GENE MUTATIONS AND EXPRESSION IN BREAST CANCER

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CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of the "General regulations for the degree of Ph.D.", I, as supervisor of the candidate H. DONNINGER, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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CONTENTS

DEDICATION

To my parents, for all their love and support.

ABSTRACT

Breast cancer is the most common cause of death amongst women, with the incidence of the disease varying between countries. Like all other cancers, breast cancer is a multigenic disorder with mutations in oncogenes and tumour suppressor genes playing an important role in cellular transformation and ultimately in tumour formation. In this study, 40 breast cancer patients from the Western Cape province in South Africa and 4 breast cancer cell lines were screened for mutations in the human Ha-*ras* oncogene and the p53 tumour suppressor gene. The majority of the patients were of mixed ancestry comprising 39 females and one male. DNA was isolated from both fresh tissue biopsies and formalin fixed paraffin sections and screened for mutations by PCR-SSCP and DNA sequence analysis. Since mutations in codons 12, 13 and 61 of the Ha-ras gene constitutively activate RAS, exons 1 and 2 (encompassing codons 12, 13 and 61) were sequenced and shown to harbour no mutations at these sites. A polymorphism at codon 27 was detected, however, this was not associated with the disease condition as the allele frequency was the same as in control samples. These results agree with published data and imply that mutations in the Ha-ras gene do not play a critical role in the aetiology of breast cancer.

The p53 tumour suppressor gene is the most frequently mutated gene in human tumours, and in breast cancer the reported frequency of mutation is approximately 50%. Mutations occur over a wide region of the gene, however, hotspots have been identified in exons 5-9. Screening of exons 5-9 and 11 of this gene revealed the presence of mutations in only 10% of breast cancer patients, 2 in exon 7 and 2 in exon 8, and 2 mutations in the breast cancer cell lines. Three of the mutations in the cancer patients were novel and have not been previously documented. These novel mutations were a deletion of a single adenine residue at codon 239 (p53-ΔA), resulting in a frameshift and the introduction of a termination codon at position 263, a C → A base substitution at codon 269 (p53-S269R) resulting in the substitution of an arginine for a serine in the protein, and a 15bp duplication in exon 8 (p53-+15) leading to duplication of 5 amino acids in the protein.

Wild-type p53 has a short half-life and mutations stabilise the protein, allowing it to be detected using immunohistochemistry, which is an indirect method for detection of mutant p53. Tissue sections from these patients were also screened for mutations by immunohistochemistry and 10 patients were found to stain positive for p53. Two of these patients contained confirmed mutations in exons 7 and 8 (a codon 248 mutation and the p53-S269R mutation) while the remaining 8 patients appeared to harbour no mutations. One of the patients harbouring a mutant p53 was negative for p53 by immunohistochemistry, which is to be expected since the mutation (p53-ΔA) resulted in a truncated protein that is probably rapidly degraded. Since various tumour-derived p53 mutations have been shown to have different effects on cells, the three novel p53 mutant constructs were prepared by site-directed mutagenesis and transfected into NIH-3T3 cells to examine their effects on cell growth and response to DNA damage. The mutations did not affect the growth rate of the cells, but the p53-S269R mutant was found to alleviate the requirement for serum factors when the cells were grown in serum-free medium. The mutations also affected contact inhibition of growth of the stably transfected cells to various degrees, with the p53-S269R and p53-+15 mutations enabling the cells to grow without contact inhibition. The cells stably transfected also responded differently to DNA damage. After exposure to 7Gy of ^{60}Co radiation, only the p53-S269R mutant failed to arrest the cells in the G_1 phase of the cell cycle, while the other two transfected cell lines were still able to undergo a G_1 cell cycle arrest, albeit with lower efficiency than the untransfected cells or cells transfected with the wild-type construct. Thus only the p53-S269R mutation found in these breast cancer patients behaved like a true p53 mutant. On examination of the survival rate of these patients, it was found that the majority of the patients were alive 5 years after diagnosis, and only one patient (with the p53-S269R mutation) died from the disease. This patient harboured a C $\rightarrow$ A base substitution at codon 269, which was the mutation that behaved the most aggressively in the transfection studies. Thus it appeared that the nature of the p53 mutation may affect the survival of these patients and their response to therapy, as only the patient with the aggressive p53 mutation died from the disease, whereas the remaining patients with p53 mutations were still alive. These results also raise the possibility that the frequency of p53 mutations may differ amongst population groups and confirm that different tumour-derived p53

mutations have different effects, with some mutations being more aggressive than others. Furthermore, p53 mutations may be a prognostic indicator for breast cancer, depending on the nature of the mutation.

CHAPTER 1 INTRODUCTION

1.1 Breast Cancer Risk Factors

Breast cancer is the most common cancer in females in developed countries and recently there has been an increase in the incidence of the disease in developing countries. Although breast cancer occurs primarily in women, the disease does occur in males, however its occurrence in males is rare, with the female:male incidence ratio ranging from 70:1 to 130:1 (Henderson and Feigelson, 1997).

Many risk factors are associated with breast cancer, but substantial evidence has linked the majority of these to hormones, which play a major role in the etiology of the disease. The known risk factors such as early menarche, late onset of menopause, obesity (in post-menopausal women) and hormone replacement therapy, all result in increased exposure of the breast to estrogen, and to a lesser extent, progesterone. These hormones affect the proliferation of breast epithelial cells which is crucial in the development of a malignant phenotype. In addition to these risk factors, other criteria that result in decreased exposure to estrogen and progesterone tend to have a protective effect. Such factors include the age of the first full-term pregnancy, lactation and physical activity (Henderson and Feigelson, 1997).

1.1.1 Age

The most important demographic risk factor in the development of breast cancer is increasing age. There is a rapid increase in the risk of developing the disease from the late teens until the age of 50. After the age of 50 the risk of developing breast cancer continues to increase, but the rate of increase of this risk is less than that before the age of 50 (Pike, 1987).

There is a wide variation in breast cancer rates between various countries as well as between various population groups. The rates of breast cancer are substantially higher in first world countries such as the USA, Canada, and northern Europe compared to Asia and black populations in Africa (Henderson and Feigelson, 1997). Furthermore, rates for

black American women are comparable with their white counterparts, but not to those for black African women. These rates appear to be at least partly related to early life experiences, such as diet and physical activity, rather than solely due to differences in genetic susceptibility, as evidenced by migratory Japanese women in the USA, who have increased rates of breast cancer compared to those women who remain in Japan (Buell, 1973). If the women migrated at a young age, then their rates were comparable with their white American counterparts in the USA.

1.1.2 Age at Menarche

An early age of menarche is associated with an increased risk for developing breast cancer. Studies have indicated that the risk of developing the disease decreases by about 20% with each year that menarche is delayed (reviewed by Henderson and Feigelson, 1997). In addition, it appears that the development of regular menstrual cycles also has an effect on risk. Women who develop regular menstrual cycles within 1 year after the first menstrual period have more than double the risk of developing the disease than women whose regular menstrual cycles are delayed by 5 years or more (Henderson et al., 1988).

1.1.3 Physical Activity

Strenuous, as well as moderate, physical activity, is thought to decrease the risk of breast cancer. This is thought to occur by delaying menarche and the development of the breast. It has also been reported that physical activity significantly reduces the risk of breast cancer in young women less than 40 years of age (Bernstein et al., 1994).

1.1.4 Age at Menopause

The rate of increase in the age-specific incidence rate of breast cancer slows substantially at the time of menopause and the rate of increase in post-menopausal women is only about one-sixth the rate of increase in pre-menopausal women (Henderson and Feigelson, 1997). The age of onset of menopause also appears to be important, with women who experience early menopause (before the age of 45) having about half the risk as women who experience late menopause (after the age of 55) (Trichopoulos et al., 1972).

1.1.5 Pregnancy

Women who have children have a lower risk of developing breast cancer than childless women, and it appears that this decreased risk is primarily associated with the age of the first child-birth (MacMahon et al., 1970). Subsequent births have been shown to have only a small protective effect, whereas the main protective effect is associated with the age of the first full-term pregnancy (Yuan et al., 1988).

1.1.6 Weight

A strong relationship between weight and breast cancer risk has been established and this relationship is also dependent on age. Women under the age of 50 have little or no increased risk with increasing weight, but women who are 60 years or older have an increased risk with increasing weight (de Waard et al., 1977). It is, however, unclear whether this effect of weight on breast cancer risk is due to the increase in body fat or the increase in weight per se.

1.1.7 Other Possible Risk Factors

Dietary factors have also been implicated in breast cancer, particularly high fat consumption (Gray et al., 1979). The involvement of high fat consumption is controversial as various studies differ in their findings with respect to its effect on the risk of developing the disease (Howe et al., 1990; Hunter et al., 1996). Dietary differences may explain the changes in breast cancer rates after migration, as well as the difference in incidence rates between different population groups (Armstrong and Doll, 1975).

In contrast to fat consumption, an increase in dietary fiber may have a protective effect, although this is likely to be only a small effect, unlike in colon cancer where the protective effect of dietary fiber may be more pronounced (Willett et al., 1992). Vitamin A has also been suggested to protect against breast cancer, although no definitive studies have yet been done (Hunter et al., 1993).

Alcohol consumption has been associated with an increased risk of developing the disease. This may be brought about by altered liver function which may increase estrogen

levels (Howe et al., 1991).

1.2 Prognostic Factors in Breast Cancer

Prognostic factors in cancer can be defined as variables that play a role in recurrence of the disease, response to treatment, and patient survival. Identification of prognostic markers plays a role in understanding the molecular basis of the disease, thus aiding in treatment. At present, there is little consensus regarding the available information on all potential prognostic indicators, but there is agreement on a few prognostic factors for breast cancer, such as histologic type, stage and grade of the tumour.

1.2.1 Histologic Type

Malignant epithelial tumours of the breast can be divided into 3 categories: (1) non-invasive tumours; (2) invasive tumours of various types and (3) Paget's disease of the nipple (Williams and Buchanan, 1987 and WHO, 1981). The most common breast cancers are invasive adenocarcinomas (characterised by being poorly differentiated) followed by invasive lobular carcinomas. Invasive breast tumours are divided into various subtypes such as medullary, mucinous, tubular and papillary carcinomas, all of which are rare forms of the disease and certain of these, such as tubular carcinomas, papillary carcinomas and mucinous carcinomas have been associated with a low risk of recurrence and have favourable features, namely a low histologic grade and low rates of axillary lymph node involvement and consequently correlate with a better prognosis (Ravdin, 1997). Only a small percentage of breast carcinomas are of a non-invasive nature and include ductal in situ carcinomas and lobular in situ carcinomas (Ravdin, 1997). These non-invasive types have no metastatic potential and are associated with a low risk of recurrence.

1.2.2 Staging

The classical staging for breast tumours is the TNM system, where T refers to the tumour size, N refers to the lymph node involvement and M refers to the presence or absence of metastases. The tumour size is divided into various categories: a T_1 tumour is □ 2cm in its greatest dimension, a T_2 tumour is between 2 - 5cm in its greatest dimension and a T_3

tumour is ≥ 5cm in its greatest dimension. These various stages in turn, are subdivided into "a" or "b" categories, where "a" signifies no attachment to the underlying muscle and "b" signifies attachment to the underlying muscle. A T_4 category also exists and this refers to a tumour of any size with a direct extension to the chest wall or skin (Salvadori, 1984).

Similarly the lymph node involvement (N) is divided into various categories: N0, no metastases in the homolateral axillary nodes; N1, movable metastatic lymph nodes not fixed to one another or any other structure; N2, homolateral lymph nodes fixed to one another or any other structure and containing metastatic tumour; N3, homolateral supraclavicular or infraclavicular nodes containing tumour or oedema of the arm (Williams and Buchanan, 1987; Salvadori, 1984). The metastasis status is also sub-divided into 2 categories; M0, no known distant metastases and M1, distant metastases present (Salvadori, 1984).

In general, larger tumours and those with axillary lymph node involvement, tend to be associated with a poorer survival compared to smaller tumours which are node-negative. Furthermore, the number of lymph nodes affected are also important, with patient prognosis being poorer with increasing number of lymph nodes involved (Ravdin, 1997).

1.2.3 Histologic Grade

The histologic grade of a tumour is a subjective assessment of the degree of tubule formation, nuclear pleomorphism, elastosis and the frequency of hyperchromatic and mitotic figures in a tumour (Bloom and Richardson, 1957). It has been recognised as having potential value in predicting or evaluating prognosis, especially when combined with the tumour size and nodal status (Henson and Ries, 1991). The five year survival rate for patients decreases with increasing grade of the tumour, as well as increasing stage. When taken together, the five year survival rate of patients with stage I tumours remains the same, irrespective of the grade of the tumour. Patients with stage II tumours, however, have a poorer survival rate with increasing tumour grades. In addition to this, the survival rate is also affected by the involvement of lymph nodes. Patients with the

node-positive breast tumours of a high stage and a high grade have the poorest five year survival.

Although tumour stage and grade are independent predictors of prognosis, their effect on patient survival seems to be additive in that high stage node-positive tumours with a high histologic grade are associated with a poor prognosis.

1.2.4 Other Potential Prognostic Indicators

In addition to the classic prognostic indicators mentioned above, other factors such as patient age have been shown to be important in predicting response to chemotherapy and may be a prognostic marker for breast cancer. Numerous studies have suggested that patients with a younger age, usually those under 35, have a poorer prognosis than older patients (de la Rochefordiere et al., 1993; Bonnier et al., 1995; Nixon et al., 1994). The authors do not address whether the tumours in these younger patients are familial, but these tumours generally had the characteristics associated with poorer survival, such as a high grade, invasion of lymph nodes and necrosis. This suggests that if these tumours are familial, they are more aggressive than the sporadic form of the disease, which may account for the poorer prognosis in these patients.

Other potential prognostic indicators are the estrogen receptor (ER) and/or progesterone receptor (PR) status of the tumour. Patients who are ER or PR positive tend to have a better survival than those patients who are ER or PR negative (Silvestrini et al., 1995; Arriagada et al., 1992). This may be due to the response of the hormone receptor positive tumours to chemotherapeutic agents such as tamoxifen. These studies, however, showed that these hormone receptors were only weakly prognostic.

Measurement of the proliferative rates of cancer tissue by various techniques such as thymidine labelling index, S-phase fraction or immunohistochemistry has also been suggested to be of prognostic significance (reviewed by Ravdin, 1997). The p53 tumour suppressor has also been implicated as a potential prognostic marker for breast cancer, especially because of its role in apoptosis. This will be discussed in greater detail later in

section 1.4.2.

1.3 Hereditary Breast Cancer

Although the majority of breast cancers are sporadic, about 5% - 10% of breast cancers are familial (Claus et al., 1991). Patients with hereditary breast cancer tend to develop the disease at a relatively early age, and also tend to develop bilateral tumours. The familial nature of the disease suggests that a gene(s) could possibly be involved in this form of breast cancer.

The gene that confers susceptibility to hereditary breast and ovarian cancer has been localised to chromosome 17q (Easton et al., 1993) and a candidate gene, BRCA1, has been identified (Miki et al., 1994). It is estimated that mutations of this gene account for 45% of families with a high incidence of familial breast cancer and approximately 80% of families with an increased incidence of both early onset breast and ovarian cancers. A second locus, BRCA2, which maps to chromosome 13q, is also thought to account for a significant proportion of hereditary breast cancers, but not ovarian cancer (Wooster et al., 1994).

The BRCA1 gene has been partially characterised and encodes a protein of 1863 amino acids (Miki et al., 1994). The gene itself spans approximately 100kb of DNA and contains 22 coding exons to produce a transcript of 7.8kb. The protein has certain characteristics, such as several zinc finger motifs, which suggest that it is a transcription factor. The localisation of this protein within the cell is, however, a contentious issue. It has been reported to be expressed exclusively in the cytoplasm (Chen et al., 1995) or exclusively in the nucleus (Scully et al., 1996). Scully et al (1996), however, have provided compelling evidence that BRCA1 could be located either in the nucleus or the cytoplasm, depending on the staining technique used, which could account for the conflicting data. Given the proposed structure of the protein and the postulate that it functions as a transcription factor, one would expect to find the majority of the protein in the nucleus, although the possibility of a cytoplasmic pool of the protein cannot be totally excluded. There is further evidence that supports the idea that BRCA1 acts as a

transcription factor in that one of the ways in which it acts as a tumour suppressor is by mediating cell cycle arrest by transactivating the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner (Somasundaram et al., 1997). The exact mechanism by which this transactivation occurs, however, is not clear.

The mRNA for BRCA1 is expressed most abundantly in the testis and thymus, with smaller amounts in the breast and ovary (Miki et al., 1994).

Mutations in the gene may occur throughout the coding sequence as well as in the regulatory sequences (Miki et al., 1994; Futreal et al., 1994). In addition to playing a role in hereditary breast cancer, LOH of the BRCA1 locus has been detected in 30-70% of sporadic breast and ovarian cancers indicating that this gene may be involved in the sporadic forms of either breast or ovarian cancers (Futreal et al., 1992; Jacobs et al., 1993; Sato et al., 1991; Cropp et al., 1994). Unfortunately, very few mutations have been found in the remaining allele in sporadic cases in which there is LOH at this locus. There may be a number of reasons for this. Firstly, somatic BRCA1 mutations may fall in non-coding sequences and these may affect mRNA levels. This scenario has been found in one BRCA1 germline mutation (Miki et al., 1994). Secondly it is possible that LOH at 17q may not reflect somatic inactivation of BRCA1, but rather another potential as yet unidentified tumour suppressor gene located in the same region may be involved (Futreal et al., 1994).

Breast tumours that arise due to mutations in the BRCA1 gene tend to be slightly different histopathologically from somatic breast tumours in that they are highly proliferating tumours, with the majority of them being grade 3 tumours (Eisinger et al., 1996). This suggests that management and treatment of these tumours should be different to that of sporadic cases.

1.4 Genetic Alterations in Breast Cancer

It is clear that accumulation of genetic alterations is required for the development of the malignant phenotype. These genetic alterations can be of various types: chromosome

deletions or rearrangements, gene amplification, loss of heterozygosity (LOH) at certain alleles and point mutations, which include small insertions or deletions. It has also become apparent that these genetic alterations that play a role in tumourigenesis affect genes which are involved in the regulation of cell growth and proliferation, either in a positive manner (cellular oncogenes) or in a negative fashion (tumour suppressor genes). Oncogenes are usually activated or amplified in tumours, whereas tumour suppressor genes have been associated with inactivating mutations such as LOH and point mutations that abrogate the function of the protein.

Distinct areas of the genome have been shown to be amplified in primary human breast tumours: these include the *myc* gene located on chromosome 8q24 (Callahan and Campbell, 1989), the *int2/hst* locus, a member of the FGF gene family (Callahan and Campbell, 1989), the *bcl*1 gene on chromosome 11q13 (Callahan and Campbell, 1989; Theillet et al., 1990), the *bek* gene on chromosome 10q24 and the *flg* locus on 8p12 (Adnane et al., 1991). Amplification of c-*myc*, c-*erb*B2 and *int2/hst/bcl*1 have each been related to either aggressive or metastatic breast cancer (Slamon et al., 1987; Varley et al., 1987; Callahan and Campbell, 1989), and each of these gene amplifications may identify a particular subset of breast tumours (Adnane et al., 1991).

Certain genes have also been reported to be co-amplified in breast cancers. The c-*erb*B2 oncogene is frequently co-amplified with the c-*erb* A1 oncogene, which is a member of the steroid/thyroid hormone family of receptors (van de Vijver et al., 1987).

1.4.1 The *Ras* Genes

The *ras* gene family consists of three major members, designated H-*ras*, K-*ras* and N-*ras* (Barbacid, 1987). All three genes are located on different chromosomes; N-*ras* on chromosome 1, H-*ras* on chromosome 11 and K-*ras* on chromosome 12. These genes are ubiquitous and there is a high degree of homology between their gene products, which are commonly referred to as p21. All the genes have 4 exons, with the K-*ras* gene having an alternative 4^{th} coding exon which results in the production of 2 proteins that differ at their carboxyl terminus.

The *ras* gene promoters do not contain TATA or CCAAT elements, but rather have multiple G/C boxes characteristic of housekeeping genes. These G/C elements have been implicated in the binding of the Sp1 transcription factor (Ishii et al., 1986).

Mammalian *ras* genes can be constitutively activated and acquire properties that can lead to cellular transformation (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). This usually occurs as single point mutations in their coding sequences, especially codons 12, 13 and 61. In vitro mutagenesis studies have shown that in addition to these 3 codons, mutations in codons 63, 116, and 119 can also confer transforming properties to RAS.

The critical residue seems to be glycine at codon 12. A substitution of this residue by any other amino acid (except proline) results in constitutive activation of RAS (Seeburg et al., 1984). The glycine at position 13 is also important, although different substitutions at this site have different effects on the activation of the protein (Fasano et al., 1984). The glutamine at position 61 is also crucial as substitution of this residue with any other amino acid, with the exception of proline and glutamate, results in a constitutively active protein (Taparowsky et al., 1983; Brown et al., 1984).

1.4.1.1 How RAS Functions

The RAS proteins are involved in signal transduction, transmitting signals from the membrane into the cell. In order to do this, the *ras* p21 protein binds guanine nucleotides (both GDP and GTP) and possesses an intrinsic GTPase activity, both of which are crucial for normal RAS function (Temeles et al., 1985). Mutants which are unable to bind guanine nucleotides are incapable of transforming NIH-3T3 cells (Willumsen et al., 1986; Lacal et al., 1986), and furthermore, the GTPase activity in constitutively activated RAS is considerably less than in the wild-type protein in that mutants hydrolyse GTP to GDP with less efficiency than wild-type RAS, and consequently remain active for longer (Manne et al., 1985; Sweet et al., 1984).

The model proposed for the function of the RAS proteins proposes that the RAS protein

exists in equilibrium between an inactive and an active state (Barbacid, 1987). In the inactive state, RAS binds GDP. When a signal is received upstream of RAS, the GDP is exchanged for GTP, leading to a conformational change in the protein, resulting in activation of RAS. Once in this active state, RAS interacts with its downstream effector molecules. The intrinsic GTPase activity of the molecule ensures that once the interaction between itself and the effector has taken place, the protein returns to the inactive conformation as the GTP is hydrolysed to GDP.

1.4.1.2 RAS Protein Structure

RAS proteins contain 4 defined domains (reviewed by Barbacid, 1987). The first domain spans the amino-terminal third of the protein and is highly conserved, with the first 85 amino acids being identical between Ha-Ras, Ki-Ras and N-Ras. The second domain comprises the next 80 amino acids and is slightly less homologous (85%) between the different proteins. The third domain is highly variable, and the last domain comprises the last four amino acids with a common Cys-A-A-X motif, where A is any aliphatic amino acid and X is any amino acid. Each of these domains has a specific function that is crucial for normal RAS function. Residues 5-63, 77-92, 109-123, 139-165 and the carboxyl terminal Cys-A-A-X motif are the regions most critical for normal RAS function (Willumsen et al., 1986).

The amino terminus (residues 5-63) of RAS is involved in guanine nucleotide binding, with the glycine at position 12 playing a critical role (Barbacid, 1987). Consequently any mutations disrupting this residue would drastically alter the function of the protein, and would lead to a constitutively active form of the protein.

It has been suggested that domains 77-92, 109-123, and 139-165 are involved in interacting with the downstream molecules (or RAS effector proteins) in the signal transduction cascade.

The RAS proteins are anchored in the cytoplasmic side of the cell membrane via the carboxyl terminal Cys-A-A-X motif (Fujiyama and Tamanoi, 1986; Willumsen et al.,

1984a). Once the proteins are synthesised, the cysteine residue at position 186 becomes acetylated by palmitic acid, and this acetylation allows the proteins to anchor in the membrane. This modification is crucial for RAS function, since mutants lacking Cys^{186} remain localised in the cytoplasm and are unable to transform cells (Willumsen et al., 1984a; Willumsen et al., 1984b).

1.4.1.3 Mechanisms of Activation of the *ras* Genes

The most common way the *ras* genes acquire transforming properties is by point mutations in specific codons. However, overexpression of the wild-type *ras* gene can also lead to a certain degree of cell transformation, albeit to a lesser extent.

Activated *ras* genes have been identified in a variety of tumours such as carcinomas of the bladder, colon, breast, kidney, liver, lung, ovary, pancreas and stomach, and are possibly the most frequently mutated oncogenes in human cancer, with an incidence of 10-15%. The frequency of activated *ras* genes varies between tumour types, with a high incidence of mutated genes in pancreatic, colon and thyroid tumours and a low frequency in breast, ovary, oesophageal and gastric cancers (Bos, 1989). Activated *ras* oncogenes are not associated with any specific type of tumours and their activation does not correlate with the histopathological properties of the tumour (Barbacid, 1987).

In addition to mutations, overexpression of the wild-type RAS may play a role in the transformation process. This could be brought about by mutations in the regulatory sequences of the *ras* genes, although there is no evidence for such mutations in human tumours (Barbacid, 1987). Expression of the *ras* genes in tumours has been reported to be increased 2-10 fold relative to the normal tissue in 50% of human tumours (Barbacid, 1987; Slamon et al., 1984; Spandidos and Agnantis, 1984; Spandidos and Kerr, 1984). The significance of this overexpression is unclear at present, but it may contribute to the malignant phenotype.

Another possible mechanism that could lead to overexpression of wild-type *ras* is by gene amplification, which has been observed in a number of different tumours (Pulciani

et al., 1985; Fujita et al., 1985; Bos et al., 1986; Filmus and Buick, 1985). The overall incidence of *ras* gene amplification, however, is probably not higher than 1% (Pulciani et al., 1985).

ras Oncogenes are the most frequently activated oncogenes identified in sporadic tumours, and this frequent identification of these oncogenes raises the question of whether they are the cause of the malignant phenotype or merely a consequence of it. Some evidence suggests that *ras* oncogenes can become activated after cells have become transformed (Albino et al., 1984; Tainsky et al., 1984; Vousden and Marshall, 1984), however substantial evidence, primarily from animal tumour models, suggests that the *ras* genes play a role in the initiation of the malignant phenotype.

1.4.1.4 Carcinogen-Induced Tumours

The *ras* genes have been shown to be activated in murine carcinogen-induced tumour model systems. In rats, the H-*ras* oncogene is activated in 86% of mammary carcinomas induced by nitroso-methyl urea (NMU) (Sukumar et al., 1983; Zarbl et al., 1985). This dropped to 23%, however, when the carcinogen was dimethylbenz(a)anthracene (DMBA). In other studies, the K-*ras* gene was found to be activated in 40% of kidney tumours induced by methyl(methoxymethyl)nitrosamine (DMN) and in 74% of lung carcinomas induced by tetranitromethane (TNM) (Sukumar et al., 1986). Similar high frequencies of activated *ras* genes occur in mice treated with different carcinogens (reviewed by Barbacid, 1987). This high frequency of reproducible *ras* activation in carcinogen-induced tumours suggests that the *ras* genes play a causative role in the development of the malignant phenotype.

Most carcinogens are mutagens and a large proportion of them form adducts on the DNA (reviewed by Singer and Kusmierek, 1982). These adducts can lead to the introduction of mutations into the DNA, which could result in activation of, amongst others, the *ras* genes (if these adducts happened to form in the coding regions of the genes). In fact, it has been shown that each allele of the H-*ras* gene in NMU-induced rat mammary carcinomas contained a G $\rightarrow$ A activating mutation (Zarbl et al., 1985). This was not the

case for DMBA-induced tumours, suggesting that NMU was directly responsible for the activation of the H-*ras* gene in those mammary tumours. Furthermore, only the G in the middle postion of codon 12 (GGA) was affected, thus strengthening the suggestion that NMU is directly responsible for the activation of this gene in NMU-induced rat mammary carcinomas.

The idea that the *ras* genes are targets for chemical carcinogens is further supported by the findings that DMBA-induced skin tumours in mice involve the activation of H-ras via A $\rightarrow$ T transitions specifically at the second base of codon 61 (Quintanilla et al., 1986; Bizub et al., 1986). Taken together, these findings suggest that chemical carcinogens are directly responsible for activation of the *ras* genes, which is an early event in the process of carcinogenesis, with other genes developing subsequent mutations that would ultimately lead to the development of a neoplastic phenotype.

Although current evidence suggests that *ras* oncogenes are involved at an early stage in the initiation of tumourigenesis in animal models, and given the fact that the *ras* genes are ubiquitously expressed, it is somewhat surprising that not more tumour types contain a high frequency of *ras* gene mutations. In the case of breast cancer, the *ras* genes may only acquire mutations once the mammary gland has been stimulated to proliferate and differentiate after exposure to hormones during sexual development and these mutations may only manifest themselves during this period. Thus acquiring a *ras* gene mutation after sexual development might not lead to the induction of tumourigenesis, whereas early acquisition (before sexual development) of a *ras* gene may trigger carcinogenesis during proliferation and development of the mammary gland (Barbacid, 1987).

1.4.1.5 *Ras* and the Cell Cycle

A role for *ras* in the cell cycle comes from the evidence that transformation of NIH3T3 cells with v-H-Ras results in an altered cell cycle (Liu et al., 1995). These transformed cells grow faster, have a higher S phase content and also have a shortened cell cycle time compared to the control cells. The shortened cell cycle is due to a shortened G_1 phase that is explained by an increase in cyclin D expression. A similar shortened G_1 phase was

observed for NIH3T3 cells overexpressing cyclin D, and the shortened G_1 phase could be reversed by inhibiting the expression of cyclin D in the *ras*-transformed cells (Liu et al., 1995). This overexpression of cyclin D in the *ras*-transformed cells occurred as a result of stabilisation of the existing mRNA, rather than an increase in transcription of the gene. These results suggest that signaling via the *ras* pathway leads to an altered cell cycle and may explain one of the ways in which *ras* plays a role in cell transformation.

1.4.2 Mutations Affecting Tumour Suppressor Genes - p53

The second class of genetic alterations in cancer is the inactivation or loss of tumour suppressor genes (Seemayer and Cavenee, 1989; Weinberg, 1989; Knudson, 1989). These genes are involved in suppression of cell growth during normal development. Numerous candidate tumour suppressor genes such as p53 (probably the best studied one), WT-1 (Wilms tumour), DCC (deleted in colon carcinoma), NF-1 (Neurofibromatosis) and the retinoblastoma gene have been identified to date (reviewed by Marshall, 1991 and Weinberg, 1991). Inactivation of these genes has been proposed to occur by a "two-hit" mechanism in which loss of one allele and a point mutation in the remaining allele inactivate the gene. It has also been suggested, however, that loss of one allele is not a pre-requisite for inactivation of the gene, but rather the mutant protein may interact with the wild-type protein in a "dominant-negative" fashion and in this way abolish the normal function of the protein. This type of mechanism has been proposed for the p53 tumour suppressor gene (Baker et al., 1989).

As in the case of amplification of oncogenes, loss of genetic material has been identified at numerous loci in the genome in breast cancer patients: chromosome 1p (Genuardi et al., 1989; Devilee et al., 1991a; Bieche et al., 1993), 1q (Chen et al., 1989; Gendler et al., 1990; Devilee et al., 1991a), 3p (Callahan and Campbell, 1989), 11p (Callahan and Campbell, 1989), 13q (Lundberg et al., 1987; Sato et al., 1990), 17p (Mackay et al., 1988; Cropp et al., 1990; Sato et al., 1990), 17q and 18q (Cropp et al., 1990; Devilee et al., 1991b) are all regions frequently lost in human breast cancer. All of these loci have been implicated in other tumour types and are thus thought to harbour tumour suppressor genes. The frequencies of these individual losses vary, with chromosome 17p (the

location of the p53 tumour suppressor) being the region most frequently lost (McBride et al., 1986). It has been suggested that the large number of sites affected by allelic loss in breast cancer could signify a co-operative effect of multiple alterations which would favour progression to a malignant phenotype (Ben Cheickh et al., 1992). Loss of 1p and 11p occurs concurrently with loss of 17p, whereas there has been no association with loss of 1p and 11p (Ben Cheickh et al., 1992; Cropp et al., 1990; Devilee et al., 1991b; Sato et al., 1990). There is, however, an association of deletions on chromosome 11p with amplification of the c-*erb*B2 oncogene, and loss of 17p with amplification of the *flg* locus (Ben Cheickh et al., 1992). It therefore appears that certain genetic aberrations may co-operate in the process of tumourigenesis of breast cancer, such that specific sets of alterations may define specific sub-groups of breast tumours (Ben Cheickh et al., 1992).

The p53 gene is the best characterised tumour suppressor gene to date. It is involved in the negative regulation of cell proliferation by regulating gene transcription, monitoring DNA for damage and halting the cell cycle when damage is detected and it also plays a role in apoptosis. The human p53 gene is found on the short arm of chromosome 17 (17p13.1) and spans 20kb of DNA (Lamb and Crawford, 1986). The gene consists of 11 exons, of which exon 1 is a non-coding exon.

p53 is the most frequently mutated gene in human tumours and the majority of mutations are point mutations which fall within evolutionary conserved domains of the gene. The majority of these mutations cause conformational changes in the protein, rendering the protein inactive with respect to its normal function. Certain mutations may also lead to a gain of function in which the protein promotes cellular proliferation.

1.4.2.1 History

p53 was originally discovered in nuclear extracts from cells transformed with Simian virus 40 (SV40), where it forms an oligomeric complex with the virally encoded large T tumour antigen (Lane and Crawford, 1979). Because T antigen is needed for maintenance of the transformed state, it was suggested that this interaction between p53 and T antigen might play a role in the modulation of the transformed state and p53 became classified as

a tumour antigen.

It was later discovered that cells transformed by a variety of agents expressed increased levels of p53 when compared to the homologous non-transformed cells (Crawford et al., 1981; DeLeo et al., 1979). More than one mechanism is involved in the regulation of the level of p53 protein in the cell (Reich et al., 1983). An alteration in the amount of p53 mRNA i.e. an increase in transcription, or in the stability of the protein can lead to an increase in the amount of the protein in the cell. These mechanisms may operate independently or jointly in upregulating the levels of the p53 protein in the cell. Interaction of p53 with other proteins, such as the SV40 large T antigen, may be one explanation for the increased stability of the protein. This interaction would increase the half-life of the protein, thus increasing it's effective concentration. It was proposed that this increase in p53 levels was partially responsible for the altered growth characteristics of cell lines expressing increased levels of p53 (Reich et al., 1983).

p53 was then classified as an oncogene when it was found that a number of cDNA and genomic p53 clones could immortalise cells in culture and cooperate with the *ras* oncogene in transforming these cells (Eliyahu et al., 1984; Parada et al., 1984; Jenkins et al., 1984). It turned out, however, that all the p53 clones that could cooperate with *ras* in transforming cells in culture were mutant forms of p53 (Hinds et al., 1989). This led to the re-classification of p53 as a recessive oncogene or anti-oncogene (tumour suppressor gene), with a normal function of negatively regulating cell growth. Several diverse mutations are able to inactivate the wild-type function of the protein, resulting in cellular transformation in cooperation with *ras*. Furthermore, viral transforming proteins such as SV40 large T antigen, bind to wild-type p53 and abrogate its normal function in the cell, and in this way allow for transformation. Further evidence for the role of p53 as a tumour suppressor gene came from the introduction of the wild-type protein into a human colorectal carcinoma cell line, resulting in decreased cellular proliferation (Baker et al., 1990).

1.4.2.2 p53 Protein Structure

The p53 gene encodes a protein of 393 amino acids. There are 5 regions of the protein which are highly conserved between species. The first region occurs at the N-terminus of the protein, with the other four conserved domains in the central region of the protein.

The protein has the characteristics of a transcription factor. The N-terminal transactivation domain is acidic and is involved in the interaction with other proteins. The central region of the molecule is involved in sequence-specific DNA binding and the C-terminal region contains the nuclear localisation signal (Shaulsky et al., 1990b) and the oligomerisation domain (p53 exists as a tetramer in solution (Vogelstein and Kinzler, 1992)). In addition to this, the C-terminal domain also plays a role in non-sequence specific DNA binding.

1.4.2.3 The Function of Wild-Type p53

p53 is involved in the negative regulation of cell growth and is important in cell cycle regulation. There are numerous mechanisms by which p53 carries out these functions; p53 blocks the cell cycle in G_1 in response to various stimuli such as agents that induce DNA damage, for example UV light, γ-irradiation and certain drugs.

Initial studies have shown that p53 is a regulator of GTP synthesis, suggesting a role for the protein as a novel regulator of signal transduction, and in this way, it was able to control the cell cycle (Sherley, 1991)

There is now conclusive evidence that p53 behaves as a transcription factor. Firstly it contains an acidic domain near its N-terminus, similar to that of other well characterised transcription factors (Fields and Jang, 1990; Raycroft et al., 1999). Furthermore, when this acidic domain was fused to the GAL4 DNA-binding domain, the fusion protein could activate transcription from a GAL4 operon. This transcativation domain has been mapped to the region between codons 20 and 42 of the p53 gene (Unger et al., 1992).

Secondly, p53 has been shown to bind to two copies of the 10 base pair 5'-

PuPuPuC(A/T)(A/T)GPyPyPy-3' consensus sequence(Kern et al., 1992; Hupp et al., 1992; Vogelstein and Kinzler, 1992). One copy of this 10 base pair motif is insufficient for binding, and the two copies necessary for binding can be separated by up to 13 base pairs of random DNA to preserve binding of p53 to the DNA. The symmetry of this motif suggests that p53 may bind as a tetramer, which is consistent with biophysical data which indicate that p53 exists as a tetramer in solution (reviewed by Vogelstein and Kinzler, 1992). The fifty C-terminal amino acids of the protein have been shown to contain 2 motifs required for heterogeneous oligomerisation (Kraiss et al., 1988), and the DNA binding domain of the protein has been mapped to the C-terminal region as well. Recently, a more well-defined p53 consensus binding site has been elucidated as GGACATGCCCGGGCATGTCC (Funk et al., 1992). This sequence is a palindrome and p53 only binds to it in the presence of crude nuclear extract, suggesting that it may bind DNA after post-translational modification, or as a complex with other proteins. p53 has been shown to directly interact with the TATA binding protein (TBP) (Martin et al., 1993; Liu et al., 1993). This interaction may mediate the influence of p53 on transcription and involves TBP-associated factors (TAFs), specifically $TAF_{II}40$, $TAF_{II}60$ and $TAF_{II}230$ (Thut et al., 1995; Farmer et al., 1996). p53 also interacts weakly with TFIID, an important factor required for transcription initiation (Liu et al., 1993). The region of p53 involved in this interaction was mapped to between residues 20 and 57 located in the acidic transactivation domain.

There is evidence to suggest that p53 acts as both a transactivator (Kern et al., 1992; Funk et al., 1992; Farmer et al., 1992) and an inhibitor of gene expression (Ginsberg et al., 1991; Chin et al., 1992; Kley et al., 1992; Mack et al., 1993). It would seem that wild-type p53 behaves as an activator of transcription on genes with promoters containing p53 binding sites, and it acts as an inhibitor of transcription on genes with promoters lacking a p53 consensus binding site. Various speculations have been made as to how p53 can act as both an inhibitor and an activator of gene expression (Martin et al., 1993). When p53 is complexed to TBP it acts as a repressor i.e. p53 represses the activity of promoters whose initiation is dependent on the presence of a TATA box. This repression, however, was only observed when p53 is overexpressed and it was speculated that the conformation of

the p53-TBP complex is not favourable for interaction with other transcription factors, thereby inhibiting transcription in general (Mack et al., 1993; Martin et al., 1993). A conformation favourable for activating transcription could be achieved by a mutant p53 protein interacting with TBP, or by direct binding of wild-type p53 to the DNA. Furthermore, for promoters with p53 consensus binding sites, the presence of p53 on these promoters and its ability to bind TBP, may facilitate the nucleation of TBP on these promoters, thus enhancing transcription activity (Martin et al., 1993).

p53 has been shown to activate its own promoter (Deffie et al., 1993). The DNA response element of the p53 promoter involved in this activation is the NF-kB motif, which has a one base pair mismatch with the p53 consensus sequence. A direct interaction between this motif and p53 has, however, not been observed. p53 probably activates one or more transcription factors which do bind to this NF-kB site i.e p53 indirectly activates transcription from its own promoter (Deffie et al., 1993). Alternatively, p53 could interact directly with one of the transcription factors binding to the NF-kB sequence and thereby activate transcription. In addition to activating its own promoter, p53 has also been shown to regulate its own transactivation function. The region mapping between residues 92 and 160 has a strong inhibitory effect on the activation domain of p53, which lies between residues 20 and 92 (Liu et al., 1993).

1.4.2.4 Post-Translational Modifications of the p53 Protein

1.4.2.4.1 Phosphorylation

p53 function is regulated by phosphorylation, for which there are a number of sites distributed at both ends of the protein. Phosphorylation by casein kinase II at the C-terminal seems to be important in DNA binding activity. The C-terminal region of p53 is important in controlling its activity (Hupp et al., 1992). Removal of the 30 C-terminal amino acids constitutively activates DNA binding, suggesting that it has a negative effect on DNA binding. Other modifications in this region, such as proteolysis by trypsin, interaction with E.coli dnaK (a protein that disrupts protein-protein complexes), and phosphorylation by casein kinase II (Hupp et al., 1992) and the CDK_7-cycH-p36 kinase complex of TFIIH (Lu et al., 1997), also activate the DNA binding activity of p53, with

serines 371, 376, 378, and 392 being potential phosphorylation sites (Lu et al., 1997). In addition, mutation of Ser 386 in mouse p53 (the casein kinase II phosphorylation site) resulted in the loss of the growth suppressor function of p53, further suggesting that phosphorylation plays an important role in the anti-proliferative function of p53 (Milne et al., 1992). It has been shown that interactions with proteins such as dnaK can stabilise the protein and maintain it in a conformation that is thermostabile and capable of DNA binding (Hansen et al., 1996). In contrast, the calcium-dependant protein kinase C (PKC) phosphorylates murine p53 in vitro on several residues in the basic region of the C-terminal, but PKC appears to bind to a domain in p53 distinct from the one containing the phosphorylated residues (Delphin et al., 1997). The C-terminal region of murine p53 is able to stimulate sequence-specific DNA binding at micromolar concentrations, but this function is lost when it is phosphorylated by PKC. Human p53 is phosphorylated by protein kinase C on Ser 378 that activates the sequence-specific DNA binding of p53 (Takenaka et al., 1995). This activation of the DNA binding function of p53 is inhibited by phosphatases 1 and 2A and it is possible that these mechanisms may operate in vivo to regulate the activity of p53 as a transcription factor.

p53 is also phosphorylated at the N-terminal at serines 15 and 37 by DNA-PK, an event that is important in activating the growth-suppressive functions of p53 (Lees-Miller et al., 1992). DNA damage activates DNA-PK, which in turn phosphorylates p53, resulting in activation of the DNA binding activity of p53 and inhibition of p53 interaction and inactivation by mdm2 (Woo et al., 1998). Earlier studies also suggested that phosphorylation at the DNA-PK sites caused an alteration in the conformation of the p53 protein, which could account for its inability to interact with mdm2 (Shieh et al., 1997).

p53 may also be phosphorylated at serine 15 by the ATM protein in vitro in response to DNA damaging agents (Banin et al., 1998; Canman et al., 1998). Additional phosphorylation sites at the N-terminal have been identified in mouse p53 and these are serines 7, 9, 18 and 37 (Wang and Eckhart, 1992). Serines 7 and 9 are phosphorylated by the dsDNA-dependent kinase (DNA-PK) in vitro, but it is not clear whether this also occurs in vivo. Phosphorylation of p53 is altered during the cell cycle, with the

phosphorylation status of the protein changing as the cells progress through the cell cycle. In G_0/G_1, the protein is under-phosphorylated compared to the S phase.

Recent evidence suggests that phosphorylation of p53 at both the N- and C-termini is not crucial for the functional activity of p53 (Ashcroft et al., 1999). p53 molecules which had all of their phosphorylation sites simultaneously mutated were equally efficient at activating transcription of the CIP1, mdm2 and bax promoters as the wild-type protein. p53 proteins with only their C-terminal phosphorylation sites mutated did so with a reduced activity, which is consistent with the idea that C-terminal phosphorylation enhances DNA binding. Also, all of the mutants were as sensitive to degradation by mdm2 and were all stabilised after DNA damage by either actinomycin D treatment or UV irradiation. These results suggest that phosphorylation of p53 is not absolutely necessary for the regulation of its stability and imply that other mechanisms exist to stabilise the protein in response to DNA damage. Further studies have shown that the ATM protein that phosphorylates p53 at the N-terminus in vitro, is not required for its transactivation function or its ability to induce apoptosis suggesting that phosphorylation of the N-terminus is not a prerequisite for normal p53 function in vivo (Liao et al., 1999). These results do not, however, preclude that phosphorylation may play a subtle role in the regulation of p53 function under certain conditions.

1.4.2.4.2 Acetylation

CREB binding protein CBP/p300 and PCAF are acetyltransferases and are co-activators of p53 and are required for full transcriptional activity of p53 (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). Both of these proteins acetylate different residues of p53 in vitro (Gu and Roeder, 1997; Liu et al., 1999). PCAF acetylates Lys320 while p300 acetylates Lys373, resulting in stimulation of sequence-specific DNA binding in vitro, suggesting that p53-mediated transcriptional activation may be modulated by acetylation. Furthermore, acetylation of Lys373 by p300 and Lys320 by PCAF was found to occur in vivo in cells exposed to UV or ionising radiation, but not in unexposed cells. This suggests that acetylation of p53 plays an important role in the response to DNA damage.

1.4.2.5 DNA Binding

p53 binds to DNA in both a sequence-specific and a nonspecific manner. The sequence-specific DNA binding and transactivation of genes is essential for p53 to suppress growth (Pietenpol et al., 1994). The C-terminal 47 amino acids contain the nonspecific DNA binding region that recognises and binds to damaged DNA.

The region responsible for sequence-specific DNA binding maps to residues 102-292 in human p53, encompassing the evolutionary conserved domains II-V where most mutations have been detected (Srinivasan et al., 1993; Pavletich et al., 1993). This domain consists of a β sandwich comprising two antiparallel β sheets. A loop-sheet-helix (LSH) motif binds in the major groove and is involved in contacts with the bases in the DNA while two large loops interact such that Arg248 makes contact with the minor groove of the DNA in the A/T rich portion of the consensus binding site (Prives, 1994). The sequence-specific DNA binding of p53 is dependent on reactive sulfhydryl groups and p53 must be in a reduced state in order to bind DNA in zinc dependent manner (Hupp et al., 1993; Rainwater et al., 1995). A number of cysteine residues in this region are believed to be important for zinc binding and mutation analysis in murine p53 revealed that cysteines 173, 235, and 239 are essential for transactivation by p53 (Rainwater et al., 1995). These 3 cysteine residues interact directly with a zinc ion and it has been suggested that, by binding zinc, they can modulate the secondary structure of the protein, enabling it to adopt a conformation that allows interaction with the DNA (Rainwater et al., 1995). In human p53, the interaction of p53 with the DNA is stabilised by a zinc atom which is held in place by four residues, Cys176, His179, Cys238 and Cys242, all of which fall in the two large loops that interact such that Arg248 can make contact with the minor groove (Prives, 1994).

