrate for in-vitro mismatch repair assay

Diagramatical representation of the f1MR(n) phage construct used as a substrate in the in-vitro mismatch repair assay. The phage is 6440 base-pairs long and contains a strand specific break at base-pair 6440 and a single base-base or dinucleotide insertion/deletion mismatch at base-pair 5632. The mismatch lies within the overlapping recognition sites for the restriction endonucleases indicated. (Taken from Holmes, J. et al. 1990).

Legend.   O = mispair residing on open strand of heteroduplex within XhoI restriction site.

C = mispair residing on covalently closed strand of heteroduplex within HindIII restriction site.

mismatches. The mismatches were engineered in such a way as to lie within, and disrupt, the sequence of two overlapping restriction endonuclease recognition sites. The presence of a mismatch at these sites prevents cleavage of the DNA by the restriction enzyme. With the template used in this experiment, correction of the mismatch on the open (O) strand e.g. reversion to G/C, results in restoration of the XhoI restriction site. If the mismatch is corrected on the opposite (C) strand e.g. reversion to A/T, the HindIII site is restored.

In the mismatch repair assay, template DNA is incubated for 15 minutes with a nuclear extract of the cell line (chapter 2, section 2.10.2). The proportion of template DNA that can be digested with either of the restriction enzymes after the incubation, is taken as a measure of the repair of the mismatch in the heteroduplex. If a defect in mismatch repair is observed then complementation, using either purified mismatch repair complexes or nuclear extracts from cells with defined mismatch repair mutations, can be performed to identify the missing or mutated component of repair.

Four cell lines were used in the *in vitro* mismatch repair assay, A2780, A2780/cp70, A2780/AD and A2780/MCP1. This assay was performed by Dr. J. Drummond. Two different types of template were employed in the reaction, with either a G/T mismatch or a CA dinucleotide loop mismatch arranged with the strand break either 3' or 5' to it. The results of the assays with these cell lines can be seen in figure 4.5. In A2780 a normal level of repair is observed for both mismatches and either orientation of strand break. When the strand break is 3' to the mismatch all three drug-resistant cell lines show complete loss of detectable repair activity. When the strand break is 5' to either type of mismatch, A2780/cp70 and A2780/MCP1 have a slight reduction in repair activity compared to A2780 whereas A2780/AD repairs slightly more efficiently. This therefore suggests that a specific defect in 3' directed mismatch repair exists in these drug resistant ovarian cancer cell lines. To determine where the defect leading to abnormal 3' directed mismatch repair lay, the cell extracts were complemented with either 220ng of purified hMutSα (hMSH2/hMSH6) or 160ng of
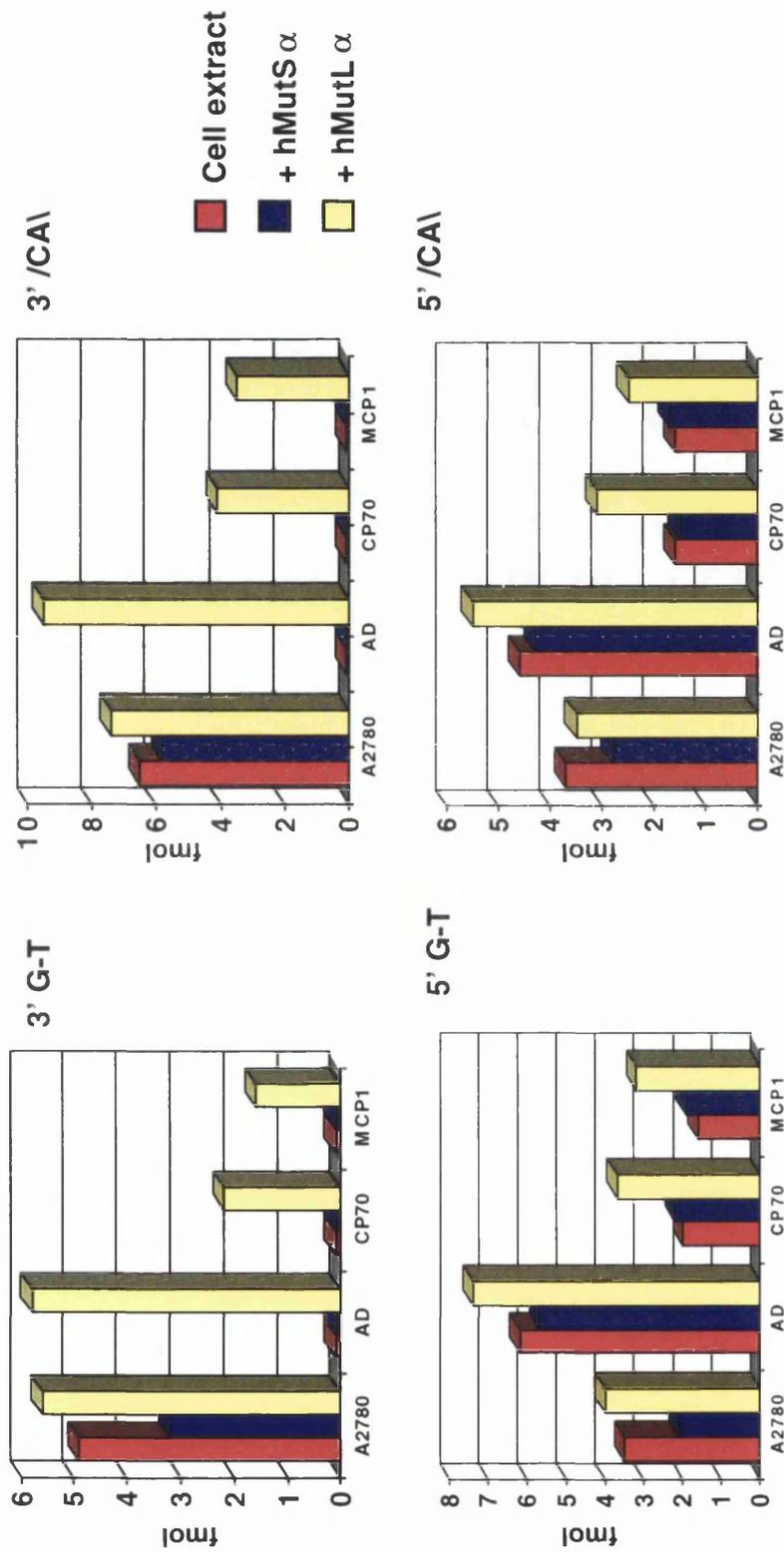
Figure 4.5. In vitro mismatch repair assay of A2780 and drug resistant derivatives.

The efficiency of DNA mismatch repair was determined by addition of a phage construct, containing the mismatches indicated, to extracts of each cell line and the amount of DNA repaired (fmol) in 15 minutes measured. Less then 0.5 fmol repaired DNA / 15 minute reaction represents defective repair. Normal levels of repair are 5 fmol/15 min and above. To define the abnormal component of repair in defective cell lines, complementation, using purified hMutS-alpha or hMutL-alpha, was performed.

hMutLα (hMLH1/hPMS2) and the repair activity measured. As can be seen in figure 4.5 complementation with hMutSα did not result in any restoration of repair function in these cell lines. Addition of hMutLα, however, fully restored the repair function in A2780/AD to levels similar to A2780 and brought about a significant increase in the levels of repair seen in A2780/cp70 and A2780/MCP1. Complementation with hMutSα when repairing mismatches from a 5' orientated strand break did not result in any change in repair activity whereas hMutLα resulted in a slight but not significant increase in repair.

To further define the defect in the mismatch repair activities of these cell lines complementation using nuclear extracts from cells with known mutations in mismatch repair genes was performed. MT1 cells have been shown to harbour defects in the GTBP subunit of hMutSα (Drummond et al. 1995). Addition of a nuclear extract of MT1 cells to extracts of A2780/cp70, A2780/AD and A2780/MCP1 restored repair of 3' orientated mismatches (Figure 4.6). RKO cells are colorectal carcinoma cells deficient in hMutLα activity, due to a homozygous, or hemizygous, chain termination mutation in hMLH1. Addition of extracts from the RKO cells did not restore the repair function of A2780 derived cell lines (Figure 4.6).This further indicates that the likely defect in 3' directed mismatch repair involves a component of hMutLα, most likely hMLH1.

4.4   IMMUNOBLOT ANALYSIS OF THE MutLα COMPONENTS hMLH1 AND hPMS2 IN DRUG RESISTANT A2780 CELL LINES.

The results of the complementation studies in the *in vitro* repair assay, implicate the hMutLα complex as the defective component of mismatch repair in A2780/cp70, A2780/AD and A2780/MCP1. Defective hMutLα function can arise from mutation in the hMLH1 or hPMS2 genes, whose products form the hMutLα heterodimer. Mutations in these genes can either result in production of a non-functional protein or a reduction
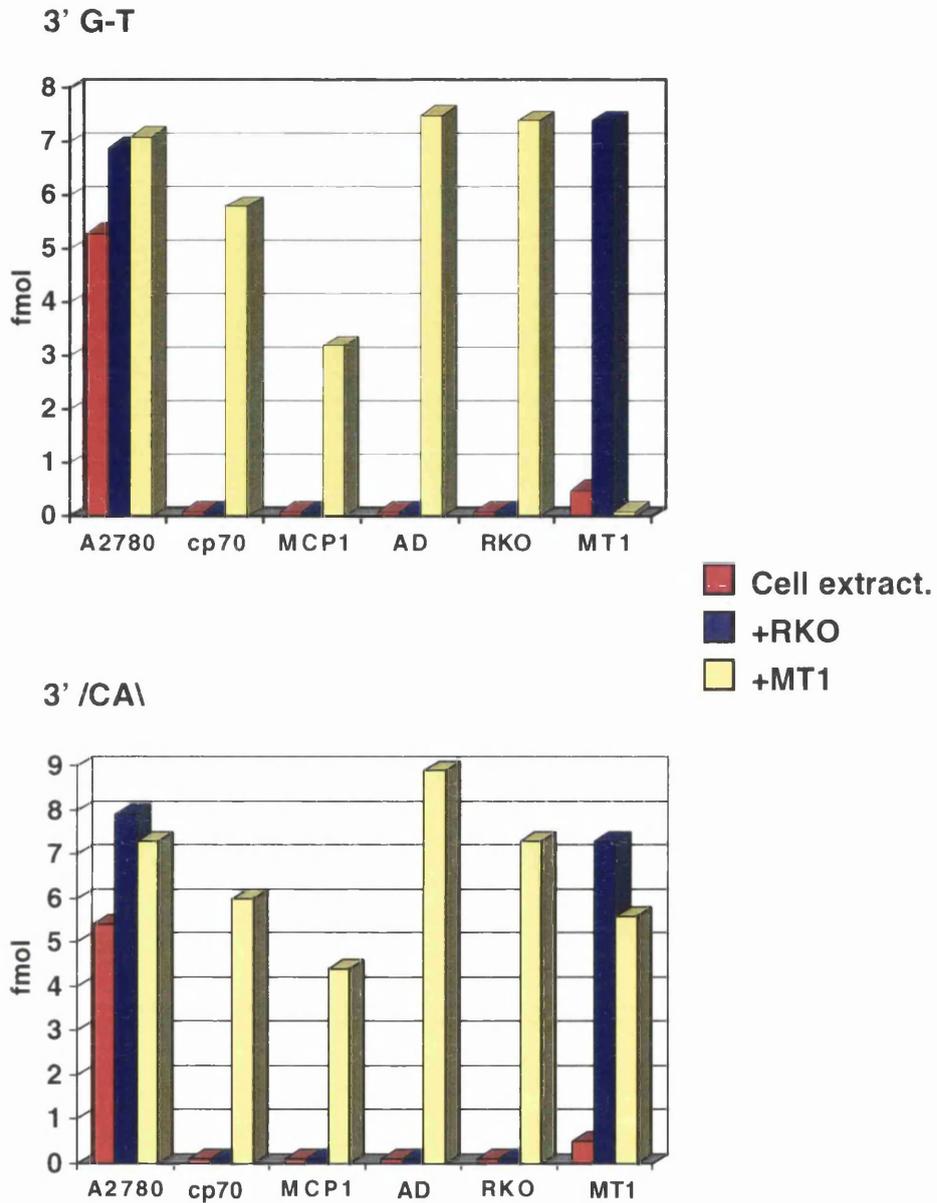
Figure 4.6 Characterisation of mismatch repair defects in A2780, drug resistant, cell lines, by complementation.

An in vitro mismatch repair assay was performed in which repair of the G-T or -CA-mismatch on the phage construct was directed from 3' to the lesion. The amount of repaired DNA, in fmol, after a 15 minute reaction was determined. Complementation of each reaction with cell extracts of RKO cells (MutL-alpha deficient) and MT1 cells (MutS-alpha deficient) enabled characterisation of the defect in 3' mismatch repair.

in the amount of protein within the cell. Reduction in the intracellular concentration of either protein can result in loss of hMutLα function.

To determine whether gross differences in the levels of hMLH1 or hPMS2 proteins exist between A2780 and its cisplatin and doxorubicin resistant derivatives, Western immunoblot analysis was performed. Specific monoclonal antibodies to hMLH1 and hPMS2 are available commercially. G168-15 (PharMingen Labs, Torreyville, CA. USA) is a monoclonal antibody raised against full length hMLH1 protein. In the immunoblot assays performed with this antibody, extracts from two different cell lines were used as controls. The MCF7 cell line was recommended as a positive hMLH1 control (manufacturers data sheet). The endometrial carcinoma cell line AN3CA has previously been shown to lack detectable hMLH1 mRNA (Umar et al. 1994) and to be defective in mismatch repair due to lack of hMLH1 function (J. Drummond. Personal Communication). This line was used, therefore, as a negative control.

The results of an hMLH1 immunoblot of cell extracts from A2780, A2780/cp70, A2780/MCP1 to MCP9 and A2780/AD, as well as positive and negative controls, is shown in figure 4.7. Three of the cell line, A2780, A2780/MCP8 and MCF7 show a specific band on the immunoblot with an apparent molecular weight of approximately 80kDa, which is that expected for hMLH1. Each of these cell lines also show a more intense band, with an apparent molecular weight of about 45kDa. This smaller band is not observed in any of the cell lines which do not display the full-sized hMLH1, even after prolonged enhanced chemiluminescence exposure. The hMLH1 immunoblot also detects two bands in the A2780/AD cell line. These have an apparent molecular weight intermediate between that of hMLH1 and the 45kDa band seen in A2780, A2780/MCP8 and MCF7. If the immunoblot is exposed to enhanced chemiluminescence and then autoradiographed for an extended period of time similar bands are observed in all cell lines. It is highly likely that these bands represent non-specific hybridisation of the antibody.

