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INVESTIGATION OF THE MOLECULAR BASIS

OF CISPLATIN SENSITIVITY IN

TESTICULAR GERM CELL TUMOURS

By

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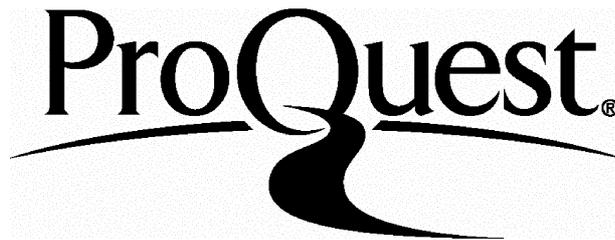**Research Laboratories**
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ABSTRACT

Over 80% of metastatic testicular germ cell tumours (TGCT) are cured using cisplatin-based chemotherapy. Five studies were undertaken to investigate the molecular basis for the hypersensitivity of testicular tumour cells to cisplatin.

Firstly, to determine the number of genes involved in the hypersensitivity of testicular tumour cells to cisplatin, four cell lines were fused with each other and complementation analysis was performed. Hybrids between testicular tumour cell lines were sensitive to cisplatin, indicating that the sensitivity might be controlled by a common mechanism and possibly by one gene.

Secondly, to try to identify the gene, a cDNA library carrying a G418-resistant marker from a cisplatin resistant human cell line was transfected into a cisplatin-sensitive mouse embryonal carcinoma cell line. G418 and cisplatin-resistant primary transfectants were selected and G418-resistant secondary transfectants were isolated. However, the secondary transfectants failed to show resistance to cisplatin indicating that the primary cisplatin and G418-resistant transfectants were non-specific.

Thirdly, to characterize a cisplatin resistant secondary transfectant, which was isolated by transfection of a cDNA library into a human testicular tumour cell line, the plasmid carrying the DNA insert was amplified and transfected into 5 tumour cell lines. However, it failed to confer resistance to cisplatin suggesting that the cisplatin resistance in the primary transfectant was non-specific.

Fourthly, to identify human chromosomes carrying genes controlling cisplatin sensitivity, a mouse embryonal carcinoma cell line was fused with a human bladder tumour cell line. However, in contrast to most of the human-mouse hybrids, the hybrids isolated in this study showed little loss of human chromosomes. Therefore, it was not possible to identify which individual human chromosomes are responsible for causing changes in the sensitivity to cisplatin in the hybrid cells.

Lastly, to compare the gene expression of testicular and bladder tumour cell lines before and after exposure to cisplatin, differential display RT-PCR was performed. Ten differentially displayed bands were characterized and sequenced. A DNA fragment located on chromosome 5q31 was found to be upregulated by cisplatin in testicular tumour cells.

DEDICATED TO MY HUSBAND AND MY PARENTS

ACKNOWLEDGEMENTS

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ABBREVIATIONS

°C: degree centigrade
α-MEM: Minimum Essential Medium
$CaCl_2$: Calcium chloride
Cisplatin: diamminedichloroplatinum(II)
CO_2: Carbon dioxide
CsCl: Caesium chloride
DD-PCR: Differential display PCR
DEPC: diethyl pyrocarbonate
DMEM: Dulbecco's Modification of Eagle's Medium
DMSO: Dimethyl sulfoxide
DNA; Deoxyribonucleic acid
dNTP: deoxyribonucleotide triphosphate
EDTA: Ethylenediaminetetra-acetic acid, disodium salt
FCS: Fetal calf serum
FISH: Fluorescent *in situ* hybridisation
FITC: Fluorescein Isothiocyanate
g: gram
G418: neomycin
HEPES: N-(2-hydroxyethl)-piperazine-N'-(2-ethane) sulphonic acid
HPLC: High-performance liquid chromatography
$IC_{50:}$: Inhibition concentration of 50% colony formation
IR: Ionizing radiation
IPTG:Isopropyl β-D-thiogalactopyranoside
Kb: kilobase
KCl: Potassium chloride
KDa: Kilodaltons
KH_2PO_4: potassium dihydrogen orthophosphate
KOAC: Potassium acetate
LB: Luria Broth
ml: millilitre
M: molar
$MgCl_2$: Magnesium chloride
Na_2HPO_4: sodium phosphate
NaCl: sodium chloride
Na-MOPS:3-[N-morpholino] propane sulfonic acid, sodium salt
NaOH: sodium hydroxide
OD: Optical density
PBS: Phosphate buffered saline
PCR: polymerase chain reaction
RNA: Ribonucleic acid
rpm: revolutions per minute
RT-PCR: Reverse transcription- polymerase chain reaction
SSC: Standard saline citrate
TE: Tris EDTA
VNTR: variable tandem repeat region
X-gal: β-D-galactopyranoside

Chapter 1

INTRODUCTION

1.1   TESTICULAR GERM CELL TUMOURS

1.1.1   Aetiology of Testicular Germ Cell Tumours

Germ cell tumours of the testis are comparatively rare and account for approximately 1% of all cancers in men. However, it is one of the most common forms of cancer in young men between the ages of 15-44 years (UK Testicular Cancer Study Group, 1994a) with a particularly high rate of incidence at 20-34 years of age (CRC factsheet, 1991). There has been a 300% increase in incidence in men aged between 25-29 years in England and Wales during this century and the numbers continue to rise (Mead, 1992).

Many risk factors have been suggested but the aetiology of testicular cancer is not well understood. The only known specific risk factor which is associated with a sizable proportion of cases is undescended testis. Approximately 10% of cases occur in men who had an undescended testis (Swerdlow, 1993).

Testicular cancer has a characteristic age distribution that has provided several risk factors. There is a smaller peak of incidence around the age of 2 years and low rates thereafter in childhood. Then there is a big peak of incidence at young adult ages, which is unlike the age distribution of most malignancies. This indicates an age-specific aetiological factor, which might relate to changes in hormone levels and sexual activity. It also has been suggested that events earlier in life may be involved in the initiation of testicular cancer (Swerdlow, 1993). As testicular cancer incidence is lower in some non-white populations, it has been suggested that increased testosterone levels in black women during early pregnancy compared with white women may provide a protective effect from testicular cancer in their male children later in life (Henderson *et al.*, 1988). Other factors, such as early age at puberty, a history of sexually transmitted diseases, low sperm count, and environmental factors such as lower amount of exercise and high socioeconomic background have been suggested to increase the risk of testicular cancer (Swerdlow, 1993; UK Testicular Cancer Study Group, 1994 a, b; Oliver *et al.*, 1993, 1996).

1.1.2 Classification of Testicular Germ Cell Tumours

Several classification systems have been used to categorize germ cell tumours. The testicular tumours are divided into two main groups, seminoma and non-seminoma (Grigor, 1993). It is thought that the majority of testicular germ cell tumours originate from malignant cells in the seminiferous tubules. These pre-invasive malignant germ cells are called carcinoma-in-situ (CIS) (Giwercman *et al.*, 1993). It has been suggested that the CIS cells can progress to a seminoma or nonseminoma (Oosterhuis *et al.*, 1993) (see Table 1.1 and Figure 1.1).

GERM CELL

↓

Carcinoma *In Situ* (CIS)

Seminoma   →   **Non-seminoma**

↓↓

Embryonal carcinoma   Teratoma

↓

Teratocarcinoma

Choriocarcinoma   Yolk Sac carcinoma
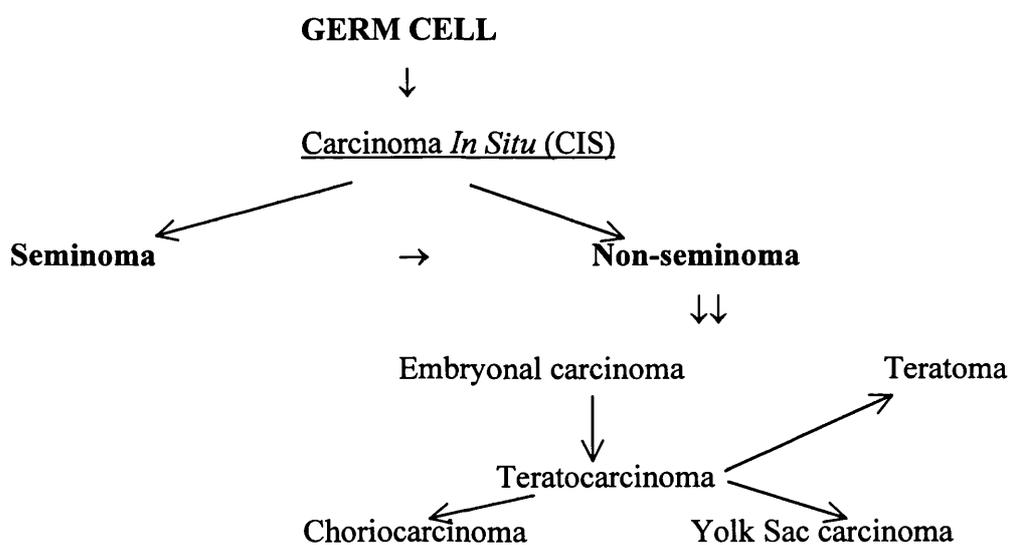
Figure 1.1 Theoretical histogenesis and classification of testicular germ cell tumours (based on Mostofi, 1980).

The majority of testicular tumour patients present with diffuse testicular pain, swelling, hardness or some combination of these three symptoms. Ninety-five percent of tumours originating in the testis are germ cell tumours. Fewer than 10% of all germ cell tumours arise from extragonadal primary sites, with the mediastinum and retroperitoneum the most common. Seminomas account for approximately half of all testicular germ cell tumours and the peak of the incidence is at the age of 40 years. 10-20% are mixed seminomas and nonseminomas. The nonseminomas occur at a peak of 30 years of age (Mead, 1992).

Computed tomographic (CT) imaging of the abdomen and pelvis and chest radiography are used for staging. Serum tumour markers are also indicators of the stage of the disease. α-fetoprotein (AFP) production is restricted to nonseminomatous germ-cell tumours,

especially embryonal carcinoma and yolk-sac tumour. Increased AFP concentrations may be seen at any stage and 40-60% of patients with metastases have increased serum concentrations. Increased serum concentrations of human chorionic gonadotrophin (hCG) may be observed in both seminomas and nonseminomatous tumours. Increased hCG concentrations are seen in 40-60% of patients with metastatic nonseminomatous germ-cell tumours and 15-20% of patients with metastatic seminomas. Serum lactate dehydrogenase (LDH) concentrations are also increased in approximately 60% of patients with nonseminomatous germ-cell tumours and 80% of those with seminomas (Bosl and Motzer, 1997).

Patients with stage I, IIA or IIB seminomas are treated with radical orchiectomy and then radiation to the retroperitoneal and ipsilateral pelvis lymph nodes (Bosl and Motzer, 1997). Relapse occurs in approximately 4% of patients with stage I seminomas and 10% of patients with stage IIA or IIB seminomas (Fossa et al., 1989). Chemotherapy cures more than 90% of patients who have a relapse after radiation therapy (Mencel et al., 1994). Approximately 99% of patients with stages I and II seminomas are ultimately cured.

Twenty percent of patients with stage I tumours have no lymphatic or vascular invasion into tunica albuginea, spermatic cord, or scrotum. Patients who are discovered to have regional lymph nodes at operation require chemotherapy (Hoskin et al., 1986). Patients with persistently increased concentrations of α-fetoprotein, hCG, or both, but without other clinical evidence of disease after orchiectomy, usually have disease outside the retroperitoneum. These patients usually undergo three or four cycles of standard chemotherapy. Stage II nonseminomas are treated initially with either retroperitoneal lymph node dissection or chemotherapy, depending on the extent of disease, serum tumour marker concentrations, and the presence or absence of tumour related symptoms (Davis et al., 1994; Saxman et al., 1996).

Initial chemotherapy is required in approximately one third of patients with germ-cell tumours. Patients with clinical stage IIC disease and primary retroperitoneal and mediastinal seminomas receive initial chemotherapy, since relapse is frequent when these patients are treated with radiation therapy only. Patients also receive initial chemotherapy if they have nonsenimomatous stage III tumours or stage II disease with multifocal retroperitoneal lymph node involvement (Zagars et al., 1987).

Table 1.1. Pathological Classification of Testicular Germ Cell Tumours (based on Peckham, 1988).

British Testicular Tumour Panel	American (Mostofi, 1980)	Histological Appearance
Seminoma	Seminoma	Composed of fairly uniform, round or polygonal cells with clear or finely granular cytoplasm
Malignant teratoma undifferentiated (MTU)	Embryonal carcinoma	Composed of tumour without mature elements, with a variable appearance ranging from a solid sheet of cells to an adenocarcinomatous pattern.
Malignant teratoma intermediate (MTI)	Embryonal carcinoma with teratoma (teratocarcinoma)	Composed of differentiated and undifferentiated tissue
Teratoma differentiated (TD)	Teratoma	Composed of fully differentiated tissue, such as cartilage, bone, muscle or squamous columnar or glandular epithelium.
Malignant teratoma trophoblastic (MTT)	Choriocarcinoma	A malignant teratoma of the general type of either MTI or MTU, but distinguished from them by containing true trophoblastic elements, that is syncytial cell mass and malignant cytotrophoblast.
Yolk sac tumour	Yolk sac tumour	Endodermal-sinus tumour, like the embryonic yolk sac histologically

Table 1.2 Classification of Testicular Germ Cell Tumour Staging (American Joint committee on Cancer) (Bosl, *et al.*, 1997)

Stage I	Tumours with or without lymphatic or vascular invasion, and subdivided into patients with persistently elevated AFP or hCG concentrations in the absence of clinical or radiographic evidence of metastatic disease.
Stage II	Includes retroperitoneal nodal disease without distant metastases, with or without increased marker levels.
Stage III	Includes presentations with distant metastases or high serum tumour-maker values

1.1.3 Development of Chemotherapy for the Treatment of Testicular Germ Cell Tumours

The cure rate of stage I and II seminomas is greater than 95% by surgical removal of the diseased testis followed by adjuvant radiotherapy in seminomas (Maier *et al.,* 1973; Doornbos *et al.,* 1975). However, before the mid 1970s, treatment for nonseminomas was less satisfactory and most young men with metastatic testicular cancer died of uncontrolled malignancy. The response rate for advanced testicular germ cell tumours was approximately 50% and the cure rate was 5-10% with standard chemotherapy consisting of dactinomycin, alone or in combination with methotrexate and chlorambucil (Mackenzie, 1966). The use of the combination of vinblastine and bleomycin later achieved a 25% long-term disease free survival (Samuels *et al.,* 1976). However, a breakthrough came when a heavy metal, cisplatin, was discovered and used as an anti-neoplastic agent in the late 1970s (Higby *et al.,* 1976). The introduction of cisplatin in combination with vinblastine and bleomycin dramatically improved the cure rate of testicular cancer to 70-80% (Peckham, 1988; Horwich, 1989). The major improvement in this combination therapy was due to cisplatin rather than the older drugs vinblastine and bleomycin (Einhorn and Donohue, 1977). Since then further improvements have been made and with the substitution of vinblastine by etoposide (Peckham *et al.,* 1983), the cure rate of metastatic testicular cancer has been reported to be over 80% (Kelty *et al.,* 1996). The high cure rate but substantial toxicity of combination chemotherapy requires an effort to identify patients more likely (good-risk) and less likely (poor-risk) to be cured with standard chemotherapy. Extent of disease and serum tumour-marker concentrations are also identified as independent predictors of prognosis (see Table 1.2 and 1.3).While the cure rate of most solid tumours is still very low, testicular cancer has become a model for curable neoplasia.

Table 1.3 Risk Classifications Used to Assign Patients in Clinical Trials (Xiao *et al.*, 1996)

Risk Group	Seminoma	Nonseminoma
Good Risk	All of following: Any hCG elevation Any LDH elevation Absence of nonpulmonary visceral metastases Any primary site	All of the following: HCG<5000mIU/ml LDH<1.5x upper limit of normal AFP<1000ng/ml Absence of nonpulmonary visceral metastases Absence of mediastinal primary site
Intermediate Risk	Any of the following: Any hCG elevation Any LDH elevation, Presence of nonpulmonary visceral metastases present Any primary site.	Any of the following: HCG5000-50,000mIU/ml LDH1.5-1.0 x upper limit of normal AFP1000-10,000 ng/ml Presence of nonpulmonary visceral metastases Presence of mediastinal primary site
Poor Risk		Any of the following: HCG>50,000mIU/ml LDH>10x upper limit of normal AFP>10,000ng/ml Presence of nonpulmonary visceral metastases Presence of mediastinal primary site

1.1.4   Molecular Genetic Basis of Testicular Germ Cell Tumours

The chromosome numbers in CIS are usually tetraploid. In seminomas, they tend to be hypertriploid, however, in nonseminomas they are usually hypotriploid. It has been suggested that an early event may be polyploidization of a dysplastic germ cell precursor which leads to *carcinoma in situ* with a tetraploid chromosome numbers with few structural abnormalities (Sandberg *et al.*, 1996).

Another early event is the formation of the iso (12p). In 80% of all types of germ cell tumours the characteristic anomaly is the presence of a copy of the i(12p) isochromosome, which consists of two short arms of chromosome 12. This cytogenetic change is highly specific for germ cell tumours of all histological varieties (Sandberg *et al.*, 1996). However, how this abnormality contributes to the development of testicular germ cell tumours is not clear. The frequency and number of copies of i(12p) have been reported to be higher in nonseminomas than in seminomas (Bosl *et al.*, 1989).

Consequently the number of copies of the i(12p) isochromosomes has been used as a prognostic indicator, with more than 3 copies being associated with tumour progression (de Jong *et al.*, 1997). Other chromosomal changes such as altered dosage of the sex chromosomes (Peltomaki *et al.*, 1991a), deletion of chromosome 12q and abnormalities on chromosomes 1, 5, 7 and 12 have also been observed in testicular tumours (Peltomaki *et al.*, 1990).

The genes on the short arm of chromosome 12 have interested many researchers. The most intensively studied genes are the *ras* gene family. This family contains three closely related cellular genes, *K-ras, H-ras* and *N-ras* (Bos, 1988). The *ras* genes encode for small GTP binding proteins that are involved in signal transduction pathways from the cell surface to the nucleus (Bos, 1989). Mutations and amplifications of the *ras* gene have been observed in many human cancers (Rodenluis, 1993). Overexpression of the *K-ras* gene may contribute to the transformation of germ cell tumours (Peltomaki *et al.*, 1991b; Samaniego *et al.*, 1990). Increased expression of the *K-ras* gene has also been correlated with the i(12p) copy number in testicular germ cell tumours. No mutations of the *H-ras* gene were detected in a panel of established cell lines (Dmitrovsky *et al.*, 1990). *K-ras* and *N-ras* mutations have been observed in less than 15% of primary testicular tumours and their derived cell lines (Moul *et al.*, 1992; Ridapaa *et al.*, 1993; Olie *et al.*, 1995).

Several other oncogenes have been studied in relation to the development of testicular tumours. The *c-Kit* gene, which encodes a tyrosine kinase receptor and its ligand *hst1*, a stem cell growth factor, have been related to the regulation of stem cell growth (Witte, 1990). The c-Kit protein has been found to be expressed in normal testis, while seminomas have higher levels than nonseminomas. It has been suggested that this oncogene may interfere with differentiation in testicular tumours (Shuin *et al.*, 1993). While the expression of the hst1 was undetectable in normal testis cells, it was overexpressed in 63% of nonseminomas (24/38) but only 4% (1/24) in seminomas (Strohmeyer, 1994). None of the nonseminomas that expressed hst1 had significant levels of *c-kit* mRNA, demonstrating the inverse relationship between expression of the *hst1* and *c-kit* oncogenes in these tumours. Expression of other oncogenes such as the platelet-derived growth factors PDGFA and PDGFB, the growth factor receptor c-erbB-1 have also been described to be significantly increased in testicular tumours compared to the normal tissues (Peltomaki *et al.*, 1991b; Shuin *et al.*, 1993), suggesting that they might be play a role in the tumorigenesis.

Identification of tumour suppressor genes in testicular tumours has also been carried out (Murty *et al.*, 1992; Leahy *et al.*, 1995; Peng *et al.*, 1995; Al-Jehani *et al.*, 1995). As deletions of the long arm of chromosome 12 are common in testicular tumours, it has been suggested that a tumour suppressor gene might be located in this region (Murty *et al.*, 1992). Other candidate regions on chromosomes 1, 4, 5q, 14, and 18 have also been suggested (Asada *et al.*, 1994; Leahy *et al.*, 1995). Mutations of common tumour suppressor genes such as *Rb, p53* and *WT-1*, are not found in testicular tumours (Peng *et al.*, 1993; Fleischhacker *et al.*, 1994; Schenkman *et al.*, 1995). So far there is no report on candidate tumour suppressor genes in testicular tumour.

Unlike the majority of solid tumours, p53 mutations in testicular tumours are rare. High levels of wild type p53 have been detected in 8 out of 8 testicular tumour cell lines (Tesch *et al.*, 1990) and in the mouse F9 teratocarcinoma cell line using antibodies raised against wild type p53 by Western blotting analysis (Lutzker and Levine, 1996). Using Single Strand Conformation Polymorphism (SSCP) followed by DNA sequencing, cells from frozen tissues from seminomas and non-seminomas (n=22, Peng *et al.*, 1993; n=40, Fleischhacker *et al.*, 1994; n=26, Schenkman *et al.*, 1995) were examined for *p53* mutations on exons 5-8, which have been shown to be "hot spots" for mutations in other human cancers (Hollstein *et al.*, 1991), and no mutations were found. However, Wei and co-workers (1993) detected point mutations in the *p53* gene in exon 5 (codon 141), 7 (codon 238) and 8 (codon 276) in 4 of 17 seminomas during DNA sequencing.

There are a number of families with more than one person affected, and recent reports estimated that the relative risk factor to brothers is increased by 7-10 fold (Heimdal and Fossa, 1994). By screening a small part of the genome on each chromosome on 35 families in which there were either 2 or 3 affected bothers, there was no highly linked loci to explain the increased relative risk factor (Leahy *et al.*, 1995), although a region on chromosome 4 was identified as a possible candidate region for a testicular cancer susceptibility gene.. This is in contradiction to the inheritance of a single recessive gene hypothesized by Nicholson and Harland (1995), who analysed the published data on the age distribution of the first tumour in bilateral disease in familial and general cases and suggested that the increased risk to brothers can be accounted for by the homozygosity of a single predisposing gene.

1.2.   MECHANISMS OF ACTION OF CISPLATIN

Cis-diamminedichloroplatinum (II) (cis-DDP or cisplatin) was discovered to inhibit bacterial cell growth in the 1960's (Rosenberg *et al.*, 1965). By the 1970s, anticancer activity by cisplatin was reported in clinical trials with terminally ill cancer patients (Higby *et al.*, 1976). Using cisplatin combination therapy, Einhorn and Donohue (1977) achieved over 80% long term survival of testicular cancer patients. This achievement resulted in its application to the treatment of other malignancies. Since the 1980s cisplatin had been used in many cancers, such as ovarian, bladder, head and neck, lung and gastric cancers. It is not only a cure for testicular cancer, but also induces a response in ovarian and breast cancer (Rosenberg, 1985; Petsko, 1995).

Cisplatin is a neutral square-planar coordination compound with two chloride and two amino groups attached to the platinum atom (see Figure 1.2). The two chloride groups are stable at extracellular chloride concentrations. After cisplatin has diffused into a cell, the chloride groups are exchanged for hydroxyl groups or water to produce a monofunctional or a bifunctional positively charged radical. This positively charged radical can then interact with negatively charged protein, RNA or DNA in the cell. Although only approximately 1% of cisplatin binds to DNA, the evidence indicates that DNA is the crucial target for its cytotoxicity (Eastman, 1987 and 1990).
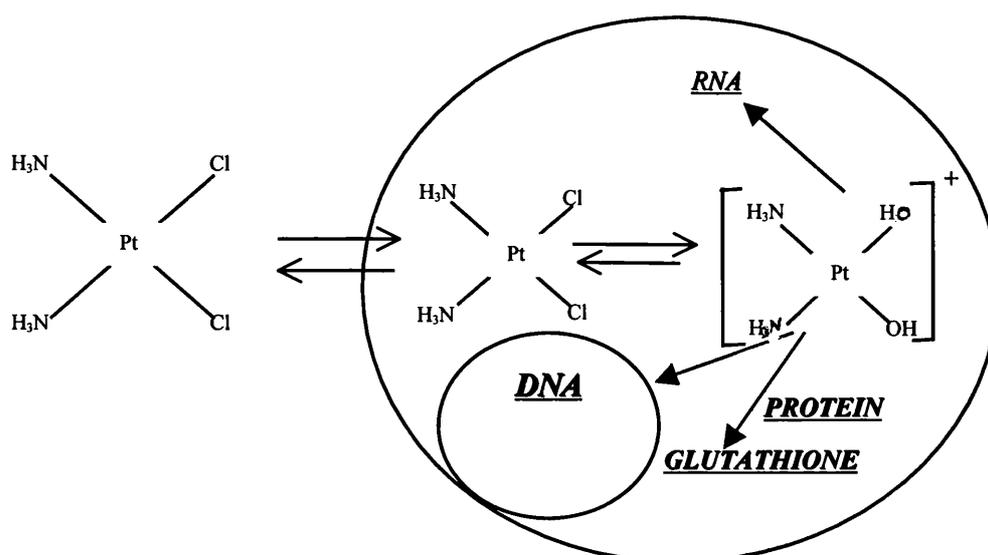
Figure 1.2 Mechanisms of cisplatin action

Cisplatin has been shown to prefer to react with G-C rich regions of DNA (Stone and Kelman, 1974). Cisplatin forms both monofunctional platinum-DNA adducts and bifunctional adducts. The monofunctional adducts are not thought to be cytotoxic because they do not cause significant distortion of the DNA double helix, and are usually rapidly detected by proteins involved in detoxification (Pinto and Lippard, 1985). The major bifunctional lesions caused by cisplatin on DNA are intrastrand cross-links between two neighbouring guanine bases [d(GpG)1.2-intrastrand cross-link)] (approximately 65%) and two neighbouring adenine and guanine bases [d(ApG) 1,2-intrastrand cross-link] (approximately 25%). In these adducts, the platinum is covalently bound to the N^7 position of the neighbouring purine bases causing distortion of the DNA helix. The fact that the cisplatin analogue trans-platin cannot form 1,2-intrastrand crosslinks suggests that such lesions might be responsible for the cytotoxicity of cisplatin (Lippard, 1993). The remaining intrastrand cross-links are between two guanine bases separated by one or more bases [d(GpNpG) 1,3- intrastrand cross-link]. Cisplatin can also bind to two DNA bases on the opposite strands forming interstrand cross-links (Fichtinger-Schepman et al., 1985). The number of interstrand cross-links has been shown to correlate with cell death (Roberts et al., 1984; Erlichman, 1991). However these lesions represent less than 1% of total DNA-cisplatin adducts.

It was believed that inhibition of DNA synthesis by cisplatin might be the critical step in its cytotoxicity (Harder and Rosenberg, 1970). However, at cisplatin concentrations that inhibit DNA synthesis, DNA-repair proficient Chinese hamster ovary (CHO) cells survive treatment but DNA-repair deficient cells die (Sorenson and Eastman, 1988b). This indicates that cell death does not always correlate with inhibition of DNA synthesis. Sorenson and Eastman (1988a) demonstrated that when murine leukemia L1210 cells were treated with cisplatin for 2 hours, they progressed through S phase of the cell cycle but arrested at G_2 phase. At low concentrations, the cells recovered within 1-3 days. At high concentrations, the cells were unable to recover and died 4-6 days after the drug treatment. This suggests that events leading to cell death by cisplatin might occur at the G_2 phase of the cell cycle. When the DNA repair-deficient and proficient CHO cells were studied (Sorenson and Eastman, 1988b), the two repair-deficient cell lines were hypersensitive to cisplatin and showed a characteristic G_2 arrest. In contrast, the DNA repair-proficient cells required much higher concentrations of cisplatin before showing any signs of G_2 arrest. Analysis of DNA strand breaks in these cell lines showed that significant numbers of double strand breaks only occurred at concentrations of cisplatin

that led to cell death. This suggests that the formation of double-strand breaks may be the first change leading to cell death by cisplatin.

The evidence suggests that the major decision point in response to cisplatin is during G_2 and that cell death caused by cisplatin may be due to the failure to overcome the block at the G_2 phase. The trigger of cisplatin induced cell death may be related to the level of DNA damage tolerated. Although numerous proteins have been shown to recognize cisplatin-induced DNA adducts, it is not clear which protein(s) is responsible for signalling the presence of cisplatin-induced damage leading to apoptosis (discussed further in section 1.3.4).

1.3. Factors Influencing Cisplatin Sensitivity

This section will describe the factors that influence cisplatin sensitivity in a range of cells and will also consider any genetic factors or molecular mechanisms that have been determined to influence this sensitivity. Section 1.4 will concentrate on the effects of cisplatin in testicular tumour cells.

Some types of common cancers such as colon cancer and non-small cell lung cancer show little response to treatment with anticancer drugs. Their resistance to chemotherapy may be influenced by humoral factors such as vascular access or drug penetration, but the most important factor is the inherent resistance of the tumour cells to chemotherapy. Other human tumours, such as breast cancer and bladder cancer respond to initial treatment, but they acquire resistance to further therapy. Inherent and acquired drug resistance are the major factors that limit the successful use of chemotherapy. Some cancers, such as Hodgkin's disease and other lymphomas, childhood acute leukemia and testicular germ-cell tumours, are sensitive to chemotherapy and their cure rate is high. These tumours show inherent sensitivity to chemotherapeutic drugs (Tannock and Hill, 1993).

Cell lines derived from inherently resistant and sensitive tumours have been established which retain their differences in sensitivity to chemotherapeutic drugs *in vitro*. For example, testicular tumour cell lines are more sensitive to DNA damaging agents than the relatively resistant bladder tumour cell lines. This suggests that the differential sensitivity between these two tumour types is inherent to the tumour cells rather than a result of

humoral factors. These model systems have been used to study the mechanisms resulting in their different sensitivities *in vitro* (Walker *et al.*, 1987; Masters *et al.*, 1993).

A group of human disorders, including xeroderma pigmentosum (XP), Cockayne's syndrome, trichothiodystrophy (TTD), Fanconi's anaemia (FA), Blooms syndrome (BS) and ataxia telangiectasia (AT) are hypersensitive to a range of DNA damaging agents such as UV, ionizing radiation and cisplatin due to deficiencies in their DNA repair pathways. Cell lines derived from these disorders are also used to study the mechanisms involved in sensitivity to DNA damaging chemotherapeutic drugs (Lehmann *et al.*, 1992)

Many cell lines were made resistant *in vitro* by either continuous exposure to low concentrations of drugs such as cisplatin for a period of a few months (for example, Hill *et al.*, 1990; Walker *et al.*, 1990).. However, whether the acquired resistant cell lines *in vitro* reflect the type of acquired cisplatin resistance found in patients treated with cisplatin is not clear. A few studies also used samples from the primary and metastatic (for example, Kashani-Sabet *et al.*, 1990). The following sections will focus on the possible mechanisms affecting cisplatin sensitivity, concentrating on cells inherently sensitive to cisplatin.

1.3.1 Cisplatin Accumulation

The mechanisms by which cisplatin enters the cell are still poorly understood. However, it has been suggested that cisplatin enters the cell largely through passive diffusion. Gale and coworkers (1973) found that the uptake of tritiated cisplatin in human tumour cells increased with drug concentration. Plotting of platinum accumulation *versus* a range of drug concentrations resulted in a straight line through the zero point, suggesting that the limiting factor for cisplatin uptake is only the concentration of the drug. This was supported by other studies by measuring cisplatin uptake using atomic absorption (Mann *et al.*, 1990; Andrews *et al.*, 1988).

Despite these observations, active uptake of cisplatin has been suggested to occur. Cisplatin accumulation may be dependent on a sodium-potassium ATPase (adenosine triphosphatase) mediated channel. When 2008/DDP cells were pre-incubated with the ATPase inhibitor ouabain for 1 hour, cisplatin uptake was inhibited by 50%, as measured by the uptake of ^{195}Pt-DDP (Andrews *et al.*, 1991). A study (Ohmori *et al.*, 1994) on an

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ouabain resistant small cell lung cancer subline, which had an overexpression of sodium-potassium ATPase mRNA levels, showed an increased cisplatin accumulation and cisplatin sensitivity compared to its parental line. A ouabain resistant Hela line, D98^{OR} was shown to be more sensitive to cisplatin than its ouabain sensitive parental line (Wang *et al.*, 1996), however the mechanisms involved were not studied.

A highly expressed membrane protein has been isolated from a cisplatin resistant lymphoma cell line (Kawai *et al.*, 1990). The 200kDa protein was identified by comparing the presence of plasma membrane proteins in the sensitive parental line and its resistant subline using Western blotting with a rabbit antibody raised against proteins of resistant cells. However, the function of this protein is not well established although it has been suggested that it might be a homologue of the cell membrane P-glycoprotein, which causes multidrug resistance.

1.3.2 Detoxification System

Once cisplatin enters the cell, the two chloride ligands can be exchanged for H_2O or OH groups to form a positively charged active form of cisplatin (see Figure 1.1). A group of intracellular non-protein or protein sulfhydryl molecules such as glutathione (GSH) or metallothioneins (MT) have been shown to play a part in the neutralization and conjugation of such cytotoxic free radicals thus protecting the cells from being damaged (Webb and Cain, 1982; Arrick and Nathan, 1984).

Glutathione (GSH) and Glutathione S-Transferase (GST)

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) with a free sulfhydryl group and is the most abundant intracellular nonprotein sulfhydryl. GSH exists in a number of interchangeable forms. As a potential nucleophile, the reduced form of GSH can react with positively charged radicals such as cisplatin (Meister, 1988). A GSH-cisplatin complex has been isolated from cisplatin treated murine L1210 cells (Ishikawa and Ali-Osman, 1993) and GSH may protect cells by reacting with cisplatin inside the cell before it reaches its target DNA (Arrick and Nathan, 1984).

Fibroblasts from an individual with 5-oxoprolinuria were deficient in glutathione as compared to cells from a healthy person (Hansson *et al.*, 1996). This rare autosomal recessive disease has a metabolic error causing a deficiency in GSH synthesis. The GSH

deficient cells were more sensitive to cisplatin, carboplatin and melphalan but not UV radiation. Depletion of GSH with buthionine sulfoximine (BSO) sensitized the normal fibroblasts to cisplatin to a similar level to the GSH deficient cells. However, the increased sensitivity to cisplatin in the GSH deficient cells cannot be explained by the increased levels of cisplatin-DNA adducts or the rate of removal of cisplatin-induced damage. The authors postulated that the sensitivity to cisplatin was the result of increased induction of the transcription factor AP-1, leading to increased apoptosis.

The conjugation of GSH with positively charged free radicals is thought to be catalyzed by the enzyme glutathione S-transferase (GST). The GSTs are a multigene family of isoenzymes that are classified as α, μ or π according to their substrate specificity or amino acid sequences (Meister, 1988). GSTs are thought to catalyze the conjugation of GSH with electrophiles and reduce active organic peroxides. However only GST-π has been shown to influence cisplatin sensitivity (Teicher et al., 1987). When GST-π antisense cDNA was transfected into a human colon cancer cell line M7609, which expresses high levels of GST-π and shows intrinsic resistance to DNA damaging agents, the resulting transfectants had approximately 50% reduced GST-π level. The sensitivities of these antisense transfectants were increased between 2-3 fold to cisplatin, melphalan and etoposide (Ban et al., 1996).

Metallothionein

Another protein family, the metallothioneins (MT), are small proteins that are important in binding and detoxifying heavy metals such as zinc and cadmium (Webb and Cain, 1982). The transcription of the *MT* gene is strongly induced by heavy metal ions and stress but not by the potentially lethal electrophiles (Karin et al., 1985). When cisplatin binds to MT, it loses its amine ligands and displaces heavy metal ions. The displacement process is thought to protect cells from chemotherapeutic agents (Kraker et al., 1985).

A study of prostate cancer cell lines by immunostaining suggested that the subcellular distribution of MT might be more important than its content. It was found that prostate cancer cell lines containing MT in their nuclei were more resistant to cisplatin than the ones containing MT in the cytoplasm (Kondo et al., 1995). Because cisplatin targets DNA, it is possible that MT in the nucleus may provide protection to DNA thus reducing

the effect of cisplatin. However, the subcellular localization of MT needs to be examined in other cancer cell lines before any conclusion can be drawn.

The evidence that cisplatin sensitive testicular tumour cells had higher levels of both constitutive and cisplatin induced MT levels than the relatively resistant bladder tumour cells suggests that it may not play an important role in determining intrinsic sensitivity (Masters *et al.*, 1996).

1.3.3 Roles of Oncogenes and the Tumour Suppressor Gene p53

A number of oncogenes are known to influence drug sensitivity and efforts have been made to study the mechanisms involved in cisplatin sensitivity.

ras gene family

The *ras* oncogene family consists of three genes, *H-ras, K-ras* and *N-ras*. All of them appear to be activated by single base mutation or overexpression (Bos, 1989). The *ras* genes encode a 21kDa GTP-binding protein that plays a role in the signal transduction process (Haubruck and McCormick, 1991). The Ras-p21 proteins differ not only in their amino acid composition (Brandt-Rauf *et al.*, 1988) but also in their function (Ballester *et al.*, 1987). The transfection of a pSV2NEO plasmid containing an activated *H-ras* gene into 3T3 cells resulted in increased sensitivity to cisplatin compared to the cells transfected with the vector alone, but transfection with the wild-type *H-ras* had no effect on cisplatin sensitivity (Gao *et al.*, 1991; Niimi *et al.*, 1995). Other studies showed that transfection of the activated *H-ras* gene into 3T3 cells resulted in cisplatin resistance. (Sklar *et al.*, 1988; Shinohara *et al.*, 1994). The overexpression of *H-ras* resulted in a reduced Na^+, K^+ ATPase activity and decreased cisplatin accumulation in these transfectants, resulting in a decreased sensitivity, which agrees with the observed effects of overexpression of ATPase in an ouabain resistant small cell lung cancer cell line (see section 1.3.1). The different results obtained in these studies might be due to the different controls used for determining cisplatin sensitivity in the transfectants.

c-fos

DNA damaging agents can induce the expression of specific genes such as the nuclear oncogenes *c-jun* and *c-fos* (Hollander and Fornace, 1989). The transcription factors c-jun and c-fos belong to the ras, raf and MAP kinase pathway, which can be activated in

response to extracellular stress (Hollander and Fornace, 1989). Activation of these two genes results in the regulation of cell proliferation, differentiation, and possibly apoptosis by transcriptional activation of other genes (Angel *et al.*, 1991). A study on fibroblast cells derived from *c-fos* knockout mice showed that *c-fos-/-* cells are hypersensitive to DNA-damaging agents such as ionizing radiation and melphalan induced apoptosis compared to the c-Fos proficient cells (Kaina *et al.*, 1997). Chromosome aberrations, determined by the visualization of chromosomal abnormalities by Giemsa staining, appeared at 18-24 hours after the drug treatment. Peak numbers of apoptotic cells appeared 72 hours after the drug treatment. The fact that *c-fos-/-* cells showed a high percentage of chromosomal abnormalities and a high rate of apoptosis after exposure to DNA damaging agents suggests that c-Fos may play a role in cellular defence against DNA damage. This protection may not be a result of activation of DNA repair genes, as the overall activities of various DNA repair enzymes such as apurine endonuclease, O^6-methylguanine-DNA methyltransferase were not altered.

c-myc

As a transcription factor, *c-myc* has been shown to transcriptionally control both cell proliferation and apoptosis (Spencer and Groudine, 1991; Evan *et al.*, 1992). Cisplatin induced *c-myc* expression was demonstrated by linking the promoter region of the *c-myc* gene to the reporter CAT gene (chloramphenicol acetyltransferase) (Eliopoulos *et al.*, 1995). When mouse erythroleukemia cells were treated with a range of cisplatin concentrations, significantly increased CAT activity was observed. As there was no increased CAT activity observed when the same experiments were performed with the reporter gene linked to the *c-Hras1* promoter in these cells, the effect of cisplatin may be specific to the *c-myc* promoter. Evidence suggests that increased c-myc may play a role in acquired cisplatin resistance and transfection of the antisense of the *c-myc* gene has been shown to restore the sensitivity of the acquired resistant cells (Van Wardenburg *et al.*, 1996).

p53

When DNA damage occurs in normal proliferating cells, they respond in one of two ways: cell cycle arrest or apoptosis, and *p53* is involved in both. For example, after 5Gy γ-irradiation, proliferating fibroblasts arrest, whereas proliferating intestinal crypt epithelium cells undergo apoptosis, both by a p53-dependent mechanism (Bellamy, 1996). Wild-type p53 is present in extremely small quantities in most cells and its half life

is short, approximately 30 minutes. It acts as a transcription factor which activates a specific set of target genes. p53 also interacts directly with cellular proteins and is itself a target of several viral proteins. p53 activity and stability are regulated post-transcriptionally and appear to be cell type dependent (Ko and Prives, 1996). DNA damage induces p53 and the presence of DNA strand breaks is crucial for this induction. Therefore it has been proposed that p53 acts as "a guardian of the genome" (Lane, 1992). It controls a G_1 checkpoint that allows cells to repair DNA damage before entering S phase or undergoing apoptosis. This is consistent with the finding that *p53* knockout mice develop tumours more rapidly than mice with wild-type *p53*. This pattern has been observed in patients with Li-Fraumeni syndrome, who have mutations in *p53* and who develop tumours with high frequency (Malkin *et al.,* 1990).

It has been shown that wild-type p53 induces apoptosis in E1A/Ras transformed mouse fibroblasts in response to radiation, adriamycin and etoposide, but the cells do not undergo apoptosis if it is absent (Lowe *et al.,* 1993b). In human cancers, it is difficult to confirm this, but the evidence seems to point in this direction. Testicular tumour, pediatric acute lymphoblastic leukemia, and Wilms' tumour (some of the most common cancers in patients under 35) are highly curable even in advanced stages and show a lack of p53 abnormalities, except for the anaplastic variant of Wilms' tumour, which contains p53 abnormalities and has a poor prognosis (Fisher, 1994). Mutations of *p53* are associated with poor prognosis in many of the most common human tumours such as lung, breast, colorectal, prostate, brain and multiple hematopoietic tumours (Hollstein *et al.*, 1991).

Different cell types vary in their susceptibility to undergo apoptosis. Thymocytes for example, undergo apoptosis when irradiated at doses that fail to kill fibroblasts (Fisher, 1994). The evidence that untransformed fibroblasts arrest from the same treatments that trigger apoptosis in their transformed counterparts may explain the selectivity of many cancer drugs (Lowe *et al.,* 1993b). Surrounding normal tissues may pause and repair the damage while tumour cells die through apoptosis.

There is evidence that lymphocytes from *p53* knockout mice are more resistant to DNA damage (Lowe *et al.,* 1993a) but fibroblasts showed no difference in radiosensitivity (Slichenmyer *et al.*, 1993) when compared to the wild type cells. This suggests that the role of p53 in mediating apoptosis in response to DNA damage may be cell type specific. Also, additional cellular proteins such as MDM-2 or viral proteins such as human

papilloma virus E6 protein may interact with p53 and functionally inactivate it. In these cases, presence or absence of *p53* mutations may not correlate with response to treatment, probably because p53 function is altered by other mechanisms (Fisher, 1994).