Several genes contain the p53 consensus-binding site, and some examples of p53 activated genes are c-erbA, CIP1 (el-Deiry et al., 1993), TGF-α (Shin et al., 1995), PCNA (Shivakumar et al., 1995; Xu and Morris, 1999), c-*fos* (Elkeles et al., 1999), matrix metalloproteinase-2 (Bian and Sun, 1997), IGF-BP3 (Buckbinder et al., 1995) and *bax* (Miyashita and Reed, 1995). Rb, *bcl*2, MDR1 (Goldsmith et al., 1995), IL-6

(Margulies and Sehgal, 1993; Wang et al., 1995), c-*jun*, DNA topoisomerase IIα (Wang et al., 1997) and β-actin on the other hand, have all been shown to be repressed by p53. The effect of p53 on the human multidrug resistance promoter (MDR1) is, however, highly contentious. Drug resistance is a major problem in cancer chemotherapy which is associated with overexpression of the multidrug resistance gene. Various studies have shown that mutant p53 stimulates expression from the MDR1 promoter, whereas wild-type p53 has no effect or represses MDR1 promoter activity in heterologous human/rodent systems and cell lines (Chin et al., 1992; Zastawny et al., 1993; Dittmer et al., 1993). When a human MDR1 CAT construct was transfected into NIH3T3 cells that are usually non-permissive for the expression of MDR1 CAT because of endogenous p53, a His275 mutant p53 was able to activate expression from the MDR1 promoter (Chin et al., 1992). Co-transfection and re-introduction of wild-type p53 reversed this activation of the MDR1 promoter by the mutant protein. These results therefore suggest that introduction of the mutant p53 into NIH3T3 cells inhibits/abolishes the negative effect of wild-type p53 on the MDR1 CAT construct because of the dominant negative mode of action, thus resulting in activation of MDR1 CAT. In another study, the opposite effect was reported where wild-type p53 stimulated expression from the MDR1 promoter whereas several mutant p53 proteins failed to do so and even slightly repressed expression of an MDR1 CAT construct (Goldsmith et al., 1995). This study utilised a human MDR1 promoter, human p53 and human cell lines, as opposed to the previous studies where heterologous systems were used. Also, maximal stimulation of the MDR1 CAT construct was observed 20-30 hours after transfection, at which time CAT activity dropped steadily and returned to basal levels 48 hours post-transfection, which is frequently used as the termination time for transient transfection studies. Thus discrepancies between the results may be due to the specific systems and cell lines used. In addition, it is possible that different p53 mutants may have different effects on the activation of the MDR1 promoter.

There also seems to be some controversy regarding the effect of wild-type p53 on the c-*fos* promoter. Some evidence indicates that p53 activates transcription of the c-*fos* gene (Elkeles et al., 1999) whereas others show that c-*fos* is repressed by p53 (Kley et al.,

1992). In the case of p53 activation of c-*fos*, p53 was shown to bind to a site within the first intron of the gene and does not involve the basal promoter, whereas repression of c-*fos* involved the basal promoter. It must be noted, however, that the repression study (Kley et al., 1992) used a transient transfection assay using a construct lacking the first intron. Thus it is likely that p53 activates c-*fos* in vivo, but in the absence of the first intron it is capable of repressing transcription from this promoter due to interactions with other transcription factors.

1.4.2.6 Stability of the Protein

In normal cells p53 has a very short half-life of 20-40 minutes and consequently is present at very low levels. The levels of the protein increase about 10- to 20-fold in late G_1 phase, just prior to the S phase. High levels of p53 may also be detected after DNA damage or following exposure to stimuli that induce differentiation or apoptosis. These high levels of protein are, however, transient.

A possible explanation for the short half-life of p53 is the ability of wild-type p53 to activate the mdm2 gene whose product targets p53 for degradation via the ubiquitin-proteosome pathway thus significantly reducing the steady state levels of p53 (Haupt et al., 1997). Binding of mdm2 to the N-terminal transactivation domain of p53 is a prerequisite for degradation (Gottlieb and Oren, 1996). The C-terminal region of p53 also appears to be important for mdm2-mediated degradation since deletion of the C-terminus renders the protein resistant to mdm2 mediated degradation, suggesting a role for oligomerisation in degradation by mdm2 (Kubbutat et al., 1998). Degradation of p53 by mdm2 more than likely occurs in cytoplasmic proteosomes since nuclear export is required for the degradation to take place (Freedman and Levine, 1998).

The exact mechanism(s) by which p53 becomes stabilised after DNA damage are unclear, but given that p53 is degraded via the ubiquitin-proteosome pathway, a possible mechanism could involve repression of ubiquitin-mediated degradation via some signalling pathways induced by DNA damaging agents. Exposure of RKO cells (colon carcinoma cells) to γ or UV radiation results in stabilisation of the p53 protein with the

half life increasing to more than 3.5 hours in the irradiated cells (Maki and Howley, 1997). Interestingly though, ubiquinated p53 was detectable in both untreated and γ irradiated cells, but not in the cells exposed to UV radiation. In fact, ubiquitinated p53 was found to disappear as early as 1 hour after exposure to UV radiation. These results suggest that stabilisation of the p53 protein in the UV irradiated cells is due to a loss of ubiquitination and further imply that different mechanisms are involved in stabilising p53 in response to various DNA damaging agents.

Proteolytic cleavage of p53 has also been implicated in regulating the stability of p53. Calpain cleaves p53 in vitro in the N-terminal domain, and inhibition of calpain by protease inhibitors results in stabilisation of p53 (Kubbutat and Vousden, 1997).

1.4.2.7 Oligomerisation of p53

p53 exists predominantly as a homotetramer in both the latent and activated forms, however, the role of oligomerisation is somewhat contentious (Vogelstein and Kinzler, 1992; Milner et al., 1991; Sturzbecher et al., 1992; Hupp and Lane, 1994). It would appear that p53 is produced as a latent tetramer, which, upon modification of the C-terminus, is converted to an active tetamer (Hupp and Lane, 1994). The C-terminal regulatory domain of p53 plays a major role in the activation of latent tetramers into active tetramers since a synthetic peptide derived from this domain has been shown to activate latent p53, supporting a model in which disruption of specific contacts between this regulatory domain and other domains of the protein results in activation of the latent tetramer (Hupp et al., 1995). Each monomeric unit of the tetramerisation domain comprises a turn (residues 324-326), a β strand (residues 326-334), a second turn (residues 335-336) and an α helix (residues 337-355). Each subunit interacts with another subunit such that the helices and β strands are antiparallel (Clore et al., 1994 and reviewed by Prives, 1994). Two of these dimers interact, resulting in a tetramer. The formation of tetramers is important for DNA binding, but it appears, at least with respect to murine p53, that p53 can also bind in a sequence-specific manner to the DNA as a dimer. It has also been suggested that tetramerisation of p53 plays a role in the recognition of DNA damage (Hupp and Lane, 1994; Wang et al., 1995).

1.4.2.8 Subcellular Localisation of p53

The localisation of p53 varies during the cell cycle. In mouse cells, newly synthesised p53 accumulates in the cytoplasm during the G_1 phase of the cell cycle and then moves to the nucleus until just prior to the S phase (Shaulsky et al., 1990a). After the initial round of DNA synthesis, the protein is translocated from the nucleus and again accumulates in the cytoplasm. The mechanism regulating the subcellular localisation of p53 is unclear, but the C-terminal region of the protein may play a role in it's localisation (Liang et al., 1998). The wild-type protein is localised in the cytoplasm, nucleus or both in a subset of MCF-7 (breast carcinoma), RKO (colon carcinoma) and Saos-2 cells. Mutation of lysine 305 results in the sequestration of p53 in the cytoplasm in the MCF-7 and RKO cells, but the mutant protein is distributed in both nuclear and cytoplasmic compartments in the Saos-2 cells. In addition, cytoplasmic sequestration of the mutant p53 is dependent on the C-terminal region (residues 326-355). These results suggest that cis-acting sequences are involved in the regulation of localisation of p53 within the cell. Phosphorylation may also play a role in modulating the localisation of the protein in the cell via a phosphorylation site within the nuclear localisation signal (reviewed by Ozbun and Butel, 1996).

A nuclear export sequence (NES) located within the C-terminal tetramerisation domain spanning residues 340-351 has recently been identified (Stommel et al., 1999). Mutations in this region result in the accumulation of p53 in the nucleus. It is critical for the cell to export p53 out of the nucleus when it is no longer required, as prolonged wild-type p53 activity may result in death of a normal cell. When a cell becomes damaged or is stressed, modifications of p53 may occur which facilitate tetramerisation of the protein. Because the NES occurs in the tetramerisation domain, formation of p53 tetramers would occlude the NES, resulting in accumulation of p53 in the nucleus. Once in the nucleus, wild-type p53 can bind DNA and transactivate genes and thus mediate a p53 stress-response. Once the cell has recovered, or in unstressed normal cells, modifications may result in dissociation of the tetramers, thereby exposing the NES. This would then lead to export of the protein from the nucleus, probably by binding to the nuclear export receptor, CRM1 (Stommel et al., 1999; Fornerod et al., 1997).

1.4.2.9 Regulation of Cell Growth by p53

It has been hypothesised that p53 can negatively regulate cell growth in three ways; (1) repressing genes whose expression is mediated by a TATA box, (2) activating genes containing p53 consensus binding sites, thereby blocking cell cycle control and (3) blocking the replication machinery and thus playing a role in DNA replication (reviewed by Ozbun and Butel, 1996). In addition, p53 binds to mismatched DNA suggesting that it may play a role in the recognition of DNA damage.

Wild-type p53 functions as a cell cycle control protein (Baker et al., 1990; Diller et al., 1990). Carcinoma cells overexpressing the wild-type gene were unable to progress through the cell cycle by becoming arrested in the G_1 phase resulting in failure of these cells to complete S phase. Further evidence suggests that growth suppression mediated by wild-type p53 occurs prior to or near the restriction point (R-point) in the late G_1 phase of the cell cycle (Lin et al., 1992; Pardee, 1989; Pardee, 1987). Once past this point, protein synthesis is no longer required for cells to enter S phase and initiate DNA synthesis. p53 is able to block the cell cycle by activating the expression of genes such as p21 (CIP1), which is an inhibitor of cyclin-dependent kinases (cdks). A p53-dependent increase in p21 results in the inhibition of cyclin E-cdk2 kinase activity causing a G_1/S cell cycle arrest (el-Deiry et al., 1993; Gu et al., 1993).

The transcription regulatory function of p53 is critical for G_1 arrest in cells, which has been shown to be inhibited by mdm2 (Chen et al., 1996). Interaction between the two proteins is required for this abrogation and has been confirmed using a mutant p53 (14/19 double-point mutant) which was unable to bind mdm2 but retained DNA binding activity (Chen et al., 1996). Mdm2 had no effect on the G_1 arrest induced by this mutant, thus it is essential that p53 be able to bind to DNA and transactivate genes in order for it to cause a G_1 cell cycle arrest.

p53 has also been implicated in the regulation of other proteins, such as pRB which is involved in cell cycle control. The phosphorylation status of pRB changes as the cells progress through the cell cycle, and in response to DNA damage. This phosphorylation

state of pRB was found to be dependent on the presence of functional p53 after exposure of cells to γ-radiation. Cells harbouring functional wild-type p53 were found to have elevated levels of p53 and underwent a G_1 arrest after exposure to γ-radiation. This correlates with a hypophosphorylated form of pRB in the cells (Slebos et al., 1994). In contrast, cells containing mutant p53 fail to arrest in G_1 after exposure to ionising radiation and no hypophosphorylated pRB is detected in these cells. Hypophosphorylation of pRB is dependent on an increase in p53 after DNA damage as inactivation of wild-type p53 by the HPV E6 protein results in a failure of these cells to arrest after DNA damage (Slebos et al., 1994). Thus it appears that p53 plays a central role in the regulation of other proteins involved in cell cycle control in order to elicit a G_1 arrest after exposure to DNA damaging agents.

Accumulation of p53 after DNA damage and the transactivation function of p53 may be regulated differently with respect to the cell cycle. The period during the cell cycle in which the cells are exposed to DNA damaging agents also seems to be important for p53 accumulation. Exposure of synchronised cells to UVC in G_0 or early to mid G_1 phases of the cell cycle results in low levels of p53 accumulation, whereas cells exposed at the G_1/S border show higher levels of p53 (Haapajarvi et al., 1997). In contrast, the DNA binding capacity of p53 is activated after UV radiation during any phase of the cell cycle, suggesting that activation of the DNA binding and transactivation functions of p53 induced by exposure to UV irradiation occurs independently of the cell cycle (Haapajarvi et al., 1997).

p53 is phosphorylated by $p60^{cdc2}$ and cyclin B^{cdc2}, and it is possible that the under-phosphorylated form of p53 exerts cell cycle control, suggesting that in order to allow progression through the cell cycle, p53 must be phosphorylated (Bischoff et al., 1990). This is supported by more recent findings which show that p53 is phosphorylated by cyclin A/Cdk2 and cyclin B /Cdc2 complexes but not by cyclin E/Cdk2 and cyclin D1/Cdk4 complexes (Wang and Prives, 1995). Since cyclin D1 and E are most abundant in early and late G_1 respectively, this implies that p53 is in an underphosphorylated form at the G_1 checkpoint. The cell cycle-specific growth suppression exhibited by p53 may

act as a check point control, hence the role of p53 may be to monitor whether a cell should continue to progress through the cell cycle (Diller et al., 1990). Loss of this function, through mutation, for example, would enable a cell to escape this growth control and begin proliferating towards a transformed phenotype. Also, by regulating the progression of cells through the cell cycle, p53 plays a role in modulating the expression of certain growth response genes that are important for the progression from G_1 into S phase.

Emerging evidence suggests that p53 plays little role in normal cell cycle control, but rather is crucial in controlling the cell cycle in stressed cells (Kastan et al., 1991). In response to x-ray or drug-induced DNA damage, p53 levels are increased in normal cells, which then become arrested in the cell cycle until the damage is repaired. A response element to genotoxic stress has been identified in the p53 promoter (Sun et al., 1995). Exposure of cells to the anticancer agents 5-fluorouracil and mitomycin or UV light results in accumulation of p53, as well as an increase in p53 mRNA levels. The region of the p53 promoter required for this response to genotoxic agents has been shown to reside in a fragment of the p53 promoter between -70 to -40 (Sun et al., 1995). Cells containing mutant p53 genes, on the other hand, fail to arrest in the cell cycle and die.

p53 may also play a role in regulating the cell cycle at a G_2 checkpoint. Treatment of cells containing wild-type p53 with nocodazole (a microtubule-disrupting agent which can trap cells in mitosis) after irradiation results in very few cells entering mitosis, consistent with a G_2 block (Bunz et al., 1998). Treatment of cells containing mutant p53, however, results in a large proportion of cells entering mitosis, indicating that p53 controls a G_2 checkpoint. This G_2 checkpoint is also dependent on the presence of p21 (CIP1), a cyclin dependent kinase inhibitor that is activated by p53.

1.4.2.10 p53 in DNA Repair

There is evidence suggesting that p53 plays a more direct role in DNA repair. Wild-type p53 has been shown to harbour an intrinsic exonuclease activity which might act in DNA replication, recombination and repair (Mummenbrauer et al., 1996; Janus et al., 1999).

This exonuclease activity has been mapped to the central core of the protein and is specific to the wild-type p53 as different mutant p53 molecules do not display this 3' → 5' exonuclease activity. The central core domain of p53 contains both the sequence-specific DNA binding activity and an exonuclease activity and these 2 functions of p53 are mutually exclusive. As is the case for the sequence-specific DNA binding activity, the exonuclease activity is regulated by the C-terminal domain of p53 since C-terminally truncated p53 showed a higher exonuclease activity than full length p53 (Janus et al., 1999). The sequence-specific DNA binding activity and the exonuclease activity, however, are oppositely regulated by the C-terminal domain: the C-terminal domain activates specific DNA binding, but negatively regulates the exonuclease activity. Thus, in the absence of stress, when p53 is thought to be in an inactive state, p53 plays a role in maintaining the integrity of the genome by repairing spontaneous DNA damage via its exonuclease activity in a non-sequence specific manner.

p53 has also been indirectly implicated in DNA repair and replication, by activating the expression of the proliferating cell nuclear antigen, PCNA, a protein which functions in DNA replication and repair (Xu and Morris, 1999). Exposure of rat embryo fibroblasts to γ radiation causes an increase in p53 levels, which in turn activates expression of PCNA, which has been shown to be brought about by p53 binding directly to the PCNA promoter (Xu and Morris, 1999).

1.4.2.11 Involvement of p53 in DNA Replication

p53 has also been implicated in directly regulating DNA replication. Wild-type p53 is able to inhibit the replication of polyomavirus constructs in an in vitro system in a sequence-specific binding manner. Mutant p53 proteins that bind poorly to DNA (Trp248 and His273) were unable to inhibit replication of the polyomavirus, suggesting that p53 inhibits replication by altering the structure of the DNA, thereby hindering the binding of transcription factors necessary for replication (Miller et al., 1995). Conversely, DNA replication may affect p53 function in that p53 is able to form a complex with the replication protein A (RP-A), a multi-subunit complex involved in both DNA replication and repair. p53 does not inhibit RP-A from binding to single-stranded DNA, however, the

presence of single-stranded DNA prevents p53 from complexing with RP-A (Miller et al., 1997). When complexed to RP-A, p53 is unable to bind to the WAF1 p53 binding site and consequently would prevent transactivation of the WAF1 gene. In the presence of single-stranded DNA, however, RP-A was not able to prevent p53 from binding to the WAF1 p53 consensus binding site (Miller et al., 1997). The data suggests that in the absence of single-stranded DNA, p53 may be bound to RP-A and thus be unable to interact with consensus binding sites in various promoters, but in the presence of single-stranded DNA that may arise due to replication or DNA repair, the p53:RP-A complex dissociates with RP-A binding to the single-stranded DNA, leaving the p53 free to interact with the promoters of various p53-responsive genes.

p53 can directly recognise DNA damage in the form of insertion or deletion mismatches. Both wild-type p53 and its 14kDa C-terminal (residues 311-393) are able to bind mismatched DNA in vitro as determined by electron microscopy, with the predominant C-terminal complexes that bound to the DNA being tetramers. Electrophoretic mobility shift assays suggested, however, that p53 could bind to the mismatched DNA as monomers, dimers, tetramers and even higher oligomers, and also suggested that the C-terminal region could bind to mismatched DNA as dimers and tetramers (Lee et al., 1995). Furthermore, these protein-DNA complexes are stable with a half-life of more than 2 hours. These results impart a further important role for the C-terminal region of p53, that of recognition of and direct binding to damaged DNA. By forming a stable complex on mismatched/damaged DNA, p53 probably undergoes a conformational change resulting in resistance to degradation. The formation of this stable complex at the site of the DNA lesion may provide a scaffold for the binding of proteins involved in DNA repair. It may also result in p53-specific activation of genes involved in cell cycle control and/or apoptosis (Lee et al., 1995). These findings are supported by evidence in vitro evidence that shows that wild-type p53 is able to induce the annealing of heat-denatured double-stranded DNA (Reed et al., 1995). The region of p53 responsible for this action is located between amino acids 318 and 393 in the C-terminal domain. This same segment of the C-terminal was also able to catalyse DNA strand transfer from duplex DNA to single-stranded linear DNA in vitro, as well as recognise damaged DNA

induced by DNaseI or ionising radiation (Reed et al., 1995).

1.4.2.12 Polymorphic Variants of p53

There are two polymorphic variants of p53 which differ at amino acid 72 (Matlashewski et al., 1987). The variants contain either proline or arginine at codon 72 arising from a single base substitution (CCC or CGC). It has been shown that these two isoforms behave slightly differently and may play a role in tumours associated with HPV. The E6 protein of HPV more efficiently targets the p53Arg for degradation via the ubiquitin pathway than the p53Pro variant (Storey et al., 1998). The majority of HPV-associated tumours analysed to date are homozygous for the Arg variant. Subtle variations in the biological functions of these two isoforms have also been found. Both variants are structurally wild-type as determined by monoclonal antibody reactivity and they both have similar affinities for p53 consensus DNA recognition sequences. The p53Pro variant, however, is a slightly stronger transcriptional activator than the p53Arg variant, which stems from differences in their interactions with various transcription factors. The Pro variant binds with a higher affinity to TAFII 32 and TAFII 70 than the Arg variant. Both isoforms suppress the growth of Saos-2 cells to a similar extent, but the Arg isoform is more efficient than the Pro isoform at suppressing colony formation in an E7-EJ-ras transformation assay. Both of the variants are capable of inducing equal levels of apoptosis, suggesting that the p53 genotype may affect design of treatment and management in those patients with p53 wild-type tumours(Thomas et al., 1999).

A silent polymorphism in the p53 gene has been identified in codon 213 (CGA to CGG). This is a rare polymorphism and occurs in only 3.2% of lung and breast cancer DNAs examined (Carbone et al., 1991).

1.4.2.13 p53 and Apoptosis

p53 promotes apoptosis only under certain circumstances, such as in response to DNA damage and may not be involved in all forms of apoptosis (Shaw et al., 1992; Lowe et al., 1993b; Clarke et al., 1993). Immature thymocytes lacking p53 die normally when exposed to agents which mimic T-cell receptor engagement, but they are resistant to

ionising radiation, that is, they do not undergo apoptosis after exposure to ionising radiation. In contrast, thymocytes containing wild-type p53 undergo apoptosis after exposure to ionising radiation (Lowe et al., 1993b; Clarke et al., 1993). Both wild-type and p53 null thymocytes, however, undergo apoptosis in response to glucocorticoids or calcium. These results suggest that p53 is not necessary for apoptosis under normal circumstances, but is required when cells are exposed to DNA damaging agents and also indicate that the triggers for p53-dependent apoptosis are different from those during normal cell development.

p53-dependent apoptosis occurs predominantly in the G_1 phase when cells are growth-arrested (reviewed by Ozbun and Butel, 1996). The mechanism by which apoptosis is promoted by p53 is unclear, but it may involve the levels of *bax* and *bcl2* in the cell. A high level of *bax* and a low level of *bcl2* results in apoptosis, and p53 is able to control this *bax*:*bcl2* ratio in the cells by activating the expression of the *bax* gene and repressing expression of the *bcl2* gene. The exact downstream effectors of p53 directly involved in apoptosis are at present not clearly defined, but some evidence suggests that caspases such as caspase-9 are involved (Soengas et al., 1999). p53 has also been implicated in the movement of Fas, a member of the TNF receptor family involved in apoptosis, from the cytoplasmic pool to the membrane such that it can interact with the Fas-ligand and initiate apoptosis (Bennett et al., 1998). A model for p53-induced apoptosis has been proposed based on the identification and characterisation of p53-induced genes (*PIG*s). Somewhat astoundingly, only 14 out of more than 7000 transcripts identified in p53-expressing cells are significantly overexpressed compared to p53 null cells after the induction of apoptosis (Polyak et al., 1997). Some of these overexpressed genes were predicted to encode proteins which could generate reactive oxygen species (ROS) and based on the genes identified and biochemical and pharmacological experiments, a three-step model was proposed: (1) activation of redox-related genes, (2) the formation of reactive oxygen species, and (3) oxidative damage of mitochondrial components, resulting in cell death (Polyak et al., 1997).

The ability of p53 to induce apoptosis plays an important role in tumourigenesis. p53 null

mice develop tumours very rapidly, and this correlates with the loss of p53-dependent apoptosis (Symonds et al., 1994). p53-dependent apoptosis is induced by abnormal cell growth and consequently, loss of wild-type p53 function contributes to the process of tumourigenesis as does mutant p53. Cells that lack p53-dependent apoptosis would be unable to respond to this abnormal cell proliferation by undergoing apoptosis, resulting in accumulation of gene mutations that may promote tumour progression.

p53-dependent apoptosis also plays an important role in the response of tumours to various anticancer agents. Cells expressing wild-type p53 are able to undergo apoptosis after exposure to various chemotherapeutic agents such as 5-fluorouracil, etoposide and adriamycin, whereas p53 null cells do not generally respond (Lowe et al., 1993a). Since many chemotherapeutic agents involve a similar mechanism of action (DNA damage), it is essential to have wild-type p53 in order to respond to the treatment. These results may explain how tumours containing mutant p53 are resistant to chemo- and radiotherapy, as these p53 mutant tumours would not undergo p53-induced apoptosis and would continue to proliferate.

1.4.2.14 p53 and Angiogenesis

Angiogenesis is important for tumour survival in order to obtain nutrients. Tumour cells secrete cytokines that stimulate angiogenesis and the new blood vessels are important in tumour cell metastasis. p53 has been implicated in the regulation of genes such as thrombospondin-1 (TSP-1) that are involved in this process. Fibroblasts from Li-Fraumeni patients secrete high levels of TSP-1 at early passages, but after loss of the normal p53 allele during passaging, the level decreases (Dameron et al., 1994). This loss of wild-type p53 and the reduced expression of TSP-1 corresponds with the loss of the culture media to inhibit angiogenesis both in vitro and in vivo in a rat corneal model. Re-introduction of wild-type p53 into these late passage cells resulted in an increase in TSP-1 levels and also restored the anti-angiogenic phenotype.

1.4.3 p53 Mutations in Human Cancers

p53 mutations are the most frequent alterations in human tumours, but the frequency of

mutations varies among tumour types. Not all tumour types have a high frequency of mutations (Peng et al., 1993; Saylors et al., 1991) but in most tumour types, mutations in the p53 gene occur in 20-50% of cases, while some tumour types have a mutation frequency as high as 80%. The mutations occur in all classes (base substitutions, insertions and deletions), however, the most prevalent mutations are point mutations resulting in amino acid substitutions.

The spectrum of p53 mutations in a given cancer type can often be accounted for by environmental or chemical carcinogens. Skin cancer is linked to exposure to UV light, which causes pyrimidine dimers to form in the DNA, thus accounting for a large proportion of p53 mutations detected in skin cancer (Burren et al., 1998; Dumaz et al., 1993). Hepatocellular carcinoma is another example in which a specific carcinogen, aflatoxin B, induces a high frequency of mutations in codon 249 of the p53 gene (Shimizu et al., 1999; Lunn et al., 1997; Aguilar et al., 1994).

p53 mutations may have a prognostic value. The stage at which p53 mutations occur varies between tumour types, but it is generally thought that p53 mutations occur at an early stage in breast, cervix, oesophageal, lung and stomach cancer, whereas in tumours of the brain, liver, ovary and thyroid, mutations occur at a later stage in the development of these tumours.

1.4.3.1 Properties of Mutant p53

Most p53 mutants lose their wild-type function i.e. they are unable to act as tumour suppressors and to suppress growth. There are two hypotheses suggesting how mutant p53 inactivates wild-type p53. Firstly, the mutant p53 acts in a dominant negative fashion whereby the mutant form abrogates the function the wild-type protein, and secondly some mutants have been found to gain a function and consequently behave as oncogenes. There is evidence to support both of these hypotheses.

1.4.3.2 Dominant Negative Theory

The fact that p53 exists as an oligomer in solution, and that it may bind to DNA as a

tetramer, could provide an explanation as to how mutant p53 acts in a dominant negative fashion. The mutant subunit could form an oligomeric complex with a wild-type subunit thus preventing the wild-type subunit from functioning normally (Levine et al., 1991). This represents a dominant loss of function mutation. This loss of function of the wild-type subunit could be due either to it not being able to bind to DNA, or, once bound, it is unable to activate (or repress) transcription. It has been shown, for example, that the His175 mutation inhibits the ability of wild-type p53 to bind DNA, thus abrogating its normal function (Kern et al., 1992). Supporting evidence for this theory comes from a codon 280 mutation found in nasopharyngeal carcinomas. This mutation commonly occurs in a heterozygous state and a thr280 point mutation showed a dosage-dependent dominant negative effect on wild-type p53 function (Sun et al., 1993). In vitro assays showed that this mutant is unable to activate transcription from a p53-responsive promoter and the Thr280 mutant inhibits wild-type p53 driven transcription in a dose-dependent manner. Some inhibition is observed even with a 3:1 ratio of wild-type:mutant and almost complete inhibition is observed at a ratio of 1:3 (wild-type:mutant). This mutant also affects the growth inhibition function of wild-type p53 in a dose-dependent fashion. Saos-2 cells transfected with the wild-type p53 undergo a growth arrest, and this arrest is inhibited by co-transfection with the Thr280 mutant. As for transcriptional inhibition, inhibition of the wild-type by the mutant occurrs as low as 3:1 (wild-type:mutant). The growth arrest induced by wild-type p53 is substantially blocked at a ratio of 1:1 (wild-type:mutant) and is completely abrogated at a ratio of 1:3 (wild-type:mutant). These results support the model that mutant monomers can form tetramers with wild-type monomers and furthermore, the more mutant monomers in the heterotetramers, the greater the effect on wild-type function.

Numerous anogenital tumour-derived p53 mutants also showed various degrees of dominant negative effects on wild-type function when co-transfected with the wild-type gene into p53 null Saos-2 cells. p53 with a Leu173 mutation showed the greatest dominant negative effect (Crook et al., 1994).

1.4.3.3 Gain of Function Mutations

The alternative hypothesis as to how mutant p53 acts is that the mutant form gains a new function which can overcome the negative regulatory properties of wild-type p53. Tumour-derived p53 mutants have been shown to activate a number of genes involved in cell proliferation, and in fact these mutants do not bind to the wild-type p53 consensus DNA binding site (Lanyi et al., 1998). Furthermore, the C-terminal region containing the oligomerisation and nonsequence-specific DNA binding domains (spanning amino acids 370 to 380) were shown to be required to activate the promoters of the EGFR, MDR1, PCNA and c-*myc* genes by these tumour-derived p53 mutants. In addition, the mechanism of activation of mutant p53 varied for the different promoters; various deletion constructs of mutant p53 lost their ability to transactivate the EGFR and MDR1 genes, but not the PCNA or c-*myc* genes (Lanyi et al., 1998; Frazier et al., 1998). Various p53 mutants, but not wild-type p53, are capable of transactivating the c-*myc* promoter in p53-null cells, demonstrating a gain of function rather than a dominant negative mode of action (Frazier et al., 1998). Furthermore, exon 1 of the c-*myc* gene is required for this transactivation, suggesting that the p53 response element in the c-*myc* gene does not act as a classical enhancer and that the sequences to which p53 binds may have to be transcribed into RNA in order for p53 to activate c-*myc* expression (Frazier et al., 1998).

A study on a large number of naturally occurring p53 mutants as well as various mutants designed to target specific regions of the protein provided further evidence that different p53 mutants have different effects with respect to transactivation and transcriptional repression (Crook et al., 1994). Deletion of conserved regions II, III, IV and V results in the loss of transcriptional activity, whereas deletion of conserved domain I and a small C-terminal truncation (Δ370) does not have a significant effect on transcritpional activation by the mutants. Of the numerous point mutations analysed, only two (Pro175 and Leu181) retained transcriptional activation activity. In addition to activating transcription, p53 also plays a role in transcriptional repression and the various mutants were examined for their ability to affect transcriptional repression by wild-type p53. All the mutants studied, except for those containing a deleted conserved domain I or the truncated C-terminal domain (p53Δ370), had a negative effect on the ability of wild-type p53 to

repress transcription (Crook et al., 1994). Furthermore, the various mutants were able to suppress growth of Saos-2 cells to various degrees, which correlated with their ability to transactivate a p53-responsive promoter. Those mutants that retain transcriptional activation activity are able to suppress the growth of Saos-2 cells, whereas those mutants that no longer retain tranactivation activity, fail to suppress growth of the Saos-2 cells. These mutants were also examined for their ability to co-operate with E7 and *ras* in transformation assays, and deletion of the conserved domains II-V results in the acquisition of transforming properties. Interestingly, both the mutants that retain transactivation function (the Pro175 and Leu181 mutants) fail to suppress transformation. Taken together, these results suggest that transcriptional activation by p53 correlates with suppression of growth in p53 null cells, but transcriptional transactivation is independent of the ability of p53 to suppress transformation by E7 and *ras*. These results imply that activation of gene expression by mutant p53 does not necessarily result the ability to suppress tumour formation.

Some fairly strong evidence argues against the dominant negative theory. Wild-type p53 is very rarely found together with mutant p53 in tumours. One allele usually has a mutation, while the remaining wild-type allele is lost. Also, introduction of mutant p53 into cells lacking endogenous p53 enhances the transformed phenotype in vitro and tumourigenicity in vivo, suggesting that the mutants gain a function and act as oncogenes (reviewed by Ozbun and Butel, 1996).

Different tumour-derived p53 mutants have different effects on cells thus while some mutants may retain some wild-type functions others may retain the ability to induce cell cycle arrest but lose the ability to induce apoptosis, whilst yet others may lose the ability to induce cell cycle arrest, but retain the ability to induce apoptosis (Mukhopadhyay and Roth, 1993; Ludwig et al., 1996). Two tumour-derived p53 mutants (Pro175 and Leu181) are able to activate expression of the CIP1 promoter, and consequently induce G_1 cell cycle arrest, whereas mutants Leu173 and p53 22/23 (an N-terminal mutant) are unable to activate expression from the CIP1 promoter and consequently unable to induce a G_1 arrest. In contrast, the Pro175 mutant has lost the ability to activate the *bax* promoter and

the IGF-BP3 promoters. The Leu181 mutant, on the other hand, retains the ability to activate the *bax* promoter and the IGF-BP3 box A promoter, but not the IGF-BP box B promoter, while the Leu173 mutant has lost the ability to transactivate all of these promoters. This loss of activation is due to the inability of the various mutants to bind to the DNA. Activation of the *bax* gene results in apoptosis, consequently cells transfected with the Pro175 and Leu173 mutants do not undergo apoptosis , whereas the Leu181 mutant containing cells are still able to undergo apoptosis, albeit at a much lower level than wild-type cells.

Codon 175 is one of the hotspots for mutations in human cancers, and a more detailed analysis of various codon 175 mutations revealed that mutations at this position can be divided into 3 classes: class I, which behaves like the wild type and retains both apoptotic and cell cycle arrest functions; class II, which retains cell cycle arrest function, but has lost apoptotic function, and class III, which has lost both activities (Ryan and Vousden, 1998). An example of a class I mutant is Cys175 with Pro175, Lys175, Ile175 and Ser175 all being class II mutants. Tyr175, Trp175, Asp175 and Phe175 have all lost both the ability to arrest the cells in G_1 and apoptotic activity, and are thus classified as class III mutants. All class I and II mutants are able to transactivate the p21(CIP1) gene which is correlated with the ability of these mutants to arrest the cell cycle. These data support the idea that different tumour-derived p53 mutants can have different effects on different p53-responsive promoters, and consequently, by retaining the ability to bind to certain promoters, these mutants may retain some wild-type function. Conversely, other p53 mutants lose all wild type function entirely. It is noteworthy and interesting that different p53 mutants which have different amino acid substitutions at the same codon behave differently.

These results are supported by similar findings with different p53 mutants (Friedlander et al., 1996a). p53Ala143 (a temperature sensitive mutant), His175 and Trp248 mutants are transcriptionally inactive at 37°C and consequently do not induce apoptosis. At the permissive temperature of 32°C, the temperature sensitive Ala143 mutant is able to activate transcription from a number of p53 response elements, including those from the

mdm*2* and CIP1 promoters. Interestingly though, even at the permissive temperature, p53Ala143 does not transactivate the *bax* promoter or the IGF-BP3 promoters. These findings suggest that this mutant form of p53 can discriminate between different p53-responsive target genes and point to discrete classes of p53-responsive genes.

Tumour-derived p53 mutants have been shown to be able to bind to p53 consensus DNA binding sites at lower temperatures (Friedlander et al., 1996b). At 25°C, a number of tumour-derived p53 mutants are able to bind to RGC (Kern et al., 1991) and GADD45 (Kastan et al., 1992) (sites in human genomic DNA containing p53 response elements) oligonucleotides, whereas at 37°C binding of the mutants to these oligonucleotides is undetectable. Furthermore, these mutants are able to transactivate the mdm2 promoter at 26°C but not at 37°C, consistent with their ability to bind to DNA at a lower temperature. Not all of the mutants tested, however, are able to bind to DNA or transactivate the mdm2 promoter at 26°C. Surprisingly, an antibody directed to the N-terminal of p53 is able to stabilise the mutants and render them capable of binding DNA at 37°C. These results can have implications for treatment of tumours containing p53 mutants. Since sequence-specific DNA binding is a critical function of wild-type p53 and the majority of p53 mutants lack the ability to bind DNA in a sequence specific manner, it is possible that development of small molecules that stabilise mutant p53 may aid in the treatment of tumours containing mutant p53 (Friedlander et al., 1996b).

1.4.3.4 Stability and Cellular Localisation of Mutant p53

Many p53 mutants have been found to accumulate in the cytoplasm. Most mutations in p53 result in a conformational change in the protein resulting in increased stability of the mutants, which can be explained to some extent by their inability to activate expression of the mdm2 gene, such that they are not targetted for rapid degradation (Kubbutat et al., 1997). In addition, in vitro studies have shown that p53 mutants lacking the ability to bind mdm2 were not down-regulated, but p53 mutants that retained the ability to interact with mdm2 were down-regulated (Haupt et al., 1997).

1.4.4 p53 Status and Response to Cancer Therapy

As a result of the involvement of p53 in the regulation of cell growth, DNA repair and apoptosis, tumours harbouring p53 mutations may respond poorly to therapy compared to tumours containing wild-type p53. Embryonic fibroblasts transformed with the adenovirus E1A and activated *ras* proteins formed tumours when injected subcutaneously into nude mice, irrespective of their p53 status (Lowe et al., 1994). The number of tumours formed and their response to therapy, however, differed depending on the p53 status. Cells containing wild-type p53 formed fewer tumours and responded better to gamma radiation than cells lacking p53. The p53 status also influenced the responsiveness to adriamycin, a chemotherapeutic agent that causes a regression of the tumours containing wild-type p53, but has little effect on those tumours lacking p53. This response to treatment was shown to be due to apoptosis, as treated tumours containing wild-type p53 showed a higher apoptotic rate than treated tumours lacking p53. More importantly, it was shown that acquired resistance to therapy was due to de novo p53 mutations (Lowe et al., 1994).

p53 status may therefore be important in prognosis and response to treatment, since it is possible that tumours with different p53 mutations may respond differently to various therapies, depending on the ability of the various mutants to abrogate the function of the wild-type protein (in instances where the wild-type allele is not lost) or the ability of the various p53 mutants to effect apoptosis by regulation of genes involved in this process.

Breast cancer, like all other types of cancer, is a multigenic and highly complex disease. It is clear, however, that genes involved in the regulation of cell growth and differentiation play a major role in the process of tumourigenesis. More specifically, it is mutations in these regulatory genes that cause these genes to function aberrantly ultimately leading to the formation of tumours. Examples of such genes include the *ras* family of genes, which are involved in the stimulation of cell growth, and tumour suppressor genes, that are important for the negative regulation of cell proliferation. Because genes such as the p53 tumour suppressor gene play a central and important role in the normal growth of cells, it is important to identify mutations in this gene which may

inactivate its normal function. Furthermore, mutations in this gene have been implicated in the response of cancer patients to therapy. Thus it is essential not only to identify these mutations, but also to determine their effect on the normal function of the protein, since it has been shown that different p53 mutations can have different effects on the wild-type function. By elucidating the nature and frequency of these mutations in cancer, it may be possible to group them into different subsets; those that may not dramatically alter the function of the protein, and those that would result in the total abrogation of wild-type function. Understanding of p53 mutations may be helpful in deciding on a treatment regimen and may also give some indication as to the patient prognosis and response to therapy.

This study is aimed at identifying such mutations in the Ha-*ras* oncogene and p53 tumour suppressor gene in a group of breast cancer patients from South Africa. The study also examined the nature of the various p53 mutations detected with respect to their ability to arrest the cells in the G_1 phase of the cell cycle, their effect on cell growth and their ability to transform cells. The results reported in this study suggest a correlation between the severity of the p53 mutations and the response of the patients to therapy and survival.

CHAPTER 2: ANALYSIS OF MUTATIONS IN THE Ha-*ras* GENE

2.1 INTRODUCTION

Breast cancer is the most common cancer amongst women, and recent epidemiological studies have suggested that one in eight women will develop the disease during their lifetime (Feuer et al., 1993). In South Africa, the risk of developing breast cancer varies among different population groups with the highest incidence found in White and Asian females (1 in 15), a lower incidence in Coloured females (1 in 33) and the lowest incidence in Black females (1 in 68) (Sitas et al., 1997).

Breast tumours develop mainly from ductal or lobular hyperplasias that acquire cumulative genetic changes, resulting in clonal outgrowths of progressively malignant cells (Dickson et al., 1991). Mutated *ras* oncogenes have been found in about 10% of human tumours and are thus far the most frequent group of mutated oncogenes identified in human cancers (reviewed by Bos, 1989; Barbacid, 1990; and Barbacid, 1987). In addition, the frequency of mutated *ras* oncogenes varies among different tumour types (Bos, 1989) with the highest incidence in tumours of the pancreas, where more than 80% of the tumours contain a mutated K-*ras* oncogene. The incidence of mutated *ras* oncogenes is also significant in tumours of the colon (K-*ras*) and thyroid (H-, K-, and N-*ras*). In several tumour types such as cancer of the breast, oesophagus, ovary, cervix, stomach, glioblastomas, neuroblastomas and lymphoid leukemias (Bos, 1989), however, activated or mutated *ras* oncogenes are rarely present.

The *ras* oncogene becomes constitutively activated by a single point mutation within the coding sequence (Taparowsky et al., 1982; Tabin et al., 1982; Reddy et al., 1982; Seeburg et al., 1984), predominantly in codons 12, 13 and 61. It has also been found, however, that overexpression (between 10- and 100-fold) of normal *ras* oncogenes can also induce certain malignant phenotypes (Chang et al., 1982).

A limited number of studies have shown that *ras* mutations occur infrequently in human breast cancer (Rochlitz et al., 1989; Miyakis et al., 1998; Clark and Der,

1995). In chemically-induced mammary carcinomas in rats, however, the Ha-*ras* gene has been implicated in the etiology of the disease (Sukumar et al., 1983; Zarbl et al., 1985), with mutations occurring mostly at either codons 12 or 13, depending on the chemical carcinogen used. Furthermore, the techniques used to identify *ras* mutations in human breast cancer varied from study to study and ranged from transfection assays to assess whether the *ras* oncogene could transform NIH-3T3 cells, to oligonucleotide hybridisation and RNase mismatch analyses. Some of these techniques (such as oligonucleotide hybridisation) are fairly insensitive at detecting a large number of mutations at various positions in the gene.

This chapter analyses Ha-*ras* gene mutations in breast cancer patients from the "Coloured community" from the Western Cape area of South Africa, as well as in breast cancer cell lines. The "Coloured community" refers to a heterogeneous group of mixed ancestry, including San, Khoi, West African Negro, Madagascar, Javanese, and Western European origin. Exons 1 and 2 (encompassing codons 12, 13 and 61) of the Ha-*ras* gene were screened for mutations using single strand conformation polymorphism analysis (SSCP), a technique that allows fairly rapid examination of the entire exon for mutations.

2.2 RESULTS

2.2.1 Breast Tumour Samples and Cell Lines

Forty primary breast tumours were obtained from the Breast Clinic at Groote Schuur hospital. Twenty three of the samples were fresh tissue samples obtained from patients who underwent mastectomies and 17 of the samples were paraffin-embedded tissue sections. Thirty nine of the samples were infiltrating ductal carcinomas of various stages and 1 sample was a fibroadenoma (table 2.1). With the exception of 1 sample from a male patient, all of the samples were from female patients. In addition, the majority of the samples (88%) were obtained from the "Coloured community", 4 of the samples were from white patients and 1 sample was from a black patient.

In addition to the 40 primary tumour samples, 4 breast cancer cell lines; T47-D, MCF-7, MDA-MB-231 and ZR-75, obtained from ATCC, were also analysed in this study. The human embryonic lung fibroblast, WI-38, was used as a normal control.

Table 2.1: Tumour samples used in this study.

TUMOUR STAGE	NO. OF SAMPLES
I	18
II	11
III	9
IV	1
Fibroadenoma	1

2.2.2 Mutation Analysis of Exon 1 of the Ha-Ras Gene

2.2.2.1 PCR-Amplification of Exon 1

DNA was isolated from the breast tumour samples and the cell lines as described in section 6.1. The polymerase chain reaction (PCR) was used to amplify exon 1 of the Ha-*ras* gene as outlined in section 6.3, using the primers listed in table 2.2, in a reaction volume of 50µl. After amplification, 5µl of the reaction mixture was fractionated on a 1.5% agarose gel to check that the exon had been correctly amplified to give the expected product of 206bp (figure 2.1).

A

1595
H12/1

GGGCCCTCCTTGGCAGGTGGGGCAGGAGACCCTGTAGGAGGACCCCGGGCCGCAGGCCCCT

Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala Gly Gly
GAGGAGCG ATG ACG GAA TAT AAG CTG GTG GTG GTG GGC GCC GGC GGT

Val Gly Lys Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe
GTG GGC AAG AGT GCG CTG ACC ATC CAG CTG ATC CAG AAC CAT TTT

Val Asp Glu Tyr Asp Pro Thr Ile Glu
GTG GAC GAA TAC GAC CCC ACT ATA GAG GTGAGCCTAGCGCCGCCGTCCAGGTG

H12/2 1800

B

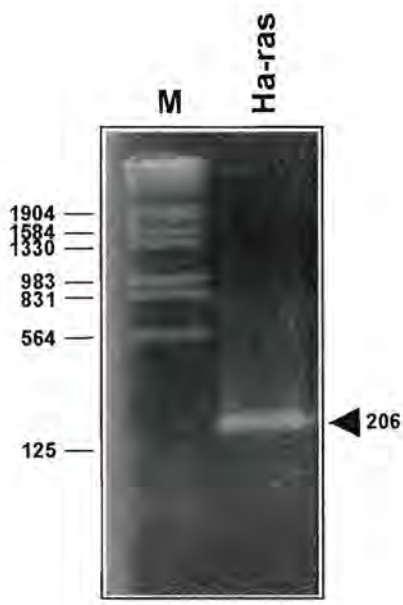


Figure 2.1: Amplification of exon 1 of the Ha-*ras* gene. (A); sequence of exon 1 with the primers H12/1 and H12/2 depicted in the boxes. The amino acid sequence is shown above the gene sequence. (B); The PCR product was electrophoresed on a 1.5% agarose gel, the gel stained with ethidium bromide (EtBr) and the bands visualised under UV light. The molecular weight markers (M) are shown on the left hand side of the figure (in base pairs) and the 206bp amplified product is indicated by the arrowhead.

