STUDIES OF THE ATAXIA TELANGIECTASIA MUTATED GENE AND ITS PRODUCT

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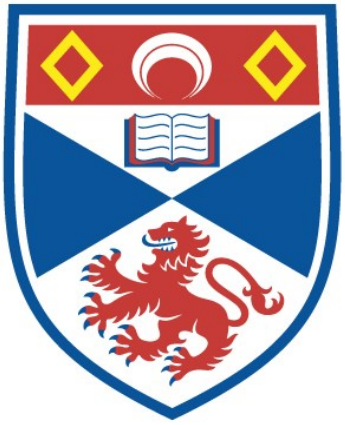

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Studies of the Ataxia Telangiectasia Mutated Gene and its Product

By

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ABSTRACT

ATM is a large and complex gene, identified as the recessive gene mutated in individuals with the childhood syndrome ataxia telangiectasia. While AT patients are rare and the disease has many severe symptoms, including cerebellar degeneration, immunodeficiency and a very high cancer risk, the relatively common carriers of one mutation are clinically normal. However, there is some evidence that these heterozygotes, too, have a high relative risk of cancer, especially breast cancer in women. The cellular radiosensitivity of carriers is intermediate between that of normal individuals and that of AT patients, and this may suggest the cells are predisposed to tumorigenic transformation. ATM is believed to be involved in DNA damage response, but the mechanisms by which it works are not fully understood.

In this study, DNA from 412 Scottish women with breast cancers were screened for two ATM mutations, known to be relatively common in Celtic populations. In attempting to estimate the burden in Scotland of breast cancers in AT heterozygotes, it is shown here that even these two most common ATM mutations in the UK account for only a small proportion of all ATM mutations. None of these mutations were found, suggesting that these particular mutations do not confer a predisposition to breast cancer.

Also presented are the results of various immunological studies. The ATM protein is shown to be localised to specific structures within the nucleus of a normal cell. It appears to be found in a number of different forms, varying in size and state of glycosylation. It sediments at a relatively low speed, suggesting that it is normally bound in a large protein complex.

This work indicates that a large-scale screening study will be required to establish whether or not AT carriers are at an increased risk of cancer, and reveals that the ATM product may be present in more than one form, depending on whether the cell is stimulated to divide.

DECLARATIONS

I, Robert Gordon Hislop, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

February 22, 1999 Signature:

I was admitted as a research student in October, 1995 and as a candidate for the degree of Doctor of Philosophy in October, 1995; the higher study for which this is a record was carried out between 1995 and 1999.

February 22, 1999 Signature:

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ABBREVIATIONS

A_{260}	Absorbance at 260nm
AP	Alkaline phosphatase
APS	Ammonium persulphate
AT	Ataxia-telangiectasia (disease)
ATM	Ataxia Telangiectasia Mutated (gene)
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ci	Curie
Da	Dalton
dNTP	Deoxyribonucleotide where N can be A: adenosine C: cytidine G: guanosine T: thimidine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gravitational field
GADD	Growth arrest and DNA damage inducible (gene family)
GalNAc	N-acetylglucosamine
Ig	Immunoglobulin
M	Molar
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
NP-40	Nonidet P-40 (detergent)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PI	Phosphatidylinositol
PMSF	Phenylmethylsulfonyl fluoride
PPi	Pyrophosphate
PVP	Polyvinylpyrrolidone
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate buffer
Taq	*Thermus aquaticus*
TBE	Tris-borate/EDTA buffer
TEMED	N,N,N',N-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane

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Introduction

1.1: Background

1.1.1: Features and symptoms of the disease.

The earliest description of patients with a disease recognisable as AT was published in 1926 by Syllaba and Henner, while a report of a single case published by Louis-Bar in 1941 has led to the less commonly used name, Louis-Bar syndrome. The term ataxia telangiectasia was first used by Boder and Sedgwick (1957) to describe a syndrome affecting seven children presenting with a distinctive set of symptoms. In a subsequent article, Boder and Sedgwick (1958) described the major features of these seven cases and one other case. Of the two eponymous symptoms, cerebellar ataxia is seen first between 10 and 14 months while telangiectasia (dilated blood vessels) in the eyes, especially the bulbar conjunctivae, and in the butterfly area of the face, usually appear between 4 and 6 years. Truncal ataxia, initially manifested in swaying of the head and trunk on standing and even sitting, and ataxia of gait, are slowly and steadily progressive. In spite of good muscular strength, an AT patient will usually require a wheelchair by 11 years of age. Other symptoms recognised by Boder and Sedgwick included frequent sinopulmonary infection and peculiarity of eye movements.

Further features of the disease have since been added to the symptomology of AT (Boder, 1985). Peripheral lymphoid tissue is decreased and the thymus is either absent or resembles an embryonic thymus. Selective deficiency of serum IgA partly explains the susceptibility to sinopulmonary infection, which is the most common cause of death, while lymphoreticular malignancy was recognised as the second most frequent cause of death. Like many characteristics of AT, this immunodeficiency varies from

patient to patient, even between affected sibs. Woods and Taylor (1992) found 27 of 70 British patients had normal immunological function while 10% had severe immunodeficiencies. Serum levels of IgG2, IgA and IgE are deficient in around 70% of patients while IgM, IgG1 and IgG3 are usually normal (Gatti *et al*, 1991a). Around half of AT patients also have T cell deficiencies. Responses to antigens are poor and T cell cytotoxicity to influenza-infected target cells is reduced. Chemotaxis of neutrophils and natural killer cell activity have been reported as reduced (Schuller and Hellman, 1981), but other studies have indicated they are unaffected (Tsuge *et al*, 1987).

The observation that lymphoreticular malignancy was a common cause of death among AT patients led Morrell *et al* (1986) to retrospectively measure cancer incidence in 263 AT sufferers. The results showed 52 primary cancers, representing a 61-fold cancer excess for white probands and a 184-fold excess for black probands. These figures are believed to under-estimate the relative cancer risk, but clearly illustrate a very strong predisposition to cancer among AT patients.

1.2: Cellular radiosensitivity in ataxia-telangiectasia

1.2.1: General cellular phenotypes

Enhanced sensitivity to ionising radiations was first shown at cellular level by Taylor et al (1975) and is a constant feature of cells from AT individuals. This is manifested as loss of colony-forming ability (a measure of cell death) and chromosomal damage to individual cells. AT cells are also hypersensitive to chemicals which cause DNA

damage through free radical action, including bleomycin, neocarzinostatin, hydrogen peroxide, streptonigrin and phorbol ester (review McKinnon, 1987) and to restriction endonucleases which produce only blunt-ended double strand breaks (Liu and Bryant, 1993; Costa and Thacker, 1993). AT cells did not prove hypersensitive to treatment with those restriction enzymes which produce cohesive ends (Liu and Bryant, 1993; Costa and Thacker, 1993), or to UV (Taylor *et al*, 1975) suggesting ATM is not involved in the response to all types of DNA damage.

It has been shown that normal cells held after irradiation in a non-growing state have some ability for potentially lethal damage repair (PLDR), while AT cells show little or no sparing from lethal effects (Cox *et al*, 1981). Further, for a given dose, at a low dose-rate and for weakly ionising radiation (such as X-rays), the difference between normal and AT cell sparing is greatest, while sparing is most similar for an acute dose from densely ionising radiation (such as α-particles) (Cox *et al*, 1982) probably because a level of damage is reached that even normal cells cannot repair effectively. These results illustrate that AT cells are incapable of recovering from radiation damage and that time is not a factor in this deficiency.

While double strand breaks (dsb) appear to be the most lethal type of DNA damage, and repair of dsb is responsible for recovery under sparing conditions (Frankenberg-Schwager and Frankenberg, 1990), it has been difficult to prove that AT cells have a break-repair defect (McKinnon, 1987). Studies showing increased levels of residual dsb in AT cells (Blocher *et al*, 1991; Foray *et al*, 1997; Cornforth and Bedford, 1985) are contradicted by studies suggesting there is no difference (Taylor *et al*, 1975, Coquerelle, 1987). Some evidence has been presented, however, indicating that after

γ-irradiation AT cells had more residual chromosome breaks than normal cells, while there was no significant difference in residual dsb (Pandita and Hittelman, 1992). Similarly, Lui and Bryant (1993, 1994) found an equal number of dsb in AT and control cells, induced by PvuII treatment, resulted in an enhanced frequency of chromatid breaks in the AT cells. Together these results indicate that the increased sensitivity to agents such as ionising radiation (though not, notably, UV or methylmethane sulphonate (Khanna and Lavin, 1993)) derives from an inability to recover from DNA breakage, leading to a higher rate of conversion of dsb into chromatid aberrations. This conversion of dsb into aberrations may, in turn, be due to an altered chromatin structure in AT cells (Hittelman and Panditta, 1994).

1.2.2: Non-conservative recombination.

In vitro recombination of dsb produced in human cells by restriction endonuclease treatment includes a number of non-conservative (sequence loss) events (Thacker *et al*, 1992). This percentage of mis-rejoining is higher in AT cells than in normal cells (North *et al*, 1993; Dar *et al*, 1997).

Meyn (1993) measured spontaneous mitotic recombination between directly repeated genes contained in vectors that had been integrated into the genomic DNA of AT, xeroderma pigmentosum (XP) and control cells. Recombination rates were also measured using a linear version of the vector, incapable of integration. They found spontaneous intrachromosomal recombination rates were 30 to 300 times higher in AT fibroblast lines than in normal cells, whereas extrachromosomal recombination frequencies were near normal. Although most of these events were conservative, the

AT lines also had twice as many non-conservative events compared to normal cell lines. Hyperrecombination appears to be specific to AT as an XP cell line (deficient in excision repair) displayed normal spontaneous recombination rates.

In AT patients of any age, approximately 10% of all circulating T lymphocytes show rearrangements, usually involving the sites of the T-cell receptor (TCR) gamma genes (7p15), TCR beta genes (7q35) and TCR alpha genes (14q11) (McFarlin *et al*, 1972; Ying and Decoteau, 1981). Chromosomal rearrangements in B lymphocytes involving the immunoglobulin heavy chain gene cluster have also been reported (reviewed in Taylor *et al*, 1996). These observations may relate to the immunodeficiency seen in all AT patients. One of the most common rearangements in T cells of AT patients, t(14;14)(q11;q32), involves the gene for the alpha subunit of the T-cell antigen receptor (TCRα at 14q11.2), and the oncogene TCL1 (T-cell lymphoma/leukaemia-1) may be located at the other breakpoint (14q32.3) (Croce et al, 1985). It is thought that the TCL1 gene may be activated by juxtaposition of TCL1 with TCRα by chromosomal inversion or translocation. This translocation is of particular interest as it is associated with many T-cell malignancies of AT patients (McCaw *et al*, 1975).

The combined evidence of elevated levels of recombination involving the T-cell receptor genes in lymphocytes from AT patients and the high levels of intrachromosomal recombination points to a general disruption of the recombination process in AT cells.

It may be argued that an increased rate of non-conservative recombination should lead to an increase in mutation frequency. Loss of heterozygosity (LOH) can be measured

in anucleate erythrocytes by antibody detection of the two co-dominant forms of glycophorin A (GPA): M and N. In an individual heterozygous for the two forms, LOH results in N∅, M∅, NN or MM varient cells. Cole and Skopek (1994) have summarised studies measuring loss of heterozygosity (LOH) in the GPA system in erythrocytes and mutations in the HPRT (hypoxanthine phosphoribosyl transferase) gene in lymphocytes. These data showed that in AT cells, LOH was elevated by a mean of ten-fold in GPA, and mutations in HPRT by a mean of four fold above the normal range. In each system, however, cell samples from individual AT patients displayed a range of frequencies from normal to 100-fold higher than normal. Studies were also carried out to measure the proportion of endonuclease-induced breaks in the replicating shuttle vector pZ189 that were incorrectly rejoined when transfected into cells. The plasmid contains the ampicillin resistance gene, allowing selection of transfected cells, and the mutagenesis target *supF* gene. Cells with correctly rejoined vectors form blue colonies in the presence of X-gal, while those with incorrectly rejoined vectors form white colonies. When the linearised plasmid, which requires joining of the DNA ends by host cell enzymes for survival, were transfected into the cells, the proportion of rejoined vectors was lower, and the number of mutations in the gene higher, in AT than normal and XP cell lines (Runger *et al*, 1992; Tatsumi-Miyajima *et al*, 1993) These studies also found the proportion of complex mutations and especially deletions was higher in AT cells than XP or control cells. Significantly, however, when 25-50Gy of γ-rays were used to generate mostly single-strand damage, no increase in mutation frequency was found in AT lymphoblastoid cells over normal cells (Sikpi *et al*, 1992). Tatsumi-Miyajima *et al* (1993) suggested AT cells may be deficient in the mechanism to protect the broken ends of DNA strands during rejoining.

These results seem to suggest that non-conservative recombination processes leading to large-scale genetic changes may relate to the stability of the genome in AT cells and ultimately to their tumorgenicity.

1.2.3: Radioresistant DNA synthesis and the S-phase checkpoint.

Normal cells show a rapid inhibition of DNA synthesis after irradiation while AT cells consistently have a delayed and/or much reduced inhibition, and synthesis returns to normal levels more rapidly than in controls (Houldsworth and Lavin, 1980). Inhibition in normal cells appears to be dose-dependent, with DNA synthesis falling sharply with radiation below 5Gy and declining little further at higher doses (Houldsworth and Lavin, 1980; Edwards and Taylor, 1980; Painter and Young, 1980). The initial slope has been equated to inhibition of replicon formation, and the final slope to blocking the elongation of DNA strand already undergoing replication (Painter, 1986). AT cells continue to undergo DNA synthesis following radiation-induced DNA damage (Painter and Young, 1980; Houldsworth and Lavin, 1980) and this radioresistant DNA synthesis (RDS) in AT cells suggests that these cells are deficient in the S phase checkpoint which inhibits replicon formation and strand elongation.

Consistent with radiosensitivity, the radioresistant DNA synthesis (RDS) phenotype of AT cells is more pronounced in response to radiomimetic drugs such as bleomycin and neocarzinostatin than to UV (McKinnon, 1987; Coquerelle *et al*, 1987).

It was initially expected that the inability to prevent replication of damaged DNA was the basis of AT radiosensitivity. However, Cox *et al* (1981) had shown AT cells held in G1 after irradiation did not display a sparing effect. Furthermore there were more chromosomal aberrations found in AT cells than in normal cells when both were irradiated in G0 (Sasaki and Taylor, 1994), and this cannot be explained by RDS. In addition, it has been shown that the sensitivity to cell killing of AT cells can be complemented by DNA-mediated gene transfer while the RDS phenotype is retained (Lehmann *et al*, 1986).

Houldsworth and Lavin (1980) and Painter and Young (1980) suggested that DNA synthesis is inhibited by ionising radiation directly damaging a chromatin 'target', such as a cluster of replicons, which is altered in AT. Edwards and Taylor's (1980) interpretation was that radiation produces a signal that halts DNA synthesis and this signalling mechanism is defective in AT cells. Later work would show that the ATM product has sequence similarity to yeast and mammalian proteins known to be involved in signal transduction (Savitsky *et al*, 1995a; Zakian, 1995) and downstream cascades would subsequently be discovered (reviewed by Westphal, 1997).

1.2.4: G1/S and G2/M checkpoints.

There are two points in the cell cycle where arrest can occur following damage to DNA by, for example, ionising radiation or radiomimetic drugs (Murray, 1992): the first, during G1 phase, prevents replication of damaged DNA by preventing progression into S phase. The second, during G2 prior to mitosis, prevents damaged

DNA being passed on to daughter cells. It is generally accepted that these checkpoint delays allow DNA repair to take place and thus limit heritable genetic errors.

AT cells were later found to show no delay in G1 to S phase progression following DNA damage (Beamish *et al*, 1994), indicating these cells are also defective in the G1 checkpoint which normally delays cell cycle progression following damage.

Cell cycle is regulated in response to ionising radiation at the G1/S and the G2/M transitions (Hartwell, 1992). These checkpoints allow damaged DNA to be repaired before entering either S-phase (when damage would be perpetuated), or M-phase (when breaks would mean the loss of genomic material). Checkpoints comprise surveillance mechanisms, signal transduction pathways and repair activity. It has been illustrated that the G1/S and G2/M checkpoints are under genetic control, with Painter and Young (1982) showing that the G1/S checkpoint is abolished in cells from AT patients. It has been found that in normal cells p53 is upregulated 3- to 5-fold post-irradiation, coincident with a delay in the G1/S transition (Kastan *et al*, 1991) and that induction of p53 does not occur in AT cells (Kastan *et al*, 1992). It was therefore concluded that ATM acts upstream of p53 in a signal transduction pathway that activates the G1/S checkpoint. Induction of GADD45 (growth arrest and DNA damage inducible 45) by ionizing radiation is also deficient in AT cells and requires wildtype p53 function (Kastan *et al*, 1992) so is involved in this pathway. It seems likely that lesions in this pathway will contribute to tumour development and Kastan *et al* (1992) pointed out that T lymphoid malignancies are the most common tumour found in both AT patients and p53 deficient mice. The G1/S checkpoint may be involved in prevention of errors during gene rearragements of lymphoid cells.

Khanna and Lavin (1993) found AT cells had a normal p53 response to UV-B, indicating that ionising radiation and UV induce p53 by different pathways. They suggested dsb damage may be necessary for ATM-mediated stabilisation of p53 protein.

It is widely accepted that AT cells also have a lesion at the G2 checkpoint (Beamish and Lavin, 1994; Zampetti-Bosseler *et al*, 1981), but some studies have contradicted this (Smith *et al*, 1985). However separate studies have shown that AT cells already in G2 at the time of irradiation enter mitosis unchecked (Scott and Zampetti-Bosseler, 1982; Paules *et al*, 1995) while AT cells irradiated prior to G2 ultimately arrest irreversibly in G2 (Bates and Lavin, 1989; Beamish and Lavin, 1994, Hong *et al*, 1994, Lavin *et al*, 1994; Beamish *et al*, 1994, Meyn *et al*, 1994). Two possible explanations have been suggested: first, cells irradiated prior to G2 are damaged to such an extent that they are unable to divide (Paules *et al*, 1995) or second, there are two checkpoints in G2, one early in G2 that is functional in AT cells and one that functions later in G2 to delay entry into mitosis and it is the latter which is defective in AT cells (Brown and Tagle, 1997).