Vinculin
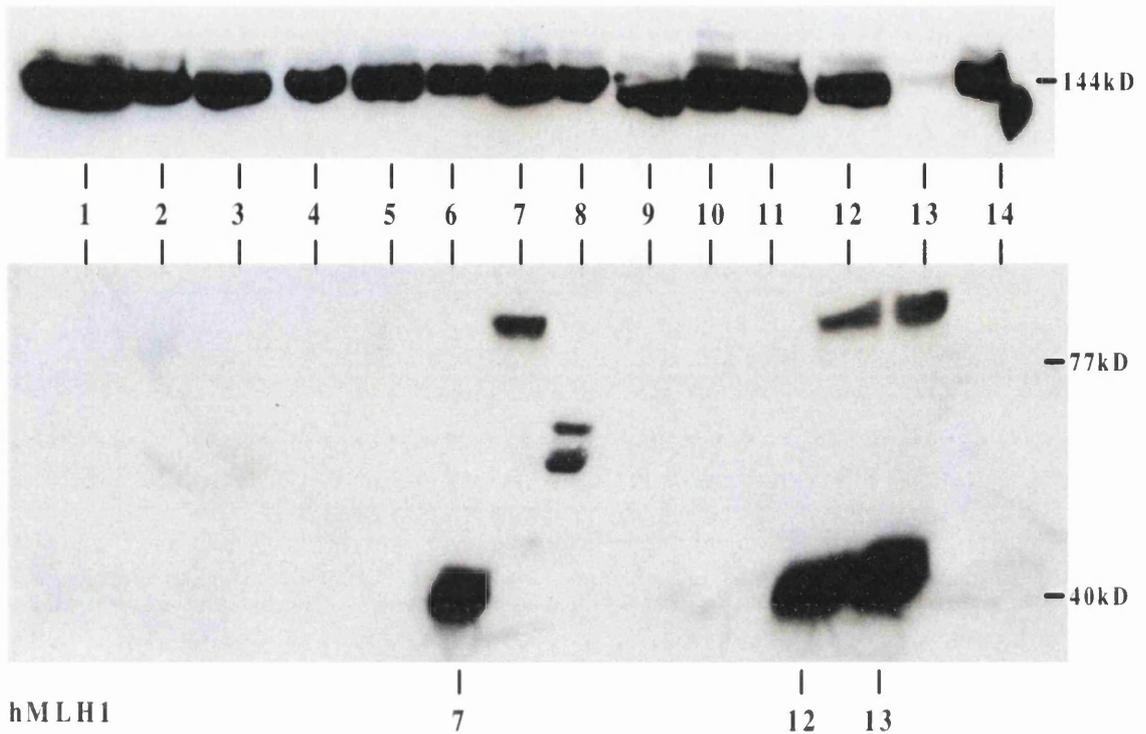
Figure 4.7 Basal levels of hMLH1 protein in A2780 and derived cell lines selected for resistance to cisplatin and doxorubicin.

Western immunoblot representing basal levels of hMLH1 and vinculin protein in the cell lines indicated. Immunodetection of vinculin was used to control for equivalence of total protein on the Western blot. MCF7 and AN3CA cell lines acted as positive and negative controls for hMLH1 respectively. Certain gel lanes are numbered top and bottom for ease of identification.

Legend.

1:- A2780/MCP1. 2:- A2780/MCP3. 3:- A2780/MCP5. 4:- A2780/MCP7.
5:- A2780/MCP9. 6:- AN3CA. 7:- A2780. 8:- A2780/AD. 9:- A2780/MCP2.
10:- A2780/MCP411:- A2780/MCP6. 12:- A2780/MCP8. 13:- MCF7.
14:- A2780/cp70

As the 45kDa band is specific only to those cell lines which possess full length hMLH1 protein it was postulated that it might represent either a truncated version of the protein or a degradation product. The cell extracts in the original immunoblot were made using a high salt extraction technique (Chapter 2, section 2.3). To determine whether the presence of the 45kDa band depended on the extraction conditions used for making cell extracts a direct cell-lysis method was used (Harlow and Lane, 1988). This technique is less likely to result in protein degradation as it involves less manipulation of the cell extract and cellular proteases are directly inhibited by the high concentration of SDS. The result of an immunoblot using the directly lysed cells shows an increase in the amount of non-specific bands of intermediate weight between hMLH1 and the 45kDa signal (data not shown). The 45kDa band is still present in the same cell lines as before although appears to be less intense than when extracted using the high salt lysis buffer. From the results of these assays it is presumed that the 45kDa band is either a specific breakdown product of hMLH1, that is not simply due to degradation, or represents a shortened form of the protein found in cells of at least two different lineages (A2780 and MCF7). In either case it is only observed in those cells which display full length hMLH1.

hMLH1 combines with hPMS2 in a 1:1 stoichiometry to form hMutLα. In view of the lack of detectable hMLH1 protein in nine out of ten cisplatin resistant A2780 cell lines and the A2780/AD line, we proceeded to look for expression of hPMS2 protein in these cells. Figure 4.8 shows the autoradiographic images from a Western immunoblot exposed to (a). hMLH1 and (b). hPMS2. The monoclonal antibody Ab-1 (Clone 9) (Calbiochem, Cambridge, Mass. USA.) raised against full length hPMS2 protein was used to detect this protein on the blots. The hMLH1 immunoblot shows a consistent result, in that detectable full length protein is only observed with A2780, A2780/MCP8 and the positive control line MCF7. Exactly the same picture is seen with hPMS2, in that only these cell lines show a band of the expected molecular weight of about 95kDa. None of the other cell lines show any evidence of reaction with this antibody. Therefore

(a) hMLH1
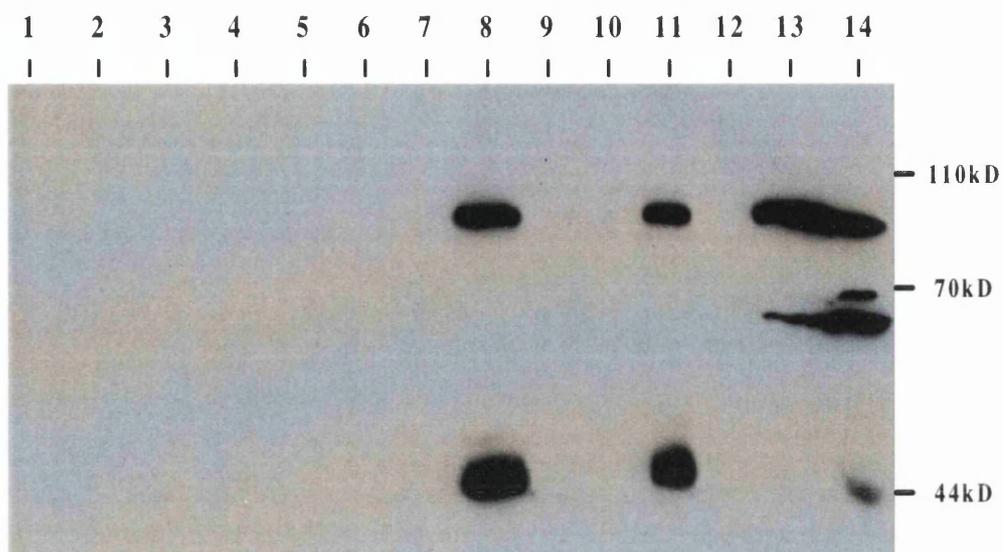
(b) hPMS2
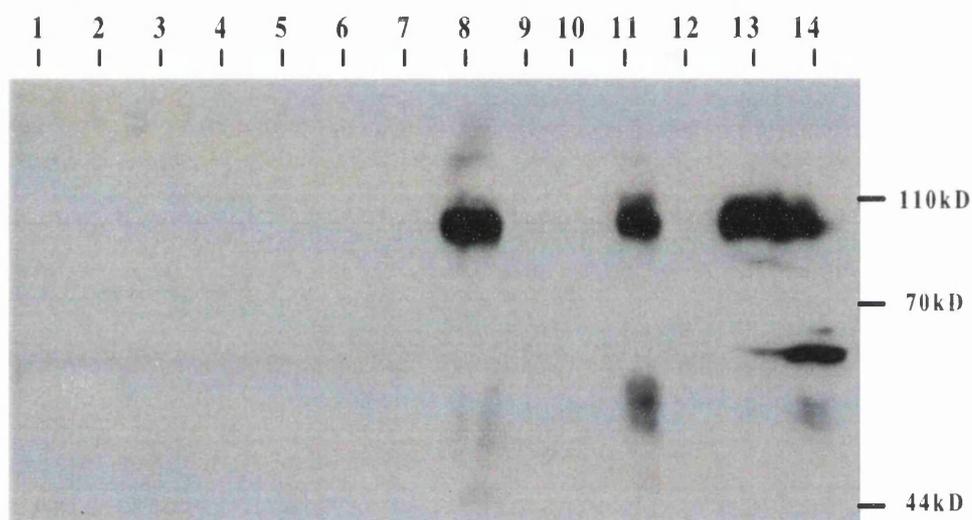
Figure 4.8 Basal levels of hMLH1 and hPMS2 in A2780 and derived cell lines selected for resistance to cisplatin and doxorubicin.

Western immunoblot representing basal levels of, (a) hMLH1 and (b) hPMS2 in the cell lines indicated. Equal amounts of cellular total protein extract were analysed per line. MCF7 and DLD1 were used as positive controls for these proteins. Protein sizes were estimated using appropriate molecular size markers.

Legend:-
1:- A2780/MCP1. 2:- A2780/MCP2. 3:- A2780/MCP3. 4:- A2780/MCP4.
5:- A2780/MCP56:- A2780/MCP6. 7:- A2780/MCP7. 8:- A2780/MCP8.
9:- A2780/MCP9. 10:- A2780/cp70 11:- A2780. 12:- A2780/AD. 13:- DLD1.
14:- MCF7.

it would appear that there is a complete absence of the components of the hMutLα heterodimer in all but one of the A2780 derived anti-cancer drug resistant cell lines.

4.5 WESTERN IMMUNOBLOT DETERMINATION OF hMSH2 AND hMLH1 IN CISPLATIN RESISTANT A2780/SCP CELL LINES.

As previously discussed the A2780/SCP cell lines were derived by exposure of A2780 cells to a single concentration of 15μM cisplatin for 24 hours. Investigation of these cell lines revealed a stable but low degree of resistance to cisplatin and the presence of microsatellite mutations in three of the lines. One of the lines, A2780/SCP6 was considered to have an RER+ phenotype in view of the high frequency of microsatellite mutation observed. Using the same antibodies described in sections 4.1 and 4.4 a number of the A2780/SCP cell lines were investigated with regards to their hMSH2 and hMLH1 status. Figure 4.9 shows the results of these investigations. The levels of hMSH2 protein detected vary little amongst the different A2780/SCP cell lines or with A2780 or A2780/cp70. A2780 shows a detectable band with hMLH1 antibody whereas A2780/cp70 shows no evidence of detectable protein. The A2780/SCP cell lines all show evidence of detectable hMLH1 protein, including A2780/SCP6, which is RER+, and A2780/SCP5 which displayed microsatellite mutation at one locus out of ten studied. These results indicate that loss of detectable hMLH1 protein may not always represent the defect in mismatch repair selected for in cisplatin resistant A2780 cells.

4.6 IMMUNOHISTOCHEMICAL DETECTION OF hMLH1 PROTEIN IN A2780 AND CISPLATIN RESISTANT A2780 DERIVED LINES.

Immunoblotting techniques are useful in determining characteristics of protein antigens such as their presence and relative quantity within a cell. However, such variables as the method of protein extraction or protein denaturation may alter the

(a) hMLH1

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

(b) hMSH2

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
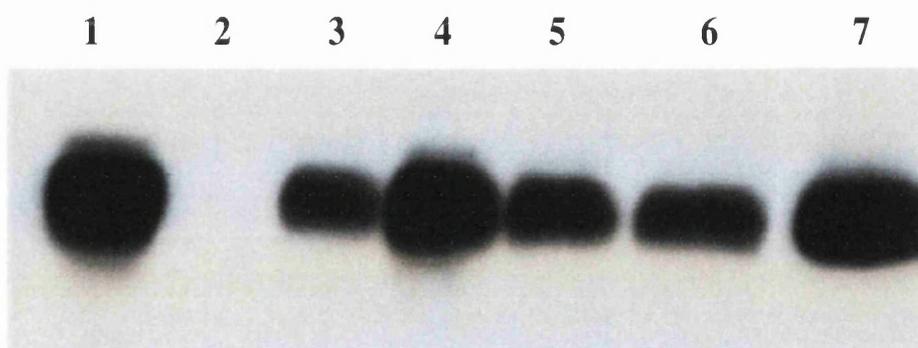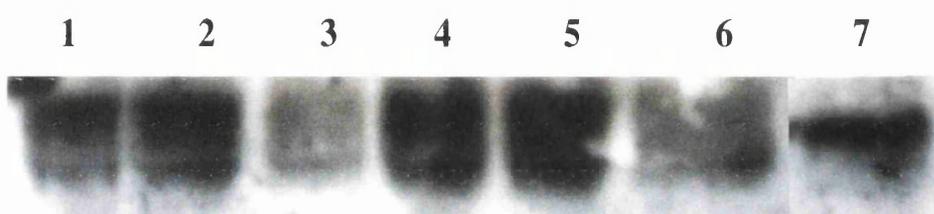
Figure 4.9   Basal levels of hMLH1 and hMSH2 in A2780 cell lines selected
for resistance to cisplatin by single step selection process.

Western immunoblot representing the basal levels of (a) hMLH1 and (b) hMSH2
in the cell lines indicated. Equal amounts of cellular total protein extract were
analysed per cell line. Autoradiographic images represent the same immunoblot
probed with the two different antibodies.

Legend.

(1) A2780   (2) A2780/cp70   (3) A2780/SCP2   (4) A2780/SCP10

(5) A2780/SCP4   (6) A2780/SCP6   (7) A2780/SCP1

sensitivity of this technique. In addition, immunoblotting gives no information as to the subcellular localisation of the protein of interest. Immunohistochemical detection of proteins within whole cells allows a determination of the subcellular localisation of the protein under investigation. It also provides another method by which the presence or absence of a particular protein within a cell can be assessed. In collaboration with A. McIlwrath in our laboratory an immunohistochemical analysis of hMLH1 was performed on the A2780 derived cell lines.

1×10^5 cells from each line were processed by the method described in chapter 2 (section 9.1). Analysis of the cells treated with hMLH1 antibody was performed using confocal microscopy. The use of propidium iodide as a counter stain enables identification of the nucleus within the cell. This allows determination of the sub-cellular localisation of the particular protein under study. An example of the results observed with A2780 and its derived cell lines is shown in figure 4.10. With A2780 intense propidium iodide staining, localised to the nucleus, is observed in all cells. The signal from hMLH1 antibody is also localised to the nucleus and again is present in all cells. A similar picture is seen with A2780/MCP8, with the majority of cells staining strongly for nuclear hMLH1. A2780/cp70, on the other hand, shows no evidence of hMLH1 staining within the cells despite a good nuclear signal for propidium iodide in the cells. In A2780/MCP2 the majority of cells displayed no staining for hMLH1. However, one or two small groups of cells did reveal a positive nuclear hMLH1 signal, suggesting some heterogeneity for the presence of hMLH1 within the cell population. To determine the heterogeneity of hMLH1 protein levels amongst cells within each line, a total of 400 cells were analysed from three different high power fields, for the presence of nuclear hMLH1 staining. The results of this are shown in table 4.1.

hMLH1   Propidium Iodide

A2780

hMLH1   Propidium Iodide

A2780/cp70

hMLH1   Propidium Iodide

A2780/MCP8

hMLH1   Propidium Iodide
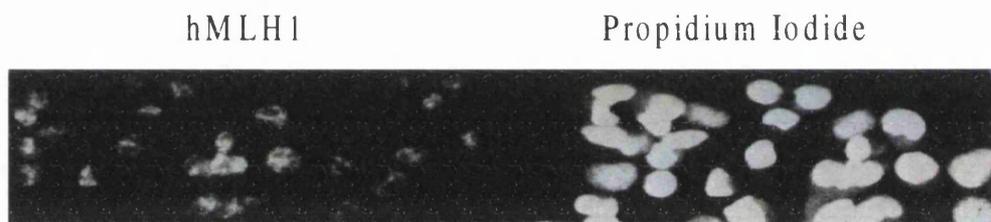
A2780/MCP2

Figure 4.10   Immunohistochemical detection of basal hMLH1 levels in A2780
                   and cisplatin resistant derived cell lines.

Confocal micrographs representing the basal expression of hMLH1 protein in fixed cells of
A2780, A2780/cp70 and A2780/MCP 8 & 2. Left and right frames in each panel represent
the same microscopic field, viewed for hMLH1 (left) or Propidium Iodide (right). The
propidium iodide binds to DNA and acts as a control for cell staining..
(These micrographs were produced in collaboration with Amanda McIlwrath).