Therefore, contradictory results have been reported relating to *p53* status and response to cisplatin. A large number of publications suggest that wild-type p53 correlates with cisplatin sensitivity, but a similar amount of evidence shows that wild-type p53 is associated with increased cisplatin resistance. For example, when the wild-type *p53* gene was transfected into a human nonsmall cell lung cancer cell line, which has a homozygous deletion of *p53*, by the recombinant adenovirus-mediated transfer, the sensitivity to cisplatin was increased up to 5 fold (Fujiwara *et al.*, 1994). It was also shown in patients with ovarian and cervical carcinomas that wild-type *p53* correlates with response to cisplatin and a high percentage of patients with either advanced disease or poor prognosis contain *p53* mutations (Righetti *et al.*, 1996; Garzetti, *et al.*, 1996; Buttitta *et al.*, 1997).

There is evidence from a study of 9 human ovarian cancer cell lines suggesting that cisplatin-induced cytotoxicity is independent of *p53* status (Feudis *et al.*, 1997). However, when p53 function in a breast cancer cell line MCF-7 was disrupted by transfection of human papillomavirus type-16 E6 gene, which encodes a protein that binds to p53 and stimulates its degradation (Scheffner *et al.*, 1990), the transfectants showed increased sensitivity to cisplatin. When a CAT reporter plasmid containing cisplatin-induced damage was transfected into these cells, they showed reduced ability to repair the cisplatin-damage carried by the plasmid. This was shown by the low levels of CAT activity when compared to the parental cells (Fan *et al.*, 1995). This was also supported by a clinical study on bladder carcinoma patients that p53 alterations correlated with good response to DNA damaging agents including cisplatin (Cote *et al.*, 1997).

The mechanism by which *p53* regulates cisplatin induced apoptosis appears to be by up-regulating genes such as *p21Waf1/Cip1*. p53-binding sites are found in the coding region of the *p21* gene (Ei-Deiry *et al.*, 1994), which functions as a cyclin-dependent kinase inhibitor (Harper *et al.*, 1993) and plays a role in cell cycle G_1 arrest in response to DNA damage (Kastan *et al.*, 1991). Murine embryonic fibroblasts from *p21* knockout mice showed increased sensitivity to cisplatin induced apoptosis and reduced repair ability of cisplatin induced damage (Fan *et al.*, 1997). This indicates that *p21* may play a negative

role in cisplatin induced apoptosis possibly by allowing the cell time to repair DNA damage before proceeding to the synthesis of new DNA. The activities of the proteins regulated by *p21* in response to DNA damage also needs to be investigated.

Recently, a number of p53-induced-genes (PIG) were isolated by screening a large number of transcripts appearing in cells induced to express p53 (Polyak *et al.*, 1997). Among them was the *p21* gene and several others that are involved in the generation or response to oxidative stress. Subsequent generation of reactive oxygen species (ROS) results in mitochondrial damage and the release of enzymes that drive the terminal events of apoptosis. As the anti-apoptotic Bcl-2 is normally anchored to the mitochondrial membrane, oxidative damage to this membrane also has consequences for cell survival. This will be discussed in the next section.

bcl-2 Gene Family

The proteins of the *bcl-2* gene family are critical regulators of apoptosis. A number of b*cl-2* related genes have been identified. Some, such as *bcl-2, bcl-$_{XL}$ and bcl-w* inhibit apoptosis, while others like *bax, bak* and *bad* promote it. The proteins encoded by these genes are homologous and can dimerise, therefore antagonizing or enhancing each other's function (reviewed by Jacobson, 1997). *Bcl-2, bcl-$_{XL}$* and *bax* so far have been associated with cisplatin sensitivity.

Bcl-2 was identified by a translocation from its normal location at chromosome 18 to the immunoglobulin heavy-chain locus at chromosome 14. This translocation results in the overexpression of *bcl-2* mRNA and the encoded protein. The Bcl-2 protein is located in the nuclear envelope, parts of the endoplastic recticulum and the outer mitochondrial membrane (Reed, 1994). Bcl-2 can protect cells from apoptosis by prolonging their life span. The fact that Bcl-2 protects against a wide variety of drugs with different mechanisms of action suggests that they may use the same final common pathway for inducing cell death and that Bcl-2 is a regulator. Bcl-2 does not prevent the entry of drugs and does not alter drug induced damage of DNA or its repair. No effects have been found on the cell cycle by Bcl-2. In Bcl-2 overexpressing cells, drugs still enter the cells and induce damage, but the damage is somehow unable to induce cell death (Reed, 1995).

In addition to its involvement in lymphomas, relatively high levels of Bcl-2 have been found in a variety of cancers such as leukemia, colon and stomach cancers regardless of

their sensitivity to chemotherapeutic drugs. Unlike B-cell lymphomas, the high levels of Bcl-2 are not associated with translocations of the bcl-2 genes (Reed, 1995). The fact that a p53 negative response element was found in the bcl-2 gene using reporter gene assays, and overexpression of p53 by transfection can lead to down-regulation of Bcl-2 suggests that p53 may act as a down-regulator of Bcl-2 (Miyashita et al., 1994 a and b). Therefore, loss of p53 functions in the majority of inherently resistant tumours may be due to the loss of down-regulation of the bcl-2 gene. In addition, in p53 knockout mice, Bcl-2 protein levels are found to be increased in some tissues such as prostate, thymus and spleen (Miyashita et al., 1994 a). However, not all tissues have increased Bcl-2 levels suggesting that this regulation may be cell type specific.

It has been suggested that the components released from the mitochondria may initiate apoptosis (Willie, 1997; Polyak et al., 1997). The p53-induced genes may cause the generation of reactive oxygen species (ROS) which are able to cause the destruction of the mitochondrial membrane. Bcl-2 is normally located on the outer surface of the mitochondria where it may regulate the flow of ions and other molecules. The Bcl-2 protein also binds to its homologue Bax, which shares 21% amino acid identity with the Bcl-2 protein (Olitvai et al., 1993). Bax is a powerful stimulator of apoptosis and its release from attachment to the mitochondria, via Bcl-2, results in its activation (Zou et al., 1997). Therefore the release of apoptosis releasing factors from within the mitochondria and the liberation of Bax from the mitochondrial membrane following the generation of ROS by PIGs may be one mechanism used for triggering cell death.

Studies on thymocytes derived from bcl-2 knockout mice showed an increased sensitivity to radiation and glucocorticoid (Nakayama et al., 1993; Veis et al., 1993). Using transfection experiments, bcl-2 has also been shown to provide protection against apoptosis induced by cisplatin (Miyashita and Reed, 1993; Minn et al., 1995; Dole et al., 1995; Simonian et al., 1997). Bcl-$_{XL}$ seems to provide better protection against cisplatin induced apoptosis than Bcl-2 as cells expressing Bcl-$_{XL}$ showed greater survival than Bcl-2 expressing cells after exposure to cisplatin (Simonian et al., 1997). Both Bcl-2 and Bcl-$_{XL}$ can work independently of p53 as it has been shown that both of them can protect against apoptosis in p53 mutated cells (Strasser et al., 1994; Boise et al., 1995). The mechanisms involved in the protection of Bcl-2 and Bcl-$_{XL}$ against cisplatin induced apoptosis seem to be by inhibition of DNA degradation and maintaining cell viability (Dole et al., 1995). However, the genes and/or biological pathways regulated by Bcl-2

and Bcl-$_{XL}$ leading to the protection of apoptosis need to be established. Increased Bcl-2 expression seems to correlate to cisplatin resistance in the majority of studies, for example, ovarian tumour patients who are resistant to cisplatin had higher levels of Bcl-2 than the relatively sensitive patients (Elipomlas *et al.*, 1995). However, in contradiction to most of the studies, a study on 71 ovarian cancer patients showed that increased Bcl-2 expression correlated to improved survival in patients (Herod *et al.*, 1996).

Another *bcl-2* family member, *bax*, was shown to accelerate cisplatin induced apoptosis and its function was independent of *bcl-$_{XL}$* (Simonia *et al.*, 1996). As sequence-specific binding sites for *p53* were found in the *bax* promoter (Miyashita and Reed, 1995), the involvement of the Bax protein in the p53-dependent apoptosis pathway has been studied. It has been found that activated wild-type p53 can induce Bax expression in a cisplatin sensitive ovarian carcinoma cell line. However, in a cisplatin resistant subline the mutant *p53* is unable to induce Bax expression and these cells failed to go through apoptosis and developed cisplatin resistance (Perego *et al.*, 1996).

The evidence from embryonic fibroblasts derived from *p53* and *bax* knockout mice (McCurrach *et al.*, 1997) showed that *p53-/-* or *bax-/-* cells were more resistant to cisplatin when compared to the wild type cells. However, the *bax-/-* cells were not as resistant to cisplatin induced apoptosis as the *p53-/-* cells. This suggests that other p53 regulated factors might also be involved in this pathway. The fact that *p53-/- bax-/-* cells showed only a slightly increased resistance to DNA damaging agents compared to the *p53-/- bax+/-* or *p53-/- bax+/+* cells suggests that Bax may function in the p53-dependent pathway.

1.3.4 DNA Repair

There is strong evidence that the cytotoxicity of cisplatin is due to its interaction with DNA (Rosenberg, 1985). Thus, the ability of cells to deal with cisplatin-induced DNA damage may determine their sensitivity. The fact that both bacterial and mammalian cells deficient in DNA repair are hypersensitive to cisplatin indicates that DNA repair may play an important role in the cytotoxicity of cisplatin (Beck, 1973; Chu, 1994).

The nucleotide excision repair (NER) pathway has a very broad specificity in recognizing DNA alterations resulting in local distortions of the DNA structure, including UV

induced pyrimidine dimers and (6-4) photoproducts, a wide range of chemical adducts and certain types of DNA crosslinks (Bootsma, 1993). When radiolabelled oligonucleotides containing cisplatin intrastrand crosslinks were incubated with DNA repair proficient HeLa cell free extract, the damage was excised, indicating the involvement of the excinuclease in the removal of cisplatin damage (Huang *et al.*, 1994). However, removal of cisplatin damage did not occur in the NER deficient XPF and G group cells lacking the excinuclease, suggesting that these cisplatin lesions are substrates for the human excision repair system.

The NER pathway consists of five steps: damage recognition, incision of the damaged strand on both sides at some distance from the lesion, excision of the injury-containing oligonucleotide, DNA synthesis using the undamaged strand as template, and ligation (Bootsma, 1993). (see Figure 1.3).

An important first step towards the cloning of the NER genes was the isolation of large numbers of UV-sensitive repair-deficient mutants from CHO (Chinese hamster ovary) cells. Complementation analysis of these mutants has revealed at least 11 complementation groups (Bootsma, 1993). Subsequent cell fusion studies showed that the repair defects in these cells could be complemented by human genes (Thompson, 1989). Transfection of human DNA has now facilitated isolation of the human genes involved. These have been designated ERCC (Excision Repair Cross Complementation) 1-6 (see Table 1.4). These human genes complement defects in nucleotide excision repair disorders. For example, ERCC2 is able to compensate for the UV-sensitivity of XP-D cells (Flejter *et al.*, 1992)

The transfection of wild type *ERCC-1* gene into an excision repair deficient CHO cell line, 43-3B (due to a mutation on ERCC-1 gene) showed the correction of its hypersensitivity to cisplatin (Larminate and Bohr, 1994). However, the ERCC-1 transfected cells did not fully regain the level of resistance compared to the wild type cells. This may be due to inefficient function of human genes in the CHO cells. However, the transfected cells showed efficient removal of cisplatin induced interstrand cross-links within 24 hours. But they were only able to remove a third of the intrastrand cross links at the same time point. This evidence supports the possibility that a deficiency in repairing intrastrand crosslinks may result in the hypersensitivity of 43-3B cells to cisplatin.

The major proteins include XPA, which is a zinc finger protein and involved in the recognition of DNA damage. XPB and XPD are helicases which unwind the DNA to facilitate incision on both sides of the lesion. The damaged strand is then excised on the 3' side by XPG nuclease and the 5' side by the ERCC-1-XPF nuclease complex (Griffin, 1996).

The consequences of a deficiency in excision repair are apparent from the clinical symptoms of the human repair disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS), trichothiodystrophy (TTD), Fanconi's anemia (FA), Bloom's syndrome and ataxia telangiectasia (AT). Patients suffering from these rare autosomal recessive diseases are hypersensitive to at least one type of DNA damaging agent and have genetic instability (Hoeijmakers and Bootsma, 1992) (see Table 1.5). Each of these rare disorders is thought to reflect a defect in a different repair pathway. For example, most of the XP patients carry a deficiency in the NER process and the majority of TDD cells are defective in the XPD gene, whose product is a helicase (a subunit of TFIIH) and involved in loading RNA polymerase II onto promoters. Although the NER process is normal in FA and CS patients, a pathway dealing with certain types of interstrand crosslinks in FA is impaired, and CS cells are deficient in repairing DNA damage in the transcribed regions of DNA. The gene responsible for AT has been cloned and encodes a protein with a carboxy-terminal domain similar to the catalytic domain of the family of

reviewed in MEYN, 1995

lipid kinases (Hoeijimakers and Bootsma, 1992; Lehmann, 1995). (see Table 1.5).
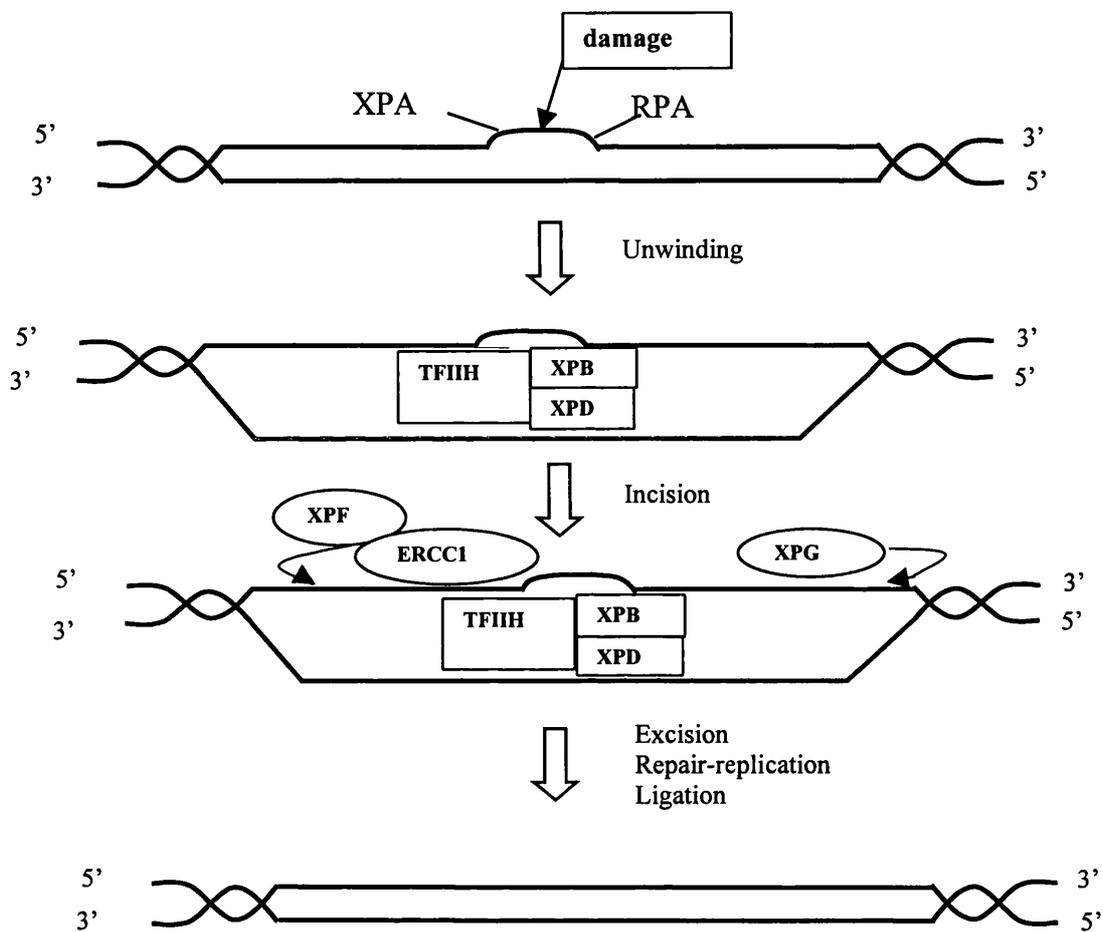
Figure 1.3. Nucleotide excision repair pathway in mammalian cells (adapted from Griffin, 1996).

Table 1.4 Identified genes required for human excision repair (based on Sancar, 1996).

Fraction	Proteins	Sequence motif	Role in repair
Excision nuclease subunits			
XPA	XPA	Zinc finger	Damage recognition
RPA	P70 P34 P11		Damage recognition
TFIIH	XPB/ERCC3 XPD/ERCC2 P62 P44 Cdk7/41 CycH/p38 P34	Helicase Helicase Zinc finger S/T kinase Cyclin Zinc finger	a) Formation of preincision complex b) Transcription-repair coupling
XPC	XPC/p125 HHR23B/p58	Ubiquitin	a) Stabilisation of preincision complex b) Protection of preincision complex from degradation
XPF	XPF/ERCC4 ERCC1/p33		5'-Incision
XPG	XPG/ERCC5/p135		3'-Incision
Repair synthesis and ligation			
RFC			
PCNA		ATPase	Molecular match-maker
RPA		Polymerase	Polymerase clamp
Polδ	P258 P55		Repair synthesis
Ligase	P102		Ligation

Table 1.5 The main human DNA repair disorders (Based on Hoeijmakers and Bootsma, 1992).

Disease	Tumour predisposition	Primary sensitivity	Defective process	Number of complementation groups
XP	Skin cancer	UV	NER/PRR*	8
CS		UV	Defective in preferential strand-selective repair	2
FA	Leukemia	Crosslinks	Crosslink repair?	4
AT	Lymphoma	X-ray	X-ray response	1
*BS	Wide variety	Many agents	DNA ligase	1

*BS: Bloom's syndrome. PRR: post replication repair.

Xeroderma Pigmentosum (XP)

Complementation studies were performed by fusing cells from different XP patients and comparing the levels of unscheduled DNA synthesis (UDS) in heterodikaryons and unfused monokaryons (Weerd-Kastelein *et al.,* 1972). It was observed that cells from different XP patients could complement one another, restoring levels of UDS to normal. The complementation analysis led to the identification of seven complementation groups, named XPA-G (Weerd-Kastelein *et al.,* 1972; Giannelly *et al.,* 1992). In addition, there is a group of patients with classic symptoms of XP but with normal excision repair however they are defective in postreplication repair. They are called XP variants (XPV) (Boyer *et al.,* 1990).

The repair capacity of XP cells following treatment with a variety of DNA damaging agents such as mitomycin C, cisplatin and nitrogen mustard is also defective (Friedberg, 1991). These DNA damaging agents generally produce bulky adducts which cause significant DNA helix distortion. The fact that XP cells showed hypersensitivity to both UV and DNA damaging agents suggests that a defect in a step common in repairing both types of damage may result in their hypersensitivity.

Cell lines from XP group A and G patients are also highly sensitive to cisplatin and were able to remove the interstrand crosslinks induced by cisplatin suggesting that this hypersensitivity may be related to the lack of repair of intrastrand crosslinks in these cells (Chu, 1994). Wild-type XPA is involved in the recognition of several types of DNA damage including that by cisplatin (Asahina *et al.,* 1994). When [35]P labelled double-stranded DNA was treated with UV-irradiation and cisplatin, and incubated with XPA

protein, it was found that XPA not only bound to the UV-irradiated DNA but also to cisplatin-induced damage with a high affinity. However, a mutant XPA protein, in which a cysteine residue was replaced by a serine, lost its binding activity to cisplatin. This indicates that as with UV damage, cisplatin induced damage may also be recognized by the XPA protein, and a defect in this recognition step may result in the sensitivity of the XPA cells to cisplatin (Jones and Wood, 1993; Asahina *et al.*, 1994).

A murine L1210/0 cell line showed deficiencies in nucleotide excision repair due to a defect on the *XPG* gene and sensitivity to cisplatin. Purified XPG protein, but not other repair proteins could restore its repair ability and increase resistance. This suggests that the sensitivity of these cells to cisplatin may be due to a deficiency of XPG protein. Expression of *XPG* mRNA was similar in L1210/0 cells and its repair proficient derivatives indicating that the defect in the XPG protein function may be due to a point mutation on the gene, which may alter the expression, processing or protein function, rather than the absence of the gene (Vilpo *et al.*, 1995).

There is evidence that p53 is involved in the regulation of repair proteins in the NER pathway. p53 binds to RPA, which forms a complex with XPA and plays a role in recognition of the damage, and TFIIH. The binding of p53 to RPA may convert RPA from a 'replication' form to a 'repair' form. By binding to TFIIH, p53 inhibits its helicase activity, resulting in the formation of a stable helicase-damaged DNA complex, which could stimulate the formation and activity of repair complex. The inhibition of the helicase activity may facilitate the initiation of NER, leading to more rapid and effective repair (Sancar, 1996).

Fanconi's Anemia (FA)

The four known mutations (FA-D) were identified among 7 patients from North America, based on the correction of hypersensitivity to DNA damaging crosslinks such mitomycin C in hybrids prepared from EBV immortalized lymphoblastoid cell lines (Duckworth-Rysiecki *et al.*, 1985; Strathdee *et al.*, 1992). A fifth group was also identified after an analysis of a group of European patients (Joenie *et al.*, 1995).

FA cells are not sensitive to UV (Poll *et al.*, 1985) and are proficient in excision of UV-induced pyrimidine dimers (Klocker *et al.*, 1985). However, FA cells have been shown to be hypersensitive to cisplatin (Ishida and Buchwald, 1982; Poll *et al.*, 1982 and 1984;

Pooly *et al.*, 1985). A different repair defect was also identified in FA cells (Poll *et al.*, 1984). Unlike XP cells, FA cells are unable to repair interstrand crosslinks such as those induced by mitomycin C (MMC) (Sasaki, 1975). Since cisplatin also generates interstrand crosslinks like MMC, the abnormal persistence of interstrand crosslinks in these cells suggests that the hypersensitivity of FA cells to cisplatin is due to the inability to repair this minor adduct rather than the intrastrand crosslinks (Plooy *et al.*, 1985; Averbeck *et al.*, 1988; Zamble and Lippard, 1995).

A study on gene specific repair of cisplatin induced DNA damage on both FA group A (FAA) and XPA cells (Zhen *et al.*, 1993) showed that while normal human fibroblast cells repair 84% of the interstrand crosslinks in the DHFR gene (dihydrofolate reductase), the FAA and XPA cells only repaired 32 and 50% of the same type of damage. A similar pattern was also observed in the repair ability of the cisplatin-induced intrastrand crosslinks. This suggests that the FAA cells are not only deficient in the gene specific repair of cisplatin interstrand crosslinks, but also in the gene specific repair of the more common cisplatin intrastrand crosslinks.

So far, one of the genes, *FAC* has been identified using functional cloning by transfection of a cDNA library into FAC cells (Strathdee *et al.*, 1992). This gene has been located on chromosome 9q22.3 (Gibson *et al.*, 1994). The *FAC* gene encodes a protein of 60kD and is localized in the cytoplasm but its function is unknown (Yamashi *et al.*, 1994). Its localization suggests that it is unlikely to have a direct role in DNA repair. Recently, a *p53* binding site was found to be in the coding region and the promoter region of the *FAC* gene (Liebetrau *et al.*, 1997; Savoia *et al.*, 1995), which can be activated by the wild-type *p53*. This suggests that the function of FAC may be involved in p53 transcriptional activation pathway (Liebetrau *et al.*, 1997).

Ataxia Telangiectasia (AT)

The hypersensitivity of cells from AT patients to ionizing radiation suggests that it may be due to a defect in a DNA-damage response. However, these cells have sensitivity to UV similar to that of normal fibroblasts. The characteristic of the AT phenotype is the failure of ionizing radiation to block DNA synthesis, which indicates a defect in controlling mechanism that stops replication when the DNA lesions are induced (Zakian *et al.*, 1995). The range of DNA lesions that might trigger a response leading to cell death

is not certain, although strand breaks and gaps containing the modified 3' termini are likely to play a major role (Lavin *et al.,* 1995). The fact that the ATM gene product is a member of a novel family of large proteins with PI 3-kinase activity implicated in the regulation of the cell cycle and response to DNA damage indicates that the major function of the ATM protein may be in signal transduction (Meyn, 1995). Recently the relationship between ATM and a nuclear tyrosine kinase c-Abl was observed (Baskaran *et al.,* 1997; Shafman *et al.,* 1997). The c-Abl has been shown to be activated by DNA damaging agents including ionizing radiation and is necessary for radiation-induced G_1 arrest (Kharbanda *et al.,* 1995). However, in AT cells and the thymocytes or fibroblasts from the ATM-knockout mice, the c-Abl was not activated in response to ionizing radiation. The defect could be corrected by expression of the ATM protein. This suggests that ATM is involved in the activation of c-Abl and the ATM-c-Abl interaction may be responsible for the induction of ionizing radiation-induced G1 arrest (Baskaran *et al.,* 1997; Shafman *et al.,* 1997).

X-Ray Sensitive Mutants (XRS)

A number of ionizing radiation sensitive rodent mutants have also been used to study the mechanisms resulting in their sensitivity. Cell fusion studies have defined four complementation groups (see Table 1.6). Defects in the repair of DNA double-strand breaks (DSB) have been found to be the common phenotype in these mutant cell lines. They are also defective in V(D)J recombination (Variable Diversity and Joining (Jackson and Jeggo, 1995), a process in which antigen receptor molecules are rearranged (Alberts *et al.,* 1994). The V(D)J recombination is initiated by the generation of blunt DNA DSBs between recombination signal sequences and coding gene sequences (Lewis, 1994).

Table 1.6 Mammalian ionizing radiation (IR) complementation groups (based on Jackson and Jeggo, 1995).

Complementation Group	Mutant Cell Lines	Ku DNA-Binding Activity	DNA-PK Activity	Chromosomal Locus	Complementing Gene/product
IR4	XR-1	Positive	Positive	5q	XRCC4
IR5	Xrs-1 to xrs6; XR-V15B, XR-V98. Sxi-3	Negative	Positive	2q33-35	XRCC-5/Ku80
IR6	Sxi-1	Negative	?	?	XRCC6
IR7	V3; Scid	Positive	Negative	8q11	XRCC7/DNA-PK

Micro-cell fusion has been used to identify the segment of human chromosomes that complement the ionizing radiation sensitivity of these cells. Both ionizing radiation sensitivity and the ability to rejoin radiation–induced DNA DSBs of IR4, 5 and 7 cells are complemented by human chromosome 5q, region q33-35 of chromosome 2 and region of q11 of chromosome 8 respectively (Jackson and Jeggo, 1995). So far the defective components in IR5 and IR7 groups have been discovered. The XRCC5 complementing gene has been identified as the 80kDa protein Ku80. This is a DNA-end binding protein that complements the sensitivity of *xrs* cells of the IR5 group by restoring their ability to repair DSBs by non-homologous end-joining mechanisms. However, repair by homologous recombination events is not affected by transfection of the gene encoding the Ku80 protein. Furthermore the XRCC7 complementation gene has been identified as the catalytic subunit of the DNA-protein kinase which binds at DSBs, in a complex with Ku80 and Ku70 (for a review see Jackson and Jeggo, 1995).

One of the *xrs* mutants, *xrs-5* has been shown to be sensitive to cisplatin compared to the parental cells (Jaffe *et al.*, 1991; Haraf *et al.*, 1991). However, when the *xrs-5* cells were transfected with a cosmid library, the ionizing radiation resistant transfectants were isolated but they failed to show resistance to cisplatin. The deficiency in DNA DBS rejoining was also complemented in the transfectants. This suggests that the DBS rejoining process might not be important in repairing cisplatin damage or the break region following cisplatin exposure might be processed by a different mechanism.

1.3.5   Other Factors

HMG Proteins

Other cellular factors have also been shown to be involved in processing cisplatin-induced damage. A family of proteins containing high mobility groups (HMG) have been shown to bind to the structural distortions caused by cisplatin crosslinks in human cell free extracts (Donahue *et al.*, 1990; Toney *et al.*, 1989; Chao *et al.*, 1991). This binding effect may make certain types of DNA damage less favorable for repair by human NER by direct shielding or by the HMG-induced changes (Chow *et al.*, 1994; Zamble and Lippard, 1995). When one of the HMG proteins, HMG1 was added to the excision repair assay containing radiolabelled DNA carrying cisplatin-adducts and Hela cell free extract, the amount of excised products decreased with increased HMG1 concentrations (Zamble *et al.*, 1996). It is possible that the inhibition of the excision of cisplatin adducts may result in the failure to repair the damage and the presence of unrepaired damage may in turn result in the generation of signals leading to cell death (Zamble *et al.*, 1996).

Mismatch Repair

Although cells are protected from cisplatin damage mainly through the NER pathway, proteins such as hMSH2 and hMLH1, which are involved in recognition of mispaired bases in mismatch repair pathways, have been shown to recognize and bind to cisplatin-DNA adducts specifically (Mello *et al.*, 1996; Zamble *et al.*, 1996; Yamada *et al.*, 1997). This suggests that the mismatch repair pathway may also play a role in cisplatin sensitivity. A hMLH1 deficient human colon cancer cell line, HCT116, due to a deletion on one allele and a mutation of the other, was more resistant to cisplatin than its subline in which chromosome 3 containing the wild type *hMLH1* had been transferred (Fink *et al.*, 1996). Increased resistance to cisplatin was also observed in a hMSH2 defective line compared to the subline containing wild type *hMLH1*. Using a gel mobility shift assay with radiolabelled DNA probes containing cisplatin-DNA adducts and hMSH2 and hMLH1 antibodies, the formation of protein-DNA damage complexes containing hMSH2 and hMLH1 were observed when cell free extracts from HeLa cells were incubated with cisplatin-DNA adducts. The recognition of hMSH2 and hMLH1 proteins bound to cisplatin-DNA adducts seems to be specific as these proteins failed to recognise DNA adducts formed with the cisplatin analogue oxaliplatin. Using a similar approach, Mello and coworkers (1997) confirmed earlier findings and demonstrated that hMSH2 was able

to recognize a 100bp probe containing a single site 1,2-d(GpG) cisplatin adduct with high affinity.

A study on embryonic fibroblasts derived from mice deficient (MSH2 -/-), heterozygous (MSH2+/-) and proficient (MSH2+/+) suggests that the mismatch repair pathway may play at least a partial role in the sensitivity to DNA-damaging agents including cisplatin (Reitmair *et al.*, 1997). In this study, the spontaneous mutation rate was found to be lower in MSH2 proficient cells compared to MSH2-/- cells. The MSH2-/- cells also showed increased resistance to cisplatin and UV than the MSH2 proficient cells, however there was no significant difference in sensitivity when compared to MSH2+/- cells. In addition, there was no significant change in sensitivity to ionizing radiation in these three cell populations. This indicates that MSH might recognise crosslinking damage and contribute to promoting cell death.

Mitochondrial DNA Damage

Other factors have been shown to influence cisplatin sensitivity. For example, cisplatin has been shown to bind to mitochondrial DNA more efficiently than to the nuclear DNA in CHO cells (Murata *et al.*, 1995). When mitochondrial and chromosomal DNA was isolated from cisplatin treated human malignant melanoma cells, it was shown by atomic absorption that 6 hours after cisplatin treatment the binding of cisplatin to mitochondria DNA was approximately 50 times greater than the binding to chromosomal DNA. This binding of mitochondrial DNA resulted in the reduction of NADH-ubiquinone reductase activity, which in turn disturbs the generation of ATP. Cisplatin also causes morphological changes in mitochondria and alters their structure and function (Rosen *et al.*, 1992). In addition, cisplatin-DNA adducts are not removed as efficiently from mitochondrial DNA as from nuclear DNA (Oliver *et al.*, 1997). This suggests that cisplatin damage to mitochondria might play a role in its cytotoxicity. However, to date, there is no evidence correlating the amount of cisplatin-induced mitochondrial DNA damage to cisplatin sensitivity in human tumour cells.

Stress Response Proteins

In response to extracellular stress, the cells can synthesise and accumulate a group of proteins, known as heat shock proteins (Hsp). The small heat shock protein, Hsp27, a cytosolic protein involved in cytoskeletal organization, is the most studied Hsp in relation to response to chemotherapy (Hettinga *et al.*, 1996). Constitutively low levels of Hsp27

have been found in testicular tumour cells and transfection of Hsp27 resulted in increased cisplatin resistance (Richards *et al.*, 1996b). It has also been shown that expression of Hsp27 increases resistance to drug induced apoptosis in promonocyte (U937) and fibrosarcoma (Wehi-s) cell lines (Samali and Cotter, 1996).

1.4 Molecular Basis of Cisplatin Sensitivity in Testicular Germ Cell Tumours

While the majority of solid tumours treated with cisplatin either do not respond or develop resistance, testicular germ cell tumours have become a model for a cancer curable with drugs. Understanding the molecular basis of the hypersensitivity of testicular tumours to DNA damaging agents such as cisplatin might provide clues for the treatment of other advanced cancers.

Many continuous cell lines derived from human testicular germ cell tumours have been characterised and studied. These cell lines show hypersensitivity to DNA-damaging agents such as cisplatin (Oosterhuis *et al.*, 1984) when compared to relatively resistant tumour cell lines such as bladder tumour cell lines (Walker *et al.*, 1987; Masters *et al.*, 1993). This suggests that the sensitivity of testicular tumour cell lines *in vitro* reflects that of testicular tumours in the clinic. These cell lines can be used to study the mechanisms involved in the hypersensitivity of testicular tumours. Mechanisms involved in cisplatin sensitivity have been studied in cell lines derived from testicular tumours (Hogan *et al.*, 1977; Bronson *et al.*, 1980; Vogelzang *et al.*, 1983; Pera *et al.*, 1987), or acquired cisplatin resistant cell lines generated by long term exposure to cisplatin (Walker *et al.*, 1990; Kelland *et al.*, 1992; Klys *et al.*, 1992; Timmer-Bosscha *et al.*, 1993). Some possible mechanisms have been suggested which might contribute to the hypersensitivity of testicular tumors to cisplatin, as discussed in the following sections.

1.4.1 Drug Accumulation

Studies comparing testicular tumour lines with their acquired cisplatin resistant sublines (Sark *et al.*, 1995; Timmer-Bosscha *et al.*, 1993; Kelland *et al.*, 1992) or with relatively cisplatin resistant tumour cell lines such as bladder tumour cell lines (Koberle *et al.*, 1997) did not show any correlation between drug accumulation and cisplatin sensitivity. The initial DNA platination in testicular tumour cells was lower than in the relatively cisplatin resistant bladder tumour cells, as measured by atomic absorption directly after cisplatin treatment (Koberle *et al.*, 1997) despite their greater sensitivity. This suggests that initial DNA damage by cisplatin may not play a role in the inherent sensitivity of testicular tumour cells.

1.4.2 Detoxification System

The detoxification system can influence the response to cisplatin as mentioned in section 1.3-2. It has been suggested that GSH can protect against germ cell mutagenesis and that this protection is dependent on enzymatic conjugation by GST (Teaf *et al.*, 1985). High levels of GST have been found in both normal human testis and testicular germ cell tumour cells (Klys *et al.*, 1992). However, GSH levels in three testicular tumour cell lines (Tera-2, 833KE and SuSa) were lower than in the three cisplatin resistant colon cancer cell lines (Sark *et al.*, 1995). GSH levels were also found to be relatively low in 5 testicular tumour cell lines compared to the bladder tumour cells (Masters *et al.*, 1996). There was also no significant difference in GST levels between testicular tumour cells and the relatively resistant bladder cells.

The role of MT in the cisplatin sensitivity of testicular tumours has not been established. A recent study of 5 testicular tumour cell lines showed that all the cell lines studied had constitutively higher levels of MT than the relative resistant bladder cancer cell lines (Masters *et al.*, 1996). These results were the opposite of what one would expect but they agreed with a previous study (Sark *et al.*, 1995) on 3 other testicular tumour cell lines.

This evidence indicates that the sensitivity of testicular tumour cells cannot be explained by the detoxification system.

1.4.3 DNA repair

Testicular tumour cells showed a sensitivity to cisplatin similar to that of cells derived from the DNA repair disorders FA and XP, suggesting that DNA repair may also play a role in the sensitivity of testicular tumour cells (Pera *et al.*, 1987).

A study on two testicular tumour cell lines, SuSa and 833K, and a cisplatin resistant bladder cancer cell line showed that both types of cell lines had similar amounts of DNA damage (Bedford *et al.*, 1988) immediately following exposure to IC_{50} (drug concentration required to inhibit survival by 50%) concentrations of cisplatin, measured by ELISA assays using antibodies raised against cisplatin-DNA adducts. This suggests that the initial DNA damage caused by cisplatin is not a crucial factor in the sensitivity of these cells to cisplatin. When the cells were given a 18-hour incubation after initial

cisplatin treatment, the cisplatin resistant bladder cancer cell line showed a 50-70% reduction of DNA-cisplatin adducts while there was no change in the amount of DNA-cisplatin adducts in the testicular tumour cell line SuSa. This indicates that the bladder cancer cells were able to repair DNA damaged by cisplatin, and that this in turn might lead to cell survival after cisplatin treatment resulting in their resistance. In contrast, the testicular tumour cell line SuSa was not able to repair the damage caused by cisplatin and this may have resulted in their sensitivity to cisplatin. However, despite having similar sensitivity to SuSa, one of the testicular cell lines, 833K, showed more efficient repair than SuSa, but the efficiency was lower than that of the bladder tumour cell lines.

A similar study was performed on 4 other testicular tumour cell lines, GCT27, GCT46, H32 and H12.1 (Hill et al., 1994), which showed similar levels of platination of DNA immediately after cisplatin treatment. Following an 18 hour incubation in the absence of cisplatin, one of the cell lines showed approximately 8% removal of total platinated DNA, while the total platination of DNA in 3 of the cell lines was higher than that detected immediately after the drug treatment. This suggests that these cell lines were totally deficient in removal of platinated DNA and additional lesions were formed but not removed during that period. When the mRNA levels of the DNA excision repair genes ERCC-1 and XPBC/ERCC3 were measured by Northern blotting analysis, there was no significant difference observed in any of the cell lines.

DNA repair rates are not uniform throughout the genome. Two pathways exist, one is the removal of lesions from the transcribed strand of active genes, such as essential genes involved in cell signalling pathways. This process is fast and efficient. The other one is slower and involved in the repair of the rest of the genome (Bohr et al., 1991). In DNA repair disorders such xeroderma pigmentosum (XP), defects have been found in one or both pathways. The Cockayne syndrome (CS) is defective in repair of damage on transcribed genes (Hoeijimakers and Bootsma, 1992). To investigate if the repair of cisplatin-induced damage on active and inactive genes contributed to the sensitivity of the testicular tumour cells to cisplatin, three testicular tumour cell lines were studied. Three relatively cisplatin resistant bladder tumour cell lines were also used as comparison. Using quantitative-PCR, part of the actively transcribed N-ras and inactively transcribed CD3δ genes were amplified in cisplatin treated and untreated cells (Koberle et al., 1996; 1997). Due to the presence of cisplatin-induced lesions on the amplified regions, the PCR reaction is inhibited, resulting in decreased production of PCR product. Using

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radiolabelled nucleotide in the PCR reaction, the PCR product can be quantified by measuring the levels of incorporation of radiolabelled nucleotide in the PCR product. By comparison to the untreated controls, the number of lesions per amplified fragment can also be estimated. This study showed that there was no significant difference in the levels of initial cisplatin damage between testicular tumour cell lines and bladder lines immediately after cisplatin treatment, which agrees with previous studies (Bedford et al., 1988; Hill et al., 1994). There was also no significant difference in initial frequency of lesions in the N-ras gene fragment when compared to the CD3δ gene fragment in all cell lines. Three bladder tumour cell lines removed cisplatin damage efficiently 24 hours after cisplatin treatment indicated by the reduction in the number of lesions compared to the lesions immediately after cisplatin treatment. In contrast, two testicular tumour lines, 833K and GCT27 showed no reduction of lesions on both gene fragments but increased damage was observed 24 hours after treatment with cisplatin, suggesting their inefficiency in the repair of cisplatin damage. However, the other testicular tumour line, SuSa was able to remove cisplatin damage from both gene fragments. However the repair capacity was lower than that of bladder tumour cell lines. This indicates that two of the testicular tumour cell lines are not only deficient in repair of cisplatin damage on the whole genome as previously reported (Bedford et al., 1988; Hill et al., 1994) but also at the specific gene level. However, there was no difference in repairing cisplatin-induced damage on the transcribed and non-transcribed genes.

The mechanism responsible for increased DNA damage after the removal of cisplatin in the testicular tumour cell lines needs further investigation. The results from one of the testicular tumour cell lines, SuSa, did not agree with 833K and GCT27 lines. This may suggest that even though DNA repair deficiency might contribute to the sensitivity of testicular tumour cells to cisplatin, other mechanisms may also be involved. The same study was also performed on the acquired cisplatin resistant testicular tumour cell lines, GCT27CisR and SuSaCP, and the evidence suggests that the resistance of these cell lines to cisplatin was not due to the increased DNA repair ability on CDδ and N-ras genes (Koberle et al., 1996).

The majority of cisplatin damage has been shown to be repaired by the nucleotide excision repair pathway, as mentioned in section 1.3.4. There is evidence that the cause of the repair deficiency might be due to the low constitutive levels of nucleotide excision repair proteins in testicular tumour cells, although the previous DNA repair studies have

their limitations. In order to measure DNA damage, the cells were treated with cisplatin and given a period of time for the repair to occur. During this period it is possible that cells were dying rather than repairing DNA. This could result in an underestimate of the repair ability of the cells. In order to circumvent this problem, *in vitro* assays were used to study the repair using cell-free extracts. When the cell free extracts were incubated with either a plasmid containing a single platinum intrastrand crosslink or a UV-treated plasmid, little damage-dependent repair synthesis was observed (Koberle *et al.*, 1997). This suggests that testicular cells may be deficient in their constitutive NER capacity.

The initial steps of the NER process were examined using an assay which detects the excision products resulting from dual incisions on either side of a single platinum lesion in a plasmid. The cell-free extracts of testicular cells gave a weak signal of excision products compared to DNA repair proficient cells, such as Hela and bladder cells (B. Koberle, personal communication). This suggests that the low constitutive levels of NER repair may be due to a deficiency in the initial steps of the repair process. Furthermore, constitutive levels of the XPA, ERCC1 and XPF proteins were found to be lower in testicular cells. When XPA alone or ERCC1 and XPF proteins were added to testicular cell free extracts they were able to restore repair capacity, confirming that a deficiency in these proteins is at least partially responsible for the reduced repair ability of testicular cells.