Jung *et al* (1995) found expression cloning of ΔIκB-α in an AT mutant fibroblast cell line corrected the radiation sensitivity and the DNA synthesis defects. ΔIκB-α is a truncated form of IκB-α, which is an inhibitor of the transcription factor NF-κB (nuclear factor-κB). The parental cell line expressed larger amounts of IκB-α than control lines, and yet NF-κB was constitutively expressed in these cells. The transfected cells, however, showed normal levels of IκB-α and regulated activation of NF-κB, including up-regulation in response to radiation. While the mechanism by

which ΔIκB-α corrects NF-κB regulation remains unclear, this is strong evidence that aberrant NF-κB regulation may contribute to the cellular defect in AT. Jung *et al* (1995) suggested that this was not the primary defect in AT, but that IκB-α operates downstream of ATM.

1.3: AT Heterozygotes

AT is inherited as a recessive disorder. While AT carriers (heterozygotes) are clinically normal, it is a long-held view that heterozygote carriers of a recessive mutation may be predisposed to some of the congenital manifestations of the disease affecting homozygotes. A number of tests were carried-out to test this hypothesis. In 1976, Swift *et al* compared the incidence of death from malignant neoplasm in 27 families of patients with AT to that expected of the general population and found that carriers have between 2 and 6- fold excess risk of dying from cancer over the general population. They also identified the most likely types of neoplasm to be stomach, leukaemia and lymphoma, ovary and breast. A follow-up study of only the obligate heterozygotes (first degree relatives of AT patients) in this group (Swift and Chase, 1983) confirmed an excess of cancer deaths among carriers, and also found heart disease to be a more common cause of death in carriers than in the general population. Welshimer and Swift (1982) studied families of AT, Fanconi's anemia (FA) and xeroderma pigmentosum (XP). Among XP relatives, mental retardation and microcephaly were higher than expected; genitourinary and limb malformations were found in FA families; and idiopathic scoliosis and vertebral anomalies were more common in AT families than in controls.

As the most striking aspect of AT is the elevated risk of lymphomas and lymphocytic leukaemias, a logical step was to assess cancer risk among carriers. Swift *et al* (1987) studied documented cases of cancer among 110 unspecified white AT families, 4 Amish AT families and 14 black AT families. They compared cancer incidence among blood relatives to that of spouses. The results suggested a dramatically elevated risk of cancer among AT heterozygotes: the estimated relative risks were 2.3 for men and 3.1 for women. The complex nature of tumorigenesis suggests that predisposition to one cancer is not necessarily associated with predisposition to another. Swift *et al* (1987) found the incidence of colon and rectal cancer to be lower in blood relatives than in spouse controls. While AT heterozygosity is unlikely to be protective for these cancers, this suggests a site-specific predisposition. Breast cancer in women was most clearly associated with AT heterozygosity and Swift *et al* (1987) estimated that between 8% and 18% of white breast cancer patients in the USA would be AT carriers. Pippard *et al* (1988) reinforced this data, finding an excess of breast cancer deaths among mothers of AT probands.

In 1990, Morrell *et al* studied a further 44 AT families. From retrospective data for 574 blood relatives and 213 spouse controls they found the risk of cancer among the carriers was 6.1 times that of the controls. Nine of the cancers observed were of the female breast, making it the most common among the heterozygotes. Gatti *et al* (1991a) reviewed the available data and concluded there was a possible excess of breast cancers among AT carriers.The results of a prospective study of 161 families by Swift *et al* (1991) showed cancer rates among the 1,599 blood relatives of AT patients were significantly elevated above those of the spouse controls. Cancer rates were most notably elevated in the obligate heterozygotes.The relative risk for all

cancers was 3.8 in men and 3.5 in women and for breast cancer in women it was 5.1. It was reported that female blood relatives with breast cancer were more likely to have been exposed to diagnostic or occupational ionising radiation than those female blood relatives without breast cancer. Blood relatives from 20 to 59 years were also found to have excess mortality from all causes in both males (3-fold) and females (2.6-fold). Swift *et al* (1991) suggested that female AT carriers are at increased risk of ionising radiation-induced breast cancer. This is consistent with the theory that radiation-sensitive genotypes are predisposed to radiation-induced cancers (Sankaranaryanan and Chakraborty, 1995).

Kuller and Modan (1992), among others, have criticised Swift's work on AT heterozygotes and breast cancer, claiming that the study included a small sample, inadequate assessment of radiation exposure and the fact that the relatives with cancer were older than the controls was inappropriate for a breast cancer study. Furthermore, the spouse controls had a low rate of breast cancer compared with numbers expected on the basis of the Surveillance Epidemiology and End-Results (SEER) Programme data.

Athma *et al* (1996) used tightly-linked CA repeat markers to determine carrier status of 775 blood relatives of AT patients in 99 AT families. Of the 33 women with breast cancer, 25 were found to be AT carriers, compared to an expected number of 14.9 carriers. Based on these results, Athma *et al* (1996) calculated the relative risk of breast cancer among female heterozygotes to be 3.8 times higher than normal individuals. Furthermore, among carriers the relative risk of breast cancer with onset before 60 years was 2.9 times normal, and with onset after 60 years was 6.4 times

normal. Overall Athma *et al* (1996) estimated that 6.6% of all breast cancers could occur in AT heterozygotes.

In synopsis of the work up to 1994, Easton (1994) developed an age-specific model of breast cancer risk in AT heterozygotes predicting that 8% of breast cancers occurring in women under 40 years of age are found in AT carriers compared with 2% of those occurring between 40 and 59 years. FitzGerald *et al* (1997) tested this hypothesis by using the protein truncation test (PTT) to detect germline mutations in the ATM gene in a population of women with early-onset breast cancer. Chain terminating mutations detected by the PTT account for 90% of all known ATM mutations. In 202 controls they found 2 heterozygotes (1%), consistent with epidemiologic studies, while only 2 heterozygotes (0.5%) were found in 401 women with early-onset breast cancer (P=0.6). They therefore concluded that heterozygous ATM mutations do not confer genetic predisposition to early-onset breast cancer.

Bishop and Hopper (1997) attempted to rationalise the discrepancy between the results of Athma *et al* (1996) and FitzGerald *et al* (1997) with respect to early-onset breast cancer. At the 95% confidence interval, they found the upper limit of early-onset breast cancers occurring in AT heterozygotes to be 2.4% in the FitzGerald study. Therefore, the two studies are consistent with one another within 95% confidence intervals (CIs).

Easton's review of studies by Swift *et al* (1987, 1991), Pippard *et al* (1988) and Borresen *et al* (1990) indicates that there is an overall 3.9 fold estimated relative risk of breast cancer in female carriers, and is still regarded as the best estimate. Although

this risk is much lower than other breast cancer susceptibility genes such as p53, BRCA1 and BRCA2 (Szabo and King, 1995), AT heterozygotes may represent a higher proportion of breast cancer cases, given the relatively high carrier frequency of AT.

In practical terms, the question arising from these data is whether female carriers should undergo regular mammography. Swift *et al* (1987) found that a substantial proportion of blood relatives with breast cancer had previously undergone myelography and in a subsequent study (Swift *et al*, 1991) found 53% of blood relatives with breast cancer and 19% of controls with breast cancer had received documented exposure to radiation. These data are consistent with the assertion that AT carriers have a lowered dose threshold at which radiation may contribute to tumorigenesis, but do not provide conclusive support.

There is evidence that individual mutations in the ATM gene may carry different risks of cancer. Stankovic *et al* (1998) identified a mutation, base change 7271T to G in the cDNA, associated with a milder clinical and cellular phenotype (allowing patients to live longer) which was present in two families with a history of familial breast cancer. In one familiy, two female probands homozygous for this mutation both had breast cancer, one bilateral, and the mother, a carrier, had breast cancer. In the second family, the father had three sisters, two of whom had breast cancer. One sister with breast cancer was confirmed as a carrier, but the second was deceased. The sister without breast cancer had not been tested for carrier status. From these results, Stankovic *et al* (1998) estimated the relative risk of breast cancer in heterozygotes to be 12.7 and in homozygotes to be 74.1. If mutations such as this are responsible for

most cases of breast cancer in AT heterozygotes, screening would be a more attainable goal.

The literature also contains other data contradicting the view that there is increased risk of breast cancer in AT heterozygotes. While Pippard *et al* (1988) and Borresen *et al* (1990) found an excess of breast cancers among the parents of AT patients, they found no such excess among the grandparents, 50% of whom should be heterozygotes. Vorechovsky *et al* (1996a) found no germline mutations in 38 consecutive breast cancer patients. In a second study, Vorechovsky *et al*, (1996b) found three ATM mutations in 88 cancer families, but found the tumour phenotype did not cosegregate witht the mutant allele. Chen *et al* (1998) found only one ATM mutation in 100 breast cancer patients with a family history of breast cancer which is consistent with the observed carrier frequency of 0.2-1.0% for AT.

Also against the association between AT heterozygosity and breast cancer is the work of Wooster *et al* (1993) who typed 5 DNA markers in the AT region of chromosome 11q. They found no evidence for linkage between breast cancer and these markers, concluding that the contribution of AT to familial breast cancer is minimal.

If an individual is heterozygous for a tumour-suppressor gene, loss of the normal allele (i.e. loss of heterozygosity or LOH) in a cell could result in uncontrolled growth. LOH at 11q22-23, the region of the ATM gene, has been demonstrated to be around 40% in breast cancer tumours (Carter *et al*, 1994; Hampton *et al*, 1994; Kerangueven *et al*, 1997). Despite loss of heterozygosity at 11q22-23 being demonstrated in 47% of cases in their study, Vorechovsky *et al* (1996a) found no

somatic ATM mutations in 38 unselected breast tumour samples, indicating that ATM is not a candidate for this tumour suppressor gene at 11q23.

A significant feature of AT heterozygotes is the cellular radiosensitivity, which is intermediate between that of normal individuals and AT patients in terms of cell survival (Ramsay *et al*, 1996) and number of aberrations per 100 cells (Scott *et al*, 1994) after irradiation. This observation was potentially significant as there has been a reported correlation between radiosensitivity and cancer proneness (Sanford *et al*, 1989), and this feature could be exploited for detection of AT heterozygotes by the G_2 phase chromosomal radiosensitivity assay (Tchirkov *et al*, 1997; Scott *et al*, 1996).

There is also evidence against the suggestion that a high proportion of breast cancer patients with an extreme reaction to radiotherapy are likely to be AT heterozygotes. Ramsay *et al* (1998) selected 15 breast cancer patients who had developed a severe late reaction to a standard radiotherapy schedule. Cellular radiosensitivity compared to controls was confirmed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay and mutation analysis performed using a protein truncation assay. No mutations were found in the test cases, indicating that mutation testing for the ATM gene is unlikely to be useful in predicting response to radiotherapy.

There is certainly strong evidence to suggest AT carriers have a predisposition to breast cancer. However, the fact that some studies have found no such link indicates that the relative risk involved may be lower than reported in some papers. The risk associated with certain ATM mutations may be greater than with others, and these

risks may be difficult to measure, due to low penetrance. In comparison to BRCA1 mutations, which can very definitely be associated with inherited breast cancer (Szabo and King, 1995), heterozygous ATM mutations may be a factor in stomach cancer, leukaemia and lymphoma as well as breast cancer (Swift *et al*, 1976).

1.4: The ATM gene

Initial studies suggested that ataxia telangiectasia was genetically heterogeneous. Houldsworth and Lavin's (1980) finding that no decrease in DNA synthesis was obtained in AT cells immediately following irradiation, prompted Jaspers and Bootsma (1982) to test complementation of the radioresistant DNA synthesis (RDS) phenotype. Using five AT cell lines and a normal cell line, RDS was measured in binucleate fusion cells. The inhibition of DNA synthesis caused by X-rays in the heterodikaryons was more pronounced than in the parental homodikaryons and was comparable to that in normal binucleate cells, indicating complementation. Jaspers and Bootsma (1982) suggested four complementation groups in AT, designated A-D. It was assumed that a different gene was mutated in each group, but later studies would find all AT mutaions would cosegregate within the one gene.

Gatti *et al* (1988) performed linkage analysis on 31 families with AT-affected members and thus mapped the AT gene for complementation group A to chromosome 11q22-23. Their results also indicated linkage of the other groups to the same region and further research confirmed this (Lambert *et al*, 1991; Ziv *et al*, 1991; Shiloh *et al*, 1995; McConville *et al*, 1993).

In order to clone ATM, Savitsky *et al* (1995) constructed a yeast artificial chromosome (YAC) contig and cosmid contigs spanning the interval from D11S384 to D11S1818, known from linkage studies (Lange *et al*, 1995) to contain the AT gene. Hybrid selection based on direct hybridisation of genomic DNA with cDNA's and exon amplification to detect splicing activity were both used to identify transcribed sequences. One cDNA clone, called 7-9, had an open reading frame that predicted a protein of 1,708 amino acids. The affected members of family ISAT9 were found to have a deletion spanning most of this clone. This led to a search for further mutations within this clone in AT patients using the restriction enzyme fingerprinting (REF) method on the cDNA. When an abnormal REF pattern was found, the relevant portion of the transcript was sequenced. Most of the mutations (7/10) identified in 14 patients were predicted to lead to truncation of the product, while three were predicted to cause in frame deletions of 1, 2 or 3 amino acids. Significantly, mutations in the region of clone 7-9 were found in patients assigned to all four complementation groups, indicating that mutations in this single sequence were solely responsible for the disorder.

Sequencing of clone 7-9 revealed a kinase domain similar to that of mammalian phosphatidylinositol-3 kinase (PI-3 K) and further similarity to the Schizosaccharomyces pombe gene, rad3. Northern blot analysis using the 7-9 clone as a probe indicated that the gene involved had a major transcript of around 12kb. Savitsky *et al* (1995a) designated this gene 'AT mutated' or ATM.

Savitsky *et al* (1995b) reported the creation of a cDNA contig spanning the entire ATM open reading frame which was found to be 9168 nucleotides long. The

predicted protein of 3,056 amino acids contained the previously reported PI-3 kinase domain (Savitsky *et al*, 1995a) and a potential leucine zipper at codons 1217-1238. It also showed significant similarity to large proteins in S. cerevisiae (TEL1, MEC1, TOR1 and TOR2), S. pombe (rad3), Drosophila (MEI-41) and mammals (RAFT1, FRAP and DNA-PK_{cs}), all of which share the PI-3 kinase domain. This family of proteins is involved in the detection of DNA damage and control of cell cycle progression. Mutations in these genes confer phenotypes similar to those displayed by AT cells in vitro. Figure 1 illustrates the position of the ATM gene on chromosome 11.

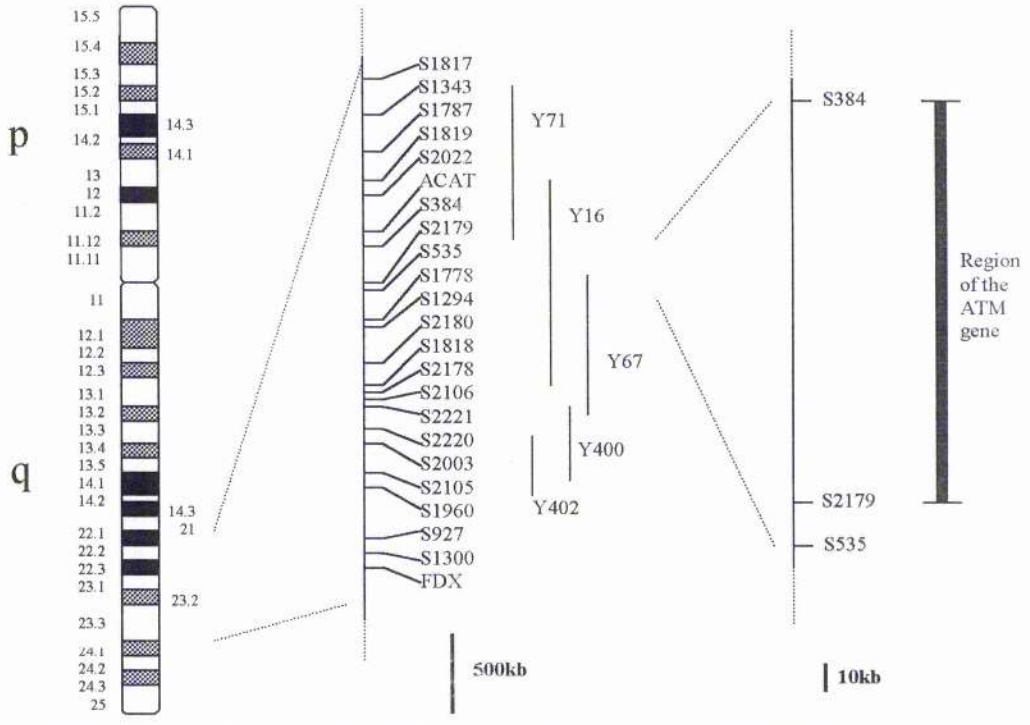


Figure 1.1: Position of the ATM gene.
ATM was initially localized to region 11q22-23 by linkage analysis (Gatti et al, 1988). Savitsky et al (1995a; 1995b) identified the 200kb of the gene by constructing a YAC cosmid across the region and using Y16 and Y67 to screen a cosmid library to construct a cosmid contig. Successive screening of randomly-primed cDNA libraries enabled construction of a cDNA contig of around 10kb.

Sequencing of the 5' half of the ATM gene revealed that the N-terminal half of the product has no homology to other proteins (Byrd *et al*, 1996). Six mutations were identified in this portion of the gene, one of which is associated to a haplotype common to four apparently unrelated families of Irish descent. This mutation was identified as a 200bp deletion in cDNA and was expected to result in protein truncation at codon 880. As with patients described in previous reports, all were compound heterozygotes. All but one of the mutations identified by Byrd *et al* (1996a) were deletions, while 6 out of 9 (including three in the 3' half of the gene) were predicted to truncate the protein.