A2780	cp70	MCP1	MCP2	MCP3	MCP4	MCP5	MCP6	MCP7	MCP8	MCP9
>90%	-ve	-ve	2.3%	-ve	-ve	0.8%	-ve	2.5%	>90%	-ve

Table 4.1 Expression of hMLH1 protein in A2780 derived cells.

Legend: The percentage of cells staining positive for hMLH1, out of 400 cells counted using confocal laser microscopy, is presented on the lower line. A -ve result indicates that no cells were observed that displayed staining for hMLH1 antibody.

It can be seen, therefore, that the majority of cell lines which showed no evidence of hMLH1 by immunoblot analysis also show no detection of protein using immunohistochemistry. A2780 and A2780/MCP8, which are the only two cell lines to show the presence of hMLH1 by immunoblot, show strong staining for the protein in nearly all cells. Three of the A2780/MCP cell lines do, however, indicate the presence of hMLH1 within a small percentage of the cell population. The A2780/MCP cell lines were derived from a clonal population of A2780 cells. However, during the selection process for resistance to cisplatin the population of cells was not further sub-cloned. The result of this is that the A2780/MCP cell lines that resulted from this selection process are not a clonal population of cells. It is likely that they consist of cells that are heterogeneous with regards to the mechanisms by which they have developed resistance to cisplatin. As indicated in the previous section, not all A2780 cells selected for cisplatin resistance, which display evidence of microsatellite mutation, need have developed this through loss of hMLH1. It is possible, therefore, that the small numbers of hMLH1 positive cells observed in A2780/MCP2, 5, and 7, arise due to the non-clonal nature of the lines.

4.7 CONFIRMATION OF THE IDENTITY OF A2780/MCP7 AND A2780/MCP8 CELL LINES USING MICROSATELLITE PCR.

The results from the immunoblot analysis of hMLH1 and hPMS2 in the A2780 derived cisplatin resistant cell lines, show that only A2780/MCP8 possessed detectable levels of either protein. This result was surprising in that A2780/MCP8 had been shown to display an increased frequency of microsatellite mutation and was also deficient in p53 functions as compared to A2780 (Chapter 3). On the other hand, A2780/MCP7 which had not shown any mutations at the microsatellite loci studied, and had levels of p53 function most similar to A2780, appeared to be deficient in hMLH1 and hPMS2. One explanation for this could have been that at some point after the initial characterisation of the microsatellite instability status of the cell lines the A2780/MCP7 and A2780/MCP8 lines had inadvertently been switched. To ascertain whether such a switch had occurred the following experiment was performed. A2780, A2780/MCP7 and A2780/MCP8 cells, from the same stocks used to produce the protein extracts for Western immunoblotting, were grown and genomic DNA extracted. This DNA was labelled as 1996 DNA. The original genomic DNA stocks used to perform the microsatellite analysis was available for these cell lines and was labelled as 1994 DNA. In the original study of microsatellite mutations in these cell lines the only locus at which A2780/MCP8 showed an alteration from A2780 was D2S123. Therefore duplicate samples of 1994 and 1996 DNA from all three cell lines were amplified at this locus using the previously described PCR protocol (Chapter 2, section 7.1) and the products electrophoresed on a polyacrylamide gel. The resulting autoradiograph is shown in figure 4.11. As can be seen there is a shift in the size of the microsatellite alleles between A2780 and A2780/MCP8 in the original 1994 DNA. No difference is observed between A2780/MCP7 and A2780. When comparison is then made to the DNA samples from 1996, no change is observed in the state of the microsatellite alleles. This would therefore suggest that no switch in these cell lines has occurred since the
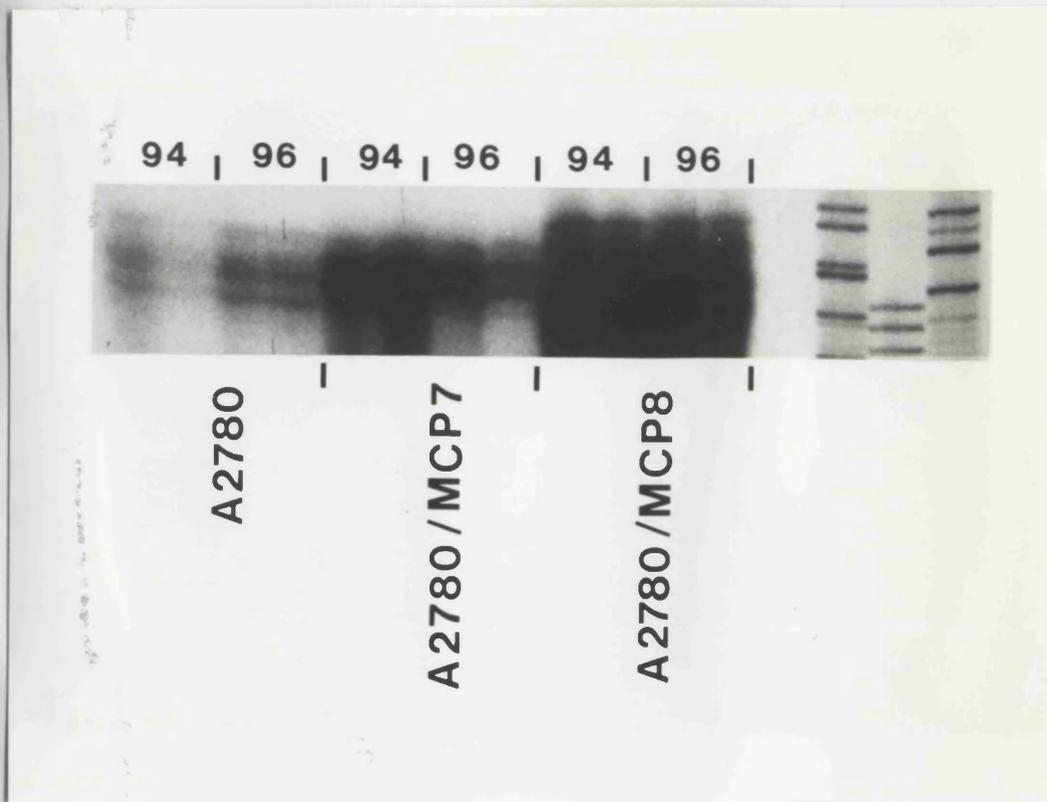
Figure 4.11 Determination of the identity of A2780/MCP cell lines using comparative analysis of microsatellite repeat sequences.

Comparison of the microsatellite allele D2S123 in DNA from A2780, A2780/MCP7 and A2780/MCP8 between 1994 to 1996. DNA from the cell lines obtained in 1994 (94) and that obtained from the same cell lines cultured in 1996 (96) was amplified by PCR using primers for the locus D2S123 and separated by gel-electrophoresis. To allow comparison of the alleles a DNA size ladder was also included in the electrophoresis.

No difference is observed in the size of the allele in each cell line between DNA from 1994 and 1996. The shift in allele size in A2780/MCP8 compared to A2780 continues to be observed.

original microsatellite analysis and that the results observed in the hMLH1 and hPMS2 immunoblots are accurate.

4.8 NORTHERN BLOT ANALYSIS OF EXPRESSION OF MISMATCH REPAIR GENE mRNA IN DRUG RESISTANT A2780 DERIVED CELL LINES.

The absence of detectable hMLH1 and hPMS2 protein in A2780/cp70 and all but one of the A2780/MCP lines is something of a surprise. It seems unlikely that whatever selection pressures are acting upon these cells should result in the loss of two genes acting within the same DNA repair pathway. It would therefore be of interest to determine whether the absence of hMLH1 and hPMS2 proteins within these cell lines is a consequence of abnormal gene expression. It has been shown previously that mutations arising within a gene, that result in certain alterations of the mRNA, render it unstable. Thus the mRNA is rapidly degraded within the cell and is undetectable by standard techniques (Lim et al. 1992). To further define the defects arising in the cisplatin resistant cell lines we proceeded to analyse the mismatch repair genes, at the level of their mRNA expression, by Northern blot analysis.

Total RNA was isolated from A2780, A2780/cp70, and A2780/MCP1-9 cells using a commercial kit based on the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA was also extracted from MCF7 and AN3CA cells to act as positive and negative controls respectively. The Northern blot was performed as decribed in Chapter 2, (section 4.1). To allow determination of the size of a particular mRNA species, an RNA ladder was electrophoresed concurrent with the other RNA samples. The distance migrated by each of the known RNA standards was measured during UV transillumination of the ethidium bromide stained gel. A plot of distance migrated versus RNA size was then constructed. This gives an approximate guide to the size of any RNA species detected.

To confirm that transfer of non-degraded total RNA from the agarose gel to the membrane was equal for all lines, the membrane was initially probed with a cDNA clone of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Tso et al. 1985). GAPDH is found in essentially constant and equivalent levels in most cells, thus allowing it to be used as an internal control for gene expression. To detect hybridisation to specific RNA species on the membrane, the GAPDH cDNA was radiolabelled with α ^{32}PdCTP by random priming (Chapter 2, section 3.2). Figure 4.12(a) shows the pattern of hybridisation of the GAPDH probe to the membrane. There were no significant differences in the levels of the signal obtained with the GAPDH probe between the cell lines, suggesting efficient transfer of the RNA to the membrane. It can be assumed, therefore, that equal amounts of non-degraded mRNA from each cell line, are present on the Northern membrane.

To determine the level of expression of hMSH2 mRNA a probe consisting of the cDNA for this gene was used to screen the membrane. hMSH2 cDNA was obtained from Prof. B. Vogelstein (Johns Hopkins University, Baltimore, USA) cloned into a pB SK(+/-) plasmid. The hMSH2 cDNA was excised from the plasmid vector by restriction digestion and purified before radiolabelling. Hybridisation to the same Northern membrane as used for GAPDH was performed and the result of this hybridisation can be observed in figure 4.12(b). A specific signal of equivalent intensity is observed with all the cell lines. The migration distance of the band gives an RNA species which is consistent with the size of the hMSH2 mRNA. These results confirm those of the Western immunoblot for hMSH2 and suggest equivalent expression of this gene among A2780 and its drug resistant derivative lines.

A cDNA probe, consisting of 750bp from the 5' end of hMLH1, was prepared from MCF7 cells and radiolabelled. The results of hybridising the Northern blot membrane with this probe are observed in Figure 4.12(c). The signal intensity from the membrane as a whole is low which might be a result of the small size, and hence lower specific activity, of the probe in this hybridisation. A number of bands with calculated

Figure 4.12   Determination of the basal levels of mRNA for hMSH2, hMLH1
and hPMS2 in A2780 and its drug resistant derived cell lines.

A comparison of the basal level of gene expression for each of the mismatch repair
genes, hMSH2, hMLH1 and hPMS2, in A2780 and its derived cell lines was made using
northern blot analysis. To control for efficient transfer of mRNA from gel to membrane,
and against degradation of RNA during processing, determination of the level of
GAPDH mRNA in each cell line was made.

The arrow in hMLH1 represents the approximate molecular weight of hMLH1 mRNA

Legend

(1) A2780 (2) A2780/cp70 (3) A2780/MCP1 (4) A2780/MCP2 (5) A2780/MCP3 (6)
A2780/MCP4 (7) A2780/MCP5 (8) A2780/MCP6 (9) A2780/MCP7

(10) A2780/MCP8 (11) A2780/MCP9 (12) MCF7 (13) AN3CA

a

1 2 3 4 5 6 7 8 9 10 11 12 13

GAPDH

b

1 2 3 4 5 6 7 8 9 10 11 12 13

hMSH₂

c

1 2 3 4 5 6 7 8 9 10 11 12 13

hMLHI

d

1 2 3 4 5 6 7 8 9 10 11 12 13

hPMS₂
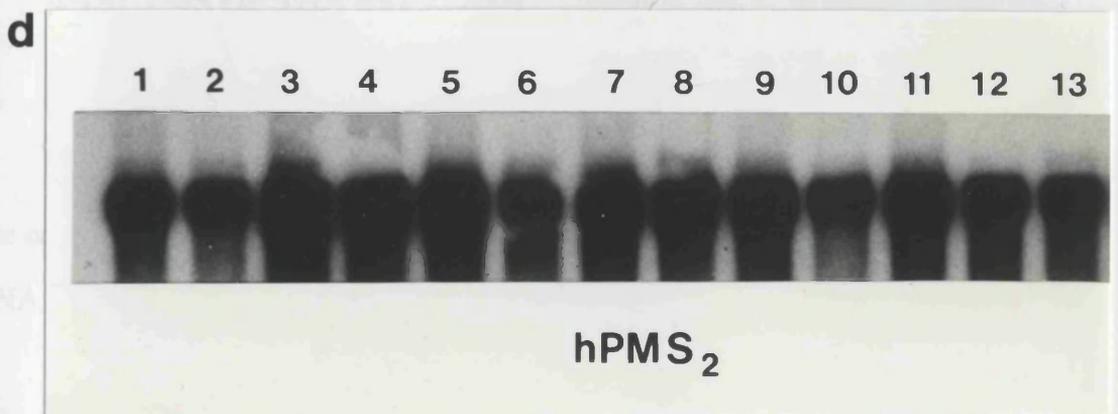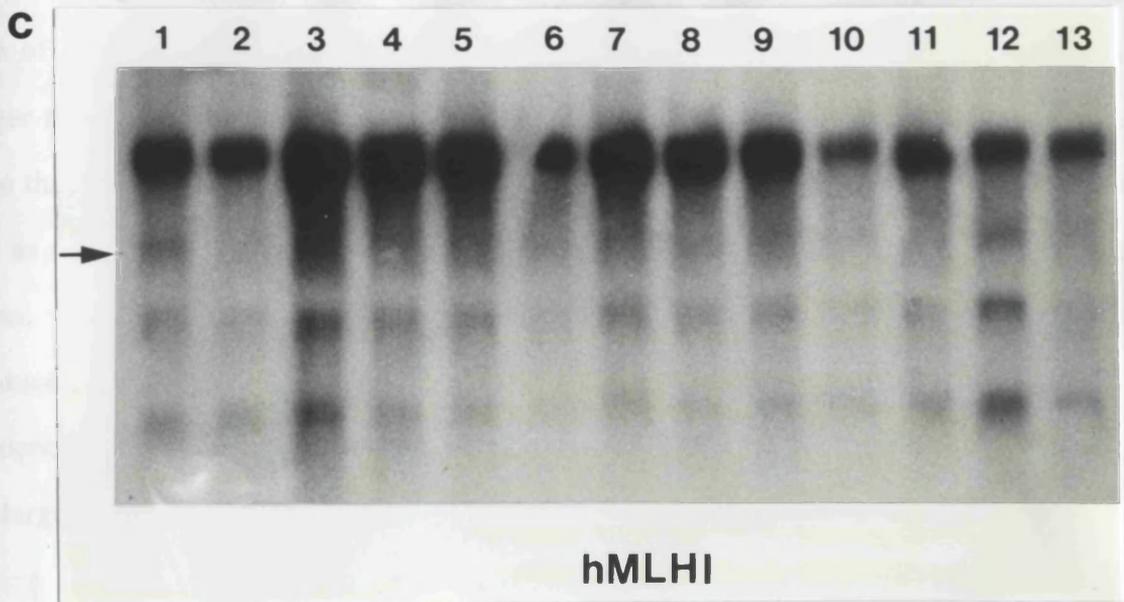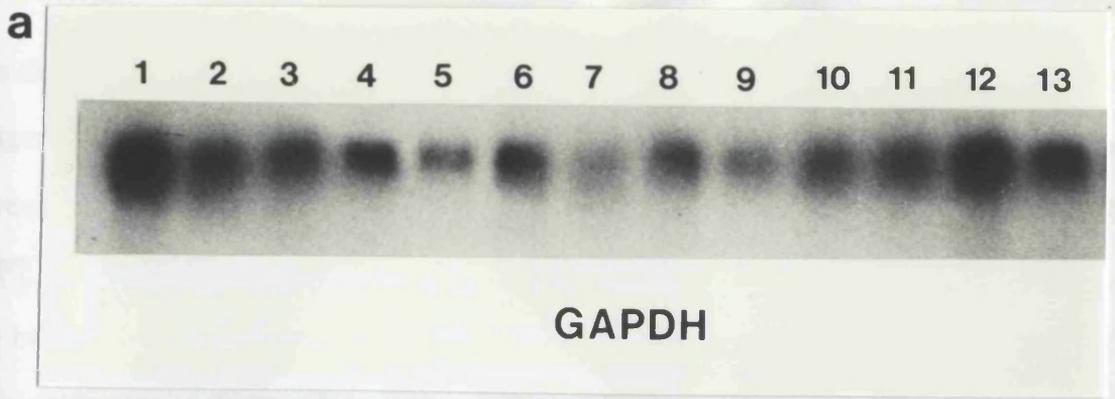
sizes different from that expected for hMLH1 mRNA are observed and persisted despite stringent washing of the membrane. These represent non-specific hybridisation of the probe. A very faint signal could be detected in the lanes corresponding to A2780 and MCF7 running at an estimated size in keeping with that expected for hMLH1 mRNA. This band could not be detected in any of the other cell lines, including A2780/MCP8. The significance of this result in terms of the levels of hMLH1 mRNA is unclear due to the difficulty in differentiating signal from background.