1.4.4 Apoptosis Pathway

While DNA repair seems to be one of the mechanisms involved in the sensitivity of testicular tumour cells to cisplatin, these tumour cells have also been associated with an increased susceptibility to undergo apoptosis (Huddart *et al.,* 1994; Chresta *et al.,* 1996). Unlike the majority of cancers, *p53* gene mutations are very rare in testicular cancers, as mentioned in section 1.1.4. This suggests that there might be a potential mechanism for their hypersensitivity, which involves p53-induced apoptosis. Constitutively high levels of the wild type p53 protein were found in human testicular tumours (Bartkova *et al.,* 1991; Schenkman *et al.,* 1995) and both human and mouse testicular tumour cell lines (Lutzker and Levine, 1996; Chresta *et al.,* 1996) by immunohistochemistry and Western blotting. This protein was suggested to be inactive, as there was a lack of induction of p53-regulated genes such as mdm-2 and *p21*. Another apoptosis suppressor gene, *bcl-2*, was almost undetectable in three testicular tumour cell lines, GH, GCT27 and 833K, but

one of its family members, the apoptosis accelerator, Bax, was highly expressed in these cell lines compared to the relatively cisplatin resistant bladder tumour cell lines (Chresta *et al.*, 1996). It is possible that the ratio of theses two proteins may contribute to the sensitivity of testicular tumour cells to DNA damaging agents.

When three testicular tumour cell lines, 833K, GH and GCT27 were treated with IC_{90} concentration of etoposide for 4 hours, apoptosis, but not G1 arrest, occurred in all cell lines characterised by the appearance of a low molecular weight DNA ladder (Chresta *et al.*, 1996). Despite high basal levels of p53 present in the untreated cells, p53 was increased 3-6 fold by etoposide at the same time point, as detected by Western blotting. The increased p53 expression was accompanied by the up-regulation of the p53-regulated gene *p21*. This suggests that p53 was transcriptionally activated by etoposide and this activation may play a role in etoposide induced apoptosis in testicular tumour cells.

Similar observations were made when a mouse embryonal carcinoma cell line, F9, was studied (Lutzker and Levine, 1996). This cell line showed 40-fold overexpression of p53 protein compared to that found in fibroblasts, as determined by Western blotting analysis. After 2 hours of exposure to etoposide, p53 protein expression was increased by 17.5-fold and remained elevated over a 12 hour period. The mRNA levels of p53-regulated genes such as *mdm-2* and *p21*, were also induced at the same time point. This again indicates that p53 was transcriptionally activated by etoposide as early as 2 hours after exposure. However, apoptosis occurred only after 24 hours following an 8-hour exposure to an IC_{99} concentration of cisplatin in F9 cells (Lutzker and Levine, 1996). When teratocarcinoma cell lines from p53 knockout mice were also treated with same concentration of etoposide, they failed to undergo apoptosis. This suggests that the apoptosis induced by etoposide in these cells might be p53-dependent. However, the cell survival in this study was measured by counting the percentage of floating cells after etoposide treatment, which may not be a sufficient approach for measuring apoptotic cells.

In a separate study, programmed cell death was also observed in three human testicular tumour cell lines treated with cisplatin and a range of chemotherapeutic drugs that act by different mechanisms. Drug induced apoptosis occurred at 9 hours after exposure to the drugs and by 24 hours, over 90% cells were dead through apoptosis characterised by the appearance of a low molecular weight DNA ladder and loss of DNA content in the apoptotic cells (Huddart *et al.*, 1995).

Recently, a study on four testicular tumour cell lines (N2, 2102EP, S2 and NCCIT) showed lack of correlation between cisplatin-induced apoptosis, p53 status and expression of Bcl-2 family proteins (Burger *et al.,* 1997). Based on IC_{50} and IC_{90} values, these cell lines showed differential sensitivity to cisplatin, which was not associated with differences in cisplatin accumulation, the levels of cisplatin-DNA adducts or repair. The induction of apoptosis was observed 48-72 hours after cisplatin treatment in three cell lines, but not in the relatively resistant line 2102EP (4-fold more resistant). When the *p53* status was studied, there was no correlation between the expression of p53 and cisplatin sensitivity as one of the sensitive lines has mutated p53 while the relatively resistant line 2102EP had wild type p53. The endogenous protein levels of the Bcl-2 family members Bcl-2, Bax, Bcl-x and Bax were not associated with the differential susceptibility to cisplatin-induced apoptosis. This study is in contradiction to previous studies on the mechanisms of cisplatin-induced apoptosis.

As mentioned in section 1.3.5, it is possible that the recognition of DNA damage by DNA mismatch repair pathways may also play a role in cisplatin-induced apoptosis (Mello *et al.,* 1996). Testis tissue has elevated levels of both *hMSH2* mRNA and protein compared to other tissues (Fink *et al.,* 1996). It is possible that this overexpression of hMSH2 in testis might help to explain their mechanism of cisplatin sensitivity. When a testis specific HMG domain protein was studied for its ability to bind to cisplatin adducts by gel mobility assay, it also showed binding and inhibition of the excision of cisplatin-adducts (Mello *et al.,* 1997). This indicates that the presence of elevated DNA damage recognition proteins in the testis may also be important in their sensitivity to DNA damaging agents. However, currently no evidence is available on the status of these proteins in testicular tumour cells.

1.4.5   Heat Shock Proteins

In response to heat shock and stress, a group of heat shock proteins (HSP) can be induced. Both constitutive and induced HSPs can provide protection against cytotoxic effects. To investigate whether the sensitivity of the testicular tumour cells to DNA damaging agents was associated with the differences in their constitutive or induced expression of HSPs, studies were performed on 3 testicular tumour cell lines. Three

bladder tumour cell lines were also used to provide a comparison (Richards *et al.*, 1995, 1996a and b). Testicular tumour cells showed increased sensitivity to heat compared to bladder tumour cells. There were lower constitutive levels of HSP27 in testicular tumour cells measured by Western blotting analysis. Transfection of a plasmid containing the full length *Hsp27* gene into 833K cells resulted in the selection of clones which overexpressed Hsp27 by 3.7-38.3 fold, and this overexpression conferred 2-3 fold increased resistance to heat, doxorubicin and cisplatin in these cells (Richards *et al.*, 1996b). This suggests that constitutively low levels of HSP27 may contribute to the hypersensitivity of the testicular tumour cells.

1.4.6   Role of Telomerase

Telomerase is an enzyme which functions by adding guanosine-rich tandem repeat sequences TTAGGG to the ends of chromosomes in order to maintain chromosome stability (Zakian, 1995). The telomerase carries an RNA component containing a TTAGGG sequence which is used as a template for DNA synthesis. Testicular tumour cells have been shown to have high levels of telomerase activity when compared to other tumour cell lines and cisplatin can inhibit telomerase activity specifically in testicular tumours (Burger *et al.*, 1997). It was demonstrated that the telomerase activity was inhibited in testicular tumour cells by IC_{50} concentrations of cisplatin 4 hours after the drug treatment but not by other drugs such as melphalan, bleomycin, and transplatin. The RNA component expression of the telomerase was also shown to be inhibited by cisplatin detected by RT-PCR. This suggests that the inhibition of telomerase activity might be due to the inhibition of the RNA component carried by the telomerase. This may be caused by the presence of cisplatin-DNA adducts on the guanine rich tandem repeat template, which might be essential for expression or maintenance of telomerase activity. This inhibition of telomerase results in its inability to maintain the chromosome stability, leading to cell death. The fact that testicular tumour cells have been shown to have high levels of telomerase that were inhibited by cisplatin (Burger *et al.*, 1997) suggests that telomerase might play a part in the hypersensitivity of testicular tumours to cisplatin. However, this evidence cannot explain the hypersensitivity of testicular tumours to other DNA damaging agents such as bleomycin and etoposide.

1.4.7   Other Factors

Other mechanisms that have also been studied in relation to the sensitivity of testicular tumours to cisplatin include mutation frequency, as a high rate of spontaneous mutation frequency has been linked to drug resistance in bacteria (Parris *et al.*, 1990), and O^6-alkyguanine-DNA-alkyltransferase (ATase) activity, because high levels of this enzyme have been shown to cause resistance to DNA-damaging agents (Walker *et al.*, 1992). However, they both failed to provide evidence to explain the hypersensitivity of testicular tumour cells to DNA damaging agents such as cisplatin. Recently high levels of the enzyme lactate dehydrogenase (LDH) have been found in 2 testicular tumour cell lines using Western blotting analysis, and the decreased expression of LDH was found in their acquired resistant sublines (Graaf *et al.*, 1997). However, the mechanisms involved were not studied.

1.5   THE OBJECTIVES OF THE PRESENT STUDY

Over 80% of metastatic testicular germ cell tumours are cured using cisplatin-based combination chemotherapy. Testicular tumour cells are hypersensitive to DNA damaging agents at similar levels to XP and FA cells (Pera *et al.*, 1987). They also show a deficiency in repairing cisplatin-induced DNA damage on the overall genome and within specific genes. In addition there is strong evidence that testicular tumour cells have constitutively low levels of NER capacity and a deficiency in the initial steps of the repair process.

In previous studies, I demonstrated that the hypersensitivity of the testicular tumour cell lines to cisplatin was complemented by another sensitive line D98 (Wang *et al.*, 1996). This indicates that the hypersensitivity has a genetic basis and the functional complementation approach used for the DNA repair disorders could be used to study the sensitivity of testicular tumour cells. In addition, it was observed that cisplatin resistance was expressed in a partially dominant manner, indicating that genes conferring cisplatin resistance might be identified by expression cloning in cisplatin-sensitive cells (Wang *et al.*, 1996).

Following the rationale in the studies on xeroderma pigmentosum and Fanconi's anemia, the first part of the present study was designed to use testicular tumour cells as recipient

cells to identify genes which could complement the hypersensitivity of testicular tumour cells to cisplatin. The second part of this study was to investigate gene regulation in response to cisplatin using differential display. The following strategies were used in this study:

1. Complementation analysis of testicular tumour cells to investigate the number of genes involved in the hypersensitivity of testicular tumour cells to cisplatin.

2. Attempt to clone genes conferring cisplatin resistance to testicular tumour cells by functional cloning after transfection of a cDNA library.

3. Characterization of a yeast genomic DNA insert from a cisplatin resistant secondary transfectant.

4. Attempt to identify human chromosomes conferring cisplatin resistance in the mouse-human hybrids.

5. Investigation of genes regulated by cisplatin in human testicular tumour cells and bladder cancer cells by differential display reverse transcription PCR.

Chapter 2

MATERIALS AND METHODS

2.1. GENERAL CELL CULTURE CONDITIONS

The cell lines used in this thesis and their culture conditions are listed in the following table:

Table 2.1 Summary of cell lines used in this study.

Cell Line	Doubling Time(hr)	Biopsy Origin	Prior Treatment of patient	Reference	Culture Medium
SuSa	20	Testis primary, embryonal carcinoma	None	Hogan *et al.*, 1977	
GH	25	Testis primary, embryonal carcinoma,	None	Lower *et al.*, 1981	
833K	22	Testis abdominal metastasis, embryonal carcinoma, teratoma	Chemo-therapy	Bronson *et al.*, 1980	α-MEM
GCT27	26	Testis primary, embryonal carcinoma	None	Pera *et al.*, 1987.	
MGHU1	20	TCC, bladder recurrence	None	Bubenik *et al.*, 1973	RPMI
RT112	24	TCC, bladder primary	None	Masters *et al.*, 1986	
HT1080	26	Fibrosarcoma metastasised to acetabulum cartilage	None	Rasheed *et al.*, 1974	DMEM
F9	14	Mouse embryonal carcinoma, OTT6050 tumour-129/J		Berstine *et al.*, 1973.	DMEM .

TCC: transitional-cell carcinoma; DMEM: Dulbecco's Modified Eagle's medium;

α-MEM: Modified Eagle's medium.

All cell culture medium was supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 2mM L-glutamine (Gibco). Penicillin (100IU/ml) and streptomycin (10μg/ml) were added to the cell culture medium used in all transfection experiments. All cell lines were maintained at 36.5°C in a humidified atmosphere of 5%CO_2/95% air. All cell lines were cultured *in vitro* continuously for no than 10 passages. To avoid cross-contamination of the cell lines used, only one cell line was handled at a time. Cell culture wares used for F9 cells were coated with 0.1% gelatin (dissolved in PBS, Sigma) to facilitate cell attachment.

2.2. DRUG TOXICITY TESTS

2.2.1. Clonogenic assay

Exponentially growing cells (70-80% confluent) were detached by incubating with 1x trypsin / EDTA (0.25% / 2%) (w/v) in a tissue culture flask for 2-5 minutes at 37°C. The detached cells were taken up in 5ml of medium and a single-cell suspension was produced by passing the cells through a 19-gauge needle prior to counting. Viable cell number was assessed using a haemocytometer by counting the number of cells in the squares excluding trypan blue (0.1% in PBS) (w/v). A minimum of 100 cells were counted. The number of cells per millilitre was calculated as follows:

Average number of viable cells per square x $(2x10^4)$ = number of cells/ml

An appropriate dilution of the cells was made to produce the final cell number required for each colony forming experiment. According to the plating efficiency, 500-3000 cells were plated in 6-cm dishes to produce approximately 150 colonies after 10-14 days growth. The single cells were left to attach overnight in cell culture medium containing 10% FCS (v/v). A range of concentrations of freshly made cisplatin were added (Sigma, P-4394, dissolved in doubled-distilled water or David Bull Labs 1886A, dissolved in 9mg/ml NaCl and 1mg/ml Mannitol). 5 concentrations of cisplatin were tested in each experiment and each concentration was tested in triplicate. 5 control dishes were set up with solvent. After 10-14 days incubation, the cells were fixed in 70% methanol (v/v) and stained with 10% Giemsa (v/v). Colonies consisting of 50 or more cells were counted and the dose-response curves plotted. Results were derived from a minimum of 3 independent assays.

The colony forming assays performed in Chapter 4 on selecting cisplatin resistant transfectants and in Chapter 6 on characterising MGF9 hybrids were performed on either 2 or 3 cisplatin concentrations. Three controls were set up in each experiment and duplicate plates were set up for each cisplatin concentration. Each experiment was repeated at least twice.

2.2.2 Selective Concentration Test

In order to determine the drug concentration for selection of transfected cells and cell hybrids, selective concentration tests were performed on the cell lines studied. $5x10^5$ cells

(counted as mentioned in 2.2.1) were plated in 6-cm dishes, and 24 hours later a range of concentrations of a selective drug was added. The medium was changed every 2-3 days with fresh drug and the cells were fixed and stained after approximately 14 days. The lowest concentration that killed all plated cells was determined and used for selecting transfectants and cell hybrids. Each experiment was repeated at least twice.

pSV2NEO and pcDNAI/NEO plasmids confer resistance to G418 in mammalian cells. pBABE plasmid confers resistance to puromycin and p220LTR plasmid confers hygromycin resistance in mammalian cells. Three selective drugs, G418 (Gibco, 066-01811), puromycin (Sigma, P-7255) and hygromycin (Calbiochem. 400051) were tested on the cell lines studied. The untransfected cells were killed within 4-14 days of adding the selective drugs. The following table shows the summary of selective drug concentrations used in this study:

Table 2..2 Summary of drug concentrations used for selecting transfectants.

Cell lines	G418 (neomycin) (μg/ml)	Puromycin (μg/ml)	Hygromycin (μg/ml)
	For selecting pSV2NEO and pcDNAI/NEO transfectants	For selecting pBABE transfectants	For selecting p220LTR transfectants
SuSa	200	1.0	50
GH	200	1.0	50
833K	300	1.0	50
GCT27	300	1.0	-
MGHU1	1000	-	250
HT1080	600	-	-
F9	575-700	1.0	350

2.2.3   Statistical Analysis

The concentration required to inhibit colony-formation by 50% of untreated controls (IC_{50}) was determined from each experiment and compared between cell lines. All statistical data was generated from at least three experiments. Unpaired student t test was used for the comparison of IC_{50}s between the cell lines studied. Two-sided P values were calculated. It was considered a significant difference if the P value was smaller than 0.05.

2.3   SOMATIC CELL FUSION

Four human testicular tumour cell lines (SuSa, 833K, GH and GCT27) transfected with pSV2NEO and pBABE plasmid were fused to generate hybrids. Either $2x10^6$ cells were co-plated, or one cell line ($2x10^6$ cells) was added to the other cell line (about 70-80% confluent) in a T25 flask. Two control flasks were set up at the same time each containing one parent cell line only. After 24 hours, the medium was removed and 2ml pre-warmed (37°C) 50% (v/v) PEG (polyethylene glycol, MW 1450, Sigma) was added to cover the cells. The PEG was left in the flask for 60 seconds with the cells and then 5ml pre-warmed medium added. The medium was removed and the cells were washed again, finishing with a final volume of 5ml of α-MEM containing 10% FCS (v/v). The longer period of exposure to PEG resulted in a high percentage of cell death in these human testicular cancer cell lines. The same fusion conditions were used for fusing F9 and HT1080 or MGHU1 cells except that the culture medium was DMEM or RPMI.

The medium was changed for all the flasks the following day. The cells were split the next morning if they were confluent (or the medium was changed if not confluent) and the selective drugs G418 and puromycin were added approximately 6 hours later. All flasks were treated with both G418 and puromycin. The cells in the G418 control flasks died 14 days after the drug was added and the puromycin controls died 4 days after the drug treatment. The hybrid colonies were visible 14 days after adding the drugs. The mixed population of hybrids was cloned either by circling individual colonies with cloning rings from the original flasks directly or by plating 200 cells per 5cm dish, and then after approximately 14 days individual colonies were picked using cloning rings and expanded. MGF9 Hybrids were cloned by picking single colonies from the original cell fusion flasks.

All human testicular cell hybrids and the hybrid between F9 and HT1080 cells were maintained in both G418 and puromycin. The hybrids between MGHU1 and F9 were selected in both G418 and puromycin but maintained in only puromycin to facilitate the loss of human chromosomes.

2.4 POLYMERASE CHAIN REACTION ANALYSIS (PCR) ON HUMAN TESTICULAR TUMOUR CELL HYBRIDS

This technique was used to confirm the origin of the testicular cancer hybrids.

2.4.1 DNA Isolation from Cultured Cells

DNA from cultured cells was isolated using a phenol/chloroform extraction method. Approximately 5×10^6 cells were trypsinized and the single cell suspension was washed once with PBS. The cells were lysed in 1 ml lysis buffer [150mM NaCl, 10mM Tris, 10mM EDTA and 0.5% SDS (w/v)] containing 0.1mg/ml proteinase K (Sigma, P-2308) and 50μg/ml RNAase (Sigma, R-9005), and the mixture was incubated at 36.5°C for 2 hours. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture was shaken for 30 minutes at room temperature. The mixture was then spun in a microcentrifuge at 13,000rpm for 10 minutes. The supernatant was removed and the same procedure was repeated again using chloroform/isoamyl alcohol (24:1). The supernatant was precipitated with 2 volumes of ice-cold ethanol and 0.2 volume of ammonium acetate. The genomic DNA was transferred to a fresh tube and washed once with 70% ethanol (v/v). The pellet was air-dried and dissolved in TE buffer (10mM Tris-HCl and 0.1mM EDTA). The DNA concentrations were measured using a spectrophotometer. The absorbance readings at a wavelength of 260nm allow calculation of the concentration of DNA in the sample. An OD of 1 corresponds to approximately 50μg/ml for double-stranded DNA. The ratio between the reading at 260nm and 280nm provides an estimate of the purity of the DNA. The DNA preparations had purity of greater than 1.7.

2.4.2 PCR amplification of VNTR (Variable Number of Tandem Repeat) Regions

The two VNTR regions analysed in Chapter 3 are highly polymorphic, with heterozygosity of over 80% in unrelated individuals (Horn et al., 1989; Budowle et al., 1991). A range of concentrations of MgCl$_2$, genomic DNA, primers and dNTPs were tested and the optimised conditions were as follows: Amplification of the pYNZ22 locus was performed with 1μM each of the primers 5'-CGAAGAGTGAAGTGCACAGG-3' and 5'-CACAGTCTTTATCTTCAGCG-3', 200μM of each dNTP, 0.75mM MgCl$_2$ and 1 unit of Taq DNA polymerase (Stratagene, 600203) in reaction buffer [10x reaction buffer contains:

500mM KCl; 100mM Tris-HCl, pH 8.4 at room temperature and 0.1% gelatin, (w/v)] in a total volume of 25µl. Genomic DNA (0.5µg) was amplified for 30 cycles using 60 sec at 94°C, 30sec at 58°C and 90sec at 72°C; one final cycle at 72°C for 5 minutes on a Perkin Elmer 480 thermal cycler. Amplification of D1S80 region was performed with 1µM each of the primers 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' and 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3', 400µM of each dNTP, 2.5mM MgCl₂ and 1 unit of Taq DNA polymerase (Stratagene, 600203) in reaction buffer (as above) in a total volume of 25µl. Genomic DNA (0.5µg) was amplified for 25 cycles using 60 sec at 95°C, 60 sec at 65°C and 480sec at 70°C.

The PCR products were separated on a 2.0% (w/v) agarose gel (Gibco, 5510UB) run at 150V for approximately 90 minutes in 1xTBE buffer (45mM Tris-Borate, 1.0mM EDTA), and then stained in 0.5µg/ml ethidium bromide and photographed. The calculation of numbers of repeats was facilitated by using a 100bp ladder and φ174 RF DNA/Hae III fragments as standards. The migration of each band of the molecular weight markers was plotted (Y-axis) against its molecular weight (X-axis) and standard curves were drawn. The migration of bands from each DNA sample was measured and the average corresponding molecular weight was extrapolated from the standard curves. Corresponding repeats numbers were then calculated (70bp per repeat for pYNZ22 locus and 30bp per repeat for D1S80 locus).

2.5.   KARYOTYPING ANALYSIS

Cells in exponential growth were treated overnight with colcemid (Sigma) at 0.1µg/ml, allowed to swell for 20 minutes in hypotonic buffer (3mg/ml KCl, 0.2mg/ml EDTA and 4.8mg/ml Hepes) at 37°C, and fixed in methanol/acetic acid (3:1). The cells were dropped on to cold slides (stored at -20°C) and stained with 4% (v/v) Giemsa prepared in 0.06M KH₂PO₄ and 0.06M Na₂HPO₄ buffer. The metaphase cells were photographed with Ilford PanF 50 films using a Zeiss microscope under a 40x or 100x objective lens. The chromosomes of each metaphase cell were counted from negatives of the films under a microscope. The number of chromosomes in at least 10 metaphase spreads was counted. The modal chromosome numbers were determined as the most frequently appeared number in the metaphase cells examined.

## 2.6.	TRANSFECTION

### 2.6.1	Plasmid Preparation for Transfections

Transformation of E. coli bacteria: 0.5μg of library plasmid DNA was added to each tube of competent cells as they started to thaw. The DNA and cells were mixed gently until the cells defrosted completely. The mixture was left on ice for 30 minutes. The DNA and cell mixture was then put on a heat block at 42°C directly from the ice for 150 seconds and then the tubes were put back on ice immediately for 5 minutes. The tubes containing the transformed cells were transferred into each Falcon tube containing 1.5ml Luria Broth on ice and mixed gently. They were incubated in a 37°C incubator for 1.0-1.5 hours with shaking at 200rpm. The cells were plated out on agar plates and incubated at 37°C overnight. The control agar plates were left open at room temperature for 20 minutes and then incubated at 37°C overnight.

Mini- preparation of plasmids from transformed bacteria: This was used to isolate plasmid DNA from a small quantity of transformed E Coli. A single bacteria colony was picked and expanded into a tube containing 10ml Luria broth (LB, Gibco) and appropriate concentrations of selective antibiotics. They were allowed to grow overnight in a 37°C shaker at a speed of 200rpm. The bacteria were centrifuged at 3000rpm for 10 minutes and the pellet was resuspended in 100μl of solution I [25mM Tris pH 8.0, 10mM EDTA pH 8.0, 1% (w/v) glucose]. After 10 minutes at room temperature, 200μl of Solution II was added [0.2M NaOH, 1% (w/v) SDS] and the bacteria were left on ice for 5 minutes. Then 150μl of Solution III (58.88g potassium acetate, 23ml glacial acetic acid, made up to 200ml with ddH$_2$O) was added and left on ice for 5 minutes. The cells then were spun at 14,000rpm for 5 minutes and the supernatant was transferred into a fresh tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the mixture was shaken for 5 minutes at room temperature. It was then spun at 14,000rpm for 5 minutes and the top layer was removed and precipitated by adding 2 volumes of 100% (v/v) ethanol. The DNA pellet was spun down and dried at room temperature. The DNA was then dissolved in 20μl of double distilled water (ddH$_2$O). The DNA was then used either for enzyme digestion analysis or for the transformation of bacteria.

Maxi-preparation of plasmids from transformed bacteria: A plasmid transformed bacterial colony was picked and expanded into 10ml Luria Broth (LB, Gibco) containing appropriate concentrations of antibiotics. They were cultured overnight at 37°C in a shaking incubator at a speed of 200rpm. The bacteria were transferred into to 1 litre of LB containing antibiotics and cultured overnight in the same incubator.

The bacteria were spun and the pellets were resuspended in 20ml of cold Solution I (See Miniprep solutions). Then 40ml of solution II was added dropwise at room temperature and the cells were left on ice for 10minutes. 30ml of Cold Solution III was added and the cells were left on ice for at least 20 minutes. The mixture was centrifuged at 7000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new centrifuge tube through a gauze (10x10cm BT Type10, Smith-Nephew) to remove the pellets. Two volumes of absolute alcohol was added to the solution and it was centrifuged at 7000 rpm at room temperature for 10 minutes. The supernatant was discarded and the pellet was dried at room temperature. Each dried pellet was then dissolved in 8.2ml ddH$_2$O containing 200µl of ethidium bromide (10mg/ml). 9.6g ultra pure CsCl (Sigma) was added to each dissolved DNA solution. The solution was spun down at 3500 rpm for 10 minutes at room temperature and the supernatant was transferred to ultra-centrifuge tubes (Quick-Seal, 13x51mm, Beckman). The samples were spun at 70,000 rpm at 20°C overnight (minimum of 16 hours) on a Beckman L-80 ultracentrifuge. The second heavy band was then removed by using a G19 needle with a 2ml syringe. The ethidium bromide was extracted three times by adding an equal volume of water-saturated isoamyl alcohol, centrifuging at 1500 rpm for 5 minutes and discarding the upper pink layer. The lower layer was then diluted with 2 volumes of ddH$_2$O, then 2 volumes of 100% ethanol was added to precipitate plasmid DNA. The DNA precipitate was pelleted at 10,000 rpm for 20 minutes at room temperature. When the pellet was dry, it was resuspended in 1.0ml of ddH$_2$O and the DNA was measured at OD$_{260nm}$. DNA Concentration (µg/ml) =OD$_{260nm}$ x 50 x dilution factor. The purity of the plasmid DNA used for transfections was greater than 1.7. All plasmid DNA aliquots were stored at - 20°C.

2.6.2   Plasmid Preparation for cDNA Library Transfection

cDNA library: The cDNA library used in Chapter 4 was derived from a human fibrosarcoma cell line (HT1080) and was a generous gift from Dr Ian Hickson, Institute of Molecular Medicine, ICRF). The cDNA library was cloned into the pcDNAI/NEO vector (see Figure 2.1) through *BstXI* sites resulting in 2×10^5 independent clones. The insert sizes ranged from 0.6Kb to 3.8Kb and one out of 30 had no insert (personal communication, Dr. Ian Hickson, Institute of Molecular Medicine, ICRF). The *neo* gene in the plasmid confers G418 resistance in mammalian cells and kanamycin resistance in E coli cells. The SupF gene in the plasmid suppresses the P3 gene in E coli MC1061/P3 strain, thus conferring ampicillin and tetracycline resistance. The cDNA library was analysed and amplified before use for transfection experiments.

Preparation of competent E coli MC1061/P3 cells: The transforming efficiencies of the E coli cells by plasmid DNA was improved by the following method. A 200μl aliquot of bacterial cell stock was transferred onto an agar plate (containing 50μg/ml kanamycin and 20mM $MgSO_4$). The bacterial culture was spread on the plate using a loop and the plate was incubated at 37°C overnight. A single colony was picked from the agar plate and transferred into a 20 ml Universal containing 20ml TYM [2% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 0.1M NaCl, 10mM $MgSO_4$) and 50μg/ml kanamycin and incubated on a shaker at 37°C overnight. The OD_{600nm} was measured until it was between 0.2 and 0.8 (midlog phase cell growth). The cells were then transferred into 100 ml TYM containing 50μg/ml kanamycin (Sigma). They were grown until the OD_{600nm} was between 0.5 and 0.9. The cells were then diluted to 500 ml TYM containing 50μg/ml kanamycin. When the OD_{600nm} reached 0.6, the flask containing the bacterial cells was put in ice-cold water to cool rapidly. Then the cells were spun at 4200 rpm at 4°C and the pellet was resuspended in 100ml cold TfB I [30mM KOAc, 50mM $MnCl_2$, 100mM KCL, 10mM $CaCl_2$, and 15% (v/v) glycerol] by gentle shaking on ice. The cells were respun in the same bottle at 4200 rpm for 8 minutes at 4°C. The supernatant was discarded, the pellet was resuspended in 20 ml cold TfB II [10mM Na-MOPS, pH7.0; 75mM $CaCl_2$; 10mM KCl, 15% (v/v) glycerol] by gentle shaking on ice. Aliquots of 0.1 to 0.5ml were frozen in microfuge tubes and stored at -70°C. The transforming efficiencies of each batch of competent cells were estimated to ensure that each agar plate (243x243x18mm) used in amplifying cDNA library contained at least 200,000 colonies.

FIGURE 2.1

MAP OF pcDNAI/NEO VECTOR
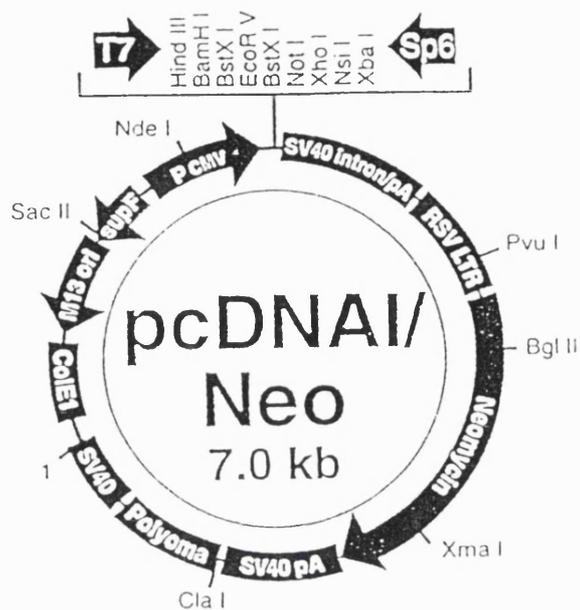
Diagram of the pcDNAI/NEO vector containing the HT1080 cDNA library. The library was inserted between the two *BstXI sites*.

Maxiprep of pcDNAI/NEO cDNA library from transformed MC1061/P3: The cDNA library was amplified on agar plates. This was to make sure that all the clones present in the library were amplified equally.

a) Preparation of agar plates: 9 bottles of stock agar (Sigma L-3522, 400ml/bottle) were melted in a microwave oven. When the agar had cooled down to approximately 50°C, ampicillin (12.5μg/ml, dissolved in 70% (v/v) ethanol; Sigma, A-0166) and tetracycline [7.5μg/ml, dissolved in 70% (v/v) ethanol; Sigma, T-7660] were added. The agar then was poured on to Bio-Assay plates (243x243x18mm, Gibco) using 300-350ml per plate. Ten plates were set up per Maxiprep. The plates were left to set and dry. Two 15cm plates were also set up as control plates.

b) Transformation of E. coli MC1061strain: 0.5 - 1.0μg library DNA was used per bacterial aliquot (0.5ml) for two agar plates. Five frozen competent cell aliquots were used and were put on ice immediately. The transformation protocol was followed as described in section 2.6.1.

c) Maxiprep: No bacterial colonies should be seen on control plates. All the Bio-assay plates should be covered with single colonies. 10ml Luria Broth (LB, containing no selective drug, Gibco) was added to each plate to suspend the bacterial cells from the agar plates. The cells were transferred into centrifuge tubes and spun down at 4200 rpm for 10 minutes at 4°C. The next steps were the same as the maxipreparation procedures mentioned in section 2.6.1.

pcDNAI/NEO plasmid DNA used for transfection modification experiments was generated from 12 randomly selected bacterial colonies on a cDNA library agar plate. Minipreps were performed to generate plasmid DNA. The mixture of the 12 miniprep DNA was used to transformed MC1061/P3 E coli strain and the DNA was amplified by performing maxipreps.

2.6.3.   Analysis of the cDNA library

Miniprep of plasmid DNA: 12 single colonies were picked randomly from an agar plate harbouring bacterial colonies containing the library and the DNA was isolated using miniprep method mentioned in section 2.6.1.

Restriction enzyme digestion: Restriction enzymes *BamHI* and *XbaI* (Gibco) were used to cut the insert cDNA from the plasmid (see Figure 2.1, Map of pcDNAI/NEO vector) and thus the insert sizes of each picked clone could be identified by restriction digestion. 5µl of DNA from each miniprep was used in each reaction and the digestion procedure was as follows: 1/10 of 10x Universal digestion buffer (Gibco), 0.5µl of enzyme (5U/µl, Gibco) and ddH$_2$O added to make a total volume of 20µl. The mixture was then incubated at 37°C for 1 hour. 10µl of the digested DNA was run on a 1% (w/v) agarose gel in TBE buffer (45mM Tris-Borate, 1.0mM EDTA) at 150V for 40 minutes. Photos were taken using a Polaroid camera. *EcoRI* and *HincII* (Gibco) were used to digest the maxiprep DNA and to provide information on the quality of the plasmid DNA.

Figure 2.2 shows the results of enzyme digestion analysis on 12 randomly selected bacterial clones from a cDNA library agar plate. Twelve single bacterial colonies from a plated HT1080 cDNA library were picked and analysed after minipreparation. As shown in Figure 2.1, the pcDNAI/NEO plasmid has a molecular weight of 7kb and the cDNAs were cloned through *BstXI* sites. There is one *BamHI* and one *XbaI* restriction site outside the cDNA cloning site in the plasmid. These two enzymes were used to analyse the size of the inserted cDNA and the percentage of plasmids containing cDNA inserts in this library.

As shown in Figure 2.2, each digested lane showed one band corresponding to a size of 7kb molecular weight, indicating the pcDNAI/NEO plasmid. The smaller band(s) in each lane indicated the sizes of the inserted cDNA. In some lanes (Lanes 1, 4 and 6), there was more than one band indicating that there were *BamHI* or *XbaI* restriction sites within the inserted cDNA. All 12 clones analysed had inserts and the sizes of the cDNA clones tested corresponded to sizes ranging from 0.8 to 3.3kb.

FIGURE 2.2

RESTRICTION ANALYSIS OF pcDNAI/NEO cDNA LIBRARY
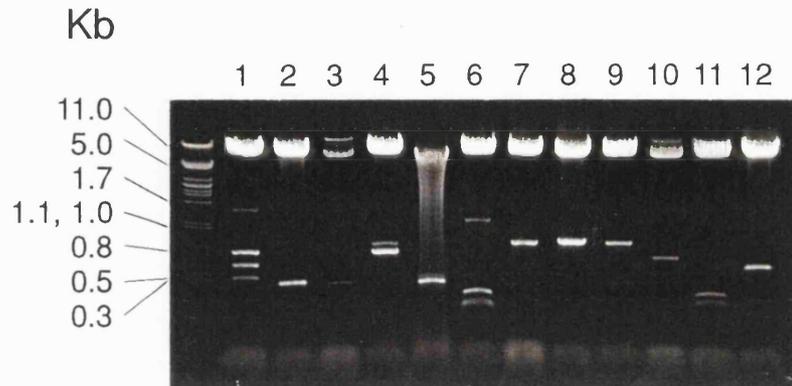
Twelve randomly selected bacterial colonies from the pcDNAI/NEO library were miniprepped and analysed. The plasmid DNA was digested using *BamHI* and *XbaI* enzymes. The lane on the left hand side is a DNA molecular weight marker. The top band of each lane (1-12) represents the pcDNAI/NEO plasmid and the smaller band(s) represent the inserted cDNA.

2.6.4. Transfection Protocols

2.6.4-1 Transfection of Human Tumour Cells

In order to generate markers for the selection of testicular cell hybrids in Chapter 3, plasmid pSV2NEO (conferring G418 resistance) and pBABE (conferring puromycin resistance) were transfected into four testis tumour cell lines (SuSa, 833K, GH and GCT27) respectively. pSV2NEO was introduced into a human fibrosarcoma cell line (HT1080) and a human bladder tumour cell line MGHU1, pBABE was also transfected into a mouse embryonal carcinoma cell line F9 for cell fusion experiments in Chapter 4 and 6 using the following protocol:

The cells were plated 2 - 3 days before the transfection and used when they were about 80% confluent. The medium was changed 24 hours before transfection [α-MEM or DMEM containing 10% (v/v) FCS].

The DNA suspension was prepared as follows:

	ddH$_2$O(μl)	2M CaCl$_2$ (μl)	DNA plasmid (μl)	2xHBS(μl)
Mock	481	69	0	550
Plasmid	461	69	20(5μg)	550

2xHBS (280mM NaCl, 10mM KCl, 1.5mM Na$_2$HPO$_4$.2H$_2$O, 12mM dextrose and 50mM HEPES; pH 7.02) was added dropwise, at the same time using an automatic pipette to bubble air gently through the solution (approximately 2 bubbles per second). The mixture was left at room temperature for 20 minutes.

The cell culture medium was changed with 5ml of medium and 1.1ml of DNA suspension was added to each flask. The cells were incubated with the suspension for 6 hours at 36.5°C in an incubator, washed once with medium gently and then 5ml fresh medium was added to each flask and incubated at 36.5°C overnight. The cells were split the next morning (if confluent) and left overnight at 37°C. The medium was then changed and selective drugs were added at pre-determined concentrations. The DNA incubation time for MGHU1 and F9 cells was 24 hours instead of 6 hours.

2.6.4-2   Modification of Transfection Efficiencies

Preliminary work was done to optimize transfection conditions. In order to transfect the entire library (approximately 200,000 clones) into F9 cells, it was aimed to obtain at least a transfection efficiency of 1 in 5000 (0.02%). At this efficiency we would be able to transfect the 200,000 clones using 10^9 F9 cells. Four techniques were tested:

Calcium-phosphate mediated transfection, originally described by Graham and Van der Eb (1973), is a simple and inexpensive method. Since then many modifications have been introduced to improve transfection efficiency, such as the pH and composition of the buffer, DNA exposure and expression time, CO_2 levels in the incubator and DNA concentrations (Chen *et al.*, 1987; O'Mahoney *et al.*, 1994; Song *et al.*, 1995; Jordan *et al.*, 1996).

An alternative to the calcium-phosphate method is lipofectin-mediated transfection which has been reported to be 5-100 fold more efficient than the calcium-phosphate method (Felgner *et a.l.*, 1987).

A high voltage electric shock to the cells leads to the formation of small pores in the mammalian cell membrane (Neumann *et al.*, 1982), and allows foreign DNA to be taken directly into cells, a technique known as electroporation (Sambrook *et al.*, 1989).

However, all of these approaches have been shown to transfect growing cells only. Poly-L-ornithine mediated transfection combined with DMSO shock has been claimed to be able to transfect both growing cells and resting cells efficiently (Dong *et al.*,1993). This method has been reported to be 5-10 times more efficient than other standard methods of transfection. The following sections summarise the transfection modification experiments on F9 cells.

Calcium-phosphate mediated transfection:

Standard calcium-phosphate mediated transfection protocol:

Day 1. 5×10^5 F9 cells were plated in each 4cm well of a 6-well plate.

Day 2. The cell culture medium was replaced with DMEM containing 10% (v/v) FCS 1-2 hours before performing the transfection. The DNA suspension was prepared as follows (in 5ml medium).

2xHBS buffer: ddH$_2$O   481µl

2M CaCl$_2$ 69µl (final concentration 0.125M)

2xHBS   550µl

When adding DNA, the volume of ddH$_2$O was reduced to keep the total volume at 1100µl. The mixture was left at room temperature for 20 minutes.

2xBBS buffer: 0.25M CaCl$_2$ 500µl (final concentration 0.125M)

2xBBS   500µl

When adding DNA (maximum of 20µl), the volume of 0.25M CaCl$_2$ was reduced to make the total volume 1000µl. The mixture was left at room temperature for 1 minute and added to the cells dropwise.

Day 3.   After 6 hours of incubation, the cells were washed with PBS and the medium replaced with DMEM containing 10% (v/v) FCS and then left for 24 hours.

Day 4. The medium was changed again and G418 was added.

Day 5 -6. The cells were washed with PBS 1-2 times to remove dead cells and the medium replaced with DMEM with 10% FCS containing G418.

Day 7 -14. The cells were washed with PBS and the medium replaced with DMEM with 10-20% FCS containing G418 every 2-3 days as necessary until the transfected colonies grew. The colonies were fixed with 70% (v/v) methanol and stained with 10% (v/v) Giemsa and counted. Transfection efficiencies were calculated as follows:

Number. of colonies ÷number. of cells plated x100%= T.E.%

In the standard procedure, it was used 15µg/plate DNA, DNA incubation time of 6 hours, expression time of 30 hours and the medium used for incubation was DMEM containing 10% FCS (v/v).

The variables tested were as follows:

Transfection buffers: 2x*BBS* :50mM BES pH 6.96, 280mM NaCl and 1.5mM Na$_2$HPO$_4$

(Chen *et al.,* [1987] and O'Mahoney *et al.,* [1994]).

2x*HBS*:   50mM HEPES, 12mM Dextrose, 1.5mM Na$_2$HPO$_4$.H$_2$O, 10mM KCl and 280mM NaCl; pH 7.02 (Sambrook *et al.,* 1989).

DNA incubation time: 6 hours and 24 hours;

DNA concentration: 2 - 4µg/ml (Chen *et al.*, 1987)

Other modifications: DMSO shock: 30% and 15% [(v/v), Troelstra *et al.*, 1990]

Glycerol shock: 15% [(v/v], Sambrook *et al.*, 1989]

Low CO_2 DNA incubation: 3% (Chen *et al.*, 1987).

The optimised transfection method resulted in 0.25% with 2xBBS buffer and 0.53% with 2xHBS with 15µg/well DNA. The DNA incubation time was 24 hours and the exposure time was 30 hours. These transfection efficiencies were at least 10 times higher than it was aimed for and was the highest among the 4 transfection methods tested so it was used for transfection of the cDNA library.

Electroporation mediated transfection:

Standard protocol

Day 1. Cells were 70%-80% confluent and the medium was changed 2-4 hours before the electroporation. Single cell suspensions were collected by trypsinisation and washed once with IMDM (Iscove's modified Dulbecco's medium) or 1xPBS without serum. The cells were adjusted to 10^7 - $3x10^7$ cells per cuvette (in 0.8ml volume) and 20µg DNA was added per cuvette. The cell-DNA mixture was left on ice for 10 minutes. The cells were then electroporated at the desired voltages and capacitances. They were then left at room temperature for 10 min and before plated in 15cm dishes at $1x10^7$ cells per dish.

Day 2. The medium was changed and the cells left for 48 hours to express the transfected vector.

Day 3 -11. The cells medium was changed with DMEM containing 10% FCS (v/v), and G418 was added. This was repeated every 2-3 days with fresh G418 until colonies were visible.