Table 2.2: PCR primers and annealing temperatures used for the amplification of exons 1 and 2 of the Ha-*ras* gene

Gene	Exon	Primer Pair	Tm	Fragment Size
Ha-ras	1	H12/1: 5' - GGGCCCTCCTTGGCAGGTGG - 3' H12/2: 5' - CACCTGGACGGCGGCGCTAG - 3'	65°C	206bp
Ha-ras	2	H61/A: 5'- GGAGAGGCTGGCTGTGTGAA - 3' H61/B: 5' - AAAAGACTTGGTGTTGTTGA - 3'	56°C	230bp

2.2.2.2 Screening of Exon 1 for Mutations

Activating mutations of the *ras* genes have been shown to occur at codons 12, 13 and 61 (Taparowsky et al., 1982; Tabin et al., 1982; Reddy et al., 1982). Codon 12 is part of an MspI restriction endonuclease site and any mutation at this position would destroy this site. Screening for mutations is thus a simple process involving amplification of the exon and digestion with MspI.

Codon 12 of the Ha-*ras* gene was therefore screened for mutations by amplifying exon 1 in the presence of ^{32}P-dCTP and digesting the PCR product overnight with MspI at 37°C in the appropriate reaction buffer. ^{32}P-dCTP was used as a tracer in order to visualise the small DNA fragments after digestion. The digested products were electrophoresed on 8% non-denaturing polyacrylamide gels, dried under vacuum and exposed to x-ray film for 16 hours. An additional MspI restriction site upstream of codon 12 served as an internal control (figure 2.2). If the MspI site at codon 12 remained intact, fragments of 105bp, 56bp and 45bp should be observed. If, however, the site is disrupted, the 56bp band would be part of a 161bp fragment. The WI-38 pattern represents the normal control and as can be seen, none of the patients showed any shift of the 56bp to 161bp, indicating that no mutations were present in codon 12 in any of the tumour samples.

2.2.2.3 SSCP Analysis of Exon 1 of the Ha-*ras* Gene

In order to screen the entire exon 1 of the Ha-*ras* gene for mutations, single strand conformation polymorphism (SSCP) analysis was used as described in section 6.3.2. Briefly, 100ng of patient or cell line DNA (section 2.2.1) was used as a template in a PCR reaction with primers H12/1 and H12/2 (table 2.2). PCR reactions were carried

A

GGGCCCTCCT TGGCAGGTGG GGCAGGAGAC CCTGTAGGAG GACC**CCGG**GC

CGCAGGCCCC TGAGGAGCGA TGACGGAATA TAAGCTGGTG GTGGTGGGCG

12

CCGGCGGTGT GGGCAAGAGT GCGCTGACCA TCCAGCTGAT CCAGAACCAT

TTTGTGGACG AATACGACCC CACTATAGAG GTGAGCCTAG CGCCGCCGTC

CAGGTG

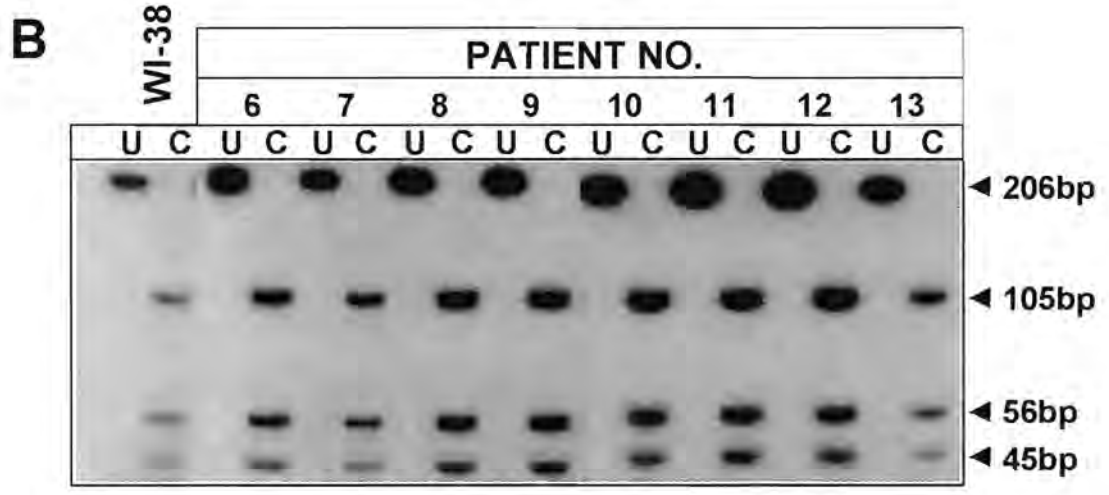


Figure 2.2: Analysis of exon 1 of the Ha-*ras* gene. (A); the sequence of the PCR product. The exon is underlined and the MspI restriction site encompassing codon 12, as well as an upstream MspI site which serves as an internal control, is depicted in bold lettering. (B); exon 1 of the Ha-*ras* gene was amplified in the presence of ^{32}P-dCTP and the PCR products digested with MspI. The digested products were resolved on a non-denaturing 8% polyacrylamide gel, the gel was dried and exposed to x-ray film for 16 hours. WI-38 is the normal control, U represents undigested DNA and C the digested DNA. The sizes of the bands are indicated by the arrowheads on the right hand side of the figure.

out in the presence of ^{32}P-dCTP in a final volume of 50µl. 3µl of the labelled PCR product was mixed with 3µl of formamide loading buffer, denatured at 95°C for 5min and loaded immediately onto non-denaturing 6% polyacrylamide gels. The samples were electrophoresed at 70W for 10min and then at 30W for 4-6 hours.

SSCP analysis is based on the theory that single-stranded DNA will migrate through a non-denaturing polyacrylamide gel as a function of it's secondary structure. This secondary structure is governed by the sequence of the DNA, thus any change in the sequence of the DNA could theoretically lead to an altered conformation and consequently an altered migration in a non-denaturing polyacrylamide gel. This, however, is not always the case in that some mutations do not alter the secondary structure of the DNA sufficiently to cause a conformational change. In order to alleviate this problem and maximise the number of mutations detectable, the non-denaturing polyacrylamide gels in this study were electrophoresed under 4 different sets of conditions; (1) room temperature, (2) room temperature in the presence of 5% (v/v) glycerol, (3) 4°C and (4) 4°C in the presence of 5% (v/v) glycerol.

All electrophoretic mobility patterns were compared to the WI-38 DNA, which represents the normal sequence. From figure 2.3(A) it is clear that the majority of the samples have a similar migration pattern compared to the normal WI-38, with only the T47-D cell line and patient 10 showing an altered electrophoretic mobility pattern. Gels run at room temperature (figure 2.3B) and at room temperature in the presence of 5% (v/v) glycerol (figure 2.3C), however, show that, in contrast to the data observed in figure 2.3(A), only patients 10, 13, 14 and 16 and the T47-D cell line are different from the other samples, but similar to the normal WI-38 cell line (arrowheads). As the T47-D cell line and patient 10 showed an altered migration pattern in figure 2.3(A) and additional changes have been detected under the conditions used in figures 2.3(B) and 2.3(C), this suggests that these samples may have mutations in exon 1. These results also suggest that the normal WI-38 sample might also harbour a change.

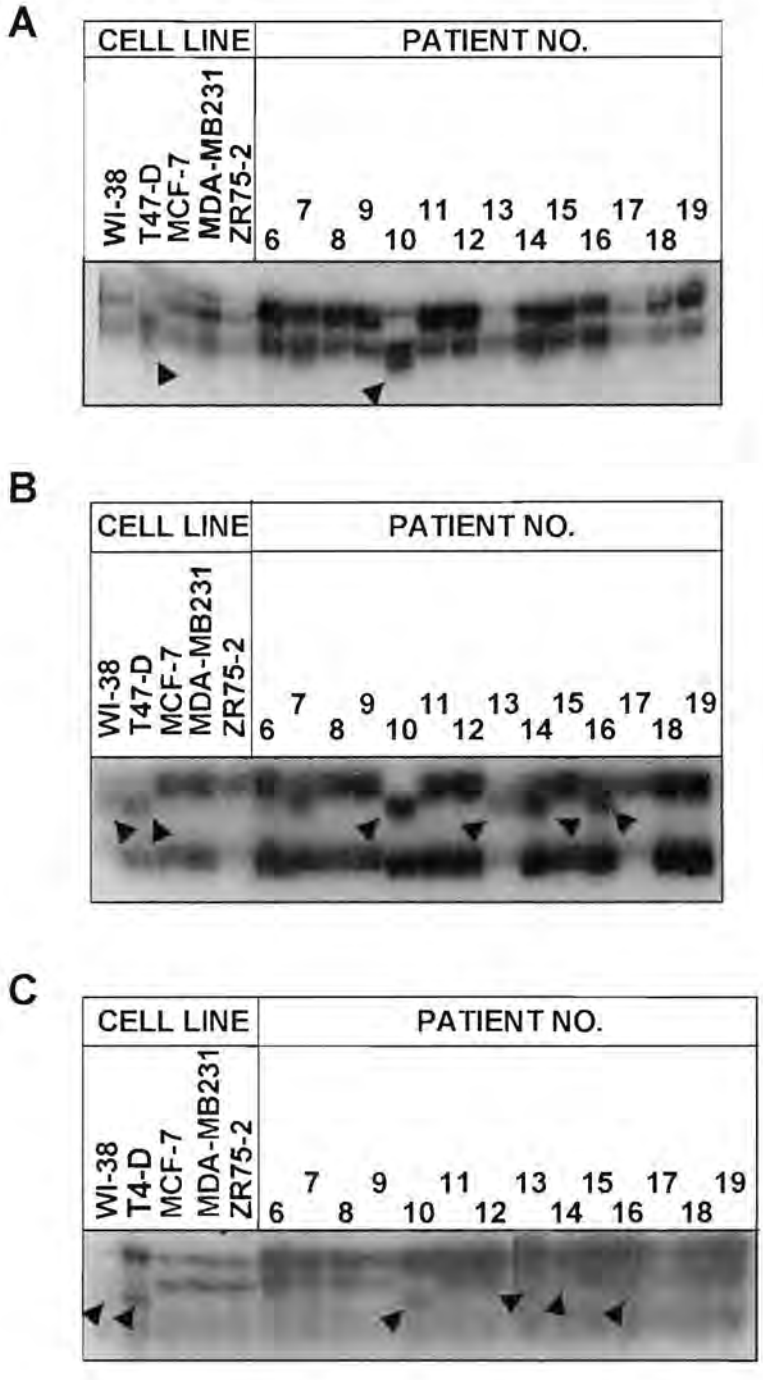


Figure 2.3: Mutation detection by SSCP. Exon 1 of the Ha-*ras* gene was PCR amplified in the presence of ^{32}P-dCTP as described in chapter 6. 3µl of the PCR product was mixed with 3µl of formamide loading buffer (section 6.14), denatured at 95°C for 5min and loaded immediately onto non-denaturing 6% polyacrylamide gels. The samples were electrophoresed at 30W for 4-6 hours at (A); 4°C, (B); room temperature and (C); room temperature in the presence of 5% glycerol. The gel were dried under a vacuum at 70°C and exposed to x-ray film for 16-48 hours. WI-38 is the normal control DNA and altered electrophoretic mobility patterns can be observed in the WI-38 and T47-D cell lines and patients #10, 13, 14 and 16 (indicated by arrowheads).

2.2.2.4 Sequence Analysis of Exon 1 of the Ha-*ras* Gene

The WI-38 and T47-D cell lines and patients 10, 13, 14 and 16, as well as a number of other samples showing an altered migration pattern in figures 2.3(B) and 2.3(C), were subjected to sequence analysis to determine the exact nature of the mutation. Sequence analysis revealed that the base change observed in these samples was a T → C transition in the wobble position of codon 27 (CAT → CAC) which did not alter the amino acid sequence (figure 2.4). Thus the assumed normal sequence in this study, namely the WI-38, has an altered sequence from that published in the Genbank database (accession #: J00277). This base substitution has been noted previously as an allelic difference in bladder cancer (Capon et al., 1983a; Taparowsky et al., 1982), and although the sequence differs from the published sequence, there is no change in the protein sequence as a result of this base substitution. These sequence data suggest that this base change is an allelic difference and signifies a polymorphism at this position in the DNA in the Ha-*ras* gene, as some samples have the T variant and others have the C variant, with both variants resulting in the same amino acid sequence.

2.2.2.5 Digestion of Ha-*ras* Exon 1 PCR Product with DraIII

The base substitution described above resulted in the creation of a DraIII restriction enzyme site (figure 2.5A), allowing rapid detection of this allele by digestion of the PCR product with DraIII. Those samples with the substitution should yield DNA fragments of 153bp and 53bp, whereas those samples without the substitution should remain uncut.

Out of a total the 40 patients screened, 16 had the T → C substitution at codon 27 giving an allele frequency of 0.4. A number of healthy control individuals (16) were also screened for this substitution and 9 of the 16 had the T → C substitution at codon 27, corresponding to an allele frequency of 0.563 (table 2.3). It appears that the C allele is more frequent in the control population than in the cancer patients, however this difference is not significant ($p=0.235$) and may be due to the small sample size. It is clear that the homozygous C allele is rare as it only occurs in 3 out of the 16 cancer patients and 1 of the 9 control samples.

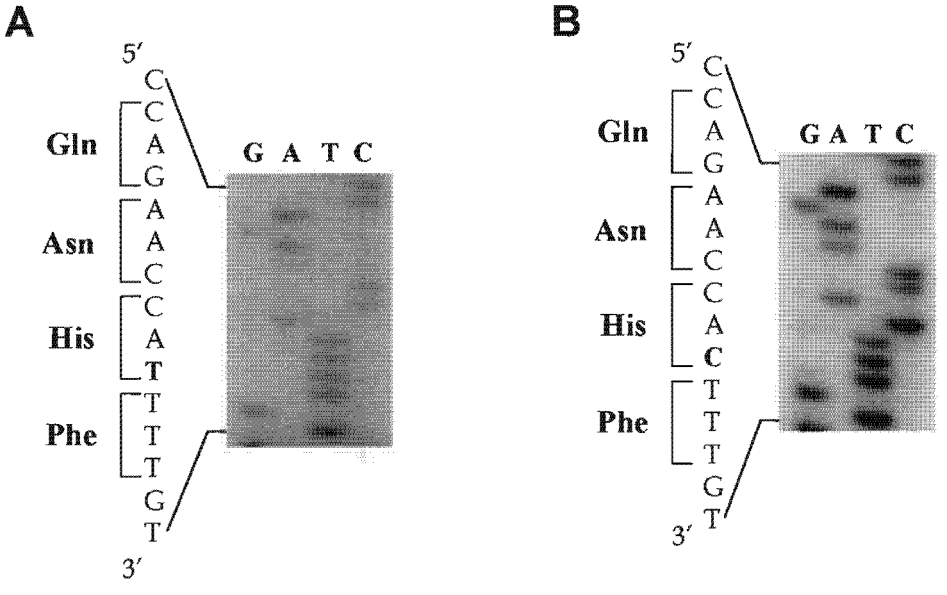


Figure 2.4: Sequence analysis of the two variants of Ha-*ras* exon 1 observed by SSCP. Sequence reactions were performed as described in section 6.6.1 and analysed on 6 % polyacrylamide gels containing 42% urea as described in section 6.6.1. The gels were dried under a vaccuum and exposed overnight to x-ray film at -70°C. The variation in sequence was found to be either a T, as shown in bold in (A) or a C, as shown in bold in (B) in the wobble position of codon 27. This base substitution was conservative and did not alter the amino acid sequence.

Table2.3: Distribution of the Ha-*ras* polymorphism at codon 27 amongst patients and controls showing the number of individuals with each allele.

SAMPLES	HA-*ras* ALLELE		
	T/T	T/C	C/C
Controls (n=16)	7	8	1
Patients (n=40)	24	13	3

2.2.3 Mutation Analysis of Exon 2 of the Ha-*ras* Gene

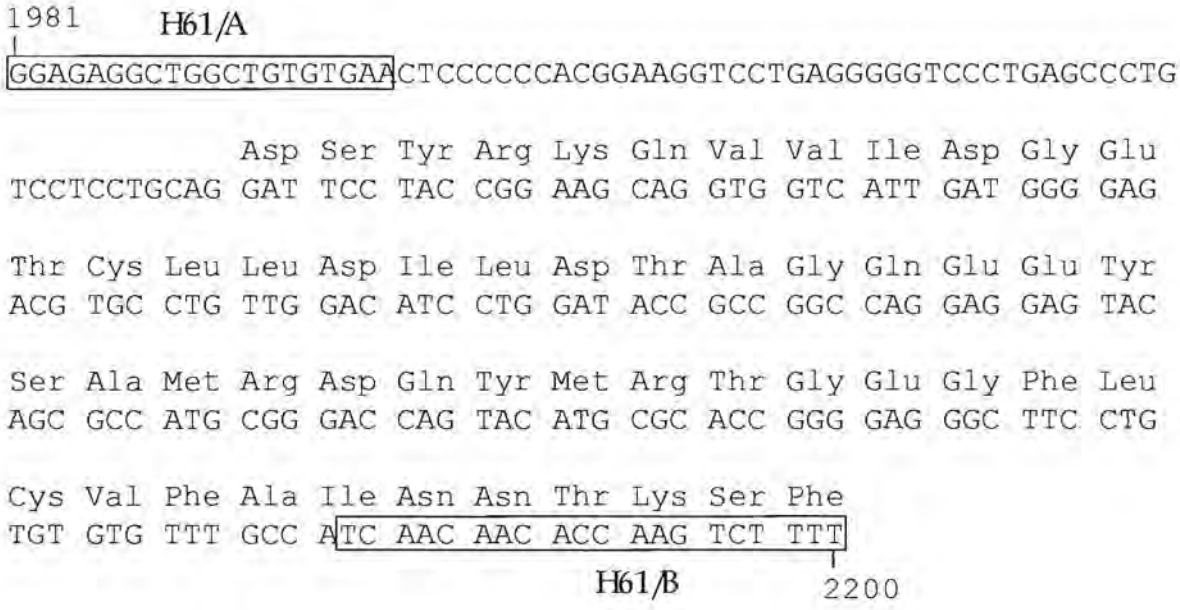
2.2.3.1 Amplification of Exon 2

Mutations in codon 61 of the Ha-*ras* gene have also been shown to constitutively activate the GTPase activity, thus exon 2 of the Ha-*ras* gene (encompassing codon 61) was PCR amplified using primers H61/A and H61/B (table 2.2) as described in section 6.3 (figure 2.6A). The PCR reaction was carried out as outlined in section 6.3.1, using 100ng of DNA as a template. In order to check for efficient amplification, 5µl of the amplified product was electrophoresed on a non-denaturing 8% polyacrylamide gel and the bands visualised under UV light after staining the gel with ethidium bromide (0.25µg/ml). Figure 2.6(B) shows an example of a polyacrylamide gel with the amplified product of 230bp indicated by the arrowhead.

2.2.3.2 Screening of Exon 2 for Mutations using SSCP Analysis

DNA from patient samples and breast cancer cell lines was amplified using primers H61/A and H61/B as described in section 2.2.3.1 in the presence of ^{32}P-dCTP. 3µl of the amplified product was mixed with 3µl of formamide loading buffer, heat-denatured at 95°C for 5min and loaded immediately onto non-denaturing 6% polyacrylamide gels. The gels were electrophoresed under the conditions indicated in figure 2.7 (A-D). Samples were initially electrophoresed into the gels at 70W for 10min, and subsequently at 30W for 4-6 hours. The gels electrophoresed at room temperature were prevented from overheating by cooling them with a fan. Under all the conditions used, no alterations in electrophoretic mobility patterns were detected between any of the breast cancer cell lines or the breast tumour samples and the normal control, indicating the absence of any mutations in exon 2 of the Ha-*ras* gene in these breast cancer patients.

A



B

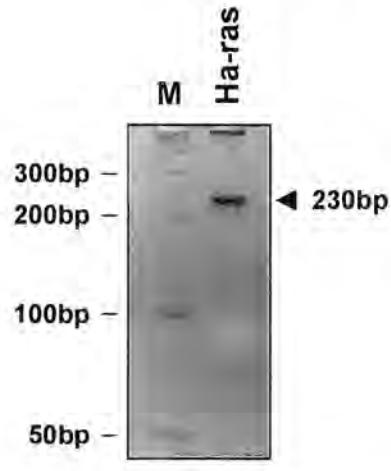


Figure 2.6: PCR amplification of exon 2 of the Ha-*ras* gene. (A); sequence of the 230bp product after PCR amplification with primers H61/A and H61/B which are boxed. The amino acid sequence is also shown above the DNA sequence. (B); after amplification, 5µl of the product was electrophoresed at 150V on a non-denaturing 8% polyacrylamide gel. Bands were visualised under UV light after staining with ethidium bromide (0.25µg/ml). Molecular weight markers are shown on the left hand side of the figure and the expected band of 230bp is indicated by the arrowhead on the right hand side of the figure.

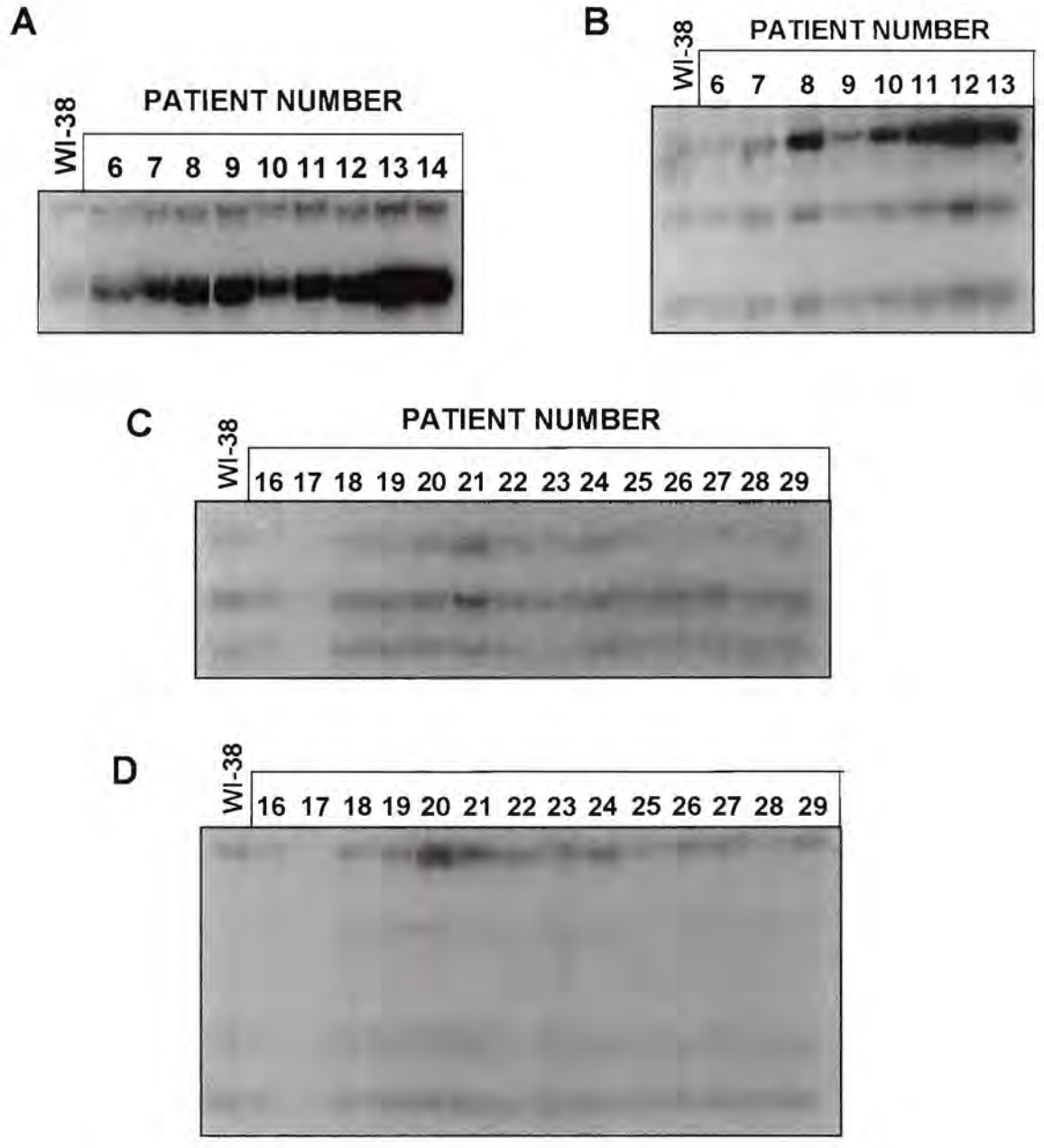


Figure 2.7: SSCP analysis of exon 2 of the Ha-*ras* gene. Exon 2 of the Ha-*ras* gene was amplified using primers H61/A and H61/B in the presence of ^{32}P-dCTP. 3µl of the PCR product was mixed with 3µl of formamide loading buffer, heat-denatured at 95°C for 5min, and loaded immediately onto non-denaturing 6% polyacrylamide gels. The gels were electrophoresed initially at a constant power of 70W for 10min, and then at a constant power of 30W for 4-6 hours under the following conditions: (A); room temperature, (B) room temperature containing 5% (v/v) glycerol, (C), 4°C and (D) 4°C in the presence of 5% (v/v) glycerol. A fan was placed in front of the gels at room temperature in order to prevent excessive heating. The gel was dried at 70°C and exposed to x-ray film for 16 hours. WI-38 represents the normal control and no differences in mobility patterns was observed between the control and any of the patients.

2.3 DISCUSSION

In this chapter, exons 1 and 2 containing the mutation hotspots in codons 12, 13 and 61 in the Ha-*ras* gene were analysed for mutations. These codons have been implicated in the constitutive activation of the Ha-*ras* protein in several tumours (Taparowsky et al., 1982; Bos, 1989; Barbacid, 1987; Tabin et al., 1982; Reddy et al., 1982).

Previous studies have shown that mutations in the Ha-*ras* gene occur very rarely in human breast cancer (Spandidos, 1987; Rochlitz et al., 1989; Barbacid, 1990). Various techniques were used in these studies to detect *ras* mutations, including oligonucleotide hybridisation, RNase mismatch cleavage analysis and restriction length polymorphism analysis. More recently SSCP has been reported as an alternative technique for detecting mutations in the *ras* genes in human tumours (Suzuki et al., 1990; Bauer-Hofmann et al., 1992). In this study, SSCP and RFLP analysis were used to detect mutations in the Ha-*ras* gene.

The C $\rightarrow$ T mutation in codon 12 that results in loss of an MspI site of the Ha-*ras* gene was shown not to be present in the patient DNA analysed in this study. In addition, on screening the entire exon for other possible mutations by SSCP analysis, no such mutations were detected. These results support the findings by Rochlitz et al (1989) and Spandidos (1987) that mutations in codon 12 of the Ha-*ras* gene are a rare event in human breast tumours. Screening of exon 2 of the Ha-*ras* gene by SSCP analysis also revealed no mutations in any of the breast tumour samples used in this study.

Other solid tumours, such as colon cancer have a high frequency of *ras* mutations (Bos et al., 1987; Vogelstein et al., 1988; Capon et al., 1983b; Laurent-Puig et al., 1991; Moerkerk et al., 1994) thus the reasons for the absence of mutations in the Ha-*ras* gene in human breast cancer are unclear. Since the *ras* genes are normally expressed in most tissues (Leon et al., 1987; Furth et al., 1987; Chesa et al., 1987) cellular factors may be involved in the normal activation of the gene during cell growth. Exposure to chemical carcinogens could also influence the activation of the Ha-*ras* gene and can lead to constitutive activation of the gene. Studies have shown

that exposure to N-nitroso-N-methylurea (NMU) and dimethylbenz(a)anthracene (DMBA) leads to the induction of mammary carcinomas in rats, and moreover, that the tumours induced by NMU all contained a G → A transition in the second nucleotide of codon 12 (Sukumar et al., 1983; Zarbl et al., 1985). The tumours induced by DMBA, on the other hand, did not contain mutations in codon 12, but had mutations in the two adenine residues of codon 61 instead. Furthermore, exposure to DMBA resulted in fewer tumours harbouring a *ras* mutation compared to the number of NMU-induced tumours, implying that DMBA-induced tumours had fewer ras mutations than NMU-induced tumours. These findings imply that exposure to chemical carcinogens can directly lead to the constitutive activation of the Ha-ras gene and subsequently the formation of a tumour. Given that the *ras* genes are expressed in all tissues and that other cellular factors may be involved in the normal activation of these genes, a possible explanation as to why mutations in the Ha-*ras* gene are infrequent in human breast cancer may be that humans are not exposed to the appropriate carcinogens in order to induce the mutation, or that the doses are not high enough, or that the carcinogens are not efficiently absorbed into the mammary cells. Another possibility is that the DNA repair mechanisms in these cells are efficient and that mutations do not accumulate.

A polymorphism, however, was detected in codon 27. Sequence analysis revealed the base change to be a T → C transition in the third position of the codon. This change was silent and did not cause any alteration in the amino acid sequence of the protein. Although the C allele of the polymorphism appeared to occur more frequently in the control group than in the patients, the difference in allele frequency was not significant and could be explained by the relatively small number of control individuals. More samples would have to be analysed in order to confirm these initial findings. Polymorphisms in the Ha-*ras* gene have been previously reported, although these have tended to be localised in the variable tandem repeat (VTR) in the 3' flanking region of the gene (Capon et al., 1983a). The literature is somewhat contradictory regarding whether a given Ha-*ras* allele is associated with the risk of developing breast cancer or not. A number of studies have reported no significant difference in the distribution of either common or rare alleles between control groups and cancer patients (Barkardottir et al., 1989; Sheng et al., 1988; White et al., 1988).

Furthermore, no association was found between rare Ha-*ras* alleles and breast cancer. Other groups on the other hand, have reported a significant association between certain Ha-*ras* alleles and breast cancer (Saglio et al., 1988; Lidereau et al., 1986) implying that patients harbouring specific Ha-*ras* alleles have an increased risk of developing breast cancer. This discrepancy may be due to ethnic or racial differences of the patients in the various studies. The data presented in this study suggest that there is no significant association between the presence of the codon 27 polymorphism in breast cancer and that this polymorphism has no affect on the relative risk of developing the disease. It is possible, however, bearing in mind that the majority of patients in this study were of a single population group, that the frequency of this allele may vary among different racial groups in South Africa.

In summary, this chapter has shown that mutations in the Ha-*ras* gene are rare in breast cancer patients, confirming previous findings (Spandidos, 1987; Rochlitz et al., 1989; Barbacid, 1990). It should be cautioned, however, that mutations might occur in exons 3 and 4 of the gene which were not analysed in this study. Although no mutations were identified, a polymorphism was detected in codon 27 and this proved to have no association with breast cancer.

CHAPTER 3: ANALYSIS OF MUTATIONS IN THE p53 GENE IN BREAST CANCER

3.1 INTRODUCTION

The p53 tumour suppressor gene codes for a nuclear phosphoprotein that negatively regulates cell proliferation. Wild-type p53 is a sequence specific DNA binding protein and acts as a transcription factor that is able to activate or repress genes containing a p53 consensus DNA binding sequence (Kern et al., 1991; el-Deiry et al., 1992; Funk et al., 1992; Margulies and Sehgal, 1993; Wang et al., 1995). p53 is the most frequently mutated gene in human tumours and most of the mutations result in loss of wild-type p53 function and consequently, loss of cell cycle control. Mutant p53 has a longer half-life than wild-type p53 and it can still form complexes with a host of cellular regulatory proteins (such as hsc70) which is thought to play a role in cell transformation (Finlay et al., 1988). In addition, some mutations abolish the wild-type function by disrupting the ability of p53 to bind to DNA and consequently abolish it's transcriptional activity (Kern et al., 1991; Raycroft et al., 1991). It has also been reported that different mutants exhibit distinct biological activities, such as gain of function, where the mutant protein binds promiscuously to DNA sequences that the wild-type protein does not normally bind to, resulting in constitutive activation of genes that may play a role in cellular transformation (Halevy et al., 1990; Ludes-Meyers et al., 1996).

Aberrations in the p53 gene have been reported in 50-55% of all human tumours (Hollstein et al., 1994; Hollstein et al., 1991; Hainaut et al., 1997) whilst in breast cancer, alterations in this gene have been detected in 45-50% of cases, depending on the technique used to detect mutations (Osborne et al., 1991; Cattoretti et al., 1988; Bartek et al., 1990a). These abberations include single base substitutions and small insertions and deletions in 25-30% of breast cancer cases, as well as loss of heterozygosity (LOH) in approximately 50% of primary breast tumours (Mackay et al., 1988; Devilee et al., 1989; Thompson et al., 1990; Lou et al., 1997).

Mutations in the p53 gene in breast tumours seem to be restricted to the common histological variants of the disease such as invasive ductal and medullary carcinomas, whereas the frequency of mutations in the rarer histological variants such as mucinous and papillary carcinomas is much lower (Marchetti et al., 1993). A probable explanation for this anomaly is that the latter variants are associated with a higher differentiation status, and thus a more favourable prognosis. It has also been suggested that the high frequency of alterations at the p53 locus in human breast cancer could allow mutations in the p53 gene to serve as a molecular marker for breast cancer (Runnebaum et al., 1991).

Mutations in the p53 gene occur over a wide region of the gene, although there are certain hotspots covering exons 5, 6, 7 and 8 (Hollstein et al., 1991). These hotspot regions encompass 4 of the 5 evolutionary conserved domains of the p53 gene (Soussi et al., 1990) and, moreover, the majority of mutations occur at codons which are conserved between species (Wang et al., 1995). Analysis of p53 mutations in breast cancer has clearly shown that they are clustered in exons 5-8, with codons 175, 248 and 273 being more frequently mutated than other codons (figure 3.1). A possible explanation for the higher frequency of codon 248 and codon 273 mutations is that they are critical for p53 function. Arg^{273} is involved in contact with the phosphate backbone of the DNA in the major groove, while Arg^{248} is involved in multiple hydrogen bonds in the minor groove (Cho et al., 1994). Any mutations at these sites would therefore have a detrimental effect on the binding of p53 to DNA.

This chapter describes the screening of the breast cancer patients and breast cancer cell lines (see chapter 2) for mutations in the p53 gene in order to determine the frequency of p53 mutations in this population of breast cancer patients, as well as to determine the nature of these mutations. PCR-SSCP and DNA sequence analysis was used to detect mutations. In addition, immunohistochemistry was used to study the levels of the p53 protein in these patients as it has previously been shown that mutant p53 protein has a longer half-life than the wild-type protein and therefore may accumulate in either the nucleus or the cytoplasm.

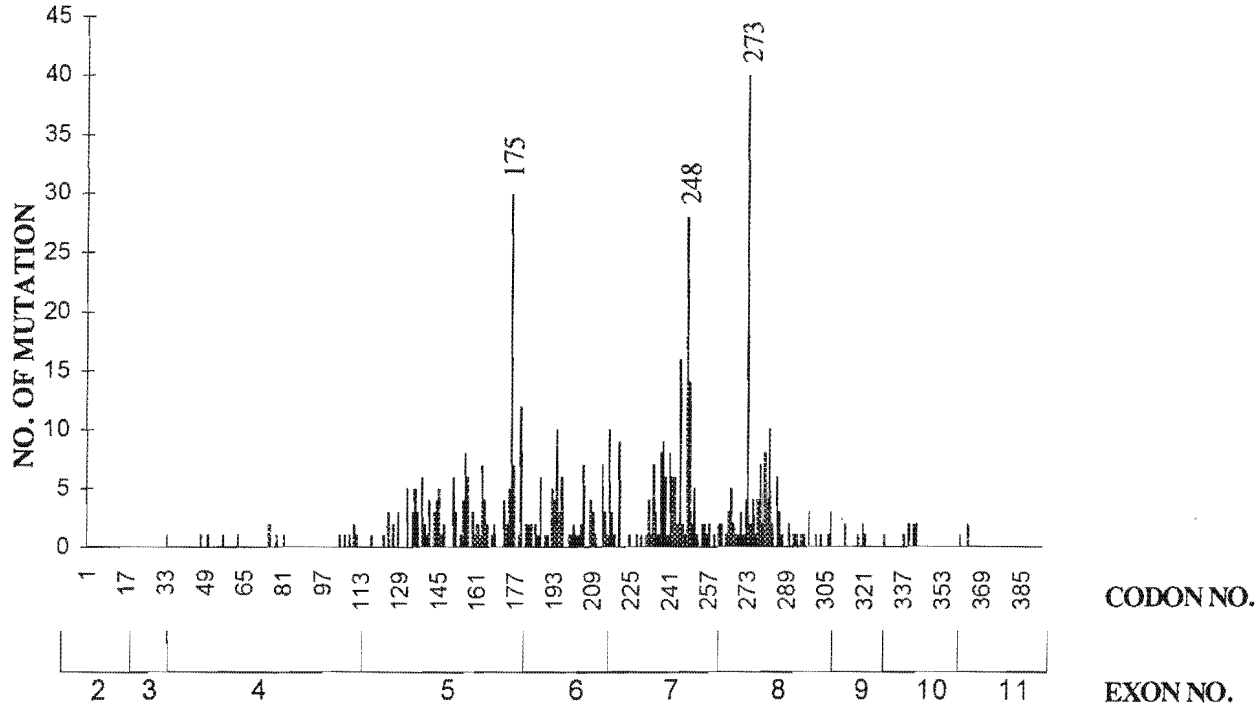


Figure 3.1: Schematic representation of the p53 mutation spectrum in breast cancer. Mutations in the p53 gene are clustered in the mutation hotspots in exons 5-8. In addition, codons 175, 248 and 273 are mutated at a much higher frequency than any other codon. (Data was collected from the p53 mutation database of Hollstein et al., 1994 and Hainaut et al., 1997).

3.2 RESULTS

DNA was isolated from the cell lines and the 40 primary breast tumour samples as described in section 6.1. The 40 primary breast tumour samples consisted of both fresh tissue samples as well as paraffin embedded tissue sections as outlined in section 2.2.1. Exons 5-9 and 11 of the p53 gene were individually amplified and screened for mutations by PCR-SSCP and sequence analysis.

3.2.1 Analysis of Exon 5 of the p53 Gene

3.2.1.1 Amplification of Exon 5

Exon 5 of the p53 gene was PCR amplified using primers p53-5A and p53-5B (section 6.3.1) as indicated in figure 3.2(A). The primers were situated within introns 4 and 6, thus ensuring amplification of the entire exon, including the splice junctions. The annealing temperature used in the PCR reaction is shown in table 6.1. In order to check for efficient amplification of exon 5, 5µl of the PCR product was electrophoresed on a 1% agarose gel and the bands were visualised under UV light after staining with ethidium bromide to confirm the presence of the expected 310bp DNA fragment of (figure 3.2B).

3.2.1.2 SSCP Analysis of Exon 5

Exon 5 was screened for mutations by SSCP analysis as described in section 6.3.2. The PCR reactions were carried out in the presence of ^{32}P-dCTP and electrophoresed through non-denaturing 6% polyacrylamide gels as previously described. Figure 3.3 shows SSCP gels using different conditions, these being 4°C in the presence of 5% (v/v) glycerol, 4°C in the absence of glycerol, room temperature in the absence of glycerol and room temperature in the presence of 5% (v/v) glycerol. As can be seen, no alterations in electrophoretic mobility patterns were detected in any of the patient or cell line samples compared to the normal WI-38 cell line. Although patients 15 and 16 appear to have an altered electrophoretic mobility pattern compared to the normal WI-38 cell line, this change was not reproducible and was considered to be artifactual.

A

13005

p53-5A

```
                                                          Tyr Ser
TGTTCACTTGTGCCCTGACTTTCAACTCTGTCTCCTTCCTCTTCCTACAG TAC TCC

Pro Ala Leu Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro
CCT GCC CTC AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC CCT

Val Gln Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val
GTG CAG CTG TGG GTT GAT TCC ACA CCC CCG CCC GGC ACC CGC GTC

Arg Ala Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val
CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT

Val Arg Arg Cys Pro His His Glu Arg Cys Ser Asp Ser Asp
GTG AGG CGC TGC CCC CAC CAT GAG CGC TGC TCA GAT AGC GAT G G
TGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGGGTCCCCAGGCCTCTGA
TTCCTCACTGATTGCT
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p53-5B

13314

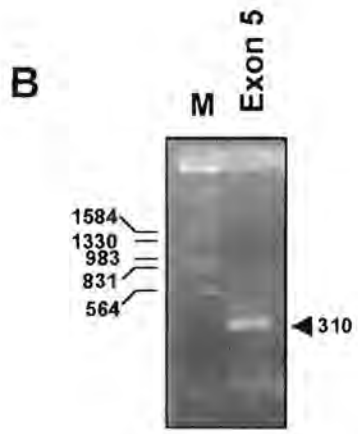


Figure 3.2: PCR-Amplification of exon 5 of the p53 gene. (A); primers p53-5A and p53-5B used in the PCR reaction to amplify the 310bp fragment of the p53 gene are boxed. (B); 100ng of DNA was used as template and the PCR reaction was carried out in a final volume of 50µl as described in section 6.3.1. To check for efficient amplification, 5µl of the PCR product was electrophoresed on a 1% agarose gel and the bands visualised under UV light after staining with ethidium bromide (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure and the expected 310bp fragment is indicated by the arrowhead on the right hand side. All sizes are in base pairs.

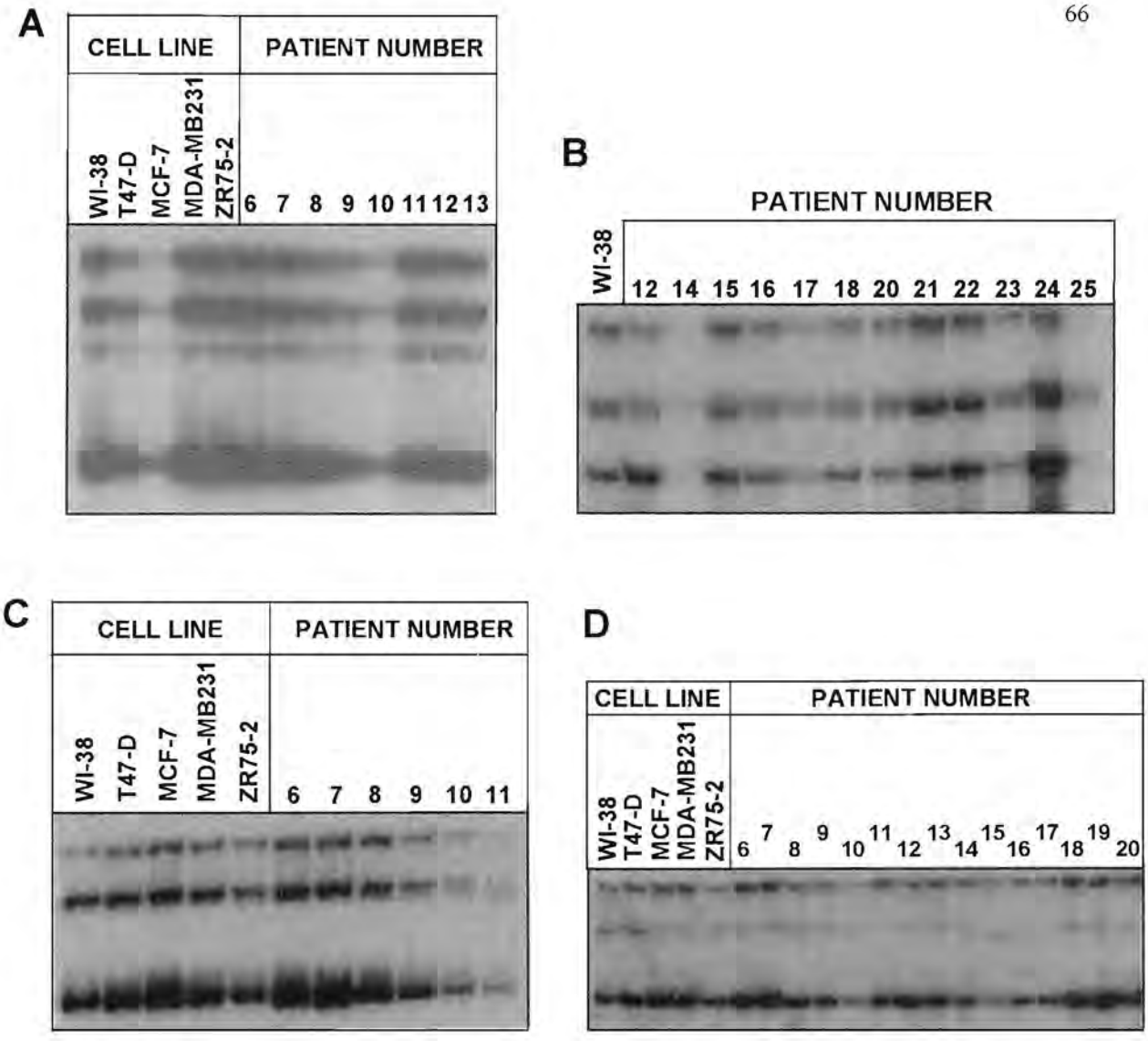


Figure 3.3: SSCP analysis of exon 5 of the p53 gene. Exon 5 was amplified using primers p53-5A and p53-5B in the presence of ^{32}P-dCTP as described in section 6.3.1. 3µl of the PCR product was mixed with 3µl of formamide loading buffer, heat-denatured at 95°C for 5 min, and then loaded onto non-denaturing 6% polyacrylamide gels. The samples were initially electrophoresed through the gel at 70W for 10 min, and then for 4-6 hours at 30W, in 1 X TBE under the following conditions: (A); 4°C in the presence of 5% (v/v) glycerol, (B); 4°C in the absence of glycerol, (C); room temperature in the absence of glycerol and (D); room temperature in the presence of 5% (v/v) glycerol. The room temperature gels were prevented from excessive heating by cooling with a fan. The gels were dried and exposed to x-ray film for 16 - 48 hours at -70°C. All electrophoretic mobility patterns should be compared to that of the WI-38 cell line, the normal control. There were no differences between any of the patient samples and the WI-38 cell line, indicating the absence of any mutations.

3.2.2 Analysis of Exon 6

3.2.2.1 PCR Amplification of Exon 6

Exon 6 of the p53 gene was amplified using primers p53-6A and p53-6B under the conditions outlined in section 6.3.1. The sequence of the amplified product, indicating the position of the primers, as well as the amino acid sequence, is shown in figure 3.4(A). As for exon 5, the primers are situated in the introns flanking exon 6, thus incorporating the splice junctions in the PCR product. 5µl of the amplified product was electrophoresed on a 1% agarose gel to check for correct amplification of the expected fragment of 201bp (figure 3.4B).

3.2.2.2 SSCP Analysis of Exon 6

SSCP analysis of exon 6 was carried out as described previously by including a trace amount of ^{32}P-dCTP in the PCR reaction and subsequent electrophoresis of the resulting PCR products through non-denaturing 6% polyacrylamide gels using different electrophoretic conditions (figures 3.5A-D). As indicated in the figures, no mutations were detected in any of the breast cancer patient samples, however an altered electrophoretic mobility pattern was found for the T47-D breast cancer cell line (arrowhead in figure 3.5B, C and D). This mutation only separated out under 3 of the conditions, but not when the gel was run at room temperature in the absence of glycerol (figure 3.5A).

In order to identify the exact nature of the mutation, the exon was re-amplified and the resulting PCR product was subjected to direct sequence analysis as described in section 6.6.1. The sequence data revealed the mutation to be a C → T transition at codon 194 resulting in a Leu → Phe amino acid substitution.

3.2.3 Analysis of Exon 7 of the p53 Gene

3.2.3.1 PCR Amplification of Exon 7

Exon 7 was PCR amplified under the conditions described in section 6.3.1 and table 6.1, using primers p53-7A and p53-7B. The sequence of the amplified product, as well as the position of the primers, are indicated in figure 3.6(A). Once again the primers flank the

A

13271
p53-6A