Byrd *et al* (1996a) also reported that the initiation codon of ATM was 681bp downstream of the initiation codon of a novel gene, E14. In a subsequent report, they found ATM and E14 are transcribed in opposite directions and found evidence of a bidirectional promoter region serving both genes, with expression directed towards ATM threefold higher than towards E14 (Byrd *et al*, 1996b). This has led to speculation that ATM and E14 (called NPAT, for Nuclear Protein mapped to the AT locus, by Imai *et al*, 1996) may operate in the same biochemical pathway (Byrd *et al*, 1996b).

Using long distance PCR, Uziel *et al* (1996) determined the genomic organisation of the ATM gene. They showed that it has 66 exons from 43bp to 634bp (excepting the 3.8kb 3' exon which is largely untranslated) which encode a transcript of 12.8kb. The first two, 1a and 1b exons, are spliced differentially into alternative transcripts. The initiation codon is in exon 4 and the final 3.6kb of exon 66 is a 3' untranslated region.

Uziel *et al* (1996) estimated the size of the gene to be about 150kb, but this size was refined later to 146kb (Platzer *et al*, 1997).

Ultimately a single full-length cDNA has been cloned in Epstein-Barr virus based vectors (Zhang *et al*, 1997, Ziv *et al*, 1997) Transfection with these constructs resulted in correction of multiple aspects of the radiosensitive phenotype of AT cells, including survival in response to ionising radiation, decreased radiation-induced chromosome aberrations, reduced RDS, partial correction of cell-cycle checkpoints and partial induction of stress-activated protein kinase. This provides further evidence of the multiplicity of effector functions performed by the ATM product and suggests possible approaches to gene therapy. Introduction of a missense mutation into the cDNA resulted in instability of the protein and an inability to correct the AT phenotype. In order to generate large quantities of recombinant protein, the same groups then independently cloned the cDNA in a baculovirus vector and successfully expressed the gene in insect cells (Scott *et al*, 1998; Ziv *et al*, 1997). Although this sytem had previously proved efficient, ATM could only be expressed at levels much lower (20ng/100ml infected cells) than was hoped for (greater than 100μg/100ml infected cells), probably because of the strain placed on a cell by over-expression of such a large protein. Meanwhile a recombinant protein had also been shown by Ziv *et al* (1997) to restore normal cellular phenotype to AT cells.

Expression of ATM may be subject to post-transcriptional modification. Savitsky *et al* (1997) characterised the 5' and 3' untranslated regions (UTR's) of ATM transcripts in several tissues and compared their sequences to those of the corresponding genomic domains of the gene. Extensive alternative splicing in the 5' UTR and differential

polyadenylation in the 3' UTR were observed. The 5' UTRs may have between 1 and 18 upstream AUGs (initiation codons) and free energy values from –27.4 to –174.2 kcal/mol, providing a range of transcripts requiring very different conditions for efficient translation. An additional putative promoter was also identified in exon 3 with a TATA box and sites for binding of trans-acting factors (Platzer *et al*, 1997). This promoter could provide a transcript with a short 5' UTR allowing basal levels of ATM translation, while translation of transcripts from the upstream promoter would be regulated.

Savitsky *et al* (1997) also found the long 3' UTR of ATM contained several AU-rich elements (AREs) and polyadenylation signals of varying efficiencies. Savitsky *et al* (1997) speculated that transcripts with different tail lengths could have different half-lives, and that the 3' UTR could also be responsible for localisation of the protein within the cell.

Platzer *et al* (1997) sequenced 184 kb of region 11q22-23 containing the complete ATM gene, 10kb of E14 and the intergenic region containing the bidirectional promoter serving these two genes. Analysis found a possible L1/L2 isochore boundary (where there is a distinct change in GC:AT content) between the main body of the gene and the PI3 kinase domain. This difference in A:T to G:C content may represent a change in compositional constraints between regions of the gene.

Nijmegen breakage syndrome (NBS) shares immunodeficiency, genome instability , clinical radiosensitivity and cancer predisposition, but not cerebellar deterioration, with ATM and cellular phenotypes are almost indistinguishable (Shiloh, 1997). A

gene for NBS has been found to map to chromosome 8q21 (Saar *et al*, 1997). The similarities between the two diseases suggest the unidentified NBS protein may act in the same pathway(s) as the ATM protein.

1.5:Mutations

The disease AT has an incidence of around 1 in 100,000 and an estimated gene frequency of 0.007 (Swift et al 1986; Easton *et al*, 1994).

Mutation detection turned out to be even more difficult than expected within a gene of 154kb and 65 exons. The results of the first studies (Byrd *et al*, 1996a; Teletar *et al*, 1996; Gilad *et al*, 1996; Wright *et al*, 1996) indicated that there were no individual mutations which are predominant, and that there are no mutation hot spots, even although a number of different methods were employed to detect mutations

On publication of the sequence of the 5' half of the ATM gene, Byrd *et al* (1996a) also reported 12 mutations within the gene, detected by restriction endonuclease fingerprinting (REF). One of these was a 200bp deletion which was found in four unrelated patients of Celtic descent.

Gilad *et al* (1996) performed REF on RT-PCR products from cultured fibroblasts or lymphoblastoid lines derived from 55 AT families. Of the 44 mutations identified, 39 (89%) were expected to inactivate the protein by truncating it, as a result of incorrect initiation or premature termination of translation or by gross deletion. Other mutations

were small in-frame deletions and an amino acid substitution in the highly-conserved PI-3 kinase domain.

Wright *et al* (1996) assayed 36 cell lines from unrelated AT patients and two control lines for ATM mutations. They detected, by SSCP, a total of 30 mutations, including two substitutions, one insertion and 27 deletions of 2 to 298 nucleotides. A 9bp deletion at codon 2546 in exon 54 was detected in 3 individuals and was the most common mutaion. This mutation had previously been reported in five different patients (Byrd *et al*, 1996a; Gilad *et al*, 1996) and represented 8% of all reported mutations at that time.

To avoid the extraction and analysis of RNA, Vorechovsky *et al* (1996c) employed an exon-scanning protocol for mutation detection in ATM. Amplification of each of the 65 exons individually and analysis by SSCP gave a 70% detection rate, but throughput was low.

McConville *et al* (1996) identified 14 families (representing about 10%-15% of AT families in the UK) in which homozygosity is associated with a less severe clinical and cellular phenotype. In ten of these families, a point mutation in intron 40 produces a cryptic splice donor site, 137 bases downstream of an acceptor-like sequence, which results in the inclusion of these 137 intronic bases. The inserted sequence has stop codons in all three reading frames and is expected to cause a truncated protein of 60% the normal size. However, while the cryptic splice donor site generated by the mutation is very strong, the acceptor-like sequence is very weak and is not always recognised (ie the mutation is 'leaky'), allowing some transcripts to be spliced

normally. Expression of low levels of a protein of normal size indicate that this is the reason for late onset and mild phenotype of the disease.

Stankovic *et al* (1998) compared genotype and phenotype in a group of British patients. Two of the 11 founder mutations in the UK confer a mild form of AT with respect to cellular degeneration and cellular features. Of these, a 7271 T→G base change mutation in the cDNA, associated with expression of a full-length protein at normal levels, was found in two families. Although homozygotes for this mutation exhibited a phenotype less severe than classical AT, both homozygotes and carriers are at a high risk of breast cancer. A compound heterozygote with this mutation has a mild phenotype, but a patient homozygous for this mutation has a phenotype even milder, in terms of longevity and fertility. This indicates a dose response effect of ATM function. One homozygote reported here is the only known AT patient to have a child.

A wide variety of ATM mutation types, including missense mutations and in-frame deletions, were seen in a group of 18 AT patients who developed leukaemia, lymphoma, preleukaemic T-cell proliferation or Hodgkins lymphoma (Stankovic *et al*, 1998).These mutations were scattered throughout the gene, indicating that no particular region is associated with lymphoid tumours. However, mutations were associated with concordance for tumour type. In families in which two homozygous siblings developed a lymphoid tumour, the same type of tumour developed at the same age. It may be significant that 8 of 15 homozygotes who had developed leukaemia or lymphoma had missense mutations or in frame deletions which resulted in expression of mutant protein.

Gilad *et al* (1998b) also found that the mild variants of AT exhibited 1%-17% of normal ATM protein level. However, an individual homozygous for a mutation resulting in truncation of the protein by only the last nine amino acids had a pronounced phenotype, which Gilad *et al* (1998b) interpreted as an illustration of the importance of the carboxy terminus of the ATM protein, a region probably harbouring the catalytic site and playing a role in stability. Gilad *et al* (1998b) also found two patients homozygous for the same mutation, one of whom did not have telangiectasia at all (though expressed other symptoms), pointing to the involvement of other physiological or genetic factors in the determination of the extent or existence of disease features.

In contrast to the mild forms of AT mentioned above, AT_{Fresno} patients, who exhibit AT phenotype with microcephaly and mental retardation (Curry *et al*, 1989), have mutations typical of the classical disease, and produce no ATM protein. As many classcal AT patients similarly express no ATM protein, it is unlikely that AT_{Fresno} is due to a particularly devastating mutation. Instead it has been suggested that this form of the disease may represent a subgroup of AT patients who have a lesion in a second gene, causing a more extreme phenotype.

Despite over 200 mutations in AT patients having been defined, Teletar *et al* (1998b) noted "we do not appear to be reaching a point of saturation for new mutations". Clinical screening for mutations would be laborious, due to the absence of a mutation hot spot and the fact that there are 65 exons over 154kb of DNA. It is also notable that Gilad *et al* (1996), Teletar *et al* (1996) and Wright *et al* (1996) between them assayed 141 patients, who must carry 282 mutations, yet detected only 82 of these. These

methods require the production of high quality cDNA, (which is not always possible from a clinical sample) and although 71% of mutations are expected to truncate the protein, only 21 out of 96 mutations could be detected by PTT (Teletar *et al*, 1996).However, Gilad *et al* (1998a) modified the REF method so that only minimal amounts of RNA were required (from 150μl of blood) and Stankovic *et al* (1998) detected 100 mutations out of 136, equivalent to the predicted 71% causing truncation, so detection efficiency is improving.

Ejima and Sasaki (1998) studied the 16 AT alleles from eight affected Japanese families and found 7 (44%) were either 4612del65 or 7883del5. Microsatellite genotyping confirmed that each of these was associated with a common haplotype. They suggested these two founder mutations may be predominant among ATM mutant alleles. It has been shown that six founder mutations account for more than 95% of all mutaions in Costa Rica (Teletar *et al*, 1998a). Extending this study, Teletar *et al* (1998b) designed rapid assays for the three most frequent of these (CRAT [A], [B] and [C]) representing 76% of Costa Rican mutations as well as further assays to detect 50% of mutations in Norwegians, 27% in Poles and 14% in Italians.

It is clear that in the detection of ATM mutations there is a compromise between throughput and sensitivity. The inefficiency of screening individuals for all ATM mutations and the expectation that a detectable percentage of heterozygotes may carry one of a small number of founder mutations suggested it would be most efficient to develop a high throughput test for these mutations.

T-cell prolymphocytic leukaemia (TPLL) is a rare clonal malignancy. Stilgenbauer *et al* (1997) found deletion of segment 11q22.3-23.1 in 15 of 24 TPLL tumours. The remaining allele of six of these 15 were anaylsed for mutations, and in all six mutations of the second ATM allele were identified. Similarly, Stoppa-Lyonnet *et al* (1998) found of loss of heterozygosity (LOH) at the ATM locus in a high proportion (10 of 15) of a series of these tumours. Four of those tumours with LOH also had a mutation (three somatic and one germline) of ATM in the remaining allele. Stoppa-Lyonnet *et al* (1998) therefore concluded that ATM is a tumour-suppressor gene whose inactivation is a key event in the development of TPLL. Vorechovsky *et al*, (1997) and also found deletion of one ATM allele and mutation of the other in TPLL tumour cells, but these mutations did not follow the distribution seen in AT patients, rather being most frequently nucleotide substitutions, clustered in the region of the kinase domain. One of the mutations detected in this study was the 9bp deletion in exon 54 reported previously by Wright *et al* (1996), Byrd *et al* (1996a) and Gilad *et al* (1996).

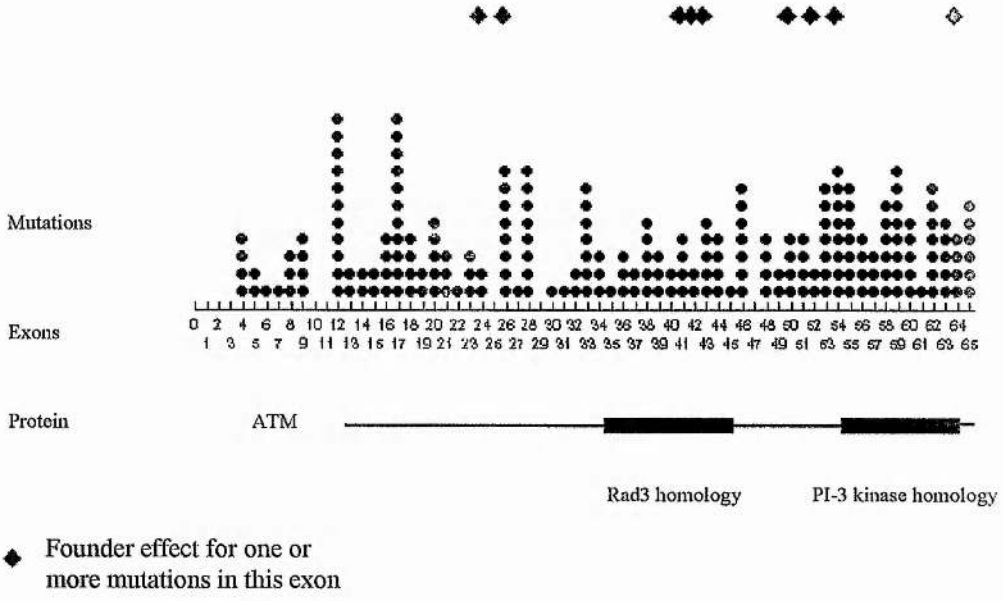


Figure 2: Mutations in the ATM gene detected in AT patients. ATM Mutation Database http://www.vmmc.org/vmrc/atm.htm

The overall picture is that ATM has a wide spectrum of mutations (illustrated in figure 2), most of which result in expression of no stable protein, and that these mutations generally cause classical AT in homozygotes (eg Stankovic *et al*, 1998). Stankovic *et al* (1998) compared phenotype and genotype in a group of patients and found that in the British Isles, 59 mutations have been observed so far (Stankovic *et al*, 1998), of which 11 are founder mutations, and the cancer risk (and type of cancer) associated with carriers varies between mutations. However, a number of mutant alleles producing a stable mutant protein are associated with a mild phenotype or late onset AT (eg Gilad *et al*, 1998b). That AT mutations cause a predisposition to tumourigenesis is seen by their predominance in TPLL (eg Vorechovsky *et al*, 1997; Stilgenbauer *et al*, 1997; Stoppa-Lyonnet *et al*, 1998).

1.6: Protein

From the cDNA sequence, the size of the ATM product was predicted to be around 350kDa (Savitsky *et al*, 1995b; Byrd *et al*, 1995). The first antibodies raised against synthetic peptides corresponding to different regions of the predicted amino acid sequence detected a protein of this size (Lakin *et al*, 1996; Watters *et al*, 1997; Brown *et al*, 1997) or slightly larger (Chen and Lee, 1996) which was absent in AT cell extracts.

Brown *et al* (1997) developed a rabbit antiserum against a synthetic peptide corresponding to amino acid residues 819-844 of the predicted primary structure of ATM. This region was believed to be unique to the ATM protein. The specificity of the antiserum, designated pAb132, was confirmed by testing its reactivity to GST

fusion peptides and to protein extracts from normal and AT cell lines. Using pAb132, Brown *et al* (1997) established that the ATM product is a high molecular weight protein predominantly confined to the nucleus of human fibroblasts but present in both the nuclear and microsomal fractions of lymphocytes and lymphoblasts. They also evaluated the location of the ATM product at various stages of the cell cycle by immunofluorescence on synchronised fibroblasts. pAb132 stained only the nucleus during the G0, G1 and G1/S phases. Following breakdown of the nuclear envelope at the onset of mitosis, ATM staining appeared diffuse and did not appear to be associated with any particular structures. Truncated ATM protein was not detected in lymphocytes from AT patients homozygous for mutations leading to premature protein termination. Abundance of ATM and p53 proteins was quantatively analysed in extracts from unirradiated cells and cells at 1, 2, 4 and 6 hours post-irradiation. The cellular levels and electrophoretic mobility of the ATM protein remained unchanged after irradiation while a quantitative increase in p53 was observed. These findings, that ATM is constitutively expressed and is predominantly nuclear, are consistant with ATM's proposed role as a signalling protein involved in DNA damage detection. That ATM has no recognisable nuclear localisation sequence (NLS) (Savitsky *et al*, 1995b) led Brown *et al* (1997) to suggest ATM has a previously unknown NLS or is imported to the nucleus by associated factors. A punctate staining pattern was observed within the nucleus of fibroblasts stained with pAb132 (Brown *et al*, 1997) which indicates that ATM may be associated with particular structures of the nucleus.

Lakin *et al* (1996) found no detectable protein in AT patients with truncating mutations, even when truncation was expected to occur downstream of the antibody binding site. Similarly Watters *et al* (1997) found no protein in the 13/23 AT cell lines

which had truncating mutations. Brown *et al* (1997) also failed to detect the presence of truncated protein by immunoblot analysis of three cell lines derived from homozygous AT patients with mutations predicted to result in premature termination of the ATM protein. The absence of truncated protein has been reported in other mutant genes and has been attributed to disruption of proper protein folding (Pakula and Sauer, 1989). The failure to detect mutant ATM protein levels in homozygous cells indicates that patients will have little or no ATM protein function. Furthermore, the predicted 50% reductions of protein levels in heterozygotes may account for the intermediate phenotype (with respect to cancer predisposition and risk of heart disease) reported in these individuals (Swift *et al*, 1991) and the intermediate radiosensitivity of heterozygous cell lines (Scott *et al*, 1994; Ramsay *et al*, 1996).