To determine the levels of hPMS2 mRNA in the cell lines a cDNA probe, consisting of an 800bp segment from the 5' end of the gene, was prepared from MCF7 cells. Figure 4.12(d) shows the result of hybridisation of the radiolabelled hPMS2 probe to the Northern membrane. A highly specific signal is obtained from each of the cell lines with little background hybridisation. This suggests that mRNA is produced from the hPMS2 gene in each of the lines, and with relatively equal efficiency. However, the size of hPMS2 mRNA calculated from the signal detected on the Northern blot was larger than would have been expected from the known size of hPMS2 cDNA. At the time that this experiment was performed no hPMS2 deficient cell line was available to use as a negative control, with the result that it could not be determined whether this signal was specific for cells with hPMS2 only. It has been shown that hPMS2 is produced in a number of different mRNA forms, varying in the size of the 5' upstream sequences and the length of the poly-A tail (Nicolaides et al. 1995b). This could explain the larger size of the mRNA species detected with this hybridisation.

4.9 ANALYSIS OF THE EXPRESSION OF MISMATCH REPAIR GENE mRNA IN A2780 DRUG RESISTANT CELL LINES USING RT-PCR.

The Northern blot analysis in the previous section would tend to suggest that those cell lines which do not show hMLH1 protein also show no expression of hMLH1 mRNA. The same cell lines, however, do express hPMS2 mRNA which does not result

in detectable protein. Unfortunately the poor signal quality from hMLH1 probes, and the unexpectedly larger estimated size of hPMS2 mRNA, makes it difficult to draw definite conclusions from these experiments. To clarify further the gene expression of hMLH1 and hPMS2 in the cisplatin resistant A2780 derived cell lines, the more sensitive technique of RT-PCR has been used. An equal amount (2 or 5µg) of total RNA extract from each cell line was used to form cDNA by reverse transcription (Chapter 2, section 7.2) using oligo-d(T) primers. It is important to determine whether reverse transcription has proceeded equally well in all cell lines. This is to ensure that differences in the levels of specific mRNA detected between lines are real and not due to differences in the efficiency of reverse transcription between lines (Rashtchian, 1994). To accomplish this, PCR primers that amplify ubiquitous genes are used. β-actin is the most commonly used control gene because its mRNA is present in high levels in nearly all cells and tissues. PCR primers which amplify a 838bp region of β-actin cDNA were used for this purpose. To detect the presence of mRNA from hMLH1, hMSH2 and hPMS2, specific PCR primers were designed to amplify a region of the corresponding cDNA. The sequences of these primers are shown in chapter 2, (section 1.10). The PCR reactions were performed using the optimal annealing temperature for the primer pair used.

When using RT-PCR in a semi-quantitative manner, it is important to determine an optimal number of cycles that lies on the exponential section of the amplification curve (Gause and Adamovicz, 1994). The reason for this is that once the reaction reaches its plateau (due to utilisation of all the primer or polymerase enzyme) no further amplification occurs. Although different cell lines might, therefore, start off with varying levels of gene specific mRNA, if too many cycles of amplification are allowed they will all attain the plateau phase thus tending to equalise the amounts of PCR product obtained. In view of the result from the Northern assay, suggesting that the majority of the cisplatin resistant cell lines displayed no hMLH1 mRNA, this RT-PCR assay was not intended to be used semi-quantitatively but instead to detect gross

differences in the levels of RNA. Notwithstanding this, however, it was observed that 35 cycles of PCR probably represented the plateau phase for all the different primer pairs and hence 30 cycles were used in most cases. An equal volume of the products from each reaction were run on a 2% agarose gel, stained with ethidium bromide and visualised under UV transillumination.

The results of these RT-PCR reactions are shown in figure 4.13. Amplification with hMLH1 specific primers shows that A2780, A2780/MCP8 and the positive control line MCF7 all express equivalent amounts of mRNA. The remainder of the cisplatin resistant cell lines, except A2780/MCP5, show no detectable PCR products, as is the case with the negative control, AN3CA. This suggests that no hMLH1 mRNA is expressed in these cells. A2780/MCP5 shows a very small amount of correctly sized PCR product suggesting that hMLH1 mRNA may be expressed but at levels greatly reduced to that found in A2780. This has been confirmed for A2780/MCP5 and some of the other cisplatin resistant cell lines (MCP2,3,6,7 and 9) by amplification with a greater number of PCR cycles. It should be remembered that A2780/MCP2, 5 and 7 were cell lines that showed small numbers of hMLH1 positive cells by immunohistochemistry. The non-clonal nature of the A2780/MCP cell lines was proposed as an explanation for this finding. The RT-PCR technique is extremely powerful in detecting small quantities of mRNA. It may be, therefore, that the very small amount of amplification obtained in some of the A2780/MCP cell lines by hMLH1 RT-PCR, arises from small numbers of cells, in the non-clonal population, which retain functional hMLH1.

RT-PCR using primers specific to hPMS2, produces equivalent levels of product DNA in each of the cell lines (figure 4.13). This suggests that there is no significant difference in the levels of hPMS2 mRNA amongst the A2780 derived cell lines. This result, along with that of the Northern blot analysis, indicates that the lack of detectable hPMS2 protein in the cisplatin resistant A2780 cell lines is not due to reduced gene expression. The results of RT-PCR using hMSH2 specific primers indicate that
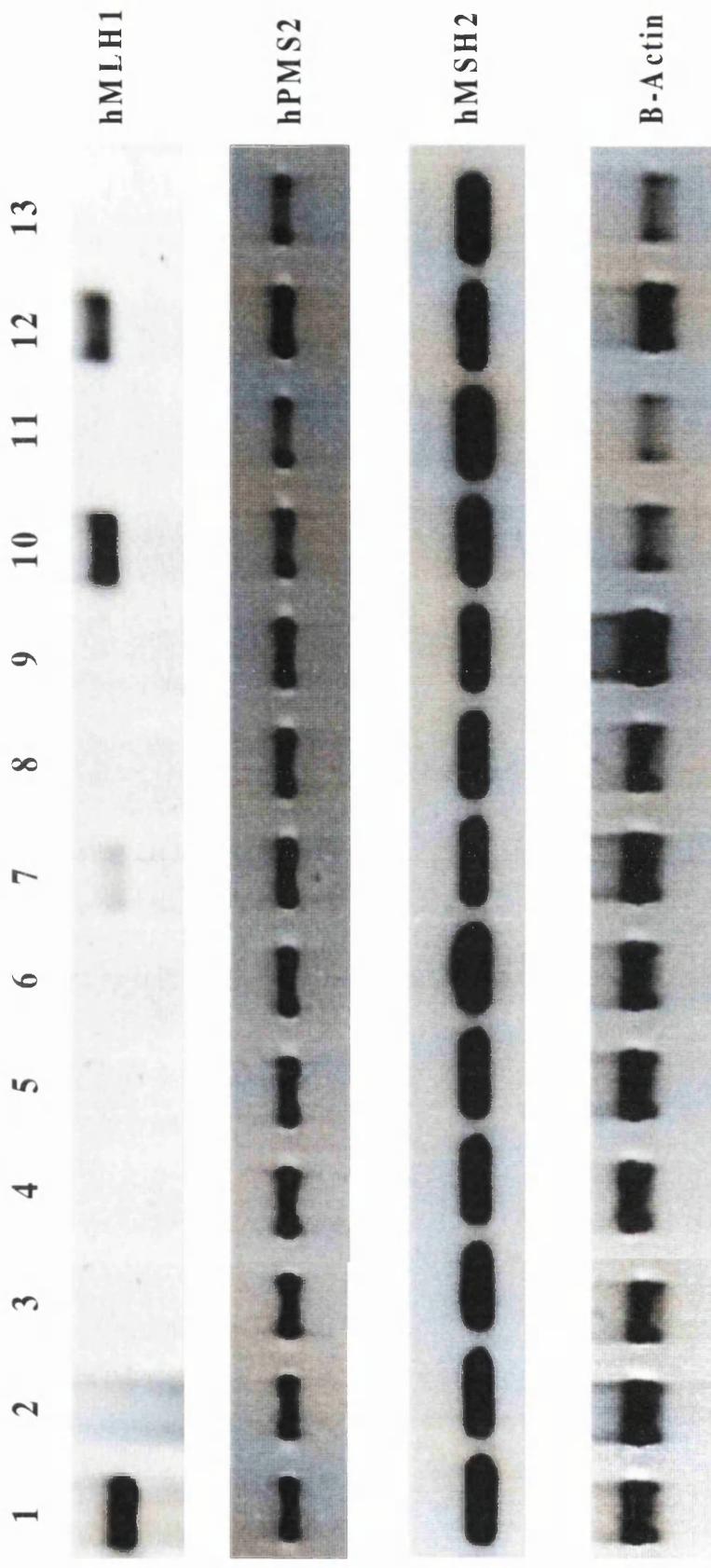
Figure 4.13   RT-PCR detection of mismatch repair gene mRNA.

PCR amplification from cell line cDNA using primers specific for hMLH1, hPMS2 and hMSH2. cDNA formed by reverse transcription of equal amounts of total cellular mRNA from each line. Amplification using primers for beta-Actin provide a control for the quality of initial mRNA and the efficiencies of reverse transcription and PCR.

Legend;   1: A2780; 2: A2780/cp70; 3: A2780/MCP1; 4: A2780/MCP2; 5: A2780/MCP3; 6: A2780/MCP4 7: A2780/MCP5; 8: A2780/MCP6; 9: A2780/MCP7; 10: A2780/MCP8; 11: A2780/MCP9. 12: MCF7; 13: AN3CA.

equivalent amounts of mRNA are observed in all of the A2780 derived cell lines and parental A2780. This result is consistent with the data from Western and Northern blot analysis for hMSH2 in these cell lines. With the β-actin primers good amplification of equivalent levels of product are observed in all lines except A2780/MCP9 and AN3CA where slightly lower amounts of product are observed. This suggests that the amount and quality of starting mRNA were relatively equal in all lines. It is therefore unlikely that the results observed in the hMLH1 RT-PCR are due to degradation of the mRNA during processing.

4.10   SOUTHERN BLOT CHARACTERISATION OF STRUCTURE OF hMLH1 GENE IN A2780 AND A2780/CP70.

The lack of hMLH1 mRNA in the cisplatin resistant A2780 cell lines could arise through gross alteration of the hMLH1 gene. To assess whether any differences existed in the genomic structure of hMLH1 between A2780 and A2780/cp70 a Southern blot was performed. 25μg of genomic DNA from both cell lines was digested to completion with the restriction enzymes EcoRI and HindIII. The final products of digestion were separated on a 0.8% agarose gel by electrophoresis. The DNA within the gel was denatured and transferred to a nylon membrane by capillary action. After blotting of the DNA onto the membrane it was fixed into position.

The primer used to probe the Southern blot was a cDNA fragment consisting of 732 bp from the 5' end of the hMLH1 gene. Purified cDNA was radiolabelled by random priming using α^{32}PdCTP. The autoradiographic results obtained from this Southern analysis are presented in figure 4.14. A specific band of approximately 6.6kb is observed in the hybridisation to EcoRI digested DNA. There is no apparent difference in the size of the bands observed from A2780 and A2780/cp70. With DNA digested using Hind III a specific band of about 4.4kb is observed in both A2780 and A2780/cp70, again showing no obvious differences. These results would suggest that no
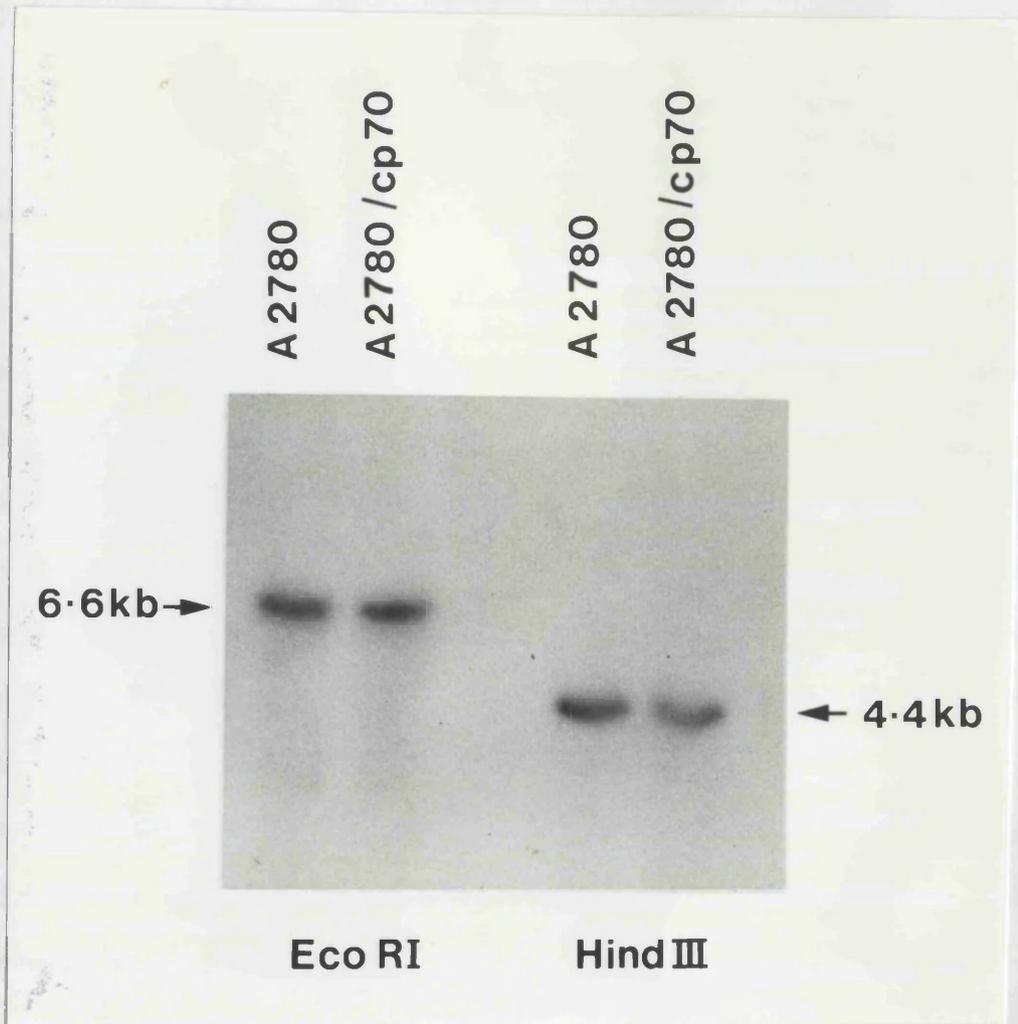
Figure 4.14 Use of Southern blot analysis to determine the gross structure of the hMLH1 gene in A2780 and A2780/cp70.