```
                                                          Leu Ala
TGGTTGCCCAGGGTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAG GT CTG GCC

Pro Pro Gln His Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu
CCT CCT CAG CAT CTT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG

Tyr Leu Asp Asp Arg Asn Thr Phe Arg His Ser Val Val Val Pro
TAT TTG GAT GAC AGA AAC ACT TTT CGA CAT AGT GTG GTG GTG CCC

Tyr Glu Pro Pro Glu
TAT GAG CCG CCT GAG GTCTGGTTTGCAACTGGGGTCTCTGGGAGGAGGGGTTAA
```

p53-6B
13471

B

Figure 3.4: PCR-amplification of exon 6 of the p53 gene. (A); exon 6 was amplified in a PCR reaction using primers p53-6A and p53-6B as indicated in the boxed areas. To check for efficient amplification, 5µl of the PCR product was electrophoresed on a 1% agarose gel (B) and the bands were visualised under a UV light after staining the gel with ethidium bromide (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure while the 201bp PCR product is indicated by the arrowhead on the right hand side. All sizes are in base pairs.

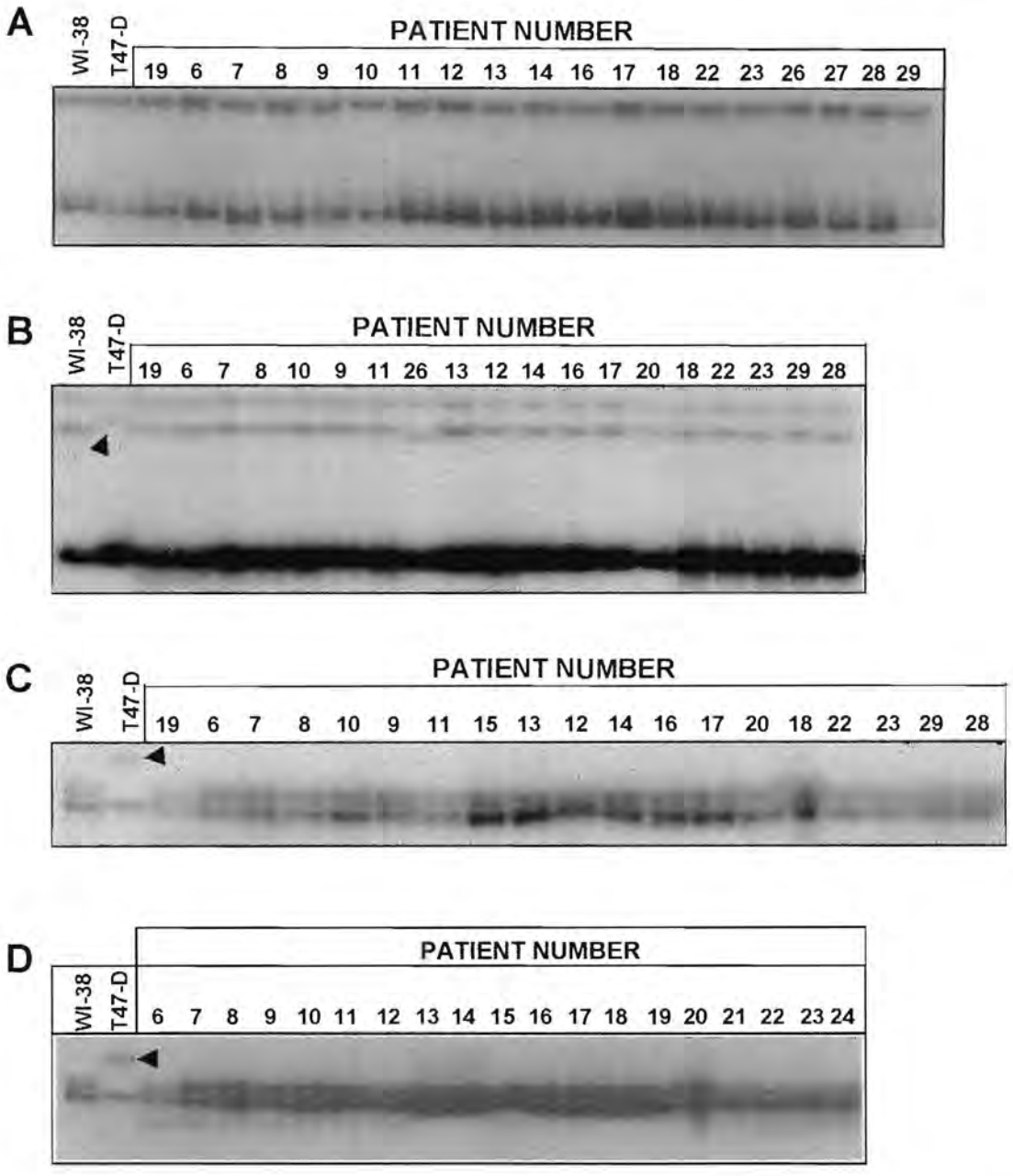
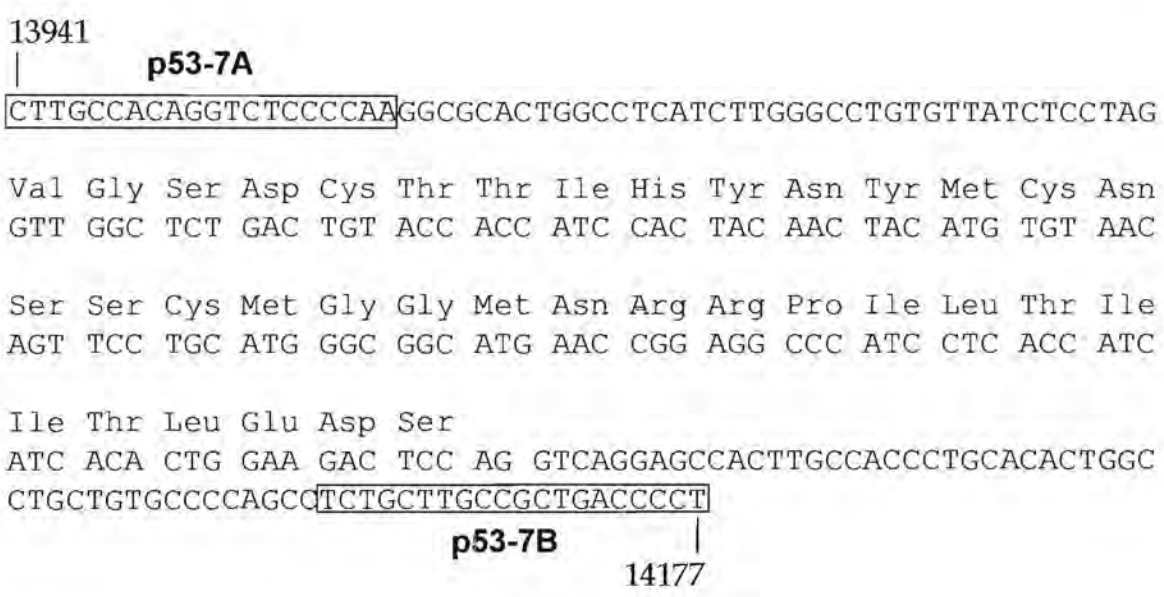


Figure 3.5: SSCP analysis of exon 6 of the p53 gene. Exon 6 was amplified in the presence of ^{32}P-dCTP using primers p53-6A and p53-6B as described in section 6.3.1. 3µl of the PCR product was mixed with 3µl of formamide loading buffer, heat-denatured at 95°C for 5 min and then loaded onto a non-denaturing 6% polyacrylamide gel. The samples were electrophoresed at 70W for 10 min and then at 30W for 4-6 hours under the following conditions: (A); room temperature, (B); room temperature in the presence of 5% (v/v) glycerol, (C); 4°C and (D); 4°C in the presence of 5% (v/v) glycerol. The room temperature gels were cooled with a fan in order to prevent excessive heating. The gels were then dried under a vacuum at 70°C for 1 hour and exposed to x-ray film for 16 - 48 hours. Under these conditions, no alterations were observed in mobility patterns in the patient DNA compared to the normal DNA.

A



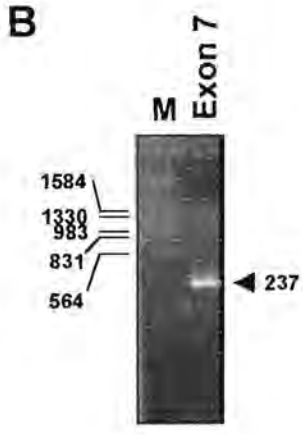


Figure 3.6: PCR-amplification of exon 7 of the p53 gene. Exon 7 was PCR amplified using primers p53-7A and p53-7B as shown in (A) and described in section 6.3.1. 5µl of the PCR product was electrophoresed on a 1% agarose gel (B) and the bands visualised under UV light after staining the gel with ethidium bromide (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure and the expected 237bp fragment is indicated by the arrowhead on the right hand side. All molecular sizes are in base pairs.

exon, thus incorporating the splice junctions in the PCR product. Analysis of the PCR product on a 1% agarose gel showed the correct 237bp product, indicated by the arrowhead on the right side of figure 3.6(B).

3.2.3.2 SSCP Analysis of Exon 7

Figure 3.7 represents SSCP gels using the different electrophoretic conditions for exon 7. Results from these SSCP experiments show that patients #6 and #27 both had altered electrophoretic mobility patterns (indicated by the arrowheads in the figures), indicating the presence of mutations in these 2 samples. The mutation in patient #6 separated out from the normal sample under all the conditions tested, however, the mutation in patient #27 could only be distinguished from the normal when the samples were electrophoresed at room temperature in the presence of glycerol (figure 3.7B) and at 4°C in the absence of glycerol (figure 3.7C). No other patients showed altered electrophoretic mobilities for exon 7.

3.2.3.3 Sequence Analysis of the Exon 7 Mutations

Since SSCP does not reveal any information about the exact nature of the mutation, sequence analysis on the mutant PCR products was done in order to determine the site and nature of the mutation. Exon 7 from patients #6 and #27 was re-amplified and the PCR products were cloned as described in section 6.4. Briefly, the PCR products were phosphorylated using T_4 polynucleotide kinase (Amersham), blunt-ended using the Klenow fragment of DNA polymerase I (Amersham) and ligated into pUC-19 previously digested with SmaI and dephosphorylated with calf intestinal phosphatase (Roche Biochemicals). The ligation reactions were transformed into competent E.Coli DK-1 cells and a number of colonies were picked and screened for inserts by either restriction endonuclease digestion or PCR, as outlined in section 6.4.6. A number of recombinant clones were subjected to DNA sequence analysis using the Pharmacia Biotech ^{T7}Sequencing^{TM} Kit as described in section 6.6.2.

Sequence analysis of the mutation from patient #6 revealed the deletion of a single adenine residue at position 239 (figure 3.8), resulting in a frameshift and the introduction

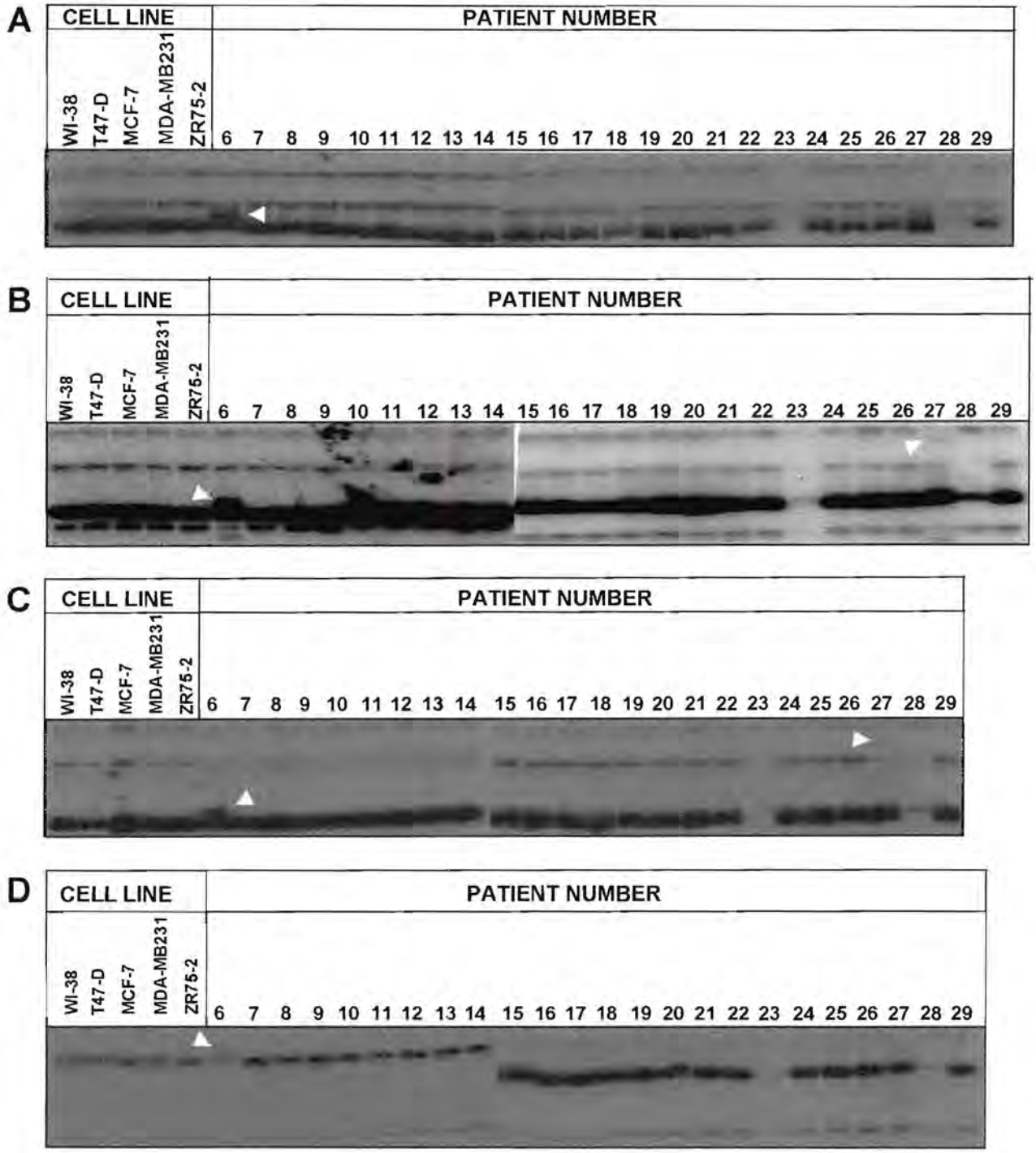


Figure 3.7: SSCP analysis of exon 7 of the p53 gene. Exon 7 was amplified in the presence of ^{32}P-dCTP using primers p53-7A and p53-7B as described in section 6.3.1. 3µl of the PCR product was mixed with 3µl of formamide loading buffer, heat-denatured at 95°C for 5 min and then loaded onto non-denaturing 6% polyacrylamide gels. The samples were electrophoresed through the gel at 70W for 10 min and then at 30W for 4-6 hours, in 1 X TBE, at room temperature (A); at room temperature in the presence of 5% (v/v) glycerol (B); at 4°C (C) and at 4°C in the presence of 5% (v/v) glycerol. The gels were dried under a vacuum at 70°C for 1 hour and exposed to x-ray film for 16-48 hours. Under these conditions, patients #6 and #27 have altered electrophoretic mobility patterns (arrowheads). No other patients or cell lines showed alterations under these conditions.

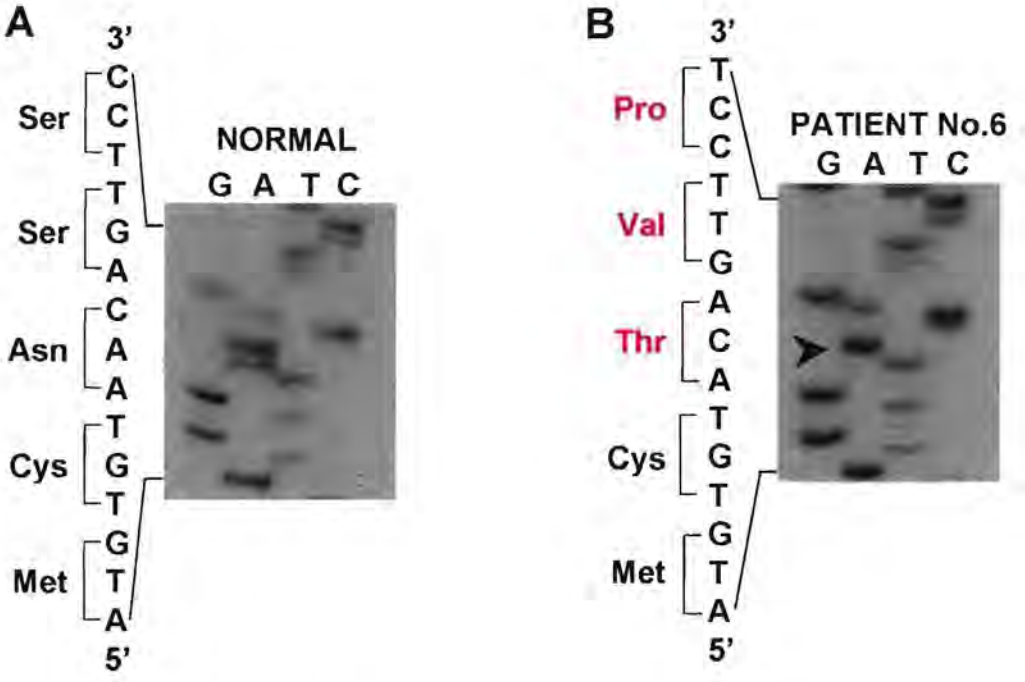


Figure 3.8: Sequence analysis of the exon 7 mutations. The exon 7 PCR product for patient #6 was cloned and sequenced as described in sections 6.4 and 6.6.2. The sequence reactions were heated at 75°C for 2min before loading onto a denaturing 6% polyacrylamide gel which had been pre-electrophoresed until the temperature of the gel had reached 50°C. The samples were electrophoresed for 3 hours after which the gel was dried and exposed to x-ray film for 16 hours. (A); the normal sequence corresponding to the site of the mutation in patient #6, (B); the deletion of an adenine residue at codon 239 in patient #6, resulting in a frameshift.

of a termination codon further downstream at position 263.

The mutation in exon 7 from patient #27 was a single base substitution of a G → A at codon 248 (figure 3.9) resulting in the substitution of a glutamine for an arginine in the protein sequence.

3.2.3.4 Restriction Endonuclease Analysis of the Exon 7 Mutations

The deletion of the adenine residue from patient #6 created a new RsaI restriction endonuclease site in exon7 (figure 3.10A). In order to confirm this mutation and as a rapid screening procedure, exon 7 was re-amplified and digested with RsaI. The resulting DNA fragments were resolved on a non-denaturing 8% polyacrylamide gel. Since there is only one RsaI site in the wild-type, 2 fragments were expected, while the introduction of the new RsaI restriction site would result in 3 fragments. The additional expected 135bp fragment created by the new restriction site can be clearly seen in the mutant lane (figure 3.10B). The small DNA fragment of 27bp between the two sites is not visible on the gel as runs off in the dye front. It is also evident from the presence of the 163bp, 135bp and 74bp bands, that there is a mixture of both normal and mutant sequences in the PCR product. There are two possible explanations for this; (1) the patient is heterozygous for the mutation or (2) the tissue sample from which the DNA was isolated was heterogeneous and contained both normal and tumour cells.

The exon 7 mutation from patient #27, on the other hand, abolished an existing MspI restriction endonuclease site as shown in figure 3.11(A). Thus to further confirm the presence of this mutation, exon 7 from patient #27 and the normal WI-38 cell line were re-amplified and equal amounts of PCR product digested with MspI. The resulting DNA fragments were resolved on a non-denaturing 8% polyacrylamide gel and as can be seen from figure 3.11(B), the expected DNA fragments of 130bp and 107bp are obtained for the wild-type sequence. The mutant also shows the digested DNA fragments observed in the wild-type, but with a large amount of undigested product. This implies that the MspI site was destroyed and suggests that the patient was either heterozygous for the mutation, or the tumour tissue from which the DNA was isolated contained some normal tissue.

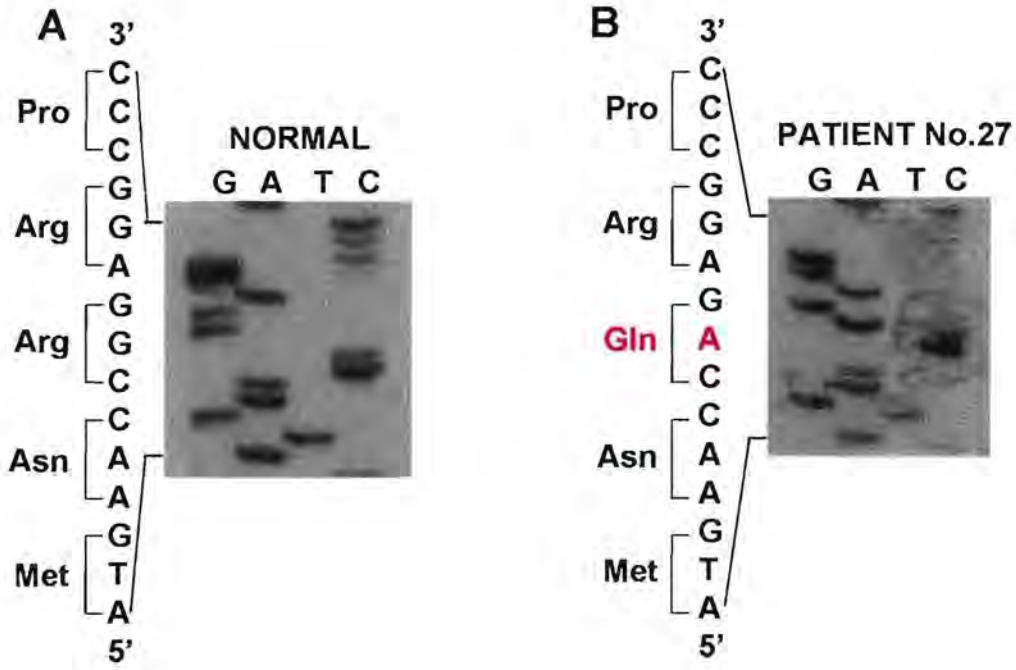
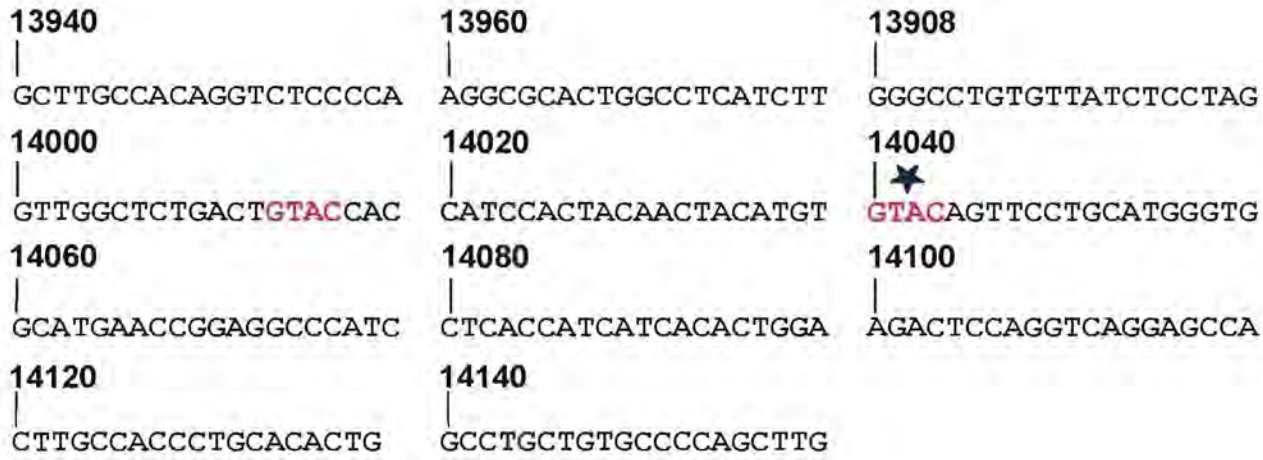


Figure 3.9: Sequence analysis of the exon 7 mutation in patient #27. The exon 7 PCR product for patient #27 was cloned and sequenced as described in sections 6.4 and 6.6.2. (A); the normal sequence corresponding to the mutation site in patient #27, (B); the mutation corresponding to a G → A base substitution at codon 248 (shown in red) resulting in the substitution of a Gln for an Arg.

A



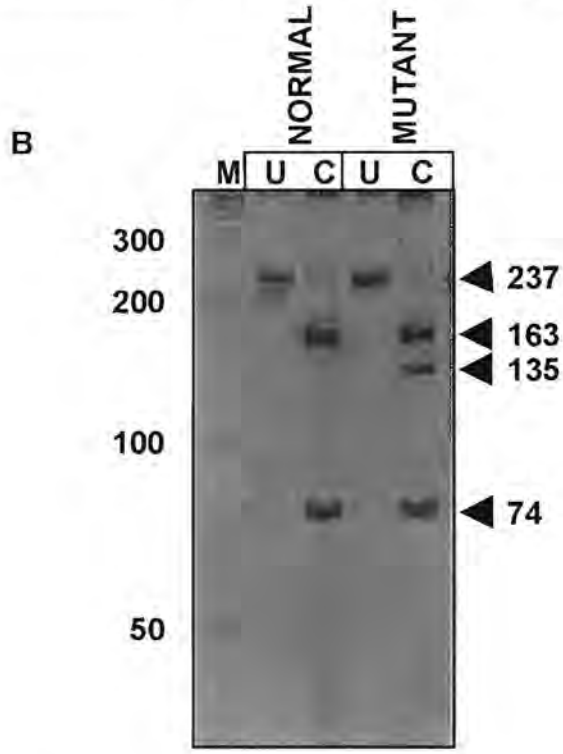


Figure 3.10: Digestion of the exon 7 PCR product from patient #6 with RsaI. (A); the mutation in patient #6 created an RsaI restriction site (asterisk) while the existing upstream RsaI site is indicated in red. (B); exon 7 from patient #6 was re-amplified and the resulting PCR product was incubated with RsaI for 16 hours at 37°C. The resulting DNA fragments were resolved on an 8% polyacrylamide gel and visualised under UV light after staining the gel with ethidium bromide (0.25μg/ml). The molecular weight markers (M) are shown on the left hand side of the figure and the fragment sizes are indicated by arrowheads on the right hand side. All molecular sizes are in base pairs. U represents uncut PCR product and C represents PCR product digested with RsaI.

A

13940
GCTTGCCACAGGTCTCCCCA
13960
AGGCGCACTGGCCTCATCTT
13908
GGGCCTGTGTTATCTCCTAG
14000
GTTGGCTCTGACTGTACCAC
14020
CATCCACTACAACTACATGT
14040
GTACAGTTCCTGCATGGGTG
14060
GCATGAACCAGAGGCCCATC
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14140
GCCTGCTGTGCCCCAGCTTG

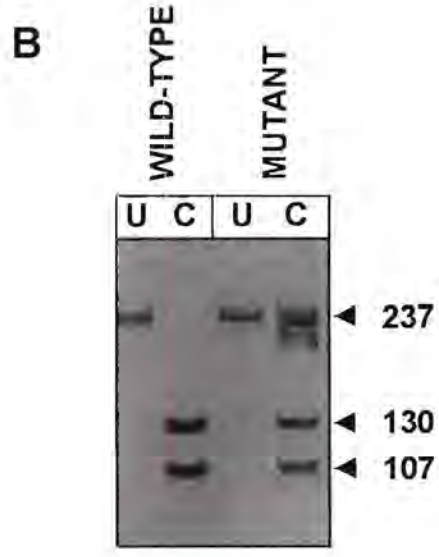


Figure 3.11: MspI digestion of the exon 7 PCR product for patient #27. (A); the G → A base substitution in codon 248 from patient #27 destroys an MspI restriction site, shown in red. (B); exon 7 from patient #27 was re-amplified and the PCR product was incubated with MspI at 37°C for 16 hours and the DNA fragments analysed on an 8% polyacrylamide gel. U represents the uncut PCR product and C represents the PCR product digested with MspI. The sizes of the DNA fragments are shown in base pairs on the right hand side of the figure.

3.2.4 Analysis of Exons 8 and 9 of the p53 Gene

3.2.4.1 PCR Amplification of Exons 8 and 9

Exons 8 and 9 of the p53 gene were co-amplified as a result of the small intron (92bp) between them, using primers p53-89A and p53-89B described in section 6.3.1 to give a PCR product of 445bp in length (figure 3.12A). The PCR product was digested with StyI to separate the two exons from each other before analysis by SSCP. The primers were situated in the flanking introns, thus incorporating the splice junctions into the PCR product. To check for the correct PCR product size, 5µl of the PCR product was electrophoresed on a non-denaturing 8% polyacrylamide gel (figure 3.12B) to confirm that exons 8 and 9 were successfully amplified by producing the expected 445bp fragment. The StyI digested fragments of 261bp and 184bp are also indicated in the figure.

3.2.4.2 SSCP Analysis of Exons 8 and 9 of the p53 Gene

Exons 8 and 9 of the p53 gene were co-amplified in the presence of ^{32}P-dCTP as described in section 6.3.1 and the PCR product digested with StyI endonuclease in order to produce two fragments, the one containing exon 8 and the other exon 9 (figure 3.12A).

Figure 3.13(A-D) represent SSCP gels using different electrophoretic conditions for exons 8 and 9. Results from this screening indicated that patient #7 had an altered electrophoretic mobility pattern that separated under all of the conditions used while patient #28 had a mutation in exon 8 that separated out from the normal only when the samples were electrophoresed in the presence of glycerol at 4°C (figure 3.13(D)). The MDA-MB231 cell line also harboured a mutation in exon 8, as evidenced by it's altered banding pattern when the samples were electrophoresed at 4°C in the presence of glycerol (figure 3.13D). This mutation and the mutation in patient #28 could not be distinguished from the wild-type when the samples were electrophoresed at room temperature in the absence of glycerol (figure 3.13A), at room temperature in the presence of glycerol (figure 3.13B) or at 4°C in the absence of glycerol (figure 3.13C).

A 14361

p53-89A

TTGGGAGTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACT

Gly Asn Leu Leu Gly Arg
GCCTCTTGCTTCTCTTTTCCTATCCTGAGTAG T GGT AAT CTA CTG GGA CGG

Asn Ser Phe Glu Val Arg Val Cys Ala Cys Pro Gly Arg Asp
AAC AGC TTT GAG GTG CGT GTT TGT GCC TGT CCT GGG AGA GAC

Arg Arg Thr Glu Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro
CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG GAG CCT

His His Glu Leu Pro Pro Gly Ser Thr Lys Arg
CAC CAC GAG CTG CCC CCA GGG AGC ACT AAG CGA G GTAAGCAAG
CAGGACAAGAAGCGGTGGAGGAGACCAAGGGTGCAGTTATGCCTCAGATTCACTT

StyI

Leu Pro Asn Asn Thr Ser
TTATCACCTTTCCTTGCCTCTTTCCTAG CA CTG CCC AAC AAC ACC AGC

Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu Tyr
TCC TCT CCC CAG CCA AAG AAG AAA CCA CTG GAT GGA GAA TAT

Phe Thr Leu Gln
TTC ACC CTT CAG GTACTAAGTCTTGGGACCTCTTATCAAGTGGAAAGTTTC
CAGTCTAACACT

p53-89A

14805

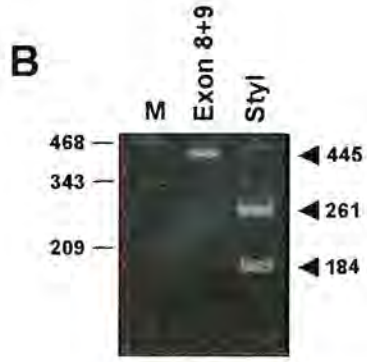
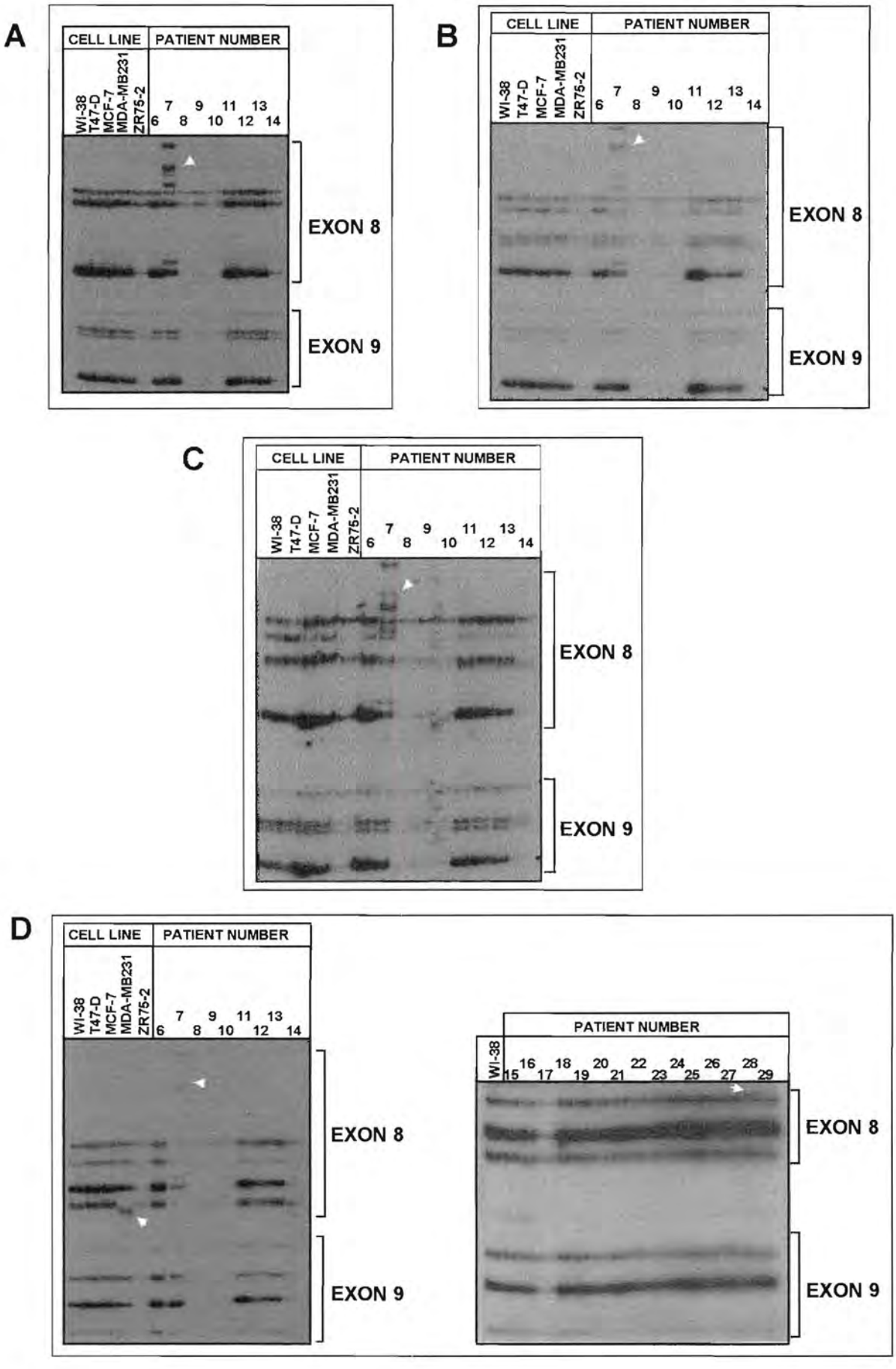


Figure 3.12: Amplification of exons 8 & 9 of the p53 gene. (A); exons 8 & 9 were co-amplified as described in section 6.3.1 using primers p53-89A and p53 89B as indicated. (B), in order to check for efficient amplification, 5µl of the PCR product was electrophoresed on a non-denaturing 8% polyacrylamide gel and the bands visualised under UV light after staining with ethidium bromide (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure and the amplified 445bp PCR product is indicated by the arrowhead on the right hand side. The 261bp and 184bp fragments obtained after digestion with StyI are also indicated by arrowheads on the right hand side. All sizes are in base pairs.


A
CELL LINE
PATIENT NUMBER
WI-38
T47-D
MCF-7
MDA-MB231
ZR75-2
6 7 8 9 10 11 12 13 14
EXON 8
EXON 9
B
CELL LINE
PATIENT NUMBER
WI-38
T47-D
MCF-7
MDA-MB231
ZR75-2
6 7 8 9 10 11 12 13 14
EXON 8
EXON 9
C
CELL LINE
PATIENT NUMBER
WI-38
T47-D
MCF-7
MDA-MB231
ZR75-2
6 7 8 9 10 11 12 13 14
EXON 8
EXON 9
D
CELL LINE
PATIENT NUMBER
WI-38
T47-D
MCF-7
MDA-MB231
ZR75-2
6 7 8 9 10 11 12 13 14
EXON 8
EXON 9
PATIENT NUMBER
WI-38
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
EXON 8
EXON 9

None of the samples showed any mutations in exon 9, although patient #27 appears to have an altered migration pattern when electrophoresed at 4°C in the presence of glycerol (figure 3.13D). This altered migration pattern, however, was a false positive as it was not reproducible.

3.2.4.3 Sequence Analysis of the Exon 8 Mutations

In order to determine the exact nature of the mutations detected by SSCP in exon 8 in the MDA-MB231 cell line and patient 7 and 28, the PCR products were subjected to DNA sequence analysis.

Exon 8 and 9 of the MDA-MB231 cell line was re-amplified and the resulting amplified product was sequenced directly as described in section 6.6.1 using a PCR Product Sequencing Kit (Amersham). DNA sequence analysis revealed the mutation to be a single G $\rightarrow$ A base substitution at position 280, resulting in an Arg $\rightarrow$ Lys amino acid substitution (results not shown). This mutation has been described previously (Bartek et al., 1990b).

The exon 8 and 9 PCR product from patient #7 was also sequenced directly but it was not possible to get an unambiguous sequence and it was decided to clone the PCR product for sequence analysis. The PCR product was cloned as outlined in section 6.4 and a number of clones were sequenced. The mutation was found to be an in frame 15bp duplication resulting in the duplication of 5 amino acids corresponding to codons 268 - 272 (figure 3.14).

As a consequence of this mutation, a new AluI restriction site was introduced into the DNA sequence (figure 3.15A). This new restriction site enabled the mutation to be easily confirmed and screened for by digesting the PCR-amplified product with AluI, which would release a 15bp DNA fragment. Exon 8 and 9 were thus re-amplified in the presence of trace amounts of ^{32}P-dCTP to visualise such a small fragment. The expected 15bp DNA fragment can be clearly seen in the mutant lane, thus confirming the presence of this duplication in patient #7 (figure 3.15B).

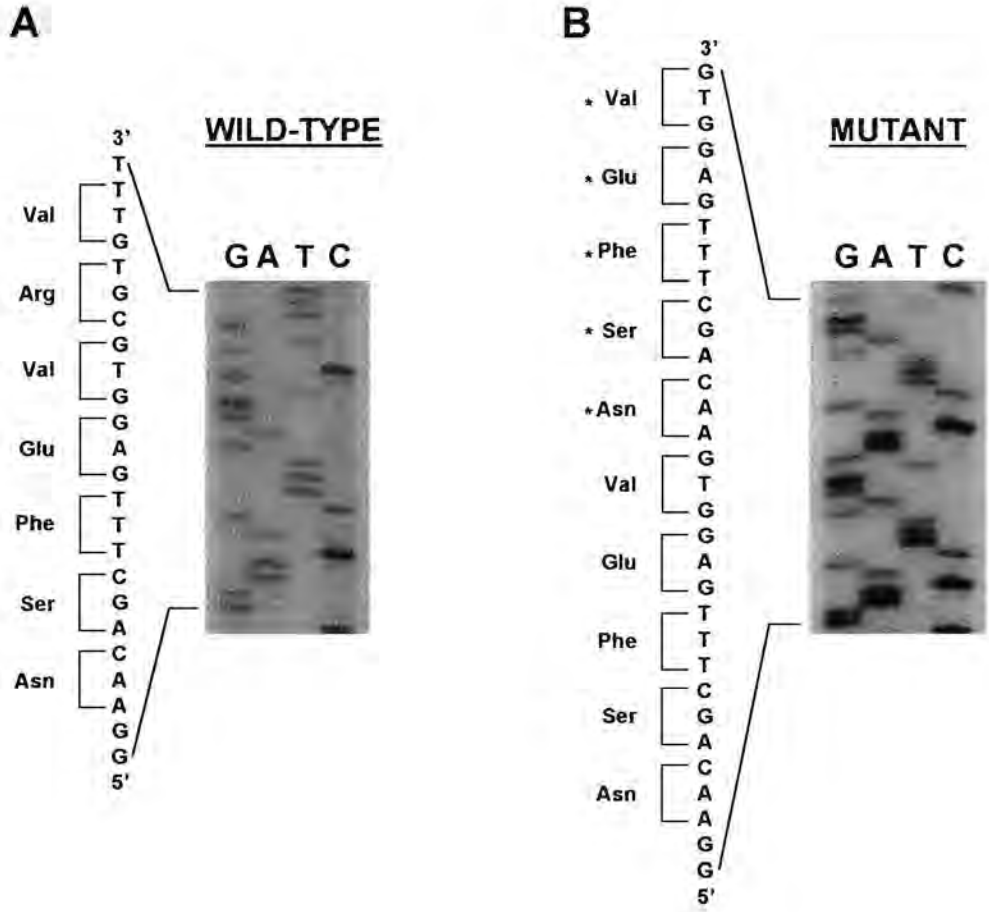


Figure 3.14: Sequence analysis of the exon 8 mutation in patient #7. The exon 8/9 PCR product of patient #7 was cloned and sequenced using the dideoxy sequencing method with the Pharmacia ^{T7}Sequencing^{TM} Kit as described in section 6.6.2. Sequence reactions were heated at 75°C for 2 min and fractionated on a denaturing 6% polyacrylamide gel, which had been pre-electrophoresed until the temperature of the gel had reached 50°C. The samples were electrophoresed through the gel for 3 hours, after which the gel was dried at 70°C under a vacuum for 1 hour and exposed to x-ray film for 16 hours at -70°C. The wild-type WI-38 sequence is shown in (A) and the mutant sequence of patient #7 in (B). The mutation is a 15 base duplication indicated by asterisks.

A

```
14361                    14381                    14401
|                        |                        |
TTGGGAGTAGATGGAGCCTG GTTTTTTAAATGGGACAGGT AGGACCTGATTTCCTTACTG

14421                    14441                    14461
|                        |                        |
CCTCTTGCTTCTCTTTTCCT ATCCTGAGTAGTGGTAATCT ACTGGGACGGAACAGCTTTG

14481                    14501                    14521
|                        |                        |
AGGTGAACAGCTTTGAGGTG CGTGTTTGTGCCTGTCCTGG GAGAGACCGGCGCACAGAGG

14541                    14561                    14581
|                        |                        |
AAGAGAATCTCCGCAAGAAA GGGGAGCCTCACCACGAGCT GCCCCCAGGGAGCACTAAGC

14601                    14621                    14641
|                        |                        |
GAGGTAAGCAAGCAGGACAA GAAGCGGTGGAGGAGACCAA GGGTGCAGTTATGCCTCAGA

14661                    14681                    14701
|                        |                        |
TTCACTTTTATCACCTTTCC TTGCCTCTTTCCTAGCACTG CCCAACAACACCAGCTCCTC

14721                    14741                    14761
|                        |                        |
TCCCCAGCCAAAGAAGAAAC CACTGGATGGAGAATATTTC ACCCTTCAGGTACTAAGTCT

14781                    14801
|                        |
TGGGACCTCTTATCAAGTGG AAAGTTTCCAGTCTAACACT
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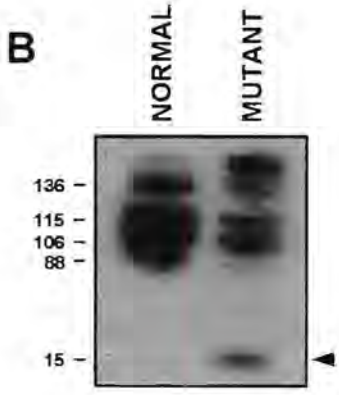


Figure 3.15: Restriction endonuclease digestion of the exon 8/9 PCR product from patient #7. (A); sequence of the exon 8 mutation in patient #7. The duplicated bases are represented by the boxed sequence. The 15 base pair duplication results in the creation of an AluI restriction site (red sequence in the boxed region). All other AluI restriction sites are depicted in red. (B); exon8/9 from patient #7 was re-amplified in the presence of ^{32}P-dCTP using primers p53-89A and p53-89B and the PCR product incubated with AluI for 16 hours at 37°C. The DNA fragments were resolved on a non-denaturing 20% polyacrylamide gel. The gel was dried under a vacuum for 1 hour at 70°C and exposed to x-ray film for 16 hours at -70°C. Molecular weights in base pairs are indicated on the left hand side of the figure. The expected 15bp fragment in the mutant is indicated by an arrowhead on the right hand side.

Direct sequence analysis of the exon 8/9 PCR product from patient #28 revealed the mutation to be a single C → A base substitution at codon 269 (figure 3.16), resulting in a serine → arginine substitution.

3.2.5 Amplification of Exon 11 of the p53 Gene

Exon 11 was amplified using primer p53-11A and p53-11B under the conditions outlined in table 6.1 and described in section 6.3.1. Figure 3.17(A) shows the sequence of the PCR-amplified product and the position of the primers flanking the exon, thus incorporating the splice junctions. Figure 3.17(B) represents a 1% agarose gel showing the 245bp product.

3.2.6 Sequencing of Exons 5-11 in all Patients

Since only a limited number of mutations had been detected by SSCP analysis, it was decided to sequence all the previously described exons, as well as exon 11 in all patients in order to ensure that no mutations had gone undetected by SSCP analysis. The sequence analysis supported the SSCP data and confirmed that no mutations had gone undetected by SSCP analysis. A summary of the SSCP and sequencing results is shown in table 3.1.