In normal cells ATM appears to be ubiquitously expressed (Lakin *et al*, 1996; Chen and Lee, 1996; Watters *et al*, 1997; Brown *et al*, 1997) and is not regulated in response to UV or ionizing radiation (Lakin *et al*, 1996) or the radiomimetic drug neocarzinostatin (Brown *et al*, 1997). However, Chen and Lee (1996) found expression levels to be higher in the embryonic lung and CNS and in the adult spleen, testis and thymus than in other tissues. This pattern of high expression of normal protein in certain tissues may relate directly to the disease, with the CNS, testis and thymus being worst affected in AT patients.

As discussed above, ATM is mostly confined to the nucleus (Lakin *et al*, 1996; Chen and Lee, 1996; Watters *et al*, 1996; Brown *et al*, 1996), despite no recognisable nuclear localisation signal being present, but it may also be present in small quantities in the cytoplasmic vesicles (Lakin *et al*, 1996; Watters *et al*, 1997). Chen and Lee

(1996) initially detected ATM in large complexes, and found the protein in a phosphoryalated form. Together these results suggested to Chen and Lee (1996) that ATM may work in a similar manner to DNA-PK_{CS}, which operates in association with the Ku antigen and is inactivated by phosphorlyation.

Mutant ATM protein appears to have a dominant negative effect on normal ATM activity, as only partial correction of cellular defects could be achieved by induced expression of normal ATM cDNA transfected into mutant cells (Zhang *et al*, 1997; Scott *et al*, 1998). In this respect, it is of note that the two cell lines (AT1ABR and AT3ABR) selected by Zhang *et al* (1997) were capable of producing near full-length or truncated protein. It would be interesting to see if complete correction of cellular defects can be achieved by expressing an exogenous normal gene in ATM knock-out cells. This would determine if the mutant protein is disrupting normal ATM processes.

1.7: Glycosylation

Synthesis of mammalian proteins takes place on ribosomes which are free in the cytosol. If the protein is destined to be secreted or targeted to an organelle, the ribosome will attach to the rough endoplasmic reticulum (RER) and the nascent protein will be delivered into the lumen of the RER as polypeptide elongation continues. Many proteins are modified by the addition of glycosyl groups and this is often related to biological function. The maturation of a typical oligosaccharide chain is represented in figure 1.3. Core N-glycosylation of proteins occurs in the RER, with a standard fourteen residue sugar being constructed on a carrier lipid (dolichol

phosphate) and then being transferred to the amide group of a specific asparagine residue in the polypeptide. The sugar chain consists of two N-acetylglucosamine (GlcNAc), nine mannose (Man) and three glucose (Glc) residues. Thereafter the three glucoses and one of the mannoses are trimmed and the glycoprotein can be transported to the Golgi apparatus, where terminal glycosylation takes place. This involves the addition of O-linked sugar units and modification of N-linked sugars, before the shuttle vesicles of the *trans*-Golgi export the protein to the appropriate destination (Stryer, 1988).

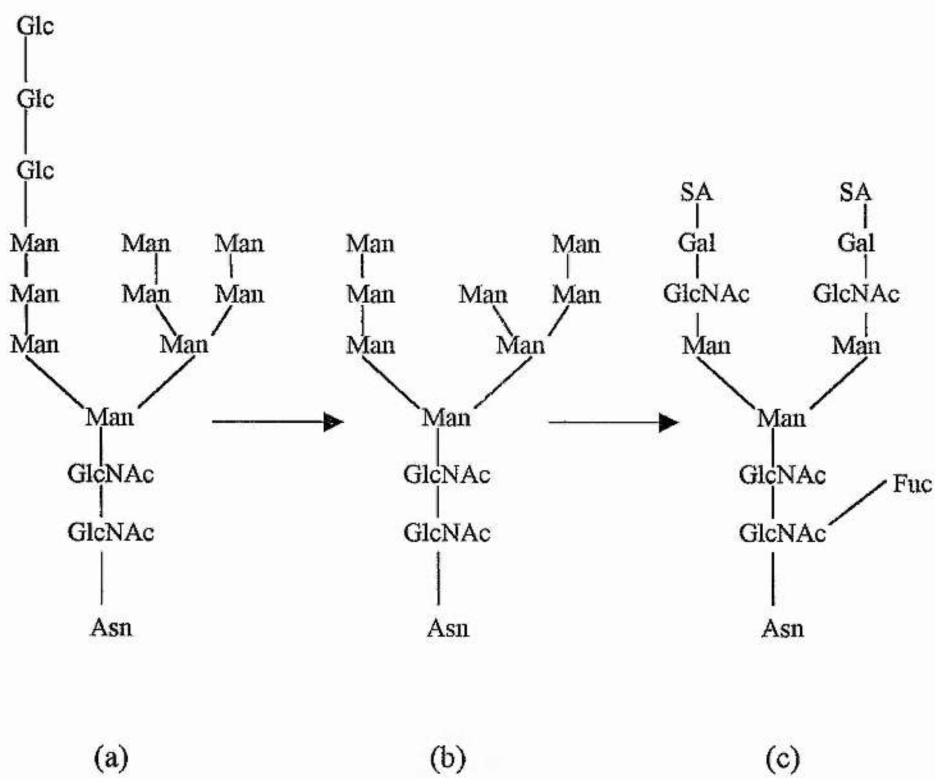


Figure 1.3: Structures of protein-linked oligosaccharide chains. (a) Core N-linked oligosaccharide, transferred to protein in the endoplasmic reticulum. (b) High mannose chain, formed by trimming of (a) in the Golgi. (c)Complex oligosaccharide formed from (b) by further trimming and sugar addition in the Golgi. From Apps, Cohen and Steel, 1992.

Oligosaccharide chains can be removed experimentally from a protein by two types of enzyme: exoglycosidases and endoglycosidases. Exoglycosidases, such as the sialidases, specifically release single monosaccharides only when they are present as terminal units on sugar chains. Endoglycosidases cleave intact oligosaccharides from proteins under mild conditions that preserve the structure of both the oligosaccharide and the protein. N-linked sugars can be removed from a glycoprotein by the use of endoglycosidase H (high mannose and hybrid chains), endoglycosidase F_2 (biantennary chains) and Peptide:N-glycosidase F (those cleaved by endo H and endo F_2 as well as tri- and tetra-antennary chains). As shown in figure 1.4, endo H cleaves the bond between the two GlcNAc residues in the core of N-linked oligosaccharides. (Freeze, 1994).

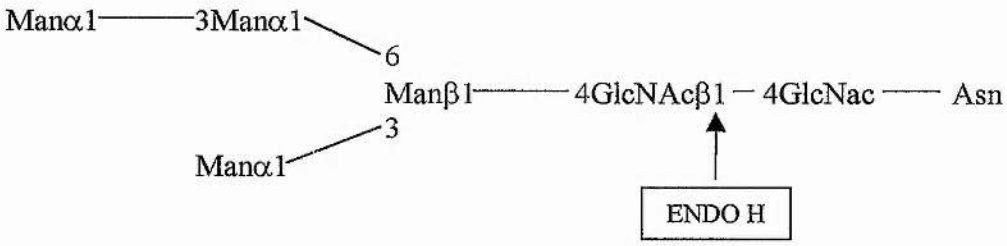


Figure 1.4: the minimal structure required for endoglycosidase H digestion.

Lectins are proteins or glycoproteins that bind with great specificity to certain carbohydrate structures and are able to agglutinate cells or precipitate complex carbohydrates. Lectins are isolated from a wide variety of natural sources, including seeds, plant roots and bark, fungi, bacteria, seaweed and sponges, molluscs, fish eggs, body fluids of invertebrates and from mammalian cell membranes. The role of lectins in nature is unknown, but the lectin binding properties of a protein can provide

evidence of the type of sugar structures attached to a protein. This can be done by probing a protein blot with labelled lectins, or by extracting proteins from a crude extract with a lectin bound to agarose beads (Freeze, 1993).

1.8: ATM-like genes

1.8.1: Mouse Models

Pecker *et al* (1996) showed that the mouse Atm gene encodes a 3,066 amino acid predicted protein with 84% identity to the human sequence. Northern blots showed a 13kb transcript in brain, skeletal muscle and testis and lower amounts in other tissues. A 10.5kb band was also seen in testis mRNA.

Mouse models of AT have been created individually by Barlow *et al* (1996), Elson *et al* (1996) and Xu and Baltimore (1996). Barlow *et al* (1996) disrupted the Atm locus by gene targeting. Mice homozygous for the disrupted allele displayed growth retardation, neurologic dysfunction, male and female infertility, defects in T-lymphocyte maturation and extreme radiosensitivity. Most of the animals developed malignant thymic lymphomas between 2 and 4 months of age. Fibroblasts from these mice showed abnormal radiation-induced G1 checkpoint function. Xu *et al* (1996) also found $Atm^{-/-}$ mice had a phenotype similar to human AT.

Westphal *et al* (1997) bred mice heterozygous for null alleles of atm and p53 to allow generation of all genotypic combinations. Mice doubly null for atm and p53 exhibited dramatically accelerated tumour formation relative to single-null mice, suggesting that

the two genes collaborate to prevent tumorigenesis. Loss of atm rendered thymocytes partially resistant to apoptosis, whereas loss of p53 caused complete resistance implying the atm and p53 apoptotic pathways are not totally superimposible. Westphal *et al* (1997) found that atm and p53 do not appear to interact in acute radiation toxicity, suggesting a separate atm effector pathway for this DNA damage response.

1.8.2: ATM-related genes

Chromosomal stability in eukaryotes is maintained at four levels. First the chromosomes themselves have cis-acting structures: replication origins, telomeres and centromeres. Second, trans-acting factors aid fidelity in replication and segregation. Third, cell cycle checkpoints delay cell progression when defects are detected in essential genetic components, to allow repair before cell division. Finally, repair mechanisms correct spontaneous or induced damage to the genome. The cellular phenotypes of ATM clearly suggest defects at one or more of these levels (Zakian, 1995).

The carboxy terminus of a conceptual ATM protein has similarity to the catalytic domains of the phosphatidylinositol 3 kinases (PI3K's) from mammals and Saccharomyces (Savitsky *et al*, 1995a). This raises the question of whether ATM, like PI3K's, functions in signal transduction. The ATM product shows even more extensive similarity to several large proteins, all of which, like ATM, have PI3K-like motifs at their carboxy-terminus (Savitsky *et al*, 1995a,b). Among the members of this family are the Saccharomyces genes TOR1 and TOR2 as well as their mammalian

homologues FRAP and rRAFT, the Schizosaccharomyces pombe rad3 gene and the Saccharomyces MEC1 gene. Saccharomyces cells that lack both Tor1p and Tor2p arrest in G1 (Kunz *et al*, 1993), indicating they are required for the G1-to-S transition. The S. pombe rad3 gene is a nonessential gene involved in both the S-M and G2-M cell cycle checkpoints and for DNA repair (Jiminez *et al*, 1992; Seaton *et al*, 1992). MEC1 is an essential Saccharomyces gene that is required for the S-M and the G2-M checkpoints as well as meiotic recombination (Weinert *et al*, 1994; Kato and Ogawa, 1994).

The prototype PI3K, with which ATM shares its kinase domain, is a heterodimer composed of an 85kDa regulatory subunit and a 100kDa catalytic subunit (Carpenter *et al*, 1990). The family of larger proteins including ATM, ATR, DNA-PK, TOR1 and FRAP are more like each other than PI3K in sequence and subdomain spacing (Hunter, 1995). Furthermore, no lipid kinase activity has been detected in DNA-PK (Hartley *et al*, 1995), TOR/FRAP (Zdzienicka *et al*, 1995) or ATM (Jung, 1997).

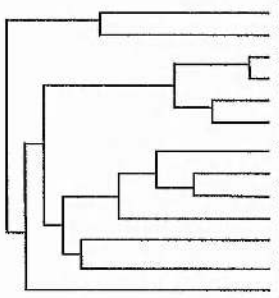
Ataxia telangiectasia and Rad3 related (ATR) protein, cloned and sequenced by Bentley *et al* (1996), is also a protein kinase essential for mediating checkpoint regulation.The 2644 amino acid ATR protein has extensive homology to *S. pombe* Rad3 and is also more closely-related to MEC1 and mei-41 than is ATM. Checkpoint responses to X-ray, UV and HU treatment are defective in *rad3* mutants (Bentley *et al*, 1996). Again like ATM, ATR can bind directly with meiosis I chromatin. ATR binds with asynapsed regions in zygotene and pachytene (Keegan *et al*, 1996).

The Saccharomyces gene TEL1 has the greatest similarity to ATM (Greenwell *et al*,1995; Morrow *et al*, 1995).However, while TEL1 is very similar to ATM in terms of sequence, yeast cells carrying TEL1 mutations are not radiosensitive and do not show signs of checkpoint defects (Greenwell *et al*, 1995; Morrow *et al*, 1995). MEC1, on the other hand, has similar cellular phenotypes to ATM, but is essential, so must perform at least one function that ATM does not (Siede *et al*, 1996).

Hari *et al* (1995) reported the cloning and sequencing of the Drosophila mei-41 gene and demonstrated that it, too, is an ATM-like gene. Hari *et al* (1995) showed that mei-41 cells, like ATM-deficient cells, fail to show an irradiation-induced delay in the entry into mitosis that is characteristic of normal cells. Cells deficient in mei-41 are also sensitive to DNA damaging agents and have elevated numbers of chromosome aberrations even in the absence of DNA damage. Thus there are phenotypic similarities between AT cells and mei-41 cells.

Human DNA-dependant protein kinase (DNA-PK_{CS}) is the only ATM-like protein whose mechanism of action has been characterised (Anderson, 1993; Hartley, 1995). The catalytic subunit becomes active when recruited to a complex by the binding of a subunit (Ku) to DNA double strand breaks, nicks or gaps. DNA-PK is a protein kinase with no detectable lipid kinase activity. Human or rodent cells lacking either Ku or the catalytic subunit are radiation sensitive, repair deficient and incapable of V(D)J recombination of immunoglobulin genes (Jeggo *et al*, 1995). ATM may operate in a similar way to the DNA-PK paradigm, but DNA-PK cannot substitute for the ATM product in the cellular response to ionizing radiation (Jongmans *et al*, 1996).

Sequence comparisons suggest that the family of large proteins containing PI3K-like domains are more related to one-another than they are to classical PI3K's (figure 1.5; Hari *et al*, 1995; Hartley *et al*, 1995). The gene with greatest similarity to ATM is the Saccharomyces gene TEL1 but the Drosophila mei-41 gene, the S. pombe rad3 gene



	Kinase		Adjacent	
	%I	%S	%I	%S
P110	24	51	15	43
Vps34	21	51	17	42
FRAP	32	59	18	43
RRAFT	33	59	19	44
Tor1p	33	58	20	44
Tor2p	35	60	22	41
MEI-41	37	59	21	45
ATR	31			
Rad3	39	60	20	47
Mec1p	37	61	21	46
ATM	100	100	100	100
Tel1p	45	66	21	46
DNA-PKcs	28	51	19	44

Figure 1.5: Percent Identity and Similarity to ATM. From Zakian (1995) except ATR figures (Bentley et al, 1996).
The figure represents relatedness of predicted proteins encoded by genes containing PI3K motifs in their COOH-terminal regions. Identity (%I) and similarity (%S) are given for both the kinase domain and the 1000 amino acids adjacent to (preceeding) the kinase domain relative to human ATM. In brief, p110 is a bovine PI3K; Vps34 is a Sacchaomyces PI3K; Frap and rRAFT are bovine and rat rapamycin-binding proteins; Tor1p and Tor2p are Saccharomyces proteins; mei41 is a Drosophila protein; Rad3 is an S. pombe protein; Mec1p and Tel1p are Saccharomyces proteins; DNA-PK$_{cs}$ is a human protein.

and the Saccharomyces geneMEC1 gene share not only a high level of sequence similarity but, judging by the phenotypes of mutants, many of the functions of ATM. Sequence similarity is based on the termimal 300 amino acid residues which contain the putative PI3K domain and on the adjacent 1000 amino acids of lesser but significant similarity. However, all of the ATM-like genes differ considereably both from each other and from ATM in their amino terminal regions. True ATM homologues may have similarity along the length of the protein.

1.9:Function

1.9.1: Diversity of ATM function

In order to begin to understand the way in which the ATM product functions, we must consider the various cellular phenotypes caused by absence of functional protein. While there is doubtless overlap between the pathways in which ATM is active, its involvement can be broken down into major cellular functions: G1/S checkpoint; G2/M checkpoint; genomic stability. How ATM function relates to these features is considered below.

1.9.2: ATM, p53 and the G1/S checkpoint

The most notable interaction of ATM is that with p53. It has been shown repeatedly that AT cells are defective in their ability to up-regulate p53 post-iradiation (Kastan *et al*, 1992; Khanna and Lavin, 1993; Canman *et al*, 1994). As $p53^{-/-}$ mice have no G1 arrest response to DNA damage (Kastan *et al*, 1992) and p53 is stabilised in normal human cells during G1 arrest (Kastan *et al*, 1991), it seems likely that the G1/S checkpoint is mediated by p53. It is believed that p53 is a transcription factor (Fields and Yang, 1990) responsible for initiating transcription of the cell's growth suppressor GADD45 (Kastan *et al*, 1992; Zhan *et al*, 1994) and $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependant kinase activity (Harper *et al*, 1993). Accumulation of $p21^{WAF1/CIP1}$ delays E-cdk2 mediated advance into S phase (Dulic *et al*, 1994). It has been shown that cyclin dependant kinases A-Cdk2, B-Cdc2 and E-Cdk2 are resistant to inhibition by ionising radiation exposure in AT cells, and this appears to be due to

insufficient induction of WAF1, which binds Cdk's, inactivating them (Beamish *et al*, 1996; Khanna *et al*, 1995).

The p53 response of AT cells to the radiosensitive drug bleomycin, to topoisomerase-targeted drugs and to nucleases is also defective (Nelson and Kastan, 1994; Canman *et al*, 1994). Significantly, p53 up-regulation in AT cells is normal following exposure to UV radiation or methylmethane sulphonate (MMS) (Khanna and Lavin, 1993; Artuso *et al*, 1995). Equally importantly, AT cells show no defect in DNA break repair (McKinnon, 1987). Taken together, these observations indicate that ATM operates in a signal transduction pathway which delays cell cycle progression in response to DNA strand breaks, but not to base damage (Nelson and Kastan, 1994).