Day12 -14. Colonies were stained and counted as mentioned in the *calcium-phosphate transfection* protocol.

According to Dr. Martin Pera and Dr. Martin Evan's personal suggestions the following conditions were tested with a Bio-Rad gene pulser:

a). At 500µF, with increases of 50v or 20v over the range 200v-450v in IMDM (Iscove's modified Dulbecco's medium) .

b). At 960µF, with increases of 50v or 20v over the range 200v-350v in PBS.

At 960μF capacitance and240V, the highest transfection efficiency of 0.0017%. was achieved. However, it was much lower than the T.E achieved with the calcium-phosphate method.

Lipofectin mediated transfection

According to the manufacturer, different concentrations of lipofection reagent (5-20μg/ml) should be tested.

Day 1. 7.5×10^6 cells were plated in a 15 cm plate in DMEM medium with 10% FCS (v/v).

Day 2. Preparation of the transfection solutions:

Solution A: The DNA (5μg) was diluted into 100μl of serum-free DMEM medium.

Solution B: The desired amount of lipofectin reagent was diluted into 100μl of serum-free DMEM medium.

The two solutions were mixed gently and incubated at room temperature for 15 minutes. Meanwhile the cells were washed once with serum-free medium. For each transfection, 0.8ml of serum-free medium was added to each tube containing the lipofectin-DNA complexes. The mixture was then overlaid onto the cells for 6 hours in an incubator and 5ml of DMEM containing 10% (v/v) FCS was added.

Day 3. The DNA containing medium was replaced with DMEM containing 10% (v/v) FCS.

Day 4. G418 was added to the cells at the predetermined concentration.

Day 5 -14. The cell culture medium containing G418 was replaced every 2-3 days until the transfected colonies grew. The colonies were then fixed and stained as described in calcium phosphate transfection protocol.

The highest T.E. of 0.002% was achieved using 20μg/ml lipofectin and 5μg DNA.

Poly-L-ornithine mediated transfection:

Day 1. The cells were plated at 7.5×10^6 per 15cm plate with DMEM containing 10% (v/v) FCS.

Day 2. The medium was replaced and 10μg of DNA was added to each plate. Then Poly-L-ornithine (Sigma, P3655) was added from a 10mg/ml sterile water stock solution. The cells were incubated at 36.5°C with gentle mixing every 1.5 hours. After 6 hours, the medium was aspirated and replaced with 30% (V/V) DMSO in DMEM for 4 minutes at room

temperature. The plates were then rapidly washed and fresh DMEM medium containing 10% FCS was added.

Day 3 -4. After 48 hours of culture, the cells were treated with G418 at the predetermined concentration.

Day 5 -14. The medium was changed every 2-3 days with DMEM containing 10% FCS and G418 until transfected colonies grew up. The colonies were then fixed and stained as mentioned in calcium phosphate transfection protocol.

The following conditions were tested: Poly-L-ornithine concentration (5μg/ml-10μg/ml), DNA concentration (10-15μg per plate) and incubation time (6 and 24 hours).

The highest T.E of 0.0052% was achieved using 15μg DNA per plate to incubate cells for 6 hours in 5μg/ml poly-L-ornithine.

The modified calcium-phosphate mediated transfection method (15μg/well DNA with either 2xHBS or 2xBBS buffer for 24 hours incubation and 30 hours expression) achieved the highest transfection efficiency of approximately 0.5% among the 4 methods tested. So it was used for transfection of pcDNAI/NEO cDNA library into F9 cells.

2.6.5   Transfection Protocols for Transfecting of cDNA Library

10^8 F9 cells were used in each library transfection to ensure transfection of the entire library at a transfection efficiency of approximately 0.2%. The modified calcium phosphate mediated transfection method was used for the transfection of the cDNA library. Controls were set up in each transfection. The cells in two 4cm wells were transfected with transfection buffer only, as G418 selection controls. Two wells were transfected with pcDNAI/NEO trial plasmid DNA and selected in G418 only, as transfection efficiency controls. Two wells were transfected with pcDNAI/NEO trial plasmid DNA and selected in both G418 and cisplatin, as cisplatin selection controls. In each of the cDNA library transfection experiments, there were no colonies visible in either of the G418 or cisplatin selection controls. The colonies in the transfection efficiency controls were stained and counted to estimate the transfection efficiency in each experiment.

Colonies surviving cisplatin selection from the primary transfections were selected again. Each primary transfectant colony was split into three wells. Two wells in a 96-well plate were used for cisplatin test and its control, and one well of a 6-well plate was used as a G418 selection. 300ng/ml cisplatin was used for the secondary selection for 2 days. The confluence of the cells in each paired well was compared between the stained cisplatin test controls and the cisplatin test wells. The cisplatin treated wells that had similar cell numbers to the control wells were considered as possible cisplatin resistant clones. The corresponding colonies that grew in G418 selecting wells were expanded. Three cisplatin concentrations were tested on the selected clones using colony-forming assays as mentioned in section 2.2.1. The IC_{50} cisplatin concentration values were determined.

2.6.6   Secondary genomic DNA transfection

Genomic DNA was isolated from cisplatin resistant primary transfectants and used for secondary transfection into parental line F9 cells.

Genomic DNA isolation for secondary transfection: The selected primary transfectants were grown in G418 and counted and washed twice in PBS. They were aliquoted at 5×10^7 cells per 1.5ml eppendorf in 1ml PBS and kept at -20°C. To isolate genomic DNA, the cells were spun down in a microcentrifuge at 13,000rpm for 5 minutes before use and resuspended in 0.5ml fresh PBS for each 5×10^7 cell tube. The next steps were as described in section 2.4.1 'DNA isolation from cultured cells'.

Generating 25-50Kb genomic DNA fragments: A 25G needle was used with a 2ml syringe to mix the DNA solution up and down in a tube 15 times and the DNA was checked on an agarose gel (containing 0.5μg/ml ethidium bromide). The gel was made as follows: 30ml of 1% (w/v) agarose was poured on a midigel in 1xTBE buffer. When the gel was set, 100ml 0.4% (w/v) agarose (containing 0.5μg/ml ethidium bromide) was poured on top of it. 350ng genomic DNA was then loaded in each 5mm thick well. A high molecular weight DNA marker (HMW, Gibco Cat.15618-101, 40μg/ml) was prepared as follows: Heat the marker DNA at 65°C for 8 minutes before loading, load 9μl per 5mm well when cool. To check the size of the isolated genomic DNA, 400ng HMW marker and genomic DNA were loaded on a agarose gel at 21V for 21 hours to result in the best separation of the HMW

marker. The gel was photographed and the size range was estimated. The DNA fragments of 25-50kb were used for generating secondary transfectants.

Secondary transfection: The secondary transfection was carried out using the optimised calcium phosphate method in 2.6.4-2. A representative photo of the generated genomic DNA fragments is shown in Figure 2.3.

Calculations: The human genome is $3x10^6$Kb in size and the smallest genomic DNA fragment could be used was 25Kb. Therefore to cover the whole genome, 120,000 fragments had to be transfected ($3x10^6$Kb/25Kb). At a transfection efficiency of 0.3%, $4x10^7$ cells needed to be used for the transfection (120,000x1000/3). To make sure that the whole genomic library was transfected, the cell numbers used in each secondary transfection were three times more than calculated, which was 10^8 cells. $5x10^5$ cells were plated in each well of 6-plates. A total of 40 plates were used for each transfection. Two G418 selection controls were set up and transfected with transfection buffer only. Transfection efficiency controls were also set up for calculating transfection efficiencies of each experiment.

FIGURE 2.3

**A REPRESENTATIVE EXAMPLE OF ISOLATED GENOMIC DNA FOR THE
SECONDARY TRANSFECTIONS**
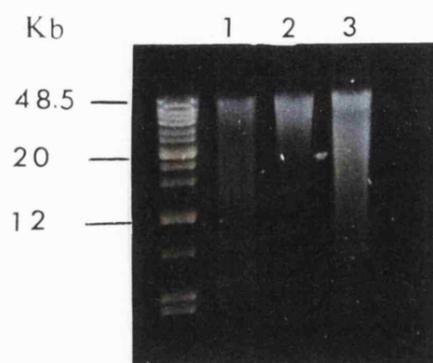
Gel electrophoresis of genomic DNA generated for secondary transfections. 0.5μg of
DNA was loaded in each lane and electrophoresed on a 0.4% agarose gel at 20V for 21
hours. DNA was visualised using ethidium bromide.

2.6.7 Plasmid Rescue from Cells Transfected with EBV-based Shuttle Vectors

A dish of cells at 80% confluence was trypsinized and re-suspended in 10ml of medium supplemented with the serum in a universal tube. The cells were pelleted at 1100rpm for 4 minutes, washed with 10ml of PBS and pelleted again. The PBS was discarded and the pellet was vortexed briefly. After adding 0.4ml of 0.4% (w/v) SDS/10mM EDTA the cells were left at room temperature to lyse. The viscous solution was gently transferred to a microfuge tube and 0.1ml of 5M NaCl was added, mixed and the suspension placed in ice-water in the cold room overnight. Following centrifugation at 13,000rpm for 10 minutes at room temperature, the supernatant was removed and recentrifuged to clarify the supernatant which was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:iosamyl alcohol (24:1). 1/10 of the volume of 3M Na-Acetate and 20μl of 0.25% (w/v) linear polyacrylamide was added and precipitated with 2 volumes of absolute ethanol at room temperature. The precipitate was collected by centrifugation and washed with 70% (v/v) ethanol and stored in 70% (v/v) ethanol at -20°C.

2.7 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

This technique was used to detect the presence of the pcDNAI/NEO plasmid (such as copy numbers, integration sites) in the primarily transfected F9 cells in Chapter 4 and the presence of human chromosomes in the human-mouse hybrids in Chapter 6. Metaphase slides were prepared as mentioned in section 2.5. The pcDNAI/NEO plasmid or human Cot-1DNA (Gibco Cat.15279-011) was labelled using the BioNick labelling system (Gibco, Cat. 18247-015). 1.0μg of probe was labelled by biotin by nick translation at 15°C for 1.5 hours. After adding 5μl of stop buffer, 2μl of sonicated herring sperm DNA (Sigma Cat. D7290, 10μg/ml) per slide was added. The labelled DNA was purified by adding 1/10 volume of 3M NH$_4$ acetate and 2 volumes of 100% (v/v) ice-cold ethanol. The DNA pellet was obtained by spinning at 14,000 rpm for 5 minutes and dried at room temperature. It was dissolved in hybridisation mix [50% (v/v) deionised formamide, 10% (w/v) dextran sulfate, and 2xSSPE pH 7.0] and denatured at 75°C for 5 minutes before adding to the slides.

The metaphase slides were treated with RNase (100μg/ml in 2xSSC, 1xSSC contains 150mM NaCl, 15mM sodium citrate) in a humidified chamber at 37°C for 1 hour. The slides were rinsed 4 times in 2xSSC in a Coplin jar at room temperature. The slides were dehydrated in 70% (v/v) ethanol for 3 minutes; 90% (v/v) ethanol for 3 minutes and 100% (v/v) ethanol for 5 minutes. The slides were then treated with proteinase K buffer (20mM Tris-HCl, 2mM $CaCl_2$, pH 7.4) for 10 minutes and 0.035μg/ml proteinase K for 7 minutes at 37°C. The slides were rinsed in 50ml PBS (Sigma, P4417 tablets) in a Coplin jar for 5 minutes on a shaker. The slides were fixed in 1% (v/v) formaldehyde for 2 minutes [1.3ml of 37% (v/v) formaldehyde (Sigma F1635) in 50ml PBS/0.5g $MgCl_2.6H_2O$, final concentration: 1%] and rinsed in PBS for 10 minutes at room temperature. The slides were then dehydrated again. They were denatured in 70% formamide in 2xSSPE (0.3M NaCl, 20mM NaH_2PO_4) at 75°C for 5 minutes in an oven and dehydrated in cold 70% (v/v) ethanol for 3 minutes, 90% (v/v) ethanol for 3 minutes and 100% (v/v) ethanol for 3 minutes. When the slide was dry, 20μl of denatured probe was added. The slide was covered with a cover slip and sealed with Cowgum and put in a humidified container overnight at a 37°C incubator.

Formamide [50% (v/v) made up in 2xSSC], pre-warmed in a 42°C waterbath, was used to wash the slides 3 times for 5 minutes each. Then the slides were washed twice with 2xSSC at 42°C for 5 minutes each. The stringency washes were performed using 0.1xSSC at 42°C twice for 2.5 minutes each. The slides were put in 0.05% (v/v) Tween20 (diluted in 4xSSC, Sigma) for 5 minutes at room temperature. Then they were incubated in 5%(w/v) Marvel milk powder (dissolved in 4xSSC) for 20 minutes at room temperature. The slides were then incubated in 100μl of 5μg/ml FITC-Avidin [Vector Labs, Cat.A3101, diluted in 5% (w/v) Marvel (dissolved in 4xSSC)] per slide at room temperature for 20 minutes. The slides were then washed 3 times for 5 minutes each in 0.05% (v/v) Tween-20 (diluted in 4XSSC) in a Coplin jar. The slides were incubated in 100μl of 5μg/ml Biotin-anti-Avidin [Vector Labs, Cat. BA0300, diluted in 5% (w/v) Marvel (dissolved in 4xSSC)] at room temperature for 20 minutes. They were washed once with 0.05% (v/v) Tween-20 (diluted in 4xSSC) for 5 minutes at room temperature. The signals were amplified again by treating the slides with 5μg/ml FITC-avidin again as described before and washed with 0.05% (v/v) Tween-20 (diluted in 4xSSC) once for 5 minutes and in PBS twice for 5 minutes each. The slides were then dehydrated again as described. They were mounted in 100μl of Vectashield Antifade (Vector Labs, Cat.

H1000) containing 0.8μg/ml Propidium Iodide (PI) (Sigma P4170) and 2.0μg/ml DAPI (4,6-Diamidino-2-phenylindole) (Sigma, D1388) and covered with a cover slip. The stained chromosomes were viewed under a confocal laser microscope. The chromosome images were captured under a 100x objective oil lens.

2.8 DIFFERENTIAL DISPLAY REVERSE TRANSCRIPTION PCR (DDRT-PCR)

2.8.1 RNA Isolation from Cultured Cells (Chomczynski and Sacchi, 1987)

Two T175 flasks containing cells at approximately 80% confluent (approximately 5×10^7 cells per flask) were treated with cisplatin at an IC_{90} concentration for 1 hour, washed once with PBS and cultured in cell culture medium for 2 hours. The control flasks were treated with cisplatin solvent only.

The cell culture medium was discarded and 4ml of Solution D (4M guanidinium; 25mM sodium citrate, pH7; 0.5% sarkosyl NL30 (v/v) (BDH) and 0.1M β-mercaptoethanol) was added to each flask. The lysed cells were transferred to a 15ml tube. Sequentially, 0.4ml of 2M sodium acetate, pH4.0, 4ml of phenol (water saturated) and 0.8ml of chloroform-isoamyl alcohol mixture (49:1) were added with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and incubated on ice for 15 minutes. Samples were centrifuged at 5000rpm for 20 minutes at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 4 ml isopropanol, and stored at 4°C for future study.

The RNA pellet was recovered by centrifuging at 5000rpm for 20 minutes at 4°C and dissolved in 100μl of DEPC water [0.1% (v/v) diethyl pyrocarbinate, Sigma]. The concentration of RNA was determined by measuring the OD_{260nm} of an aliquot of the preparation. A solution of RNA whose $OD_{260nm} = 1$ contains approximately 40μg/ml RNA. All RNA samples were adjusted to 2.0mg/ml and stored at -70°C for future analysis.

20μl (40μg) of 2.0mg/ml RNA stock was denatured in 60μl of denaturing mix at 65°C for 30 minutes (750μl of denaturing mix contains: formamide 500μl, 40% (v/v)

formaldehyde 179μl, 20x phosphate buffer 50μl, 0.5M EDTA 1μl and DEPC water 20μl) and put on ice immediately for 5 minutes to cool down. 8μl of 10x loading buffer was added to each tube and 20μg denatured RNA was loaded in each lane on a 1.2% (w/v) agarose gel containing 2.2M formaldehyde in 1x phosphate buffer (10mM Na_2HPO_4 and 10mM NaH_2PO_4, pH 7.0) made in DEPC water. The gel was left to run at 15V overnight and stained in ethidium bromide (1.0μg/ml) and photographed.

2.8.2   Removal of DNA Contamination from RNA Samples

It is crucial that total RNA should be absolutely free of DNA contamination before being used for mRNA Differential Display. The RNA was cleaned with DNase treatment using MessageClean kit (GenHunter).

DNase I digestion: The following components were added in order and incubate for 30 minutes at 37°C.

Total RNA	50 μl (10-50μg)
10xReaction buffer	5.7 μl
Dnase I (10U/μl)	1μl

The RNA was re-extracted by phenol/chloroform (3:1). 40μl of phenol/chloroform was added and the mixture was vortexed for 30 seconds. It was left on ice for 10 minutes and centrifuged at 13,000rpm for 5 minutes. The upper phase was collected and the RNA was re-precipitated by adding 5μl of 3M NaOAC and 200μl of 100% (v/v) ethanol. After incubation at -70°C overnight, the RNA was pelleted by centrifuging at 13,000rpm for 1 hour at 4°C. The pellet was removed and washed with 0.5ml cold 85% (v/v) ethanol (in DEPC water, stored at -20°C) once. The RNA pellet was air-dried and then dissolved in 20μl of DEPC water. The RNA was quantitated by measuring OD_{260nm} after 1:100 dilution. The RNA was diluted to 0.5mg/ml and stored as 1μg aliquots at -70°C. The RNA was diluted to 0.1μg/μl with DEPC water before use for differential display. Diluted RNA was not reused after freezing and thawing.

2.8.3 Differential Display PCR

2.8.3-1 Step I: First Strand cDNA synthesis

Two reverse transcriptases were compared and one of them (Superscript II) was used in differential display experiments.

a) RNAimage kit (GenHuter) was used as follows: The following components were defrosted and kept on ice. Three reverse transcription reactions were set up for each RNA sample in three PCR tubes, each contains one of the 3 different one-base-anchored H-T$_{11}$ M primers (where M may be G, A or C). mRNAs containing polyA tails will be transcripted to cDNAs using the following reaction components.

For 20µl final volume

dH$_2$O	9.4µl
5xRT buffer	4.0µl (125mM Tris-Cl, pH8.3; 188mM KCl, 7.5mM MgCl$_2$ and 25mM DDT)
dNTP (250µM)	1.6µl
Total RNA (DNA-free)	2.0µl (0.1µg/µl, freshly diluted)
H-T$_{11}$M (2µM)	2.0µl

The mixture was incubated at 65°C for 5 minutes, 37°C for 60 minutes, 75°C for 5 minutes. After 10 minutes at 37°C, 1µl (100U/µl) of MMLV reverse transcriptase was added to each tube, and mixed quickly by finger tapping and the incubation was continued. At the end of the reverse transcription, the tubes were spun down briefly to collect condensation. The tubes were set on ice for PCR or stored at -20°C for later use.

b) SuperScript II (Gibco Cat. 18064-014) RNase H Reverse Transcriptase: First strand synthesis.

A 20µl reaction volume was used for 1µg total RNA. The following components were added to a nuclease-free microcentrifuge tube:

1µg of total RNA diluted in 10µl of DEPC ddH$_2$O
2µl of Oligo(dT)$_{11}$ A, C or G primers (2µM)

The mixture was heated to 70°C for 10 minutes and chilled quickly on ice. The tube was spun briefly and the following components were added:

4μl 5x first strand buffer (250mM Tris-HCl, pH 8.3; 375mM KCl; 15mM MgCl$_2$)
2μl 0.1M DTT
1μl 10mM dNTP mix (10mM each dNTPs)

The reagents were mixed well and incubated at 42°C for 2 minutes. Then 1μl (200units) of Superscript II was added and mixed by pipetting up and down. It was then incubated at 42°C for 50 minutes. Then the reaction was inactivated by heating at 70°C for 15 minutes.

The cDNA was diluted in water (HPLC grade) at 1:5 dilution before PCR reactions. The cDNA samples are stored at -70°C.

2.8.3-2   Step II: PCR Reaction

RNAimage Kit (GenHunter) was used as follows:

The components indicated below were defrosted and set on ice. 20μl final volume was used for each primer set combination:

	(μl)
ddH2O	9.7
10xPCR buffer	2.0 [100mM Tris-Cl, pH8.4, 500mM KCl, 15mM MgCl$_2$ and 0.01% gelatin (v/v)].
dNTP (25μM)	1.6
Arbitrary primer (2μM)	2.0
H-T$_{11}$M (2μM)	2.0
RT-mix from Step I (it has to contain the same H-T$_{11}$M used for PCR)	2.0
α-[^{35}S] dATP (1200Ci/mmole)	0.5
AmpliTaq Gold (Perkin-Elmer)	0.2

50μl of oil was added and the PCR was set at 94°C for 10 minutes for one cycle, 94°C for 0.5 minute, 40°C for 2 minutes and 72°C for 0.5 minute for 40 cycles, and 72°C for 5 minutes for one cycle.

The sequences of primers used in differential display:

Arbitrary primers: OPE 16: 5'-GGTGACTGTA-3'

                        OPE17: 5'-CTACTGCCGT-3'

                        OPE18: 5'-GGACTGCAGA-3'

OPE19: 5'-ACGGCGTATG-3'

OPE20: 5'-AACGGTGACC-3'

Oligo-dT primers: H-T$_{11}$A: 5'-AAGCTTTTTTTTTTTA-3'

H-T$_{11}$C: 5'-AAGCTTTTTTTTTTTC-3'

H-T$_{11}$G: 5'-AAGCTTTTTTTTTTTG-3'

2.8.3-3   Step III: 6% (w/v) Denaturing Polyacrylamide Electrophoresis

A 6% (v/v) polyacrylamide sequencing gel (0.4mm thickness) was prepared as follows: 60ml Sequagel-6, 15ml Sequagel Complete (National Diagnostics), and 0.6ml of 10% APS (Ammonium persulfate in dd H$_2$O, BDH). The gel was left polymerize for at least 2 hours before use. The gel was prerun for 1 hour in 1xTBE buffer at 1700v (50mA and 60W). 5µl of PCR product and 3µl of 3x sequencing loading buffer [50% (v/v) glycerol, 1.0mM EDTA, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF] were mixed in a tube. The mixture was denatured at 80°C for 3 minutes and then 6µl was loaded in each lane. The gel was left to run at 1700v for 3 hours.

The gel was placed on 3MM paper and covered with Saran film. It was then dried at 80°C for one and half-hours on a gel drier and left to cool down for another half an hour. The dried gel was exposed to a film overnight and developed. Differential displayed bands were compared and picked for further analysis.

2.8.4   Reamplification of Differentially Displayed Bands

The bands of interest were located by punching through the film with a needle at the four corners of each band of interest. The bands were then cut out with a clean razor blade. The gel slice along with the 3MM paper was soaked in 100µl of double distilled water for at least 4 hours at 37°C. The tube was then boiled for 15 minutes with the cap tightly closed. It was then centrifuged at 13,000 rpm for 2 minutes to collect any condensation and to pellet the gel and paper debris. The supernatant was transferred to a new microfuge tube and 10µl of 3M sodium acetate, 5µl of glycogen (10mg/ml), and 450µl of 100% (v/v) ethanol were added. The DNA was precipitated overnight at –80°C. The DNA was pelleted by spinning at 13,000 rpm at 4°C for 10 minutes. The DNA pellet was rinsed with 200µl of ice-cold 85% (v/v) ethanol and dried and dissolved in 10µl of double distilled water (10µl for each

picked band). Reamplification PCR was performed using the same primer set and PCR conditions except the final dNTP concentration was 200µM. The PCR reaction was set up as follows:

ddH$_2$O	19.6µl
10xPCR buffer	4.0µl
dNTPs(2mM)	4.0µl
H-AP primer (2µM)	4.0µl
H-T$_{11}$M (2µM)	4.0µl
cDNA template	4.0µl
AmpliTaq(Perkin-Elmer)	0.4µl
Total	40µl

2.8.5   Cloning the PCR Products

The PCR products were cloned into pCR-Script Amp SK(+) cloning vector. The purified PCR products were incubated with the predigested pCR-Script Amp SK(+) cloning vector, *Srf I* restriction enzyme, and T4 ligase. Using the restriction enzyme in the ligation reaction maintained a high-steady-state concentration of digested vector DNA. The DNA was also blunt-ended using *Pfu* DNA polymerase to improve cloning efficiency. The ligation efficiency of the blunt-ended DNA fragments was also increased by the simultaneous, opposite reactions of the *Srf I* restriction enzyme and T4 ligase on nonrecombinat vector DNA. *Srf I* is a rare-cutting enzyme that recognises the sequences 5'-GCCCGGGC-3'. The pCR-Script Amp SK(+) cloning vector includes an ampicillin-resistant gene, a *lac* promoter for gene expression and T3 and T7 promoters to facilitate DNA sequencing. A map of the pCR-Script Amp SK(+) cloning vector is shown in Figure 2.4.

FIGURE 2.4

MAP OF pCR-Script Amp SK(+) PLASMID
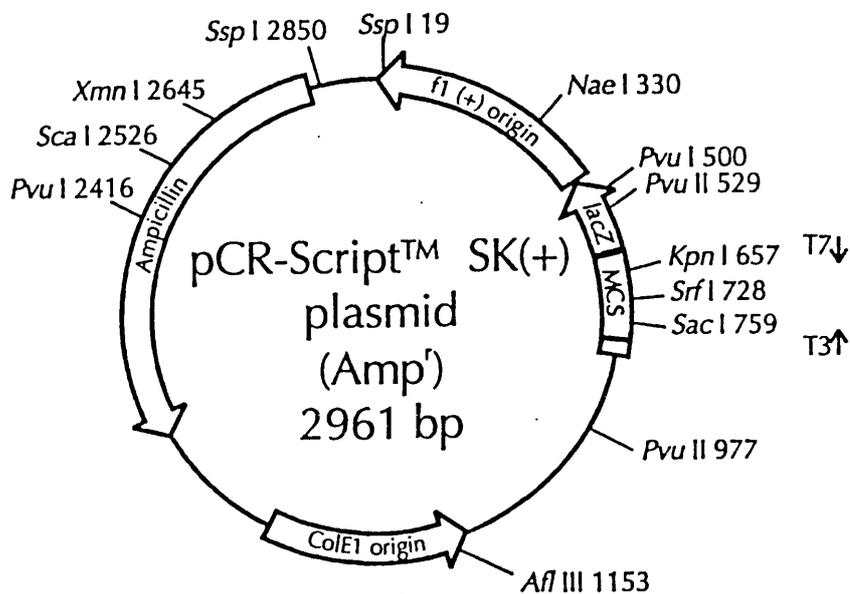
PCR products to be sequenced were polished with *pfu* polymerase before blunt end ligation into *Srf*I digested plasmid. The restriction enzyme *srf*I was included in the ligation reaction to reduce self-ligation of the vector. The annealing positions of the T3 and T7 sequencing primers are indicated.

2.8.5-1   Purification of PCR products

This method purifies the insert selectively by removing excess PCR primers from the PCR products. The PCR product was transferred into a fresh tube and 1/10 volume of 10xSTE (1.0M NaCl, 100mM EDTA, 200mM Tris-Cl, pH 7.5) buffer and an equal volume of 4M ammonium acetate were added. 2.5 volumes of 100% (v/v) ethanol were added to precipitate the PCR product. The tube was centrifuged in a microcentrifuge at 13,000 rpm for 20 minutes at room temperature. The supernatant was carefully removed and the DNA pellet was washed with 200μl of 70% (v/v) ethanol. The pellet was dried at room temperature and resuspended in 25μl of TE buffer (10mM Tris-Cl, 1.0mM, pH 7.4) and kept at 4°C for future use.

2.8.5-2   Polishing the Purified PCR Product by Pfu DNA Polymerase:

The *Pfu* DNA polymerase is a proofreading enzyme with 3'→5' exonuclease activity and after a polishing reaction it ensures a blunt-ended PCR product which enables efficient cloning into the pCRScript Amp SK(+) cloning vector. To prepare the polishing reaction, the following components were added in order to a 0.5ml tube:

purified PCR product	10μl
10mM dNTP mix (2.5mM each)	1μl
10x polishing buffer (provided in the kit)	1.3μl
cloned *Pfu* DNA polymerase (0.5U)	1μl

The polishing reaction was incubated at 72°C in a water bath for 30 minutes. The polished PCR product was either used directly in the cloning reaction or stored at 4°C.

2.8.5-3   Cloning the polished PCR product into pCR-Script Amp SK(+) cloning vector

The polished PCR product was incubated with the predigested pCR-Script cloning vector, *Srf*I, and T4 ligase. To prepare the cloning reaction, the following components were added in order in a 0.5ml tube:

1μl	pCR-Script Amp SK (+) cloning vector (10ng/μl)
1μl	pCR-Script 10x reaction buffer (provided with the enzyme)
0.5μl	10mM rATP
5.5μl	blunt-ended PCR product
1μl	*Srf* I restriction enzyme (5U/μl)

1μl   T4 DNA ligase (4U/μl)

10μl

Two controls were set up, vector only as a negative control and test insert DNA (provided in the kit) as a positive control.

The reaction was mixed gently and incubated at room temperature for 1 hour. It was heated for 10 minutes at 65°C and put on ice for 5 minutes. The reaction mixture was then used for bacterial transformation or stored at 4°C.

Transformation of E Coli

Falcon 2059 polypropylene tubes were used for the transformation. E Coli XL-1 blue MRF⁻ Kan supercompetent cells were thawed on ice. 20μl of the E Coli cells (provided in the kit) was transferred into pre-chilled Falcon tubes. To prepare the transformation reaction, 0.35μl of β-mercaptoethanol (BDH) was added to the E Coli tube to yield a final concentration of 25mM. The transformation reaction was swirled gently and incubated on ice for 10 minutes with mixing every 2 minutes. 2μl of the ligation reaction was added to the transformation reaction tube and incubated on ice for 30 minutes. The transformation reaction was heated in a 42°C water bath for 45 seconds and then put on ice for 2 minutes. 0.45ml of Luria Broth was added to each tube and the bacteria were incubated at 37°C for 1 hour with shaking at 225-250rpm. The transformed bacteria were then plated out onto LB-ampicillin agar plates (50μg/ml) containing X-gal (40μg/ml) and IPTG (30μg/ml). The plates were incubated overnight. Controls plates were set up as follows: bacteria only to test the ampicillin selection procedure and the pUC18 plasmid (provided in the kit) only to test the transformation efficiency.

Analysis of Selected Bacterial Colonies:

The control plates were examined first:

Vector only control:.>90% blue colonies with some white colonies.
Test insert control:.>50% white colonies with blue colonies as background.
Bacteria only control: no colonies.

Toothpicks were used to pick up 30 white colonies from each plate and 10 blue colonies from the vector only control plate. The colonies were transferred onto fresh plates

containing ampicillin, X-gal and IPTG to amplify each picked colony. The plated were incubated at 37°C overnight.

Colony lysis of bacteria

10 white colonies on the amplified agar plates were picked using a yellow tip and put in 50µl of lysis buffer [1% (v/v) Tween20 in PBS] in 1.0ml tubes. The picked bacterial colonies were marked on both the agar plates and the lysate tubes. The mixture was boiled for 10 minutes and spun in a microfuge for 1 minute at 13,000 rpm. The lysate was ready to use for PCR amplification.

PCR amplification of colony lysates

A cDNA insert in the pCR-Script vector (carrying T3 and T7 cloning sites) from a lysed bacterial colony was amplified by PCR reaction as follows:

Master Mix for one reaction	µl
ddH$_2$O	11.8
10xbuffer	2
2mM dNTPs	2
2µM T3	1
2µM T7	1
AmpliTaq(Perkin Elmer)	0.2
Colony lysate	2

PCR cycles: 94°C   5 minutes, for one cycle,

94°C   30sec.
55°C   1minute.
72°C   2 minutes for 30 cycles,

72°C   5minutes for one cycle.

5 µl of the PCR product was loaded on 1% (v/v) agarose TBE mini gel with a molecular marker and a positive cDNA insert control to view the PCR product.

Minipreparation of the transformed bacteria

The colonies which had the correct size PCR product were grown in 10ml LB containing ampicillin (50µg/ml) overnight at a 37°C shaker at 200rpm. The plasmid DNA was isolated using the Qiagene miniprep kit (QIAprep 8 miniprep kit, Cat No. 27144) and used to set up sequencing reaction PCR.

2.8.6   Sequencing the Cloned PCR Product

The chain-termination method involves the synthesis of a DNA strand by a DNA polymerase using a single-stranded DNA template. The pCR-Script containing cloned DNA fragments were sequenced using T3 and T7 as primers. The sequencing reaction is initiated at one site of the cloned DNA fragment where the T3 or T7 primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analogue that will not support continued DNA elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalysed polymerisation will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information. A radioactively labelled ^{35}S-dATP is included in the synthesis, so the labelled chains of various lengths can be visualised by autoradiography after separation by high-resolution electrophoresis. Each set of sequencing data was derived from three identical sequences of one template. Each template was sequenced from both sense and reverse directions.

T3 primer: 5' AATTAACCCTCACTAAAGGG 3'
T7 primer: 5' GTAATACGACTCACTATAGGGC 3'

Manual sequencing: (Sequenase Version 2.0 DNA sequencing Kit, Amersham Life Science)

The double stranded plasmid DNA was denatured by adding 0.1 volumes of 2M NaOH, 2mM EDTA and incubating 30 minutes at 37°C. The mixture was neutralised by adding 0.1 volumes of 3M sodium acetate (pH 4.5) and the DNA was precipitated with 2 volumes of 100% (v/v) ethanol and left at -70°C for 15 minutes. The DNA was pelleted by spinning at 13,000 rpm for 20 minutes and washed once with 70% (v/v) ethanol. The air-dried pellet was dissolved in 20-30µl of ddH$_2$O. The DNA was sequenced following the manufacturers instructions.

Automatic sequencing:

The double stranded plasmids containing inserted fragments were sequenced using dRhodamine terminator cycle by Department of Surgery, University College London.

2.8.7   Reverse Transcription PCR

2.8.7-1   Reverse Transcription

1μg of DNA free RNA was used in each reverse transcription experiment and the experimental details were followed as described in 2.8.3-1 except that the oligo-dT primers used were a mixture of 20μM of each oligo-dT$_{11}$ G, A,C.

2.8.7-2   PCR Amplification of RNA Transcripts

Standard PCR conditions were used as follows: 200nM of each primer, 100μM of each dNTP, 1.0 unit of Taq DNA polymerase (Perkin Elmer) and 1.0μl of RNA transcripts were amplified at 94°C 5 minutes for one cycle, 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes for 16-30 cycles, and finally 72°C 5 minutes for 1 cycle. The total volume PCR reaction volume was 50μl and 5μl of PCR product was electrophoresed.

The actin gene was amplified as an internal control. The degree of amplification was judged by eye and the cDNA samples adjusted to ensure equal loading of template in the following analytical PCR reactions.

The PCR cycles were also optimised to ensure that the PCR reaction was terminated during the exponential phase of the reaction. The number of PCR cycles used for the actin and TC1 was 24.

The sequences of the primers are listed below. PCR products were electrophoresed and photographed as described previously.

Primer	Sequence
TC1	
Sense	5'-TAAACTGGCCAGCTTCACATC-3'
Antisense	5'-CATTTGGCTTTATGTCCCGAG -3'
Actin	
Antisense	5'-GCCGAGCGGGAAATCGTGCGTG-3'
Sense	5'-CGGTGGACGATGGGAGGGGAAG-3'

Chapter 3

COMPLEMENTATION ANALYSIS OF TESTICULAR GERM CELL TUMOUR CELLS

3.1   INTRODUCTION

Complementation analysis was first used to determine the number of genes involved in DNA-repair disorders such as xeroderma pigmentosum (XP) and Fanconi's anaemia (FA) (de Weerd-Kastelein *et al.*, 1972; Duckworth-Rysiecki *et al.*, 1985). Cell lines derived from XP or FA patients were fused. Hybrids that had acquired DNA repair capacity, as shown by the acquisition of unscheduled DNA synthesis, were complemented and thus were assigned to different complementation groups. Where there was no complementation, this suggested that the two cell lines had the same mutation and belonged to the same complementation group (Giannelli *et al.*, 1982; Buchwald, 1995). Using this approach, a number of complementation groups involved in DNA repair in human cells have been defined and some of the genes controlling each of the complementation groups have been identified (Sancar, 1995; Lehmann, 1995).

Testicular germ cell tumours are hypersensitive to cisplatin-based combination chemotherapy in the clinic. Cell lines derived from testicular tumours are similar to cells derived from patients with xeroderma pigmentosum in their relative sensitivity to DNA-damaging agents (Pera *et al.*, 1987; Walker *et al.*, 1987; Parris *et al.*, 1988; Masters *et al.*, 1993), and in their low capacity for repair of DNA damage (Bedford *et al.*, 1988; Koberle *et al.*, 1996).

In this study, we planned to use complementation analysis to determine how many complementation groups control the sensitivity of testicular tumour cells to cisplatin. We had already shown that complementation can occur when we fused testicular tumour cells with a cisplatin-sensitive cell line derived from a different cell type (Wang, *et al.*, 1996). Hybrids between testicular tumour cell lines and a cisplatin-sensitive HeLa subline were more resistant to cisplatin than both parental cell types. This suggests that testicular tumour cells can be complemented by cisplatin-sensitive cell lines when different mechanisms are responsible for cisplatin sensitivity. In this study, we fused four testicular tumour cell lines with each other to determine how many complementation groups control the sensitivity of testicular tumour cells to cisplatin. The parentage of each hybrid was confirmed by karyotyping chromosome analysis and PCR amplification of VNTR (Variable Number of Tandem Repeats) regions. The sensitivities of the hybrids to cisplatin were compared to those of the parental cells.

3.2   RESULTS

3.2.1   Generating Sublines with Selectable Markers

Four testicular germ tumour cell lines: namely, SuSa, GH, 833K and GCT27 were used in this study. Firstly they were transfected independently with pSV2NEO (conferring neomycin, G418 resistance) and pBABE (conferring puromycin resistance) to generate selective markers for the fusion. A total of eight transfected cell lines were generated (see Table 3.1).

Calcium phosphate mediated transfection method was used for generating transfectants. Conditions such as DNA concentration, time of exposure, expression period, time of expression and serum concentration were modified to increase transfection efficiency. The highest transfection efficiency achieved in this study was approximately 1 in 50,000 cells with modified conditions. The transfected cell lines were given the suffix ^{P} or ^{N} for cells transfected with genes conferring puromycin or neomycin (G418) resistance respectively.

The neomycin and puromycin resistant sublines derived from the same cell line had similar cisplatin sensitivities (see Table 3.1, $P>0.05$). For example, the cisplatin IC_{50} concentration (the concentration required to inhibit colony-formation by 50% of untreated controls) of S^N was 17.4ng/ml and was 18.2ng/ml for S^P. However, the G^N line appeared to show more resistance ($IC_{50} = 22.8$ng/ml) to cisplatin than G^P ($IC_{50} =17.4$ng/ml), but this was not statistically significant ($P>0.05$, two-sided student t test). A summary of the cisplatin sensitivities of the transfected cell lines is shown in Table 3.1.

3.2.2   Generation of Cell Hybrids of Four Testicular Tumour Cell Lines

The four testicular tumour cell lines were fused with each other, generating 16 hybrids as summarised in Table 3.2. The cell lines (e.g. S^N and 8^P) were co-plated and fused using 50% PEG. The hybrids were selected in both G418 (200μg/ml) and puromycin (1.0μg/ml). Hybrid colonies were visible 14 days after the fusion. The same drug concentrations killed the control parental cells within 10 days. Using optimized hybridization conditions, approximately 50 hybrid colonies were obtained from $2x10^6$ co-plated cells. Each hybrid clone was expanded from a single hybrid colony.

TABLE.3.1

**SUMMARY OF CISPLATIN SENSITIVITIES OF TGCT CELL LINES
TRANSFECTED WITH SELECTABLE MARKERS**

Cell Lines	$IC_{50} \pm SD$ (ng/ml cisplatin)
S^P	18.2 ± 5.0
S^N	17.4 ± 7.3
8^P	16.3 ± 1.5
8^N	14.5 ± 2.8
G^P	17.4 ± 2.7
G^N	22.8 ± 2.8
C^P	17.6 ± 5.8
C^N	11.8 ± 4.0

The concentration required to inhibit colony-formation by 50% of untreated controls (IC_{50}) was determined from 3 separate experiments. The results are the means $\pm$ SD.

TABLE 3.2

HYBRIDS GENERATED BY FUSING NEOMYCIN AND PUROMYCIN RESISTANT TESTICULAR TUMOUR CELL LINES

Parental Testicular Cell Lines	S^N	8^N	G^N	C^N
S^P	S^PS^N S^PS^N-1	S^P8^N	S^PG^N	S^PC^N
8^P	8^PS^N 8^PS^N-1	8^P8^N	8^PG^N	8^PC^N
G^P	G^PS^N	G^P8^N G^P8^N-1	G^PG^N	G^PC^N
C^P	C^PS^N	C^P8^N	C^PG^N	C^PC^N

Neomycin (G418, e.g. S^N) and puromycin resistant (e.g. S^P) testicular tumour cell lines were plated and fused by exposing to 50% PEG for 1 minute. The hybrids were selected in both G418 (200μg/ml) and puromycin (1.0μg/ml). One hybrid clone from each fusion was expanded for future studies. The three repeated hybrids were given THE names S^NS^P-1, S^P8^N-1 and G^P8^N-1.

Self-crosses (e.g. $C^P \times C^N$) were used to control for the effects of gene dosage. Reverse crosses (e.g. $C^P \times 8^N$ and $C^N \times 8^P$) provided internal controls for consistency of complementation and to exclude any influence of the selective marker on individual hybrids. In three cases where inconsistent data were obtained, the cell fusion was repeated. An additional hybrid was cloned and given the appendix -1. The three repeats were: (1) the self-cross between S^N and S^P, designated $S^N S^P$-1, (2) the hybrid between $S^P \times 8^N$, designated $S^P 8^N$-1 and (3) the hybrid between $G^P \times 8^N$, designated $G^P 8^N$-1.

3.2.3   Karyotyping Analysis of Somatic Cell Hybrids

In order to investigate if there was loss of chromosomes during cell fusion, the chromosome numbers of all the parental cell lines, the self-crosses and one each of the duplicate hybrids were analyzed (see Table 3.3).

The chromosome numbers in at least 15 metaphase cells were determined. The modal chromosome numbers were chosen as the most frequently appearing number among the metaphase cells analyzed. The mean chromosome number of each cell line was close to its modal number. For example, the mean chromosome number of G^N was 54.8 and the modal number was 55. The percentage of parental chromosome numbers of the hybrids were calculated as follows: number of chromosomes in hybrid cell line ÷ number of chromosomes in both parental cell lines x100.

The neomycin and puromycin resistant transfectants derived from the same cell line had similar chromosome numbers (e.g. G^N=55, G^P=56), except that 8^N had a modal chromosome number of 52, whereas 8^P had 57. The hybrids appeared to retain the majority of chromosomes from both parents, with percentages ranging from 88.0% to 106.5%.