Although the number of mutations was very small, there was no correlation between the presence of the mutations and the stage of the tumour. Although 3 of the patients with mutations were under 50 years of age, there was also no significant association ($p=0.1323$) between the age of the patient and the presence of mutations. These results are summarised in table 3.2.

3.2.7 Analysis of Patient Survival

The survival rates of this population of breast cancer patients was compared with their oestrogen receptor, p53 and lymph node status (figure 3.18). The overall survival of this group of patients was quite good, with over 70% of the patients still alive after 5 years of being diagnosed with the disease (figure 3.18A). It is clear that there is no significant difference between the survival of patients with or without p53 mutations, although this result might be slightly skewed due to the low number of mutations in this population

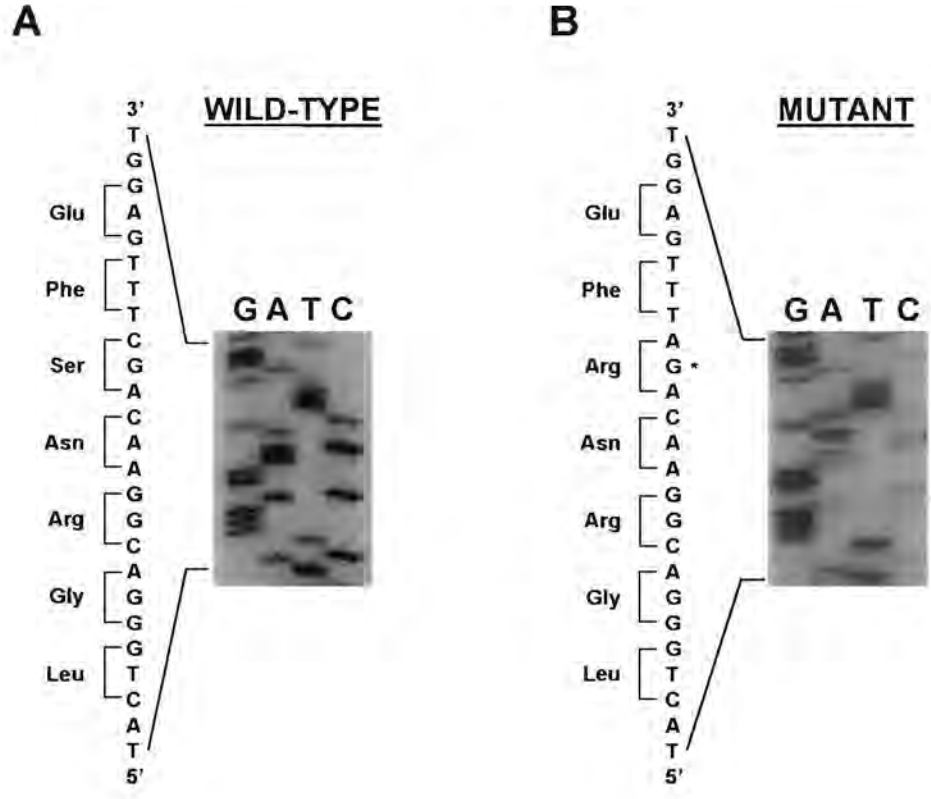


Figure 3.16: Sequence analysis of the exon 8 mutation in patient #28. Exon 8/9 was re-amplified and the PCR product subjected to direct sequence analysis as described in section 6.6.1. The sequence reactions were heated at 75°C for 2 min and loaded onto a denaturing 6% polyacrylamide gel, which had been pre-electrophoresed in order to heat it to 50°C. The samples were electrophoresed at a constant power of 50W for 3 hours, after which the gel was dried and exposed to x-ray film for 16 hours at -70°C. The wild-type sequence is shown in (A) and the mutant sequence shown in (B). Sequence analysis revealed the mutation to be a C → A base substitution at codon 269, as indicated by the asterisk, resulting in a Ser → Arg amino acid substitution.

A

18545
| **p53-11A** His
AGACCCTCTCACTCATGTGATGTCATCTCTCCTCCCTGCTTCTGTCTCCTACAG C CAC

Leu Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu
CTG AAG TCC AAA AAG GGT CAG TCT ACC TCC CGC CAT AAA AAA CTC

Met Phe Lys Thr Glu Gly Pro Asp Ser Asp Ter
ATG TTC AAG ACA GAA GGG CCT GAC TCA GAC TGA CATTCTCCACTTCTT
GTTCCCCACTGACAGCCTCCCTCCCCCATCTCTCCCTCCCCTGCCATTTTGGGTTTTGG
GTCTTTGAACCCTTGCTTGCAATAGGTGTGCGTCA
p53-11B |
18789

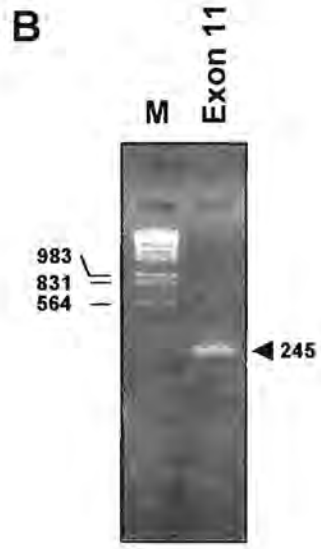


Figure 3.17: Amplification of exon 11 of the p53 gene. Exon 11 was PCR-amplified using primers p53-11A and p53-11B (A) as described in section 6.3.1. (B); 5µl of the PCR product was electrophoresed on a 1% agarose gel and the bands visualised under UV light after staining with ethidium bromide (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure with the amplified product indicated by the arrowhead on the right hand side. All sizes are in base pairs.

Table 3.1: Summary of the p53 mutations detected in this study using SSCP and DNA sequence analysis.

EXON	NO. OF MUTATIONS	SAMPLE*	BASE CHANGE#	AMINO ACID CHANGE
5	0	-	-	-
6	1	T47-D	C → T (194)	LEU → PHE
7	2	6	ΔA (239)	Frameshift (Stop at codon 263)
		27	G → A (248)	ARG → GLN
8	3	28	C → A (269)	SER → ARG
		7	15bp duplication	5 amino acid duplication
		MDA-MB-231	G → A (280)	ARG → LYS
9	0	-	-	-
11	0	-	-	-

* numbers indicate patient number

\# numbers in brackets indicate codon number

Table 3.2: Summary of data obtained in this study. Only 4 of the 39 patients had mutations in the p53 gene. One patient had a fibroadenoma with a normal p53 gene. There was no clear association between tumour stage or age of the patient with p53 mutations.

TUMOUR STAGE	NO. OF SAMPLES	NO. OF MUTATIONS	AGE OF PATIENT
I	14	2	44 30
II	13	1	40
III	10	1	62

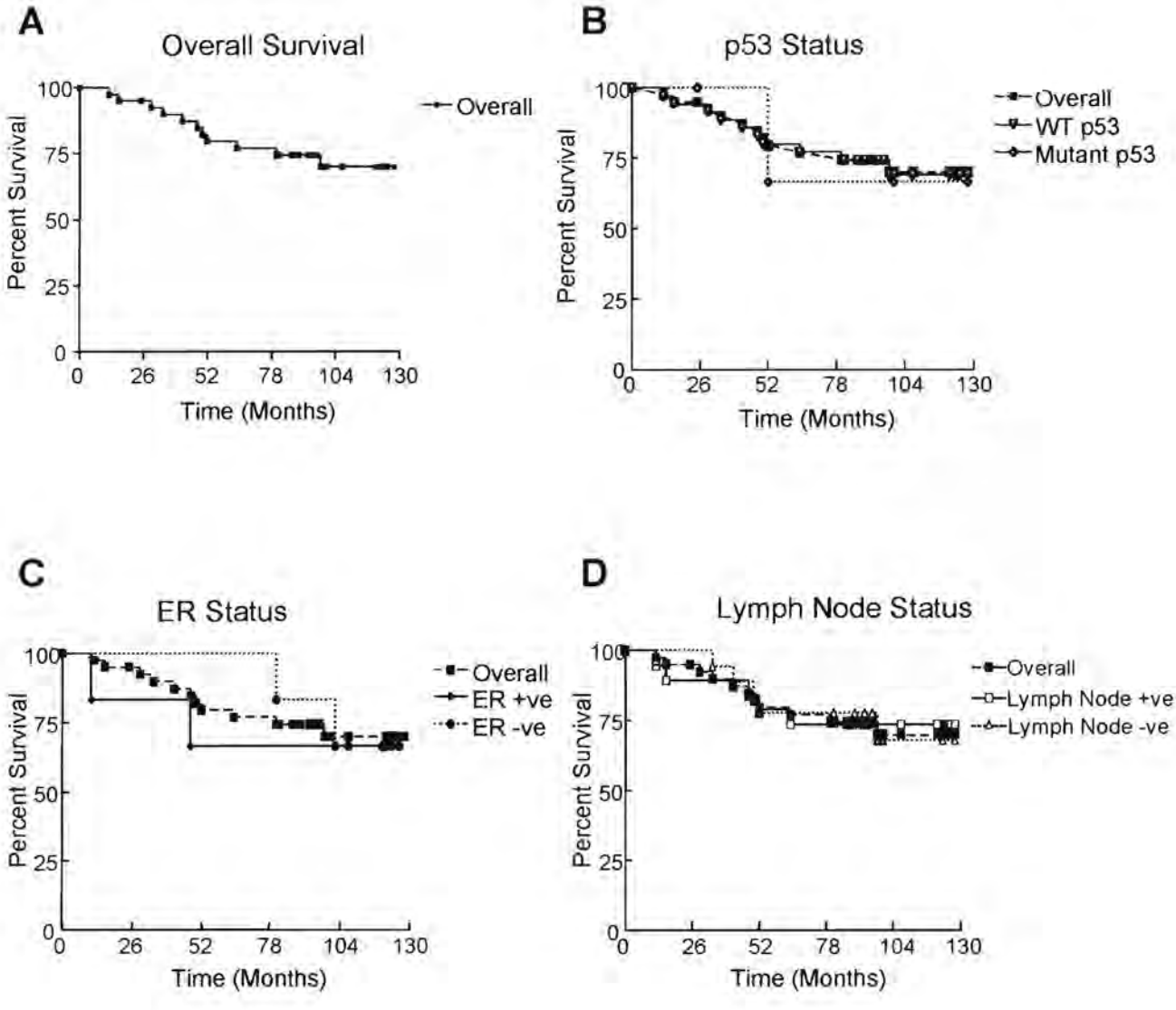


Figure 3.18: Patient survival curves. Survival times were estimated using the method of Kaplan and Meier for (A) the overall survival, (B); patients without (-▽-) and with(-◇-) p53 mutations, (C); oestrogen receptor (ER) positive (-◆-) and ER negative (-●-) tumours and (D); lymph node positive (-□-) and lymph node negative (-Δ-) tumours). The overall survival (-■-) is shown in each figure for comparison.

(figure 3.18B). Similarly, there was no significant difference in patient survival with respect to either the oestrogen receptor status or involvement of lymph nodes (figures 3.18(D) and (E) respectively).

3.2.8 Immunohistochemical Detection of p53 Protein

Wild-type p53 protein is, in the majority of cases, undetectable by immunohistochemistry, and one of the characteristics of mutant p53 is that it has an extended half life, rendering it detectable by either Western blotting or immunohistochemical staining. Since only a small number of p53 mutations were detected by SSCP and DNA sequence analysis of exons 5-11, the patient tumour samples were screened for p53 protein using immunohistochemistry in order to determine whether a higher frequency of mutations may be present in the regions of the gene not screened by SSCP and sequence analysis in this population.

Tissue sections were probed for p53 protein using an anti-p53 antibody that recognises an epitope in the N-terminus of both the human wild-type and mutant forms of the protein. Sections were treated as described in section 6.11. With this technique, 10 patients showed a positive result. It is clear from figure 3.19 that there are various intensities of p53 staining, with some tumours staining only very weakly (B) and (E) and other tumour samples staining very strongly (C), (D) and (F). The tissue sections for patient #27 harbouring the G $\rightarrow$ A base substitution at codon 248, and patient #28 with the C $\rightarrow$ A substitution at codon 269 are shown in figures 3.19(B) and (C) respectively. Patient #6 with the deletion of a single A residue at codon 239 came up negative for the presence of p53 protein which may be explained by the rapid degradation of the truncated protein. Unfortunately, no material was available for patient #7 with the 15bp insertion. A summary of the immunohistochemistry results is shown in table 3.3.

Of the 10 patients staining positive for p53, only two had mutations in the exons analysed. Thus in order to determine whether the remaining eight immunohistochemically positive patients contained mutations in the N-terminal region of the protein, exons 2 and 4 of these patients were amplified by PCR and sequenced, but no

mutations were detected in these 2 exons either.

Table 3.3: Summary of the p53 immunohistochemical positive patients

TUMOUR STAGE	NO. OF SAMPLES	p53 MUTATION STATUS*
I	2	1 Wild-type 1 Mutant
II	3	All wild-type
III	4	3 Wild-type 1 Mutant
IV	1	Wild-type

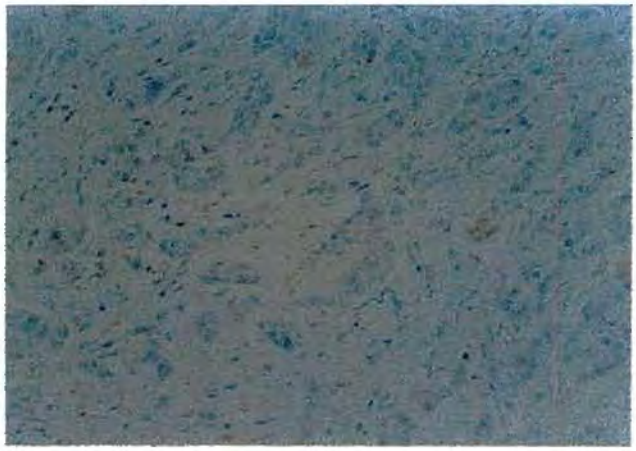
* p53 mutation status for exons 2, 4, 5-9 and 11

From the table, it is clear that p53 protein was detected more frequently in higher stage tumours (II-IV), with the majority of cases occurring in stages II and III. The staining intensity of some patients varied, which may have something to do with the nature of the mutation (figure 3.19(B) and (C)). In addition to patient #28 with the C $\rightarrow$ A mutation at codon 269, only one other patient who showed detectable levels of p53 protein had died from the disease, whilst another patient (#16) died from other causes.

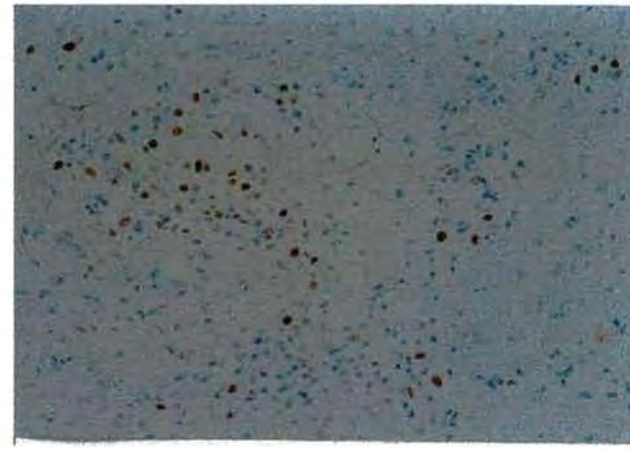
In summary, of the 40 breast cancer patients screened for mutations in the p53 gene, only 4 were found to harbour mutations in exons 5-9 and 11, and only an additional 8 patients showed detectable levels of p53 protein when screened by immunohistochemistry.

Figure 3.19: Immunohistochemical staining of p53 protein. Tissue sections from the breast cancer patients were processed as described in section 6.11 and probed for p53 protein using the DO-7 anti-p53 antibody, which recognises an epitope on the N-terminus of the protein. The antibody was detected using DAB as a substrate. (A); a section from a patient who is negative for p53 protein, (B); p53 staining from patient #27, (C); a tissue section from patient #28, (D); patient #31, (E); patient #17 and (F); patient #14. Patient #27 is a stage I tumour, #31 a stage II tumour, and 14, 17 and 28 are stage III tumours. All photographs were taken with 400 X magnification.

A



B



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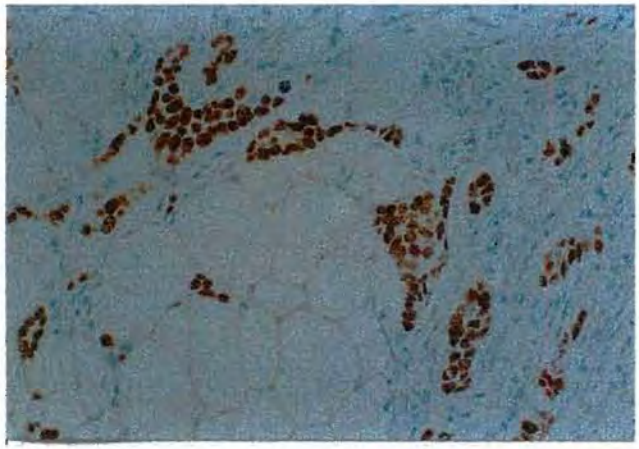
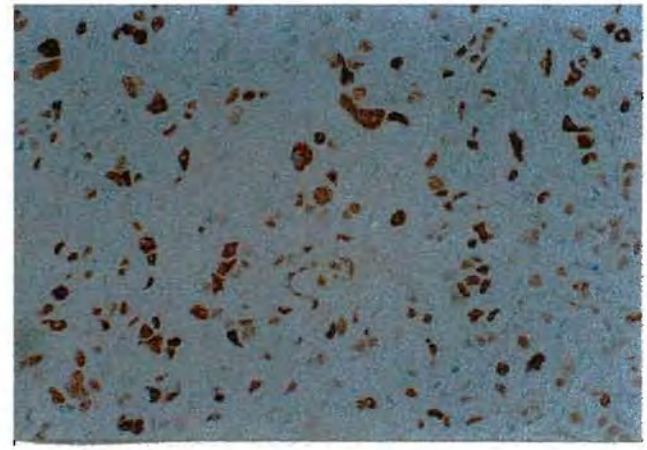
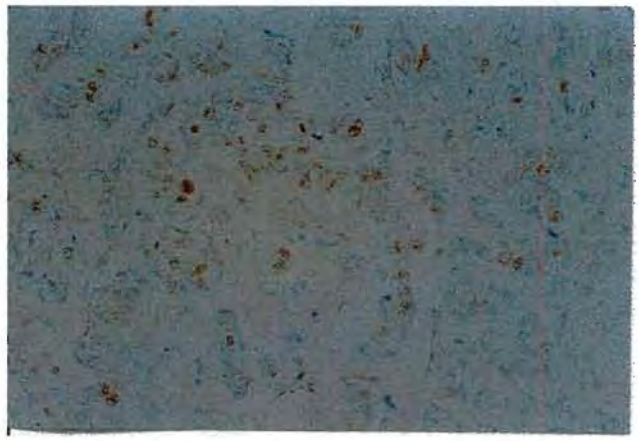


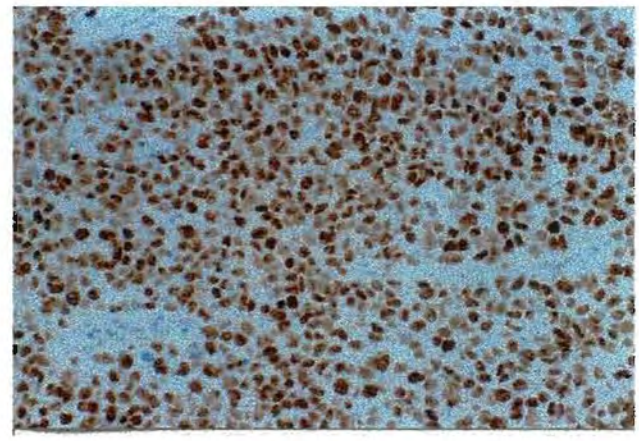
D



E



F



3.3 DISCUSSION

The frequency of alterations in the p53 gene has been reported to be between 40 - 50%, and more specifically, the frequency of point mutations (including small deletions and insertions) has been reported to be about 25 - 30% (Lou et al., 1997; Runnebaum et al., 1991; Sjörgen et al., 1996). In this study of South African breast cancer patients, a fairly low frequency of p53 mutations were found in primary breast tumours. Only 4 out of 40 (10%) primary breast tumours examined contained mutations in the p53 gene. Moreover, no mutations were present in the previously reported hotspot regions of exons 5 and 6. The mutations were restricted to exons 7 and 8. It cannot, however, be ruled out that mutations do exist in the regions (exons 2, 3, 4 and 10) not investigated in this study.

Of the 4 mutations identified in the breast cancer patients in this study, three have not previously been described. These are the single base deletion in codon 239, the point mutation at codon 269 and the 15bp duplication of codons 268-272. Previous studies have shown a large deletion, as well as a point mutation at codon 239 (Aas et al., 1996; Andersen et al., 1993). The only codon 269 mutation previously reported for breast cancer is a silent change in patients with familial breast cancer (Glebov et al., 1994) and only one point mutation has been reported for codon 268 (Seth et al., 1994). The fourth mutation at codon 248 has been reported in many studies and has been found to be frequently mutated (Aas et al., 1996; Andersen et al., 1993; Shiao et al., 1995; Davidoff et al., 1991; Faille et al., 1994). Codon 248 has been shown to be highly methylated (Rideout et al., 1990), thus deamination of a methylcytosine could account for the high frequency of mutations at this site (Magewu and Jones, 1994). The two mutations identified in the breast cancer cell lines (T47-D and MDA-MB231) have been previously documented (Nigro et al., 1989; Bartek et al., 1990b).

Although PCR-SSCP analysis is frequently used to screen for mutations, it is not 100% effective and even under different conditions, it is possible to miss some mutations. It was therefore decided to use direct DNA sequence analysis on all the PCR products in order to minimise the possibility that some mutations may have escaped detection by SSCP. The sequence data correlated 100% with the SSCP data in that all mutations

detected by SSCP were also detected by sequence analysis, and no further mutations were detected by direct sequence analysis of the PCR products. Thus the discrepancy between the results presented in this chapter and those reported by others is not due to the possibility that some mutations may have gone undetected.

Immunohistochemical techniques have also been utilised to detect p53 protein accumulation, which has been associated with p53 gene mutations (Bartek et al., 1991). It has, however, been shown that immunohistochemical detection of p53 expression is not an absolute indicator of a p53 gene mutation (Sjörgen et al., 1996; Chakravarty et al., 1996). In this study, only 10 out of the 40 (25%) patients were positive for p53 protein accumulation as determined by immunohistochemistry, with two of these having point mutations. Interestingly, those patients showing detectable levels of p53 protein tended to be skewed towards stages II-IV. A possible explanation for the patients having detectable p53 protein levels, but lacking mutations, may be that p53 in these patients is not being targetted for degradation. It has been shown that the N-terminal domain of p53 is critical for its interaction with mdm2 and its subsequent degradation (Gottlieb and Oren, 1996). Therefore, in order to determine whether the N-terminal domain of p53 in these patients contained mutations that may impede its interaction with the mdm2 protein, exons 2 and 4 from these patients were analysed for mutations by sequence analysis, but no mutations were detected. The possibility cannot be ruled out, however, that those patients with detectable levels of p53 protein and without mutations in the exons screened, may harbour mutations in the remaining exons (3 and 10) of the gene. The presence of p53 mutations in tumours has been associated with a poor prognosis and can be a prognostic indicator for recurrence and survival and it has been suggested that detection of p53 mutations has a greater prognostic value than immunohistochemical detection for overexpression of the protein (Kovach et al., 1996).

Although mutation of the p53 gene is one of the most frequent and common genetic aberrations in human tumours, not all tumours contain p53 mutations. A low frequency of mutations has been found in both testicular (Peng et al., 1993) and medulloblastomas (Saylors et al., 1991), both of which are embryonically derived tumours.

The possibility exists that the low frequency of p53 mutations detected in this study could be due to differences in mutation spectrum between different race groups, since the majority of the patients in this study were of a single population group. Reports from the United States have shown that there is a difference in survival between Black and White patients with breast cancer, and also that the mutation status of the p53 gene can vary between different race groups having the same tumour type (Shiao et al., 1996; Shiao et al., 1995). Black patients with p53 mutations had a significantly poorer survival rate than those without p53 mutations, which did not apply to White patients. Contrary to this, White patients who had p53 protein accumulation had a poorer survival rate than those without protein accumulation, which did not apply to Black patients. Interestingly, of those patients who died from the disease, Blacks often had p53 mutations without detectable levels of the mutant protein, whereas Whites often had p53 protein accumulation but lacked mutations. In the patients used in this study (of which the majority were of a mixed race), p53 accumulation did not significantly alter the survival rate. Only 1 patient (a black female) with p53 protein accumulation and no accompanying mutation, died from the disease, and only 1 patient (of mixed ancestry) with both p53 protein accumulation and a mutation died from the disease.

The results presented in this chapter suggest that the majority (90%) of the patients would have a good prognosis as their tumours lacked a p53 mutation. The survival curves indicate that a large proportion (82%) of these individuals were still alive 4 years after they were initially diagnosed with the disease. This result compares favourably with patients lacking p53 mutations in other studies (Kovach et al., 1996). The results presented here differ from those reported by Kovach et al 1996 in that they found that p53 mutations were associated with a poor prognosis, whereas results in this study show that the presence of p53 mutations did not significantly effect patient survival. These results may be skewed, though, as a result of the low number of mutations detected.

It may also be possible that the nature of the mutation may play a role in determining whether tumours with a particular type of mutation would have a poor prognosis. This will be investigated to some extent in the following chapter. It has been shown that both

the frequency and the nature of the p53 mutations differ amongst race groups (Shiao et al., 1995). Black patients showed a high frequency (80%) of G:C to A:T transitions at non-CpG sites, whereas this type of mutation only occurred in 62.5% of White patients with p53 mutations. p53 mutations have also been associated with a poorer survival in Black patients, but not in White patients, with Black patients harbouring p53 mutations having an increased risk of death from the disease as compared to Black patients without mutations (Shiao et al., 1995). In South Africa, little is known about the survival rates between race groups, but results from elsewhere suggest that the survival of patients from different race groups may vary as well. It is quite clear from the data presented in this chapter that the p53 status might vary among different race groups, with the "Coloured" population showing a low frequency of mutations, corresponding to a good survival rate.

The majority of mutations detected in the p53 gene are point mutations (Osborne et al., 1991; Finlay et al., 1988). In this study, however, 2 out of the 4 (50%) of the mutations were microdeletions/insertions. An unusually high frequency of these types of mutations has been previously reported (Kovach et al., 1996) in a different group of breast cancer patients from the Midwestern region of the United States. The reason for this high number of insertions/deletions is unclear, but it has been suggested that exogenous and endogenous factors could be implicated in the generation of these types of mutations (reviewed by Krawczak and Cooper, 1991 and Jego et al., 1993).

Existing evidence has linked p53 mutations to increased resistance to radiotherapy and chemotherapeutic drugs (Lowe et al., 1993a; Lowe et al., 1994). This is probably due to a defect in p53-dependant apoptosis, which is induced by many anticancer drugs. Consequently, chemotherapy in a patient with a p53 mutation would be less successful than in a patient lacking a p53 mutation. Thus it should be expected that the majority of the patients in this study would respond favourably to treatment. All of these patients were subjected to surgery (mastectomy and axillary clearance) and in addition, 17 patients underwent tamoxifen treatment, 12 patients had chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil) while 9 patients had no additional treatment. Only 8 of the 40 patients in this study died from the disease, 4 of whom were

subjected to tamoxifen treatment and 4 to chemotherapy. Surprisingly, none of the patients who had surgery only died from the disease. Of the 4 patients with p53 mutations, one had no treatment other than surgery while 2 had hormone therapy in addition to surgery and the fourth had chemotherapy in addition to surgery. Of the 9 patients who died, only 1 had a p53 mutation accompanied by p53 protein accumulation, while another patient, also undergoing hormone treatment, had p53 protein accumulation, but without a p53 mutation. In addition to the 9 patients that died from the disease, a further 2 died from other causes and of the remaining 29 patients only 2 have shown a recurrence of the disease. The majority of the patients are alive with no evidence of disease. In this study p53 mutations or p53 protein accumulation did not appear to have a significant effect on patient survival and did not appear to be important in the choice of treatment.

In summary, the results in this chapter suggest that mutant p53 does not play a significant role in the aetiology of breast cancer in the "Coloured" population in South Africa, and raise the possibility that this population group could be "resistant" to mutations in this gene. This may be due to the patients not being exposed to certain environmental carcinogens which could result in more widespread mutation in the p53 gene. These results also raise the possibility that tumours of the same type can be genetically diverse in that tumours of the same type may arise from different genetic aberrations which vary among different population groups.

CHAPTER 4: FUNCTIONAL ANALYSIS OF NOVEL p53 MUTANTS

4.1 INTRODUCTION

Development of a neoplastic cell or a malignant tumour is a multistep process, in which immortalisation of the cell is thought to be a critical event (Farber, 1984). In addition to immortalisation, clonal expansion and the accumulation of multiple genetic aberrations also play an important role in the progression of a normal cell into a cancerous one. p53 plays an important role in both these events, which explains why mutations in this gene are the most common genetic alterations found in sporadic cancers (Vogelstein, 1990).

Amongst it's many functions, p53 induces cell cycle arrest near the restriction point in late G_1 (Lin et al., 1992), or apoptosis, thus preventing the replication of defective DNA (Clarke et al., 1993; Lane, 1992; Kastan et al., 1991; Lowe et al., 1993a; Livingstone et al., 1992). This is achieved by the induction of the p21/WAF1 gene (el-Deiry et al., 1993), an inhibitor of most of the cyclin-dependent kinases (Xiong et al., 1993). This function of p53 is critical in its ability to arrest cells in the G_1 phase of the cell cycle in response to DNA damage, (Kastan et al., 1991; Kuerbitz et al., 1992) thus allowing DNA repair to take place prior to DNA replication in order to ensure that mutations are eliminated from the genome. In addition to arresting the cells in late G_1 phase of the cell cycle, p53 can also cause growth arrest in the G_2/M phase in the absence of DNA damage (Agarwal et al., 1995), probably via the same mechanism as for the G_1 arrest that activates p21/WAF1.

It has been demonstrated that immortalisation of primary human mammary epithelial cells by the E6 gene of HPV-16 is accompanied by a marked degradation of the p53 protein (Band et al., 1991), a phenomenon that has also been observed in primary human mammary epithelial cells after γ-radiation (Wazer et al., 1994). These results confirm that inactivation of p53 plays an important role in the immortalisation of these cells.

Based on the evidence that loss of p53 protein is associated with immortalisation of cells, it is possible that mutated p53 proteins also participate in the immortalisation process. It has been shown that various p53 mutants can immortalise cells by abrogating the G_1 cell cycle arrest induced after DNA damage (Gao et al., 1996; Fushimi et al., 1997; Kikuchi-Yanoshita et al., 1995). However, it has also been shown that certain p53 mutants are not capable of transforming cells, even though they impart some growth advantage (Williams et al., 1995; Dumenco et al., 1995). It seems that only certain p53 mutants are capable of immortalising cells, implying that the nature of the mutation is important.

This chapter is aimed at determining whether the three novel p53 mutations detected in the breast cancer patients (chapter 3) are capable of imparting a growth advantage to normal cells and whether the various mutants have the ability to arrest cells in late G_1 phase after exposure to ionising radiation.

4.2 RESULTS

In order to elucidate the effect of the mutant p53's described in chapter 3, it was first necessary to construct the various p53 mutants by site directed mutagenesis, and to transfect the mutant genes into cells to determine their effects on growth rate and their ability to arrest the cells in the G_1 phase of the cell cycle after DNA damage.

4.2.1 Construction of p53 Mutants by Site-Directed Mutagenesis

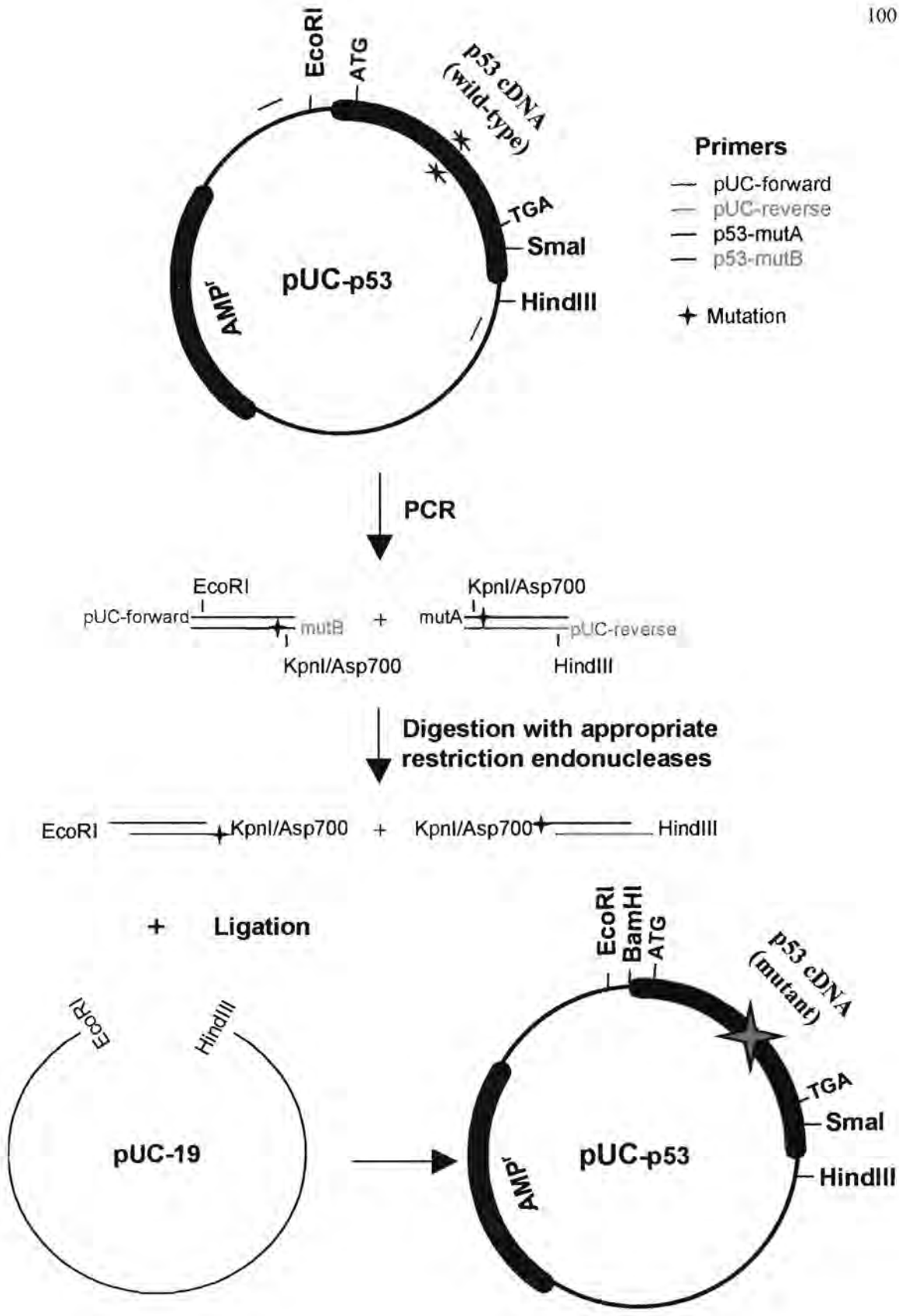
A wild-type p53 cDNA as well as a mutant cDNA (p53-SCX3) which contains a Val → Ala substitution at codon 143, were kindly provided by Dr B Vogelstein (Baker et al., 1990). Site-directed mutagenesis was carried out on the wild-type cDNA as described below.

4.2.1.1 Subcloning of the Wild-Type p53 cDNA into pUC-19

The wild-type p53 cDNA was supplied in a pCMV-Neo-Bam expression vector (Baker et al., 1990) and had to be subcloned into pUC-19 since no primers were available for the parent vector. The wild-type p53 cDNA was released from pCMV-Neo-Bam using BamHI restriction endonuclease. The released 1.8kb fragment was cloned into BamHI digested pUC-19 as described in section 6.9.1. After transformation of competent E.coli cells, a number of colonies were screened for the presence of inserts and the orientation of the inserts was determined by DNA sequence analysis using universal pUC primers.

4.2.1.2 Site-Directed Mutagenesis

Once the presence and orientation of the wild-type p53 cDNA in pUC-19 was confirmed, the clone was used as a template for site-directed mutagenesis. Primers containing the mutation of interest and silent mutations to create unique restriction endonuclease sites were synthesised and used in conjunction with the forward and reverse primers of pUC-19 in a PCR reaction, as described in section 6.9.2 and outlined in figure 4.1. The primers used for the p53-ΔA mutant had a KpnI restriction site and those for the p53-S269R and p53-+15 mutants contained Asp700 restriction sites, as indicated in table 6.2. Figure 4.2(A) shows a schematic representation of the position of the various mutant primers in the p53 sequence, as well as the pUC forward (Fp) and reverse (Rp) primers. The PCR


EcoRI
ATG
p53 cDNA (wild-type)
TGA
SmaI
HindIII
AMP'
pUC-p53
Primers
pUC-forward
pUC-reverse
p53-mutA
p53-mutB
Mutation
PCR
EcoRI
pUC-forward
mutB
KpnI/Asp700
KpnI/Asp700
mutA
pUC-reverse
HindIII
Digestion with appropriate restriction endonucleases
EcoRI
KpnI/Asp700
KpnI/Asp700
HindIII
+ Ligation
EcoRI
HindIII
pUC-19
EcoRI
BamHI
ATG
p53 cDNA (mutant)
TGA
SmaI
HindIII
AMP'
pUC-p53

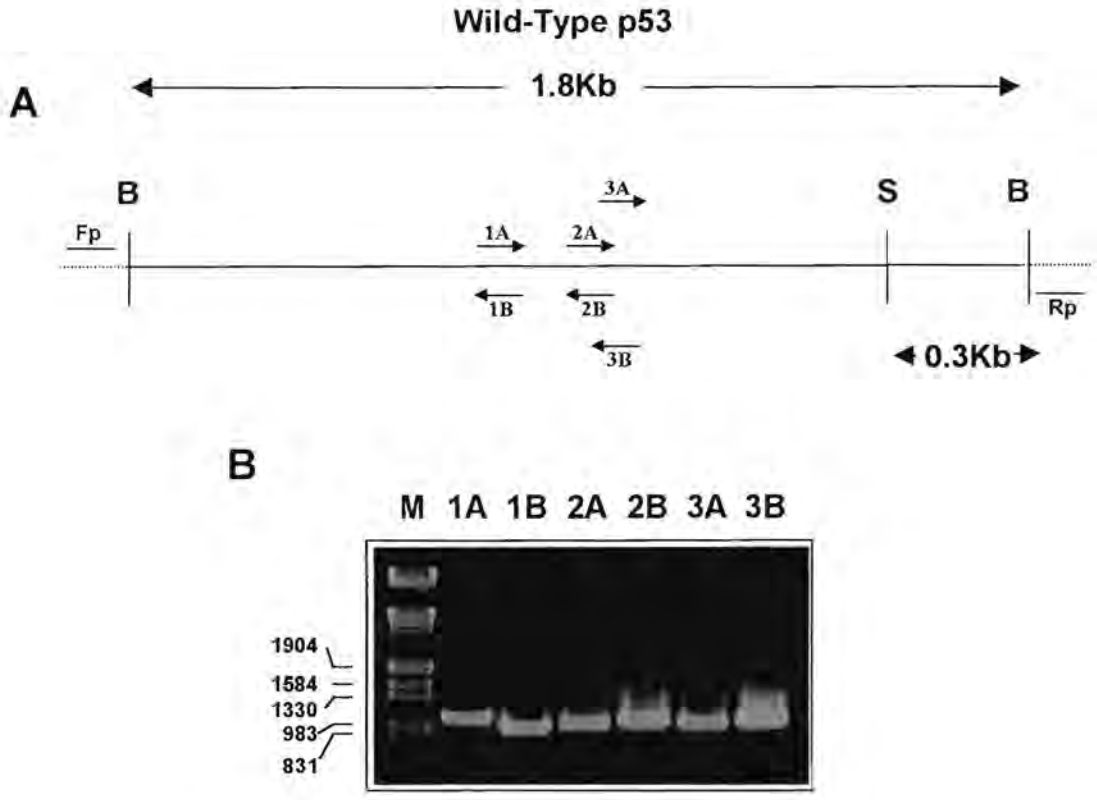


Figure 4.2: Amplification of mutant PCR products. The wild-type p53 cDNA was cloned into pUC-19 and used as a template to amplify various fragments containing the introduced mutations. (A); the relative positions of the mutant p53 primers described in section 6.9.2 in the p53 cDNA with the wild-type p53 cDNA depicted as the solid line and the pUC-19 vector sequence by the dotted line. Primers 1A and 1B correspond to the single A deletion mutant from patient #6 (chapter 3) and contain an introduced KpnI restriction endonuclease site; primers 2A and 2B correspond to the C → A base substitution at codon 269 from patient #28 (chapter 3); and primers 3A and 3B correspond to the 15bp insertion mutant from patient #7 (chapter 3). Primers 2A and 2B as well as primers 3A and 3B contain an introduced Asp700 restriction endonuclease site. Also shown is the position of the pUC-19 forward (Fp) and reverse (Rp) primers. B are BamHI sites and S is a SmaI site. Each A mutant primer was used in a PCR reaction with the Rp primer to generate the mut(A) PCR products, and each B mutant primer was used together with the Fp primer to generate the mut(B) PCR products. Amplification was carried out in a final volume of 100µl using Deep Vent polmerase (New England Biolabs) in order to minimise the number of mismatches which might be inserted randomly by the enzyme. (B); 10µl of the PCR products were electrophoresed on a 1% agarose gel and the bands visualised under UV light after staining the gel with EtBr (0.25µg/ml). The molecular weight markers (M) are shown on the left hand side of the figure, and are represented in base pairs. The various PCR products obtained were of the expected size.

amplified products for each mutant are shown in figure 4.2(B).

4.2.1.3 Ligation of PCR Products into pUC-19

The PCR products shown in figure 4.2(B) were digested with the restriction endonuclease corresponding to the site created in the primers as indicated in figure 4.1. The mut1(A) PCR product was digested with KpnI and HindIII, the mut1(B) PCR product with KpnI and EcoRI while the mut2(A) and 3(A) PCR products were digested with Asp700 and HindIII, and the mut2(B) and 3(B) PCR products with Asp700 and EcoRI. Both of the digested PCR products for each mutant were incubated with pUC-19, which had been digested with EcoRI and HindIII, in a triple ligation reaction in order to regenerate the full length (1.8Kb) p53 cDNA in pUC-19. The ligation reactions were used to transform competent E.coli Dk-1 cells (section 6.4.5) and colonies were screened for inserts by PCR (section 6.4.6.2). 2μl of a liquid culture of the colonies was used as a template for PCR in a 50μl reaction together with the pUC primers. Figure 4.3 shows representative agarose gels of the PCR products from the various colonies containing the p53-ΔA, p53-S269R and the p53-+15 mutants. CT represents a control PCR with the pUC-p53 construct (wild-type p53 cDNA cloned into pUC-19) to indicate the expected size of the PCR product. All the clones screened for the p53-ΔA mutant had inserts of the correct size whereas both the p53-S269R and p53-+15 mutants had incorrect sized inserts as well.

4.2.1.4 Sequence Analysis of the Mutant p53 cDNAs

In order to confirm that the correct mutations were created and that no other changes had been introduced by the PCR, the clones were analysed by sequence analysis as described in section 6.6.2. A number of clones were sequenced in order to obtain one of each that contained the correct sequence. The p53-ΔA mutant had the correct sequence, with the deleted A residue indicated by the arrowhead and the newly created KpnI restriction site which did not alter the amino acid sequence also indicated in figure 4.4(A). The sequence around the site of the mutation for the p53-S269R mutant and the mutated base are shown in figure 4.4(B), while the sequence containing the p53-+15 mutant is shown in figure 4.4(C).

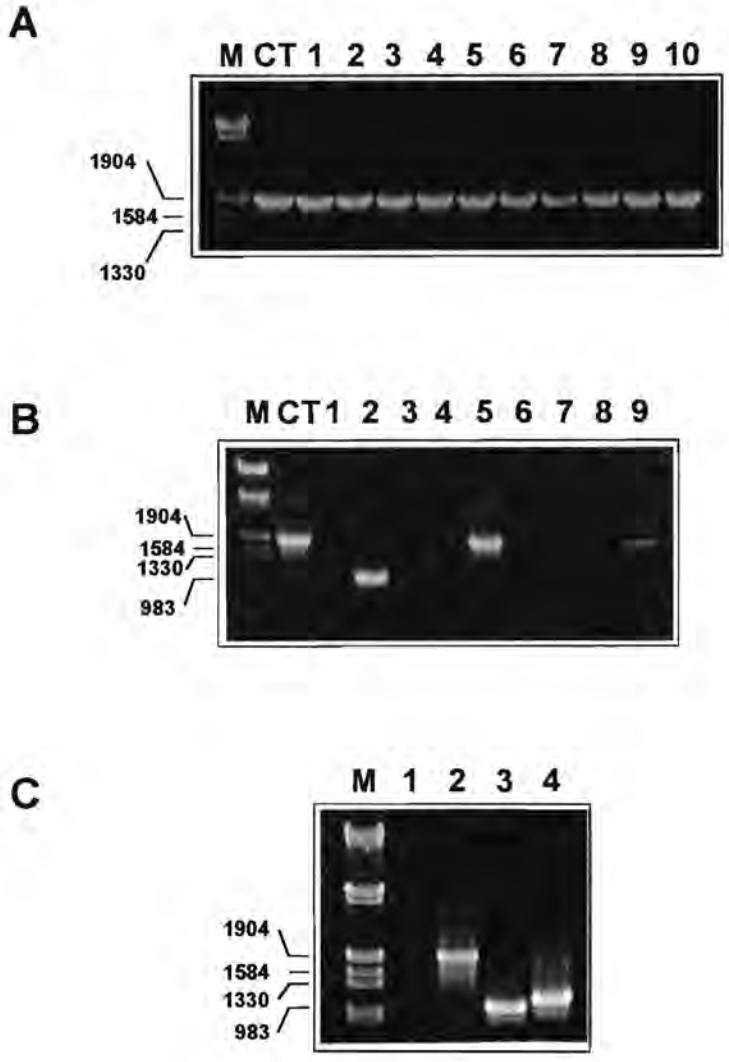


Figure 4.3: Cloning of mutant cDNA's. The mutant PCR products shown in figure 4.2 were digested with various restriction enzymes and ligated to pUC-19 digested with EcoRI and HindIII in a triple ligation reaction as outlined in 4.2.1.3. 5µl of the ligation reaction was used to transform competent E.coli DK-1 cells. Colonies were picked from agar plates and propagated as described in section 6.4.6.2. An aliquot of the cultures was screened for inserts by PCR with the pUC primers. 5µl of the PCR products were electrophoresed on 1.5% agarose gels and the bands visualised under UV light after staining the gels with EtBr (0.25µg/ml). (A); the ΔA mutant; (B); the S269R mutant and (C); the 15bp duplication mutant. The molecular weight markers (M) are shown in base pairs on the left hand side of the figures and a control reaction (CT) with the wild-type p53 cloned into pUC-19 as a template is shown in (A) and (B) to indicate the size of the expected product. The numbers at the top indicate clone numbers.