The connection between ATM and p53 may be one of the underlying causes of cancer predisposition in AT patients. Tumours are the result of one cell acquiring abnormal proliferative capability. This multi-step process involves increased activity of proto-oncogenes and reduced activity of tumour-suppressor genes (Fearon and Vogelstein, 1990). As it is central to cell cycle delay in response to genome damage (Lane, 1992), p53 has been designated as a tumour-suppressor gene. AT cells suffering damage to their genome are unable to up-regulate p53 and so this damage will remain unrepaired and lesions will be inherited by daughter cells. This process increases the chance of a cell aquiring sufficient mutations to become tumorigenic.

The presence of a PI3K domain suggests one mode of action ATM undertakes is to identify and phosphorylate specific substrates in response to DNA damage. The G1/S checkpoint defect in conjunction with the reduced p53 response (Kastan *et al* ,1992)

of AT cells post-irradiation identified p53 as a possible substrate. Watters *et al* (1997) immunoprecipitated cell lysates with anti-p53 then immunoblotted the product with anti-ATM antibody and found ATM protein associating constitutively with p53 protein. This association was absent or very limited in AT cells. Jung *et al*, (1997) showed that IκB is a substrate for phosphorylation by ATM but could not demonstrate phosphorylation of a GST-p53 construct by ATM. Waterman *et al* (1998) found, however that ATM dephosphorylates serine 376 of p53. If ATM does phosphorylate or dephosphorylate p53 in damage response, this modification may explain the post-irradiation stabilisation of the p53 protein.

1.9.3: The G2 checkpoint

While evidence has been presented both in support of (Beamish and Lavin, 1994; Zampetti-Bosseler *et al*, 1981) and contradicting (Smith *et al*, 1985) a defect in the G2/M checkpoint response of AT cells, it now appears that some AT cells lines are capable of normal G2 arrest and others are defective in this response (Skog *et al*, 1997). It seems likely that this is another feature of the disease which varies from patient to patient, depending on other genetic factors. This is supported by the observation that cell lines from two brothers are both incapable of normal G2 arrest (Skog *et al*, 1997).

It has been thought that G2 arrest is unaffected by p53 status (Kastan *et al*, 1991) but Beamish *et al* (1996) found WAF1-mediated cdk inhibition to be a feature of the G2/M checkpoint and have suggested that p53 is required for WAF1 induction. AT cells irradiated in G1 or S phase ultimately arrest irreversibly in G2 18-24 hours post-

irradiation (Beamish and Lavin, 1994; Meyn *et al*, 1994) and Skog *et al* (1997) point to the fact that the aberrent p53 response of AT cells does include a reduced and late p53/WAF1 induction (Kastan *et al*, 1992).

1.9.4: ATM/ c-Abl interaction

CAbl is a non-receptor tyrosine kinase that is activated by ionising radiation (Kharbanda *et al*, 1995). Overexpression of c-Abl induces expression of WAF1, downregulates cyclin dependant kinase 2 (cdk2) and blocks cells in G1 phase (Yuan *et al*, 1996). Cells lacking c-Abl are impaired in their ability to downregulate Cdk2 and are thus unable to undergo G1 arrest in response to ionising radiation (Yuan *et al*, 1996). This parallels a defect of AT, presenting the possibility that ATM and c-Abl are active in a common pathway.

In normal cells ATM constitutively binds c-Abl and following irradiation, ATM activates c-Abl by phosphorylation (Shafman *et al*, 1997). Binding of c-Abl is not observed in AT cells and this results in diminished radiation-induced c-Abl tyrosine kinase activity (Shafman *et al*, 1997). In support of a role for ATM in direct activation of c-Abl kinase, Baskaran *et al* (1997) demonstrated a restored ability to phosphorylate c-Abl in AT cells using a recombinant protein corresponding to the PI3K domain alone. Further evidence of the interaction between the two proteins is that both ATM and c-Abl are found to associate directly with the axes of synapsed chromosomes in meiosis I (Kharbanda *et al*, 1998; Keegan *et al*, 1996). Although the function of ATM in meiosis is unknown, Keegan *et al* (1996) speculated that it may

be involved in recognising and responding to DNA strand interruptions occurring during meiotic recombination.

1.9.5: Genomic instability

ATM must have more than one function. While checkpoint defects can explain cancer predisposition, thay cannot be responsible for cerebellar degeneration in patients as neurones are non-cycling cells permanently in G0. Also, p53 deficient mice show no neurological degeneration, indicating that disruption of the p53/ATM interaction is not the cause. Further evidence of the multiplicity of roles ATM plays is the partial correction of the AT phentype in cell lines achieved by Zhang *et al* (1997) by transfecting the cells with a full-length clone of ATM cDNA. Overexpression of ATM cDNA in AT cells improved survival rates after irradiation, decreased radiation-induced chromosmal aberrations, reduced radioresistant DNA synthesis and partially corrected defective cell cycle checkpoints and induction of stress-activated protein kinase.

As AT cells are radiosensitive even when not dividing, it is accepted that the checkpoint defect in AT is not primarily responsible for radiosensitivity (Thacker, 1994; Meyn, 1995). In addition, p53-/- mice do not show radiosensitivity (Donehower *et al*, 1992).

Meyn *et al* (1994) found AT cells are sensitive to DNA damage-induced apoptosis due to X-rays or streptonigrin, but not UV. Humar *et al* (1997) found that shortly after γ-irradiation, a larger proportion of AT cells than control cells were committed to p53-

independent apoptosis. Those cells undergoing apoptosis have a higher number of DNA breaks, while non-apoptotic cells have normal rejoining capacity. This could explain the deleterious reaction of AT patients to therapeutic radiation.

Sensitivity to ionising radiation is a major hallmark of ataxia telangiectasia, having been illustrated *in vivo* after patients have been exposed to therapeutic doses of radiation and in cultured cells. It seems likely that an understanding of this aspect of AT may be central to comprehending the disease as well as the mechanisms of leukaemia and lymphoma formation. However, radiosensitivity may simply be the best understood aspect of a general sensitivity of AT cells to oxidative stress caused by endogenous reactive oxygen intermediates (ROIs) generated in mitochondria as by-products of energy metabolism (Vuillaume, 1987). Lavin (1998) suggested a model whereby the cytoplasmic fraction of ATM protein is involved in response to ROIs while the nuclear fraction interacts with p53 and other proteins, including c-Abl (Shaffman *et al*, 1997) in response to DNA lesions. A possible clue to the radiosensitivity of AT cells is that IκB-α, an inhibitor of NF-κB which transcriptionally activates oxidative stress genes, is expressed at high levels in AT cells (Jung *et al*, 1995). The radiosensitive phenotype is corrected by transfecting AT cells with cDNA coding a mutant form of IκB-α, which is constitutively activated in AT cells (Jung *et al*, 1995). Cells with the corrected phenotype showed normal levels of IκB-α and normal regulation of NF-κB. While the mechanism of correction is unknown, it is clear that aberrant NF-κB regulation may contribute to the radiosensitivity of AT cells.

Induction of stress-activated protein kinases (SAPK) in response to DNA damage caused by γ-irradiation is defective in AT cells, while SAPK induction is normal in response to UV damage (Shafman *et al*, 1995), pointing to the range of responses in which the ATM protein mediates. More recently it has been shown that Ku86 autoantigen related protein-1 (KARP-1) is induced following DNA damage in a response dependent upon ATM and p53 proteins (Myung *et al*, 1998). KARP-1 is expressed from the human Ku86 locus (normally encoding the 86kDa subunit of DNA-PK), using an upstream promoter which includes additional exons and encodes a larger protein (Myung *et al*, 1997). Myung *et al* (1998) suggested that KARP-1 is a factor involved in damage induced DNA-PK upregulation. In the absence of ATM or p53, the DNA-PK pathway would be impaired, but double strand break repair would be carried-out by RAD51-dependent pathways.

Shiekhattar *et al* have evidence, as yet unpublished, that ATM may be part of the human RNA polymerase II (RNAPII) complex. This complex contains transcriptional co-activators, and some components of the RNAPII complex participate in DNA repair (Maldonado *et al*, 1996). Replication protein A (RPA) co-elutes with the RNAPII (Maldonado *et al*, 1996), and it has been shown that radiation-induced phosphorylation of RPA p34 is deficient in AT cells (Cheng *et al*, 1996). ATM and RPA have also been found to co-localise along synapsed meiotic chromosomes (Plug *et al*, 1997). It therefore appears that ATM and RPA interact, probably at points of recombination during meiosis, and as DNA repair components of RNAPII. A summary of the interactions and pathways in which ATM is is believed to be involved is given in figure 1.6.

1.10: Aims

The aims of this project were two-fold: to investigate the prevalence of AT carriers among breast cancer patients; and to analyse the ATM protein with a view to indentifying any differences in ATM expression which may exist between different cell types.

The first section of the project required the design of appropriate methods to screen a population of Scottish breast cancer patients for heterozygous mutations in the ATM gene and thus estimate the burden of breast cancer in AT carriers in Scotland. This information could provide important evidence whether or not screening breast cancer patients for ATM mutations is viable. The high radiosensitivity of AT carriers could be taken into account when considering diagnostic or therapeutic radiation. Chapter 3 reports on the results of this section of the project. Of two ATM mutations reported to be present at a high rate in Celtic populations, one was only known at the cDNA level. The first step was to sequence this mutation so as to determine the genomic lesion. Rapid tests were then designed to easily detect these two 'Celtic' mutations from blood DNA. Finally, with these tests it was hoped to screen over 500 Scottish breast cancer patients.

As further studies were published, it became clear that these specific mutations represented a smaller proportion of ATM mutations than was initially thought and it was therefore decided to screen for carriers of most other mutations by western blot detection of mutant protein. This was expected to detect the 71-89% of ATM mutations, which were predicted to express a truncated product (Gilad *et al*, 1996;

Stankovic *et al*, 1998). However, the initial results which indicated that no stable mutant protein was produced (Lakin *et al*, 1996), were later confirmed by Watters *et al* (1997) and Brown *et al* (1997). In light of the large number of reports of screening for ATM mutations in breast cancer patients, the screening process was curtailed.

In the latter part of the study (chapter 4) anti-ATM antibodies were to be used in a number of immunological techniques to investigate the distribution of ATM and structural modifications, which might be related to its function. It was then intended to compare the characteristics of ATM expression, such as glycosylation, in normal individuals, AT heterozygotes and a series of breast cancer patients.

1.11: Summary

The ATM gene is large and complex. Mutations can be found in any of its 65 exons, and mutant alleles usually do not express a stable protein. There is strong evidence to suggest that individuals heterozygous for an ATM mutation have an excess risk of certain cancers, including breast cancer. The mechanism by which the protein is involved in tumorgenicity is unknown, but its large size suggests it may have several modes of action. It is known to interact with p53 and c-Abl in controlling cell cycle checkpoints; its involvement with NF-κB regulation may relate to genomic stability.

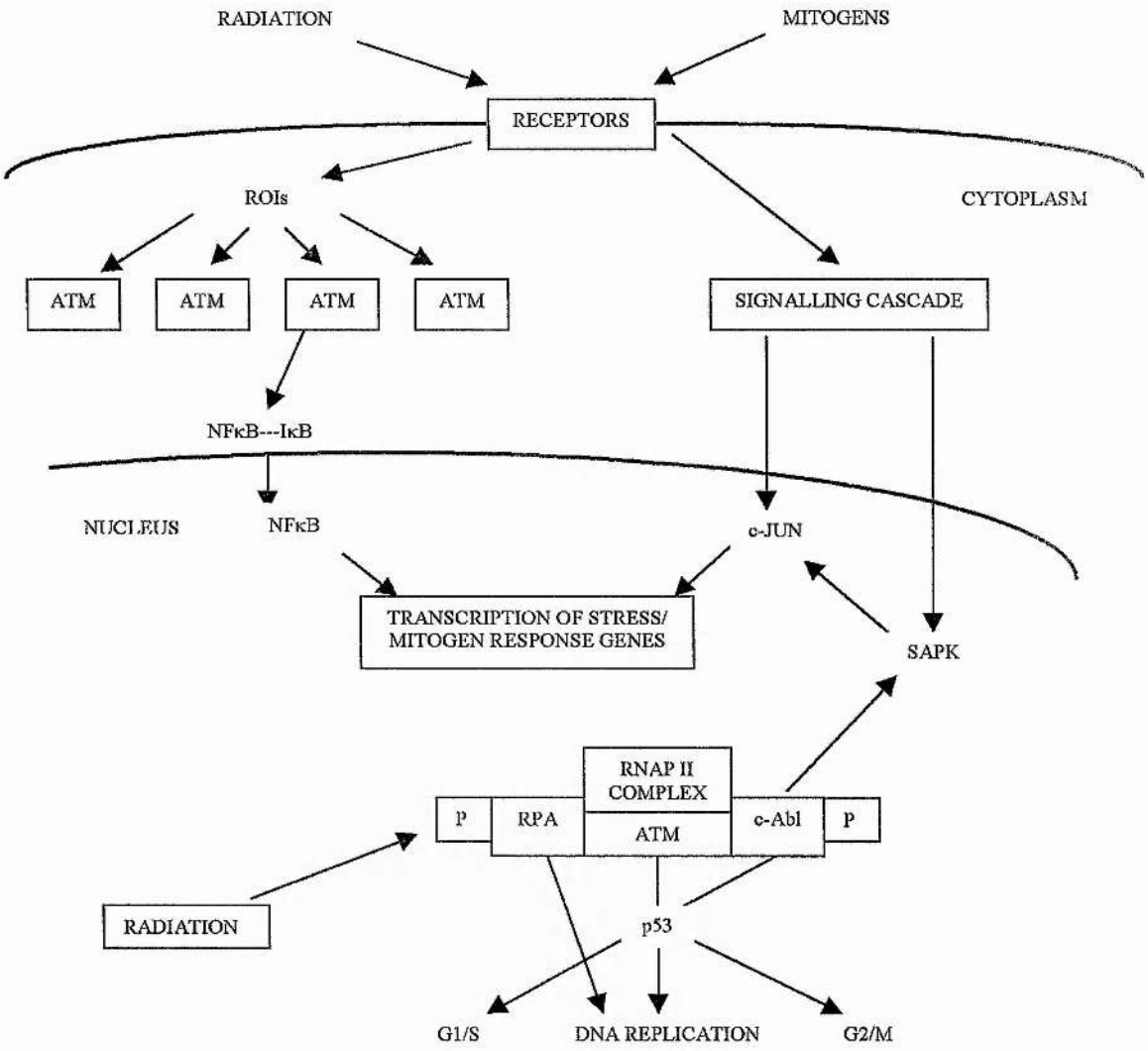


Figure 1.6: Role of ATM in signal transduction induced by radiation and mitogens/ growth factors. The presence of ATM both in the nucleus and cytoplasm suggests that it may play a role not only in detecting damage in DNA but also in transmitting signals initiated at the level of the plasma membrane. A common intermediatefrom membrane receptor signalling could be a reactive oxygen intermediate (ROI), which ATM may be able to respond to as part of a redox system. In the nucleus ATM appears to be part of the RNAPII compel (Ramin Shiekhattar, unpublished data). Recent studies show that it interacts with both with p53 and c-Abl as part of radiation induced signalling pathways. RNAPII: RNA polymerase II; RPA: replication protein A; P: unspecified protein; SAPK: stress activated protein kinases.
From Lavin, 1998.

Chapter 2:

Materials and Methods

2.1: Enzyme mismatch cleavage using T4 endonuclease VII

Genomic DNA was amplified in the region of interest for each of the following: wild type, the sample to be screened and a sample containing a known mutation as a positive control. To check that the PCR was successful, an aliquot of each was run on an agarose gel and the samples were then purified using a Promega Wizard Kit. A 15µl sample of each PCR product was radioactively end-labelled. After adding 10µl of unlabelled wild type PCR product to the 15µl of each end-labelled DNA (test or positive or negative control), the samples were precipitated by adding 2.5µl of 3M NaAc and 500µl of ethanol and incubating on ice for 30 minutes.After centrifugation at 12,000g for 15 minutes and two washes with 70% ethanol, the samples were resuspended in 25µl of 1x annealing buffer (6mM Tris-Cl pH 7.7, 600mM NaCl, 7mM $MgCl_2$). Heteroduplexes were formed by heating to 95°C for 5 minutes, 65°C for 1 hour and allowing to cool to room temperature.

To precipitate the heteroduplexes, 1.5ml of ice cold ethanol was added to the 25µl reaction mix and incubated on ice for 30 minutes. The products were collected by centrifugation at 12,000g for 15 minutes. After 2 washes with 70% ethanol, the pellets were resuspended in a total volume of 40µl, containing 1000 units of endonuclease VII in 1x reaction buffer (50mM Tris-Cl pH 8.0, 10mM $MgCl_2$, 5mM DTT, 100µg/ml nuclease free BSA). Enzyme cleavage was carried out at 37°C for 1 hour.

To a 10µl sample of each reaction, 5µl of formamide loading buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added. The

samples were run on a sequencing-type gel, which was dried, and the cleavage products were visualised by autoradiography.

2.2: RNA Extraction and Reverse Transcription

Total RNA was extracted from cultured cells using a Purescript (Flowgen, Kent) kit following the manufacturer's protocol. Around $2x10^6$ cells were rinsed in PBS and collected by centrifugation The pellet was resuspended in the lysis solution provided. DNA and protein were removed by adding the appropriate solution. After precipitation with isopropanol, the RNA was washed with ethanol and resuspended in the RNA hydration solution at approximately 200μg/ml. Samples were stored at -70°C.

The Gibco BRL SuperScript II kit was used to reverse transcribe RNA. First strand synthesis was performed by incubating 3μg of RNA for 50 minutes at 42°C in a reaction volume of 20μl containing 1x first strand buffer; 25μg/ml oligo-dT; 10mM DTT; 0.5mM each dNTP; 200 units of Superscript II enzyme. The primer and RNA were initially heated to 70°C for 10 minutes and snap cooled and the other components added. Subsequently, 2μl of the reverse transcription product was PCR amplified as described above.