Total genomic DNA from A2780 and A2780/cp70 was digested to completion using the restriction endonucleases EcoRI and HindIII. After electrophoresis the DNA fragments were Southern blotted and hMLH1 gene fragments detected using an end-labelled probe specific for the 5' end of the gene. The specific gene fragments detected by hybridisation to the probe show no difference between A2780 and A2780/cp70 The approximate size of each gene fragment (in kilobasepairs) is indicated

specific gross alterations have occurred in the region of hMLH1 genomic DNA covered by the specific probe.

4.11   CONCLUSIONS.

Defects of mismatch repair are considered to be responsible for development of the RER+ phenotype in tumour cells. The data presented in this chapter help to characterise the defects in the mismatch repair system responsible for the RER+ phenotype observed in the cisplatin and doxorubicin resistant derivatives of A2780. The results show that the majority of A2780 cell lines with an RER+ phenotype, display complete absence of the protein components of the hMutLα complex involved in mismatch repair. This results in a specific defect in the repair of single base/base and insertion/deletion mismatches, when repair is directed from 3' to the damage. It is also shown that there is a lack of expression of the hMLH1 gene in these cells, with no obvious gross rearrangement of the gene to account for it. Wild-type levels of mRNA for the hPMS2 component of hMutLα appears to be expressed.

The results of the *in vitro* mismatch repair assay, provide definitive evidence that the RER+ phenotype observed in the cisplatin and doxorubicin resistant A2780 cell lines, is a result of defective mismatch repair. However, the evidence that purified hMutLα complements the mismatch repair defect in these cell lines is surprising, in view of the results of the mismatch binding assay. The A2780/cp70 cell line shows loss of a specific G/T mismatch binding complex that is present in parental A2780 and A2780/AD cells. It would also appear, from preliminary studies, that the A2780/MCP cell lines display no loss of G/T mismatch binding as defined by the same assay (A.McIlwrath & R.Brown. personal communication). However, the results with A2780/cp70 are reproducible using independently isolated cell extracts. The anomaly arises that, under the conditions of the mismatch repair assay, A2780/cp70 cells can repair G/T mismatches once hMutLα has been added to the extracts. This indicates that

under these conditions there is recognition of G/T mismatches, whereas under the conditions of the mismatch binding assay no binding is observed.

The results of Western blot studies on A2780/cp70 cells show that they would appear to possess hMSH2 and hMSH6 proteins at levels equivalent to those in A2780. The A2780/cp70 cells have also been shown to lack expression of hMLH1 at both protein and mRNA levels, further suggesting that this is the cause of defective mismatch repair in these cells. One possible explanation for the anomaly existing between the results of the *in vitro* repair assay and the mismatch binding assay, is that hMutSα cannot bind stably to a mismatch in the absence of hMutLα, under the conditions existing in the assay. However, if this is the case then it must be specific only for A2780/cp70, as the other cell lines with loss of hMutLα activity show no loss of G/T mismatch binding. One way to test for this would be to perform a mismatch binding assay in A2780/cp70 cell extracts that have been complemented with hMutLα and see if it restores binding to G/T mismatches. Another possibility is that the conditions present in the mismatch binding assay inhibit the binding of hMutSα to G/T mismatches, despite there being no abnormality in the complex itself. Again this does not explain the specificity of this finding to A2780/cp70 cells.

A2780/cp70 has been cultured repeatedly since its original isolation. If, as is highly possible, the RER+ phenotype developed early during the selection process, then the increased genomic mutation rate could have resulted in the accumulation of mutations in functional genes, as well as in microsatellite and other repeat sequences. It is possible, therefore, that A2780/cp70 harbours a mutation or mutations in other genes which effect, directly or indirectly, the binding of G/T mismatches, leading to the results observed in the mismatch binding assay. It is now known that other proteins involved in mismatch binding exist (Gaffney and Jones, 1989), and that these may be involved in determining the specificity of mismatch binding within a cell (Risinger et al. 1996).

Confirmation of the defect in the hMutLα component of mismatch repair in the RER+ A2780 cell lines is provided by the Western immunoblot and immunohistochemistry data. This reveals that in all but one of the cisplatin resistant cell lines, and in the doxorubicin resistant line, derived from A2780 there is essentially no detectable hMLH1 or hPMS2 protein. As stated previously, it would appear highly unlikely that under the selection pressures exerted by exposure to cisplatin or doxorubicin, independent mutations would occur in two components of the same repair pathway. Northern blot and RT-PCR studies show that, at the level of gene expression, those cells which show no hPMS2 protein produce levels of mRNA similar to that found in A2780. In cells in which no hMLH1 protein is detected there is an absence of hMLH1 mRNA. This finding would appear to exclude the possibility that hMLH1 protein is involved in the transcriptional regulation of hPMS2, as the gene is expressed in the absence of hMLH1 protein. It would also tend to rule out expression of the hMLH1 and hPMS2 genes occurring in a conjugate manner.

Perhaps a more likely explanation of these results is that the stability of hPMS2 protein is, in some way, dependent on the presence of hMLH1 protein and in its absence is degraded. It has been suggested that the individual components of hMutSα are less stable than when present in the heterodimeric complex (Drummond et al. 1995). To determine whether reduced hPMS2 stability in the absence of hMLH1 is possible, it would be interesting to study the expression of hPMS2 in other cell lines which lack hMLH1 expression. The endometrial carcinoma cell line, AN3CA, is an ideal candidate as it is known to lack hMLH1 expression at the level of the mRNA. There is some evidence to suggest that this other hMLH1 deficient cell line shows absence of hPMS2 protein (Drummond et al. 1996). It would also be interesting to determine whether hMLH1 is unstable in cell lines with an inherent defect of hPMS2 expression. Such cell lines have now been identified, e.g. HEC-1-A and evidence suggests that they possess normal levels of full length hMLH1 protein (Drummond et al. 1996).

The lack of hMLH1 protein would appear to arise from a lack of expression of the gene, in view of the absence of detectable mRNA in the RER+ cell lines. The Southern blot analysis of A2780 and A2780/cp70 suggests that there has been no gross structural rearrangement of the hMLH1 gene within the latter line, such as a deletion, to account for the lack of expression. Previous studies have shown that mis-sense or nonsense mutations within a gene can result in synthesis of a mRNA species that is quickly degraded within the cell (Lim et al. 1992). This would appear to be the most likely candidate for the type of mutational lesion occurring within the hMLH1 gene, in view of the lack of mRNA. Characterisation of the hMLH1 gene, in the RER+, A2780 cell lines, by SSCP and DNA sequencing, for example, will hopefully determine whether such mutations exist.

One major anomaly arises from the results in this section. The results in chapter 3 revealed that of all the A2780/MCP cell lines derived by selection against cisplatin, A2780/MCP7 was the one that showed no evidence of microsatellite instability and had the least abnormality of p53 function. In the results presented in this section, however, A2780/MCP7, although not having been shown to have defective mismatch repair in the *in vitro* assay, has no detectable hMLH1 gene expression or hMutLα protein. A2780/MCP8, on the other hand, which displayed microsatellite mutation at one locus on chromosome 2, and appeared to have defective p53 function, displays levels of hMutLα protein equal to that in A2780. The most likely cause of this anomaly was an inadvertent swapping of the cell lines at some point after the original microsatellite analysis. This does not appear to be the case, as repeat analysis of the microsatellite locus originally displaying mutation in A2780/MCP8 continued to display this difference in DNA obtained from A2780/MCP8 cells used in the Western immunoblot assay. The A2780/MCP7 cells used in the Western blot assay also retained the same pattern of microsatellite alleles as in the original analysis. A possible explanation for the microsatellite instability and loss of p53 dependent functions in A2780/MCP8 is that the defect in mismatch repair exists in a component of the repair apparatus other than

hMLH1. This cell line has not been subjected to the *in vitro* repair assay and therefore the actual component which is responsible for the defective repair is unknown. It is harder to define a possible explanation for the results observed with A2780/MCP7. Although no microsatellite mutations were observed in the original study, with this cell line, it is possible that by looking at a greater number of loci, evidence of microsatellite instability would have been detected. However, why there has not been an associated loss of p53 dependent functions in this line, as in all the others that have loss of hMutLα , is unclear.

The selective loss of 3' directed mismatch repair observed with the RER+, A2780 drug resistant cell lines, is a novel finding having only previously been detected in the hMLH1 deficient AN3CA cell line (J. Drummond. Personal communication). It differs from the biochemical phenotype of other hMLH1 deficient cell lines, such as the colorectal tumour line H6, which is unable to correct mismatches when repair is directed from either 3' or 5' to the lesion (Parsons et al. 1993). The basis of this selective loss of 3' directed mismatch repair in hMLH1 and hPMS2 deficient, A2780 cell lines is unclear. Another way of looking at the same question is to ask why the RER+, A2780 cell lines retain 5' directed repair, despite lacking hMLH1 and hPMS2. As in the case of eukaryotic MutS homologues, it is thought that the MutL component of eukaryotic mismatch repair is more complicated than in the prokaryotic situation (Kolodner, 1996). It may be that there are a number of different mammalian proteins that are involved in the steps of mismatch repair performed by prokaryotic MutL. Some of these may determine the directionality of repair. It is possible that the RER+ A2780 cells possess an activity that supports 5' directed mismatch repair in the absence of hMutLα. This activity may not be present in other hMLH1 null cells such as the H6 cell line. Another explanation could arise from the different types of hMLH1 mutation in these cells. It would appear that the RER+ A2780 cells express no hMLH1 protein. H6 cells, on the other hand, possess a hemizygous or homozygous UAA chain termination mutation and produce an amino terminal fragment of the hMLH1 polypeptide. It could be that the

presence of such hMLH1 fragments in H6 cells inhibit 5' directed repair, resulting in total loss of mismatch repair. Further characterisation of the defects in mismatch repair by *in vitro* repair assay, in tumour cell lines with different mismatch repair gene mutations, may help characterise the determinants of repair directionality.

CHAPTER 5

GENERAL DISCUSSION

The results presented in this thesis can be summarised in two main conclusions;

(1). An association exists in certain tumour cell lines between resistance to the clinically important anti-cancer drugs cisplatin and doxorubicin, and the loss of mismatch repair as characterised by microsatellite instability. The development of an RER+ phenotype occurs at high frequency during the selection for cisplatin resistance, either by single or multiple exposures to the drug, in the A2780 ovarian carcinoma cell line.

(2). The RER+ phenotype observed in the cisplatin resistant A2780 cell lines is the result of a loss of mismatch repair activity, specific for repair occurring from a direction 3' to the DNA mismatch. In nearly all cases this occurred concurrently with loss of expression of hMLH1, at both the mRNA and protein levels, and of hPMS2 protein. Those cells with loss of mismatch repair also display a reduction in specific p53 mediated functions; ionising radiation induced G1 arrest, basal and induced expression of Cip1 and an apoptotic response to cisplatin induced DNA damage.

The following general discussion will attempt to define the relevance of these findings in the context of our current understanding of mismatch repair and cisplatin resistance. It will also explore the potential clinical implications of an association between mismatch repair and drug resistance in ovarian and other tumours.

5.1 MISMATCH REPAIR DEFICIENT CELL LINES

5.1.1 Frequency of selection of RER+ and drug resistant cell lines

The discovery that cisplatin and doxorubicin resistant clones of independently derived ovarian and breast carcinoma cell lines display high levels of microsatellite mutation compared to parent lines is an intriguing result. Defects in mismatch repair with associated microsatellite instability have been detected frequently in ovarian tumour cell lines (Boyer et al. 1995; Orth et al. 1994). However, the RER status of the A2780 cell line had not previously been characterised and the results presented show that it has a proficient mismatch repair activity. The RER status of the other tumour cell lines used in the initial investigation, MCF7 and OV1/p (IGROV1), has also not been characterised to date. It has been shown, by immunoblotting, that MCF7 has normal levels of several mismatch repair proteins (A.McIlwrath, personal communication) and would therefore be assumed to have normal repair function. Confirmation of this, however, awaits clonal analysis of microsatellite mutations in MCF7 and MCF7/AD or measurement of the *in vitro* mismatch repair function of these cell lines. Therefore, although there is a reasonable likelihood that the microsatellite instability observed in MCF7/AD and OV1/DDP is a feature of the resistant and not the parental lines this, as yet, can only be defined categorically for A2780/cp70, A2780/MCP1-9 and A2780/AD. It should be noted that not every tumour cell line selected for resistance to cisplatin or doxorubicin displays evidence of microsatellite instability. In a study of each of two ovarian, lung and testicular cell lines, and their cisplatin resistant derivatives, no evidence of microsatellite mutation was observed at a variety of dinucleotide repeat sequences (G.Hirst. personal communication.).

One of the most significant observations to arise from this work, was the high frequency with which an RER+ phenotype developed during selection for cisplatin resistance in A2780 cells. This raised the possibility that an association might exist

between the two phenomena. A similar high frequency of RER+ phenotype development has been suggested from studies on other drug resistant cell lines. In cells that lack methyltransferase activity, exposure to alkylating agents, such as MNNG, is toxic. An association between tolerance to methylating agents and defects in the mismatch repair system was originally proposed from studies performed on E.coli (Holmes et al. 1990) and has subsequently been shown in mammalian cell lines which are tolerant to O^6MeG and 6-TG (Kat et al. 1993; Branch et al. 1993). In a series of MGMT deficient HeLa cells exposed to cytotoxic levels of MNNG, 5 out of 8 subclones displayed resistance not due to re-induction of the methyltransferase activity (Gothgoldstein, 1987). This resistance in the HeLa cells was shown to be due to tolerance of the methylation damage, induced by MNNG, as similar levels of DNA methylation were observed in the resistant and parental cell lines. A similarly high frequency of selection of methylation tolerance, 6 out of 9 clones, was observed in Chinese hamster ovary cells selected for resistance to increasing concentrations of 6TG over a period of months (Aquilina et al. 1990). Cross resistance was also shown to MNU, with no evidence of methyltransferase activity as a cause. This phenotype has been shown to be due to loss of a specific G-T mismatch binding complex (Branch et al. 1993).

5.1.2 Mechanism of development of drug resistant, RER+ phenotype.

Thus the evidence suggests that selection for resistance to certain types of alkylating agent can be associated with the appearance of an RER+ phenotype at high frequency. By what mechanism does exposure to alkylating agent appears to result in development of an RER+ phenotype? Most of the alkylating agents in question are mutagenic as well as cytotoxic. Therefore it could be that exposure of tumour cells to these agents results in mutations which lead to an RER+ phenotype. Subclones of an $MGMT^-$ HeLa cell line, pre-treated with a sub-lethal dose of MNNG, were shown to

display greater resistance to an additional exposure to MNNG than non-pre-treated subclones (Gothgoldstein, 1987). This was taken to suggest that resistance could be induced by MNNG treatment. Many of the cell lines studied for cisplatin and methylation tolerance have been developed by exposure to increasing concentrations of drug. Exposure to the lower drug doses could result in the occurrence of mutations which increase the resistance to subsequent higher doses. This has been suggested as the basis for induction of cisplatin tolerance in ovarian 2008 cancer cells (Aebi et al. 1996). However, the results from the A2780 clones selected by a single exposure to cisplatin show that this can also result in an RER+ phenotype. This would, perhaps, argue against drug tolerance being induced by exposure to the specific alkylating agent.