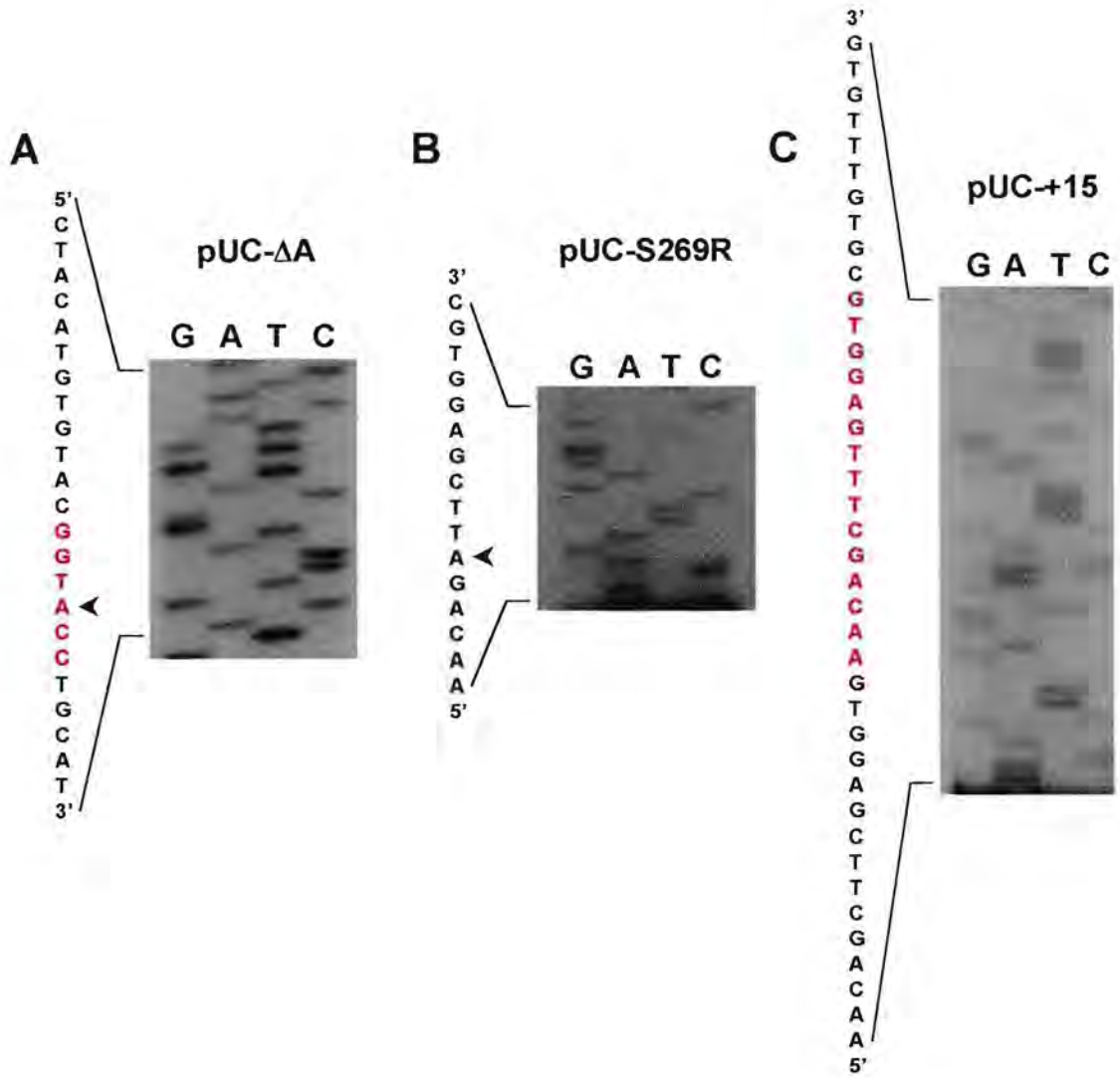


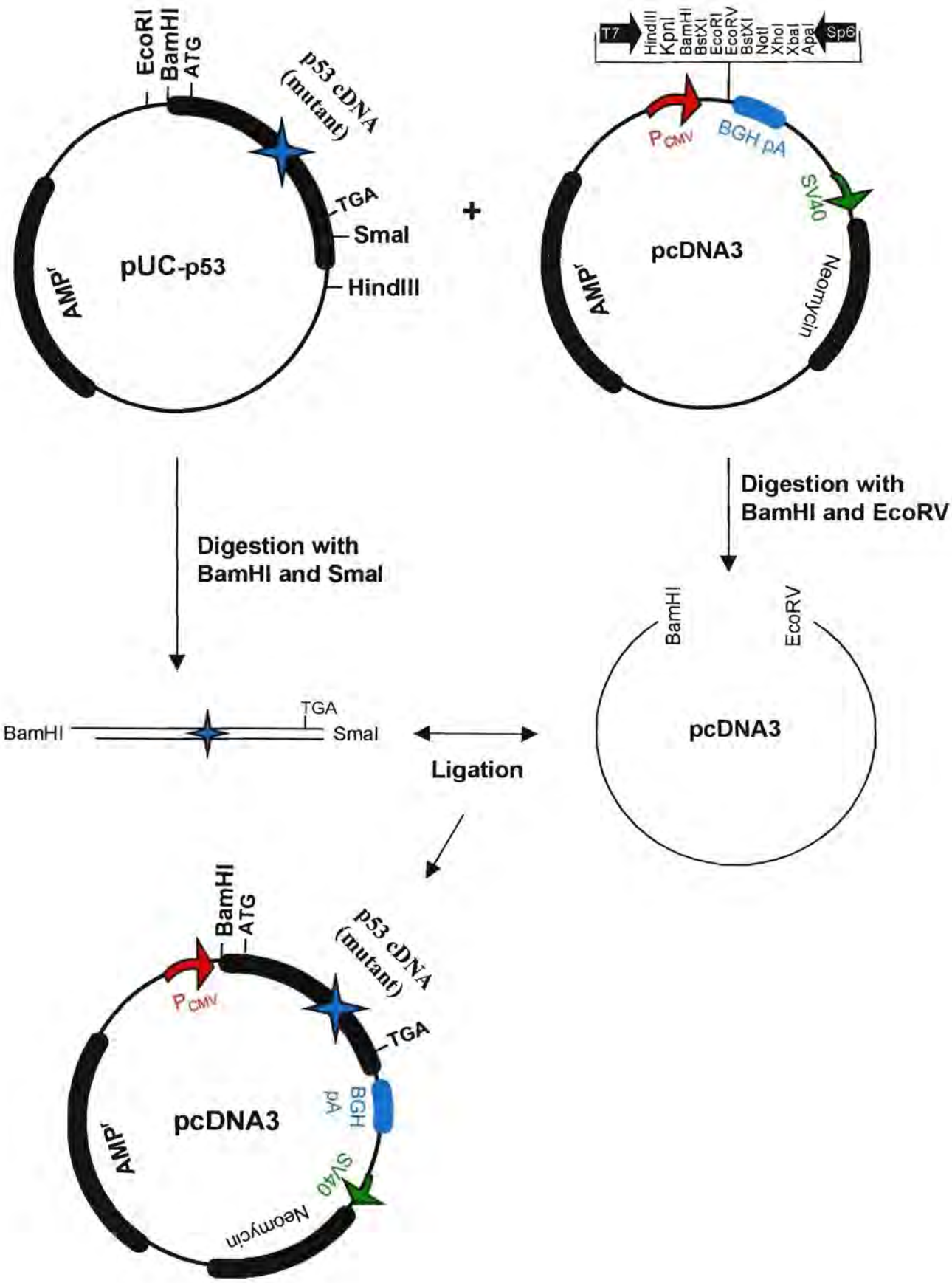
Figure 4.4: Sequence analysis of mutant p53 clones. The clones containing the correct sized inserts in figure 4.3 were subjected to sequence analysis as described in section 6.6.2. The sequencing reactions were separated on denaturing 6% polyacrylamide gels which were then dried and exposed to x-ray film. (A); the sequence corresponding to the ΔA mutant with the point mutation indicated by an arrowhead and the newly created KpnI restriction site depicted in red, (B); the sequence of the S269R mutation, with the point mutation indicated by an arrowhead and (C); the sequence of the 15bp insertion mutant with the duplicated bases depicted in red.

In order to ensure that no other mismatches or incorrect bases were present in the various cDNAs, the entire cDNA for each clone containing the correct mutation was sequenced.

4.2.1.5 Subcloning of the Mutant p53 cDNAs into pcDNA3 Expression Vector

In order to express the various p53 mutant cDNAs, they had to be subcloned into a mammalian expression vector. pcDNA3 was used as it contains the ampicillin resistance gene, providing a selectable marker when grown in bacteria, as well as the neomycin resistance gene as a selectable marker in mammalian cells. pcDNA3 also contains a multiple cloning site downstream of the CMV promoter and a bovine growth hormone polyadenylation site downstream of the multiple cloning site (figure 4.5).

The p53-ΔA, p53-S269R and p53-+15 mutant clones in pUC-19 were digested with BamHI to release the full length cDNA and then subsequently with SmaI (which shortens the cDNA by about 300bp) in order to enable the insert to be cloned in the correct orientation into the expression vector (figure 4.5). The SmaI site is located just downstream of the termination codon in the 3' untranslated region. This also allows the mRNA from the transfected gene to be distinguished from the endogenous message. The digested mutant p53 cDNAs, as well as the wild-type p53 cDNA and the SCX mutant cDNA, were ligated into pcDNA3 which had been digested with BamHI and EcoRV. Colonies were screened for the presence of inserts after transformation of the ligation reactions into competent E.coli cells. Plasmid DNA was isolated from a number of colonies and screened for inserts by digestion with BamHI and XbaI (the XbaI site is downstream of the EcoRV restriction endonuclease site, figure 4.5) and analysis on 1.5% agarose gels (figure 4.6). The correct insert size is 1.5kb, as indicated by the arrowheads. Similar screening was carried out for the wild-type p53 cDNA and the p53-SCX mutant and clones which yielded similar results (data not shown).


EcoRI
BamHI
ATG
p53 cDNA (mutant)
TGA
SmaI
HindIII
pUC-p53
AMPr
+
T7
HindIII
KpnI
BamHI
BstXI
EcoRI
EcoRV
BstXI
NotI
XhoI
XbaI
ApaI
Sp6
PCMV
BGH pA
SV40
pcDNA3
AMPr
Neomycin
Digestion with BamHI and SmaI
Digestion with BamHI and EcoRV
BamHI
TGA
SmaI
Ligation
BamHI
EcoRV
pcDNA3
BamHI
ATG
p53 cDNA (mutant)
PCMV
TGA
BGH pA
SV40
pcDNA3
AMPr
Neomycin

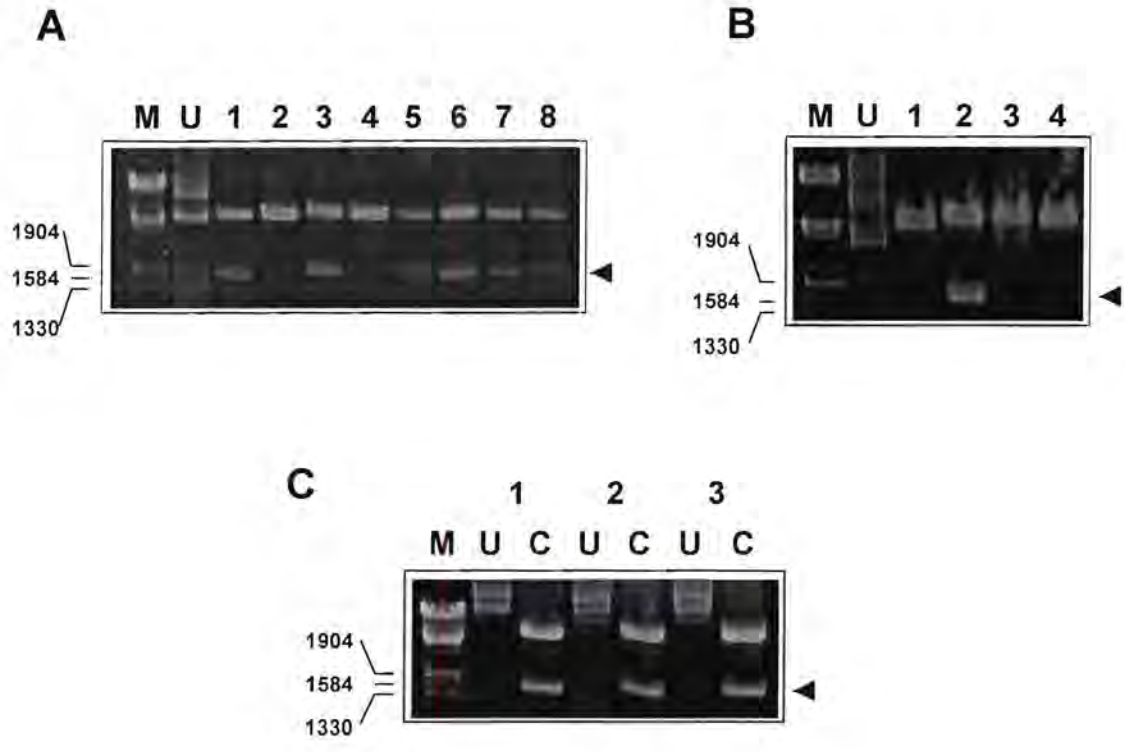


Figure 4.6: Subcloning of the mutant cDNA's into pcDNA-3. The mutant p53 cDNA's cloned into pUC-19 (figure 4.4) were subcloned into pcDNA-3 after release from pUC-19 by BamHI and SmaI digestion and ligation into pcDNA-3 digested with BamHI and EcoRV. The ligation reactions were transformed into competent E.coli cells and colonies screened for inserts by restriction endonuclease digestion. DNA from overnight cultures was isolated and incubated with HindIII, which cuts 5' of the cDNA, and XbaI, which is situated downstream of the EcoRV site in pcDNA-3. The resulting DNA fragments were resolved on 1.5% agarose gels and the bands visualised under UV light. (A); the p53-ΔA mutant, (B); the p53-S269R mutant, and (C); the p53-+15bp mutant. U represents uncut plasmid in all the figures and the numbers indicate various clones digested with HindIII and XbaI. C represents the digested DNA. The molecular weight markers are shown in base pairs on the left hand side of the figures and the expected 1.5Kb fragments are indicated by the arrowheads on the right hand side.

4.2.2 Transfection of p53 Constructs into NIH-3T3 Cells

In order to ascertain whether the mutant p53 affected the growth of cells, the constructs described above were transfected into NIH-3T3 cells. Initially the constructs were transfected into MCF-12A cells, which are normal human breast epithelial cells, but after isolating a number of stable clones, no expression of any of the constructs could be detected. No expression of the constructs was detected in stably transfected FG0 cells (a human skin fibroblast cell line) either and it was decided to transfect the constructs into NIH-3T3 cells, a mouse fibroblast cell line. The calcium phosphate method was used to transfect the DNA into the cells (section 6.10).

4.2.3 Northern Blot Analysis of RNA from Stable Transfectants

Northern blot analysis was used to determine whether the stably transfected clones described above were expressing p53 mRNA. RNA was isolated from the clones as described in section 6.7.2 and 5μg was electrophoresed on 1% agarose gels containing 8% formaldehyde, transferred onto Hybond-N nylon membranes and probed with a radiolabelled fragment of the p53 cDNA. As a control, and also to determine whether the constructs were active, the p53-SCX mutant cDNA was transiently transfected into SVWI-38 cells (transformed human embryonic lung fibroblasts). The RNA from these cells was separated in parallel with the RNA from the NIH-3T3 transfectants (figure 4.7). In the control (SV) lanes, the middle band corresponds to the endogenous transcript and the bottom band corresponds to the transcript from the transgene. The top band is non-specific cross hybridisation of the probe. In the top panel the 3T3 lane represents RNA from untransfected NIH-3T3 cells, and as can be seen, the human probe for p53 used to screen the blots does not detect the mouse p53 RNA. The specific transcripts for the various constructs are indicated by arrowheads on the right hand side of the panels. It is clear from figure 4.7 that some clones strongly express the RNA, whereas others express it weakly (compare clones S3 and S9 with clone S6 for the p53-S269R mutant and clones W2 and W5 with clone W3 for the wild-type). A possible explanation for this is that the number of copies of the plasmid may be different in the various clones. Based on the above data, various clones were selected for further analysis. These were clone #W2 for the wild-type; clone #A6 for the p53-ΔA mutant; clone #S3 for the p53-S269R mutant;

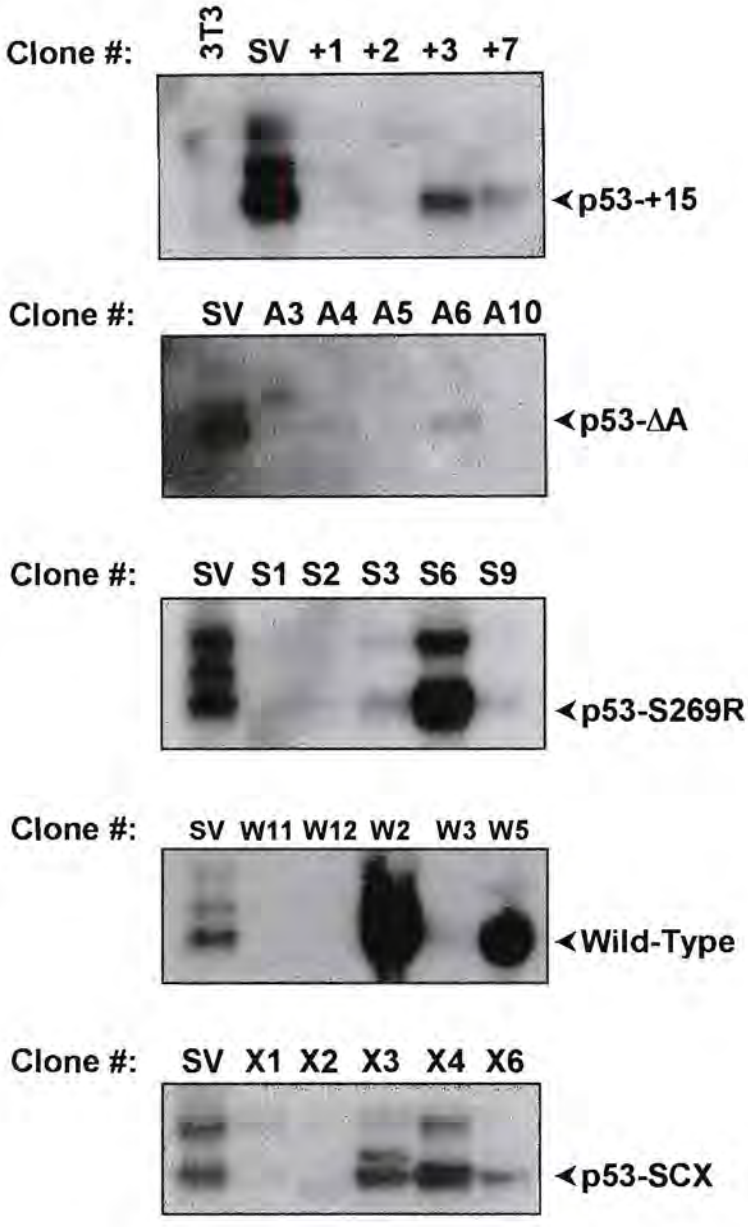


Figure 4.7: Northern blot analysis for p53 mRNA in stably transfected NIH-3T3 clones. RNA was isolated from the clones as described in section 6.7 and 5μg electrophoresed on a 1% agarose gel containing 8% formaldehyde. The RNA was transferred onto Hybond-N membranes and the blots probed with a ^{32}P-labelled fragment of the p53 cDNA. After hybridisation, the blots were washed and exposed to X-ray film for 16-48 hours. The various panels show examples of northern blots for each of the constructs indicated on the right hand side of the figures. 3T3 in the top panel represents the untransfected cells and SV represents a transiently transfected transformed human embryonic lung fibroblast cell line (SVWI-38). The transfected transcripts are indicated by arrowheads on the right hand side of the figures. The middle band in the control lanes corresponds to the transcript of the endogenous p53 gene and the top band is non-specific cross hybridisation of the probe. Clone numbers represent different clones for each construct.

clone #+3 for the p53-+15 mutant and clone #X4 for the p53-SCX mutant.

4.2.4 Analysis of Transfected p53 Protein in the Stably Transfected Clones

Once the expression of the p53 plasmid in the clones had been established, the clones were analysed for protein levels using immunohstochemistry as described in section 6.11.1. Cells were plated on glass slides and allowed to attach and grow overnight at 37°C. The cells were then fixed and probed with the D0-7 anti-p53 antibody (Dako), a mouse monoclonal antibody recognising the N-terminus of both human and mouse p53. The antibody was detected using DAB as a substrate, resulting in a brown colour. Figure 4.8(A) represents immunohistochemistry on the untransfected NIH-3T3 cells, and as expected, no detectable p53 protein was observed as the cells have only wild-type p53 and under normal growth conditions, the protein levels are too low for detection by immunohistochemistry, or Western blots. p53 was located predominantly in the nucleus of the NIH-3T3 cells transfected with the wild-type protein, whilst faint staining was observed in the cytoplasm as well (figure 4.8B). In the p53-ΔA mutant, however, it was clear that p53 was expressed only in the cytoplasm since there was no brown staining in the nuclei at all (figure 4.8C). This is not unexpected as the mutation results in a frameshift which introduces a termination codon at position 263 resulting in a protein that has lost it's nuclear localisation signal, located at the C-terminal end of the protein. Consequently the protein remained in the cytoplasm. The p53-S269R mutant was present in both the nucleus, and to a lesser degree in the cytoplasm (figure 4.8D). It is unclear as to why all of the cells failed to stain for the protein, but since the cells were not treated in a pressure cooker as an antigen retrieval step, the epitope might not be fully accessible to the antibody, or the cells might not have been permeabilised sufficiently to allow the antibody to enter. The p53-+15 mutant protein stained strongly in the cytoplasm, with weak staining in the nuclei whilst the p53-SCX protein was detected only cytoplasmically (figures 4.8 E and F).

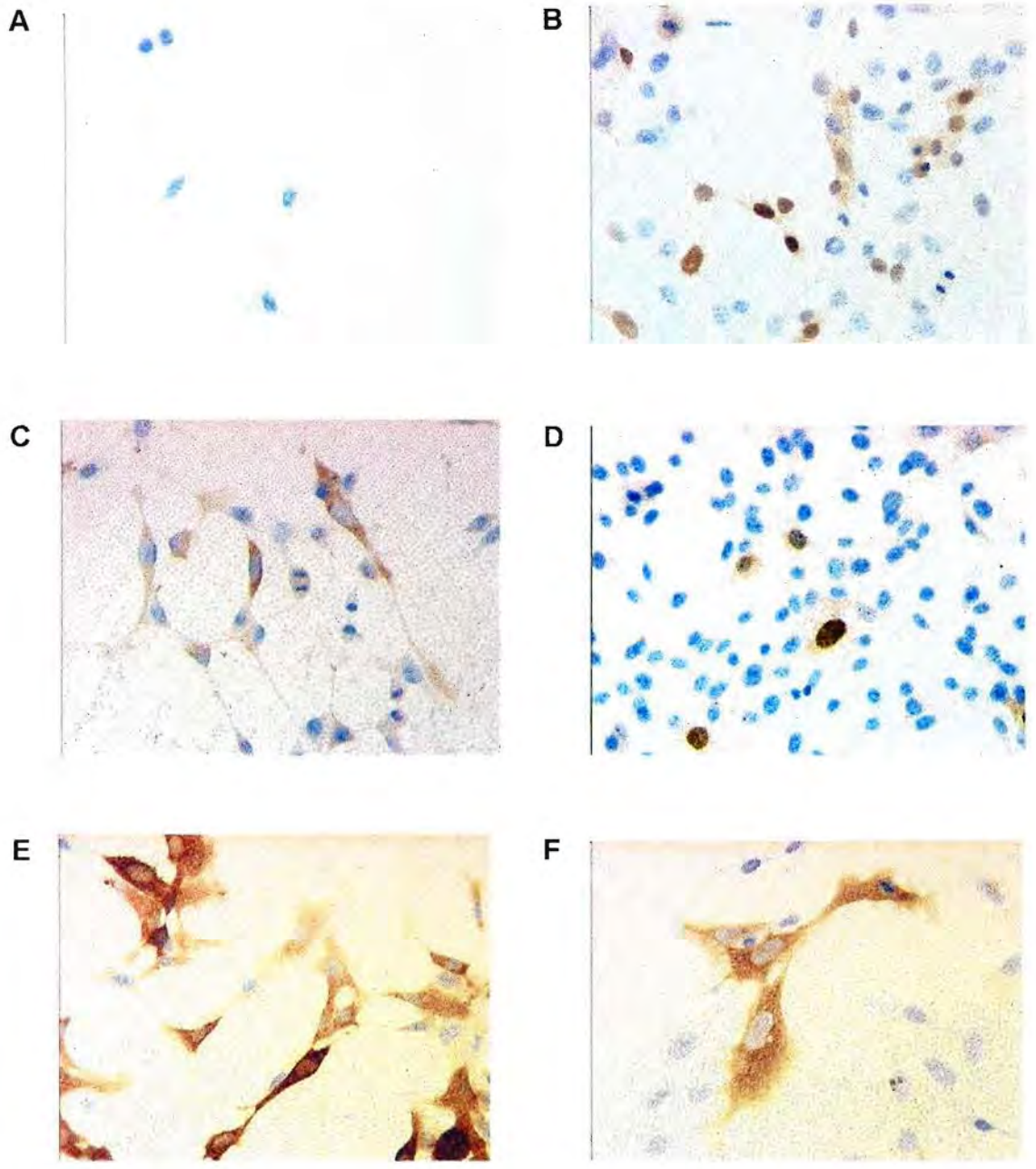


Figure 4.8: Expression of the transfected p53 constructs in NIH-3T3 cells. The stable clones expressing the transfected plasmids were screened by immunohistochemistry as described in section 6.11.1. Cells were plated on glass slides and incubated at 37°C overnight and p53 was detected using antibody DO-7 (Dako). (A); untransfected NIH-3T3 cells, (B); cells transfected with the wild-type construct, (C); cells transfected with the p53-ΔA construct, (D); cells transfected with the p53-S269R construct, (E); cells transfected with the p53-+15 construct and (F); cells transfected with the p53-SCX construct. The brown staining indicated a positive result and the blue staining is haemotoxylin which stains the nuclei.

It was also clear that the different mutants had different effects on the morphology of the cells. The wild-type p53 as well as the p53-ΔA and p53-+15 mutants did not seem to alter the morphology of the cells drastically, in that they remained thin and spindly. The p53-S269R and p53-SCX mutants, however, altered the morphology in that the cells became slightly more rounded and flat while in the case of the p53-SCX mutant, the cells seemed to be bigger in size than the untransfected cells.

4.2.5 Analysis of the Growth Rate of the Transfected Cells

4.2.5.1 Growth in Complete Medium

In order to determine whether expression of the mutant p53 proteins had any effect on the inherent growth rate of the cells, a fixed number of cells were plated in medium supplemented with 10% foetal bovine serum (FBS) and harvested at various times after plating. The doubling time of the various clones was analysed and calculated using Graphpad Prism software (table 4.1). The growth curves are shown in figure 4.9. Although the transfectants all seemed to have a slightly faster doubling time than the untransfected cells, this difference, however, was not significant (table 4.1).

4.2.5.2 Growth in Serum-Free Medium

The growth of the cells in serum-free medium was also analysed, since transformed cells tend to have a lower dependance on serum factors for growth. Once again, 30 000 cells per well were plated in 6-well plates in full medium and allowed to adhere to the dishes. After 24 hours, the medium was removed and replaced with serum-free medium. The cells were harvested 24, 48, 72 and 96 hours after seeding and the cell number determined for calculation of the doubling times as shown in table 4.1. Although there was a slight difference in doubling time between the various clones, these differences were not significant (figure 4.10).

Comparison of the growth rates of the various clones grown in complete medium and serum free medium showed a significant increase in doubling time for the wild-type clone ($p = 0.0077$) as well as the p53-ΔA mutant (0.0284), the p53-+15 mutant ($p = 0.0483$) and the p53-SCX mutant ($p = 0.0304$). There was no significant increase in the doubling time

for the p53-S269R mutant (p = 0.2990) when cultured in serum-free medium versus to full medium.

Table 4.1: Doubling time of the various stable transfectants in full and serum free media. Doubling times were calculated using Graphpad Prism software. 30 000 cells were plated per well in 6-well plates and incubated at 37°C for various time intervals after which the cells were harvested by trypsinisation and the cell number determined using a Coulter counter

GROWTH CONDITIONS	CELL LINE	DOUBLING TIME (HOURS)	P VALUE
Full Medium			
	NIH-3T3	21.14 ± 1.28	
	Wild-type	19.83 ± 3.72	0.5961
	p53-ΔA	19.01 ± 1.73	0.7478
	p53-S269R	19.75 ± 0.44	0.9711
	p53-+15	19.15 ± 0.83	0.7729
	p53-SCX	20.30 ± 0.85	0.8427
Serum-Free Medium			
	NIH-3T3	27.95 ± 4.94	
	Wild-type	33.46 ± 2.97	0.1735 (0.0077)
	p53-ΔA	54.03 ± 17.98	0.1223 (0.0284)
	p53-S269R	24.98 ± 7.58	0.1457 (0.2990)
	p53-+15	33.22 ± 8.63	0.9659 (0.0483)
	p53-SCX	27.37 ± 3.64	0.0880 (0.0304)

P values are for the doubling times of the p53 transfected cells compared to the untransfected cells. P values in brackets represent the comparison of the growth of the different clones in complete medium and serum-free medium.

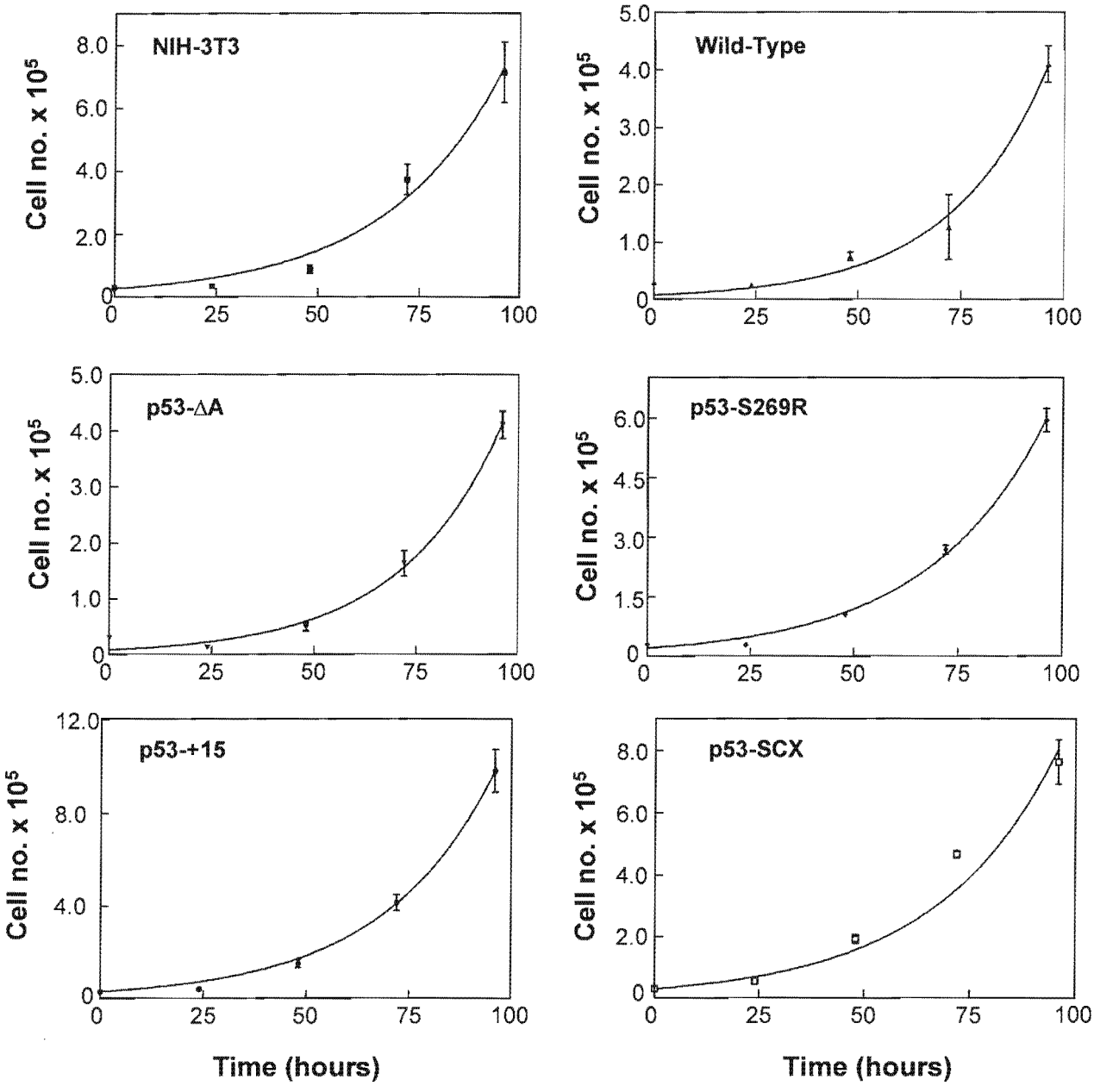


Figure 4.9: Cell growth of cells transfected with the various mutant p53 constructs. Cells were seeded in 6-well plates at a density of 30 000 cells/well in complete medium containing 10% foetal bovine serum (FBS) and incubated at 37°C. Cells were harvested 24, 48, 72 and 96 hours after plating and counted in a Coulter counter. The data were processed using Graphpad Prism software in order to calculate doubling times.

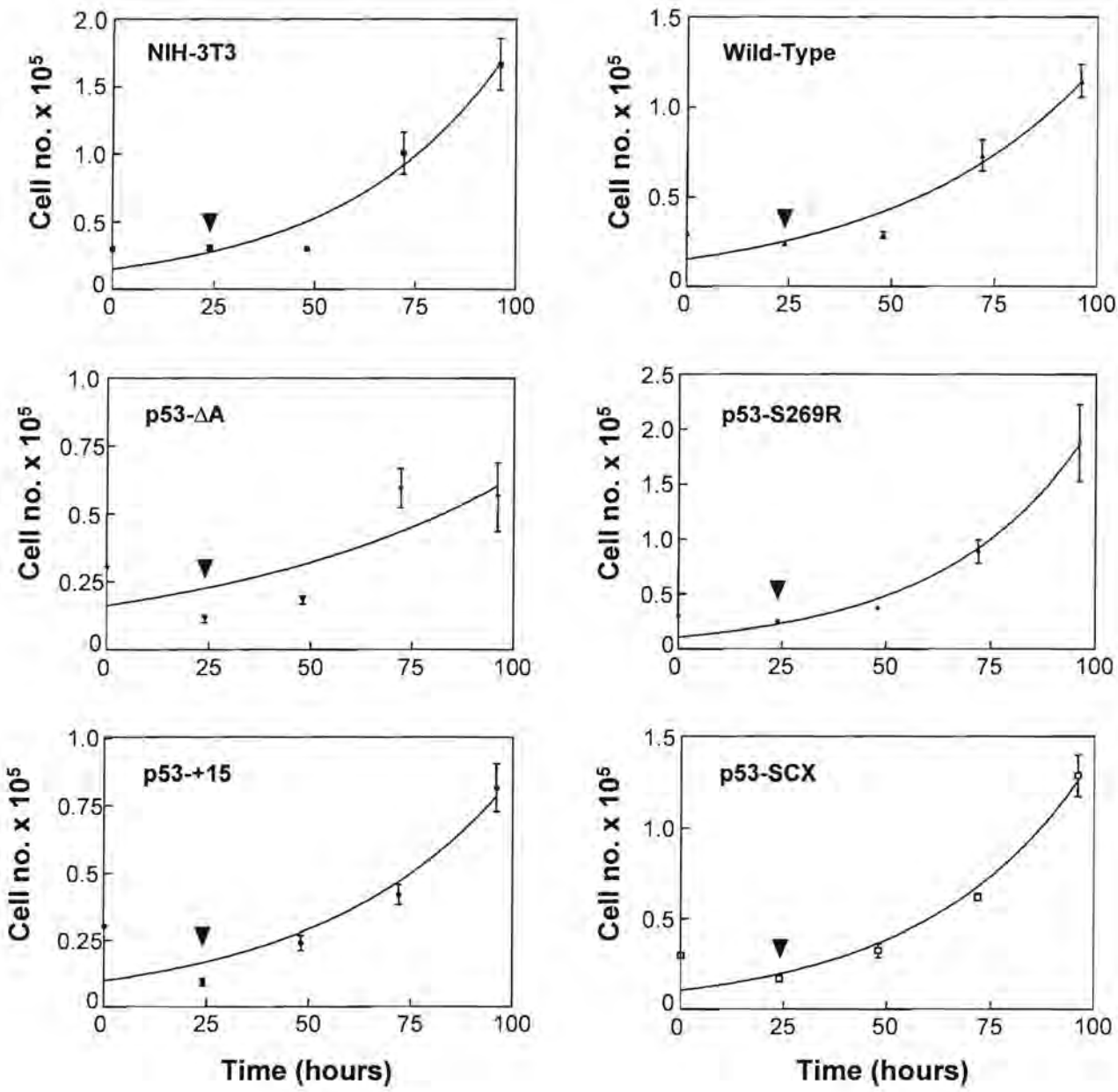


Figure 4.10: Growth rate of cells transfected with the various mutant p53 constructs in serum-free medium. 30 000 cells were seeded into 6-well plates in full growth medium and incubated for 24 hours at 37°C in a humidified incubator. The cells from one well of each dish were harvested and the medium removed from the other wells in the plate. Serum-free medium was added and the cells were returned to 37°C and harvested 48, 72 and 96 hours after plating. The arrowheads in each panel indicate the addition of serum-free medium.

4.2.5.3 Effect of Mutant p53 on Contact Inhibition of Growth

Normal cells become contact inhibited when they reach confluency and it has been shown that transformed or tumour cells do not display this feature when they become confluent. In order to determine the effect of overexpression of the various mutants on contact inhibition of growth, cells harbouring the mutant p53 genes were plated at a high density (200 000 cells) in 60mm tissue culture dishes. The cells were incubated at 37°C and harvested at 24, 48, 72 and 96 hours after plating for determination of cell numbers. As expected, both the untransfected cells and the cells transfected with the wild-type construct exhibited contact inhibition of growth, although the wild-type cells appeared to be contact inhibited at a slightly higher cell density than the untransfected cells (figure 4.11). The p53-S269R mutant and the p53-+15 mutant however, did not seem to show any contact inhibition over the time course of the study. No significant conclusion can be reached on the p53-ΔA mutant data as at the end of the study (96 hours) there were less cells than the wild-type transfectants and they had not become contact inhibited. A possible explanation is that at high densities these cells grow more slowly than those transfected with the wild-type plasmid. This result was consistent and reproducible and it might be that the cells would become contact inhibited if allowed to grow for longer than 96 hours. The p53-SCX mutant behaved like the wild-type transfectant in that it exhibited contact inhibition at a high cell density.

4.2.6 Anchorage-Independent Growth of the Transfected Cells

One of the characteristics of a transformed cell is the ability to grow and form colonies in soft agar in the absence of a solid support. 5 $x10^3$ cells were mixed with 5ml of 0.35% agar (Sigma) in normal growth medium and overlayed onto a 0.6% agar base (also in normal growth medium) in 60mm dishes as described in section 6.12. After 3 weeks the plates were examined for the presence of colonies. The untransfected cells (figure 4.12A) formed numerous small colonies, however, no colonies were bigger than 0.2mm in diameter. Similarly the cells transfected with the wild-type p53 construct formed numerous small colonies that were only slightly larger than the untransfected cells (figure 4.12B). The p53-ΔA mutant formed very few colonies, and those that did form were relatively small, whereas the remaining three cell lines all formed colonies

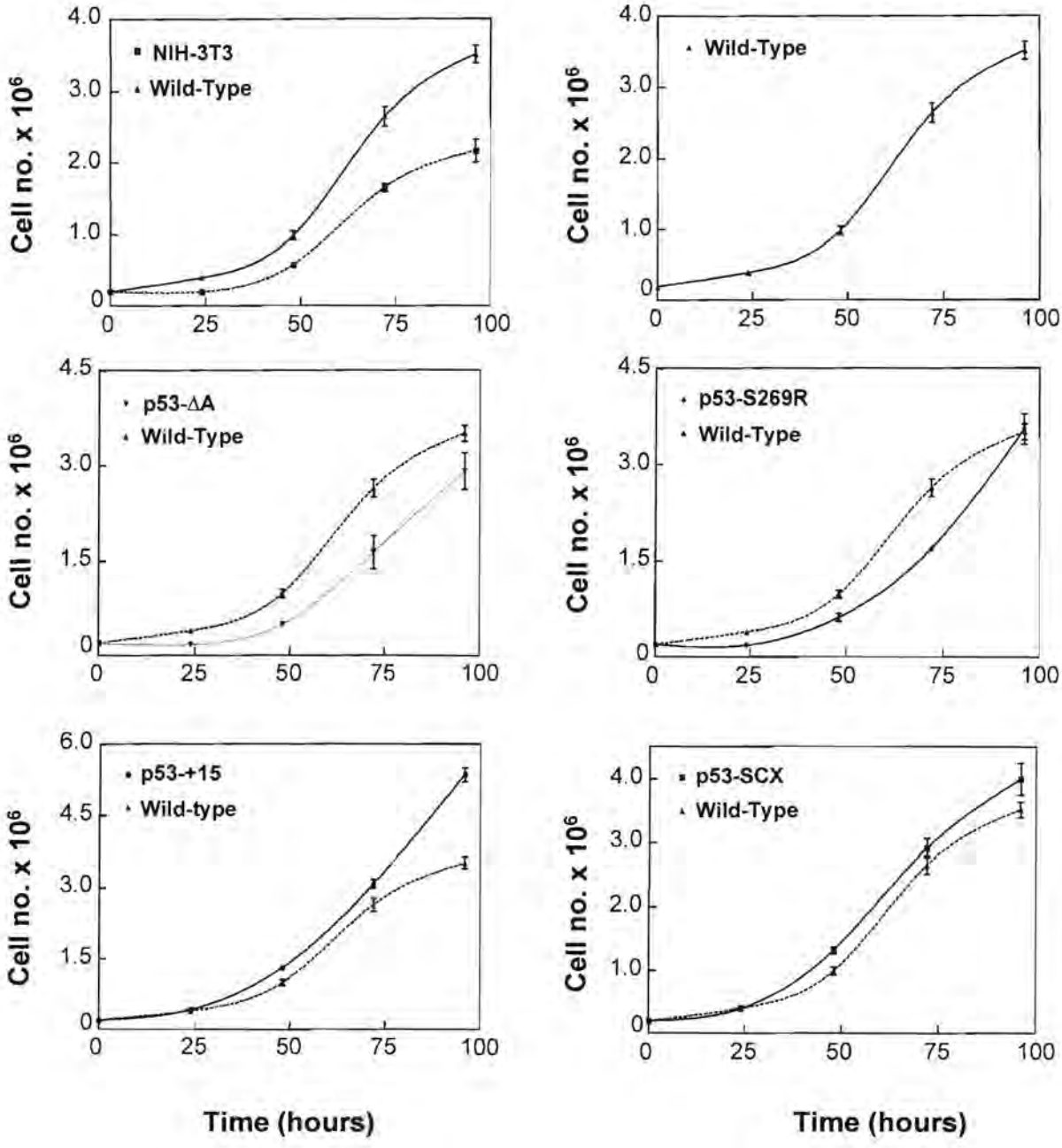
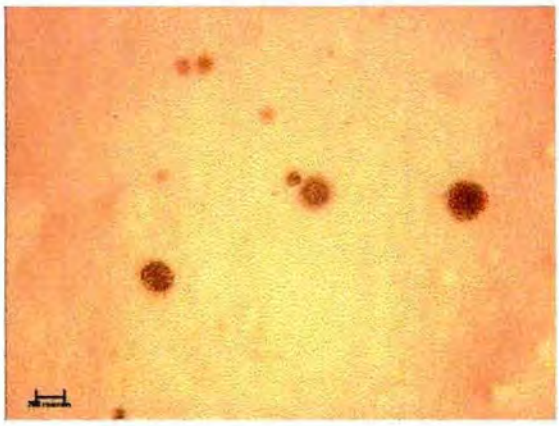
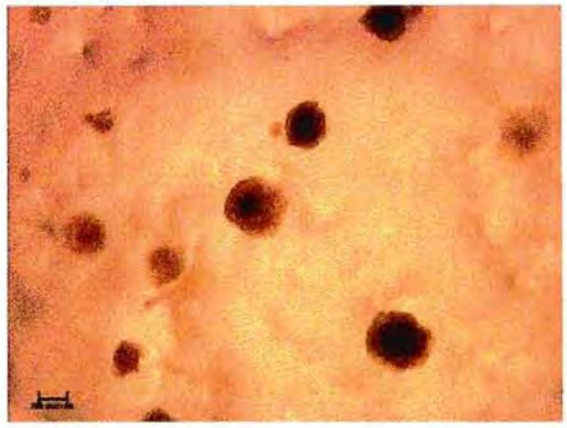


Figure 4.11: Contact inhibition of growth of cells harbouring mutant p53 genes. Cells were plated at a density of 200 000 cells per 60mm tissue culture dish, in complete medium containing 10% FBS, and incubated at 37°C for 24, 48, 72 and 96 hours, after which the cells were harvested and cell number determined in a Coulter counter. The curve for the wild-type cells is plotted with the cells harbouring the various mutants in each panel for comparison. Each time point was done in triplicate.