3.2.4   PCR Analysis of VNTR Regions in the Cell Hybrids

In order to confirm the origin of the hybrids, two polymorphic VNTR loci (pYNZ22 and D1S80) on chromosome 17p13.3 and chromosome 1p36.31 were amplified respectively. The two regions chosen in this study are highly polymorphic, with heterozygosity in excess of 80% in unrelated individuals (Horn *et al.*, 1989; Budowle *et al.*, 1991).

The PCR products amplified from these two loci in the parental cell lines and their hybrids are summarized in Table 3.4. A representative gel is shown in Figure 3.1. The products of

TABLE 3.3

CHROMOSOME ANALYSIS OF TESTICULAR TUMOUR CELL LINES AND THEIR HYBRIDS.

Cell Line	Range Chromosome Numbers	Mean Number Chromosomes ± SD	Modal Chromosome Numbers	% Parental Chromosome Numbers
TRANSFECTED PARENTS				
G^N	53 - 56	54.8 ± 1.0	55	
G^P	49 - 56	53.1 ± 3.1	56	
S^N	43 - 54	47.1 ± 3.5	46	
S^P	43 - 48	45.7 ± 1.6	47	
8^N	49 - 58	52.1 ± 3.6	52	
8^P	51 - 61	57.1 ± 2.9	57	
C^N	56 - 57	56.3 ± 0.7	56	
C^P	48 - 59	56.2 ± 4.4	59	
SELF-CROSSES				
$G^N G^P$	103 - 107	105.2 ± 1.6	103	92.8
$S^N S^P$	93 - 97	94.6 ± 1.8	95	102.1
$S^N S^P$-1	95 - 102	99.4 ± 2.0	99	106.5
$8^N 8^P$	93 - 99	95.6 ± 2.2	96	88.0
$C^N C^P$	103 - 110	105.5 ± 1.6	103	92.8
HYBRIDS				
$G^P C^N$	101 - 110	104.5 ± 2.8	106	94.6
$G^N S^P$	95 - 97	95.8 ± 1.5	96	94.1
$G^P 8^N$	106 - 112	109.4 ± 2.0	108	100.0
$G^P 8^N$-1	103 - 109	106.7 ± 1.7	107	99.1
$S^P 8^N$	97 - 101	99.1 ± 1.2	99	100.0
$S^P 8^N$-1	98 - 105	101.2 ± 2.0	101	102.2
$8^N C^P$	99 - 112	108.5 ± 4.4	110	99.0
$C^N S^P$	94 - 99	96.0 ± 1.8	95	92.2

TABLE 3.4.

SUMMARY OF PCR PRODUCTS AMPLIFIED IN THE PARENT AND HYBRID CELL LINES, SHOWING THE NUMBER OF REPEATS AT EACH ALLELE

CELL LINES	PYNZ22 REPEATS	D1S80 REPEATS
PARENTAL LINES		
GH	8, 4	17
SuSa	4, 3	18, 15
833K	5, 4	18, 15
GCT27	10	18, 16
SELF-CROSSES		
$G^N G^P$	8, 4	17
$S^N S^P$	4, 3	18, 15
$S^N S^P$-1	4, 3	18, 15
$8^N 8^P$	5, 4	18, 15
$C^N C^P$	10	18, 16
HYBRIDS		
$G^P C^N$	10, 8, 4	18, 17, 16
$G^N S^P$	8, 4, 3	18, 17, 15
$G^P 8^N$	5, 4	18, 17, 15
$G^P 8^N$-1	8, 5, 4	18, 17, 15
$S^P 8^N$	5, 4, 3	18, 15
$S^P 8^N$-1	5, 4, 3	18, 15
$8^N C^P$	5, 4	18, 16, 15
$C^N S^P$	4, 3	18, 16, 15

Two VNTR regions were amplified by PCR. The molecular weights of the products were estimated from the agarose gel by comparison to the standard markers. The number of repeats were calculated according to the criteria in Horn *et al.,* (1989) and Budowle *et al.,* (1991).

FIGURE 3.1

SEPARATION OF PCR PRODUCTS FOLLOWING AMPLIFICATION OF VNTR REGIONS IN TESTICULAR TUMOUR CELL LINES AND THEIR HYBRIDS
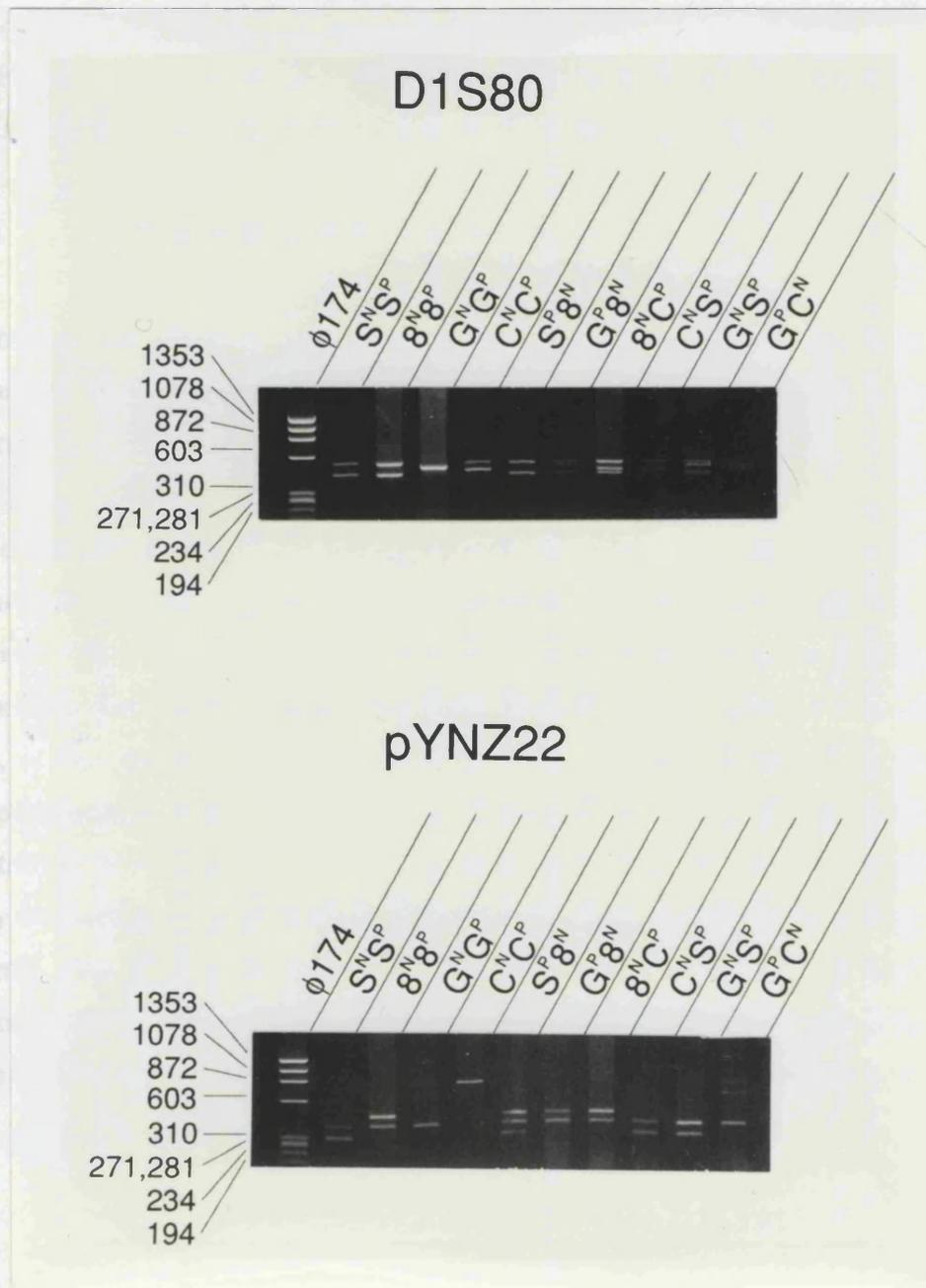
Photograph of agarose gels following electrophoresis of PCR products obtained using primers directed to the (top) D1S80 locus (on chromosome 1p) and (bottom) pYNZ22 locus (chromosome 17p) of self-crosses (S^NS^P, 8^N8^P, G^NG^P and C^NC^P) and representatives of each cross, demonstrating that the hybrids contain markers of both parental cells. The numbers on the left indicate the size of the marker bands (in base pairs) in the left-hand lanes.

the pYNZ22 region in these cell lines ranged from 310bp to 870bp, representing 3 to 10 repeats of 70bp per repeat. The products of the D1S80 region in these cell lines were in the range 310bp to 603bp, representing 15 to 18 repeats of 30bp per repeat (see Table 3.4). All the hybrids had bands corresponding to those of their parent cell lines. For example, on pYNZ22 locus, hybrid S^P8^N had three bands corresponding to the self-crosses S^NS^P and 8^N8^P (see Figure 3.1). However, the hybrids 8^NC^P, C^NS^P had lost one pYNZ22 allele of GCT27, and G^P8^N had lost one of the pYNZ22 alleles of GH (see Table 3.4).

3.2.5 Cisplatin Sensitivity

To measure the cisplatin sensitivities of the hybrids cells, colony forming assays were performed. The cisplatin sensitivities of the parent cell lines and their hybrids are shown in Figure 3.2 and Table 3.5.

One of the self-crosses (S^NS^P) was approximately 3 times more resistant (IC_{50}=58.4ng/ml), as measured by the IC_{50} value, than its parental sublines (IC_{50}=17.4ng/ml, 18.2ng/ml). However, when this cross was repeated (S^NS^P-1), the hybrid was similar in its sensitivity to the parental line (IC_{50}=13.2ng/ml, see Table 3.5). Of the 12 hybrids between different cell lines, 10 were similar in cisplatin sensitivity to their parental cell lines, indicating a lack of complementation. While the first cross between G^P and 8^N (G^P8^N) was nearly 2x more resistant (IC_{50}=37.2ng/ml) than the parental cell lines G^P (IC_{50}=17.4ng/ml) and 8^N (IC_{50}=14.5ng/ml), a second hybrid (G^P8^N-1, IC_{50}=21.8ng/ml) was similar in sensitivity to the parental cells. The hybrid generated between S^P and 8^N was relatively resistant in two separate fusions (S^P8^N, IC_{50}=43.5ng/ml and S^P8^N-1, IC_{50}=34.9ng/ml). However, the reverse cross (S^N8^P, IC_{50}=12.9ng/ml) was similar in sensitivity to the parental lines.

FIGURE 3.2 CISPLATIN SENSITIVITIES OF TESTICULAR HYBRID CELL LINES
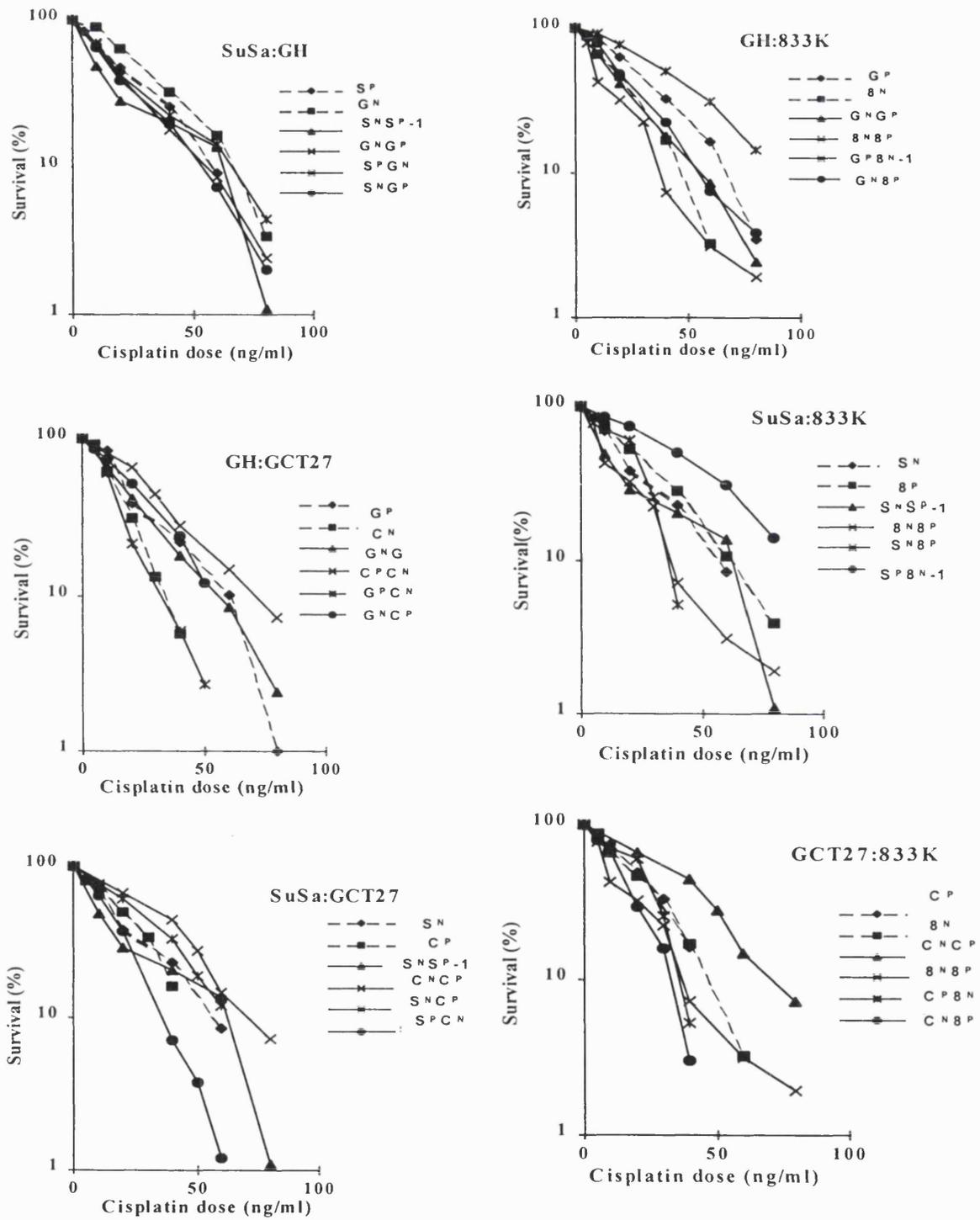
Dose-response curves obtained by clonogenic assay following continuous exposure of the six possible crosses between the four testicular tumour cell lines to a range of concentrations of cisplatin. Each graph shows the surviving fractions of the parental cells (dashed lines) transfected with genes conferring resistance to G418 (appended [N]) and puromycin (appended [P]), control self-crosses and hybrids (continuous lines).

TABLE 3.5

SUMMARY OF THE SENSITIVITIES TO CISPLATIN AND THE COLONY FORMING ABILITY OF PARENTAL AND HYBRID CELL LINES.

CELL LINES	IC50 ± SD (ng ml^{-1} CISPLATIN)	COLONY FORMING ABILITY (% ± SD)
<u>PARENTAL LINES</u>		
G^N	22.8 ± 2.8	13.5 ± 7.3
G^P	17.4 ± 2.7	3.3 ± 0.9
S^N	17.4 ± 7.3	6.2 ± 0.5
S^P	18.2 ± 5.0	6.0 ± 0.6
8^N	14.5 ± 2.8	5.2 ± 2.4
8^P	16.3 ± 1.5	9.3 ± 4.4
C^N	11.8 ± 4.0	7.2 ± 0.9
C^P	17.6 ± 5.8	16.1 ± 4.0
<u>SELF-CROSSES</u>		
$G^N G^P$	16.7 ± 0.5	3.7 ± 0.4
$S^N S^P$	58.4 ± 5.2	9.1 ± 0.6
$S^N S^P$-1	13.2 ± 3.7	5.0 ± 2.1
$8^N 8^P$	10.3 ± 2.1	6.3 ± 0.9
$C^N C^P$	27.3 ± 5.8	7.4 ± 1.7
<u>HYBRIDS</u>		
$G^P C^N$	10.6 ± 3.2	9.8 ± 3.8
$G^N C^P$	20.3 ± 2.2	7.7 ± 0.7
$G^N S^P$	16.1 ± 1.9	7.4 ± 0.1
$G^P S^N$	18.4 ± 4.4	1.8 ± 1.2
$G^P 8^N$	37.2 ± 5.9	9.8 ± 5.3
$G^P 8^N$-1	21.8 ± 5.6	4.9 ± 1.0
$G^N 8^P$	19.2 ± 5.9	5.1 ± 2.8
$S^P 8^N$	43.5 ± 7.7	12.5 ± 4.6
$S^P 8^N$-1	34.9 ± 10.9	10.3 ± 0.9
$S^N 8^P$	12.9 ± 1.9	4.6 ± 3.6
$8^N C^P$	14.4 ± 3.0	1.7 ± 0.4
$8^P C^N$	11.4 ± 0.6	7.0 ± 2.0
$C^N S^P$	12.6 ± 2.6	10.6 ± 3.0
$C^P S^N$	23.1 ± 7.7	6.9 ± 2.9

3.3   DISCUSSION

In this study, cell fusions were performed between four human testicular tumour cell lines and 16 hybrids were generated. The origin of the hybrids was confirmed by karyotyping and PCR analysis of VNTRs. Karyotyping analysis showed that the hybrids contained chromosome numbers similar to the sum of the numbers of chromosomes in the parental lines, indicating that there was little or no loss of genetic material as a result of cell fusion. PCR analysis suggested that in a small proportion of the hybrids one of the VNTR repeats was lost, as previously observed (Wang *et al.*, 1996). However, in every case the bands obtained from the hybrid cells matched those of the parental cells, and as two independent loci were examined it was possible to confirm the origin the hybrids.

Had complementation occurred, we would have expected the hybrids to be more resistant than the parental cells, as shown in our previous study when testicular cells were fused with a cisplatin sensitive cell line $D98^{OR}$ (Wang *et al.*, 1996). In these previous studies, the ratios of the IC_{50}s of the hybrid to the parental testicular lines ranged from 2.1 to 6.8 fold. Cisplatin IC_{50}s ranged from 43.7 to 146 ng/ml for the hybrids, compared to 20.6 to 21.4 ng/ml for the 3 testicular tumour cell lines (SuSa, 833K, GH). These earlier data (Wang *et al.*, 1996) provide a positive control for the present study, indicating how complementation can occur when the mechanisms controlling sensitivity to cisplatin differ.

The fusions in this study were made between cell lines containing a dominant selectable marker. The transfected sublines retained their hypersensitivity to cisplatin, indicating that neither transfection nor the presence of the dominant selectable marker influenced drug sensitivity (data not shown). Self-crosses were used to control for the influence of gene dosage on drug sensitivity. In one self-cross ($S^{P}S^{N}$) the resulting hybrids were resistant to cisplatin, both in the uncloned parent population and in clones isolated from this population (data not shown). The anomalous result was therefore not due to clonal variation. However, on repeating the fusion, the cloned hybrid cells ($S^{P}S^{N}$-1) were similar to the parental cells in their sensitivity to cisplatin. It is possible that the cisplatin resistance in the first fusion was due to a mutation or genetic loss in the hybrid cells, but further analysis would be necessary to identify the cause of the resistance. All the other three self-crosses gave hybrids with cisplatin sensitivities similar to the parent cells.

All four testicular tumour cell lines were hybridized with one another in all possible combinations. Consequently, for each cross, two pairs of cell lines were generated containing the opposite selection markers. If the genetic basis of sensitivity had been different in one of the cell lines, this could have been confirmed in two ways. Firstly, the matched hybrid with the reverse selection markers should also have been resistant. Secondly, all hybrids generated between this cell line and all other lines with a different mechanism controlling cisplatin sensitivity should have been relatively resistant. Neither of these patterns were observed, and with two exceptions all the hybrids were similar in cisplatin sensitivity to the parental cells, indicating that complementation had not occurred. The implication of these data is that a single or common genes control the sensitivity of all 4 testicular tumour cell lines to cisplatin.

Although consistent evidence of complementation was not observed, two clones were generated that were relatively resistant. Consequently these fusions were repeated and independent clones generated. In one case (G^P8^N), the second clone was sensitive to cisplatin. The first clone (G^P8^N) may have contained a mutation or genetic loss as previously suggested for S^NS^P, but further work would be necessary to determine the mechanism of resistance. In the second case (S^P8^N), the second independent clone was also more resistant to cisplatin, although the reverse cross (S^N8^P) was sensitive. It seems unlikely that this anomalous result was generated by chance on two separate occasions, and it is possible that one of the parental lines contained a mutation which could result, under appropriate circumstances, in cisplatin resistance. Both the hybrids showing the anomalous resistance contained 8^N, and it is conceivable that the neomycin gene had been incorporated in the host DNA at a site that could result in aberrant gene expression.

The data in this study showed that the hybrids between four testicular cancer cell lines had similar sensitivity to cisplatin as their parents. This indicates that there was no complementation between the four testicular cancer cell lines studied. It is therefore possible that the sensitivity of these cell lines to cisplatin could be accounted for by a single mechanism.

Chapter 4

TRANSFECTION OF A HUMAN cDNA LIBRARY INTO A MOUSE EMBRYONAL CARCINOMA CELL LINE

4.1   INTRODUCTION

The aim of this study was to isolate genes conferring cisplatin resistance by using functional expression cloning.

Functional cloning has been used to clone several human DNA repair genes (Hoeijmakers and Bootsma, 1992; Jeggo *et al.*, 1994). This approach is based on the 'correction' of repair deficient mutants by the transfer of normal genes from repair competent cells. Transfectants that have incorporated the 'correcting' gene are selected from the transfected mutant population by treatment with UV light or DNA-damaging agents. The selection step is facilitated when the genomic DNA from the normal cells is linked to vectors containing a dominant selective marker. This strategy was successfully used in repair defective mutants of Chinese hamster ovary cells (CHO) (see Table 4.1). For example, the human excision repair gene, ERCC5, was isolated by co-transfection of human genomic DNA with a dominant selective marker plasmid pSV2gpt [conferring mycophenolic acid (MPA) resistance] into a DNA repair defective (CHO) line, UV135 (Mudgett and McInnes, 1990). The transfectants were selected first for MPA resistance, and then for DNA repair proficiency. MPA and UV light resistant primary transfectants were isolated and secondary transfections were performed by transfecting genomic DNA of the primary transfectants into the UV135 cell line. The human ERCC5 gene was cloned by probing for human-specific repeat sequences in the CHO transfectant.

Functional cloning using a genomic library has the disadvantage that a large number of cells have to be transfected because the library contains large numbers of DNA fragments. Also, the size of genomic DNA fragments is large (approximately 40-50kb), making transfection technically difficult. In addition, most of the genomic libraries available do not contain a selectable marker, and therefore co-transfection is necessary, reducing the efficiency of this approach. As the transfected DNA is integrated into the host genome, isolation of the DNA of interest can be difficult.

The goal of the work described in this chapter was to clone genes conferring cisplatin resistance to testicular tumour cells. In order to achieve this aim, the plan was to transfect a human cDNA library into a mouse embryonal carcinoma cell line, F9. This cell line has a sensitivity to cisplatin similar to that of human testicular tumour cells (Oosterhuis *et al.*,

TABLE 4.1

SUMMARY OF TRANSFECTION STRATEGIES USED IN CLONING HUMAN DNA REPAIR GENES.

Gene	Gene Size (kb)	Cloning Strategy	Reference
ERCC1	15-17	Transfection of Hela genomic DNA ligated to pSV3gptH marker into CHO 43-3B repair mutant cells	Westerveld *et al.*, (1984)
XPDC	20	Co-transfection of human-hamster hybrid genomic DNA with pSV2NEO marker into CHO UV5 mutant cells	Weber *et al.*, (1990) Flejer *et al.*, (1992) Johnson *et al.*, (1992)
XPBC	45	Transfection of Hela genomic DNA ligated to pSV3gptH marker into CHO 27-1 mutant	Weeda *et al.*, (1990a) (1990b), Weeda *et al.*, (1991) Schaeffer *et al.*, (1993)
XPG/ ERCC5	32	Co-transfection of human skin fibroblast genomic DNA with pSV2gpt marker into CHO UV135 mutant.	Mudgett and McInnes (1990) Scherly, *et al.*, (1993) O'Donovan *et al.*, (1993) MacInnes *et al.*, (1993)
ERCC6	85	Co-transfection of Hela genomic DNA library with pSV3gptH marker into CHO UV61 mutant cells	Troelstra *et al.*, (1990) Troelstra *et a.l*, (1992)
XPAC	25	Human XP cells were co-transfected with mouse genomic library and a pSV2gpt marker.	Tanaka *et al.*, (1990) Miura *et al.*, (1991) Bankmann *et al.*, (1992)
XPCC	24	Human XP cells were transfected with a pEBS7 based human cDNA library containing a hygromycin-resistant marker	Legerski and Peterson, (1992), Li *et al.* (1996).
FACC	4.5*	Human FA cells (HSC536) were transfected with pREP4 based human cDNA library containing a hygromycin-resistant marker	Strathdee *et al.*,(1990), Barnes *et al.*, (1992).

* Size of the mRNA.

1984; Pera *et al.*, 1987). This mouse cell line was used in preference to human testicular tumour cell lines because F9 cells have a much higher rate of stable DNA transfection (personal experience). A human cDNA library, constructed from mRNA isolated from HT1080 cells (a human fibrosarcoma cell line), was used. This library was used because, unlike most commercially available cDNA libraries, the vector contains a neomycin gene that allows G418 to be used to select the positive transfectants.

Before transfection of the HT1080 cDNA library preliminary work was carried out to determine if the fusion of HT1080 cells with sensitive F9 cells resulted in cisplatin resistance in the hybrids. If the resulting hybrids between these two cell lines showed more cisplatin resistance than F9 cells, this would suggest that genes from HT1080 cells (and therefore possibly from the cDNA library as well) could confer resistance to cisplatin in F9 cells.

As there are 200,000 clones in the HT1080 cDNA library preliminary work was done to optimize the transfection conditions. In order to transfect the entire library into F9 cells, I aimed to obtain a transfection efficiency of at least 1 in 5000 (0.02%). At this efficiency it would be possible to transfect the 200,000 clones using 10^9 F9 cells. Four transfection methods with various modifications were tested using F9 cells in order to achieve high transfection efficiencies. After the optimization of transfection experimental procedures, a transfection efficiency was achieved at 10 times higher than was aimed for.

A cDNA library generated from HT1080 cells was then used to transfect F9 cells using the optimized transfection method and conditions. A series of primary and secondary transfections were performed. As the cDNA library was cloned into a pcDNAI/NEO vector which contains a *neo* marker (conferring G418 resistance), the primary stable transfectants were selected first for resistance to G418 and then to cisplatin. Five cisplatin-resistant primary transfectants were isolated. Secondary transfections were also performed using genomic DNA isolated from the cisplatin-resistant primary transfectants and G418 resistant secondary transfectants were isolated. However, none of them conferred cisplatin resistance in the secondary transfectants. The following diagram outlines the strategy used in this chapter:

Fusion of F9 and HT1080 cells

Demonstration of partial dominance of cisplatin resistance in the hybrids

↓↓

Modification of transfection procedures to achieve high efficiency in F9 cells

↓↓

Transfection of HT1080 cDNA library into F9 cells and selection of primary cisplatin resistant transfectants

↓↓

Secondary selection of primarily isolated transfectants

↓↓

Secondary transfection using genomic DNA from cisplatin resistant primary transfectants

↓↓

Characterization of secondary transfectants

4.2   RESULTS

4.2.1   Cisplatin Sensitivity of F9 and HT1080 Cell Hybrids

Generation of transfectants

Selectable markers were introduced into F9 and HT1080 cell lines in order to permit hybrid selection. F9 cells were transfected with the plasmid pBABE, which confers puromycin resistance, and HT1080 cells were transfected with pSV2NEO, which confers neomycin (G418) resistance.

The transfection was performed using the calcium phosphate method. Unlike human testicular tumour cells, F9 cells were able to tolerate overnight incubation with plasmid DNA and calcium phosphate. The transfection efficiency of F9 cells was approximately 1 in 5000 cells with 1μg/ml plasmid DNA (approximately 10x higher than human testicular tumour cells with a 6-hour incubation). Overnight incubation with DNA-calcium phosphate precipitation caused more than 90% cell death in HT1080 cells. However, with a 6-hour incubation, this cell line had a similar transfection efficiency to F9 cells. The transfectants were expanded to generate F9pBABE and HT1080pSV2NEO cell lines. F9pBABE transfectants grew in 1.0μg/ml puromycin and HT1080pSV2NEO transfectants grew in 600μg/ml G418. At these concentrations all the parental cells were killed by G418 within 10 days and by puromycin within 5 days of exposure.

Transfection of the selectable markers did not alter cisplatin sensitivity significantly in these two cell lines (see Table 4.2 and Figure 4.1). F9pBABE showed a decreased colony forming efficiency (CFE) when compared to F9 cells ($P<0.05$, student t test). Compared to F9 cells, HT1080 cells had a low CFE and it was difficult to perform colony-forming assays. Single HT1080 cells attached to the culture surface when plated but only a small percentage (2.6 ± 1.0%) grew to form colonies. HT1080pSV2NEO had similar CFEs and cisplatin sensitivity to the untransfected HT1080 cells, as shown in Table 4.2.

TABLE 4.2

COMPARISON OF THE CISPLATIN SENSITIVITY OF THE PARENTAL CELL LINES AND THEIR TRANSFECTANTS

Cell Lines	$IC_{50} \pm SD$ (ng/ml cisplatin) and P values	Colony Forming Efficiency (% ± SD) and P values
F9	28.6 ± 6.1	62.5 ± 3.3
F9pBABE	15.9 ± 5.7	23.6 ± 4.9
	P= 0.057	P= 0.018
HT1080	87.8 ± 18.7	2.6 ± 1.0
HT1080pSV2NEO	115.4 ± 15.5	3.3 ± 0.6
	P= 0.118	P= 0.35
HF	70.6 ± 4.4	5.7 ± 1.9
HFc6	70.6 ± 13.8	4.7 ± 0.1
HFc8	58.3 ± 6.8	7.8 ± 1.8

The concentration required to reduce colony-formation to 50% of that of untreated controls (IC_{50}) was determined from 3 separate experiments.

FIGURE 4.1

CISPLATIN SENSITIVITY OF F9 AND HT1080 CELL LINES AND THEIR TRANSFECTANTS
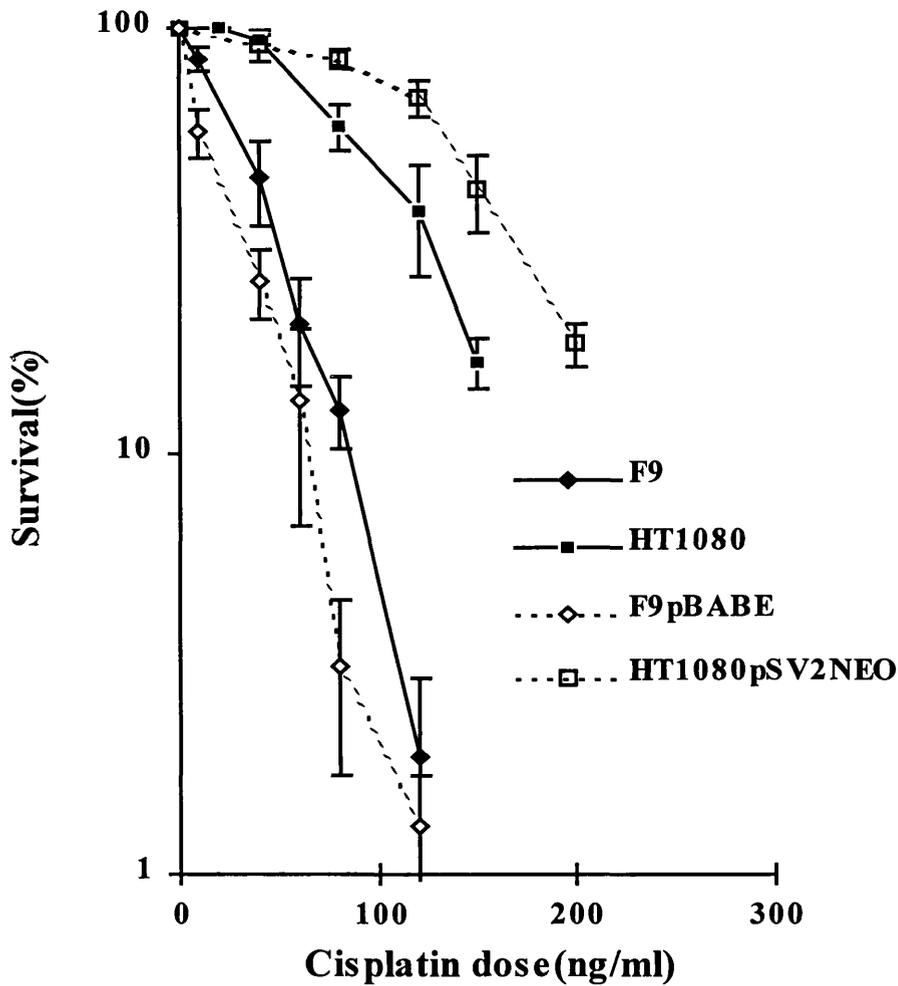
Dose response curves obtained by clonogenic assay following continuous exposure of F9 and HT1080 cell lines and their pBABE and pSV2NEO transfected cell lines to a range of concentrations of cisplatin. Results are expressed as a percentage of colony formation in untreated controls (means ± SEM). Each graph shows the surviving fractions of the parental cell lines (continuous lines) and transfected cell lines (dashed lines).

Fusion of HT1080 and F9 cells

F9pBABE and HT1080pSV2NEO cells were co-plated and fused using 50% PEG. The hybrids were selected in 1.0μg/ml puromycin and 600μg/ml G418. Both parental controls were killed at these concentrations. Ten days after the fusion, hybrid colonies were visible. Two single colonies were picked and expanded to generate two hybrid cell lines, HFc6 and HFc8. The mixed population of the rest of the hybrid colonies was also expanded to generate the hybrid cell line HF.

Unlike their parental F9 cell line, the hybrids attached to the plastic surface strongly and did not require a gelatin substrate. The hybrids were larger than both of their parental cells and their morphology was similar to that of HT1080 cells (i.e. thin and elongated) (see Figure 4.2). However they grew faster than the HT1080pSV2NEO line (see Figure 4.2).

Cisplatin sensitivity tests

The three hybrid cell lines were tested for cisplatin sensitivity. All three hybrid cell lines tested showed similar CFEs at 5.7, 4.7 and 7.8%. These CFEs were lower than that of F9pBABE line but similar to that of HT1080pSV2NEO (see Table 4.2 and 4.3). The hybrid HFc8 showed a higher CFE than HT1080pSV2NEO line. Comparing cisplatin IC_{50} concentrations, the hybrid cell lines were more resistant than F9pBABE. However, they were more sensitive than HT1080pSV2NEO but similar to HT1080 cells (see Tables 4.2, 4.3 and Figure 4.3).

TABLE 4.3

SUMMARY OF STATISTICAL STUDIES ON THE PARENTAL AND HYBRID CELL LINES.

Cell Lines Compared	Comparison of Cisplatin IC_{50} Values	Comparison of Colony Forming Efficiencies
F9pBABE		
and HF	P=0.0002	P=0.004
HFc6	P=0.003	P=0.003
HFc8	P=0.001	P=0.005
HT1080pSV2NEO		
and HF	P=0.009	P=0.122
HFc6	P=0.020	P=0.033
HFc8	P=0.004	P=0.010

The statistical analysis was carried out using a two-sided student t test. The P values were determined by comparing the IC_{50} values of each transfected cell line to those of the parental cell lines.

FIGURE 4.2

MORPHOLOGY OF PARENTAL CELL LINES AND THEIR HYBRIDS
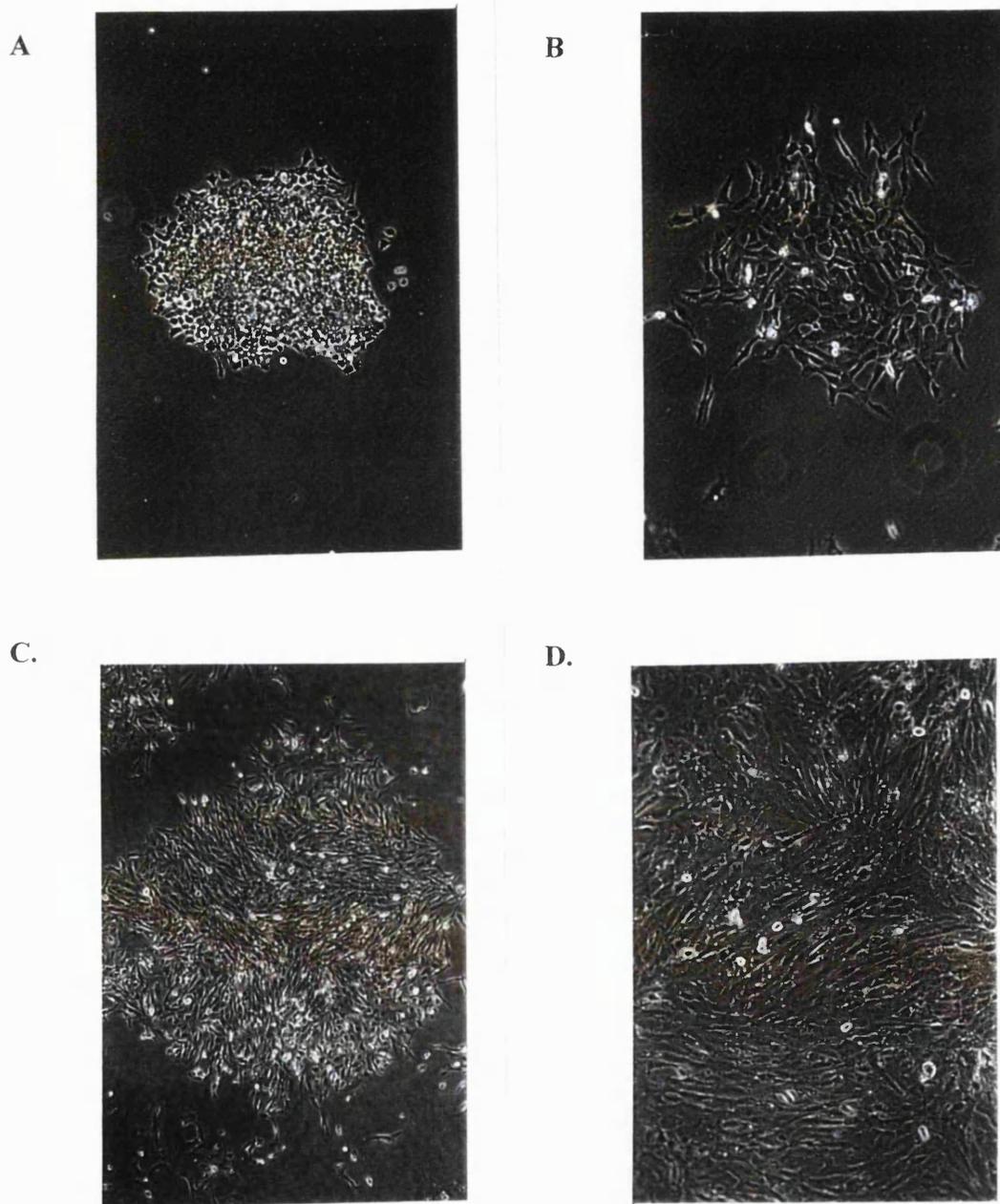
Single cells were plated and cultured for 12 days in DMEM medium containing 10% FCS. F9pBABE (A) was cultured in a 0.1% gelatin coated flask. F9pBABE (A), HT1080pSV2NEO (B) and HF (D) were photographed on an inverted phase contrast microscope using a 10x objective. HF (C) was taken under a 4x objective in order to show a complete colony.

FIGURE 4.3

CISPLATIN SENSITIVITY OF F9pBABE AND HT1080pSV2NEO CELL LINES AND THEIR HYBRIDS
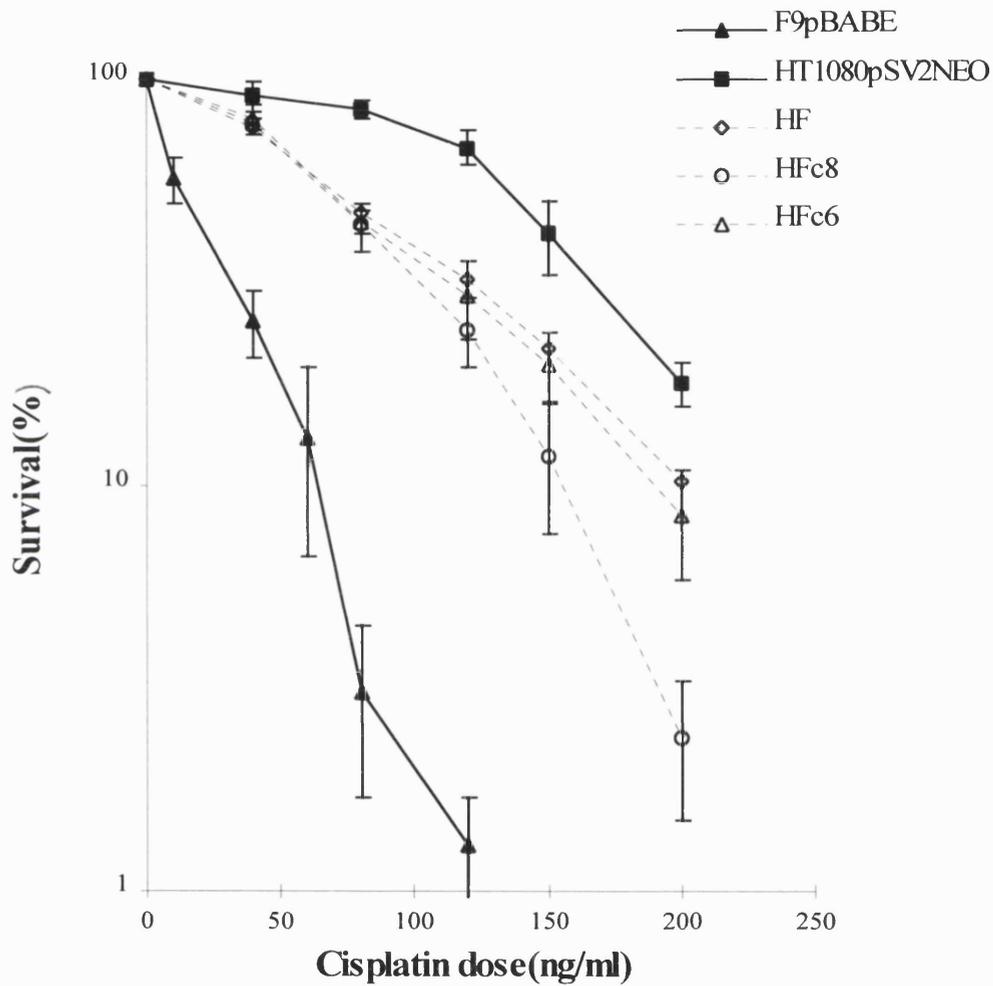
Dose response curves obtained by clonogenic assay following continuous exposure of F9pBABE and HT1080pSV2NEO cell lines and their hybrid cell lines (HFc6, HFc8 and HF) to a range of concentrations of cisplatin. Results are expressed as a percentage of colony formation in untreated controls (means ± SEM). Each graph shows the surviving fractions of the parental cell lines (continuous lines) and the hybrids (dashed lines).