The other possibility is that cells with an RER+ phenotype arise spontaneously in the tumour cell population. These would have a strong survival advantage under the selection pressures induced by exposure to specific drugs. Why such cells should arise so frequently in the tumour population is unclear. However the cell lines studied have all been cultured, *in vitro*, over long periods of time, with the attendant likelihood of accumulating multiple mutations.

5.2   MISMATCH REPAIR AND DRUG RESISTANCE

5.2 1   Mismatch repair and cellular sensitivity to cisplatin.

The data presented in this thesis supports the premise that defects in the mismatch repair apparatus of the cell may contribute to the development of resistance to cisplatin. Corroboration of this concept has come from a recent study using a different ovarian carcinoma cell line and its cisplatin resistant derivative. Aebi et al (Aebi et al. 1996) used the 2008 cell line and two cisplatin resistant derivatives, selected by exposure to increasing concentrations of drug. 2008 cells and those of the cisplatin

resistant derivative 2008/C13*5.25 both displayed detectable hMSH2 and hMLH1 by Western immunoblot and showed no evidence of microsatellite instability. Both lines were sensitive to the methylating agent MNNG and to 6-TG. Cells of the cisplatin resistant derivative 2008/A, however, were shown to have lost hMLH1 protein; displayed microsatellite instability; demonstrated an increased mutation rate at the *HPRT* locus and were also resistant to MNNG. As it has recently been shown that the A2780/cp70 and A2780/MCP1 cell lines are resistant to MNU and have an increased HPRT mutation rate (G.Hirst. Pers. comm.) the 2008/A cell line virtually mirrors the phenotypic characteristics of the cisplatin resistant derivatives of A2780.

The same study also presents further evidence for the importance of mismatch repair in determining the sensitivity to cisplatin. Tumour cell lines that were isogenic apart from the presence or absence of functional mismatch repair, were studied for their resistance to cisplatin. HCT116 colorectal carcinoma cells lack functional hMLH1 and HEC59 endometrial carcinoma cells lack hMSH2, both cell lines being deficient in mismatch repair. These lines displayed about 2 fold greater resistance to cisplatin compared to identical lines where functional mismatch repair had been restored by introduction of the required mismatch repair genes using chromosome transfer (chromosome 3 in the case of HCT116 and chromosome 2 for HEC59). Although the mismatch repair deficient cells were only two fold resistant to cisplatin changes of < 2-fold in cisplatin sensitivity are sufficient to result in the clinical failure of this drug (Andrews et al. 1990) and are similar to the levels of resistance observed with the A2780/MCP lines. Therefore an association between defective mismatch repair and cisplatin resistance has been observed not only in resistant derivatives selected from independent ovarian carcinoma cell lines but also in different tumour cell lines with no prior exposure to cisplatin.

5.2.2 Recognition of platinated DNA by mismatch repair proteins.

The bacterial mismatch repair pathway has been implicated in the cellular response to a variety of different types of DNA damage. Thus it is able to recognise damage arising from the action of MNNG (Karran and Marinus, 1982), cisplatin (Fram et al. 1985) and UV irradiation (Feng and Hays, 1995; Feng et al. 1991). If, as the evidence presented in chapters 3, 4 and above suggests, the mismatch repair system in humans also plays a role in the response to DNA damage induced by cisplatin, then recognition of such damage would seem to be required as the first step.

It has been well established that bacterial MutS protein, and its homologues in yeast and human cells, is able to recognise and bind to mismatches in DNA (Drummond et al. 1995; Su et al. 1988). It has also been shown that mismatch recognition complexes in cell extracts can bind to base pairs containing O^6-MeG and 6-TG (Griffin et al. 1994). There is now evidence to support the concept that mismatch recognition complexes from human cells can recognise DNA damage caused by cisplatin. Specific oligonucleotides containing single base mismatches, methylated base mismatches or a variety of platinated DNA adducts, were constructed in one study and their recognition by purified hMutSα determined by retardation of the oligonucleotide during gel electrophoresis (Duckett et al. 1996). Purified hMutSα recognised O^6-MeG/C, O^6-MeG/T and O^4-MeT/A adducts with an affinity one order of magnitude less than the recognition of G/T mispairs in the oligonucleotides studied. Oligonucleotides that contained a 1,2-d(GpG) intrastrand platinum crosslink, where the platinated bases were paired with dCpC on the complementary strand, were also bound by purified hMutSα with a similar level of affinity. Oligonucleotides with 1,2-d(ApG) or 1,3-d(GpTpG) platinated adducts were bound very poorly by hMutSα.

A similar selectivity has also been demonstrated for the binding of purified hMSH2 protein to platinated oligonucleotides *in vitro* (Mello et al. 1996). Those oligonucleotides treated with the clinically active compounds cisplatin and Pt(en)Cl$_2$

were found to bind to purified hMSH2 protein. Such compounds are able to form 1,2 intrastrand cross-links at adjacent purine nucleotides. Oligonucleotides formed by treatment with transplatin or , compounds ₩hich cannot form 1,2 intrastrand links and show no clinical activity, did not bind to hMSH2 protein. It was also shown that hMSH2 protein bound specifically to a 100bp oligonucleotide containing a single 1,2 d(GpG) platinum adduct.

A further gel retardation study has shown that the presence of a cisplatin adduct alone might not be sufficient to bring about mismatch protein binding. It was observed that cell extracts of mismatch repair competent cells, and purified hMutSα, only bound to a 1,2 d(GpG) cisplatin adduct when it was paired with a d(CpT) on the complementary strand (Karran,P. pers. comm.). That is when it was in the form of a mismatch. The combination of a bulky DNA adduct associated with a mismatch has been called a compound lesion (Freidberg 1995). When d(CpC) were the complementary bases, very little binding to the platinated oligonucleotides was observed. Binding was specific for this form of mismatched cisplatin adduct, as other combination of bases complementary to 1,2 intrastrand crosslink resulted in no detectable binding. It should be noted that extracts from cells lacking hMSH6 and hMSH2 showed no evidence of binding to platinated DNA.

What significance can be obtained from these results and how do they relate to the data presented in this thesis? Perhaps most importantly they confirm that DNA platinum adducts can be recognised by the mismatch repair system. It may be that it is not the platinum adduct per se that is recognised by mismatch repair proteins, but rather the conformational change in DNA structure that they produce. It has been shown, using X-ray crystallography, that introduction of a 1,2 d(GpG) cisplatin cross-link into an oligonucleotide leads to bending of the helix with formation of a unique fusion between A and B forms of DNA (Takahara et al. 1995). There is conflicting evidence as to the degree of distortion caused in DNA by simple base/base mismatches (Kalnik et al. 1988; Bhattacharyya and Lilley, 1989). However, it does appear that larger

insertion/deletion mismatches do substantially distort the DNA helix (Bhattacharyya and Lilley, 1989b). Therefore, it is very possible that mismatch proteins, that recognise the structural DNA alterations caused by mismatches, could also recognise that caused by cisplatin DNA adducts. The evidence that mismatch repair proteins can bind to cisplatin adducts in DNA provides evidence, of a mechanistic nature, to link mismatch repair and cisplatin sensitivity.

5.2.3 DNA replication, damage processing and mismatch repair.

The suggestion that mismatch recognition proteins may bind to cisplatin adducts, especially as part of a mismatched complex lesion, is interesting. In the case of methylating agents, it has been shown previously that the O^6MeG lesion produced by methylation of DNA, is not in itself toxic to the cell (Aquilina et al. 1993). A model has been constructed to explain the mechanism of cytotoxicity of such lesions and the basis of methylation tolerance in mismatch repair deficient cells (Karran and Bignami, 1992). In this model O^6MeG, when formed by DNA methylation, will remain paired with cytosine until replication, in the absence of repair by MGMT. At replication there is a strong possibility that O^6MeG will pair with thymine instead of cytosine, as the thermal stability's of each base pair are about the same (Gaffney and Jones, 1989). The O^6MeG/T base pair is recognised as a mismatch by the mismatch repair system, which removes the thymine from the newly synthesised daughter strand and attempts to repair the lesion. However, it is most likely to fill the gap with another thymine, for the reasons discussed above, and it is proposed that repeated cycles of this futile mismatch repair may lead to the formation of further DNA damage, perhaps strand breaks, resulting in cell death (Karran and Bignami, 1992).

Could such a mechanism also be involved in the cytotoxicity observed after exposure to cisplatin? Circumstantial evidence supports the possibility that replication of platinated DNA is required before toxicity is observed. Studies in immature rat

thymocytes have shown that the ability of cisplatin to induce apoptosis, and thus its cytotoxicity, is much greater with proliferating cells as opposed to quiescent cells (Evans et al. 1994). It was shown that this phenomenon was not related to alterations in the levels of DNA platination or repair. The proposal was made that the molecular events that coupled DNA damage to the engagement of apoptosis were not active in quiescent thymocytes. A dependence on replication was not observed for other cytotoxic agents, such as etoposide. One possible explanation for this phenomenon is that replication of platinated DNA is required to produce a cytotoxic lesion, whereas it is not required for agents that produce more directly toxic forms of damage such as strand breaks. If replication of cisplatin adducts is required for induction of cytotoxicity, in a manner analogous to O^6-MeG, then it could explain the enhanced binding of hMutSα to intrastrand d(GpG) adducts paired with d(CpT) (Karran,P. Pers. Comm.). Cisplatin induced formation of intrastrand d(GpG) adducts, in non-replicated DNA, may not act as a strong enough signal for hMSH2 binding. Post- replication, however, the formation of mispairs in the newly synthesised strand opposite the d(GpG) adduct could result in enhanced hMSH2 binding, thus inducing futile attempts at mismatch repair.

One argument raised against such a model for replicative processing of cisplatin damage is that platinated adducts in the DNA would act as an absolute block to progression of the replication complex. There is, however, increasing evidence to indicate that replication can proceed past bulky adducts in the DNA. A steady state replication assay, which measures the inhibition of DNA chain elongation as a function of the number of platinum-DNA adducts present following cisplatin treatment, was used to study the ability of cells to replicate past cisplatin adducts (Mamenta et al. 1994). Using the ovarian carcinoma cell lines 2008 and A2780 it was shown that the replication apparatus was able to bypass cisplatin adducts more readily in cisplatin resistant derivatives, 2008.C13 and A2780/DDP, than in the parental lines. This phenomenon has also been observed for A2780/cp70 cells (S. Channey, Pers. Comm.).

This "replicative bypass" was proposed to account for part of the cisplatin tolerance seen in the resistant cell lines.

A number of biochemical studies have looked at the ability of DNA polymerases to bypass different lesions in the DNA during replication. DNA polymerase δ has been shown to bypass UV radiation induced DNA adducts, under the influence of proliferating cell nuclear antigen (PCNA) (Oday et al. 1992). The use of anti-PCNA antibodies have also implicated the importance of DNA polymerase δ or ε in the processing of O^6MeG lesions in DNA (Ceccotti et al. 1996). More recently, it has been shown using cell free systems, that whereas chain elongation by DNA polymerase α, δ and ε are effectively terminated by bulky adducts such as d(GpG) cisplatin or N-2-acetylaminofluorene (AAF), DNA polymerase β can replicate past such lesions (Thomas et al. 1995; Hoffmann et al. 1995). DNA polymerase β, which is predominantly involved in DNA repair synthesis, can also extend the arrested replication products of the other three DNA polymerases (Hoffmann et al. 1995). Another example of replication past cisplatin adducts has been observed using structures that resemble the DNA replication fork, *in vitro*. The presence of d(GpG) cisplatin adducts in such a 'forked DNA' construct did not act as a barrier to replication. Cisplatin adducts on single stranded DNA effectively inhibited replication (Hoffmann et al. 1996).

The ability to replicate past an adduct in the DNA is also referred to as translesion synthesis. Such translesion synthesis of DNA occurs with either the insertion of the correct base, or bases, opposite the adduct or with insertion of mismatched bases. The evidence presented above suggests that replication past cisplatin adducts in DNA is possible with concomitant formation of mismatches.

5.2.4 Nucleotide excision repair, Mismatch repair and cisplatin adducts.

The preceding sections have presented evidence that mismatch repair proteins can recognise platinum adducts in DNA, in the form of 1,2 intrastrand crosslinks. It has also suggested that such lesions do not act as an absolute block to replication but form mismatches, once replicated past, which may represent a more complex form of DNA damage. Mismatch proteins have been shown to bind to such complex damage. It is generally accepted that nucleotide excision repair (NER) is the main process by which cisplatin adducts are removed from DNA. Research referred to in chapter 1 also suggests that differences in NER between cell lines might be involved in the determination of cisplatin sensitivity or resistance. Is there any evidence to suggest that the efficiency of repair of 'complex' cisplatin adducts from replicated DNA, that is those paired with non-complementary bases, determines cisplatin sensitivity? Could binding of mismatch proteins alter the repair of such complex lesions?

Cisplatin forms a number of different types of DNA adduct. The majority are 1,2 intrastrand adducts, either between adjacent guanines (65%) or an adenine and guanine (25%) (Fichtinger-Schepman et al. 1985). Although it has generally been though that, due to their prevalence, these adducts are the determinants of cisplatin cytotoxicity, it is also possible that one or more of the less common adducts is more important (Eastman, 1987b). Although repair of platinum adducts has been shown to occur using cell extracts *in vitro*, there is evidence to suggest that 1,2 d(GpG) adducts are not efficiently repaired (Bedford et al. 1988). An *in vitro* assay using M13-phage with a single 1,2 d(GpG) cisplatin adduct showed no repair, using HeLa cell extracts which could efficiently repair an AAF lesion on the same DNA construct (Szymkowski et al. 1992). It was shown that this lack of repair occurred at the stage of recognition of the DNA damage by the repairosome complex formed in excision repair. It was proposed that this could be due to blocking of recognition by proteins binding to the DNA damage, for example HMG group proteins. It has been shown recently that HMG proteins can block excision repair of 1,2 d(GpG) and 1,2 d(ApG) cisplatin adducts on DNA (Zamble et al. 1996).

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However, the same study also demonstrated that 1,2 d(GpG) cisplatin adducts were repaired 3× less efficiently than 1,3 d(GpNpG) cisplatin lesions in cell free systems containing no HMG protein. The 1,2 d(GpG) adduct is known to cause less conformational disruption to the DNA helix than 1,3 d(GpNpG) (Bellon et al. 1991). It may be that the reduced conformational disturbance caused by 1,2 d(GpG) results in lack of recognition by the exinuclease system.

Such a hypothesis has been given support by work showing that 1,2 d(GpG) and 1,2 d(ApG) cisplatin adducts are repaired up to 15-fold less efficiently than 1,3 d(GpNpG) adducts (Moggs et al. 1997).This difference was also shown to arise at a step preceding, or coincident with, endonucleolytic cleavage. Fractionation of cell extracts to remove HMG proteins did not alter the differential rates of repair. When the 1,2 d(GpG) cisplatin adduct was mismatched with a thymine on the complementary strand, to form a complex lesion which is presumed to cause greater distortion to the DNA helix, the rate of repair increased dramatically. This occurred for cell extracts from mismatch repair proficient and deficient cells suggesting that mismatch repair proteins do not influence the NER of complex lesions. In confirmation of this last point, it has been shown that mismatch repair deficient cells repair complex lesions containing UV photoproducts and 1,2 d(GpG) cisplatin adducts as well as wild type HeLa cells (Mu et al. 1997). Addition of purified hMutSα or hMutLα to complement the mismatch repair defect did not enhance the NER of complex lesions.