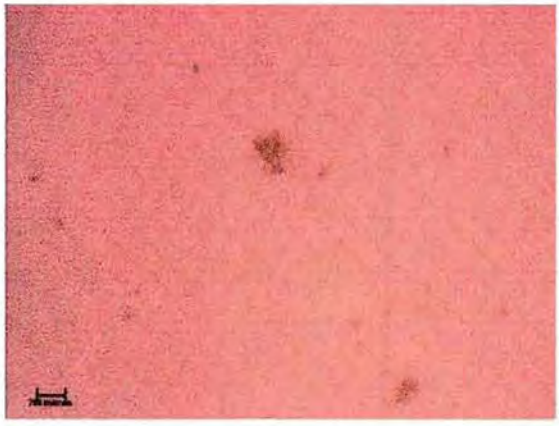
A NIH-3T3



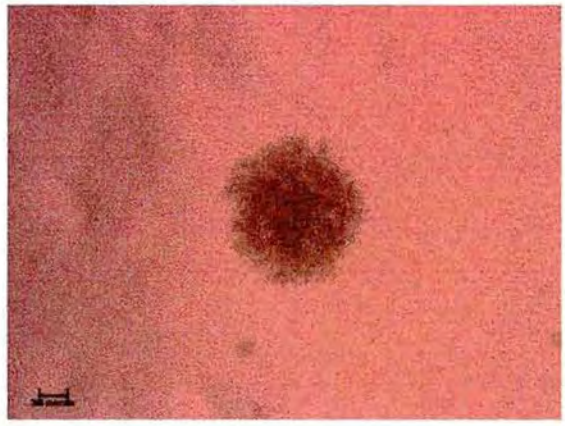
B Wild-Type



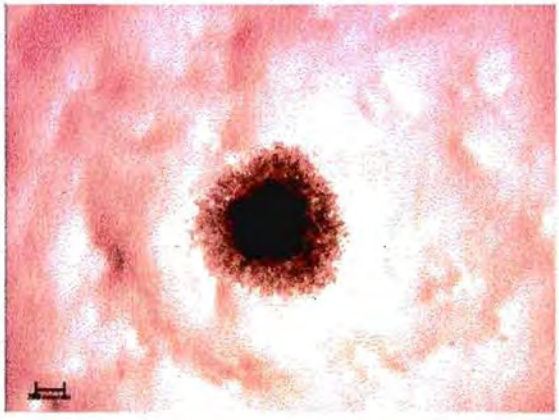
C p53-ΔA



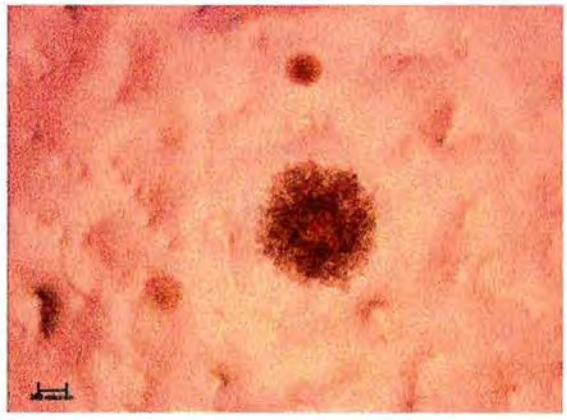
D p53-S269R



E p53-+15



F p53-SCX



significantly larger than 0.2mm in diameter, with the p53-S269R mutant having an average of 15 large colonies per plate, the p53-+15 mutant having an average of 4 large colonies per plate and the p53-SCX mutant an average of 6 colonies per plate (figures 4.12 C-F and table 4.2). In addition, the colonies formed from the latter 3 mutants were more amorphous in shape than those formed by the untransfected and wild-type clones (compare figure 4.12 A and B with D, E and F). The wild-type and untransfected colonies were more round than the mutant colonies.

4.2.7 Effect of Ionising Radiation on the Cell Cycle Kinetics

One of the roles of p53 is that it responds to various shocks to the cell to prevent the cell from entering the S phase of the cell cycle when exposed to stress. When normal cells harbouring wild-type p53 are exposed to ionising radiation, approximately 50% of the cells will arrest in the G_1 phase and the other 50% will arrest in the G_2 phase with the time taken to reach the maximum G_2 block being dependent on the cell type (Baker et al., 1990; Diller et al., 1990). Cells harbouring a mutant p53 do not exhibit the G_1 block and the majority of the cells will arrest in the G_2 phase of the cell cycle (Ludwig et al., 1996; Friedlander et al., 1996; Ryan and Vousden, 1998). In order to determine if the novel tumour-derived p53 mutants identified in chapter 3 behave like "true" p53 mutants, the various transfected cells were exposed to ionising radiation (7Gy of ^{60}Co) and analysed at various time points after exposure to determine the number of cells in the G_1 and G_2 phases of the cell cycle.

The results for the various cell lines in this study are shown in figures 4.13-4.18. The untransfected NIH-3T3 cells display normal behaviour when exposed to ionising radiation, reaching a maximum G_2 block at 6 hours after exposure to the radiation (figure 4.13) with approximately 43% of the cells being in the G_2 phase. This block did not last very long and after 12 hours the cells had returned to their normal cycling pattern (in comparison with the un-irradiated control).

The cells transfected with the wild-type p53 behaved exactly the same as the untransfected cells and also reached a maximum G_2 block at 6 hours after irradiation

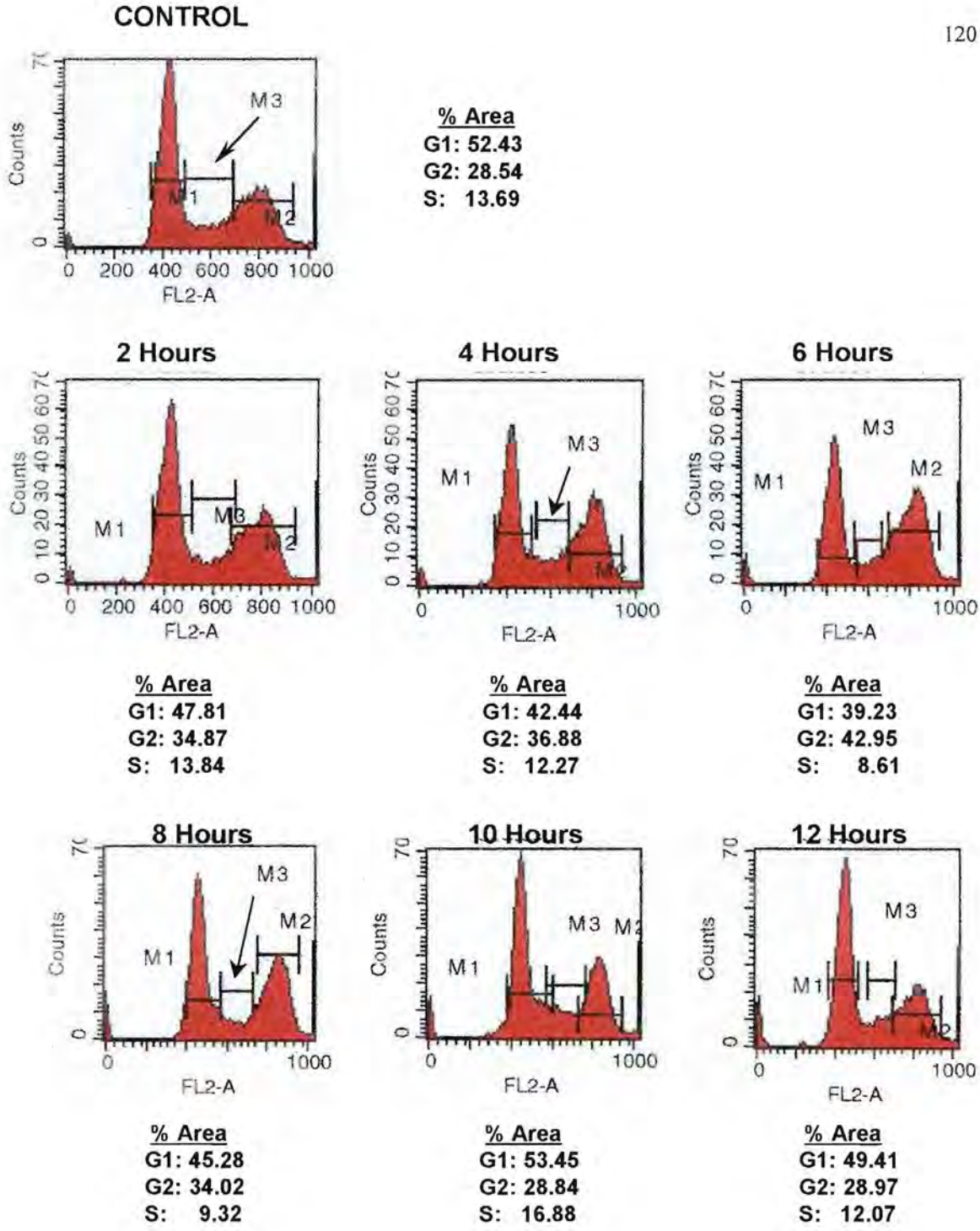


Figure 4.13: Effect of ionising radiation on the cell cycle of the untransfected NIH-3T3 cells. 200 000 cells were seeded into 25ml tissue culture flasks in full medium and incubated at 37°C overnight in a humidified incubator to allow them to adhere to the flask. Cells were then exposed to 7Gy of ^{60}Co radiation and returned to 37°C. The cells were harvested at the various times indicated in the figure and stored overnight in ice-cold 70% EtOH. The cells were then incubated in the presence of RNAse A and propidium iodide and sorted on a flow cytometer as described in section 6.13. The number of events are shown on the Y-axis and fluorescence on the X-axis. Marker M1 corresponds to G_1 phase cells, M2 to G_2 cells and M3 to S phase cells. The area under the curves is given as a percentage of the total area.

(figure 4.14) with approximately 43% of the cells occurring in the G_2 phase of the cell cycle. As with the untransfected cells, the G_2 block did not last very long and by 12 hours the cells had returned to their normal cycling pattern.

The p53-ΔA mutant (figure 4.15) showed a slightly different pattern in that the un-irradiated cells had a slightly higher population of cells in the G_2 phase compared to the untransfected cells or cells transfected with wild-type p53. This cell line, however, had a maximum G_2 block at 4 hours post-irradiation with slightly more than 50% of the cells occurring in the G_2 phase, although there were still a significant number of cells in the G_1 phase (23%). Once again, the cells had returned to normal after 12 hours. This suggested that this mutant did not behave as one would expect of a "true" mutant.

The p53-S269R mutant (figure 4.16) in turn had a different profile, with more cells occurring in the G_2 phase of the cell cycle in the un-irradiated control cells. This cell line attained a maximum G_2 block between 6 and 8 hours post-irradiation, and behaved like a mutant p53 in that the majority of the cells (73%) were in the G_2 phase with only a small percentage (13%) in the G_1 phase. Furthermore, the G_2 block was prolonged as even 12 hours after irradiation there were still a significant number of cells remaining in the G_2 phase.

Both the p53-+15 and p53-SCX mutants (figures 4.17 and 4.18 respectively) behaved in a similar fashion after exposure to ^{60}Co radiation. Both showed maximum G_2 blocks at 6 hours after exposure to radiation with approximately 60% of the cells occurring in the G_2 phase for the p53-+15 mutant and 49% of the cells for the p53-SCX mutant. The blocks in both cases did not last long and by 12 hours post irradiation both cell lines had resumed to normal cycling, compared to the control. Both of these mutants did not behave exactly as "true" mutants, however, they also did not behave in a similar fashion to the wild-type.

In all the figures, the percentages given did not add up to 100%, due to cell debris and other smaller peaks which occurred in each profile. In summary, the untransfected and

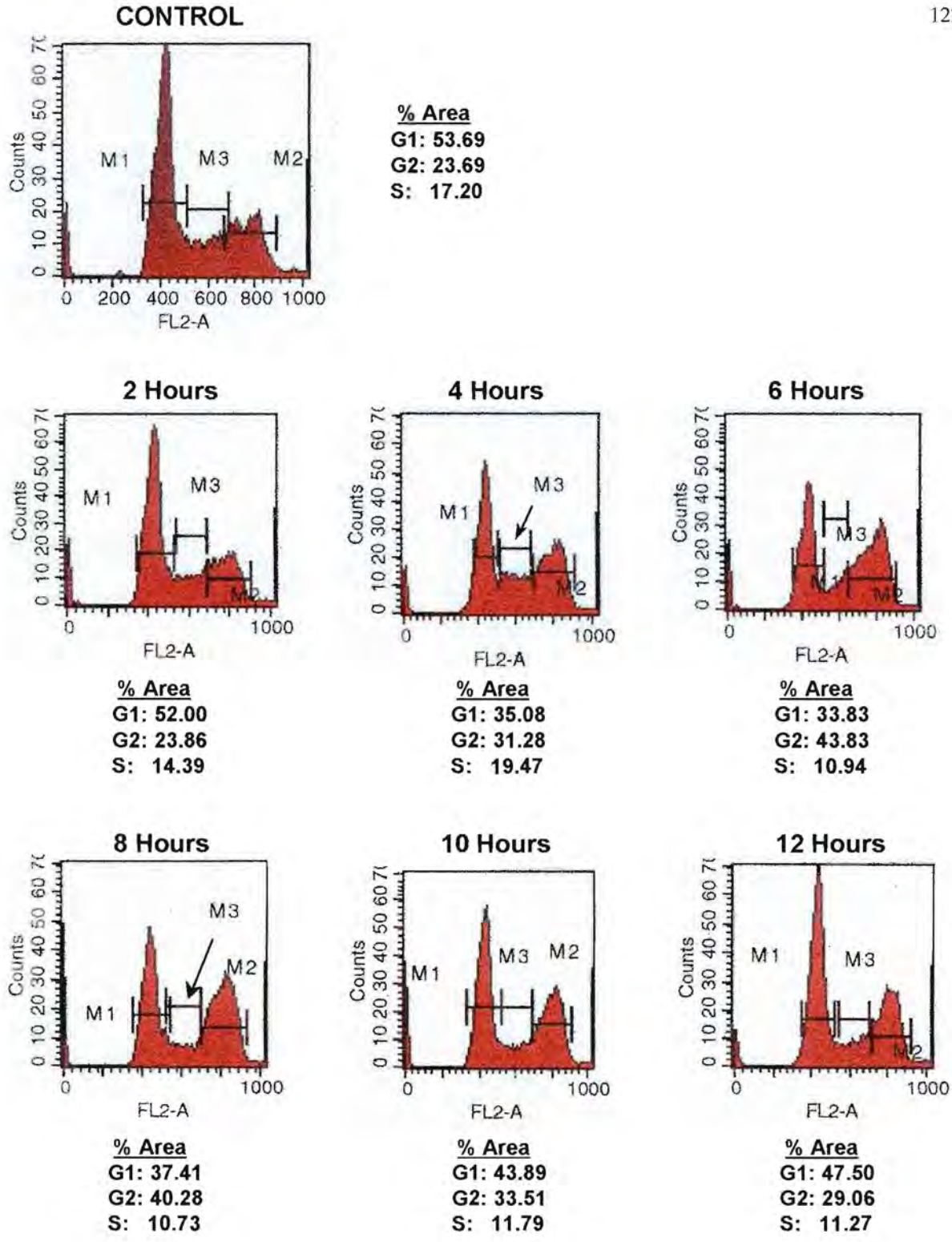


Figure 4.14: Effect of ionising radiation on the cell cycle of cells transfected with wild-type p53. The cells were treated as described in the legend to figure 4.13, except the cells used were NIH-3T3 cells stably transfected with the wild-type p53 cDNA. All markers are as described in figure 4.13.

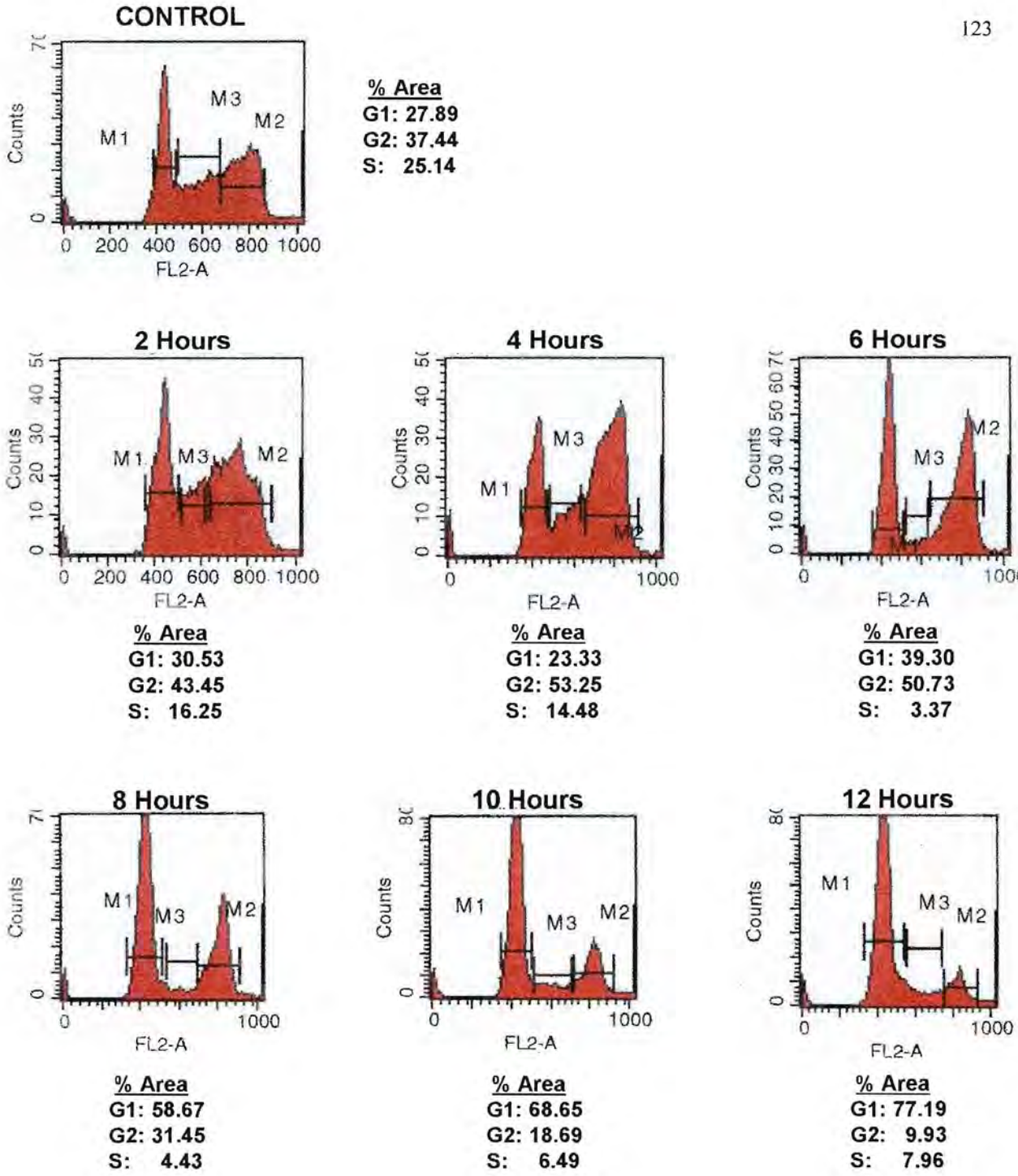
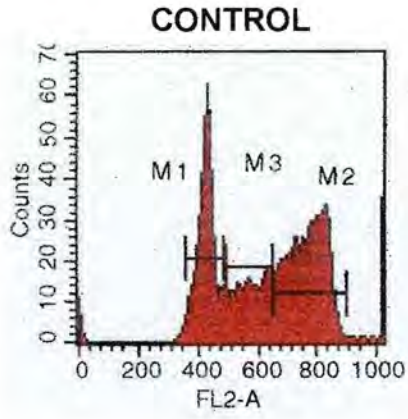
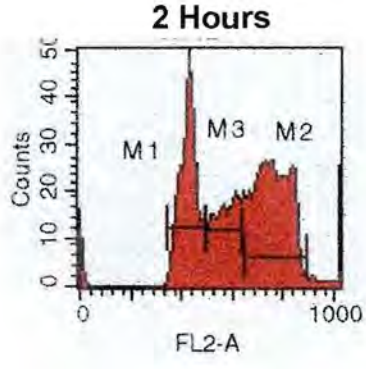


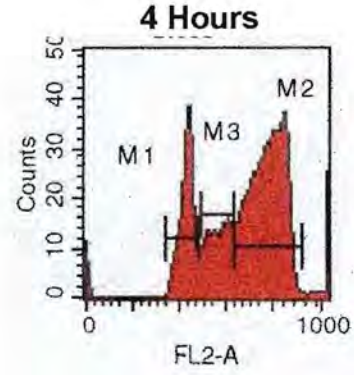
Figure 4.15: Effect of ionising radiation on the cell cycle of cells transfected with thep53-ΔA mutant. The NIH-3T3 cells stably transfected with the p53- ΔA mutant were exposed to ionising radiation as described in the legend to figure 4.13 and the percentage of cells in each phase of the cell cycle was determined as previously described. All markers are as for figure 4.13.



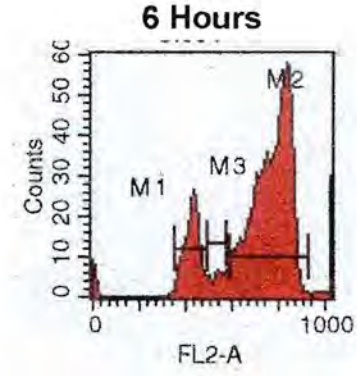
% Area
G1: 29.18
G2: 44.26
S: 19.59



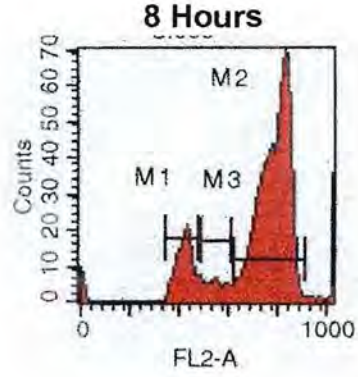
% Area
G1: 29.42
G2: 41.85
S: 19.91



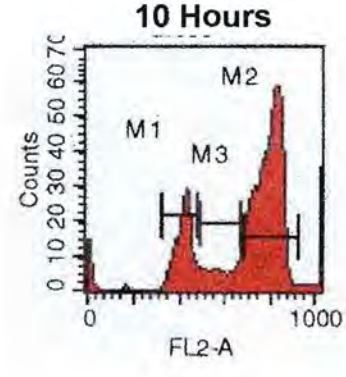
% Area
G1: 19.78
G2: 56.37
S: 16.84



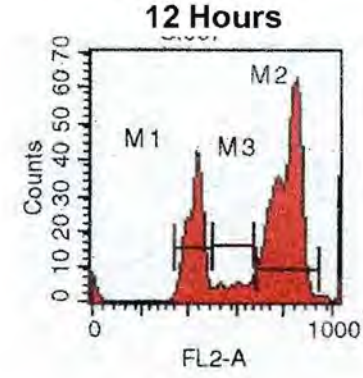
% Area
G1: 15.62
G2: 73.42
S: 3.51



% Area
G1: 13.07
G2: 73.49
S: 4.30



% Area
G1: 17.25
G2: 62.57
S: 10.09



% Area
G1: 25.07
G2: 61.50
S: 5.81

Figure 4.16: Effect of ionising radiation on the cell cycle of cells transfected with the p53-S269R mutant construct. NIH-3T3 cells which were stably transfected with the p53-S269R mutant p53 cDNA were exposed to ionising radiation as previously described and the fraction of cells in each phase of the cell cycle was determined. All markers are as described in the legend to figure 4.13.

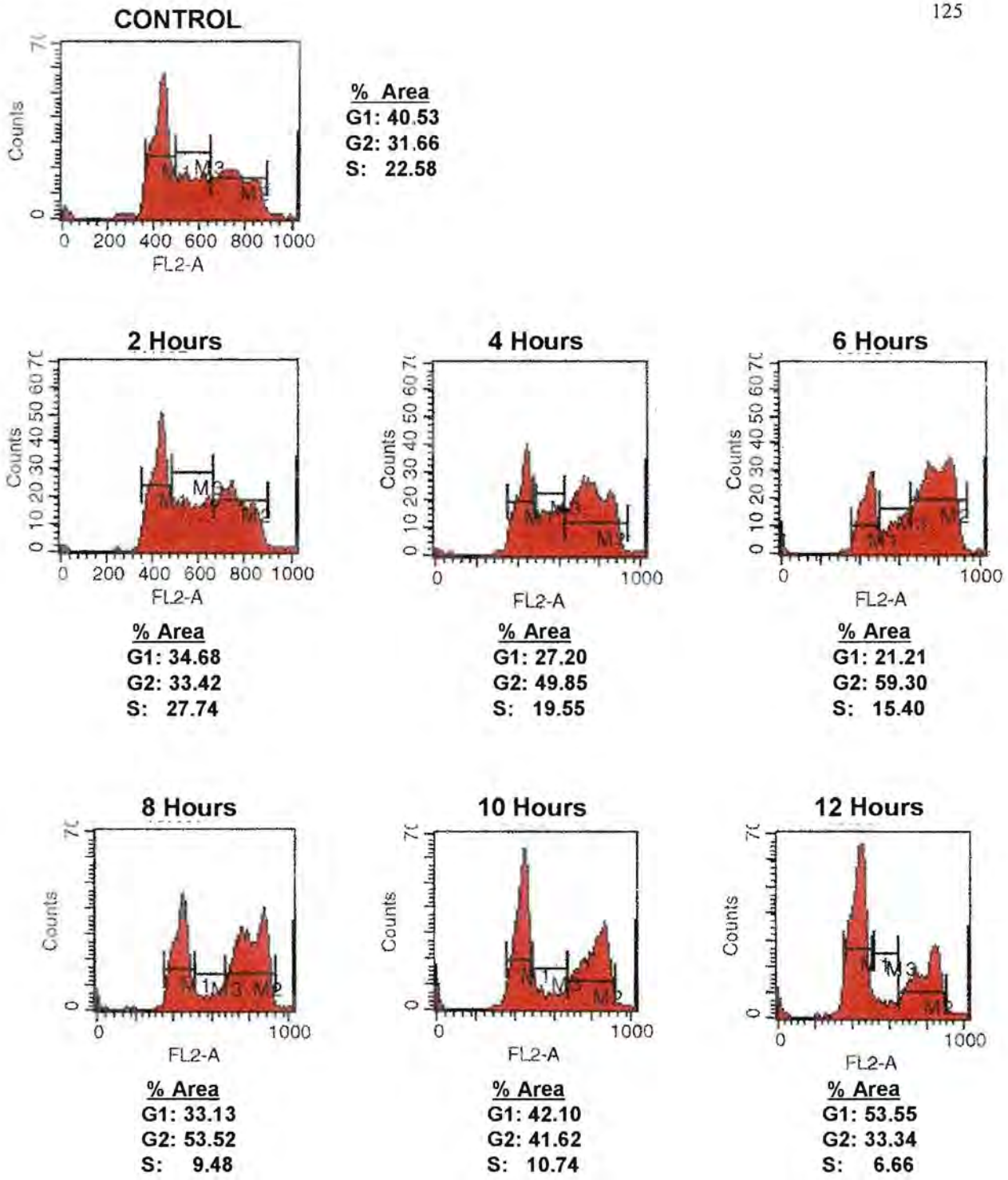
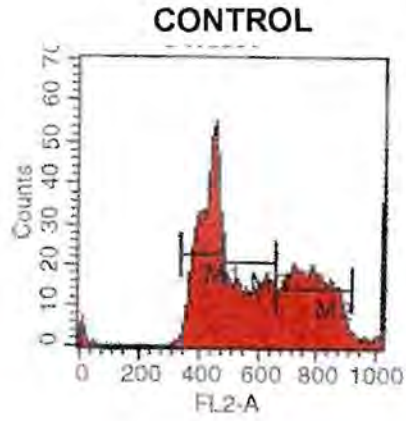
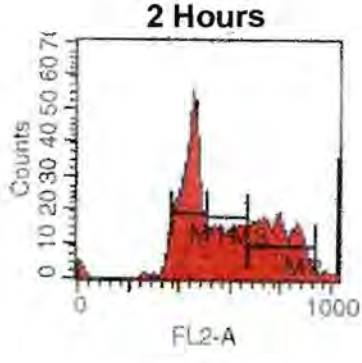


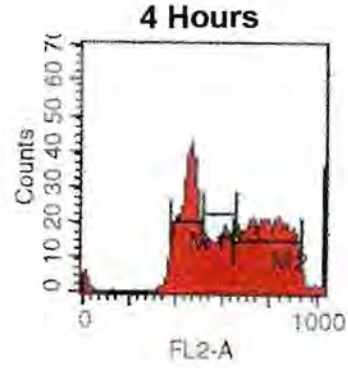
Figure 4.17: Effect of ionising radiation on the cell cycle of cells transfected with the p53-+15 mutant construct. NIH-3T3 cells which were stably transfected with the p53-+15 mutant cDNA were exposed to 7Gy of ^{60}Co as previously described, and the cells were then sorted on a flow cytometer after being incubated at 37°C for the various time points indicated on the figure. All markers are as described in the legend to figure 4.13.



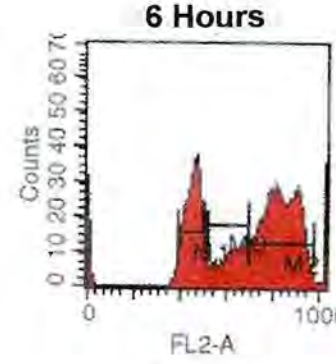
% Area
G1: 38.86
G2: 32.46
S: 23.78



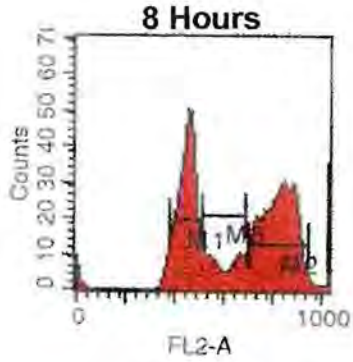
% Area
G1: 38.94
G2: 31.40
S: 20.74



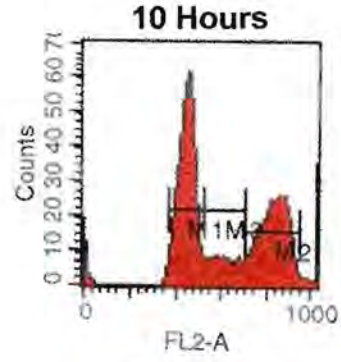
% Area
G1: 30.72
G2: 43.58
S: 18.37



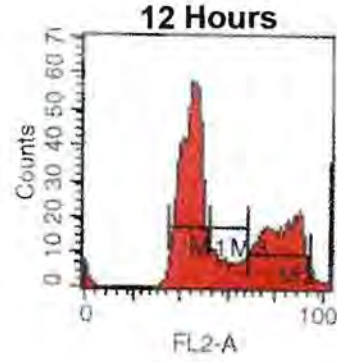
% Area
G1: 24.79
G2: 49.14
S: 15.14



% Area
G1: 34.76
G2: 45.00
S: 11.28



% Area
G1: 45.24
G2: 35.43
S: 11.50



% Area
G1: 52.45
G2: 32.31
S: 8.94

Figure 4.18: Effect of ionising radiation on the cell cycle of cells transfected with the p53-SCX mutant construct. NIH-3T3 cells stably transfected with the p53-SCX mutant cDNA were exposed to ionising radiation as described in the legend to figure 4.13 and the percentage of cells in each phase of the cell cycle was determined after the cells were harvested at the various time points after exposure to the radiation. The markers M1, M2 and M3 are as described in the legend to figure 4.13.

wild-type transfected cells behaved like normal cells, as expected, whereas the p53-ΔA, p53-+15 and p53-SCX mutants behaved differently, although they did not behave exactly like "true" mutants. This could possibly be due to the fact that the p53 protein was expressed predominantly in the cytoplasm, rather than in the nucleus. The p53-S269R mutant, on the other hand, did behave like a "true" p53 mutant in that there were very few cells left in the G_1 phase of the cell cycle after exposure to radiation.

Table 4.2: Summary of the anchorage-independent growth studies. Each cell line was tested in triplicate and the number of colonies in each plate for the various cell lines is shown.

CELL LINE	NO. OF COLONIES
Untransfected	-[a]
Wild-type	-
p53-ΔA	1
	0
	2
p53-S269R	17
	16
	12
p53-+15	2
	6
	4
p53-SCX	5
	5
	8

a The number of colonies for the untransfected and cells transfected with the wild-type were too numerous to count

4.3 DISCUSSION

The p53 tumour suppressor protein is involved in a number of cellular processes such as transcription, DNA repair, cell cycling and apoptosis (Harris, 1996). Mutations in the gene encoding this protein result in mutant forms of the protein that are not able to carry out these functions and thus contribute to the process of tumourigenesis.

The work in this chapter focussed on analysing the functional defects of the three novel tumour-derived p53 mutants described in chapter 3, especially their effects on the growth rate, response to radiation-induced DNA damage and transformation of normal cells. The three mutants were constructed by site-directed mutagenesis and stably transfected into NIH-3T3 cells. In addition to the three mutants described in chapter 3, the wild-type form of the gene as well as a known mutant, p53-SCX (Baker et al., 1990) were also transfected into NIH-3T3 cells. These various forms of the gene were originally transfected into normal breast epithelial cells, but after isolating a number of stable clones, none were found to express the transgene. It was thus decided to use NIH-3T3 cells.

While the wild-type form of the protein was predominantly located in the nucleus, the expression patterns of the various mutants varied. The p53-S269R mutant was also located predominantly in the nucleus, with some cytoplasmic expression, albeit far less. The p53-+15 mutant was located predominantly in the cytoplasm, with some minor nuclear location, while the p53-ΔA and p53-SCX mutants were located solely in the cytoplasm. The cytoplasmic location of the p53-ΔA mutant was not surprising since the mutation results in a frameshift which introduces a termination codon at position 263. This results in a truncated protein lacking the C-terminal region containing the nuclear localisation signal. The protein is therefore not translocated to the nucleus after translation.

The presence of p53 in the cytoplasm is not a novel finding since previous studies have reported the accumulation of p53 in the cytoplasm in tumours. Cytoplasmic p53 has been reported to be a prognostic indicator in colorectal cancer (Sun et al., 1992; Flamini et al.,

1996) and is correlated with poor survival and advanced stage of the disease. In contrast, however, reports on endometrial carcinoma have shown that cytoplasmic overexpression of p53 correlates with a better survival than nuclear expression (Soong et al., 1996). Cytoplasmic p53 accumulation has also been detected in primary breast cancers (Moll et al., 1992; Domagala et al., 1993; Stenmark-Askmalm et al., 1994) where it occurred mainly in lobular carcinomas (Domagala et al., 1993). It seems that cytoplasmic accumulation of p53 is a fairly common occurrence in human tumours and it's effect on survival and prognosis seems to vary between tumour types.

The significance of cytoplasmic p53 is controversial in that many studies have shown that, in the majority of cases, cytoplasmic p53 protein is wild-type (Moll et al., 1992; Moll et al., 1995; Ali et al., 1994). It has been suggested that cellular proteins may bind to p53 and in this way sequester it in the cytoplasm (Moll et al., 1992), which may represent an alternate mechanism to mutational inactivation of p53 function. It has also been suggested that the conformation of the protein plays a role in its intracellular distribution (Zerrhan et al., 1992). Thus it is possible that the p53-+15 and p53-SCX mutants described in this study may be confined to the cytoplasm as a result of some conformational change brought about by the mutation. This is certainly a strong possibility in the case of the p53-+15 mutant as an additional five amino acids have been added to the protein and it is conceivable that the addition of these extra amino acids may alter the conformation of the protein. It may be that altering the conformation of the protein enables it to interact more readily with other cellular proteins which would then sequester them to the cytoplasm. It is still possible that these p53 mutants which remain in the cytoplasm can still act in a dominant negative fashion in that they may form tetramers with the endogenous wild-type form of the protein, and thus assist in sequestering it in the cytoplasm. One of the amino acids duplicated in the 5 amino acid insertion is a serine, and this may introduce a new potential phosphorylation site. Thus another explanation for the cytoplasmic localisation of the mutant protein is that the phosphorylation status may be altered, and it has been suggested that phosphorylation may be involved in the subcellular localisation of the protein in the cell (reviewed by Ozbun and Butel, 1996). It is therefore possible that the p53-+15 mutant is retained in the

cytoplasm due to aberrant phosphorylation.

No significant difference was detected in the growth rate of any of the cells when grown in the presence of 10% serum, however, when the cells were grown in the absence of serum, there was a significant increase in the doubling times for the NIH-3T3 cells transfected with the wild-type p53, the p53-ΔA, the p53-+15 and the p53-SCX mutants, as well as for the untransfected cells. There was, however, no significant change in the doubling time for the p53-S269R mutant. These results suggest that cells showing a decreased growth rate in the absence of serum had a dependance on serum factors for growth, whereas the p53-S269R mutant, which showed no change in growth rate in the absence of serum, had no dependance on serum factors for growth. A previous study has shown that a human hepatocellular carcinoma (HCC) cell line transfected with the wild-type p53 also showed no alteration in growth rate when compared to the same cell line transfected with the p53-SCX mutant if the cells were grown in the presence of 10% serum. However, if the two cell lines were grown in the absence of serum, there was a remarkable change in the growth rate of the wild-type transfected cells, but not of the p53-SCX transfected cells (Terai et al., 1997). Furthermore, there were distinct morphological changes in the wild-type transfected HCC cells as opposed to the p53-SCX transfected cells. No such morphological changes were observed in the NIH-3T3 transfected cells in this study when the cells were switched from medium containing serum to serum-free medium. This is probably due to the different cell types used in the two studies.

Although the various mutants did not have any effect on the growth rate of the cells when grown in the presence of serum, they did, however, to various degrees, affect the contact inhibition of the cells when grown at high cell density. Both the untransfected cells and those transfected with the wild-type p53 exhibited contact inhibition of growth when the cells were grown at a high density, whereas the p53-S269R and p53-+15 mutants did not show any decrease in growth and continued to proliferate in an exponential fashion, even at very high cell numbers. It cannot be ruled out, however, that the cells would eventually become contact inhibited if grown for longer than the 96 hours incubation described in

this study. The p53-SCX mutant behaved differently from the other 2 mutants in that the cells did become contact inhibited when grown at a high cell density. These results suggest that the different p53 mutants have differing effects on the growth of the cells at high cell densities and imply that the p53-+15 and p53-S269R mutants impart some property to the cells which enable them to grow more aggressively at a high cell density than the other mutants, and have allowed the cells to lose their ability to regulate their growth when in contact with neighbouring cells.

In addition to causing loss of contact inhibition of growth, the p53-S269R, p53-+15 and p53-SCX mutants allowed growth in soft agar, while cells transfected with the p53-ΔA mutant were not able to. This ability to grow in an anchorage-independent manner further demonstrates the aggressive growth of the p53-S269R, p53-+15 and the p53-SCX mutant cell lines since the ability to form colonies in soft agar is an indication of the transformed status of a cell.

Although the p53-S269R, p53-+15 and p53-SCX mutants could all form colonies in soft agar and the colonies were of a similar size, the number of colonies for each cell line differed. The p53-S269R mutant was able to form the highest number of colonies with an average of 15 colonies per plate, and the p53-+15 mutant the least, with an average of 3 colonies per plate. This suggests that the degree to which these cells have been transformed differs according to the specific mutant they harbour. Although the p53-SCX mutant cells did not result in any significant growth advantage when grown in tissue culture dishes nor was contact inhibition of growth at high density affected, it did affect the in vitro growth of the cells in soft agar. This result contradicts a previous report which showed that the p53-SCX mutant did not affect the in vitro growth of a colonic epithelial cell line (Williams et al., 1994). These contradictory findings suggest that the same mutant p53 might affect cells from different origins, and possibly different species, in different ways. Other studies have also demonstrated that transfection of cells with a murine p53 mutant construct corresponding to a common human codon 249 mutation in hepatocellular carcinomas results in a growth advantage of the transfected cells in serum-free medium, but the cells were unable to form colonies in soft agar (Dumenco et al.,

1995). This result contradicts the findings for the p53-+15 and p53-SCX mutants, which did not give the cells a growth advantage in serum-free medium, but did, however, allow them to form colonies in soft agar, while the p53-S269R mutation gave the cells a growth advantage in serum-free medium as well as allowing colony formation in soft agar. Taken together, these results suggest that various p53 mutations impart different growth advantages to cells.

The various mutants described in this chapter also have differing effects on the growth arrest of the cells following exposure to ionising radiation. The untransfected cells, as well as the cells transfected with the wild-type p53, both showed a characteristic G_1 arrest and a G_2 block, which reached a maximum at 6 hours after irradiation. The p53-+15 and p53-SCX mutants also showed a maximum G_2 block 6 hours post-irradiation, and although the fraction of cells arrested in the G_1 phase of the cell cycle decreased when compared to the control cells, a significant number of cells were G_1 arrested, which is more characteristic of a wild-type protein than a mutant. This finding is in agreement with other studies that have shown that not all p53 mutations affect the ability of the protein to elicit a G_1 cell cycle arrest. Two other tumour-derived p53 mutants, Pro175 and Leu181 were also able to arrest cells in G_1 (Ludwig et al., 1996) by retaining the ability to activate the $p21^{CIP1}$ gene, a cyclin kinase (CDK) inhibitor. It is possible that the large number of cells still present in G_1 after irradiation is due to the presence of the endogenous murine p53 gene and a reason for the decrease in the proportion of cells arrested at G_1 as compared to the control cells is that the mutant p53 interacts and forms tetramers with the endogenous wild-type protein. Human p53 has been shown to interact with and form oligomers with murine p53 and in fact these heterologous oligomers have a similar affinity for DNA as both human or mouse oligomers alone (Hall and Milner, 1995). The fact that a significant number of cells were G_1 arrested suggests, however, that these two mutants are not completely dominant over the wild-type and as a result of the mutations may have altered conformations, decreasing their affinity to form complexes with the endogenous wild-type protein.

The p53-ΔA mutant cells showed a maximum G_2 block at 4 hours post-irradiation with a

significant population of cells arrested in G_1. The reasons for this could be similar to those suggested above. The p53-S269R mutant, however, showed the characteristics of a true p53 mutant, at least with respect to it's ability to affect the cell cycle after irradiation, in that the G_1 arrest was almost completely abrogated. Furthermore, while in the control cells and the other mutants the G_2 block was transient and fairly short (by 12 hours post-irradiation the cells had resumed cycling), the block in the p53-S269R transfected cells was more prolonged and 12 hours after exposure to radiation, the majority of the cells were still blocked in the G_2 phase of the cell cycle. This suggests that these cells might have a better chance of survival on exposure to radiation in that they remain in G_2 for a longer time and consequently may be more unlikely to undergo apoptosis, which occurs predominantly when the cells are growth-arrested in G_1 (reviewed by Ozbun and Butel, 1996). Consequently, mutations that may have arisen as a result of the radiation would be propagated and may result in further growth advantage of the cells. The small number of cells which were arrested in the G_1 phase of the cell cycle for the p53-S269R mutant could have arisen due to the presence of the endogenous wild-type p53 protein. This mutation behaved in a similar manner to other tumour-derived p53 mutants which were also incapable of arresting the cells in G_1 (Ludwig et al., 1996). A Leu173 and an N-terminal mutant also failed to arrest the cells in G_1 as a result of their inability to activate the $p21^{CIP1}$ gene.

The findings that the different p53 mutants affected the G_1 arrest of the cell cycle differently agree with other studies which have also demonstrated that the p53-SCX mutant, as well as a 175(His) and a 248(Trp) mutant p53 do not inhibit the p53-dependent G_1 arrest after exposure to gamma radiation, whereas a 273(His) p53 mutant did affect the cell cycle (Williams et al., 1995; Pocard et al., 1996). Theses studies also found that loss of wild-type p53 function alone was not sufficient to directly affect the growth rate of the cells and the authors suggested that the mutation may contribute to malignant progression by creating genomic instability due to the inhibition of the G_1 arrest. Furthermore, other mutations would be required to get a clonal outgrowth of the p53 mutant cells. The results in this chapter support this finding and confirm that different p53 mutants can affect the cells in different ways. It has also been shown that different

mutations at codon 175, a frequently mutated codon in human tumours, can behave differently with respect to the ability of the various mutants to abrogate the G_1 cell cycle arrest (Ryan and Vousden, 1998), and a codon 248 mutation retains tumour suppressor functions (Mukhopadhyay and Roth, 1993). The results presented in this chapter with the 3 novel p53 mutants support previous findings that different mutations have different effects on the function of the protein, where some mutations allow cells to lose the ability to regulate their growth while others have little or no effect on the function of the protein. The fact that different mutations at a single codon can affect the function of the protein to varying degrees (Ryan and Vousden, 1998) suggests that the nature of the mutation is important in determining the effect that it will have. The results presented in this chapter support this idea as the 3 novel mutations described are all different types of mutations (a deletion, an insertion and a point mutation) that all affect the function of the protein differently.

In summary, all the mutants did not significantly affect the inherent growth rate of the cells, but had differing effects on the contact inhibition of the cells. They also affected the G_1 arrest of the cells to various degrees after exposure to ^{60}Co radiation and were all capable of growth and colony formation in soft agar, with the exception of the p53-ΔA mutant. The results obtained suggest that the p53-S269R mutant behaves in the most aggressive manner as it shows no dependance on serum factors and it also almost completely abrogates the G_1 block of cells after exposure to radiation. Taken together, the results presented in this chapter and those reported by others, clearly demonstrate that different p53 mutations have different biological effects on normal cells and the potential of different p53 mutations to behave in a dominant negative fashion can vary, depending on the nature of the specific mutation.

CHAPTER 5: CONCLUSION

5.1 CONCLUSION

Breast cancer is a disease responsible for the majority of deaths among women worldwide. Like all other types of cancer, it is a multigenic disorder with an accumulation of mutations that results in the transformation of a normal cell and subsequent clonal expansion of this cell into a tumour. These mutations occur in genes that either positively (oncogenes) or negatively (tumour suppressor genes) control cell growth and proliferation. Mutations in oncogenes result in the constitutive activation of these genes, while mutations in tumour suppressor genes lead to their inactivation or loss of function. Thus by the simultaneous activation of genes which stimulate cell growth and the inactivation of genes which retard cell growth, these mutations result in the loss of cellular growth control and ultimately in the formation of a tumour.

This study focussed on mutations in two such genes, the Ha-*ras* oncogene and the p53 tumour suppressor gene in a population of breast cancer patients from the Western Cape region of South Africa. The majority of these patients were of a mixed race (Coloured), and of the 40 patients studied, 39 were female and one was male.

Mutations in codons 12, 13 and 61 of the Ha-*ras* oncogene have been shown to constitutively activate the protein. No activating mutations were found in any of these codons in any of the patients in this study. These results support other findings that mutations in the *ras* genes are a rare occurrence in breast cancer and suggest that mutations in other oncogenes may play a role in breast cancer (Kraus et al., 1984; Spandidos, 1987).