For each sample, RT-PCR was carried-out using eight overlapping pairs of primers spanning the length of the ATM open reading frame (Taylor, personal communication). Primer pairs, 5' to 3' were: VI GTG TTC TGA AAT TGT GAA CCA TGA GTC TAG T and TGG TAT CTT CAT TAA AAA CCT GGT GAC AG;

VII AGT AGA GGG AAG TAT TCT TCA GGA TTT CG and CGT TTG CAT CAC TAA CAC TAC TAT CAG; VIII GAG GTG GAG GAT CAG TCA TCC ATG AAT C and GCG ATG GAA AAT GAG GTG GAT TAG GAG CAG; II CAG AGA TTG TGG TGG AGT TAT TG and GCA TTA TGA TGG TCC ACT GAA G; I AGC AGC TTC CAA CAG CCT CTA CTA G and CCA TAC AAA CTA TCT GGC TCC; III CTG GAA TAA GTT TAC AGG ATC TTC and GAT GAT TTC ATG TAG TTT TCA ATT C; V GAT GGA GAA AGT AGT GAT GAG C and AGT CAC CAG ATT TCC ATA TTC TC; IV AAG ATG TTG TTG TCC CTA CTA TG and AAG GCT GAA TGA AAG GGT AAT TC. Reactions were carried-out in a final volume of 100µl containing 1x PCR buffer; 1.5mM Mg Cl_2; 200µM each dNTP; 100nM each primer; 5 units *Taq* polymerase; 2µl first strand product. Mixes were heated to 94°C for 4 minutes followed by 35 cycles of (94°C 45 seconds; 55°C 45 seconds; 72°C 45 seconds).

2.3: Agarose Gel Electrophoresis

A gel mix containing the appropriate amount of agarose (according to the size of DNA fragments to be analysed) was heated in 130ml of 1x TBE until it dissolved. To this 8µl of 10µg/ml ethidium bromide solution was added. The ends of a gel tray were sealed with masking tape and a 16-tooth comb inserted. The gel was poured and allowed to set. 10x loading dye was added to samples (9µl sample, 1µl loading dye) and a 50bp ladder (2µl GibcoBRL ladder, 1µl loading dye, 8µl water). Gels were run at 120V for about 40 minutes then viewed under UV light.

2.4: Polymerase chain reaction (PCR)

PCR was performed using a kit supplied by GibcoBRL (Paisley) containing DNA polymerase, 10x buffer and 50mM magnesium chloride. Aerosol resistant pipette tips were used. The components were kept on ice before being added to the reaction mix with the enzyme being added last. Reactions were carried-out in a total volume of 50µl containing: 1x PCR buffer; 1.5mM MgCl2; 0.2mM each dNTP; 1µM forward primer; 1µM reverse primer; 1 unit of taq polymerase; 20ng genomic DNA of template DNA. Reaction mixtures were overlayed with mineral oil and cycled in a Omnigene thermocycler (Hybaid, Middlesex) as follows: 94°C for 4 minutes; 35x (94°C 45 seconds; 55°C 45 seconds; 72°C 45 seconds). The product was stored at 4°C

2.5: Heteroduplex analysis

Each of the eight RT-PCR products was digested individually with the restriction endonucleases AluI, HinfI, DdeI and HaeIII according to the manufacturer's instructions (GibcoBRL, Paisley). Digests were heated to 99°C for one minute then cooled at 1°C/minute to 20°C. Heteroduplexes were run under non-denaturing conditions on 9% polyacrylamide gels in a TBE continuous buffer system, stained for one hour with 1ng/ml ethidium bromide and viewed under UV light.

2.6: ^{32}P-End Labelling of Sequencing Primers

To 5µl (about 50ng) of purified DNA (sample, control or marker) was added: 1µl (10µCi) of [γ-^{32}P] ATP (cat. no. AA0018, Amersham, Bucks) 1.5µl of 10x

polynucleotide kinase buffer and 10 units of 5' T4 polynucleotide kinase (Boehringer Mannheim, Lewes, East Sussex) and made up with water to a total volume of 15µl. The reaction mixture was incubated at 37°C for 45 minutes. To precipitate the DNA, 1/10 volume of 3M sodium acetate and 500µl of cold 95% ethanol was added. The DNA was collected by centrifugation at 15,000xg for 10 minutes and the supernatant discarded as radioactive waste. The pellet was rinsed twice with cold 70 % ethanol, collected by centrifugation and dried under vacuum.

2.7: DNA Sequencing

The region to be sequenced was amplified in two separate reactions, one using a biotinylated forward primer and one using a biotinylated reverse primer. PCR products were attached to streptavidin beads (Dynal, Wirral, Merseyside) and, following the manufacturer's protocol, the duplex melted and the non-biotinylated strand removed. Sequencing reactions were performed using a Sequenase 2.0 kit (USB, Cambridge Bioscience, Cambridge) according to the supplied protocol. Sequencing primers labelled with γ-^{32}P were added and annealed to the template by heating to 65°C for 2 minutes and allowing to cool to room temperature. For each sample, four termination tubes were prepared containing 3µl of ddATP, ddCTP, ddGTP or ddTTP The beads were collected, the supernatant removed and a reaction mix created by adding 10µl of 1x reaction buffer, 1µl of DTT and 2µl of diluted enzyme. To each termination mix, 3µl of the reaction mix was added and incubated at 37°C for 5 minutes. After discarding the supernatant, the beads were heated to 72°C for 3 minutes in 4µl of stop buffer. The stop buffer containing the sequencing products was removed and run on a sequencing gel. Samples were heated to 95°C,

cooled on ice and were run on standard 6% acrylamide / 8M urea sequencing gels and were then fixed for 20 minutes in 5% methanol/ 5% acetic acid, dried onto Whatman 3MM paper and exposed to Kodak X-Omat imaging film.

2.8: Blotting and probing of a DNA gel.

Once run, the plates of a sequencing gel were separated and the plate with the gel attached was layed flat. A piece of Hybond N membrane (presoaked in water) was layed over the gel and any bubbles rolled out. Two pieces of 3MM filter paper, then the second glass plate and a weight were placed on top. Blotting was allowed to proceed overnight. A concentrated stock of sodium chloride/ sodium citrate (SSC) buffer (20x: 3M NaCl, 0.3M Na_3 citrate.$2H_2O$, pH to 7.0 with HCl) was prepared as a base for the hybridisation solutions.

The blot was UV cross-linked and pre-hybridised in Quickhyb (0.05% BSA, 0.05% PVP, 0.05% Ficoll 400, 0.1% SDS, 0.1% PPi, 5x SSC) at 48°C. A ^{32}P-labelled probe was added to 15ml of prewarmed Quickhyb, and this added to the prehybridisation solution. Probing was carried out for 5hrs (probe Y2) or overnight (probe FAT54) at 48°C. The hybridisation solution was removed and the blot washed three times for an hour at 48°C in wash buffer (0.1% SDS, 0.1% PPi, 4x SSC). Results were viewed by autoradiography.

2.9: Restriction mapping of sequences

Changes in the restriction map of a DNA sequence due to the presence of a mutation were analysed using the Wisconsin Package Version 7.0, Genetics Computer Group (GCG), Madison, Wisc. The gain or loss of a restriction site in mutant DNA was detected by using the Map programme, while the anticipated products of digestion were compared using the Mapplot programme.

2.10: Tissue Culture

Cells were grown in diposable plastic flasks (Nunc) at 37°C in an incubator gassed with 5% CO_2. Cultures were maintained in Dulbecco's Modified Eagle Medium (D-MEM) or RPMI 1640, each containing 50μg/ml penicillin, 50μg/ml streptomycin, 292μg/ml L-glutamine and 10% heat inactivated fetal calf serum (all from GibcoBRL, Paisley).

2.11: Isolation of lymphocytes from Whole Blood

In a 30ml universal tube, 10ml whole blood was layered carefully over about 8ml Ficoll Paque (Pharmacia, St. Albans, Herts.). After centrifugation at 3000rpm for 15 minutes, most of the lymphocytes could be recovered with a sterile pastette from the interface between the Ficoll Paque and the plasma. The cells were diluted to 10ml with medium and collected by centrifugation at 1200rpm for 5 minutes. The supernatant was removed and the cells resuspended in 10ml fresh medium containing 5μg/ml phytohaemagglutinin (PHA; Sigma, Poole, Dorset) and cultured at 37°C.

2.12: Protein Extraction

Approximately $2x10^7$ cells were collected by 1200rpm centrifugation at room temperature and the medium was carefully removed. The pellet was washed twice in PBS. 1ml of radio-immunoprecipitation assay (RIPA) lysis buffer (1xPBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS), including freshly-added Protease Inhibitor Cocktail (Sigma, Poole, Dorset), was added to the cell pellet, the mixture transferred to a microcentrifuge tube and incubated on ice for 30 minutes. Solubilisation was helped by passing the suspension through a 21-gauge needle followed by standing in ice for a further 30 mins. After centrifuging at 15,000g for 20 mins at 4°C, the supernatant containing the total soluble cell lysate was aliquoted into fresh tubes and stored at –70°C.

2.13: Ethanol precipitation of protein samples

To a 150µl sample of protein, 1.5ml of ethanol was added and the mixture incubated at 4°C for one hour. The precipitated protein was collected by centrifugation at 12,800g for 2 minutes. The pellet was washed with 1ml of ethanol followed by 1ml of acetone, collecting each time by centrifugation at 12,800g for 2 minutes. The protein was air dried and then resuspended in the desired volume of RIPA buffer or 1x SDS loading buffer. From the method of McIntosh *et al* (1984).

2.14: SDS Polyacrylamide Gel Electrophoresis

Electrophoresis of proteins under denaturing conditions was performed according to Laemmli (1970). Standard proteins of known molecular mass (Rainbow Markers, Amersham, Aylesbury, Bucks.) were run in adjacent lanes for comparison. Electrophoresis was carried-out in a Bio-Rad (Hemel-Hempstead, Hertfordshire) Protean 16cm apparatus.

2.15: Western Blot Analysis

Protein was transferred to supported nitrocellulose (Electran, BDH, Poole, Dorset) as described by Towbin *et al* (1979) and stained using ponceau S to check transfer efficiency. After destaining with PBS, the membrane was blocked using 2.5% BSA in TTBS. Antibodies were diluted in blocking solution to concentrations suggested by the supplier. Incubation of the membrane with primary antibody for 1hr was followed by extensive washing with TTBS then incubation with alkaline phosphatase conjugated secondary antibody (Sigma, Poole, Dorset) for 1hr. After washing with TBS, the membrane was treated with 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Sigma, Poole, Dorset). Blocking conditions, antibody concentrations and incubation times were subsequently adjusted to optimize specificity (see results section).

2.16: Separation of proteins by lectin binding

A selection of lectins immobilised on agarose beads (Sigma, Poole, Dorset) were used for anaylsis. The targets of these lectins included a wide range of glycosyl groups. 100µl of the suspension was centrifuged briefly at 10,000g and the storage solution removed, leaving approximately 50µl of beads. The beads were rinsed three times in RIPA buffer. To the pelleted beads, 100µl of protein extract was added, and the suspension tumbled overnight at 4°C. After centrifugation, the supernatant (unbound fraction) was removed and stored at –70°C. The beads were rinsed three times in RIPA buffer before the unbound fraction was removed from the lectin by boiling in 65µl of 1.5x SDS sample buffer (including the volume remaing in the compacted beads, this is approximately equal to 100µl, the volume of the original sample).

2.17: Endoglycosidase treatment of proteins

Endoglycosidase F and endoglycosidase H enzymes (Boehringer Mannheim, Lewes, East Sussex) were used to remove glycosyl groups from proteins. A sample of 50µl of protein extract was be treated with 5µl (0.025 units) of endo H or 5µl (1 unit) of endo F. 5µl of protease inhibitor cocktail (Sigma, Poole, Dorset) was added and the mxiture incubated overnight at room temperature. The pH of a sample could be reduced to the appropriate range by addition of 1M sodium acetate, pH5.5.

2.18: Velocity sedimentation

Sucrose gradients, of 16mls from 8% to 25% in RIPA buffer, were prepares in 17ml Ultra Clear tubes (Beckman, Palo Alto, California) using a peristaltic pump (Watson-Marlow, Falmouth, Cornwall). A 1ml protein sample was carefully layered over the gradient using a pipette. Velocity sedimentation was performed in a Beckman L7-65 ultracentrifuge with a SW28 rotor at 28,000rpm overnight, unless otherwise stated. The resultant gradient was removed from the bottom of the tube using a needle connected to a peristaltic pump and collected in 1ml samples. Samples were analysed by immunoblotting either neat or having first been ethanol precipitated and resuspended in a smaller volume.

2.19: Immunostaining

Adherent cells were grown in RPMI1640 overnight on a sterile coverslip in a petri dish. Cells were washed with PBS and then fixed in methanol at $-10^{\circ}C$ for 5 minutes. Three further washes with PBS were performed. Subsequent steps were carried-out in a humidified container, and reagents removed by suction. The cells were incubated with 10% blocking serum (normal goat serum in PBS) for 20 minutes at room temperature and the blocking solution removed. The coverslip was incubated with the primary antibody diluted to 0.5μg/ml in blocking solution for 1hr, followed by three five-minute washes with PBS. The cells were then incubated for 1hr with the fluorescein isothiocyanate conjugated (FITC-conjugated) goat anti-rabbit secondary antibody (Scottish Antibody Production Unit) diluted 1/200 in blocking solution,

followed by three washes with PBS. Coverslips were mounted on slides with Vector Shield (Vector Laboratories, Peterborough) and viewed under a fluoresence microscope.

Chapter 3: Studies of ATM mutations in a population of Scottish breast cancer patients

3.1: Introduction

Early studies relating to the predisposition to breast cancer among AT heterozygotes were restricted to reports of cancer rates among relatives of AT patients (reviewed in Easton, 1994). However, once the ATM gene had been cloned and sequenced (Savitsky *et al*, 1995a; 1995b), the opportunity to identify AT heterozygotes among a population of breast cancer patients arose.

3.2: An investigation into methods of detecting mutations in the ATM gene

Preliminary experiments were carried out to evaluate an existing method of mutation detection (heteroduplex formation) and a less well established method (Resolvase treatment).

3.2.1: Heterodulplex analysis

RNA was extracted from lymphoblastoid cells of four members of an AT family and amplified by RT-PCR, using eight sets of primers spanning the ATM transcript (sets I-III: Byrd et al, 1996; sets IV-VIII: Malcolm Taylor, personal communication). Each of the PCR products was then treated with one of a range of restriction endonucleases to reduce the size of the fragments to be analysed by heteroduplex formation. The proband of the family (BP) must have two mutations, one of which must be shared with the father (BF) and the other with the mother (BM). The sibling (BS), clinically normal, may be heterozygous for one of the two ATM mutations or may have two normal ATM alleles.

A mutation detected by digestion with HinfI was found in BP and BF (see figure 3.1). The second mutation was not detected in seven sections of the transcript (the eighth did not amplify) with AluI, HaeIII, HinfI or DdeI cleavage.

3.2.2: T4 endonuclease VII (Resolvase) mismatch detection

Gene 49 of the bacteriophage T4 encodes an endonuclease believed to be involved in the resolution of Holliday structures (Mizuuchi *et al*, 1982). This enzyme appears to recognise anomalies of DNA secondary strucure, with Solaro *et al* (1993) showing that endonuclease VII can cleave DNA molecules with a range of heteroduplex loops and mismatches. An insertion of 8nt was found to stimulate 100% cleavage efficiency and a 4nt insertion 65% cleavage. Single nucleotide insertions caused 30-70% cleavage and mismatches between 10% and 50% cleavage, depending on the base and the context. Youil *et al* (1995) reported similar results for single base change and for small deletions, but could not detect a deletion of 7bp or a deletion of 33bp.

Experiments to test the efficiency of the endonuclease VII method were based on two unknown ATM mutations, an 18bp deletion in p53, and two p53 point mutations. No cleavage of the ATM heterodulpexes was observed and cleavage of only one of the p53 point mutations was detected (not shown), and this using up to five times the recommended concentration of Resolvase enzyme.

These results, and similar findings from another laboratory (David Baty, Dundee, personal communication), indicated that the enzyme mismatch cleavage method of mutation detection had not yet been fully optimised and was not user friendly.

3.3: Identification of specific ATM mutations

Early results indicated that two ATM mutations, a 200bp deletion in the cDNA and a 9bp deletion, were more common than any others in the British Isles (Byrd *et al*, 1996; Teletar *et al*, 1996; Gilad *et al*, 1996; Wright *et al*, 1996). At the time this study began, these two mutations represented over 11% of all identified mutations (12 of 103), and most individuals (7 of 12) carrying these mutations were of Celtic descent (Wright *et al*, 1996).

It seemed likely that these two mutations could be detected in genomic DNA using straightforward assays and that they may form an appreciable proportion of ATM mutations in Celtic populations. If the two mutations represent 11% of all mutations in the UK, and this figure is enriched in Celtic populations, screening for them may be an efficient way of estimating the number of AT carriers among breast cancer patients. The aims of this study were threefold: to characterise the 200bp deletion at the genomic level, to develop rapid, genomic DNA-based tests for the two Celtic mutations and to apply these tests to a sample of over 500 Scottish breast cancer patients.

The 200bp deletion was first detected by Byrd *et al* (1996) as a deletion in section VIII of the cDNA. On publication of the genomic organisation of the gene (Uziel *et*

al, 1996), this was found to represent the absence of exon 20 from the cDNA and, therefore, from the transcript. PCR amplification showed no such deletion in the genomic DNA and so it was suggested that the deletion could be the result of a mutation in the splice acceptor site of intron 19 (Taylor, personal communication).

The other common mutation, a 9bp deletion in exon 54, was one of the first mutations reported on publication of the 3' part of the ATM gene (Savitsky *et al*, 1995a). The expected result of this mutation is the in frame deletion of amino acids 2546-2548. These two mutations will be referred to as the exon-skipping mutation and the Δ9bp deletion respectively.

3.4: Characterisation of the exon-skipping mutation

Genomic DNA (supplied by Malcolm Taylor, Birmingham) from two individuals, one known to be heterozygous for the 200bp exon-skipping mutation and the other known to be wild type for ATM, were sequenced.