4.2.2   Transfection of a cDNA Library and Selection of Cisplatin Resistant Primary Transfectants

Four transfection methods: calcium phosphate, electroporation, lipofection and poly-L-ornithine mediated transfections were tested on F9 cells using the pcDNAI/NEO vector. The calcium-phosphate method resulted in the highest transfection efficiency of approximately 0.5% (see chapter 2.6.4-2 for details).

The pcDNAI/NEO-based cDNA library was transfected into F9 cells using the optimized calcium phosphate mediated transfection method mentioned in chapter 2.6.4-2. Transfection controls were set up for each transfection using the pcDNAI/NEO plasmid DNA as described in chapter 2.6.5. Cells in two 4cm wells were transfected without DNA, as G418 selection controls. Two wells were transfected with pcDNAI/NEO plasmid DNA and selected in G418 only, as transfection efficiency controls. Two wells were transfected with pcDNAI/NEO plasmid DNA and selected in both G418 and cisplatin, as cisplatin selection controls. In each of these control experiments, there were no colonies visible in either the G418 or cisplatin selection control wells. The colonies in the transfection efficiency controls were stained and counted to estimate the transfection efficiency in each experiment.

Cisplatin selective concentrations were estimated from the colony forming assays on F9 and HT1080 cells (see Figure 4.1 and Figure 4.3). It has been suggested from previous functional cloning studies that the optimum selecting concentrations for primary transfectants would be a concentration that kills approximately 99% of the sensitive cells (Westerveld *et al.*, 1984; Weber *et al.*, 1990; Weeda *et al.*, 1990; Tanaka *et al.*, 1990; Strathdee *et al.*, 1990 and Legerski and Peterson, 1992). This concentration would give a maximum selection of resistant primary transfectants although it could result in non-specific survival of some cells. In this study, at 120ng/ml and 150ng/ml continuous exposure approximately 98-99% F9 cells were killed (see Figure 4.1). These two concentrations were used for the selection of cisplatin-resistant F9 cells after the transfection of the cDNA library derived from HT1080 cells. A series of transfections were carried out using these two doses of cisplatin with 2-3 days intervals to allow more rapid selection, but still corresponding to the continuous exposure data.

cDNA library transfection number 1

9.5×10^7 F9 cells in 189 x4cm wells were plated and transfected 24 hours later with 2.9mg of cDNA library plasmid DNA at 15µg/well. Using the transfection efficiency (T.E.) of approximately 0.3% (calculated from 2 transfection control wells), a total of approximately 2.8×10^5 G418 resistant transfectants were generated (the entire cDNA library contains 2×10^5 clones). After 6 days of selection in G418, the transfected colonies were treated with 150ng/ml cisplatin for 2 days and then fresh cisplatin was added for a further 2 days. None of the G418 resistant cells survived the cisplatin treatment.

cDNA library transfection number 2

As library transfection number 1 failed to generate any cisplatin resistant colonies after selection in 150ng/ml cisplatin, the cisplatin selection was modified. Instead of using two doses of 150ng/ml cisplatin, one dose of 120ng/ml and one dose of 150ng/ml cisplatin were used.

1.05×10^8 F9 cells in 208x4cm wells were transfected with 3.1mg of cDNA library plasmid DNA at 15µg/well. At the T.E. of approximately 0.3% (calculated from the 2 transfection control wells), 3.15×10^5 G418 resistant colonies were generated. The cells were treated with 120ng/ml cisplatin for 2 days, given 2 days to recover and then treated with another dose of cisplatin at 150ng/ml for 2 days. 157 cisplatin-resistant colonies were obtained.

cDNA library transfection number 3

As the cisplatin selection in library transfection number 2 generated 157 colonies, to reduce the colony number another library transfection was performed using a higher cisplatin concentration. The same numbers of cells were transfected but the T.E. was reduced to 0.13% indicating that only approximately 1.3×10^5 G418 resistant clones were generated. The cells were then treated with 150ng/ml cisplatin for 2 days, were given 2 days to recover and treated with another dose of cisplatin at 150ng/ml for 2 days. A total of 118 cisplatin-resistant colonies were isolated.

Control transfection

In order to test if the selection procedures would result in any non-specific resistant primary transfectants a control transfection was performed. 10^8 F9 cells were plated in 133x4cm wells of 6-well plates. 2.8mg pcDNAI/NEO plasmid was transfected at 15μg/well concentration using the modified calcium-phosphate transfection method. The transfected cells were selected in G418 for 6 days and then in cisplatin. They were firstly treated with 150ng/ml cisplatin for 2 days, then given 2 days to recover and treated with another dose of cisplatin at 150ng/ml for a further 2 days. The surviving cells were selected in G418 for 12 days and all the wells were stained. 42 colonies were obtained.

Secondary selection of the cisplatin resistant clones

All colonies surviving cisplatin selection from the second and third transfections were selected again. Each primary transfectant colony was split into three wells as shown in the following diagram:

```
                    ┌─────────────────────────────────┐
                    │   Primary transfectant colony   │
                    └─────────────────────────────────┘
        ┌──────────────────────┼──────────────────────┐

1xwell/96-well plate      1xwell/96-well plate      1xwell/6-well plate
Cisplatin test control       Cisplatin test             G418 test
        │                         │                         │

     No drug             Cisplatin 300ng/ml               G418
        │                         │                         │

   Cultured for        Exposed to cisplatin for 2 days,  Cultured in G418 for 10 days
  7 days and stained   cultured for 7 days and stained
```
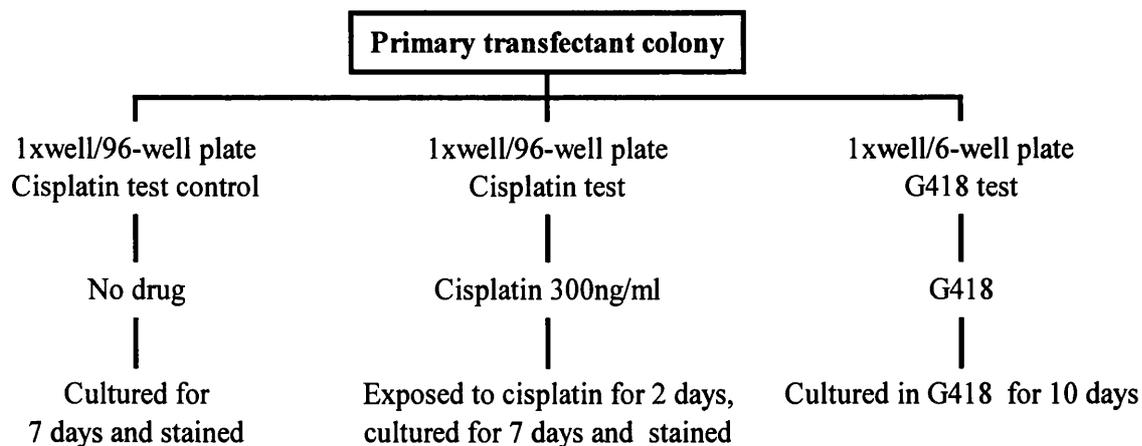
Cell growth was estimated in the stained control and the cisplatin treated wells. Cisplatin treated wells which had similar cell numbers to the untreated control wells were considered as possible cisplatin-resistant clones. The corresponding clones, which grew in G418 selecting wells, were expanded. Ten out of 152 clones from cDNA library transfection number 2 and 15 colonies out of 118 from cDNA library transfection number 3 were selected. Colony-forming assays were performed on the total of 25 selected clones (named F9cp...) to test their sensitivities to cisplatin.

Three clones F9cpc53, c87 and c133 from transfection number 2 and two clones F9cp2c9 and c13 from transfection number 3 showing increased resistance to cisplatin compared to the parental F9 cells (see Table 4.4 and Figure 4.4) were selected for further study.

TABLE 4.4

COMPARISON OF CISPLATIN SENSITIVITIES BETWEEN F9 AND THE PRIMARY TRANSFECTANTS

Cell Line	$IC_{50} \pm SD$ (ng/ml) cisplatin	P values compared to F9
F9	28.6 ± 6.1	
F9cpc53	95.8 ± 11.7	P=0.001
F9cpc87	92.5 ± 19.9	P=0.022
F9cpc133	84.0 ± 28.2	P=0.030
F9cp2c9	104.9 ± 38.6	P=0.021
F9cp2c13	75.9 ± 11.7	P=0.003

The concentration required to reduce colony formation to 50% of that of untreated controls (IC_{50}) was determined from 3 separate experiments. The statistical studies were carried out using a two-sided student t test. The P values were determined by comparing the IC_{50} values of each cell line to the parental F9 cells.

FIGURE 4.4

CISPLATIN SENSITIVITIES OF THE SELECTED PRIMARY TRANSFECTANTS AND F9 CELLS
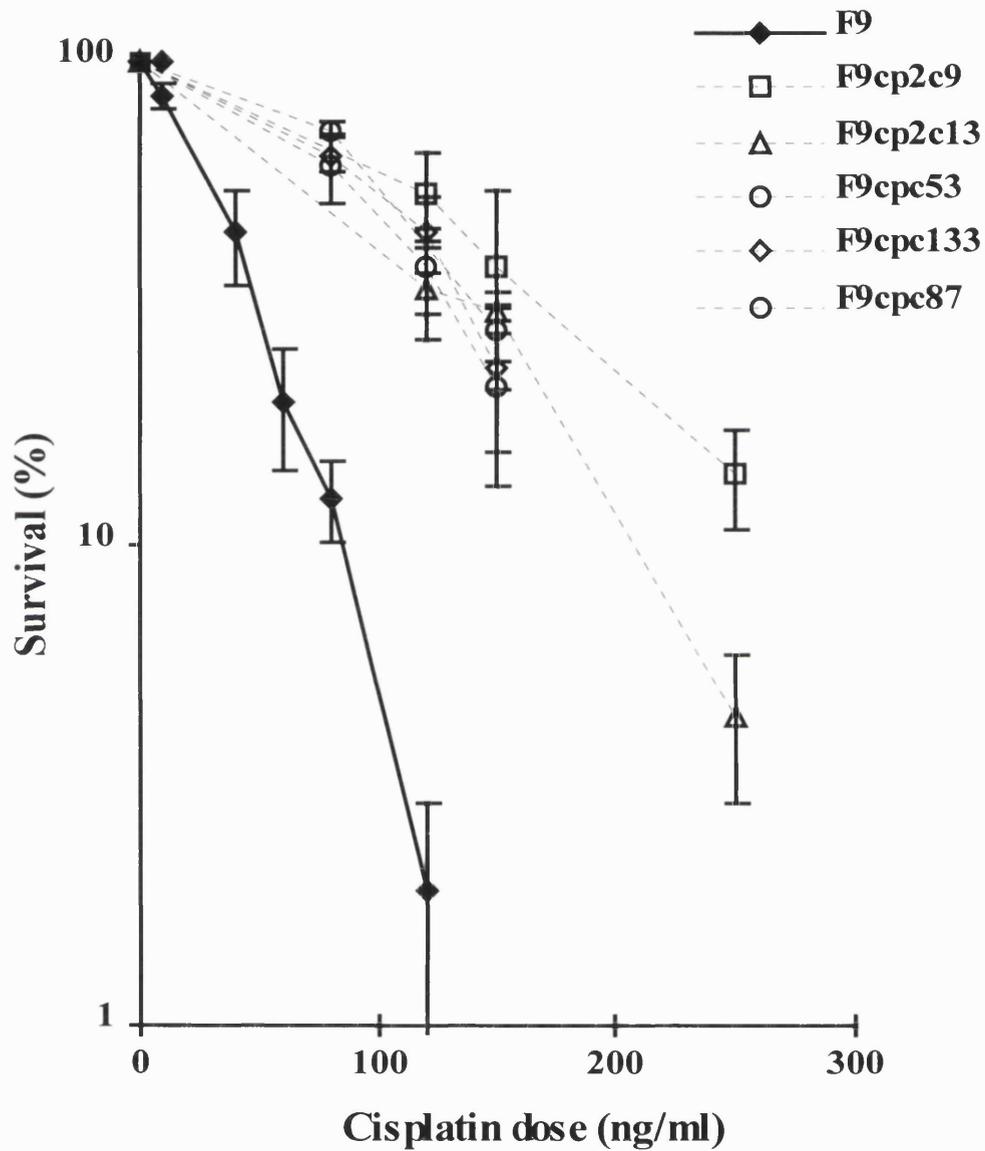
Dose-response curves obtained by clonogenic assay following continuous exposure of the five selected primary transfectants and the parental line F9 to 3 doses of cisplatin. Results are expressed as a percentage of colony formation in untreated controls (means ± SEM).

4.2.3   Secondary Transfection of Genomic DNA Isolated from the Cisplatin Resistant Primary Transfectants.

Genomic DNA from the selected cisplatin resistant transfectants was isolated and transfected back to the parental line F9 to see if it would result in cisplatin resistance in the secondary transfectants. 25-50kb genomic DNA fragments were generated. This size was selected to facilitate the transfection efficiency but not to disturb the insert DNA in the 7kb pcDNAI/NEO plasmid (the maximum size of plasmid and the insert is approximately 12kb).

Secondary transfections were performed using genomic DNA isolated from the cisplatin-resistant primary transfectants F9cpc53, c87 and c133 on F9 cells by the modified calcium-phosphate transfection method. To transfect the whole genome (3×10^6 Kb in size) into the F9 cells, approximately 120,000 transfectant clones must be generated. At a transfection efficiency of 0.3%, 4×10^7 cells were needed to generate 120,000 clones ($120,000 \times 1000 \div 3$). To try to ensure that the whole genome of each primary transfectant was transfected, 10^8 cells were used in each transfection experiment. Approximately 200x4cm wells were used in each transfection to transfect 3.0mg of genomic DNA at 15µg/well. The transfectants containing the pcDNAI/NEO plasmid were selected in G418 only to avoid exposure to cisplatin and tested for cisplatin sensitivity individually.

Cells were plated in two 4cm wells and transfected with buffer only as G418 selection controls. No colonies were visible in these control wells after G418 treatment. Two transfection efficiency controls were also set up for the calculation of transfection efficiencies in each experiment. Secondary transfection of genomic DNA from F9cpc53 was performed twice, generating 2 and 42 G418-resistant colonies respectively; secondary transfection of genomic DNA from F9cpc87 and cpc133 cells generated 16 and 28 G418-resistant colonies respectively.

The G418-resistant colonies were expanded and tested for cisplatin sensitivity. Two-point curves were performed at 80ng/ml and 150ng/ml of cisplatin using F9, F9cpc53, F9cpc87 and F9cpc133 as controls. All the selected clones were tested and each experiment was repeated at least twice. None of the clones showed resistance to cisplatin when compared to F9 cells.

4.2.4   FISH Analysis of the Primary Transfectants

To investigate the number of copies of pcDNAI/NEO plasmid integrated into the primary transfectants F9cpc53, c87 and c133, FISH analysis was performed. This experiment allowed us to determine if the number of isolated secondary transfectants in section 4.2.3 represented the number of plasmids in the primary transfectants. Previous studies have shown that a transfected plasmid could be maintained stably at copy numbers up to 50-100 in the transfectants (Folger *et al.,* 1992). It is possible that a high number of plasmid copies could have been maintained in the primary transfectants isolated in this study.

FISH analysis was performed using the pcDNAI/NEO plasmid as probe on the 3 primary transfectants F9cpc53, c87 and c133, as shown in Figure 4.5a-c. In Figure 4.5a and b, there are two signals of pcDNAI/NEO on the same metaphase chromosomes of F9cpc87 and c133 (green dots). As each metaphase chromosome consists of two identical chromatids, the results indicate that there is one integration site of pcDNAI/NEO plasmid in these two transfectants respectively. In Figure 4.5c, in the transfectant F9cpc53, it seems that tetraploidization (chromosome doubling without cell division) had occurred in the transfectant F9cpc53 after the transfection. This is because: a) this cell line had twice as many chromosomes as the other two transfectants, F9cpc87 and F9cpc133 (10 metaphase cells of F9cpc53 were examined and same results observed); b) morphologically, the two metaphase chromosomes carrying the hybridized signals seemed to be identical, c) the location of the signals on both chromosomes looked similar (the enlarged image of F9cpc53 is shown in Figure 4.5d). This suggests that after transfection, chromosome doubling occurred and this transfectant failed to divide in culture resulting in its tetraploid chromosome numbers. This would explain why the location of the signals and the morphology of the chromosomes carrying the signals seemed to be identical. Thus although 4 signals were detected in F9cpc53, they may only represent one integration site of the plasmid pcDNAI/NEO.

FIGURE 4.5

FISH ANALYSIS OF THE CISPLATIN RESISTANT PRIMARY TRANSFECTANTS
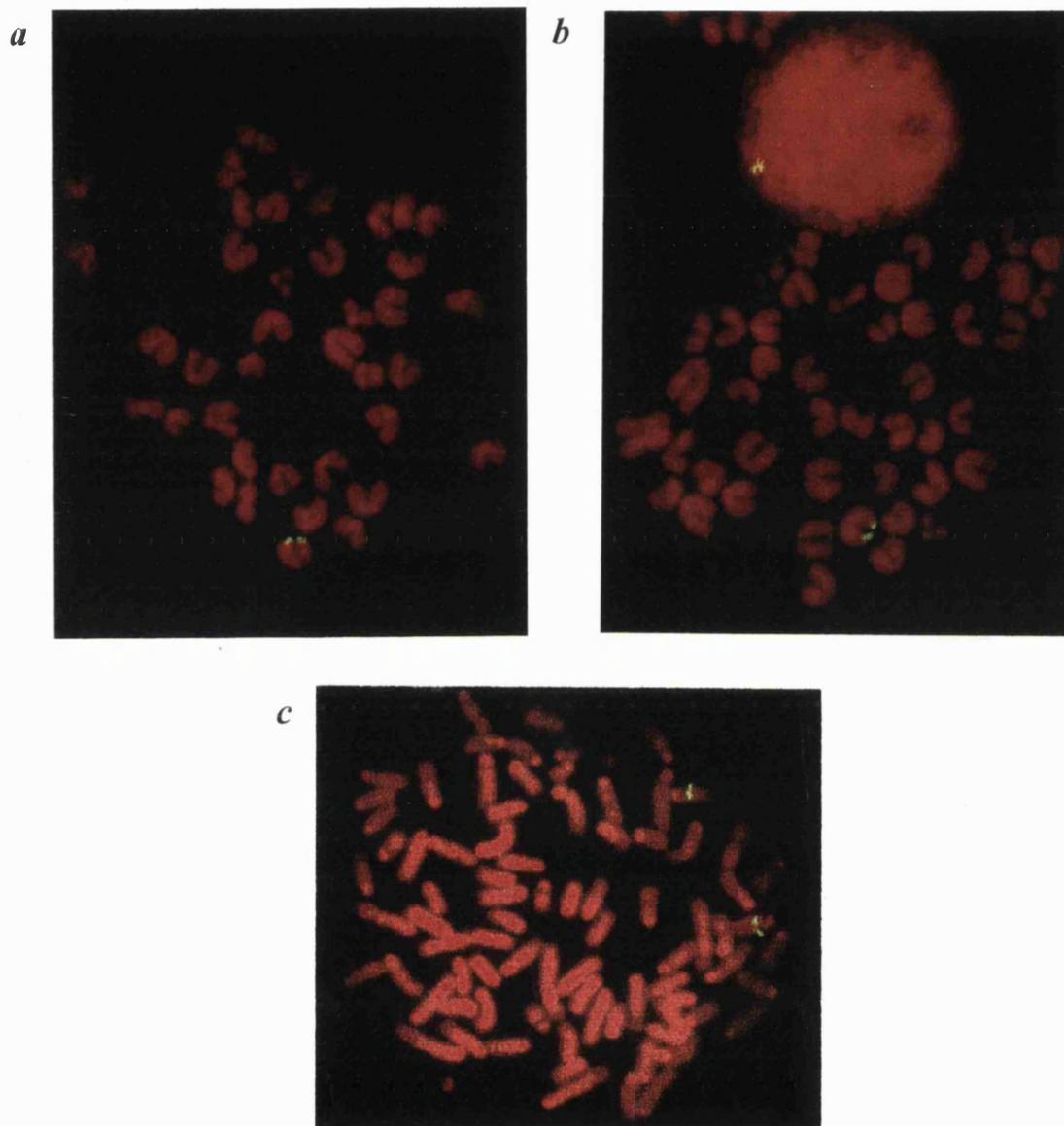
Metaphase cells were prepared from three cisplatin resistant primary transfectants F9cpc53, c87 and c133. pcDNAI/NEO plasmid was labelled with biotin and used as a probe for detecting the presence of the pcDNAI/NEO plasmid in these cell lines. Images *a* and *b* were captured using a MRC 600 (BioRad) laser confocal microscope under an oil-merged 100x objective lens. Image *c* was captured from a CCD camera using a Zeiss fluorescence microscope and a software from VISYS.

FIGURE 4.5d

ENLARGED IMAGE OF F9cpc53

The image was captured from a CCD camera using a Zeiss fluorescence microscope and software from VISYS under a 100x oil-merged objective.

4.3   DISCUSSION

The aim of this study was to clone genes conferring cisplatin resistance to F9 cells. Firstly, the conditions for fusion of F9 and HT1080 cells were established and the cisplatin sensitivity of the resulting hybrids was measured. The results indicated that the hybrids were more resistant to cisplatin than F9 cells. The preliminary experiments indicated that HT1080 cells contain genetic material that confers greater resistance to cisplatin than F9 cells.

The fact that the control transfection resulted in 42 colonies suggested that the selection procedures applied in this study allowed a low background of parental cell survival. This provided an opportunity for selection of cisplatin-resistant primary transfectants after the transfection of the cDNA library. However, this selection resulted in the total isolation of 270 primary transfectant colonies indicating that the cisplatin selection could be harsher so that the amount of secondary selection work would be reduced. If there were genes conferring cisplatin resistance to F9 cells presented in the HT1080 cDNA library, they would have been expected to be included amongst the 270 colonies isolated.

Following the secondary selection, five primary G418 resistant transfectants, which showed increased resistance to cisplatin compared to the untransfected parental cell line F9, were isolated. FISH analysis showed that a maximum of 3 different integration sites of the pcDNAI/NEO plasmid were detected in the 3 primary transfectants. Despite the fact that a large number (88) of the secondary transfectants were tested, none of them showed cisplatin resistance when compared to F9. These results suggest that the cisplatin resistance in the primary transfectants was not due to the effect of the transfected DNA from the library.

It is possible that the inserted DNA was damaged during the generation of genomic DNA from the primary transfectant and that this resulted in its inability to induce cisplatin resistance in the secondary transfectants. However, the maximum size of the cloning plasmid pcDNAI/NEO plus the inserted DNA in the library was 12kb, and a high percentage DNA isolated from the primary transfectants was sheared into fragments of 25-50Kb (see Figure 2.3). This meant that the G418 and cisplatin resistance genes remained associated in a large proportion of the fragments. It is also therefore likely that the cDNA

postulated to confer increased cisplatin resistance in the primary transfectants had also been transferred to the secondary transfectants. Even if events such as transfection-induced mutations, unexpected recombination of the transfected plasmid or instability of the transfectants had occurred during the secondary transfection, as reported in previous studies (Drinkwater *et al.*, 1986; Roth *et al.*, 1985; Wigler *et al.*, 1979), the number of secondary transfectants tested (88 clones) should have been enough to exclude these possibilities.

There are several possible explanations for generating of cisplatin-resistant primary transfectants that are not due to the effect of the transfected insert DNA.

It has been shown that spontaneous mutations occur in tumour cells *in vitro* which could result in drug resistant variants in the tumour cell population (Goldie and Goldman, 1979; Cifone and Fidler, 1981; Sager *et al.*, 1985; Otto *et al.*, 1989). It is possible that the cisplatin resistant primary transfectants isolated in this study might be cells carrying mutations in the transfected cell population. These pre-existing mutant cells were then selected by cisplatin. Thus we were unable to transfer cisplatin resistance to the secondary transfectants.

Acquired cisplatin resistance can also be generated by continuous exposure of cisplatin to the sensitive cells over a period of a few months (Kelland *et al.*, 1992). It is also possible that the cisplatin resistant primary transfectants were generated by the cisplatin selection procedures used in this study. However, the transfected F9 cells were only exposed to two doses of cisplatin over a 4 day period so it is unlikely to induce acquired resistance. This is supported by a study on doxorubicin induced resistant cells (Chen *et al.*, 1994). Human sarcoma cells were treated with a single dose of doxorubicin and the resistant colonies were selected. Using Luria-Delbruck fluctuation analysis, it found that these resistant cells were spontaneous mutant cells selected by doxorubicin and not caused by the treatment of doxorubicin. This suggests that a single dose doxorubicin might not be able to cause either mutations or acquired resistance.

Another possible reason for the generation of cisplatin resistance in F9 cells might include the random integration of the transfected plasmid. It has been shown that transfection of pSV2NEO and pCMVNEO markers into rat embryonal cells generated radioresistance in some of the transfectants (Pardo *et al.*, 1991). This group showed that the *neo* transfection

and the clonal selection process could modulate the radiation sensitivity of both mouse and human cells. Similar results have been observed in other studies (Arlett *et al.*, 1988; Green *et al.*, 1985; Murnane *et al.*, 1985). Such effects could depend on a variety of factors such as plasmid integration into a regulatory element. In the present study, it is possible that the pcDNAI/NEO vector integrated randomly into the genome. Consequently a gene involved in the acquisition of cisplatin resistance may have been mutated or activated by insertion of the plasmid DNA within the gene or adjacent to it, resulting in cisplatin resistance in the primary transfectants. When the secondary transfection was performed, the plasmid integrated into a different site in the genome, thus failing to confer cisplatin resistance.

It has been shown that when a plasmid containing 2 selective markers was transfected into human cells and the transfectants were selected for only one marker, only a small percentage of the transfectants retained the unselected marker in the cellular DNA after 4-8 weeks of culture (Mayne *et al.*, 1988). This suggests that while transfectants are selected in the selective medium, only the selective marker gene remains intact. It is possible that other genes linked to the marker might be altered, as they are not selected for. In the present study, the primary transfectants were selected for the *neo* marker for up to 8 weeks before isolation of DNA for secondary transfections. It is possible that some genetic changes had occurred during that period on the inserted DNA. However, all the primary transfectants retained their resistance to cisplatin even 5 months after they were isolated. This suggests that neither the non-selective conditions for the insert DNA nor long term culture had any effect on their resistance to cisplatin.

It is also possible that gene(s) which complement the sensitivity of F9 cells to cisplatin were either not present in the HT1080 cDNA library or that human genes might not function well in mouse cells resulting in the failure of this study. This was observed in the HF hybrids that showed less resistance than the parental line HT1080 (see Figure 4.3). However, the fact that hybrids of F9 and HT1080 cells showed increased resistance to cisplatin compared to F9 allowed the selection of the resistant transfectants from the F9 cell background. This raises another possibility that there might be more than one gene involved in the sensitivity of F9 cells to cisplatin. It may explain the fact that while cell hybrids of F9 and HT1080 showed some complementation of the sensitivity of F9 to cisplatin, we failed to achieve it when individual cDNAs from HT1080 library were transfected into F9 cells.

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Future studies should also include using the same approach with a different cDNA library derived from a human cell line that is more resistant to cisplatin than HT1080 cells. This may allow more efficient selection of the transfectants. Or a mouse library instead of human library should be used to allow the full complementation of cisplatin sensitivity in F9 cells. Alternatively, different approaches can be used for identifying genes responsible for complementing the sensitivity of testicular tumour cells. The following chapters will outline several alternative strategies.

Chapter 5

CHARACTERIZATION OF A CISPLATIN RESISTANT SECONDARY TRANSFECTANT GENERATED BY YEAST GENOMIC LIBRARY TRANSFEC TION

5.1 INTRODUCTION

In parallel with the attempts to transfect a human cDNA library into mouse cells, my colleague Dr. Majid Hafezparast, had been trying to isolate genes controlling cisplatin sensitivity by expression cloning in human testicular tumour cells. Using a yeast genomic library in an EBV-based vector, cisplatin resistance was conferred and the DNA insert recovered and used to isolate a secondary transfectant also resistant to cisplatin. In this chapter, the characterization of this secondary transfectant will be described.

EBV-based expression systems have been used to clone the human DNA repair genes XPCC and FACC (Legerski and Peterson, 1992; Strathdee *et al.*, 1990). EBV-based systems have advantages for the functional cloning of genes, because they can be transfected with high efficiency into EBNA-1 expressing human cells, and can be recovered readily as they replicate episomally in the cytoplasm (Swirski *et al.*, 1992).

The transfection efficiency of human cells to the EBV-based vectors can be increased by constitutive expression of the EBNA-1 (Epstein-Barr Nuclear Antigen-1) gene in the recipient cell line (Yates *et al.*, 1985). In the present study, the EBNA-1 gene was stably transfected into SuSa, a testicular tumour cell line, creating SuSaE46. This increased the transfection efficiency by 75-fold (data not shown) and this cell line was transfected with a yeast genomic library. In this chapter, the cisplatin-resistant secondary transformant (SuSaE46Yc1a) was characterised and the introduced DNA was recovered and amplified. The genomic insert DNA was characterised by transfecting into cisplatin resistant and sensitive tumour cell lines. Cisplatin sensitivities were measured.

5.2   RESULTS

5.2.1   Measurement of the Cisplatin Sensitivity of the Secondary Transfectant SuSaE46pYc1a

The yeast genomic library was cloned into an EBV-based vector p220LTR. The p220LTR cloning vector confers resistance to hygromycin which was used to select and maintain transfectants.

The p220LTR cloning vector containing the candidate insert in the secondary transfectant SuSaE46pYc1a was isolated and amplified. As shown in Figure 5.1, the SusaE46pYc1a cell line had a genomic DNA insert of approximately 6kb.

This cell line showed greater cisplatin resistance than the cloning plasmid p220LTR transfected control cell line SuSaE46p220LTR (see Figure 5.2). SuSaE46pYc1a displayed a different morphology to SuSaE46p220LTR, being smaller and forming smaller and tighter colonies (Figure 5.3). SuSaE46pYc1a was significantly more resistant to cisplatin than SuSE46p220LTR (IC_{50}s 158±32.2ng/ml and 89.8±19.6ng/ml respectively, $P<0.05$, Figure 5.2).

5.2.2   Transfection of YC1 Fragment into Other Tumour Cell Lines

The episomal plasmid DNA containing the insert was isolated from SuSaE46pYc1a cell line and amplified using the maxiprep method. The amplified plasmid DNA (pYc1b) was transfected into five cancer cell lines and the transfectants were selected in 50-350μg/ml hygromycin, depending on the cell line. The cell lines transfected were the human testicular germ cell tumour cell lines, SuSa, 833K and GH, the mouse embryonal carcinoma cell line, F9, and the human bladder cancer cell line, MGHU1. The cloning vector, p220LTR was also transfected into three of these cell lines to generate controls for cisplatin sensitivity. In two cases, GH and SuSa, the parental cell lines were used as controls. Cisplatin resistance was induced in one of the testicular tumour cell lines, 833K, but not in the other four cell lines (see Figure 5.4 and Table 5.1). GH cells became more sensitive ($P<0.05$).

FIGURE 5.1

RESTRICTION ANALYSIS OF pYc1 PLASMID
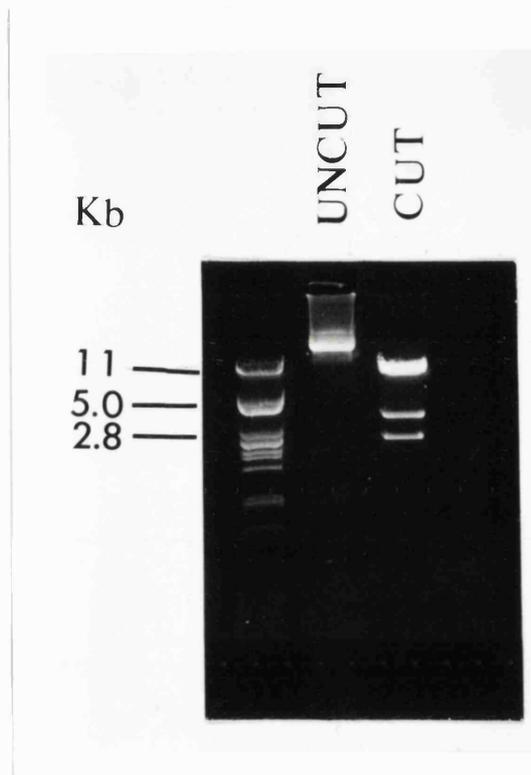
The plasmid containing pYc1 insert was recovered from the SuSaE46pYc1a cell line and amplified by minipreparation. The pYc1 plasmid was digested by *BamHI* at its cloning sites. Lane 1 is *PstI* digested λ DNA marker; Lane 2 is undigested and Lane 3 is BamHI digested pYc1a plasmid. The top band in Lane 3 is p220LTR vector, the two bottom bands represent the yeast genomic DNA insert suggesting that the inserted DNA has a *BamHI* restriction site.

FIGURE 5.2

CISPLATIN   DOSE-RESPONSE   CURVES   OF   SuSaE46p220LTR   and SuSaE46pYc1a.
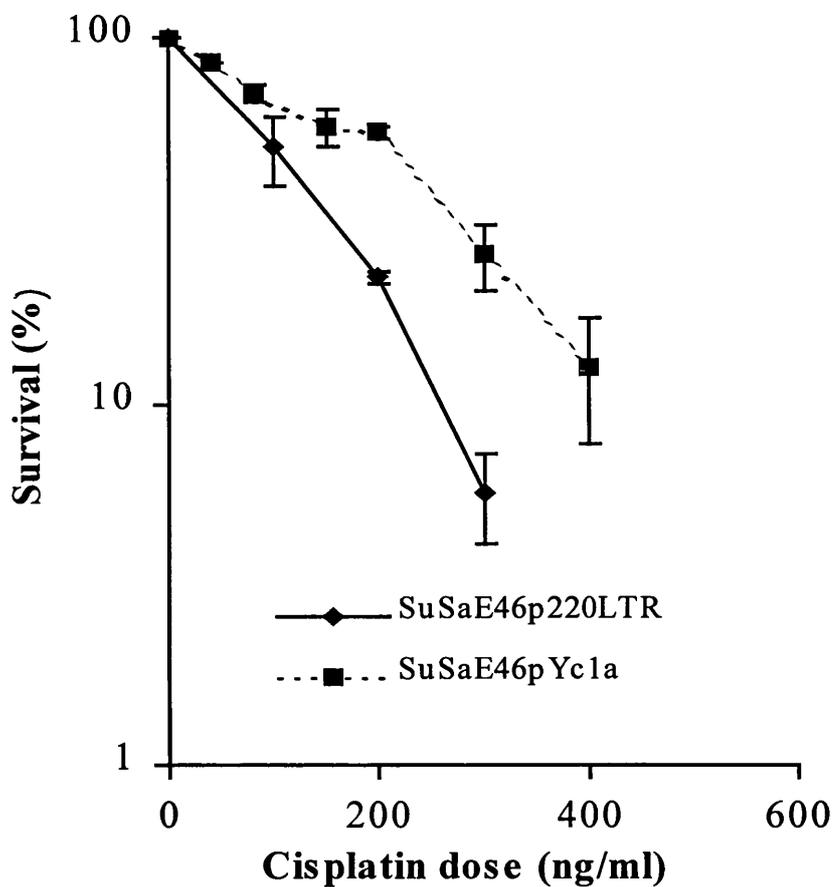
Dose-response curves obtained by clonogenic assay following continuous exposure of the secondary transfectant SuSaE46pYc1a and the control cell line SuSaE46p220LTR to a range of concentrations of cisplatin. Each dose-response curve is derived from a minimum of three independent experiments.

FIGURE 5.3

MORPHOLOGIES OF SuSaE46p220LTR AND SuSaE46pYc1a CELL LINES

SuSaE46p220LTR

SuSaE46pYc1a

Single cells were plated and cultured for 12 days in DMEM medium containing 10% FCS. The photos were taken under an inverted phase contrast microscope using a 10x objective lens. SuSaE46pYc1a had different morphology and growth rate from the control SuSaE46p220LTR.

FIGURE 5.4

CISPLATIN DOSE-RESPONSE CURVES OF THE TRANSFECTED CELL LINES AND THEIR CONTROLS
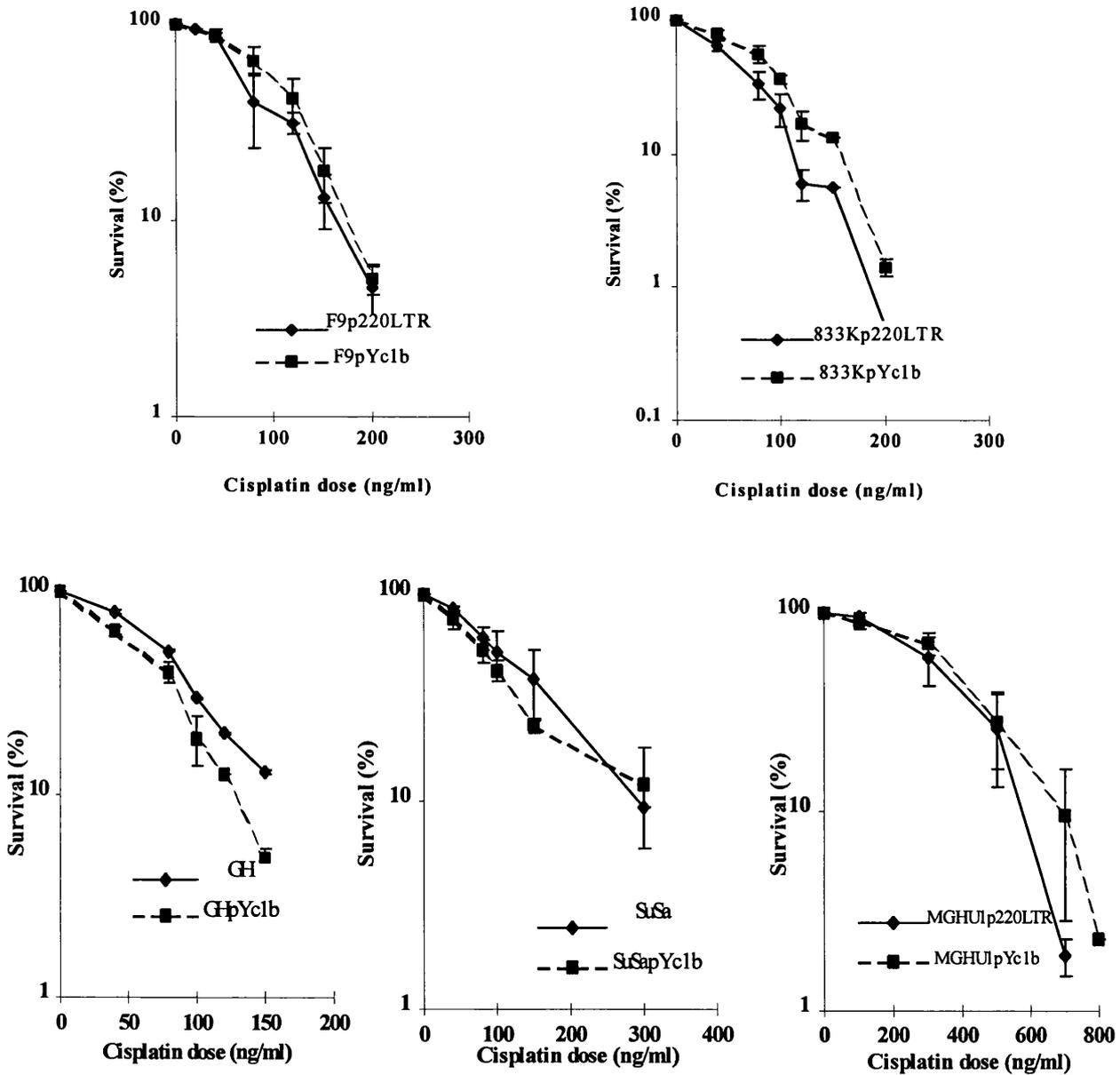
Dose-response curves obtained by clonogenic assays following continuous exposure of the 5 cancer cell lines transfected with the insert conferring cisplatin resistance and their control cell lines to a range of concentrations of cisplatin. Each dose-response curve is derived from 3 independent experiments. The solid lines represent parental controls and the dashed lines are transfected cell lines.

TABLE 5.1

SUMMARY OF THE SENSITIVITIES TO CISPLATIN OF THE PARENTAL AND TRANSFECTED CELL LINES.

Cell lines	IC50 ± SD (ng/ml) cisplatin	P value
SuSa SuSapYc1b	108.9±23.1 82.0±22.9	0.2258
833Kp220LTR 833KpYc1b	44.9±8.1 61.4±2.8	0.0294
GH GHpYc1b	62.5±0.8 47.3±6.8	0.0237
F9p220LTR F9pYc1b	72.9±13.0 65.8±15.9	0.5823
MGHU1p220LTR MGHU1pYc1b	222.2±42.6 253.2±67.0	0.5360

The concentration required to inhibit colony-formation to 50% of untreated controls (IC_{50}) was determined from at least 3 separate experiments. The results are the means ± SD. The statistical studies were carried out using a two-sided student t test. The P values were determined by comparing the IC_{50} values of each transfected cell line to those of the parental cell lines.

5.2.3 Re-confirmation of the Effect of Yc1a Insert on SuSaE46

The insert was re-transfected into the parental cell line SuSaE46, generating SuSaE46pYc1a'. And another transfection was also performed on SuSaE46 cells using the amplified pYc1 plasmid (pYc1b) to generate the transfectant line SuSaE46pYc1b. The transfectants were selected in 50µg/ml hygromycin as in previous transfections. Cisplatin sensitivities were determined by colony-forming assays on the transfectants and compared to the control cell line SuSaE46p220LTR (IC_{50}=89.8±19.6ng/ml cisplatin) (see Figure 5.5). Both SuSaE46pYc1a' (IC_{50}=91.2±16.5ng/ml cisplatin) and SuSaE46pYc1b (92.4±19.3ng/ml cisplatin) failed to show increased cisplatin resistance when compared to the parental cells (P>0.05).

5.2.4 Re-rescue of Plasmid Yc1 from SuSaE46pYc1a Cell Line and the Secondary Testing on Cisplatin Sensitivity

To confirm that the Yc1 insert had no effect on cisplatin sensitivity of SuSaE46 cells, the Yc1 plasmid was isolated from the original cisplatin resistant secondary transfectant SuSaE46pYc1a (see Figure 5.2). This secondary isolated plasmid was named Yc2. A transfection was performed using Yc2 on SuSaE46 cells. Two morphologically different colonies were observed in the generated transfectants. One of them had similar morphology to SuSaE46pYc1a (considerably smaller compared to SuSaE46p220LTR and other one was similar to SuSaE46p220LTR (see Figure 5.3 for morphological details). For further investigation of the morphological features on the effect of cisplatin sensitivity, these two morphologically different colonies were isolated and expanded independently to generate two transfectant cell lines, SuSaE46pYc2a (smaller cells) and SuSaE46pYc2b (larger cells). Colony-forming assays were performed on these two cell lines and there was no significant difference in SuSaE46pYc2a (IC_{50}=99.1±2.4ng/ml cisplatin) and SuSaE46pYc2b's (IC_{50}=97.3±19.3ng/ml cisplatin) sensitivity to cisplatin (P>0.05) (see Figure 5.6).