Although no activating mutations were found in the Ha-*ras* gene, a polymorphism was detected in codon 27. This silent mutation resulted in the creation of a new restriction endonuclease site, thus enabling rapid screening of the patients for this polymorphism. The polymorphism, however, was not associated with the cancer, as the allele frequency

was similar to that of healthy control samples. It must be noted, however, that only a small number of control samples could be obtained and that larger numbers of healthy controls should be examined for this polymorphism in order to get statistically significant results.

The tumour suppressor gene screened in this study was the p53 gene. This gene encodes a nuclear phosphoprotein that plays a critical role in negatively regulating cell growth. It also detects DNA damage and arrests the damaged cells in the G_1 phase of the cell cycle. If the damage is too extensive, then p53 can also induce apoptosis of the damaged cell to ensure that any genetic mutations that have arisen due to the damage are not propagated. In addition to these functions, p53 has also been directly implicated in DNA repair.

It is the most frequently mutated gene found in human tumours, with the frequency of mutations varying between different tumour types.The data presented in this study showed that only a small number of mutations (10% of patients) in the p53 gene were detected by SSCP and DNA sequence analysis. Of four mutations detected, three have not previously been described. These results contrast with other studies that have shown that p53 mutations are a relatively frequent occurrence in breast cancer (Osborne et al., 1991; Cattoretti et al., 1988; Bartek et al., 1990a) and also suggest that mutations in this gene do not play a role in the aetiology of the disease in the group of patients investigated in this study. It is possible that mutations in other tumour suppressor genes are involved in tumourigenesis in these patients.

Immunohistochemical staining, which has also been used as a tool to detect p53 mutations, revealed a total of 10 patients with detectable levels of p53 protein. Unfortunately there was no material for immunohistochemistry on the patient with the 15bp insertion, but two of the remaining three patients with p53 mutations had detectable levels of p53 protein. The patient with the deletion resulting in a truncated protein (p53-ΔA) failed to show detectable levels of p53 protein, and this may be due to it's rapid degradation. Thus eight patients showed an accumulation of p53 protein, but did not contain mutations in the exons screened. This phenomenon of p53 accumulation without

the presence of mutations has been noted previously (Shiao et al., 1996; Shiao et al., 1995) and may be due to a defect in the p53 degradation pathway. p53 is degraded via the ubiquitin pathway when it interacts with mdm2 (Haupt et al., 1997). This interaction occurs at the N-terminal of p53 (Gottlieb and Oren, 1996) coded for by exons 2-4. The eight patients that were positive for p53 by immunohistochemistry were screened for mutations in exons 2 and 4 in order to ascertain whether these exons contained mutations and none were found. It is possible that the mdm2 protein is defective in these patients, and since the stability of p53 is to a large extent governed by its interaction with mdm2, a defective mdm2 protein would result in the stabilisation of p53 and consequently high levels of the protein were detected in these patients. The possibility cannot, however, be ruled out, that mutations exist in the exons not screened (exons 3 and 10) in these patients.

Depending on the nature of the mutation, various tumour-derived p53 mutants may have different effects on normal cells. Some mutations may totally abrogate the wild-type function, whereas others may have little effect, thereby enabling the protein to retain it's normal tumour suppressor function (Mukhopadhyay and Roth, 1993). It has also been documented that different tumour-derived p53 mutants can affect the function of the wild-type protein to various degrees (Ludwig et al., 1996; Friedlander et al., 1996a; Ryan and Vousden, 1998). Therefore, to examine the effect of the three new mutations identified in this study, the mutant cDNAs were transfected into NIH3T3 cells and stable clones expressing the mutant proteins were selected. None of the mutants had any significant effect on the growth rate of the cells when grown in full medium. When the cells were grown in serum-free medium, however, all of the transfectants exhibited a significant decrease in growth rate, with the exception of the S269R mutant, which showed no difference in growth rate in the presence or absence of serum. Furthermore, this mutant resulted in lack of contact inhibition of growth when seeded at a high density.

The ability of cells to form colonies on soft agar is an indication of anchorage-independent growth. When cells expressing the various mutants were cultured on soft agar, their ability to form colonies varied, as well as their ability to cause a G_1 arrest after

DNA damage after exposure to ionising radiation. Taken together, these results indicate that the different p53 mutants have different effects on normal cells and have differing potentials in their ability to aid in cellular transformation. This may be due to the nature of the mutation, as it has been shown that even different mutations at the same position may have widely differing effects (Ryan and Vousden, 1998). It must be remembered that these three p53 mutants were expressed in cells containing endogenous wild-type p53 as well, thus it may be that the varying effects on growth rate and response to DNA damage may, in part, be governed by the ability of the different mutants to interact with and inactivate the wild-type protein in a dominant negative fashion. Nevertheless, the results presented in this study are consistent with those of others in that different tumour-derived p53 mutants can have different effects on cells (Ludwig et al., 1996; Friedlander et al., 1996).

The p53 status of a tumour may also play a role in the response of patients to both chemo- and radiotherapy. The disease-free survival of the patients used in this study was found to be very high, with over 70% of the patients still alive 5 years after diagnosis. Lymph node status, oestrogen receptor status, and p53 status did not seem to affect the survival of the patients. The reason for the lack of association between the p53 status and patient survival may be due to the small number of mutations detected. Interestingly, only one patient with a p53 mutation died from the disease, this was the patient with the S269R mutation, which had the most detrimental affect on the control of cell growth when transfected into normal cells. Although only a small number of mutations were examined in this study, these preliminary results suggest that the p53 status of a tumour can play a role in the response of a patient to therapy, as the majority of the group did not have p53 mutations and most of the patients were still alive 5 years after diagnosis. Furthermore, of those patients with p53 mutations, only one died from the disease, with this being the most aggressive mutation. The other three mutations did not have any significantly detrimental affect on cell growth or response to DNA damage, thus one could say that they retained some wild-type function and consequently the patients were able to respond favourably to treatment. It would be unwise to claim that p53 status alone could be a predictor of response to treatment, other factors must surely also play a role.

But the status of the p53 gene in breast tumours may be a key factor in the response of a patient to therapy.

In summary, the results in this study imply that genetic mutations in breast cancer may vary between population groups, and it may be possible that different population groups may accumulate different genetic alterations, but still develop the same type of tumour. Furthermore, with respect to p53 mutations, the data presented here demonstrate that the p53 status of a tumour may play a role in the response of patients to therapy. The data also show that different p53 mutations have different effects on cells, and consequently different p53 mutations may affect the outcome of a patient to varying degrees. This makes it important to identify p53 mutations in tumours and to investigate their functional significance, as they may have a critical bearing on patient survival. By analysing various p53 mutants, it may be possible to develop some classification of mutations that would aid in the choice of treatment modality.

5.2 FUTURE WORK ARISING FROM THIS STUDY

1) Since very few mutations were detected in these patients, it would be important to screen other population groups, such as whites or blacks, with breast cancer, in order to ascertain whether this low frequency of p53 mutations is specific for the "Coloured" population, or whether it is common to other race groups in South Africa.

2) It would be necessary to investigate different population groups from various geographical locations to determine the nature and frequency of p53 mutations in these people, as different populations may be exposed to different environmental/chemical mutagens and carcinogens which may result in a specific type of mutation.

3) Further studies could be done on the three new mutations identified in this study to determine their effect on p53 responsive genes, such as $p21^{CIP1}$, and to elucidate the exact mechanism(s) by which these mutants exert their effects on cells.

4) The effect of various chemotherapeutic agents on stably transfected cells could be determined to see whether any of the mutants are able to impart some sort of drug resistance to the cells. This may also aid in determining the kind of agent that would be most effective against the tumour.

CHAPTER 6: MATERIALS AND METHODS

6.1 ISOLATION OF DNA

6.1.1 Isolation of DNA from Fresh Tissue

Tissue samples were frozen in liquid nitrogen in the operating theatre immediately after excision, and stored at -70°C until required. The tissue samples were homogenised in 2 volumes of digestion buffer (section 6.14) using an Ultra-Turrex homogeniser and incubated overnight at 50°C. The next day, 2ul of RNase A (10mg/ml) was added and the samples were incubated at 50°C for another 30min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the samples were shaken vigorously and centrifuged at 5000rpm for 10min in a Beckman J-21C centrifuge. After centrifugation, the aqueous phase was mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged as above. The DNA was precipitated by the addition of 2 volumes of 96% ethanol (-20°C) to the aqueous phase, washed in 70% ethanol and dried under vacuum in a Savant SVC100 Speedvac. Finally the DNA was resuspended in a suitable volume of 1X TE buffer and stored at -20°C until required.

6.1.2 Isolation of DNA from Cell Lines

Confluent 150mm dishes of cells were rinsed with PBS followed by the addition of 6ml of 0.05% trypsin in PBS containing 10mM EDTA and incubation at 37°C until the cells lifted from the dish (approximately 3min). The cells were pelleted by brief centrifugation in a Spinette benchtop centrifuge (Damon/IEC Division), resuspend in 1 volume digestion buffer (section 6.14) and incubated overnight at 55°C with gentle agitation. The samples were then treated as described in section 6.1.1.

6.1.3 Isolation of DNA from Paraffin-Embedded Tissue Sections

5μM thin tissue sections were cut from wax blocks, placed into eppendorf tubes and stored at 4°C until required. The sections were dewaxed by the addition of 1ml xylene and vigorous shaking for 30min at room temperature. The tissue was pelleted by centrifugation in a microfuge at full speed for 5min, the xylene removed, 1ml of fresh

xylene was added to the tissue pellet and the above procedure was repeated. After removal of the xylene, the sections were dehydrated by the addition of 0.5ml of 96% ethanol and mixed by several inversions. The tissue was pelleted as described above, fresh ethanol was added, the contents were again mixed by inverting the tubes followed by centrifugation and removal of the ethanol. The samples were dried under vacuum (as described in section 6.1.1) followed by the addition of 200µl of digestion buffer (containing 200µg/ml Proteinase K) was and incubated overnight at 55°C. The samples were incubated at 95°C for 8min to inactivate the protease and stored at -20°C until required.

6.2 PROPAGATION OF CELL LINES

The cell lines used were:

(1) WI-38: a human embryonic lung fibroblast cell line, obtained from the American Type Culture Collection (ATCC # CCL-75).

(2) MCF-7: an epithelial breast tumour cell line obtained from ATCC (ATCC # HTB-22).

(3) T-47D: an epithelial breast tumour cell line obtained from ATCC (ATCC # HTB-133).

(4) MDA-MB-231: an epithelial breast tumour cell line obtained from ATCC (ATCC # HTB-26).

(5) ZR-75-2: an epithelial breast tumour cell line obtained from ATCC (ATCC # CRL-1500).

WI-38 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated foetal bovine serum, 100 units penicillin and 100µg/ml streptomycin. The 4 breast tumour cell lines were cultured in DMEM supplemented with 5% heat-inactivated foetal bovine serum, 100 units penicillin and 100µg/ml streptomycin. All the cell lines were incubated at 37°C with 5% CO_2 humidity. On reaching confluency, the cells were rinsed with PBS and harvested by incubation with 0.05% trypsin in PBS containing 10mM EDTA at 37°C for 3-5min. The cells were pelleted by brief centrifugation (as described in section 6.1.2), resuspended in DMEM and split at a ratio of 1:2 (WI-38 cells) or 1:3 (MCF-7, T47-D, MDA-MB231 and ZR75-2).

6.3 POLYMERASE CHAIN REACTION (PCR) - SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

6.3.1 Polymerase Chain Reaction (PCR)

Exons 2, 4-9 and 11 of the p53 gene and exons 1 and 2 of the Ha-ras gene were individually amplified using the primer pairs and annealing temperatures indicated in tables 2.1 and 6.1. PCR was performed using 100ng of template DNA (isolated in section 6.1) in 1 X PCR buffer (section 6.14) containing 30pmol of each primer, the four deoxynucleotide phosphates (dNTP's; dGTP, dATP, dTTP and dCTP) at a final concentration of 0.2mM each and 2 units of *Taq* DNA Polymerase (Roche Biochemicals) in an Omnigene thermocycler (Hybaid). PCR reactions were carried out in a final volume of 50µl for 35 cycles of 1min denaturation (94°C), 1min annealing (see temperature in tables 2.1 and 6.1) and 2min extension (72°C). After amplification, the PCR products were stored at 4°C until required.

Table 6.1: PCR primers and annealing temperatures.
Primers were synthesised in a Beckman 1000A DNA synthesiser. The annealing Temperature for each primer pair is indicated (Tm).

Gene	Exon	Primer Pair	Tm	Fragment Size
p53	5	p53-5A: 5' - TGTTCACTTGTGCCCTGACT - 3' p53-5B: 5'- AGCAATCAGTGAGGAATCAG - 3'	61°C	310bp
p53	6	p53-6A: 5' - TGGTTGCCCAGGGTCCCCAG - 3' p53-6B: 5' - TTAACCCCTCCTCCCAGAGA - 3'	58°C	201bp
p53	7	p53-7A: 5' - CTTGCCACAGGTCTCCCCAA - 3' p53-7B: 5' - AGGGGTCAGCGGCAAGCAGA - 3'	60°C	237bp
p53	8+9	p53-89A: 5' - TTGGGAGTAGATGGAGCCTG - 3' p53-89B: 5' - AGTGTTAGACTGGAAACTTT - 3'	58°C	445bp
p53	11	p53-11A: 5' - AGACCCTCTCACTCATGTGA - 3' p53-11B: 5' - TGACGCACACCTATTGCAAG - 3'	58°C	245bp
Ha-ras	1	H12/1: 5' - GGGCCCTCCTTGGCAGGTGG - 3' H12/2: 5' - CACCTGGACGGCGGCGCTAG - 3'	65°C	206bp
Ha-ras	2	H61/A: 5' - GGAGAGGCTGGCTGTGTGAA - 3' H61/B: 5' - AAAAGACTTGGTGTTGTTGA - 3'	56°C	230bp

6.3.2 Single Strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis was performed essentially as described by Orita et al., 1989. Exons 5-7

and exon 11 of the p53 gene and exons 1 and 2 of the Ha-ras gene were individually amplified, and exons 8 and 9 of the p53 gene were amplified as one fragment as described in section 6.3.1. PCR reactions were performed in the presence of 1μCi α[^{32}P]dCTP (3000Ci/mmol) (ICN). After amplification of exons 8 and 9, the PCR product was incubated at 37°C overnight with the restriction endonuclease StyI to yield digestion products of 262bp (incorporating exon 8) and 183bp (incorporating exon 9).

3μl of the labelled PCR products (or digestion products in the case of exons 8 and 9) were mixed with 3μl of formamide loading buffer (section 6.14), denatured by heating at 95°C for 5min and loaded immediately on a non-denaturing 6% polyacrylamide gel (42cm long, 0.2mm thick) in the presence or absence of 5% (v/v) glycerol. Electrophoresis was performed in 1 X TBE running buffer (section 6.14), at either room temperature (using a fan to cool the gel to prevent excessive heating) or at 4°C. The samples were electrophoresed through the gels for 5-6 hours at 30W, after which the gels were dried under vacuum and exposed to x-ray film (Cronex) at -70°C for 16-48 hours.

6.4 CLONING OF PCR PRODUCTS

6.4.1 Preparation of Competent Cells

5ml of Luria broth (section 6.14) was inoculated with 50μl of a glycerol stock of E.coli DK-1 cells and incubated at 37°C with vigourous shaking for 16 hours. 300ml of Luria broth was inoculated with 300μl of the overnight culture and incubated at 37°C with vigorous shaking until the OD_{650} of the culture was between 0.2 and 0.4. The cells were pelleted by centrifugation for 10min in a JA-10 rotor at 5000rpm at 4°C. The cell pellet was gently resuspended in 40ml of cold 60mM $Cacl_2$, 10mM Pipes, pH 7.2 and incubated on ice for 20min. The solution was transferred to 30ml Corex™ tubes and the cells were pelleted by centrifugation at 5000rpm in a JA-20 rotor at 4°C. The cells were then resuspended in 4ml of cold 60mM $CaCl_2$, 10mM Pipes, pH 7.2, 15% glycerol, frozen in liquid nitrogen in 200μl aliqouts and stored at -70°C.

6.4.2 - Preparation of pUC-19 Vector

10μg of pUC-19 was incubated for 16 hours at room temperature with 20 units of restriction endonuclease SmaI, in the appropriate digestion buffer (section 6.14). The 5' ends of the digested vector were then dephosphorylated by incubation for 1 hour at 50°C with calf intestinal phosphatase (Roche Biochemicals) at a final concentration of 1unit/pmol DNA ends in 1 X CIP buffer (section 6.14). The phosphatase was heat inactivated at 65°C for 10min and the prepared vector stored at 4°C until required.

6.4.3 Preparation of PCR Products

6.4.3.1 Purification of PCR Products

The PCR products (section 6.3) to be cloned were combined with 1/10 vol of DNA stop buffer (section 6.14) and electrophoresed on a 1% low-melting agarose gel at 25mA in 1 X TAE buffer for 2-3 hours. The bands were visualised by staining the gel with ethidium bromide (EtBr) at a final concentration of 0.25μg/ml. The bands of interest were excised from the gel, placed in microfuge tubes and the DNA isolated using Qiaex beads (Qiagen) as per the manufacturer's instructions. Briefly, 300μl of solution QX1 and 10μl Qiaex beads were added to the agarose which was then solubilised at 50°C for 10min with brief vortexing every 2min. The beads were pelleted by centrifugation in a microfuge for 1min, washed twice with solution QX2 and twice with solution QX3. After the last wash the beads were dried under a vacuum for 5min, resuspended in 20μl of sterile H_2O and incubated at room temperature for 5min. The beads were pelleted by centrifugation for 1min in a microfuge and the supernatant containing the DNA was transferred to a fresh microfuge tube. This final step was repeated with another 20μl of sterile H_2O. The resulting 40μl of DNA was dried down in a speed-vac and resuspended in 10μl of sterile H_2O.

6.4.3.2 Phosphorylation of PCR Products

The purified PCR products from section 6.4.3.1 were phosphorylated at the 5' end by incubation with 3 units of T_4 polynucleotide kinase (Amersham) for 1 hour at 37°C in 1 X T_4 kinase buffer containing 1mM ATP. The enzyme was heat-inactivated at 65°C for 10min.

6.4.3.3 Blunt-ending of Kinased PCR Products

The kinased PCR products (section 6.4.3.2) were blunt-ended using the Klenow fragment of DNA polymerase I (Amersham) as described by Sambrook et al., 1989. PCR products were incubated at room temperature for 30min with 5units of Klenow DNA polymerase in the presence of 0.25mM of each dNTP in 1 X Klenow buffer (section 6.14). The enzyme was then heat-inactivated at 65°C for 10min.

6.4.4 In-gel Ligation of PCR Products to pUC-19

The prepared pUC-19 vector (section 6.4.1) and the kinased and blunt-ended PCR products (section 6.3) were combined with 1/10 vol of DNA stop buffer and electrophoresed on a 1% low-melting agarose gel in 1 X TAE running buffer. The gel was stained with ethidium bromide (0.25µg/ml) and the DNA visualised under UV light. The bands of interest were excised from the gel and placed in microfuge tubes. The gel slices were melted at 70°C for 10min, followed by incubation at 37°C for 5min. Vector and insert DNA were combined at ratios of 1:8, 2:7 and 3:6 with 1 unit of T_4 DNA ligase (Amersham) in 1 X T_4 DNA ligase buffer (section 6.14) and incubated at room temperature for 16 hours in a final volume of 20µl.

6.4.5 Transformation of Competent Cells

Competent E-coli DK-1 cells (section 6.4.1) were thawed on ice. The ligation reactions (section 6.4.4) were heated at 70°C for 10min then incubated at 37°C for 5min, and 5µl of the ligation reaction was added to 100µl of competent cells. The cells/DNA mixture was incubated on ice for 30min and heat-shocked at 42°C for 2min. 1ml of Luria broth (pre-warmed to 37°C) was added to the cells which were then incubated for 1 hour at 37°C. Aliquots of the transformation mix were plated on Luria-agar plates (section 6.14) containing 0.05mg/ml ampicillin and incubated for 16 hours at 37°C.

6.4.6 Screening of Bacterial Colonies

6.4.6.1 Screening using Plasmid Minipreps

An alkaline lysis method of plasmid isolation was used to rapidly screen recombinant clones as described (Birnboim and Doly, 1979; Birnboim, 1983). 10ml Luria broth was inoculated with a bacterial colony picked from the agar plates (section 6.4.5) and

incubated for 16 hours at 37°C with vigourous shaking. 500µl of the overnight culture was mixed with 500µl glycerol to make a glycerol stock. The glycerol stock was incubated at room temperature for 4 hours and then stored at -70°C.

The remainder of the overnight culture was centrifuged at 3000rpm in a Beckman TJ-6 benchtop centrifuge for 10min at 4°C. The cell pellet was resuspended in 200µl "RAPS" solution 1 (section 6.14), transferred to 1.5ml microfuge tubes and incubated at room temperature for 5min. The cells were then lysed by the addition of 400µl "RAPS" solution 2 (section 6.14) and kept on ice for 5min. 300µl of "RAPS" solution 3 (section 6.14) was added and the cells were incubated on ice for a further 10min. The samples were centrifuged at 4°C for 5min at maximum speed in a microfuge. The supernatant was transferred to a fresh 1.5ml microfuge tube and the samples were centrifuged again for 5min at 4°C. The supernatant was transferred to a fresh 1.5ml microfuge tube and the plasmid DNA was precipitated by the addition of 600µl isopropanol and kept at -20°C for 1 hour. The DNA was pelleted by centrifugation at room temperature for 10min in a microfuge, washed twice with 70% ethanol, dried under a vacuum and resuspended in a 80µl of 1 X TE.

25µl of the plasmid DNA was digested with the restriction endonucleases EcoRI and HindIII at 37°C for 16 hours and checked for the presence of an insert by agarose gel electrophoresis.

6.4.6.2 Screening using PCR

5ml of Luria broth was inoculated with a single bacterial colony and incubated at 37°C with vigorous shaking for 16 hours. 2µl of the overnight culture was heated at 95°C for 5min and used directly as a template in a PCR reaction. The lysate was incubated in 1 X PCR buffer (section 6.14) in the presence of the 4 dNTP's at a final concentration of 0.2mM, 2units of *Taq* DNA polymerase (Roche Biochemicals) and 30pmol of the following primers (USB Sequenase PCR Product Sequencing Kit):

Forward Primer: 5' - GTTTTCCCAGTCACGACGTTGTA - 3'

Reverse Primer: 5' -TTGTGAGCGGATAACAATTTC - 3'

PCR reactions were carried out in a final volume of 50µl with an initial denaturation

step of 2min at 95°C, followed by 35 cycles of denaturation at 95°C (1min); annealing at 60°C (1min) and extension at 72°C (1min). 10µl of the PCR reaction was mixed with 2µl of DNA stop buffer (section 6.14) and electrophoresed on an 1.5% agarose gel in 1 X TBE. The gel was stained with ethidium bromide (0.25µg/ml) and the bands visualised under a UV light.

6.5 LARGE-SCALE PLASMID PREPARATION

6.5.1 Qiagen Columns

Qiagen columns (Qiagen) were used to prepare plasmid DNA from 200ml overnight cultures as described by the manufacturers. Briefly, overnight bacterial cultures were lysed essentially as described in section 6.4.6.1 and the cleared lysate was passed over an ion exchange column to purify the plasmid DNA. Generally 0.2-0.5mg plasmid was obtained from a 200ml overnight culture, and the DNA was sufficiently pure to be used for transfections.

6.5.2 Cs-Cl/Ethidium Bromide Equilibrium Centrifugation

Plasmid DNA was isolated using CsCl/ethidium bromide centrifugation based on the method by Radloff et al., 1967. Five hundred ml of overnight bacterial cultures were harvested by centrifugation at 5000rpm in a Beckman JA-10 rotor at 4°C for 10min. The bacterial pellet was resuspended in 10ml of "RAPS" solution 1 (section 6.14) after which 5 ml of "RAPS" solution 1 containing lysozyme (final concentration of 13.3mg/ml) was added. The samples were mixed well by pipetting and incubated at room temperature for 30min. The cells were lysed by the addition of 30ml "RAPS" solution 2 (section 6.14) on ice for 30min. Fifteen ml of "RAPS" solution 3 (section 6.14) was added, the solution was mixed by gentle swirling and incubated on ice for 20min. The cell lysate was cleared by centrifugation at 8000rpm in a Beckman JA-10 rotor at 4°C for 10min. The supernatant was transferred to a clean 250ml plastic bottle and the plasmid DNA was precipitated by the addition of 40ml of isopropanol. The DNA was pelleted by centrifugation at 8000rpm in a Beckman JA-10 rotor for 10min at 4°C, the pellet was air-dried and resuspended in 4.2ml of 1 X TE (section 6.14). After the pellet was completely resuspended, the solution was transferred to 10ml a sterile Falcon tube and 4.6g CsCl was added. The CsCl was dissolved completely by inversion of the tube. 0.2ml of EtBr (10mg/ml) was added and the solution mixed well

by inverting the tubes followed by incubation on ice for 10min. The samples were centrifuged at 10000rpm in a Beckman JA-20 rotor at 4°C for 10min, after which the supernatant was transferred to 5ml Quick-Seal ultracentrifuge tubes (Beckman) and centrifuged at 50 000rpm for 20 hours at 20°C in a Beckman NVT-90 rotor.

The plasmid band was collected by inserting an 18-G needle into the top of the tube, and then inserting a 20-G needle connected to a 2ml syringe below the plasmid band and gently aspirating the band. The solution was transferred to a second Quick-Seal tube, which was filled with 0.8g/ml of CsCl, and centrifuged in a Beckman NVT-90 rotor at 64 000rpm for 4-6 hours. The plasmid band was removed as described above and transferred into a sterile 10ml Falcon tube.

EtBr was removed by adding 10ml of H_2O-saturated 1-butanol, mixing and centrifugation at room temperature in a Sigam-302K benchtop centrifuge. The butanol layer containing the EtBr was aspirated and the butanol extraction of the aqueous layer repeated until the EtBr was completely removed. The plasmid DNA was precipitated by the addition of an equal volume of isopropanol and incubation on ice for 1 hour. The DNA was pelleted by centrifugation at 10 000rpm in a JA-20 rotor for 15min at 4°C, the pellet air-dried and resuspended in a suitable volume of sterile H_2O

6.6 DNA SEQUENCE ANALYSIS

6.6.1 Direct Sequencing of PCR Products

Sequencing reactions were based on the Sanger dideoxy chain-termination method of sequencing (Sanger et al., 1977). PCR products were sequenced directly using the USB Sequenase PCR Product Sequencing Kit (Amersham) as per the manufacturer's instructions. Briefly, 5µl of the PCR product to be sequenced was treated with exonuclease I and shrimp alkaline phosphatase at 37°C for 15min and then subjected to sequence analysis. The resulting sequencing reactions were separated on a 6% polyacrylamide gel containing 7M urea in 1 X TBE. The gel was dried under vacuum and exposed to x-ray film (Cronex) for 16-48 hours at -70°C.

6.6.2 Sequencing of Cloned PCR Products

Cloned PCR products were sequenced using the dideoxy chain termination method

(Sanger et al., 1977) with the ^{T7}Sequencing^{TM} Kit (Pharmacia Biotech) as recommended by the manufacturer. Sequence reactions were separated on a 6% polyacrylamide gel containing 7M urea as described above.

6.7 ISOLATION OF RNA FROM CELL LINES

RNA was isolated based on the method described by Chomczynski and Sacchi, 1987. Confluent dishes of cells were rinsed once in PBS before the addition of 2ml solution D (section 6.14) at 4°C. The cells were transferred to 10ml Falcon tubes and 200µl 2M Na Acetate, pH 4.0; 2ml H_2O-saturated phenol and 400µl C:IAA (49:1) (section 6.14) was added. The solution was mixed well by vortexing and then allowed to incubate on ice for 15min. The samples were then centrifuged at 10 000rpm in a Beckman JA-20 rotor at 4°C for 20min after which the aqueous phase was transferred to a fresh Falcon tube. The RNA was precipitated by the addition of an equal volume of isopropanol and left overnight at -20°C. RNA was pelleted by centrifugation at 10 000rpm in a Beckman JA-20 rotor at 4°C for 20min, washed twice in 70% ethanol, dried under a vacuum and resuspended in a suitable volume of DEPC-treated H_2O. The samples were stored at -70°C until required.

The concentration of the RNA was determined spectrophotometrically using a Beckman DU 650 spectrophotometer.

6.8 NORTHERN BLOT ANALYSIS

6.8.1 Transfer of RNA onto Hybond-N Membranes

5-10µg of total RNA was mixed with 16.5µl RNA loading buffer (section 6.14), heated at 65°C for 5min and loaded onto a 1% agarose gel containing 8% formaldehyde. The gel was electrophoresed at 30mA in 1 X RNA running buffer (section 6.14) for 2-3 hours. After electrophoresis, the gel was placed upside down on 3 pieces of Whatman 3MM paper, cut to the same size as the gel and pre-soaked in 10 X SSC (section 6.14). A piece of Hybond-N nylon membrane (Amersham), also pre-soaked in 10 X SSC and cut to the same size as the gel, was placed on top of the gel, and finally another piece of Whatman 3MM paper, cut to the size of the gel and pre-soaked in 10 X SSC, was placed on top of the nylon membrane. A wad of tissues was placed on top, the blot was covered with a glass plate and lead weight and the RNA

was allowed to transfer to the membrane by capillary action at room temperature overnight.

The next day, the blot was disassembled and the membrane rinsed briefly in 6 X SSC, air-dried and UV-crosslinked in a Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation).

6.8.2 Preparation of Radiolabelled Probe

25ng of linear DNA was used as a template to synthesise radioactive probes for northern analysis. Prior to labelling, the double-stranded DNA was separated into single strands by heating at 95°C for 5min. Radioactive probes were generated using the Multiprime DNA Labelling System (Amersham) as recommended by the manufacturer. Briefly, the single-stranded DNA template was incubated with random primers and Klenow enzyme in the presence of 50μCi α^{32}P-dCTP for 1 hour at 37°C. The reaction mix was passed over a Sephadex G-50 column and fractions containing the radiolabelled probe were pooled. An aliqout of this was counted on a Packard Tri-carb 4640 scintillation counter to calculate the specific activity of the probe.

6.8.3 Hybridisation of Probe to Nylon Membrane

Membranes were pre-hybridised for 4 hours at 42°C in a Hybaid Dual Hybridisation Oven in 10 ml of pre-hybridisation solution (section 6.14). Radiolabelled probe was then added at a final concentration of 2 X 10^6 cpm/ml and the membranes were incubated at 42°C for 16 hours.

The next day the membranes were washed twice at room temperature with wash solution 1 (section 6.14) for 15min each, and twice at 65°C with wash solution 2 (section 6.14) for 15min each. Membranes were then exposed to X-ray film for 16-48 hours at -70°C.

6.9 SITE-DIRECTED MUTAGENESIS

6.9.1 Sub-cloning of wild-type p53 cDNA

6.9.1.1 Preparation of Insert

pC53-SN3, a 1.8kb cDNA clone of wild-type p53 in pCMV-Neo-Bam (Baker et al.,

1990), was obtained as a gift from Dr B. Vogelstein. The 1.8kb wild-type p53 insert was released from the vector by digestion with the restriction endonuclease BamHI (2 units/μg DNA) at 37°C for 1 hour in the appropriate reaction buffer.

6.9.1.2 Preparation of pUC-19 Vector

pUC-19 was digested with the restriction endonuclease BamHI (2 units/μg DNA) at 37°C in the appropriate reaction buffer for 1 hour. The 5' ends were dephosphorylated with calf intestinal phosphatase (CIP) (0.01 units/pmol DNA ends) at 37°C for 1 hour. The enzyme was inactivated at by heating at 65°C for 10min.

6.9.1.3 In-gel Ligation of Vector and Insert

Ligation reactions were performed as described in section 6.4.4, except the reactions were incubated for 16 hours at 15°C.

Ligation reactions were transformed into competent E-coli DK-1 cells, and bacterial colonies were screened for inserts by PCR, as described in section 6.4. Those clones containing inserts were sequenced as described in section 6.6.2 using the pUC primers (section 6.4.6.2) to check their orientation and to confirm the sequence.

6.9.2 Mutagenesis

A PCR based method of mutagenesis based on a modification of the methods by McCracken et al., 1988 and Kohli, 1998 was used to incorporate the desired mutations into the wild-type sequence using the primers outlined in table 6.2.

Silent mutations that generated restriction endonuclease sites were incorporated in the primers together with the required mutations. The restriction sites generated by the silent mutations were a KpnI site for the ΔA mutation and an Asp700 site for both the S269R and +15bp mutations.

The wild-type p53 cDNA in pUC-19 was PCR amplified in separate reactions using the mutA primers (table 6.2) and the pUC-reverse primer (section 6.4.6.2), or the mutB primers (table 6.2) and the pUC-forward primer (section 6.4.6.2). Amplification reactions were done in a final volume of 100μl in 1 X Thermopol reaction buffer

(section 6.14) in the presence of 0.2mM dNTP's and 60pmol of each primer, using 1 unit of Deep Vent DNA Polymerase (New England Biolabs) with an initial denaturation step of 94°C for 2min, followed by 35 cycles of denaturation (94°C, 1min); annealing (60°C, 1min) and extension (72°C, 1min) for all the primers except p53-mut1B. The reaction conditions for p53-mut1B were exactly the same as for the other primers, except the annealing temperature was 55°C.

Table 6.2: Primers used for site-directed mutagenesis. Primers were synthesised on A Beckman 1000A DNA synthesiser. Silent mutations generating restriction endonuclease sites are underlined.

MUTATION	PRIMER	SEQUENCE
ΔA	p53-mut1A	5'-ATGTGTACGGTACCTGCATGGGC-3'
	p53-mut1B	5'-TGCAGGTACCGTACACATGTAGTT-3'
S269R	p53-mut2A	5'-GGACGGAACAGATTCGAGGTGCGT-3'
	p53-mut2B	5'-CTCGAATCTGTTCCGTCCCA-3'
+15bp	p53-mut3A	5'-GGACGGAACAGCTTCGAGGTGAACAGCTTTGAGGTG-3'
	p53-mut3B	5'-CTCGAAGCTGTTCCGTCCCA-3'

6.9.3 Cloning of Mutated PCR Products into pUC-19

6.9.3.1 Preparation of Vector

pUC-19 was incubated with the restriction endonucleases EcoRI and HindIII for 1 hour at 37°C in the appropriate reaction buffer. The 5' ends of the digested vector were then dephosphorylated as described in section 6.9.1.2.

6.9.3.2 Preparation of PCR Products

PCR products were purified from unincorporated dNTP's and free primers with the High Pure PCR Product Purification Kit (Roche Biochemicals). The purified PCR products were then digested with either HindIII and the created restriction endonuclease site (KpnI or Asp700 as described in section 6.9.2) or EcoRI and the created restriction endonuclease site. HindIII was used when the pUC-reverse primer was used in the PCR reaction, and EcoRI was used when the PCR was done using the pUC-forward primer. All digestion reactions were carried out for 1 hour at 37°C in the appropriate reaction buffer.

6.9.3.3 In-gel Ligation of Digested PCR Products and Vector

The digested PCR products were ligated to the prepared pUC-19 vector by a triple ligation reaction. The 2 PCR products making up the full length cDNA were added to the vector and ligated at 15°C for 16 hours as described in section 6.9.1.3.

Ligation reactions were transformed into competent E-coli DK-1 cells and bacterial colonies were screened for inserts using PCR as described in section 6.4.

Clones containing inserts were sequenced (as described in section 6.3.2) to confirm that the mutation had been inserted correctly, as well as to ensure that no other mutations were erroneously incorporated into the cDNA.

6.9.4 Subcloning of Mutated cDNA into pcDNA-3 Expression Vector

6.9.4.1 Preparation of Vector

The pcDNA-3 vector was digested with BamHI and EcoRV (2 units/μg DNA) at 37°C for 1 hour in the approriate buffer. The 5' ends of the digested vector were dephosphorylated as described in section 6.9.1.2.

6.9.4.2 Preparation and Ligation of Inserts with Expression Vector

The mutated p53 cDNA was released from pUC-19 by incubating the clones with BamHI and SmaI at 37°C for 1 hour in the appropriate reaction buffer. The inserts were ligated to pcDNA-3 as described in section 6.9.1.3 and transformed into competent E.coli DK-1 cells as described in section 6.4.

6.10 TRANSFECTION OF PLASMID DNA

6.10.1 Preparation of Cells

NIH-3T3 cells were grown to confluency in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 100 units penicillin and 100μg/ml streptomycin in 100mm petri dishes. The day prior to the transfection, the cells were split 1:12 in DMEM and incubated overnight at 37°C. 2-4 hours prior to the transfection, the medium was removed and replaced with 9ml of fresh DMEM and the cells returned to 37°C until required.

6.10.2 Preparation of DNA

Cells were transfected as described (Chen and Okayama, 1988). The plasmid DNA to be transfected was isolated from overnight cultures of DK-1 cells using Qiagen columns as described in section 6.5, except that the DNA was suspended in sterile H_2O instead of 1 X TE buffer. 10-20μg of plasmid DNA in a final volume of 450μl sterile H_2O was mixed with 50μl of 2.5M $CaCl_2$. The DNA/$CaCl_2$ mixture was then added dropwise to 500μl of 2 X Hepes buffered saline (HeBS), pH 7.05 (section 6.14), while bubbling air through the HeBS. The solution was then left at room temperature for 15min, then added dropwise to the cells which were then returned to 37°C and incubated for 16 hours.

6.10.3 Selection of Stable Clones

After the overnight incubation in the presence of the calcium phosphate/DNA precipitate, the medium was removed and the cells were washed 3 times with PBS (section 6.14) and fresh medium was added. The cells were returned to 37°C for two days before the addition of selective medium containing 400μg/ml G418 (Gibco). The medium was changed every 4 days and replaced with fresh selective medium until discreet colonies were visible on the plates.

6.10.3.1 Propagation of stable clones

Once the clones had grown sufficiently such that they were visible to the naked eye, individual clones were picked using sterile cotton-wool swabs dipped in 0.05% trypsin and transferred into 24-well plates. Once the cells had become confluent in the 24-well plates they were transferred to 60mm petri dishes until confluent, from where they were transferred to 100mm petri dishes and again grown to confluency. Cells were trypsinised and frozen in 1ml aliqouts in the presence of 10% dimethyl sulphoxide (DMSO). The cells were initially frozen at -70°C for 16 hours and then transferred to liquid nitrogen.

6.11 IMMUNOHISTOCHEMISTRY

6.11.1 Immunohistochemistry using Cell Lines

Cells were grown overnight on glass slides at 37°C in DMEM. The cells were fixed in acetone for 5min and then air-dried after which they were washed in PBS, pH 7.6.

They were then blocked with goat non-immune serum diluted, 1:20 in PBS, for 10min at room temperature. The serum was drained off and the anti-p53 primary antibody, clone DO-7 (Dako), was diluted 1:100 in PBS and added to the cells. The cells were incubated for 30min at room temperature with the primary antibody, after which they were washed very well with PBS and drained. The Envision detection system (Dako) was used to detect the primary antibody as per the manufacturer's instructions, and the chromagen used was 3'-3'-Diaminobenzidine (DAB) which was also obtained from Dako. After the colour had developed, the slides were washed and the DAB signal was enhanced by incubating the slides in a 1% copper sulphate solution. The slides were washed again and then lightly counterstained with Haematoxylin. They were then "blued" in Scotts tap water substitute and dehydrated through graded alcohols, eventually to xylol, and mounted in Entelan.

6.11.2 Immunohistochemistry Using Tissue Sections

5μm thin tissue sections were cut onto glass slides and treated essentially as described for the cell lines (section 6.11.1) except that prior to blocking, an antigen retrieval step was carried out. The sections were heated in a pressure cooker at full pressure for 2min in 0.1M citrate buffer (pH 6.0). All subsequent steps were as described in section 6.11.1. Each section was scored for the intensity of p53 staining from 0 (negative) to 3+, and the percentage of p53 positive cells for each section was determined.

6.12 ANCHORAGE-INDEPENDENT GROWTH STUDIES

Colony formation in soft agar was carried out as described previously (Rhim et al., 1997; Tavoloni and Inoue, 1997). Briefly, a suspension of either 1 X 10^5 or 3 X 10^3 cells in 5ml of 0.35% agar-select (Sigma) made up in normal growth medium (DMEM) was overlaid onto a base of 0.6 % agar-select in a 60mm dish. The cells were incubated at 37°C in a humidified incubator and the plates examined for colony formation 21 days later.

6.13 CELL CYCLE ANALYSIS AFTER EXPOSURE TO IONISING RADIATION

200 000 cells were seeded into T-25 tissue culture flasks and incubated overnight in

DMEM (NIH-3T3) or DMEM containing 800μg/ml G418 (NIH-3T3 transfectants) at 37°C in a humidified incubator. Prior to irradiation, the medium was removed and fresh DMEM was added to all the cells. The cells were irradiated with 7Gy of ^{60}Co and incubated at 37°C until ready for harvesting. At various time points after exposure to the radiation, the cells were harvested by removing the medium and rinsing them with PBS. They were trypsinised and the cells pelleted by brief centrifugation in a bench top centrifuge and resuspended in 200μl 1 X PBS. 2ml of ice-cold (-20°C) 70% ethanol was added to the cells, which were then incubated at -20°C until required (a minimum of 30min).

When ready for analysis, the cells were pelleted at 2000rpm for 10min in a bench top centrifuge and the ethanol removed. The cell pellet was briefly vortexed and resuspended in 100μl RNAse A (1mg/ml); 10μl propidium iodide (1mg/ml) in a final volume of 1ml PBS. The solution was passed 2-3 times through a 25 gauge needle and incubated in the dark for 30min at 37°C. The reaction was stopped by transferring the samples to 4°C. The samples were sorted on a Becton Dickinson Facscan to determine the fraction of cells in the various stages of the cell cycle.

6.14 BUFFERS AND SOLUTIONS

40% ACRYLAMIDE STOCK SOLUTION (38:2)

38% Acrylamide

2% Bis

2% Ion exchanger V

The solution was mixed on a roller at 4°C for 16 hours, filtered and then stored at 4°C.

Chlorophorm:Isoamyl Alcohol (CIAA) (49:1)

49ml chloroform

1ml isoamyl alcohol

10 X CIP DEPHOSPHORYLATION BUFFER

10mM $ZnCl_2$
10mM $MgCl_2$
100mM Tris-Cl, pH 8.3

100 X DENHARDT SOLUTION

10g Ficoll 400

10g Polyvinylpyrrolidone

10g Bovine Serum Albumin (Fraction V)

H_2O to 500ml

DEPC-TREATED H_2O

0.1% DEPC

Sterilise by autoclaving.

DIGESTION BUFFER

100mM NaCl

10mM Tris-Cl, pH 8.0

25mM EDTA, pH 8.0

0.1mg/ml Proteinase K (added fresh with each use)

DIGESTION BUFFER (PARAFFIN SECTIONS)

50mM Tris, pH 8.5

1mM EDTA

0.5% Tween 20

10 X DNA STOP BUFFER

20% Ficoll 400

0.1M EDTA, pH 8.0

1% SDS

0.25% Bromophenol blue

0.25% Xylene cyanol

ETHIDIUM BROMIDE (10mg/ml)

10 mg ethidium bromide

1ml sterile H_2O

10 X FORMALDEHYDE LOADING BUFFER

1mM EDTA

0.25% Bromophenol blue

0.25% Xylene cyanol

50% Glycerol

FORMAMIDE LOADING BUFFER

95% Formamide

10mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol

2 X HEPES-BUFFERED SALINE

0.28M NaCl

0.05M Hepes

1.5mM Na_2HPO_4

Titrate to pH 7.05 with 5M NaOH

Filter sterilise and store in 10ml aliquots at -20°C

10 X KLENOW BUFFER

500mM Tris-Cl, pH 7.5

100mM $MgCl_2$

10mM DTT

500µg/ml BSA

LURIA AGAR

10g/l Tryptone

5g/l Yeast extract

5g/l NaCl

15g/l Agar

LURIA BROTH

10g/l Tryptone

5g/l Yeast extract

5g/l NaCl

1 X PCR BUFFER

10mM Tris-Cl, pH 8.3

50mM KCl

1.5mM $MgCl_2$

PHOSPHATE BUFFERED SALINE

137mM NaCl

2.7mM KCl

4.3mM $Na_2HPO_4.7H_2O$ (pH 7.4)

1.4mM KH_2PO_4

PRE-HYBRIDISATION SOLUTION (NORTHERN ANALYSIS)

5 X SSC

10% Dextran Sulphate

50mM Sodium Pyrophosphate, pH 6.5

50% Formamide

5 X Denhardt's solution

0.1mg/ml Sonicated Herring Sperm DNA

0.1% SDS

"RAPS" SOLUTION 1

25mM Tris-Cl, pH 8.0

10mM EDTA

50mM Glucose

"RAPS" SOLUTION 2

200mM NaOH

1% SDS

"RAPS" SOLUTION 3

3M Potassium Acetate, pH 4.8

RNA LOADING BUFFER

1 X RNA Running buffer

45% deionised formamide

6% formaldehyde

1 X Formaldehyde loading buffer

10 X RNA RUNNING BUFFER

41.8g 3-[N-morpholino]propanesulfonic acid (MOPS) to 800ml DEPC-treated H_2O

Adjust to pH 7.0 with NaOH

16.6ml 3M Na Acetate, pH 5.5

20ml 0.5M EDTA, pH 8.0

Make up to 1 litre with DEPC-treated H_2O

SOLUTION D

4.2M Guanidine thiocyanate salt (GITC)

300mM Na Citrate, pH 7.0

0.5% Sarcosyl

Add 360µl 2-mercaptoethanol to 50ml solution D prior to use.

20 X SSC

3M NaCl

0.3M Na_3 citrate

10 X T_4 DNA LIGASE BUFFER

660mM Tris-Cl, pH 7.6

66mM $MgCl_2$

100mM DTT

660µM ATP

10 X T_4 POLYNUCLEOTIDE KINASE BUFFER

0.5M Tris-Cl, pH 7.6

100mM $MgCl_2$

100mM 2-mercaptoethanol

10 X TAE BUFEER

0.4M Tris

0.01M EDTA

pH to 7.8 with acetic acid

10 X TBE BUFFER

890mM Tris

890mM Boric acid

25mM EDTA

pH 8.3

1 X TE (Tris/EDTA) BUFFER

10mM Tris-Cl, pH 8.0

1mM EDTA, pH 8.0

1 X THERMOPOL REACTION BUFFER

10mM KCl

10mM $(NH_4)_2SO_4$

20mM Tris-Cl

2mM $MgSO_4$

0.1% Triton X-100

pH 8.8

WASH SOLUTION 1 (NORTHERN ANALYSIS)

2 X SSC

0.1% SDS

WASH SOLUTION 2 (NORTHERN ANALYSIS)

0.1 X SSC

0.1% SDS

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