Sequencing was initially performed using forward primer 8145, (GAG GTG GAG GAT CAG TCA TCC ATG AAT C), within exon 19, and reverse primer X (GAG CAT GTC TAA GAA AAG TAG ATC), within exon 20. From the region sequenced, a further primer, Y1, was designed within intron 19. Sequencing with X and Y1 led to yet another primer, Y2, close to the intron 19/ exon 20 boundary to be designed. Amplimers generated with 8145 and X were used as sequencing templates and Y1 and Y2 as internal sequencing primers.

The results of the sequencing experiments are seen in figure 3.2. Figure 3.2 shows the autoradiograph of the two sequencing reactions (normal and heterozygote) run in parallel. It can clearly be seen that, reading from intron 19 towards exon 20, the sequences are the same until an extra "G" (indicated by an arrow) is found in the heterozygote sequence. From this point on, the heterozygote sample has a normal sequence with a second sequence superimposed over it. Reading the sequence of the heterozygote revealed a deletion of three bases after genomic nucleotide number 56068, assigned the name g.56068-56070delAAT (or IVS19-22delAAT) according to the Nomenclature Working Group recommendations (Antonarakis *et al*, 1998)

3.5: Tests for detection of the Celtic mutations by changes in restriction pattern- a rapid test

Normal and mutant amplimer sequences were entered into the MAP and MAPPLOT programmes of the Wisconsin software package to allow comparison of the restriction enzyme recognition site patterns. Fortunately, these proved to be different and an enzyme was chosen that had at least one site consistent between the normal and mutant sequences (to act as an internal control) and one site which is lost or gained as a result of the mutation. The results of each search are reported individually below.

3.5.1: Exon-skipping mutation

The restriction maps of the 218 bases of normal DNA and 215 bases of mutant DNA amplified by primers Y2 and X revealed the loss of an MseI restriction site in the mutant DNA. Digestion of the normal amplimer with MseI was expected to generate

fragments of 28bp, 61bp, 41bp, 30bp and 58bp (figure 3.3a), while digestion of the mutant amplimer would generate fragments of 28bp, 61bp, 68bp and 58bp (figure 3.3b). Figure 3.3c illustrates that the difference in digestion pattern can easily be seen on an agarose gel.

3.5.2: Δ9bp mutation

The 205bp of normal DNA amplified with primers FAT54 (AAT CTA ATA GTT CTT TTC TTA CAG C) and RAT54 (TAC GTA TGT TTA ATC CAA ATA CC) was found to produce three restriction fragments (of 35bp, 68bp and 102bp) when digested with ApoI (see figure 3.4). Digestion of this amplimer containing the Δ9bp Celtic mutation produces only two restriction products, of 94bp and 102bp.

3.6: Detection of the two common Celtic mutations in a sample of 412 Scottish breast cancer patients

The sample of breast cancer patients included 162 sporadic cases, 193 familial cases and 57 early-onset cases. Details of theses series are given in Table 3.2. The series of familial cases had been variously tested for BRCA1 and BRCA2 mutations; for the purpose of this screen, those samples in which a BRCA mutation had been detected were eliminated.

DNA derived from a blood sample was available for each patient. This was amplified in two reactions, one using primers X and Y2, the other with primers FAT54 and

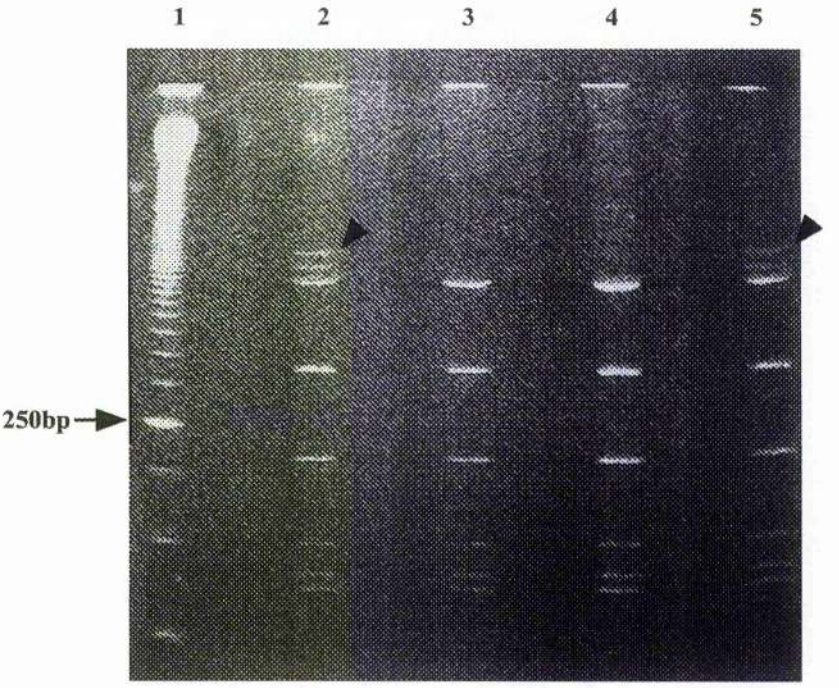
RAT54 The products were digested with the appropriate enzyme, run on a 6% acrylamide gel and viewed under UV light (not shown).

It was found during the screening process that running the undigested PCR products on an 8% sequencing gel the size difference (for either mutation) between normal and mutant DNA could be seen. By sequential loading, of up to four samples per well, as many as 380 samples could be screened on one gel. Figures 3.5 and 3.6 show examples of analysis on sequencing-type gels of DNA from breast cancer patients amplified in the regions containing the Celtic mutations.

3.7: Results

The results of screening 412 breast cancer patients for the two Celtic mutations are presented in table 3.1. No mutations were found in 162 sporadic cases, 193 non-BRCA1-associated familial cases or 57 early-onset cases of breast cancer. The expected results (based on figures accepted at the time of the start of this experiment) suggested that 2.5 carriers of Celtic mutations should be detected among 400 breast cancer cases. It was expected that this figure would be elevated further in a population of Scottish breast cancer patients.

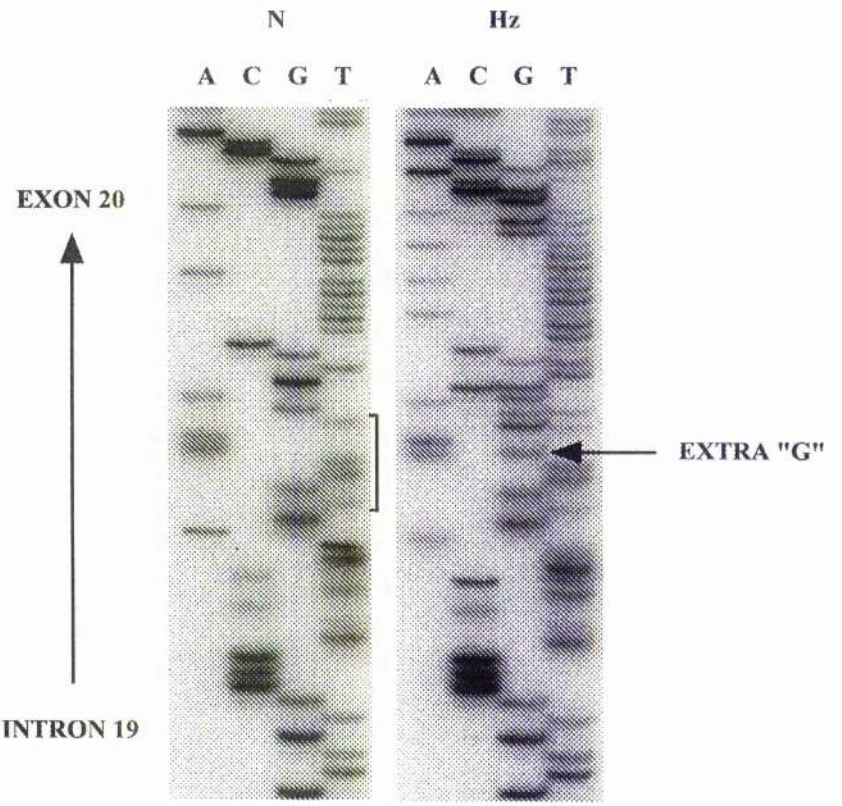
Figure 3.1: Heteroduplex analysis of DNA from an AT family.



Lane 1: 50bp ladder
Lane 2: father
Lane 3: mother
Lane 4: sibling
Lane 5: proband

Fragment VII (see section 2.8) of the ATM cDNA from each member of an AT family was digested with HinfI. The products were subjected to conditions for heteroduplex formation and run on a 9% non-denaturing polyacrylamide gel. Heteroduplexes, indicated by arrowheads, are seen in lanes 2 and 5, showing that a mutation in this fragment has been inherited by the proband from the father.

Figure 3.2: sequence analysis revealing the genomic lesion resulting in skipping of exon 20 of the ATM gene.



Genomic DNA from an individual normal (N) for the ATM gene and an individual heterozygous (Hz) for the exon-skipping mutation was amplified with primers X-biotin and Y. Sequencing was performed with the radiolabelled primer Y2. The extra "G" represents the first base of the mutant sequence, which is superimposed over the normal sequence of the heterozygous sample. The putative splice acceptor site is bracketed in the normal sequence.

A comparison of the sequences in the region of the extra "G" reveals a deletion of bases AAT in the mutant sequence. Reading from intron 19 towards exon 20:

Normal seuqence
...CCCTTCTCTTAGTGTTAATGAGTGCTTTTT...

Heterozygous sequences
...CCCTTCTCTTAGTGTTAATGAGTGCTTTTT...
...CCCTTCTCTTAGTGTTGAGTGCTTTTT...

This deletion occurs in a region corresponding to the general form of the splice branch site:

Py N Py Py Pu A Py
. . . T G T T A A T . . .

Figure 3.3a: Analysis of the normal fragment amplified by primers X and Y2.

i) MseI restriction sites detected by the MAP programme
ii) The resultant fragment sizes, in genomic order and in order of size, calculated by MAPSORT

3.3a i) NORMAL:

```
                                  M
                                  s
                                  e
                                  I
    AAGACTTTTGAAGCTTTCAGTATATAATTAATTTCACTATAATTTTGCTTTTCATATACT
  1 ---------+---------+---------+---------+---------+---------+ 60
    TTCTGAAAACTTCGAAAGTCATATATTAATTAAAGTGATATTAAAACGAAAAGTATATGA

                                  M
                                  s
                                  e
                                  I
    TTTTTTTGTGAAGAGGAGGAAATTTGAGTTAATATGACTATATATGGCTGTTGTGCCCTT
 61 ---------+---------+---------+---------+---------+---------+ 120
    AAAAAAACACTTCTCCTCCTTTAAACTCAATTATACTGATATATACCGACAACACGGGAA

            M                                 M
            s                                 s
            e                                 e
            I                                 I
    CTCTTAGTGTTAATGAGTGCTTTTTATTTTTAGGTGCCATTAATCCTTTAGCTGAAGAAT
121 ---------+---------+---------+---------+---------+---------+ 180
    GAGAATCACAATTACTCACGAAAAATAAAAATCCACGGTAATTAGGAAATCGACTTCTTA

    ATCTGTCAAAGCAAGATCTACTTTTCTTAGACATGCTC
181 ---------+---------+---------+-------- 218
    TAGACAGTTTCGTTCTAGATGAAAAGAATCTGTACGAG
```

3.3a ii) NORMAL:

```
MseI T'TA_A

Cuts at:      0      28      89     130     160     218
   Size:         28      61      41      30      58

Fragments arranged by size:

                61      58      41      30      28
```

Figure 3.3b: Analysis of the mutant (Δaat) fragment amplified by primers X and Y2.

i) MAP results showing MseI restriction sites. The site at base 130 of the normal sequence is lost.

ii) MAPSORT showing that the mutation results in a different restriction fragment pattern.

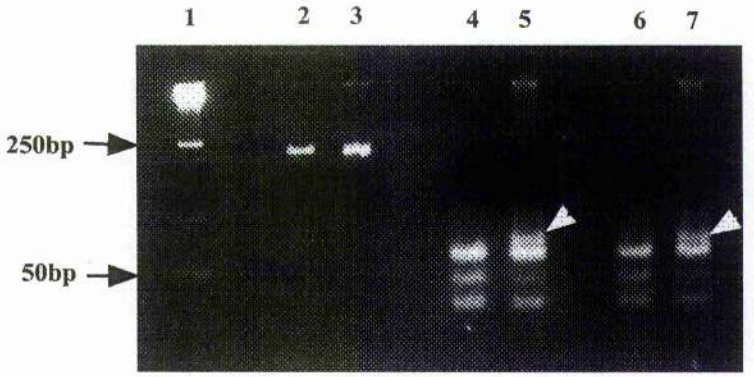
3.3b i) MUTANT:

```
                                    M
                                    s
                                    e
                                    I
    AAGACTTTTGAAGCTTTCAGTATATAATTAATTTCACTATAATTTTGCTTTTCATATACT
  1 ---------+---------+---------+---------+---------+---------+ 60
    TTCTGAAAACTTCGAAAGTCATATATTAATTAAAGTGATATTAAAACGAAAAGTATATGA

                                    M
                                    s
                                    e
                                    I
    TTTTTTTGTGAAGAGGAGGAAATTTGAGTTAATATGACTATATATGGCTGTTGTGCCCTT
 61 ---------+---------+---------+---------+---------+---------+ 120
    AAAAAAACACTTCTCCTCCTTTAAACTCAATTATACTGATATATACCGACAACACGGGAA

                                          M
                                          s
                                          e
                                          I
    CTCTTAGTGTTGAGTGCTTTTTATTTTTAGGTGCCATTAATCCTTTAGCTGAAGAATATC
121 ---------+---------+---------+---------+---------+---------+ 180
    GAGAATCACAACTCACGAAAAATAAAAATCCACGGTAATTAGGAAATCGACTTCTTATAG

    TGTCAAAGCAAGATCTACTTTTCTTAGACATGCTC
181 ---------+---------+---------+----- 215
    ACAGTTTCGTTCTAGATGAAAAGAATCTGTACGAG
```

3.3b ii) MUTANT:

```
MseI T'TA_A

Cuts at:      0      28      89     157     215
   Size:         28      61      68      58

  Fragments arranged by size:

                68      61      58      28
```

Figure 3.3c: Restriction fragment length polymorphism analysis for detection of the exon-skipping mutation.



Lane 1: 50bp ladder

Lane 2: undigested normal DNA
Lane 3: undigested DNA heterozygous for the 3bp deletion

Lane 4: normal DNA digested with MseI
Lane 5: DNA heterozygous for the 3bp deletion digested with MseI

Lanes 6 and 7: as lanes 4 and 5, but using twice the recommended concentration of enzyme

Normal DNA and DNA heterozygous for the exon-skipping mutation were amplified with primers X and Y2. The products were treated with the restriction enzyme MseI and run on a 2% agarose gel.

Figure 3.4a: Analysis of the normal and mutant fragments amplified by primers FAT54 and RAT54.

i) ApoI restriction sites in the normal sequence detected by the MAP programme
ii) The resultant fragment sizes, in genomic order, and in order of size, calculated by MAPSORT

3.4a i) NORMAL:

```
                                        A
                                        p
                                        o
                                        I
    AATCTAATAGTTCTTTTCTTACAGCTAATCTCTAGAATTTCAATGGATCACCCCCATCAC
  1 ---------+---------+---------+---------+---------+---------+ 60
    TTAGATTATCAAGAAAAGAATGTCGATTAGAGATCTTAAAGTTACCTAGTGGGGGTAGTG

                                                A
                                                p
                                                o
                                                I
    ACTTTGTTTATTATACTGGCCTTAGCAAATGCAAACAGAGATGAATTTCTGACTAAACCA
 61 ---------+---------+---------+---------+---------+---------+ 120
    TGAAACAAATAATATGACCGGAATCGTTTACGTTTGTCTCTACTTAAAGACTGATTTGGT

    GAGGTAGCCAGAAGAAGCAGAATAACTAAAAATGTGCCTAAACAAAGCTCTCAGCTTGAT
121 ---------+---------+---------+---------+---------+---------+ 180
    CTCCATCGGTCTTCTTCGTCTTATTGATTTTTACACGGATTTGTTTCGAGAGTCGAACTA

    GAGGTATTTGGATTAAACATACGTA
181 ---------+---------+----- 205
    CTCCATAAACCTAATTTGTATGCAT
```

3.4a ii) NORMAL:

```
ApoI r'AATT_y

Cuts at:      0      35     103     205
   Size:         35      68     102


  Fragments arranged by size:

                102      68      35
```

Figure 3.4b: Analysis of the Δ9bp mutant sequence amplified by primers FAT54 and RAT54.

i) MAP results showing ApoI restriction sites. The site at base 35 of the normal sequence is lost.

ii) MAPSORT showing that the Δ9bp mutation results in a different restriction fragment pattern.

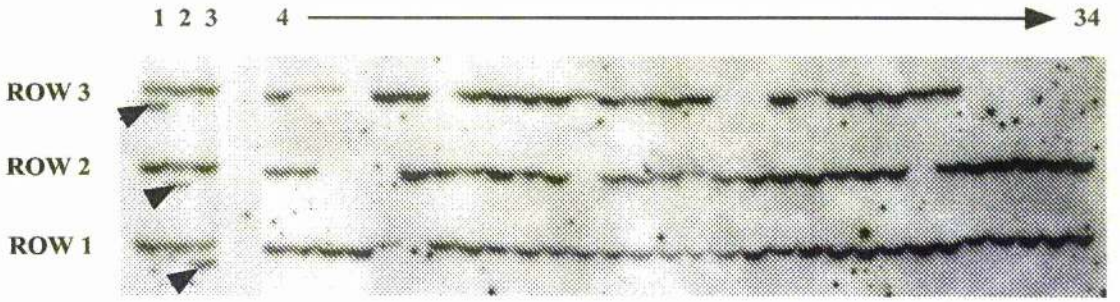
3.4b i) MUTANT:

```
    AATCTAATAGTTCTTTTCTTACAGCTAATCTCAATGGATCACCCCCATCACACTTTGTTT
  1 ---------+---------+---------+---------+---------+---------+ 60
    TTAGATTATCAAGAAAAGAATGTCGATTAGAGTTACCTAGTGGGGGTAGTGTGAAACAAA

                                        A
                                        p
                                        o
                                        I
    ATTATACTGGCCTTAGCAAATGCAAACAGAGATGAATTTCTGACTAAACCAGAGGTAGCC
 61 ---------+---------+---------+---------+---------+---------+ 120
    TAATATGACCGGAATCGTTTACGTTTGTCTCTACTTAAAGACTGATTTGGTCTCCATCGG

    AGAAGAAGCAGAATAACTAAAAATGTGCCTAAACAAAGCTCTCAGCTTGATGAGGTATTT
121 ---------+---------+---------+---------+---------+---------+ 180
    TCTTCTTCGTCTTATTGATTTTTACACGGATTTGTTTCGAGAGTCGAACTACTCCATAAA

    GGATTAAACATACGTA
181 ---------+------ 196
    CCTAATTTGTATGCAT
```

3.4b ii) MUTANT:

```
ApoI r'AATT_y

Cuts at:       0       94      196
   Size:          94      102
```

Figure 3.5: An example of a sequencing-type gel used to screen DNA samples from breast cancer patients for the exon-skipping mutation.