FIGURE 5.5

CISPLATIN DOSE-RESPONSE CURVES OF THE REPEATED TRANSFECTANTS
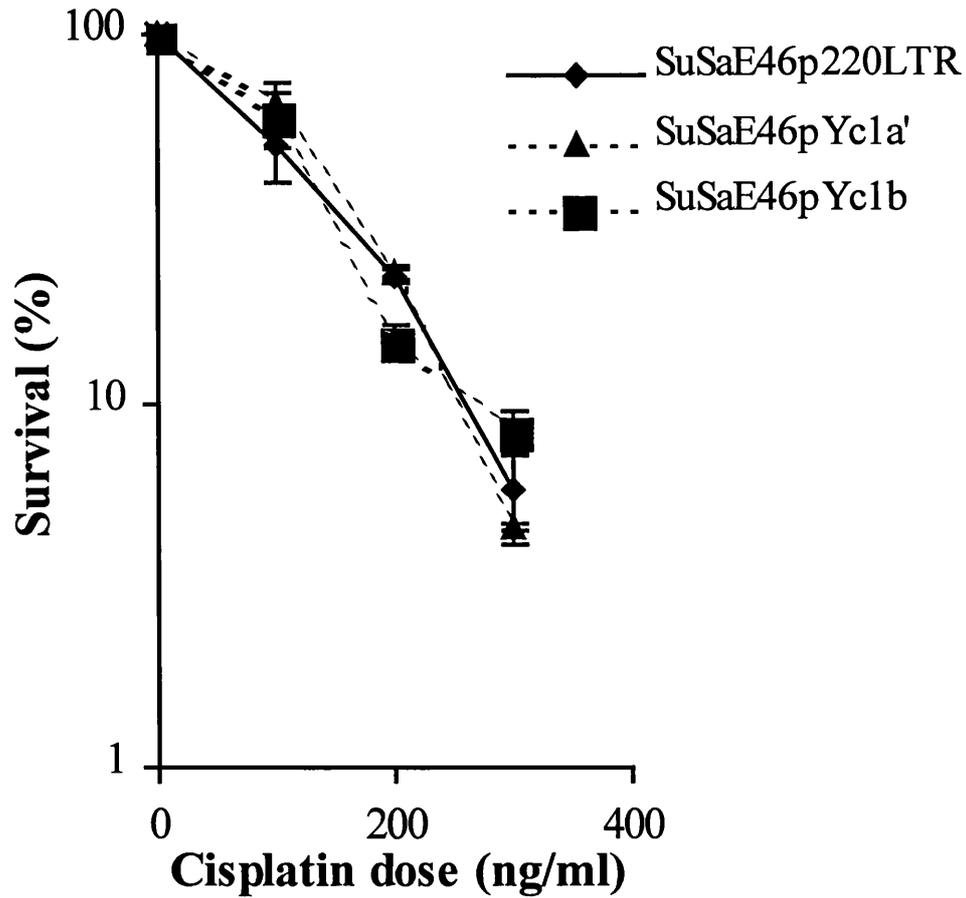
Dose-response curves obtained by clonogenic assays following continuous exposure of the repeated transfectant SuSaE46pYc1a', SuSaE46pYc1b and the control cell line SuSaE46p220LTR to a range of concentrations of cisplatin. Each dose-response curve is derived from a minimum of three independent experiments.
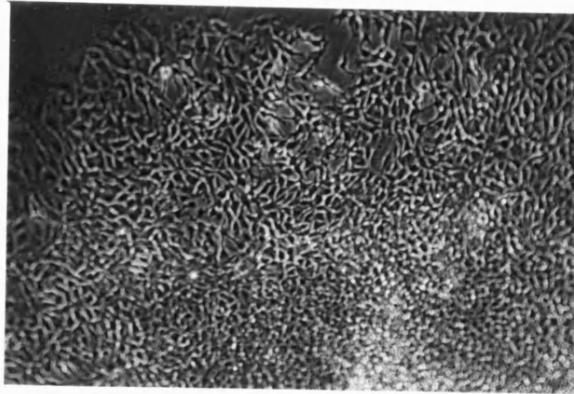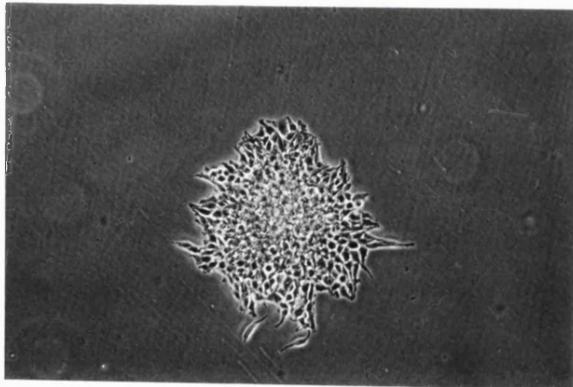
FIGURE 5.6

CISPLATIN DOSE-RESPONSE CURVES OF SuSaE46pYc2a AND SuSaE46pYc2b
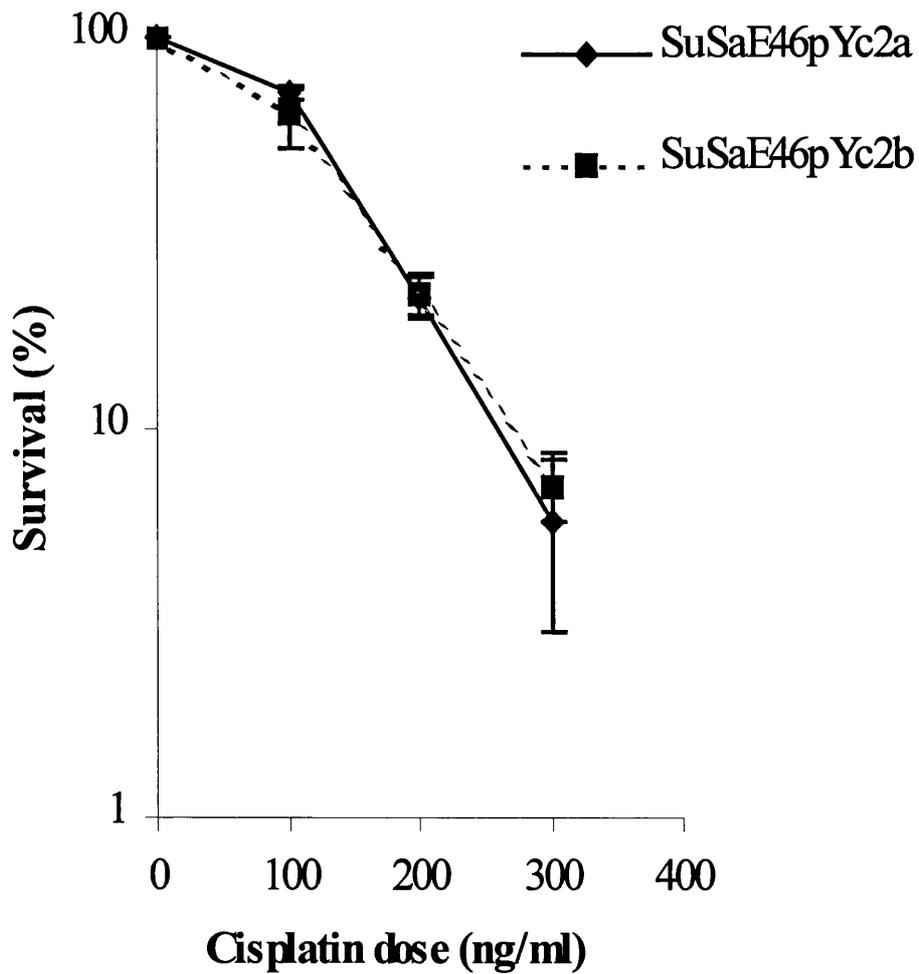
Dose-response curves obtained by clonogenic assays following continuous exposure of the transfectants SuSaE46pYc2a and SuSaE46pYc2b to a range of concentrations of cisplatin. Each dose-response curve is derived from a minimum of three independent experiments.

5.3 DISCUSSION

In this chapter, a cisplatin-resistant secondary transfectant SuSaE46pYc1a, which was isolated by transfection of an EBV-based yeast genomic library, was characterized.

The original secondary genomic library transfectant SuSaE46pYc1a cell line showed a significant increase in resistance to cisplatin compared to the control line, SuSaE46p220LTR (P<0.05). When the Yc1 insert was rescued and transfected into five tumour cell lines, it failed to produce cisplatin resistance in 4 of them compared to their control lines (P>0.05). However, the transfected 833KpYc1b showed increased resistance to cisplatin when compared to its control line 833Kp220LTR (P<0.05). Previous studies (Wang *et al.*, 1997) indicate that the human testicular tumour cell lines SuSa, 833K and GH have a common mechanism for their sensitivity to cisplatin. This suggests that if the effects of the Yc1 insert on 833K cell line were due to the complementation of its defects, we would have observed a similar effect on SuSa and GH cell lines. However, the Yc1 insert did not confer cisplatin resistance in SuSa and GH cell lines. In contrast, it made GH cells more sensitive (see Figure 5.4). This indicates that the effect of the Yc1 insert on 833K may be non-specific. In addition, the Yc1 insert failed to induce cisplatin resistance in F9, a mouse embryonal carcinoma cell line. The results indicate that the Yc1 gene was unable to confer cisplatin resistance in the transfected cell lines.

Morphological changes and a slower growth rate were observed for SuSaE46pYc1a cells suggesting that these could be due to the transfection of Yc1 insert (see Figure 5.3). These alterations might be related to the changes of cisplatin sensitivity in SuSaE46pYc1a cells. However, these morphological changes were not observed in any of the five transfected tumour cell lines. When the plasmid was isolated again from the original secondary transfectant SuSaE46pYc1a and transfected into SuSaE46, it also failed to induce cisplatin resistance. However, two morphologically different types of transfectants (SuSaE46pYc2a and SuSaE46Yc2b) were observed and studied. There was no difference when they were tested for cisplatin sensitivity (see Figure 5.6) indicating that the morphological differences of the transfectants were not related to their cisplatin sensitivity.

The potential causes of failure to induce cisplatin resistance by the Yc1 insert were investigated and eliminated. It has been reported that even though the majority of the EBV-based vectors are stably maintained in cells grown under selective conditions, they were subject to a low spontaneous mutation rate at approximately 2×10^{-6} per plasmid per cell (Drinkwater and Klinedinst, 1986). To eliminate the possibility that the pYc1b plasmid may contain a mutation and may have contributed to the failure of inducing cisplatin resistance in the five transfected tumour cell lines, the transfection was repeated on SuSaE46 cells using the original plasmid DNA pYc1a. The transfectant SuSaE46pYc1a' failed to show resistance to cisplatin when compared to the control line SuSaE46p220LTR ($P > 0.05$). This indicates that the failure to induce cisplatin resistance might be due to the Yc1 gene itself rather than mutation after its re-amplification. It also showed that the primary cisplatin resistance caused by Yc1 insert was not repeatable and may not have been specific. In addition, there were no morphological changes observed in these repeated transfectant SuSaE46pYc1a' cells as was observed previously on SuSaE46pYc1a.

Although EBV-based vectors have been shown to be maintained episomally in the transfected cells (Yates *et al*, 1985; Swirski *et al.*, 1992; Belt *et al.*, 1989), integration of these vectors was also observed (Heller *et al.*, 1990; Kioussis *et al.*, 1987; Swirski *et al.*, 1992; Morgoskee *et al.*, 1988). The random integration of plasmid into the host genome could also result in nonspecific effects on the recipient cells. However, the fact that we were able to rescue the pYc1 plasmid from the transfected SuSaE46 suggests that the plasmid was maintained episomally and not integrated.

Deletions and rearrangements of some EBV-based vectors have also been found in transfected human cells (Dean *et al.*, 1989; Heller *et al.*, 1990). However, the p220LTR vector used in this study was modified and has been shown to stably replicate episomally for up to 6 months in a non-rearranged form (Swirski *et al.*, 1992). Thus it seems unlikely that the failure of the pYc1 plasmid to induce cisplatin resistance in human cells was due to the changes of the plasmid in the transfected cells as these cells were maintained in culture continuously for less than 10 passages. However, the reason why the Yc1 insert conferred cisplatin resistance in SuSaE46pYc1a cells is still unclear. We cannot exclude the possibility that the cisplatin selection procedures in the primary and secondary transfections might have contributed to its nonspecificity since it has been reported that

transient exposure of chemotherapeutic drugs could result in the induction of multidrug resistance in human cells (Chandhary and Roninson, 1993).

Chapter 6

CHARACTERIZATION OF HYBRIDS GENERATED BY FUSION OF CISPLATIN-RESISTANT HUMAN TUMOUR CELLS AND CISPLATIN-SENSITIVE MOUSE EMBRYONAL CARCINOMA CELLS

6.1 INTRODUCTION

As an alternative strategy to that described in the previous chapters, a strategy was chosen to investigate the possibility of identifying human chromosomes conferring cisplatin resistance by hybridizing cisplatin-resistant human bladder cancer cells and cisplatin-sensitive mouse embryonal carcinoma cells. The strategy was to determine which human chromosomes were retained in cisplatin-resistant hybrids.

Mouse-human somatic cell hybrids tend to retain mouse chromosomes and lose human chromosomes (Illmensee *et al.*, 1978). This is characteristic of human-mouse hybrids and provides a tool for studying the effects of human chromosomes on mouse cells. Because human chromosomes tend to be lost in mouse-human interspecies hybrids, the human chromosomes that remain can be correlated to the phenotype of the hybrids. For example, this approach has been used to study human chromosomes involved in metastasis by the fusion of tumorigenic, but non-metastatic mouse cells, with normal human cells (Collard *et al.*, 1987; Cassingena *et al.*, 1992) as it has been suggested that normal cells might contain active genes responsible for metastasis (Roos *et al.*, 1985). The human-mouse hybrid cells were then generated and injected into animal models and the changes in the metastatic potential of the hybrids were analysed. Human chromosome 7 has been identified as necessary for invasiveness and metastatic potential of the hybrids (Collard *et al.*, 1987). A similar approach was also used to identify human chromosomes controlling cisplatin resistance in mouse-human hybrids (Mirakhur *et al.*, 1996). In that study, a cisplatin resistant human ovarian carcinoma cell line (2008/C13*) and a cisplatin sensitive mouse fibroblast cell line (A9) were fused and the hybrids were tested for their sensitivity to cisplatin. Two human chromosomes, chromosome 11 and 16, were identified as being involved in the acquisition of cisplatin resistance in the hybrids and both had to be retained.

The aim of the present study was to identify human chromosomes responsible for cisplatin resistance in human-mouse hybrids. The experimental plan was to generate hybrids between a cisplatin-resistant human bladder tumour cell line and a cisplatin-sensitive mouse embryonal carcinoma cell line. The cisplatin sensitivity of the hybrids would then be tested and the most cisplatin resistant and sensitive hybrids would be

selected. After a period of culturing, the cisplatin sensitivity of the hybrids would be determined. Also, the presence of human chromosomes would be examined in the hybrids by FISH analysis. As loss of human chromosomes has been observed in mouse-human hybrids, the remaining human chromosomes in the hybrids would be identified and correlated to the changes in cisplatin sensitivity.

The selective markers were firstly introduced into a cisplatin-resistant human bladder tumour cell line MGHU1 and a cisplatin-sensitive mouse embryonal carcinoma cell line F9 by transfecting pSV2NEO (conferring G418 resistance) and pBABE (conferring puromycin resistance) plasmids respectively. These cell lines were fused and the resulting 30 hybrid clones were tested for cisplatin sensitivity. From these hybrids, two relatively cisplatin sensitive and 4 relatively resistant hybrids were selected. At the tenth passage, the presence of human chromosomes in these 6 hybrid cell lines was studied using FISH analysis. The FISH showed that there was little loss of human chromosomes in the hybrids studied.

6.2 RESULTS

6.2.1 Generation of Human-Mouse Hybrids

Two plasmids, pSV2NEO (conferring neomycin, G418 resistance) and pBABE (conferring puromycin resistance), were transfected independently into a cisplatin-resistant human bladder tumour cell line MGHU1 and a cisplatin-sensitive mouse embryonal carcinoma cell line F9 respectively to generate selective markers for cell fusion. The calcium phosphate mediated transfection method was used for generating the transfectants F9pBABE and MGHU1pSV2NEO. MGHU1 is more resistant to cisplatin than F9 cells (see Table 6.1, $P>0.05$). The transfected cell lines showed sensitivities to cisplatin similar to that of their parental lines (see Table 6.1 and Figure 6.1) suggesting that neither the transfection nor the introduction of the selective markers had a significant effect on their sensitivities to cisplatin.

MGHU1pSV2NEO and F9 pBABE cells were co-plated and fused using 50% PEG. The hybrids were selected in 1.0μg/ml puromycin and 1000μg/ml G418. Both parental controls were killed at these concentrations, by puromycin within 4 days and by G418 within 10 days. Hybrid colonies were visible 10 days after the fusion. Thirty single colonies were isolated and expanded to generate hybrid cell lines. The hybrid cells differed morphologically from F9, being larger and more similar to the MGHU1 parent (see Figure 6.2). However, they seemed to grow faster than MGHU1 as observed from the sizes of colonies in clonogenic assays (see Figure 6.2). The hybrids were cultured in 1.0μg/ml puromycin only, in order to permit the loss of human chromosomes.

6.2.2 Selection of Cisplatin Resistant and Sensitive Hybrid Clones

Cisplatin sensitivity was measured by colony forming assays on the hybrids using the two parental lines as controls. Three cisplatin concentrations were tested on each hybrid. All of the hybrids showed greater resistance to cisplatin than the sensitive parental line F9pBABE. Among the 30 hybrid clones tested, the 4 clones (MGF9c3, c4, c5, and c6) showing most resistance, being between 7.2-17.1-fold more resistant than F9pBABE, were selected for further study. One of them, MGF9c5, was more resistant than MGHU1pSV2NEO. Two clones (MGF9c1 and c2) were relatively sensitive, only 3.8 and 4.1-fold more resistant than

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TABLE 6.1

CISPLATIN SENSITIVITY OF PARENTAL LINES AND THEIR TRANSFECTANTS.

Cell lines	$IC_{50}s$ (ng/ml) $\pm SD$	P value
F9	28.6±6.1	
F9pBABE	15.9±5.7	P=0.057
MGHU1	294.2±27.0	
MGHU1pSV2NEO	232.1±51.7	P=0.139

The concentration required to reduce colony forming ability to 50% of untreated controls (IC_{50}) was determined from 3 separate experiments. The results are the means ± SD. The statistical studies were carried out using a two-sided student t test. The P values were determined by comparing the IC_{50} values of each transfected cell line to those of the parental cell lines.

FIGURE 6.1

CISPLATIN DOSE-RESPONSE CURVES OF THE PARENTAL LINES (F9 AND MGHU1) AND THE TRANSFECTED CELL LINES
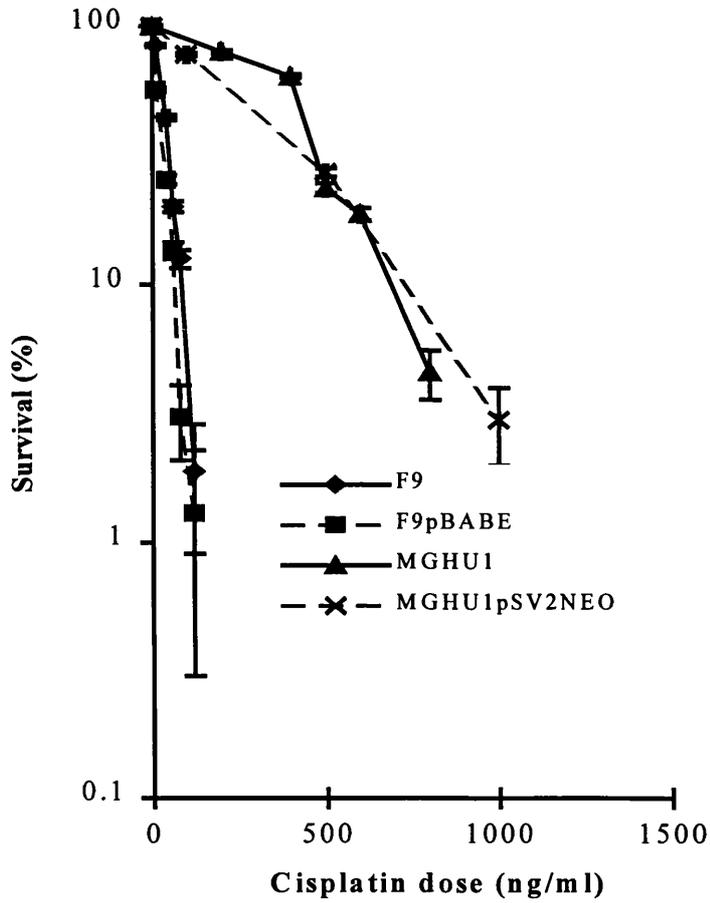
Dose-response curves obtained by clonogenic assay following continuous exposure of the parental lines (F9 and MGHU1) and the transfected cell lines (F9pBABE and MGHU1pSV2NEO) to a range of concentrations of cisplatin. Each graph shows the surviving fractions of the parental cells (continuous lines) and the transfected lines (dashed lines).

FIGURE 6.2

MORPHOLOGY OF PARENTAL (F9 AND MGHU1) CELL LINES AND THEIR HYBRIDS
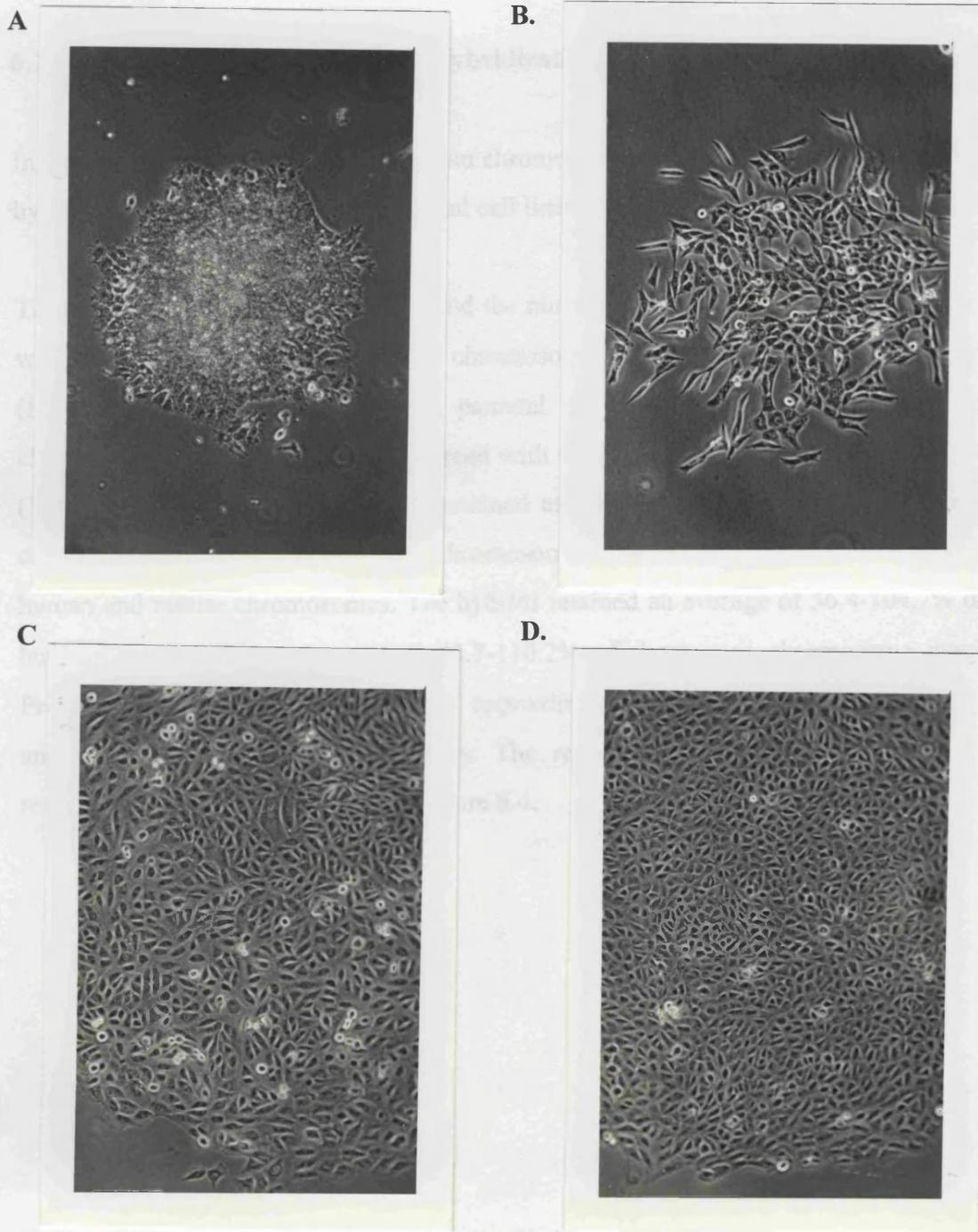
Single cells were plated and cultured for 12 days in either DMEM or RPMI medium containing 10% FCS. F9pBABE cells were cultured on a 0.1% gelatin coated dish. F9pBABE (A), MGHU1pSV2NEO (B) and MGF9c1 and 4 (C, D) were photographed on an inverted phase contrast microscope using a 10x objective lens.

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F9pBABE (see Figure 6.3.) and were also selected for further study. The results are summarized in Table 6.2. These 6 clones were grown to passage number 10 and the presence of human chromosomes in these 6 hybrid clones was studied by FISH.

6.2.3   Fluorescence In Situ Hybridization (FISH)

In order to analyze the number of human chromosomes in the hybrids, FISH was used on hybrid clone MGF9c1-6 and the parental cell lines using human Cot-1 DNA as a probe.

Ten metaphase cells were examined and the number of mouse and human chromosomes was counted. F9pBABE had a modal chromosome number of 39 as previously reported (Bernstine *et al.*, 1973). The other parental line, MGHU1pSV2NEO, had a modal chromosome number of 85, which agreed with results in our previous study (Modal=86) (Wang *et al.*, 1996). The hybrids contained an average of between 48 and 89 human chromosomes and 19 to 43 mouse chromosomes. There was evidence of loss of both human and mouse chromosomes. The hybrids retained an average of 56.4-104.7% of the human parental chromosomes and 48.7-110.2% of the mouse chromosome numbers. Proportionally all 6 hybrids contained approximately 70% of the human chromosomes and 30% of the mouse chromosomes. The results are shown in Table 6.3 and the representative photos are shown in Figure 6.4.

FIGURE 6.3

CISPLATIN DOSE-RESPONSE CURVES OF THE PARENTAL LINES AND THEIR HYBRIDS
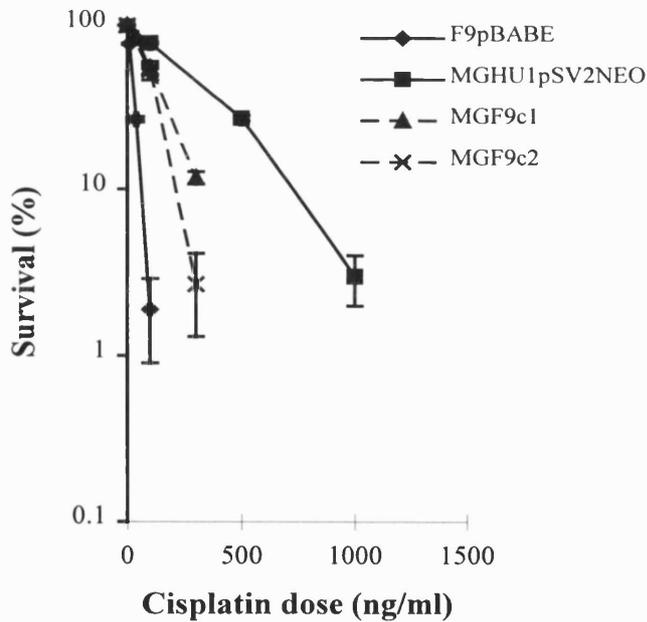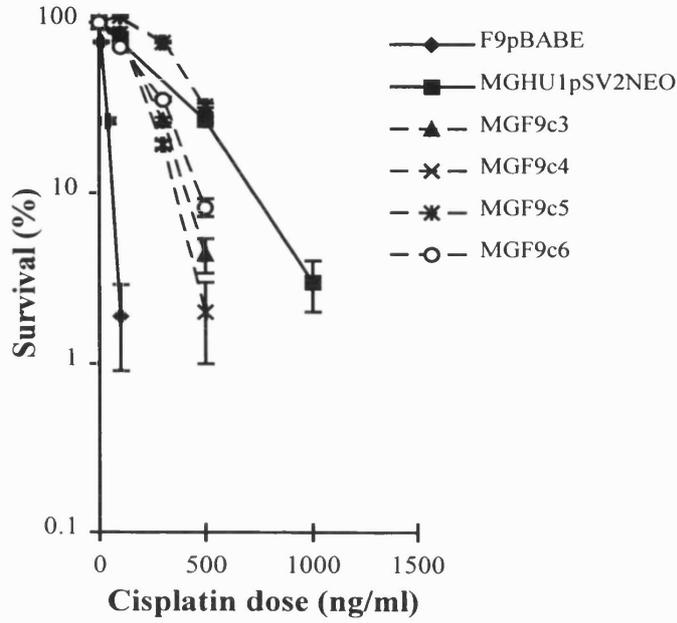
Dose-response curves obtained by clonogenic assay following continuous exposure of the parental lines F9pBABE and MGHU1pSV2NEO and their hybrids to a range of concentrations of cisplatin. Each graph shows the surviving fractions of the parental cells (continuous lines) and the hybrid lines (dashed lines).

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TABLE 6.2
COMPARISON OF CISPLATIN SENSITIVITIES OF THE SELECTED HYBRIDS AND THEIR PARENTAL CELL LINES

Cell Lines	IC_{50} Values (ng/ml)	Fold Increased Resistance Compare to F9pBABE
F9pBABE	19.7 ± 2.1	1
MGHU1pSV2NEO	232.1 ± 51.7	11
MGF9c1	84.1 ± 5.5	4.1
MGF9c2	74.9 ± 13.1	3.8
MGF9c3	163.6 ± 28.6	8.3
MGF9c4	142.6 ± 23.2	7.2
MGF9c5	336.7 ± 120.7	17.1
MGF9c6	170.8 ± 33.3	8.6

The concentration required to inhibit colony-formation to 50% of untreated controls (IC_{50}) was determined from 3 separate experiments. The results are the means $\pm$ SD.

TABLE 6.3

SUMMARY OF FISH RESULTS

Hybrids	Mouse Chromosome Number			Human Chromosome Number		
	Average ±SD	Proportion of mouse parental chr.(%)	Proportion of Mouse chr. In the hybrid (%)	Average±SD	Proportion of human parental chr.(%)	Proportion of Human chr. In the hybrid (%)
F9pBABE	39.5±1.5					
MGHU1 pSV2NEO				82.8±5.3		
MGF9c1	31.7±5.9	79.9±15.2	29.5±4.4	75.4±6.2	88.7±7.3	70.4±3.7
MGF9c2	32.1±5.5	82.2±13.2	29.3±2.5	78.6±6.9	92.4±8.2	71.2±2.9
MGF9c3	32.7±7.0	77.1±14.5	29.8±2.6	79.8±14.5	86.9±11.1	70.2±2.6
MGF9c4	29.9±5.5	76.7±14.1	28.3±4.1	75.3±6.1	87.5±7.1	71.7±4.1
MGF9c5	31.0±4.6	79.4±11.9	29.8±1.4	73.5±13	85.5±15.1	70.7±1.7
MGF9c6	34.1±6.9	84.9±16.3	30.5±2.9	75.1±7.3	87.3±8.4	69.5±2.9

Results were derived from minimum of 10 metaphase cells of each cell line.
Chr. Chromosome

FIGURE 6.4

CHROMOSOME ANALYSIS OF THE HYBRIDS AND THEIR PARENTAL CELL LINES

F9pBABE
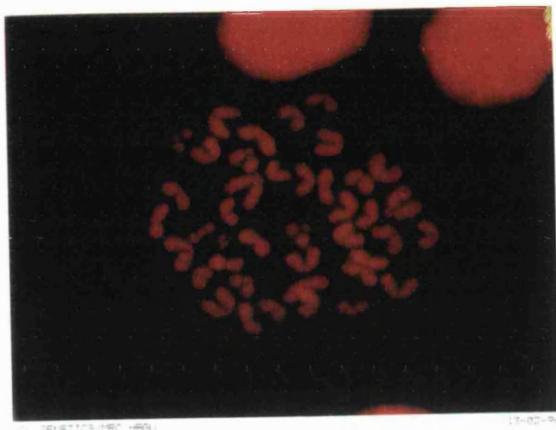
MGHU1pSV2NEO

MGF9c2
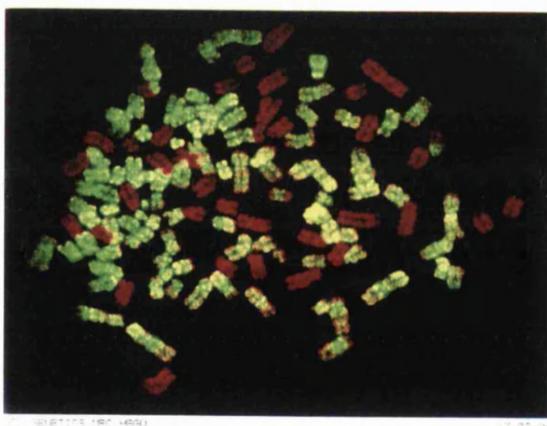
MGF9c5
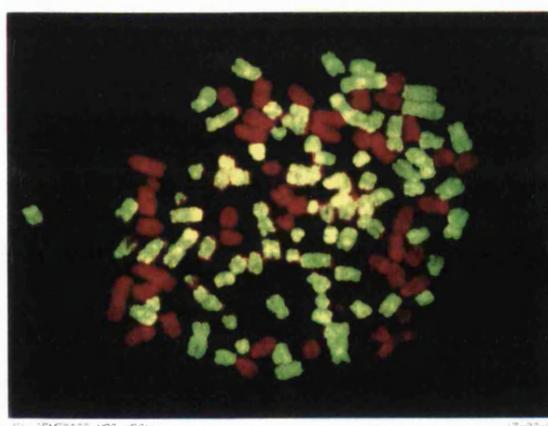
FISH was performed on the parental and hybrid metaphase cells using Biotin-labeled human cot-1 DNA as probes. Two representative graphs of the hybrids are shown. The fluorescence-stained chromosomes (green) are human and the red-stained chromosomes are mouse in origin.

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6.3 DISCUSSION

In this study, hybrids between a cisplatin-sensitive mouse embryonal carcinoma cell line (F9) and a cisplatin-resistant human bladder cancer cell line (MGHU1) were generated with the aim of identifying human chromosomes responsible for cisplatin resistance. The resistant parental line MGHU1pSV2NEO was approximately 11-fold more resistant than the sensitive parental line F9pBABE, providing a large difference in sensitivity to cisplatin.

All the hybrids except MGF9c5 were intermediate in cisplatin sensitivity between F9pBABE and MGHU1pSV2NEO, as observed in previous studies when F9 was fused with another human tumour cell line HT1080 (see Chapter 4.2.1). The four most resistant hybrids (MGF9c3-6) and the two most sensitive hybrids (MGF9c1 and 2) were studied for the presence of human chromosomes using FISH. The results indicated that by passage 10 there was loss of both mouse and human chromosomes in the hybrids. The results are in contrast to previous studies that found little loss of mouse chromosomes in mouse-human hybrids (Illmensee *et al.*, 1978; Cassingena *et al.*, 1992; Mirakhur *et al.*, 1996).

The reason why MGF9 hybrids retained the majority of human chromosomes and lost some mouse chromosomes is unknown. All of the six hybrids showed similar results. Interestingly, although different numbers of human and mouse chromosomes were observed in each hybrid, the proportional distribution of the chromosomes in all six hybrids was similar at approximately 70% of human chromosomes and 30% of mouse chromosomes. Technical artifacts could result in some metaphase chromosomes being lost during the preparation. However, a total of 60 metaphase cells from 6 independent hybrid cell lines were examined and consistent data were obtained.

In a previous study (Illemensee *et al.*, 1978), it was shown that when mouse teratocarcinoma cells were fused with a human fibrosarcoma cell line (HT1080-6TG), the resulting hybrid cells segregated human chromosomes quickly and retained only one to three human chromosomes stably. Similar observations were also reported when the HT1080 cells were fused with established mouse cell lines (Croce, 1976). However, contradictory results were obtained by Miller and co-workers (1976), when these experiments were repeated and a preferential loss of mouse chromosomes was observed. Another study of the hybrids between HT1080-6TG and mouse teratocarcinoma cells (Ajiro *et al.*, 1978) showed that

some hybrids underwent segregation of human chromosomes while others showed loss of mouse chromosomes. However, all these studies showed that there was a higher percentage of loss of human chromosomes than mouse chromosomes in the hybrids.

In previous studies, the techniques used to identify human chromosomes, such as trypsin/Giemsa staining or C-banding (chromosomes stained by formamide standard saline citrate), might not have been sufficiently sensitive. In present study, human Cot-1 DNA was used as a probe to detect human chromosomes in the hybrids. This probe hybridizes to human specific repeat sequences. In a similar study, however, loss of human chromosomes was observed in hybrids between a mouse fibroblast cell line (A9) and a human ovarian carcinoma cell line (2008/C13*) (Mirakhur *et al.*, 1996). The human chromosomes were detected by FISH analysis using human total DNA as a probe.

It has also been claimed that it is difficult to generate hybrids between human normal somatic cells and mouse embryonal carcinoma (EC) cells due to their phylogenetic distance and differences in differentiation potential (reviewed by Takagi, 1997). It was first noticed by Featherstone and McBurney (1981) that cell fusion between mouse EC cells and diploid somatic cells from other species failed to generate hybrids. They also failed to obtain any hybrids between mouse EC cells with human cell line such as Hela or a diploid human cell line IMR90 (McBurney and Rogers, 1982). When Takagi (1997) tried to generate hybrids between mouse EC cells with human lymphocytes, it was also failed. Several hybrids are known between mouse EC cells and human cells have been all generated from two cell lines, mouse embryonal carcinoma cell line, OTT6050 and human fibrosarcoma cell line, HT1080-6TG (Croce, 1976; Meller *et al.*, 1976, Ajiro *et al.*, 1978; Illmensee *et al.*, 1978). However, I had no difficulty generating such hybrids in this study and in the fusion performed in chapter 4 between F9 cells and the human fibrosarcoma cell line HT1080.

The aim of this study was to take advantage of the expected loss of human genetic material from the human-mouse hybrid cells in order to observe the effect of the remaining chromosomes on cisplatin sensitivity. Contrary to expectation and previously published data, the generated hybrids retained a significant proportion of human chromosomes. Consequently, even though changes in sensitivity to cisplatin were observed, the high number of retained human chromosomes precluded the possibility that changes in drug sensitivity could be correlated to any individual human chromosomes in the hybrids.

Chapter 7

INVESTIGATION OF GENE REGULATION BY CISPLATIN USING DIFFERENTIAL DISPLAY PCR

7.1   INTRODUCTION

Differential display reverse transcription PCR (DDRT-PCR or DD-PCR) was first described by Liang and Pardee (1992) as a tool to identify differences in mRNA expression between cells. This method is based on the assumption that, in principle, every individual mRNA molecule in a mammalian cell can be reverse transcribed and amplified by PCR. In combination with a 10 base pair oligonucleotide of arbitrary sequence, which in theory can hybridize to any mRNA, the 15,000 individual mRNAs expressed in a cell can be amplified by PCR at a low annealing temperature of 40°C. The amplified cDNA fragments can be separated on a DNA sequencing gel. Side-by-side comparison of the amplified cDNAs from different cells will allow differentially expressed genes to be identified. Therefore, changes in cellular function or behavior that are regulated by altered expression of proteins can be followed by analysis of the expression pattern of mRNA.

Cellular responses following DNA damage may include the induction of genes that play roles in self-protection, DNA repair, cell cycle arrest and apoptosis (Fram *et al.*, 1988; Fornace, 1992). Studies have been carried out to identify early-induced genes following DNA damage, using Northern blotting analysis. For example, after exposure to cisplatin (300μM) for 30 minutes, c-*jun* mRNA levels were induced in the human melanoma cell line, RPMI-8322 and reached maximum levels 2 hours after the exposure (Rabo *et al.*, 1996). The induction of the c-*jun* mRNA was shown to be sensitive to the tyrosine-kinase inhibitor herbimycin A, an inhibitor of Src-family tyrosine kinases (Chae *et al.*, 1993). This suggests that the Src type tyrosine kinase related pathway may be involved in regulating early responses to cisplatin in these melanoma cells. Using a similar approach, RhoB mRNA, encoding a Ras-related GTP-binding protein, was found to be induced to maximum levels (3-4 fold increase) 30 minutes after an IC_{90} dose of cisplatin or UV irradiation (Fritz, *et al.*, 1995). The levels of c-*fos* mRNA were also enhanced at the same time point. Induction of both RhoB and c-*fos* mRNA was blocked by inhibitors of protein kinase A or C, indicating a general involvement of these kinases in DNA-damage-induced early response. The immediate-early induction of RhoB, c-*jun* and c-*fos* indicates that the Ras-related pathway might be involved in the acute response to DNA damage. The disadvantage of the Northern blotting analysis used in these studies is the requirement for a specific cDNA probe, which therefore limits the study to known genes.

In recent studies using the differential display technique, mRNAs from three tumour cell lines, PC-3 (prostate), T24 (bladder) and KCP-4 (head and neck), were amplified and compared with those derived from their acquired cisplatin resistant sublines (Hisano *et al.*, 1996). Using a total of 15 arbitrary primers in combination with oligo-dT primers, 150 bands differentially displayed between the parental cells and the cisplatin resistant lines were recovered. Of these, 62 hybridized to cellular RNA, as detected by Northern blotting analysis. Twenty-four of the clones were confirmed to be expressed differentially between the sensitive and the resistant cell lines. One of them, the *T-plastin* gene, which encodes an actin binding protein, was expressed at levels 12-fold higher than the parental bladder tumour cell line, T24. However, this difference was not observed in the other two pairs of cell lines. The transfection of a vector containing full-length T-plastin antisense RNA resulted in decreased *T-plastin* mRNA expression and increased sensitivity to cisplatin in the resistance T24 subline (T24/DDP10). This subline also showed decreased drug accumulation but transfection with the antisense T-plastin gene resulted in increased drug accumulation. It has therefore been suggested that the overexpression of T-plastin might affect the activity of the transporter that mediated cisplatin efflux or influx.

The aim of this study was to identify genes regulated by cisplatin in testicular tumour cells using differential display technology. To study the cellular response to cisplatin, the cells were treated with IC_{90} concentrations of cisplatin for one hour and incubated for two hours before total RNA was isolated.