In total this evidence tends to suggest that formation of a complex cisplatin adduct, perhaps by translesion synthesis, can enhance its rate of repair and that this is absolutely dependent on NER, with no involvement of mismatch repair. It would also suggest that binding of mismatch repair proteins to cisplatin adducts, either matched or mismatched on the complementary strand, does not shield such lesions from the exinuclease. Since the mismatch repair status does not appear to influence the efficiency of NER of cisplatin adducts, it suggests that differential repair of these lesions does not

underlie cisplatin resistance in the A2780 cell lines. It will be of interest to characterise the NER of simple and complex cisplatin adduct lesions using A2780 cell line extracts.

5.2.5 Cell cycle checkpoints, Mismatch repair and Cisplatin resistance.

The previous section has presented evidence that cisplatin adducts with mismatched bases on the complementary strand are repaired exclusively by NER. However, it is known that mismatch repair proteins bind to cisplatin DNA adducts and that defects in mismatch repair can result in increased resistance to the drug. It may be that the mismatch repair system is not involved with repair of these adducts but, as has been suggested previously (Kat et al. 1993), signals the presence of DNA damage to downstream effectors of repair and/or cell death. If this is the case, is there any evidence linking defects in mismatch repair with alterations in the cellular processes that monitor DNA damage, and ultimately result in cell survival or cell death?

The suggestion that cisplatin resistant cells with abnormal mismatch repair may also display abnormalities of the DNA damage response pathway arose from known features of the A2780/cp70 cell line. A2780/cp70, with no difference in the sequence of the p53 gene compared to its parental line, displays a loss of the p53 dependent G1 arrest after exposure to ionising radiation or cisplatin (McIlwrath et al. 1994a). This suggests that p53 mediated functions are inhibited by a mechanism other than mutation of the gene in these cells. The results presented in this thesis confirm the loss of the radiation induced G1 cell cycle arrest in A2780/cp70, and also show that these cells have altered induction of the Cip1 gene after exposure to cisplatin and a reduction in apoptosis after cisplatin. The RER+ A2780/MCP cell lines also show this phenotype suggestive of altered p53 function, although it is not known for certain whether these lines possess wild-type p53 gene sequence or not. The one A2780/MCP cell line which was RER− displayed p53 mediated responses similar to that of parental A2780 cells.

This suggests that there is a correlation between the loss of mismatch repair and abnormalities of p53 function.

Evidence exists to support an association between loss of mismatch repair and defects in cell cycle damage response mechanisms in different cell types. Goldmacher et al in their initial studies on methylation tolerance displayed differences in the cell cycle progression of MT1 and TK6 cells exposed to isotoxic doses of MNNG (Goldmacher et al. 1986). On exposure to MNNG parental, RER−, TK6 cells underwent a single cell division and arrested at or immediately after their second S phase. At this "G2" arrest, a proportion of cells died whereas the remainder appeared to be able to regain a normal cell cycle. MT1 cells, since shown to have an RER+ phenotype, appeared to arrest cell division immediately after addition of MNNG, and this "arrest" lasted for up to 4 days. The cells then started to grow but initially at a slower rate compared to controls.

More recently the response of colorectal tumour cells lines to 6TG and MNNG has been studied with regards to cell cycle response and cell viability. The HCT116 cell line (defective hMLH1) and its derivatives, with either wild-type chromosome 3 or 2 introduced by chromosome transfer, were grown in the presence of 6TG (Hawn et al. 1995) The RER+ cell lines, HCT116 and HCT116+chr2, displayed greater resistance to the effects of 6TG than RER− HCT116+chr3 cells. This was not associated with differences in the incorporation of 6TG into the DNA but did correlate directly to the status of the mismatch repair system. When the cell cycle characteristics of these lines was determined it was observed that HCT116+chr3 displayed a marked arrest in G2 after exposure to 6TG whereas HCT116 and HCT116+chr2 cells accumulated in G1. Additional studies on these, and additional, cell lines by the same group have displayed the loss of a damage induced G2 arrest in RER+ cells after treatment with MNNG (Carethers et al. 1996). HCT116+chr3 and another RER− colorectal cell line, SW480, arrest in G2 after exposure to MNNG. This arrest occurs at the first G2 period after damage, unlike the situation observed with TK6. HCT116, HCT116+chr2 and 2774 (an

ovarian carcinoma cell line with no functional hMSH2) cells, which show loss of this MNNG induced G2 arrest although do not appear to accumulate in G1.

Full cell cycle profiles have been performed on A2780, A2780/cp70 and A2780/MCP1 after exposure to varying concentrations of cisplatin (Brown,R. et al. Oncogene, in press). A2780 cells show a marked increase in the proportion of G2-M cells after treatment with 20μM and 40μM cisplatin, indicative of a G2 arrest. A2780/cp70 and A2780/MCP1 show considerably less of a G2 arrest at these cisplatin doses. A2780 cells also show a cisplatin induced reduction in the number of cells entering DNA replication from G1 (early S-phase cells), which is lost in the cisplatin resistant lines. Therefore, it would appear that cells deficient in mismatch repair, lose the ability to arrest the cell cycle after DNA damage induced by both methylating agents and cisplatin.

The loss of the G2 arrest in mismatch repair deficient cells, can potentially be explained if mismatch repair is involved in processing of DNA damage during replication, as proposed above. That is, if futile attempts to repair the mismatch generated by a methyl adduct,or a cisplatin intrastrand crosslink, could result in the generation of strand breaks. These act as potent signals to the damage recognition pathway with a resultant arrest at G2 with concomitant repair or cell death by apoptosis. Obviously, if the mismatch repair system cannot process the DNA damage then no signal to arrest in G2 is produced and the cell cycle proceeds. This could also account for the reduced apoptosis observed in the RER+ A2780/MCP cell lines as the G2 arrest is the point at which entry into apoptosis is initiated. Loss of the G1 checkpoint in the cisplatin resistant A2780 cell lines, as a consequence of defective mismatch repair, is more difficult to explain. The G1 checkpoint is dependent on the function of p53 and genes transcriptionally transactivated by it. As shown previously (Brown et al. 1993), A2780/cp70 has wild type p53 sequence. The p53 sequence of the A2780/MCP cell lines has not yet been established although those which are RER+ display a p53 phenotype similar to that of A2780/cp70. Evidence is now accumulating which suggests

that p53 may be more closely involved in certain DNA repair processes, such as mismatch repair, than was previously recognised.

5.2.6   The involvement of p53 in DNA repair.

As indicated previously, the involvement of p53 in regulation of cell cycle checkpoints and induction of apoptosis is well established if not fully understood. However, there have been a number of recent reports that suggest that p53 may have a more direct role in DNA damage recognition and repair, indicating that it has functions upstream of its role in cell-cycle checkpoint regulation. It has been shown by a number of groups that p53, and specifically the C-terminal portion of the protein, can bind to DNA (and RNA) single strands and termini (Oberosler et al. 1993; Bakalkin et al. 1994). This DNA binding activity of p53 is also associated with DNA renaturation and strand transfer. As DNA ends, and single strands, are the type of structural damage that would be expected after, for example, exposure to ionising radiation, it has been suggested that p53 might concentrate at the sites of such damage and play a role in DNA repair or recognition. Another recent finding is that the transcriptional transactivation function of p53 can be activated by binding of short (16-40 base) oligonucleotides to the C-terminal domain (Jayaraman and Prives, 1995). The suggestion is made that the release of short oligonucleotides, of about 30 bases, during nucleotide excision repair (NER) could lead to the functional activation of p53. The basis for this hypothesis is that p53 has been shown to interact with the XPB and XPD proteins, both of which are components of the TFIIH complex involved in NER and transcription (Wang et al. 1995). XPB and XPD are helicases involved in unwinding the DNA duplex, allowing access to the damaged DNA of other proteins involved in NER. Binding of p53 to XPB and XPD, again via its C-terminal domain, inhibits the function of the helicases. This would appear to be important for normal NER as fibroblasts from Li-Fraumeni patients (heterozygous for mutant p53) have deficient repair of UV-induced pyrimidime dimers

by NER (Wang et al. 1995). This cumulative evidence suggests that p53 can play a direct role in recognition and repair of certain forms of DNA damage.

Is there any evidence that p53 is involved in mismatch repair at a point upstream of its cell-cycle effects? Electron microscopy and gel-retardation studies have shown that p53 can bind directly to certain forms of mismatched DNA (Lee et al. 1995). In this analysis it was shown that full length p53, and a 14kD C-terminal fragment, could bind to $\overset{CCC}{\diagup\diagdown}$ or $\overset{CACACACA}{\diagup\diagdown}$ DNA loops *in vitro*, in the form of monomers, dimers and tetramers. The binding appeared to be specific for mismatched DNA and was not just due to the single strand nature of the DNA in the mismatch. Perhaps surprisingly p53 did not bind to single base G/T mismatches under the same conditions. There is no evidence, as yet, as to whether such binding of p53 to these mismatches results in alteration of p53 function.

In cells with defective mismatch repair, it can be postulated that post-replication mismatches persist within the cell until the next round of replication. If this were the case then they would be present at higher numbers than in mismatch repair proficient cells. If p53 can bind to mismatched DNA the possibility arises that it will undergo sequestration by the mismatches in RER+ cells. This could result in accumulation of p53 in the nucleus, as bound p53 is less likely to undergo Ubiquitin mediated degradation than if in the free state. Furthermore, if p53 bound to mismatches was not activated by binding then it could result in abnormalities of p53 mediated functions such as cell-cycle checkpoint control and apoptosis. These correlate with the phenotypic characteristics of A2780/cp70, with wild type sequence p53 which shows nuclear accumulation (W. Gallagher. Pers. communication) and loss of various p53 mediated functions. There is no additional evidence to suggest that this is the reason for the abnormal p53 function in A2780/cp70 and no reports of nuclear p53 accumulation with associated loss of p53 mediated functions have come from other RER+ cell lines, although they may not have been looked for.

A more direct strand of evidence linking p53 directly with mismatch repair is the identification of sequences homologous to the p53 consensus binding sequence in the promoter region of the hMSH2 gene (Scherer et al. 1996). Using gel mobility shift experiments it was shown that purified wild type p53 bound to the hMSH2 promoter consensus sequences *in vitro* with an affinity stronger than that of the classical consensus sequence. The effects of binding of p53 to these consensus sequences is as yet unknown.

It is becoming increasingly obvious from the results of a wide range of studies, that components of specific DNA repair processes may have functions within other pathways of DNA repair with which they have not previously been associated. As indicated above p53 interacts with XPB and XPD to influence NER and probably transcription coupled NER. In a study in E.coli to determine which genes were important in regulating transcription coupled NER, it was shown that mutations in mutS and mutL resulted in the loss of the preferential repair of the transcribed strands of active genes (Mellon and Champe, 1996). The proposal is made that the mutS and mutL proteins are involved in coupling of NER to RNA polymerase, stalled by a lesion on the actively transcribed strand of a gene, perhaps by stabilising some form of repair intermediate. Perhaps more importantly, it has subsequently been shown that there is a similar link between mismatch repair and transcription coupled NER in humans. Tumour cell lines with mutations in hMSH2, hMLH1 or hPMS2 were proven to have loss of transcription coupled repair of UV induced pyrimidine dimers (Mellon et al. 1996). Chromosome transfer of a normal copy of hMLH1, into a cell with no functional copy of the gene, restored preferential repair of the actively transcribed DNA strands. Although no evidence exists to indicate that p53 and mismatch repair proteins interact in the process of transcription coupled repair it is another potential point of overlap.

There exist a number of other tenuous links between mismatch repair and p53 which, however, add to the overall impression that there may be some form of more or less direct interaction. These will be touched upon in the next section.

5.2.7 Cisplatin resistance and non-mismatch binding components of mismatch repair

The discussion presented so far pertains to data showing that platinated DNA can be bound by mismatch binding proteins. It also raises the possibility that the mismatch repair pathway is in some way involved with processing this form of DNA damage leading to interaction with downstream effectors of the damage response pathway. However, the data presented in chapter 4 shows that differences in the sensitivity to cisplatin between sensitive and resistant derivatives of A2780 are due to defects in hMutLα and not the mismatch binding components of mismatch repair. The most intriguing result is that of the *in vitro* mismatch repair assay, showing that the RER+ cisplatin resistant A2780/cp70 and MCP1 cells have a specific defect in repair of mismatches, when repair is directed from the 3' direction. The central question thrown up by these results is how a defect in hMutLα results in loss of mismatch repair directed from one side of a lesion but not the other.

As far as is known hMutLα does not possess any function that would explain the selective loss of 3' directed mismatch repair observed in the cisplatin resistant A2780 cell lines. However, it is highly likely that hMutLα does interact with other components of the mismatch repair apparatus (Kolodner, 1996). Some of these will be involved specifically in repair occurring from either a 3' or 5' direction, and could include the signal which determines repair strand selectivity, as well as the repair exonucleases. There is no clear data as to how the eukaryotic mismatch repair apparatus selects the correct DNA strand to repair. However, several recent reports have shed some light on the identity of the exonucleases that could, potentially, be involved in mismatch repair. It has been suggested that the exonuclease function of human mismatch repair will display redundancy (Kolodner, 1996). That is, the possible involvement of more than one form of exonuclease in both 3' and 5' directed reactions.

Studies in the yeasts *S.cereviseae* and *S.pombe* have identified several proteins which could act as exonucleases in the mismatch repair reaction. The RAD27 protein, which is a 5'→3' endo/exonuclease, has been proposed to be associated with mismatch repair as mutations in the gene cause a mutator phenotype (Johnson et al. 1995). However, mutations in RAD27 have a 3 to 5-fold synergistic effect with mutations in the other, known, mismatch repair genes, suggesting that they work in different pathways. RAD27 has been shown to be involved in processing other forms of DNA damage and also the 5' ends of Okazaki fragments (Sommers et al. 1995). Additional studies have shown that RAD27 is important in DNA replication and repair, with mutations resulting in increases in the spontaneous mutation rate, increased sensitivity to DNA-damaging agents, increased recombination and alterations of the cell cycle (Vallen and Cross, 1995). The redundancy in exonuclease function can be seen by the fact that the EXO1 gene has been proposed as another 5'→ 3' exonuclease in *S.cereviseae*. This gene was identified using yeast two-hybrid screening techniques, with MSH2 as a bait protein, and by coimmunoprecipitation (Kolodner, 1996). Mutations in this gene lead to a weak mutator phenotype and it is likely that it acts in an MSH2 dependent pathway.

A human candidate for a mismatch repair 5'→ 3' exonuclease has recently come to light by yeast two-hybrid experiments using PCNA protein. This acts as a processivity factor in cellular functions such as DNA replication and nucleotide excision repair. It was shown that PCNA binds to hMLH1 and, to a lesser extent, hMSH2 in the yeast two-hybrid system (Umar et al. 1996). A mis-sense mutation in yeast PCNA also confers a strong mutator phenotype, and results from yeast PCNA/MLH1 double mutants would suggest that PCNA acts in the mismatch repair pathway. Using an *in vitro* human mismatch repair assay it was shown that PCNA acts at a step in repair prior to synthesis of the new DNA strand. Interestingly, it has been shown that PCNA can bind to, and stimulate the activity of, FEN1 (Li et al. 1995). FEN1 is a 5' exonuclease that is structure specific and has been shown to be the equivalent of the yeast RAD27

exonuclease mentioned above (Harrington and Lieber, 1994). It would be interesting to hypothesise that the lack of hMLH1 in cisplatin resistant A2780 cells might disrupt the recruitment of PCNA, FEN1 and, possibly, other proteins involved in exonucleolytic removal of mismatched DNA.