Two disadvantages of differential display techniques are the underrepresentation of certain mRNA species and a high number of false positives (Vogele-Lange *et al.*, 1996). In previous differential display studies, twelve possible two-base anchored oligo-dT primers ($T_{12}MN$ where M may be dG, dA or dC and N may be any one of the 4 deoxynucleotides) were used in combination with at least 20 different 10 base arbitrary primers to display 10-15,000 mRNA species (Liang and Pardee, 1992). It was then found that it was the last base from the 3' end of the oligo-dT primer which provided the specificity. Therefore instead of using all 12 $T_{12}MN$ primers, four degenerate primers that differ only in the last base were used (Liang *et al.*, 1993). This significantly reduced the number of reverse transcription reactions. Further improvements were made using one-base anchored oligo-dT to reduce the number of reverse transcription reactions (Liang *et al.*, 1994). Therefore one base-anchored oligodT primers were used instead of the two-base anchored primers, which minimises the redundancy and underrepresentation of

certain RNA species due to the degeneracy of the primers (Liang *et al.,* 1994). As low annealing temperature was used in the PCR reactions (40°C), false positives are often observed. To reduce the possibility of generating non-specific differentially displayed bands, two testicular tumour cell lines were used for side-by side comparison. Two bladder tumour cell lines were also studied to investigate the differences between genes regulated by cisplatin in testicular tumour cells and cisplatin resistant bladder tumour cells.

Each set of primers displays approximately 100-150 bands. In theory, 50 arbitrary primers are needed to amplify the potential 10-15,000 mRNA species. In this study, five arbitrary primers were used in combination with three H-T_{11} primers to amplify the mRNA transcripts by PCR, and approximately 1500-2000 mRNAs were screened. The PCR products were displayed on DNA sequencing gels and differentially displayed bands were selected. To minimize the selection of false positive bands, the following criteria were established:

1) There must be at least one common band visible in all samples with a given set of primers and the density of the common band should be similar in all samples. This provides an internal control for even loading of samples and excludes false positive down (or up)-regulated bands generated by uneven loading. All the positive bands selected in this study had internal control bands using the same primer combinations.

2) A band was considered to be specifically displayed in testicular tumour cells when it appeared in all four testicular tumour cell samples (GH untreated, treated; SuSa untreated, treated with cisplatin).

3) A cisplatin-regulated band was defined when it appeared in both cell lines of the same tumour origin.

The following experimental plan was used:

Bladder tumour cell lines, MGHU1 and RT112,

Testicular tumour cell lines, GH and SuSa

↓↓

Treatment with IC_{90} concentration of cisplatin for 1 hour, followed by a two hour drug free incubation period

↓↓

Total RNA isolation

↓↓

Reverse transcription of mRNA using oligoH-T_{11}A, C and G primers, Divide the RNA transcripts into 3 sub-populations

mRNA

CAAAAAAAAAAAAAn
3'-*GTTTTTTTTTTTCGAA-5*'

GAAAAAAAAAAAAAn
3'-*CTTTTTTTTTTTCGAA-5*'

TAAAAAAAAAAAAAn
3'-*ATTTTTTTTTT CGAA-5*'

primers

↓↓

PCR amplification (low annealing temperature of 40°C) of cDNAs for each of the three cDNA pools using combinations of oligo H-T_{11}A, C and G primers and 5'arbitrary primers.

cDNA

Arbitrary primer

5

CAAAAAAAAAAAAA 3'
GTTTTTTTTTTTCGAA

Oligo dTprimer

↓↓ amplification

GTTTTTTTTTTTCGA
GTTTTTTTTTTTCGA
GTTTTTTTTTTTCGA

↓↓

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Denaturing polyacrylamide gel

(Arrows indicate the possible positive clones, a cisplatin regulated band is selected when it appears in both cell lines of the same tumour origin)

↓↓

Identification, isolation and re-amplification of the differentially displayed bands (The differentially expressed cDNA fragments were cut out from the gel and re-amplified)

↓↓

Cloning of reamplified fragments into vector pCR-Script

↓↓

Sequencing and GenBank search for homologues

↓↓

Confirmation of the differential expression of the identified DNA sequences by reverse transcription PCR.

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7.2 RESULTS

7.2.1 Cisplatin Sensitivity of Testicular Tumour Cell Lines and Bladder Tumour Cell Lines

The cisplatin sensitivities of two bladder tumour cell lines, MGHU1 and RT112 and two testicular tumour cell lines, GH and SuSa were determined. The single cells were treated with cisplatin for 1 hour and given 14 days to form colonies. The IC_{90} concentrations of cisplatin were calculated and these concentrations of cisplatin were used to treat the cells for differential display experiments (see Figure 7.1 and Table 7.1). The two bladder tumour cell lines showed 3-6 fold more resistance to cisplatin than the two testicular tumour cell lines.

7.2.2 Isolation of Total RNA

To study the expression of genes following cisplatin treatment, the treated cells were given two hours to allow for gene expression after cisplatin treatment. Total RNA was isolated from treated and from untreated controls. Due to the possibility of accidental introduction of trace amounts of RNAse, the integrity of the RNA was estimated before use to ensure degradation had not occurred. As approximately 80-85% of RNA in a mammalian cell is ribosomal RNA, the visualization of two major ribosomal RNA bands on a denatured electrophoresis gel indicates that the majority of isolated RNA in all cell lines is intact (see Figure 7.2). The two major bands represent the 28S and 18S ribosomal RNA species (Sambrook *et al.*, 1989).

7.2.3 Differential Display Reverse Transcription PCR (DD-PCR)

To avoid interference by contaminating genomic DNA in the PCR experiment, the RNA samples were treated with DNAse. RNA samples of two bladder cancer cell lines (MHU1, RT112) and two testicular tumour cell lines (GH, SuSa) were used for DD-PCR experiments. As eukaryotic mRNAs carry at their 3' termini a poly-adenine tail, the mRNAs in the total RNA population can be reverse transcribed in the presence of poly-thymine oligo primers by reverse transcriptase. The mRNA was divided into three aliquots and three one-base-anchored oligo-dT primers ($H\text{-}T_{11}G$, $H\text{-}T_{11}C$, $H\text{-}T_{11}A$) were used for reverse transcription. These primers were also linked to a *Hind III* restriction site

FIGURE 7.1

CISPLATIN SENSITIVITY OF TESTICULAR AND BLADDER TUMOUR CELL LINES
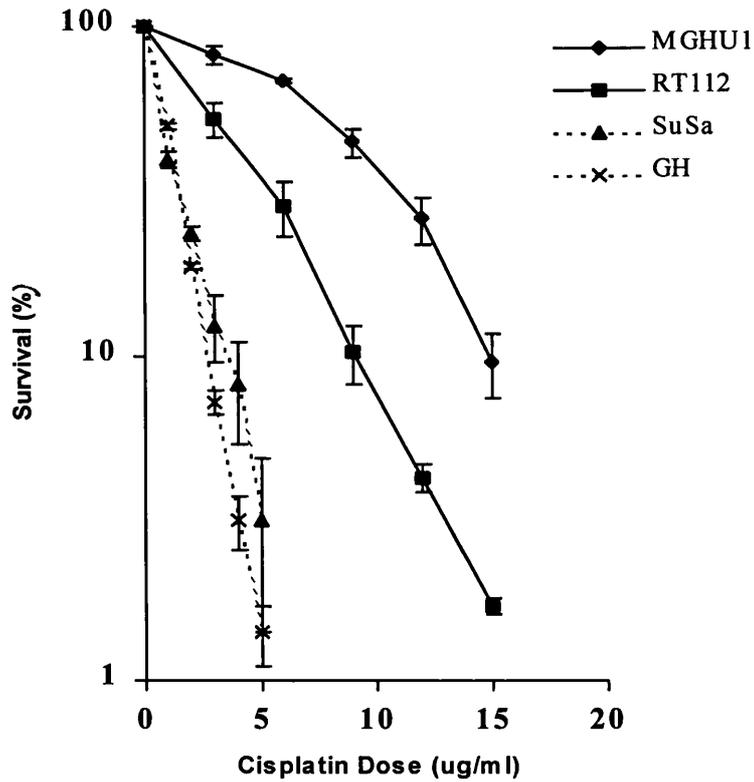
Dose-response curves obtained by clonogenic assay following 1 hour exposure of 2 testicular tumour cell lines, SuSa and GH (dashed lines), and 2 bladder tumour cell lines, RT112 and MGHU1 (continuous lines) to a range of concentrations of cisplatin. Each dose-response curve is derived from a minimum of three independent experiments.

TABLE 7.1

SUMMARY OF CISPLATIN SENSITIVITY OF TWO BLADDER AND TWO TESTICULAR TUMOUR CELL LINES

Cell Lines	IC_{90} Concentration of Cisplatin (μg/ml) $\pm$ SD
Bladder tumour cell lines **MGHU1**	17.5 ± 1.1
RT112	8.8 ± 0.6
Testicular Tumour Cell Lines **GH**	2.7 ± 0.2
SuSa	3.3 ± 0.8

The cells were treated with cisplatin for one hour and the IC_{90} concentrations of cisplatin were calculated from 3 independent clonogenic assays.

FIGURE 7.2

ELECTROPHORESIS OF TOTAL RNA ISOLATED FROM TWO BLADDER AND TWO TESTICULAR TUMOUR CELL LINES
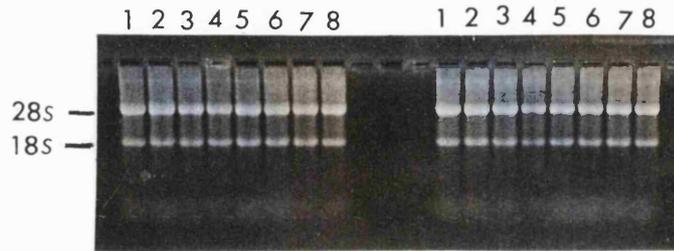
RNA preparations (20μg) were separated on a formaldehyde-agarose (1.2%) gel at 15V for approximately 20 hours and stained with ethidium bromide. The two major bands in each sample represent the 28S and 18S ribosomal RNA indicating a minimum amount of RNA degradation. Samples numbered 1-4 are bladder tumour cell lines, MGHU1 (1,2) and RT112 (3, 4), before (1, 3) and after (2, 4) cisplatin treatment; samples numbered 5-8 are testicular tumour cell lines, GH (5, 6), SuSa (7, 8), before (5, 7) and after (6, 8) cisplatin treatment.

(AAGCTT) at the 5' end to facilitate cloning the transcripts of interest. Following comparison of two reverse transcriptases, it was found that SuperScript™ (Gibco) resulted in the display of more mRNA bands than the MMLV reverse transcriptase (GenHunter) (data not shown). The SuperScript™ was used in subsequent differential display experiments.

Ten differentially displayed bands were selected using five arbitrary primers in combination with oligo-dT primers. Five bands appeared in testicular tumour cell lines, but were absent in the bladder tumour lines, regardless of cisplatin treatment, and these were selected as internal positive controls. Five bands appeared to be regulated in either both of the testicular tumour cell lines or the bladder tumour cell lines were also selected for further study. Table 7.2 summarizes the differential displayed bands selected for further analysis. Figure 7.3 shows representative photographs of differential display gels.

The ten differentially displayed bands were excised from the display gel and re-amplified using the same primer set and PCR conditions as used in the differential display experiments. The re-amplified PCR products are shown in Figure 7.4. The sizes of the amplified fragments, numbered 1-10, are estimated to be between 150 and 450bp (see Table 7.2).

7.2.4.   Cloning the Re-amplified PCR Product

The re-amplified PCR products were purified and cloned into pCR-Script™ Amp SK (+) cloning plasmid (pCR-Script) (see Figure 2.4) at the *SrfI* restriction site by blunt-end cloning. The bacterial colonies (white) containing cloned insert were selected on agar plates containing X-gal and IPTG. From each cloned insert, 15 white bacterial colonies were screened and the insert amplified by PCR using the T3 and T7 sequences on the pCR-Script plasmid as primers.

The size of amplified PCR product was compared to the original insert size. The positive clones were identified as those whose size was the original insert size plus the cloning site of the pCR-Script (approximately 170bp) (see Figure 7.5). Three positive clones of each insert were sequenced from both ends using T3 and T7 as sequencing primers by manual or automatic sequencing. The differential display primers

TABLE 7.2

SUMMARY OF THE RE-AMPLIFIED DIFFERENTIAL DISPLAYED BANDS

Sample Number	Differences Shown on Differential Display Gel	Estimated Size of Re-amplified Product (bp)	Re-Named Sample
1	Band visible in untreated bladder tumour cells but not in cisplatin treated bladder cells (Cisplatin regulated band in bladder cells)	450	BC1
2	As above	350	BC2
3	As above	150	BC3
4	Band visible in testicular tumour cells only (Testis specific band)	350	T1
5	As above	350	T2
6	As above	150	T3
7	As above	250	T4
8	Band visible in untreated bladder tumour cells but not in cisplatin treated bladder cells (Cisplatin regulated band in bladder cells)	400	BC4
9	Band visible in testicular tumour cells only (Testis specific band)	250	T5
10	Band visible in cisplatin treated in testicular cells (Cisplatin regulated band in testicular tumour cells)	250	TC1

FIGURE 7.3

REPRESENTITAVE DIFFERENTIAL DISPLAY OF PCR AMPLIFIED cDNAs FROM CISPLATIN TREATED AND UNTREATED BLADDER AND TESTICULAR TUMOUR CELL LINES
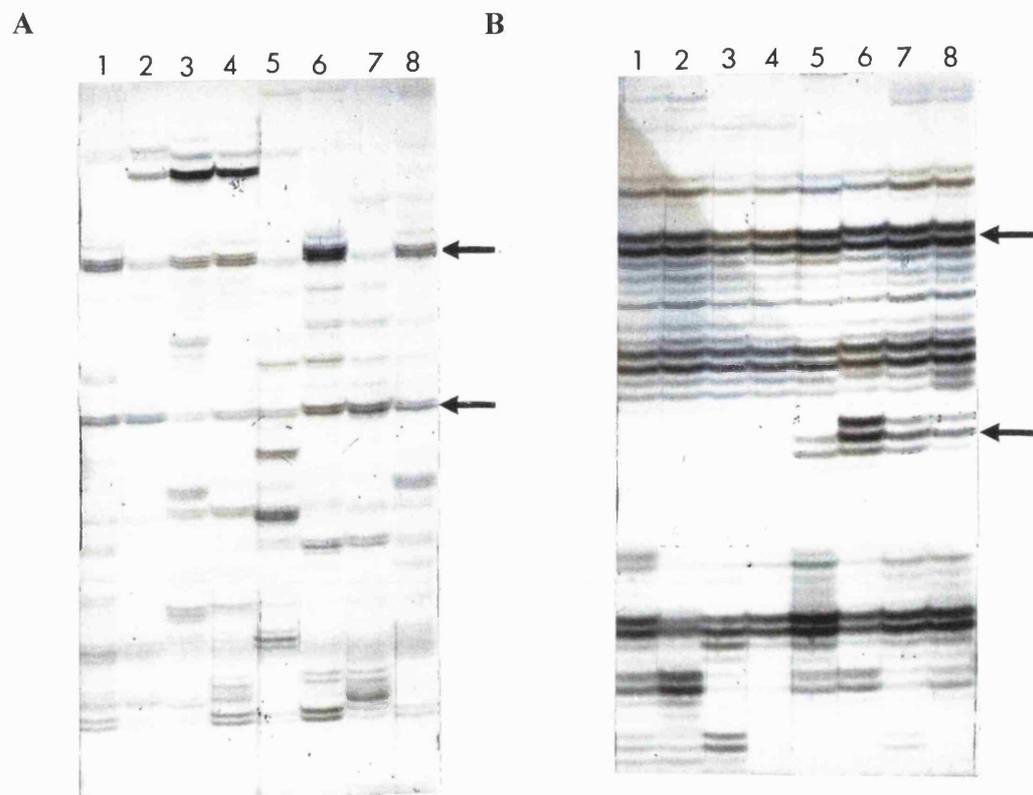
The mRNA transcripts were amplified by PCR using the combination of OPE18 arbitrary and oligo $HT_{11}C$ primers. A) A differentially displayed band in testicular tumour cell lines GH (5, 6) and SuSa (7, 8) before (5, 7) and after (6, 8) cisplatin treatment. B) A differentially displayed band between bladder tumour cell lines (1-4) and testicular tumour cell lines (5-8). The arrows indicate either the internal control bands (appeared in all samples) or the differential displayed bands. MGHU1(1,2) AND RT112(3,4) BEFORE(1,3) OR AFTER (2,4) CISPLATIN TREATMENT

FIGURE 7.4

RE-AMPLIFICATION OF THE RECOVERED DIFERENTIAL DISPLAYED BANDS
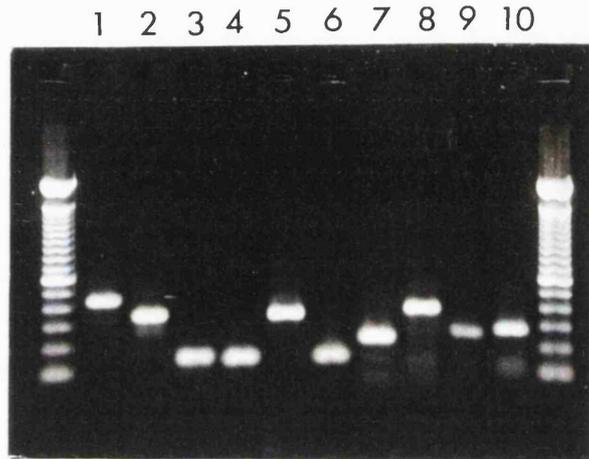
Ten differentially displayed bands were re-amplified using the same set of primers as in the original PCR reaction. 100bp ladder markers were loaded in the far left and right lanes. The sample numbers correlate to the ones listed in Table 7.2.

FIGURE 7.5

AMPLIFICATION OF CLONED PCR PRODUCTS FROM TRANSFORMED BACTERIAL COLONIES
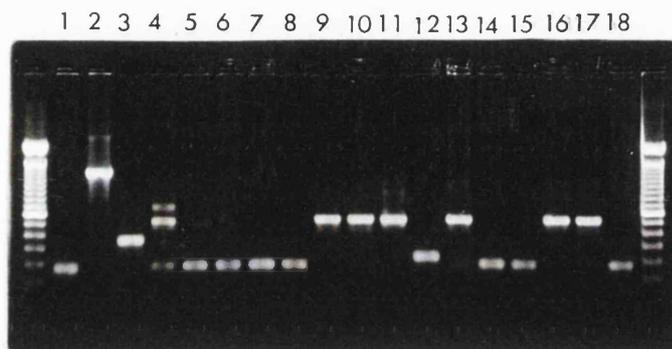
E Coli XL-Blue bacteria were transformed by pCR-Script plasmid containing inserted DNA fragment. 15 white colonies from each X-gal agar plate were lysed and the inserted DNA was amplified by PCR. This representative photograph shows the results of one of the cloned DNA fragments, clone No.2 (approximately 350bp). The two lanes on the left and right of the gel are 100bp ladder markers. *Lane 1*: Vector only, showing an amplified cloning site on the pCR-Script plasmid of approximately 150bp. *Lane 2*: a positive control with a known insert size of approximately 1.1kb. *Lane 3*: DNA insert before cloning into pCR-Script. *Lanes 4-18*: PCR amplified white bacterial colonies. *Lanes 5-8, 12, 14, 15, 18* are considered as false positive with no insert and *lanes 9-11, 13, 16, 17* are considered to be positive as their sizes are correspond to the combined size of both fragments in *Lane 1* and *Lane 3* (approximately 500bp).

were observed in each of the sequences from either the T3 or T7 end. Each differential displayed band represents one DNA sequence. Homologous genes or sequences were identified when the sequences from both ends of the insert matched the same gene or DNA sequence. The sequencing data was compared to the BLAST (Basic Local Alignment Search Tool) non-redundant GenBank database. Figure 7.6 is an example of how the identity of a cloned fragment was determined.

As shown in Figure 7.6, the arbitrary (OPE18) and oligo-dT$_{11}$C primers are seen on the ends (in bold) of the TC1 fragment identified from either T3 or T7 end sequence. The overlapping sequences between the T3 and T7 sequencing primers were also identified if possible. Due to the low annealing temperature (40°C) used in the differential display PCR experiments, the sequences corresponding to the arbitrary primers usually have 7-8 perfect matches with 1-3 internal or 5' end mismatches (Ayala *et al.*, 1995). This was also observed with the isolated TC1 sequence (Figure 7.6, the OPE 18 primer is shown in bold). Two bases in the middle of the primer are mismatched and one of the bases is missing. However, even with the mismatches, the primers are still able to recognise a specific cDNA species, while other bands are also amplified as well, because of the reduced primer specificity (Liang *et al.*, 1995). This also suggests that a 10mer actually hybridizes as a 7mer, which indicates that it may amplify more fragments than expected. The oligo-dT$_{11}$C primer is also shown in bold at the other end of the TC1 sequence.

The sequencing data from both T3 and T7 ends were compared to the available sequences in the using a BLAST (Basic Local Alignment Search Tool) search. The homologous sequences in the GenBank are shown in underlined letters. The BLAST search resulted in a high percentage match of a genomic DNA sequence located on chromosome 5q31 when both T3 and T7 ends of the sequencing data were compared separately. The accuracy of match is suggested by the BLAST search as shown in Table 7.3. This suggests that the TC1 fragment is a homologue of this genomic DNA sequence.

As shown in Figure 7.6, TC1 matches two fragments of the known genomic DNA sequence, leaving a gap of approximately 1500bp in between. This indicates that there is an intron in the genomic DNA sequence. The RT-PCR results described in section 7.2.5 confirmed this finding.

FIGURE 7.6

THE SEQUENCE OF TC1 FRAGMENT

5'-AAGCTTTTTTTTTT TC-3'

TC1   TTTTTTTTTT TCAATAAA C TGGCCAGCTT CACATCTTTT TTGGTCAGTT

                   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

GenBank   967   CAATAAACT TGGCCAGCTT CACATCTTTT TTGGTCAGTT

TC1   CACCACAGTC AGTGAGGTGA GAG

                | | | | | | | | | | | | | | | | | | | | | | | | |

GenBank   CACCACAGTC AGTGAGGTGA GAG   1029

TC1   TTACTGGAC CTTGTGTATA CATTGAACCA TTCTGGGTGA TGATTCATCT

                    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

GenBank   2497   TTAGTTACC TTGGTGTATA CATTGAACCA TTCTGGGTGA TGATTCATCT

TC1   TCTCTGCTTG TAGGGCAACT CGGGACAAAA   GCCAAAGTCC TGATTAAAAT

               | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

GenBank   TCTCTGCTTG TAGGGCAACT CGGGACAAAA AGCCAAAGTC   TGCATAGAAT

                                      3'- AGACGTCAGG-5' (OPE18)

TC1   TGTGGAAGGA GAATTCTTTG AGATGGATCT CTCTCC

               | | | | | | | | | | | | | | | | | | | | |

GenBank   TGTCGAAGAA TAATTTAT G ACATTGA2632

The sequences in bold represent the DD-PCR primers. The underlined sequence is the homologous gene sequence from the GenBank BLAST search. The dotted lines indicate the sequences of the primers used for amplification of the TC1 fragment by RT-PCR.

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TABLE 7.3

RESULTS OF DNA SEQUENCING DATA AND BLAST NON-REDUNDANT GENBANK SEARCH

Sample Name	Confirmed Sequence (bp) (approximately)	Most likely Human Homologue Gene	Percentage Match*
BC1	T3 end: 213 T7 end: 189	Human chaperone -like protein (HTR3) mRNA (1685bp), matching 1447-1640bp of the gene Function: involved in amino acid transport	90.5%
BC2	T3 end: 216 T7 end: 252	Matches 167-214 bp of a human EST#, A448519 (413bp) Matches 90-131bp of a human EST, AA080121 532bp)	79% 76%
BC3	T3 end:110 T7 end:177	Human mRNA Fau gene (518b), matching 393-507 bp of the gene. Function: Antisense of murine sarcoma virus	99%
T1	T3 end: 150 T7 end:111	Matches 408-468bp of a human EST, AA527389 (474bp)	65%
T2	T3 end: 138 T7 end::142	Human Duchenne muscular dystrophy, Exon 17 Function: cell membrane protein	95%
T3	T3 end: 150 No matching sequence found		
T4	---	----	
BC4	T3 end: 150 T7 end: 180	Matches 3358-3508bp of a human EST KIAA0035 .	97.5%
T5	T3 end: 210	Matches 42-245bp of a human EST HSC3KF062 (270bp)	90%
TC1	T3 end: 135 T7 end:192	Human 4481bp DNA sequence (sequence of human chromosome 5q), matching 967-1029, 2497-2632 bp of the sequence. Function: unknown	91%

The DNA sequence of each sample was determined as the shared sequence in at least two of the sequenced clones. The homologue nucleotide sequences were matched in non-redundant GenBank database programme through Internet (http://www.ncbi.nlm.nih.gov).
*Represents the average of the results of all sequences searched from each fragment.
#EST: Expressed sequence tag.

As also shown in Figure 7.6, the oligo-dT end of the TC1 matches the 5' end of the known genomic DNA sequence. Due to the low annealing temperature used in the PCR reaction (40°C), the oligo-dT primer annealed to the cDNA to initiate DNA synthesis as a forward primer with the OPE 18 primer acted as a reverse primer. This resulted in the amplification of the TC1 fragment as shown in Figure 7.6.

All the cloned fragments were sequenced and compared to the GenBank sequence as described for TC1 (see Table 7.3). Like TC1, some of the sequences (eg. BC2 and T2) did not match the 3' end of the known genes or sequences suggesting that the oligo-dT primers may have annealed to the middle of the RNA transcript rather than the polyA tail. Seven out of eight of the fragments (T4 was not cloned) were found to be homologous to either a known gene or an expressed sequence tag (EST), although two of them (BC2 and T1) had a relatively low percentage of matching sequences.

There is no known function relating to DNA damage or cisplatin response among the identified homologues to the cisplatin regulated fragments in bladder tumour cell lines. Three out of 4 of the testicular specific fragments matched EST, however the functions are unknown. The DNA sequences will allow us to design specific RT-PCR primers to confirm the expression of these fragments by RT-PCR.

7.2.5   Reverse Transcription PCR (RT-PCR)

To confirm that the isolated DNA sequences were differentially expressed, RT-PCR was performed using TC1 specific 20bp primers on the cisplatin treated and untreated bladder and testicular tumour samples. The TC1 fragment was studied first as its expression in testicular tumour cell samples increased following cisplatin treatment and was therefore likely to be a candidate for involvement in the regulation of cisplatin sensitivity in testicular tumour cells. The two specific primers were designed as shown in Figure 7.6 by dotted-underlined letters. Unlike the differential display PCR conditions, the annealing temperature for these primers was raised to 60°C in order to ensure specific amplification of the TC1 fragment.

To overcome the possibility of varying amounts of DNA template being used in the PCR experiment, the concentrations of the reverse transcribed RNA samples were optimized by using the amplified actin gene product as a loading control, as used in previous studies

(Kawakami *et al.,* 1997). Firstly, the appropriate number of PCR cycles was determined to ensure the recovery of the PCR products while still in the exponential phase period of the reaction. This reduced the possibility of misleading PCR results due to uneven amplification rates of PCR products in different cell lines. The DNA samples were also diluted to concentrations which resulted in the production of similar amounts of actin PCR product in all 8 samples using the determined number of cycles (judged by the density of the PCR bands on an agarose gel).

The optimised PCR conditions were then used to amplify the TC1 fragment (see Figure 7.7). The density of the PCR bands of the amplified actin gene was similar in all samples, suggesting the quantity of initial template was similar in all samples. The amplification of the TC1 fragment produced different amounts of PCR products between testicular tumour samples treated and untreated with cisplatin in both cell lines (see Figure 7.7, lane 5-8). This suggests that the gene containing this fragment is upregulated by cisplatin. The combination of differential display and RT-PCR results on TC1 suggests that the mRNA level of the gene containing this fragment may be induced in response to cisplatin in testicular tumour cells.

There is no amplification of TC1 in one of the bladder tumour cell lines MGHU1 (Figure 7.7, Lane 1 and 2) suggesting that it is neither expressed before cisplatin treatment nor induced by cisplatin. The other bladder tumour cell line RT112 showed a small increase in TC1 expression in response to cisplatin (Figure 7.7 Lane 3 and 4). However, the degree of upregulation in RT112 seems to be smaller than in the two testicular tumour cell lines judged by the density of the PCR bands. This regulation in RT112 by cisplatin may be explained by its intermediate sensitivity to cisplatin between MGHU1 and the two testicular tumour cell lines (see Figure 7.1).

FIGURE 7.7

ELECTROPHORETIC ANALYSIS OF RT-PCR PRUDUCTS
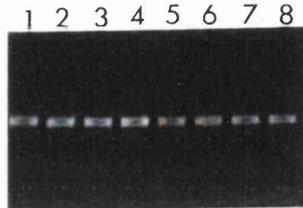
Actin

TC1
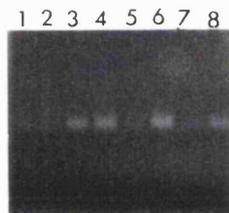
Gel electrophoresis of RT-PCR products of actin and TC1 after 24 cycles. 5μl of PCR product (from 50μl total volume) was loaded in each lane and electrophoresed using 1.5% agarose gel containing 0.5μg/ml ethidium bromide. Samples number 1-4 are bladder tumour cell lines, MGHU1 (1,2) and RT112 (3, 4), before (1, 3) and after (2, 4) cisplatin treatment; samples number 5-8 are testicular tumour cell lines, GH (5, 6), SuSa (7, 8), before (5, 7) and after (6, 8) cisplatin treatment.

7.3　　　DISCUSSION

Differential display was used to study genes regulated in testicular and bladder tumour cells after exposure to cisplatin. One DNA fragment TC1 was identified as a possible candidate for a response gene following cisplatin exposure in testicular tumour cells.

In DD-PCR experiments, the expression of TC1 mRNA was not detectable in testicular tumour cells before the cells were exposed to cisplatin, but was induced 2 hours after exposure to an IC_{90} concentration of cisplatin. It was shown by RT-PCR experiments that in the bladder tumour cell line MGHU1, the TC1 mRNA is not differentially expressed before and after exposure to cisplatin (see Figure 7.7). There seems to be a small degree of TC1 upregulation by cisplatin in one of the bladder tumour cell lines RT112, which may correlate to its relatively less resistance to cisplatin compared to MGHU1.

The homologue of TC1 is a 4481bp DNA sequence located at chromosome region 5q31 (sequence submitted by human genome centre, Lawrence, Berkeley National Laboratory, CA), which was identified by sequencing a 680kb genomic DNA fragment in a 1Mb region of chromosome 5q31. The sequencing data was then compared with the available DNA sequences in the GenBank (Frazer *et al.*, 1997). Using this method, 35 genes were identified whose predicted functions include a transcription factor, a protein involved in DNA repair and several other members of a family of transport proteins. However, there was no homology with any of the currently existing sequences in the database and the TC1 fragment, whose function is unknown. The chromosome region 5q31 also contains a family of cytokine genes, including IL-3 (interleukin), IL-4, IL-5 and IL-13, clustered over a 1Mb region, which play roles in inflammatory and immune responses (Frazer *et al.*, 1997). Currently, there are no reported abnormalities in this region in either testicular or bladder tumours (reviewed by Mitelman *et al.*, 1997).

As discussed in section 7.1, the RhoB, c-jun and c-fos are early response genes that are induced 30 minutes after exposure to cisplatin (Rabo *et al.*, 1996; Frizt *et al.*, 1995). When the induction of *c-jun* was compared with that in an acquired cisplatin resistant subline of a melanoma line, RPMI-8322, the level of *c-jun* was higher in the sensitive parental line. The low level of *c-jun* induction was also paralleled by the low level of cisplatin induced apoptosis in the resistant cells (Zhao *et al.*, 1995). This suggests that the stimulation of signal transduction pathways by DNA damaging agents, including cisplatin, may be an early event triggering cell death. Other genes such as p53 are also

induced after DNA damage. Its induction is observed 2-4 hours after the initial drug treatment in testicular tumour cells (Chresta *et al.*, 1996; Lutzker and Levine, 1996). A continuous time course of TC1 regulation immediately after cisplatin treatment should be studied in order to investigate the precise starting time point of its induction.

The full-length TC1 gene must be isolated before any further functional studies can be carried out. This will involve screening a cDNA library generated from the testicular tumour cells using the TC1 fragment as a probe. This will ensure that the TC1 fragment is the gene already identified at 5q31. To study the effects of TC1 on cisplatin sensitivity in testicular tumour cells, an antisense copy of the gene could be transfected into testicular tumour cells to see if inhibition of TC1 expression will result in an increased resistance to cisplatin. The expression of TC1 protein can also be studied by generating antibodies for Western blot analysis. This would provide an opportunity to study protein expression and determine whether this correlates to the upregulation in the mRNA levels that we observed. Appropriately labelled antibodies could also be used to identify the subcellular localization of the protein. This would provide important clues as to whether the protein was acting inside the nucleus, possibly as a transcription factor or in a DNA repair pathway, or outside the nucleus, in a signal transduction pathway or controlling the entry and exit of cisplatin from the cell.

Due to the different origins of the bladder and testicular tumour cell lines, differentially expressed genes were expected to be found providing positive controls for differential display. One of the testis specific bands, T2 (see Table 7.2), was found to be a homologue of human Duchenne muscular dystrophin gene (DMD) which is expressed predominantly in skeletal, cardiac and smooth muscle cells (Hoffman *et al.*, 1987). The apo-dystrophin mRNA which encodes a protein in the same reading frame as dystrophin but of a smaller size, has also been shown to be expressed in testis (Blake *et al.*, 1994). As the bladder tumour cell lines used in this study were carcinomas of epithelial cell origin, it is not a surprise that the DMD band appeared in testicular cell lines only. Three other testis specific bands, T1, T3 and T5, were either novel genes or their matching genes had unknown function in the BLAST GenBank database. It is necessary to carry out experiments to confirm the expression of the differential expression pattern of these fragments before any further study can be carried out.

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Four bands regulated by cisplatin in bladder tumour cells were also identified on the differential display gels. Investigation of these candidate genes may also provide clues to the differences in the cellular response to cisplatin resulting in different sensitivity. In contrast to TC1, these bands are visible in cisplatin untreated cells but disappeared after cisplatin treatment. This suggests that while cisplatin upregulates some gene(s) at this time point it down-regulates others. Although three match known human genes (see Table 7.3), there is no published evidence that they are involved in response to DNA damage. Should RT-PCR confirm the differential display results it would be interesting to discover whether down-regulation of these genes in cisplatin sensitive testicular tumour cells can increase their resistance.

As this study focused on the relatively early response genes regulated by cisplatin, future work could include studying the effects of cisplatin at different time points. It has been shown that bladder but not testicular tumour cells repair cisplatin damage at later time points (Koberle *et al.*, 1997). The changes in mRNA levels of known DNA repair proteins could be studied so that the differences in genes involved in DNA repair might provide information on why the bladder tumour cells are able to repair DNA damage while testicular tumour cells fail to do so. By comparing the changes in mRNA levels at various time points after cisplatin exposure, information will be obtained on gene regulation in the period between the cells first exposure to cisplatin and when they eventually die.

Chapter 8   Concluding Remarks and Future Work

The aim of this study was to investigate the molecular basis of cisplatin sensitivity in testicular germ cell tumour cells with the aim of identifying the genes responsible.

This project was based on previous studies which showed that the cisplatin sensitivity of testicular tumour cells was complemented by fusion with another cisplatin-sensitive human cell line, D98^{OR}C1 (Wang *et al.*, 1996). To investigate how many complementation groups control the sensitivity of the testicular tumour cells, four cell lines were fused (chapter 3). The lack of complementation indicated that there is a common mechanism shared in all four testicular tumour cell lines in regard to their sensitivity to cisplatin (Wang *et al.*, 1997). Unlike other human DNA-repair deficient diseases such as xeroderma pigmentosum (XP) and Fanconi's anemia (FA), where many complementation groups have been identified (Jeggo *et al.*, 1994), the results on testicular tumour cell lines indicate that there may be either one gene or a set of common genes controlling the sensitivity of testicular germ cell tumours to cisplatin.

Functional complementation cloning by transfection of a DNA library was the primary strategy chosen to identify the gene(s) involved. F9 is a mouse embryonal carcinoma cell line and was chosen because it has higher transfection efficiencies than human testicular tumour cells. Five primary transfectants which showed increased resistance to cisplatin compared to the parental line F9 were isolated. However we were unable to generate any cisplatin resistant secondary transfectants. The possible reasons for this failure include: 1) the gene which complements the sensitivity of F9 cells might not be present in the HT1080 library; 2) The human genes might not function adequately in mouse cells; 3) the spontaneous cisplatin-resistant mutants or the random integration of the plasmid may have conferred primary cisplatin resistance in the primary transfectants (also see chapter 4.3).

Although gene(s) controlling cisplatin sensitivity were not isolated using the functional cloning strategy, modifications could be made to improve this approach. For example, a mouse DNA library could be used instead of the human library, as this may allow more efficient gene complementation in the mouse F9 cells. In addition, the cisplatin resistance factor between F9 cells and the cells which are used to generate the DNA library could be larger to allow more efficient selection of the cisplatin resistant transfectants. To

overcome the non-specific cisplatin resistance caused by random integration of plasmid (resulting in the promotion or disruption of genes leading to cisplatin resistance), an episomal plasmid (not available when we started) could be used as one is now available for mouse cells (Gassmann *et al.*, 1995). This also allows easier recovery of the transfected plasmid.

An alternative way of achieving my aim came when one of my colleagues isolated a cisplatin resistant secondary transfectant in a human testicular tumour cell line using a yeast genomic library (chapter 5). It was confirmed that the yeast genomic DNA insert conferred cisplatin resistance in the secondary transfectant, but it did not result in cisplatin resistance in three other human testicular tumour cell lines and the mouse line F9. Consequently, when the secondary transfection experiments were repeated, increased cisplatin resistance was not observed. In combination with the results in other cell lines, this indicates that the cisplatin resistance induced in the first secondary transfectant might not be due to the effect of the yeast genomic DNA insert. However we are unable to explain the mechanisms resulting in cisplatin resistance in the secondary transfectant.

Complementation of cisplatin sensitivity was not observed between testicular tumour cell lines. As we could not identify a single gene, it is possible that multiple factors, such as the lack of expression of a group of genes or a reduction in the constitutive levels of proteins involved in DNA repair, which are shared in all four cell lines, control their sensitivity to DNA damaging agents.

Turning to an alternative strategy, I tried to identify human chromosomes carrying genes responsible for conferring cisplatin resistance using human-mouse hybrids. This is a less straightforward method of cloning genes compared to functional cloning using a DNA library. However, if successful, it would allow the identification of a human chromosome that carries the gene of interest. This procedure depends on the loss of the majority of human chromosomes from human-mouse hybrids, allowing the altered biological function to be attributed to the expression of gene(s) from the remaining human chromosomes. However, contrary to expectations, the mouse-human hybrids generated in this study retained the majority of both mouse and human chromosomes (see chapter 6). The characterization of the mouse-human hybrids in this study places doubt on the general assumption that human chromosomes are usually lost in mouse-human hybrids (Abbott and Povey, 1995). This study also showed that the fusion of mouse embryonal

carcinoma cells with human cells was possible and practical, unlike claims in other studies (Stone and McBurney, 1981; McBurney and Rogers, 1982; Takagi, 1997).

The last part of this thesis used a different approach to study the molecular basis of cisplatin sensitivity in testicular tumour cells. Differential display was used to compare testicular and bladder tumour cells mRNA expression before and following exposure to cisplatin. Two main improvements were made to previous techniques. Firstly, we used one-base-anchored oligo-dT-primers to subdivide the mRNA population into 3 populations (Liang *et al.*, 1994), unlike earlier differential display studies where twelve combinations of two-base-anchored oligo-dT-primers were used to subdivide total mRNA into 12 fractions (Banuer *et al.*, 1993; Liang *et al.*, 1993). This improvement allows potentially under-represented sub-populations of mRNA to be amplified. Secondly, by using two cell lines, the possibility of generating false positives was reduced. Further improvements to this technique could include using duplicated RNA samples of each cell line to generate higher specificity.

Only small scale experiments were carried out in chapter 7, screening approximately 2000 of the potential 15,000 mRNA species. Ten differentially expressed bands were isolated and sequenced. It was confirmed by RT-PCR that in two testicular tumour cell lines, the level of one of the mRNA species isolated (TC1) is upregulated 2 hours after exposure to cisplatin. Database comparisons confirmed that the DNA sequence homologous to *TC1* has been sequenced and localized to chromosome 5q31. However, no function for the protein encoded by this gene has been described. Identification of this gene will enable us to discover its role in the response of testicular tumour cells to cisplatin. However, the full-length *TC1* gene must first be isolated. Further differential display experiments could examine the time course of TC1 expression to determine how rapidly TC1 is expressed after cellular interaction with cisplatin.

It would also be interesting to discover the effect of toxic doses of cisplatin on gene regulation at the time points when testicular tumour cells undergo apoptosis. This may provide information concerning which genes are involved in triggering apoptosis by cisplatin in testicular tumour cells. This requires studying the mRNA expression from the time point when the cells are exposed to cisplatin until the time they begin to die. There is already evidence that p53 and Bcl-2 are involved in cisplatin-induced apoptosis in

testicular tumour cells (Chresta *et al.*, 1996; Lutzker and Levine, 1996) and the differential display technique may confirm this and allow the discovery of further genes.

Alternatively, sublethal doses of cisplatin could be used to study whether a different set of genes are regulated to help cells overcome cisplatin damage and survive. Testicular tumour cells are also sensitive to other DNA damaging agents such as bleomycin, etoposide and UV irradiation. Differential display can also be performed on cells treated with these agents to study if the same genes or pathways are regulated as in cisplatin treated cells. This information may help us to understand why the testicular tumour cells are hypersensitive to all DNA damaging agents even though these agents damage the cells in different ways and can be repaired by different pathways.

There is strong evidence indicating that testicular tumour cells not only have reduced ability to repair DNA damage over the whole genome (Bedford *et al.*, 1988; Hill *et al.*, 1994), but also at the level of specific genes (Koberle *et al.*, 1996; 1997). In addition, these tumour cells show low levels of constitutive nucleotide excision repair, similar to cells derived from DNA damage sensitivity disorders such as XP (B. Koberle, personal communication). This suggests that defect(s) in DNA repair pathways may contribute to their hypersensitivity to DNA-damaging agents. Testicular tumour cells have high basal levels of wild-type p53 and Bax, and low levels of Bcl-2, suggesting that the apoptosis pathway may also play a role in their sensitivity (Chresta *et al.*, 1996; Lutzker and Levine, 1996). It is possible that wild-type p53 promotes apoptosis in response to DNA damage, contributing to their hypersensitivity. When a mouse embryonal carcinoma cell line, F9 and a human fibrosarcoma cell line HT1080 were fused (chapter 4.2.1), the hybrids showed intermediate sensitivity to cisplatin between these two parental lines. If the hypersensitivity of testicular tumour cells was due to their defect in DNA repair only, this relatively resistant sarcoma cell line would have complemented this defect resulting in cisplatin resistance in the hybrids, although there is a possibility of lack of complementation due to the species difference of the cells used. Therefore, it is possible that there are some factors in testicular tumour cells which promote DNA damage-induced cell death, resulting in the intermediate sensitivity in the hybrids, such as high levels of p53 and Bax proteins and low levels of the Bcl-2 protein.

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