In passing, it is also interesting to remember that PCNA interacts with p21, the product of the Cip1 gene, which plays a pivotal role in regulating arrest of the cell cycle after DNA damage. The interaction between mismatch repair proteins and PCNA could link this form of DNA repair to cell cycle regulation, a link that appears to be disrupted in the hMLH1 deficient A2780 cell lines.

If loss of hMLH1 brings about a defect in 3'→5' mismatch repair this raises the possibility that hMLH1 interacts, directly or indirectly, with a 3'→5' exonuclease. Although to date no exonuclease with this specificity has been directly linked to mismatch repair, perhaps the most intriguing candidate for such an exonuclease is p53 protein. It has been shown recently that wild type, but not mutant, p53 has a 3'→5' exonuclease action on double stranded oligonucleotides. This action was mapped to the central core of the p53 molecule (Mummenbrauer et al. 1996). Although p53 appears to display multiple functions from the same protein, it has been shown to have a DNA binding domain with a structure similar to that of the catalytic domain of the major apurinic/apyrimidinic repair endonuclease in E.coli. (Cho et al. 1994). The possibility arises that p53 could play a role as a 3'→5' exonuclease in mismatch repair. If the enrolment of p53 into this exonuclease function was dependent on the presence of wild type hMLH1, then mutations in the latter protein could result in a specific loss of 3'→5' mismatch repair. This would be in keeping with the results from A2780/cp70 and A2780/MCP1. Although no evidence exists to show that p53 directly interacts with hMLH1 protein, or with hMutLα, such interaction could be investigated by coimmunoprecipitation reactions or using the yeast two-hybrid approach.

5.3   MISMATCH REPAIR DEFECTS AND CLINICAL DRUG RESISTANCE

The original association between mutation of microsatellite sequences, mutations in genes of the mismatch repair system and the familial cancer syndrome HNPCC opened a new chapter in the characterisation of the causes of cancer. Of equal importance however was the discovery that an increased frequency of microsatellite mutation was also found in a significant percentage of sporadic colorectal tumours (Kim et al. 1994). This suggested that the mechanisms underlying the inherited cancer predisposition could also be responsible for tumours arising purely from somatic mutations. This has been reinforced by an ever increasing number of reports describing the presence of microsatellite mutations in sporadic tumours from a range of different tissues. One question arising from these findings is whether the association between defects in mismatch repair and the development of tumours, both sporadic and hereditary, could be utilised in a clinically beneficial manner.

5.3.1   Analyses of microsatellite instability in sporadic tumours.

Microsatellite instability has been detected as a feature of tumours from a wide variety of tissue types. These have included tumours of the pancreas and stomach (Rhyu et al. 1994; Han et al. 1993); endometrium and uterus (Risinger et al. 1995; Risinger et al. 1993); ovary (King et al. 1995); kidney (Thrashbingham et al. 1995; Uchida et al. 1994); lung (Shridhar et al. 1994; Merlo et al. 1994); Bladder (Gonzalez-Zulueta et al. 1993); breast (Yee et al. 1994; Aldaz et al. 1995); cervix (Larson et al. 1996) and lymphoma (Peng et al. 1996). The results presented in this thesis suggest that defects in mismatch repair, resulting in microsatellite instability, are associated with resistance to clinically important anticancer agents. This, along with data showing that RER+ tumours also display resistance to methylating agents, suggests that tumours with

microsatellite instability might respond less well to treatment and thus carry a worse prognosis. Is there any evidence from the studies into microsatellite instability in different sporadic tumour types that this feature of the tumours carries prognostic significance?

One of the problems encountered in using microsatellite instability as a prognostic tool, is that there appears to be a significant variation in the frequency with which this phenotype arises within tumours of the same tissue type. For example, in carcinoma of the breast several studies have detected evidence of microsatellite mutation in around 20% of tumours (Contegiacomo et al. 1995; Yee et al. 1994) whereas others have shown negligible mutation levels (Jonsson et al. 1995). Several factors have been proposed as potential sources of such variation. Firstly there have been suggestions that defects in mismatch repair may be more significant as a cause of malignancy within certain histological subgroups of certain tumours. One study in breast carcinoma has detected an increased frequency of microsatellite mutation in invasive lobular carcinoma as opposed to invasive ductal carcinoma (Aldaz et al. 1995). In ovarian carcinoma there is a suggestion that endometriod and less common histological subtypes are associated more frequently with defects in mismatch repair (King et al. 1995; Fujita et al. 1995). It may be, therefore, that observed differences in the frequency of microsatellite instability in the same tumour type arise due to differences in the histological subtypes studied. Secondly the methods for detection of microsatellite mutations and their interpretation often differ between studies. This is observed with regard to the number and type of microsatellite loci studied, their chromosomal distribution and interpretation of subtle shifts in the size of alleles.

A third cause of differences in the reported rates of microsatellite instability within the same tumour type is that often various criteria have been used to determine what constitutes microsatellite instability. This latter point is important as it is becoming clear that microsatellite mutations can arise despite functional mismatch repair. Recent studies of spontaneous mutation at microsatellite repeats in cells without defects in

mismatch repair show frequencies in the order of 3×10^{-3} mutations per allele (Hackman et al. 1995; Shibata et al. 1994). This is of the same order as that seen in a number of reports of microsatellite instability in sporadic cancer (Wooster et al. 1994).

Most tumours are clonal expansions of a single cell. Analysis of tumour DNA, therefore, can detect spontaneous somatic mutations that have arisen in a microsatellite sequence during previous replications of the precursor cell, before or during early clonal expansions of the tumour. The greater the observed frequency of microsatellite mutations within a tumour the more likely that a defect in mismatch repair is the underlying cause. Mutation rates in HNPCC are two orders of magnitude higher than the spontaneous background mutation rate mentioned above. A number of studies in colorectal cancer revealed that those tumours with only one microsatellite mutation detected had clinicopathological characteristics similar to those tumours with no evidence of microsatellite mutation (Lothe et al. 1993). This was different from the situation observed for tumours with more than one microsatellite mutation. As a result of this, several studies have used the criterion of requiring mutations at a minimum of two out of five microsatellite loci within a tumour to qualify for displaying microsatellite mutation.

A recent report from the 87th AACR meeting (Perucho, 1996) has suggested an even more stringent set of criteria for classification of microsatellite instability. This would involve the presence of more than one microsatellite alteration together with mutations in cancer genes that are targets of microsatellite instability, such as the type II TGFβ gene. A perceived difficulty of this approach is in determining which of the genes, that are targets for microsatellite instability, are important in tumour development and, therefore, should be included in the analysis. To establish the prognostic significance of microsatellite instability in different forms of sporadic cancer future studies will have to attempt to standardise the methods and criteria used to detect microsatellite instability.

5.3.2 Prognostic significance of microsatellite instability in colorectal cancer.

The first indications that the presence of microsatellite instability might have prognostic significance were observed in the initial reports associating this feature with hereditary and sporadic colorectal cancer (Lothe et al. 1993). The majority of tumours arising in hereditary non-polyposis colorectal cancer (HNPCC) do so in the proximal colon (Lynch et al. 1993). Unexpectedly it was also found that significantly more of the sporadic tumours which displayed an RER+ phenotype were from the colon proximal to the splenic flexure as opposed to the distal colon (Lothe et al. 1993). Colonic adenocarcinoma is, in general, more frequently found in the distal colon and rectum. Subsequently it has been shown that sporadic colorectal cancers which display an RER+ phenotype tend to share certain other clinico-pathological features. These include incidence at a younger age than in RER− tumours; diploid state; exophytic growth; large size at diagnosis; poor differentiation; extracellular mucin production and lymphoid reaction around the tumour (Kim et al. 1994). Intuitively it would be thought that tumours with characteristics of poor differentiation and large size at diagnosis would have a poor prognosis. However, the results of several independent studies now overwhelmingly suggest that RER+ colonic tumours have a significantly better prognosis than those that are RER− (Bubb et al. 1996; Kim et al. 1994; Lothe et al. 1993).

It has also been shown that the presence of microsatellite instability has independent prognostic significance in sporadic colorectal cancer (Bubb et al. 1996). One explanation put forward for the better prognosis in these tumours arises from the observed inflammatory cell infiltrate surrounding the tumour cells. It may be that this represents a greater immunological host response to the RER+ colonic tumours which might prevent local or metastatic spread of the tumour (Kim et al. 1994). The hypothesis has been presented that mutations arising in RER+ tumours may affect genes which

influence expression of tumour-associated antigens that elicit host immune responses. One report has shown that colonic carcinoma cells which are RER+ have an increased frequency of mutation in the β2-microglobulin gene (Bicknell et al. 1996). This gene encodes for a protein involved in antigen presentation on the surface of a cell. As most of the β2-microglobulin gene mutations in these cells are frameshifts, occurring at repeat sequences, it may be that the loss of mismatch repair increases the likelihood of mutation in this gene. Although the clinical results of loss of such antigen presentation are not clear, intuitively it would be thought that it would be associated with a poorer prognosis. However, it has also been suggested that cells with defects in DNA repair are better targets for cytotoxic T-lymphocytes (Townsend et al. 1994). This places a very strong selection pressure on such cells for those with mutations in antigen presentation. It may be that those tumours with additional mutations in the β2-microglobulin gene will represent a poor prognosis sub-group of RER+ colorectal cancers.

Another interesting feature of RER+ sporadic colonic tumours is that they tend to express wild-type p53 protein, as detected immunohistologically (Bubb et al. 1996), and display little evidence of significant loss of heterozygosity throughout the genome (Kim et al. 1994). This is significantly different from other colorectal tumours in general and specifically from RER− tumours of the proximal colon. It would therefore appear that RER+ sporadic colonic tumours represent a class separate from RER− sporadic tumours, in terms of prognosis and molecular carcinogenesis.

5.3.3   Prognostic significance of microsatellite instability in breast and ovarian cancer.

The prognostic implications of microsatellite instability and abnormal mismatch repair have been investigated in tumour types other than colon, of which breast and ovary have, perhaps, accumulated the most data. Microsatellite instability has been reported to occur in up to 20% of sporadic breast carcinomas. Several of the studies

have investigated whether the presence of microsatellite instability correlates with particular clinico-pathological parameters of the breast tumours. Two separate studies have identified that microsatellite instability is more likely to occur in invasive lobular carcinoma of the breast as opposed to invasive ductal carcinoma (Aldaz et al. 1995; Contegiacomo et al. 1995). Invasive lobular breast carcinoma has a greater risk of development of multifocal or subsequent contralateral breast tumours compared to ductal carcinoma (Silverstein et al. 1994). Microsatellite instability has also been shown to be associated with high S-phase fraction and aneuploidy (Karnik et al. 1995), lymph-node involvement and tumour size above 2cm at diagnosis (Contegiacomo et al. 1995). All these features tend to be associated with a worse prognosis. It should be noted that many of these studies have involved limited numbers of patients; therefore the significance of the results has to be viewed with some caution in view of this.

Only one study to date has looked at the association between microsatellite instability and survival in breast cancer (Paulson et al. 1996). This study is significant in that stringent criteria were used to determine the presence of microsatellite instability. A positive association was observed between microsatellite instability and larger tumour size, positive lymph-node status, aneuploidy and higher pathological stage at diagnosis. These poor prognostic features were reflected in the observation that the RER+ breast cancers were associated with reduced disease free and overall survival as compared to RER− tumours.

Microsatellite instability has been observed in up to 20% of ovarian carcinoma in a number of different studies. In common with the results from other tumour types most of these studies relate to small numbers of patients and often use different criteria to define microsatellite instability. However certain characteristics of RER+ ovarian tumours can be inferred from the accumulated data. Microsatellite instability would appear to occur more commonly in the genesis of the less common histological types of ovarian cancer. These include endometriod (Fujita et al. 1995), mixed mesodermal (Risinger et al. 1995) and immature teratoma (King et al. 1995) forms of ovarian

tumour. No definite association between presence of an RER+ phenotype and good or bad prognostic features has yet been determined for ovarian carcinoma. On the one hand microsatellite instability was found in a greater number of stage I tumours (King et al. 1995) or in borderline epithelial ovarian tumours (Tangir et al. 1996) suggesting improved prognostic significance. On the other hand, an RER+ phenotype was significantly associated with advanced clinical stage in endometriod ovarian carcinoma (Fujita et al. 1995).

No study has yet attempted to correlate the presence of microsatellite instability with survival for ovarian carcinoma. However the possibility that defects in mismatch repair, along with other abnormalities of DNA damage control such as non-functional p53, could result in resistance to chemotherapy and thus to poorer survival is raised in a study on gynaecological sarcomas (Risinger et al. 1995). The application of measures of DNA repair, damage recognition or cell cycle progression as prognostic indicators in ovarian cancer, particularly with regard to response to treatment, is currently under investigation. There are indications that mutant or non-functional p53 are associated with reduced survival in ovarian cancer (van der Zee et al. 1995) and with resistance to cisplatin chemotherapy (Righetti et al. 1996b). From the data presented in this thesis it is possible that microsatellite instability might represent a determinant of resistance to chemotherapy that could contribute to providing better prognostic information in ovarian cancer. For example, it has been suggested that the detection of defective mismatch repair in tumour cells might determine the type of chemotherapy administered to a particular patient. This arises from the discovery that ovarian carcinoma cells with defective mismatch repair were resistant to cisplatin and carboplatin but not to other platinum based drugs such as oxaliplatin and JM-216 (Fink et al. 1996).

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5.3.4 Potential therapeutic implications of mismatch repair defects in tumours.

Could the presence of defective mismatch repair in tumour cells, as well as having potential prognostic significance, be utilized as a selective target for different forms of therapy? The "Holy Grail" of cancer therapy is a form of treatment toxic to tumour cells but with no deleterious effect on normal cells. Anything characteristic of tumour cells that distinguishes them from non-malignant cells could act as a target that would allow selective toxicity. Tumour cells that display an RER+ phenotype would be expected to have an increased number of mismatches in the DNA at any given time as compared to cells with functional mismatch repair. One possibility would be to develop treatments that targeted mismatches in DNA as these would be expected to accumulate in tumour cells to a greater extent than in normal cells. The use of peptides that can bind to mismatches as a vehicle to target drugs or radio-isotopes, with short LET characteristics, to tumour cells is one possible avenue to be explored.

5.4 CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has provided evidence that defects in the mismatch repair system are associated with development of resistance to cisplatin and doxorubicin in ovarian and breast tumour cell lines. It has localised the defect in one series of cisplatin resistant ovarian cancer cell lines to the hMLH1 protein. This defect has been characterised , at a functional level, as a specific loss in mismatch repair directed from 3' to the lesion. In the context of recently published research it has been proposed that a functioning mismatch repair system may be involved in determining the sensitivity of a cell to DNA damaging agents such as cisplatin. Components of the mismatch repair system may act

as a general sensor of DNA damage and interact with downstream effectors of DNA repair, cell cycle control and apoptosis.

The future investigation of the role of DNA mismatch repair in determining cellular drug sensitivity or resistance is likely to follow two main themes. Further characterisation of the mechanisms by which mismatch repair proteins are involved in recognising and processing chemical DNA damage is required. The way in which DNA damage recognition by mismatch repair proteins interacts with regulators of the cell cycle and apoptosis needs further clarification. In the cisplatin resistant A2780 cell lines, *in vitro* assays of NER and mismatch repair using DNA containing cisplatin adducts, either simple or mismatched, should help to answer some outstanding questions.

If the association between defective repair and cisplatin resistance is to be more than just a laboratory curiosity then investigation of its clinical significance is required. There is already early data on the relationship between tumour levels of mismatch repair proteins and response to cisplatin chemotherapy in ovarian cancer (R. Brown. Pers. Comm.). Extension of this form of analysis to include different tumour types and larger numbers of patients will determine whether mismatch repair status will signify another important prognostic indicator in the management of cancer.

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