Row 3

Lane 1: 3bp deletion control (heterozygote)
Lane 2: normal control
Lane 3: normal control
Lanes 4 to 29: breast cancer samples

Row 2

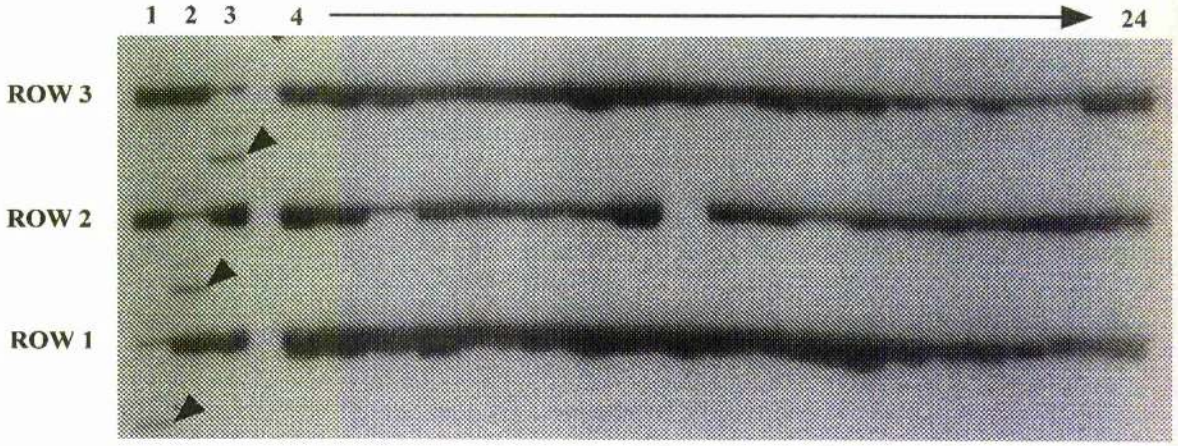
Lane 1: normal control
Lane 2: 3bp deletion control (heterozygote)
Lane 3: normal control
Lanes 4 to 34: breast cancer samples

Row 1

Lane 1: normal control
Lane 2: normal control
Lane 3: 3bp deletion control (heterozygote)
Lanes 4 to 34: breast cancer samples

Blood DNA samples from breast cancer patients were amplified across the intron 19/ exon 20 boundary using primers X and Y2. Three rows of PCR products were sequentially run on a 9% sequencing-type gel. Arrowheads indicate mutant DNA strands.

Figure 3.6: An example of a sequencing-type gel used to screen DNA samples from breast cancer patients for the 9bp Celtic deletion.



Row 3

Lane 1: normal control
Lane 2: normal control
Lane 3: 9bp deletion control (heterozygote)
Lanes 4 to 24: breast cancer DNAs

Row 2

Lane 1: normal control
Lane 2: 9bp deletion control (heterozygote)
Lane 3: normal control
Lanes 4 to 24: breast cancer DNAs

Row 1

Lane 1: 9bp deletion control (heterozygote)
Lane 2: normal control
Lane 3: normal control
Lanes 4 to 24: breast cancer DNAs

DNA samples from breast cancer patients were amplified in the region of exon 54 using primers FAT54 and RAT54. Samples were sequentially run in three rows on a 9% sequencing-type gel. Arrowheads indicate mutant DNA strands.

Table 3.1: Results of screening a sample of Scottish breast cancer patients for the Celtic ATM mutations.

	Sporadic	Familial	Early-Onset
Number Tested	162	193	57
Exon 54 Δ9bp	0	0	0
Intron 19 Δ3bp	0	0	0

Anticipated Results

Frequency of "Celtic" mutations In AT homozygotes	12/103	11.7%
Frequency of ATM mutations In the general population		1.4%
Relative risk of breast cancer Among AT carriers		3.9x
Expected number of Celtic mutations In 400 breast cancer cases		**2.5**

Table 3.2: Details of breast cancer cases used in screeing for the Celtic mutations

Sporadic:	Number	162
	Ascertainment	A consecutive series of cases classified as sporadic from the Edinburgh and Dundee (Ninewells) symptomatic breast cancer clinics.
Familial:	Number	193
	Acertainment	Cases of familial breast cancer, classified according to the Scottish Office bulletin "Cancer Genetics Service, Scotland" (1998). Collected from the Edinburgh and Dundee (Ninewells) symptomatic breast cancer clinics.
Early-onset:	Number	57
	Acertainment	Cases presenting under the age of 50 years to the Longmore Hospital (Edinburgh), collected by Daniel Porter.

Chapter 4:

ATM protein studies

4.1: Introduction

As more information about ATM mutations was published, and in light of the results obtained by screening for the Celtic mutations (as reported in chapter 3, no Celtic mutations were found in more than 400 Scottish breast cancer patients), it became clear that screening for individual mutations was not an efficient approach to estimating the percentage of breast cancer patients who are heterozygous for the ATM gene. However it was also noted that as many as 80% of ATM mutations were predicted to result in expression of a truncated protein (Gilad *et al*, 1996). Since, at the time, there had only been limited evidence that no mutant ATM protein was expressed, it was intended to detect AT heterozygotes by the presence of both a full-length ATM protein and a truncated protein. The most efficient way of detecting a truncated ATM protein is to use an anti-ATM antibody targeting the N-terminal half of the protein in western blot analysis. The same techniques might also allow ATM expression patterns in cells from a normal individual and a breast cancer patient to be compared, as even in cases where ATM itself is not mutated, another member of an ATM-mediated pathway may be defective, in turn affecting ATM expression.

Preliminary experiments with several antibodies including Ab-3, which targets residues 819-844, failed to detect any component which could correspond to a truncated protein (results not shown). These results supported the early observation by Lakin *et al* (1996) that no truncated protein was expressed and later publications have since confirmed this (Brown *et al*, 1997).

4.2: Comparison of anti-ATM antibodies

4.2.1: Materials and methods

Five antibodies raised against synthetic peptides corresponding to regions of the putative ATM protein (see table 4.1) were compared for efficiency and specificity of detection. These included four polyclonal antibodies raised in rabbit which were called ATM.V and ATM.B (both supplied by Dr. Nick Lakin, Cambridge University), FP8 (supplied by Prof. Malcolm Taylor, University of Birmingham) and Ab-3 (Oncogene Research Products, Cambridge, Massachusetts). The fifth was a monoclonal antibody, 2C1 (GeneTex Inc., San Antonio, TX). A summary of information about antibodies used to detect other proteins (as size markers and to confirm protein extraction has been successful) is also included in table 4.1.

TARGET	ANTIBODY	TYPE	PEPTIDE	SUPPLIER
ATM	ATM.V	Rabbit polyclonal	1391-1693	Nick Lakin (Cambridge)
ATM	ATM.B	Rabbit polyclonal	1980-2337	Nick Lakin (Cambridge)
ATM	FP8	Rabbit polyclonal	992-1144	Malcolm Taylor (Birmingham)
ATM	Ab-3	Rabbit polyclonal	819-844	Oncogene (Massachusetts)
ATM	2C1	Mouse monoclonal	2577-3056	GeneTex (Texas)
c-Abl	K-12	Rabbit polyclonal	Unavailable	Santa Cruz (California)
RNA polII	N-20	Rabbit polyclonal	Unavailable	Santa Cruz (California)
MHC Class II	DA6.147	Mouse momoclonal	Unavailable	Brian Cohen (St. Andrews)

Table 4.1: anti-ATM antibodies and antibodies against other proteins used as controls. The peptide against which each was raised is given in terms of the residue numbers of the putative ATM protein.

4.2.2: Results

Western blot analysis of protein extracts from a normal lymphoblastoid cell line (MR) and from cell lines heterozygous for the ATM gene (MA, FA, and DA) using the five antibodies indicated that the monoclonal 2C1 provided the best results. The polyclonal antibodies were able to detect the ATM product efficiently, but 2C1 consistently gave the lowest background and was most sensitive. A comparison of the polyclonal antibodies is shown in figure 4.1. A product of equal size, around 350kDa, was detected in normal extracts with each antibody, but ATM.V, ATM.B and FP8 produced relatively weak bands and gave especially unsatisfactory results with less concentrated protein samples. Ab-3 gave a stronger signal (lanes 1 and 2) than the other polyclonals, but a background banding pattern, including a band of similar size to ATM, present even in homozygous mutants, was also seen (lane 3). 2C1 gave a clean ATM band with an intensity approximately two or three times that of ATM.B or FP8.

4.2.3: Conclusions

Optimal conditions for probing a western blot with 2C1, with respect to all variable parameters, were determined and thereafter experiments were performed as follows: blocking for 1hr in TTBS/ 2.5% BSA; incubation for 1hr with 2C1 at 1μgml^{-1} in TTBS/BSA; three washes with TTBS; incubation for 1hr with anti-mouse IgG alkaline phosphatase conjugate (Sigma, Poole, Dorset) diluted 1/1000 in TTBS/BSA; three washes with TBS; staining with BCIP/NBT.

4.3: Distribution

4.3.1: Background

The literature indicates that the ATM protein is expressed ubiquitously in all tissues and many cell lines tested. Due to problems associated with using cell lines (culture time, cost, contamination), placenta was identified as a possible source of large quantities of protein. As placenta was previously untested for ATM expression, an experiment was carried out to determine if ATM is indeed expressed in placenta.

Preliminary results indicated that ATM was present in placenta but, when the experiment was repeated using fresh extracts, very little or no ATM was detected. Due to the fibrous nature of placental tissue, the possibility that the extraction procedure was not efficiently solubilizing the ATM protein was investigated.

4.3.2: Materials and methods

A protein extract was prepared (using approximately 50 grams of placental tissue and 50mls of RIPA buffer) and initially cleared of debris by centrifugation for 6 minutes at 2,000rpm in an Eppendorf 1540 centrifuge. 0.25ml aliquots of the supernatant were centrifuged at 15,000rpm at 4°C for varying lengths of time. Pellets were resuspended in 333µl (equivalent to the volume of supernatant, 250µl, plus 4x loading buffer, 83µl) of 1x SDS loading buffer, boiled and then spun briefly to clarify. A 60µl sample of each pellet and supernatant was run on an SDS polyacrylamide gel.

4.3.3: Results

As illustrated in figure 4.2, faint ATM protein bands were detected in the supernatants but not in the pellets, even after the longest spin time (lanes 13 and 14). The different results obtained with different samples could be due to the heterogeneous nature of the placenta and could also depend on the amount of blood, which is extracted along with the placental tissue, or the number of circulating lymphocytes in the blood. The inconsistency with which ATM was detected in placental protein preparations, and the low levels which were detected means that placenta proved not to be a prolific source of ATM protein.

4.4: PHA-stimulated T-lymphocytes

4.4.1: Background

As the results reported in section 4.3 indicated that only a small amount of ATM was expressed in placenta, and that in some placental samples no ATM was present, it was proposed that ATM expression depends on the state of the cells. Placenta, having outlived its usefulness by the time it is delivered, may have a low requirement for DNA replication and this could be reflected in the absence of ATM protein. In order to test this, cells in different states of growth stimulation were tested for ATM expression.

4.4.2: Materials and methods

Human T lymphocytes, both stimulated with phytohaemagluttinin (PHA) and unstimulated, were tested, as were cells from hamsters showing the ionising radiation sensitive phenotypes 1 and 2 ("irs1" and "irs2"). The irs2 phenotype, in particular, is very similar at a cellular level to human AT. Stimulated T cells produced a different pattern with 2C1 than unstimulated T cells or normal lymphoblastoid cells. As this feature may represent a normal growth response which could be deficient in some cancer cells, it was investigated further as follows:

A 50ml sample of fresh blood was spun over Ficoll Paque to isolate the lymphocytes, which were set up in four 10ml cultures in RPMI1640. Protein was extracted from one sample without stimulation, while the other three of the samples were stimulated with PHA and protein extracts made at 24, 48 and 72 hours post-stimulation respectively. These were analysed by immunoblotting.

4.4.3: Results

2C1 detected a protein of the same size as the human ATM protein in both types of hamster cell (figure 4.3), suggesting that hamster cells do express a direct analogue of the ATM gene, but that neither the irs1 nor the irs2 phenotype is a model for the human disease AT. The results of the PHA time course experiment are seen in figure 4.4. An additional band is detected in the sample from cells 72hr post-stimulation, which is not seen in the other samples. A possible explanation for this observation is that differently spliced transcripts are translated, depending on the state of stimulation

of the cell or that post-translational processing (including degradation) results in proteins with different mobilities in an SDS gel.

4.5: Lectin binding properties and glycosylation

4.5.1: Background

The observation of a different size of protein detected by an anti-ATM antibody in PHA-stimulated T cells led to an investigation to determine whether ATM is glycosylated and, if so, does the protein have different migration properties in an SDS gel according to its level of glycosylation. It has been shown that the activity and stability of molecules can be affected by glycosylation (Meynial, Longo and Combes, 1995), so it was possible that the two bands observed in PHA-stimulated T cells represented ATM molecules in different states of activation. If the ATM molecule is glycosylated then it should bind to a lectin. An experiment was carried out with a variety of immobilised lectins to determine their ATM binding properties.

4.5.2: Lectin-binding specificity of the ATM product

Materials and methods

Samples of normal protein were incubated with an immobilized lectin and the supernatant (unbound fraction) removed. The lectin beads were washed thoroughly in RIPA buffer and then boiled in 1x SDS loading buffer to free any bound glycoproteins (bound fraction). These fractions were run on a 5% SDS gel and analysed by western blot.

Results

The results are shown in figure 4.5. ATM has bound efficiently to Dolichos biflorus (horse gram) and less efficiently to Glycine max (soybean). The binding specificity of these lectins is α-galNAc and galNAc respectively. ATM did not bind to Concanavalin A (specific for α-man and α-glc), Lens culinaris (specific for α-man), Tetragonolobus purpureas (specific for α-L-fuc) or Triticum vulgaris, (specific for $(glcNAc)_2$ and NeuNAc).

4.5.3: Endoglycosidase treatment confirms ATM is glycosylated

Materials and methods

To confirm that these binding properties are due to glycosyl groups rather than a non-specific reaction, samples of normal protein were treated with endoglycosidase H and then incubated with immobilized dolichos biflorus.

Results

Figure 4.6a illustrates that ATM treated with endoglycosidase H does not bind to Dolichos biflorus, while untreated ATM does: the treated protein (lane 1) shows no difference in migration from the untreated protein (lane 7), but it does not bind to the lectin beads (lane 2), instead remaining in the unbound fraction (lane 3); lanes 4, 5 and 6 mirror these results, with the protein being acididfied to the optimal range for endoglycosidase activity prior to treatment; lane 8 shows that untreated ATM does bind to the lectin; the portion of untreated ATM unbound to Dolichos biflorus (lane 9) may be due to the presence of some unglycosylated protein in the cell, or the capacity of the beads was not great enough to extract all glycosylated protein; the absence of

any band from lanes 10, 11 and 12 (ATM homozygote protein) confirm that ATM is the protein being detected. Figure 4.6b is a western blot probed for MHC class II and confirms that the deglycosylation protocol was efficient: the MHC class II protein treated with endoglycosidase (lane 2) is clearly smaller than the untreated samples (lanes 1 and 3).

4.5.4: Conclusions

The results of experiments 4.5.2 and 4.5.3 confirm that the ATM protein is glycosylated, or at least that a considerable portion of it is. There was no difference in size visible on a 5% SDS gel, however (lanes 1 and 7 of figure 4.6a), meaning that glycosylation cannot explain the extra band observed in PHA stimulated T cells (section 4.4).

4.6: Velocity sedimentation

4.6.1: Background

ATM has been reported as being part of a large complex (Chen and Lee, 1996)and studies have suggested it is bound to p53 (Watters *et al*, 1997) and c-Abl (Shafman *et al*, 1997) in the cell. To confirm the approximate size of this complex and to find out if ATM is present in more than one complex, or is also found free in the nucleus, velocity sedimentation experiments were carried out.

4.6.2: Materials and methods

A sample of protein from a normal lymphoblastoid cell line and a sample of normal blood serum (with haemoglobin added as a 4S marker) were each centrifuged over a 8% to 25% sucrose gradient for 16hrs at 28,000rpm (Beckman S7 ultracentrifuge with SW28 rotor). Fractions from the run of whole cell extract were run on an SDS gel. The fractions of serum were dotted, in duplicate, on strips of nitrocellulose which were blocked with BSA and probed with anti-human IgG and anti-human IgM. Absorbance at 418nm of the serum fractions was also measured.

4.6.3: Results

Results (shown in figure 4.7) indicate that most ATM protein is found in the bottom fraction (lane 2) of the gradient after 16hrs centrifugation. This contradicts the expected result taking into account the relative positions of the IgG (7S), IgM (19S) and haemoglobin (4S) peaks found in the serum gradient.

4.6.4: Conclusions

There are a number of possible explanations for the apparently high sedimentation coefficient: the ATM protein is associated with the microsomal fraction of the cell; ATM is bound with other proteins in a large complex; ATM is not completely solubilised in RIPA buffer. However, previously published material strongly indicates that there is only a minimal amount of ATM in the microsomal fraction (Brown *et al*, 1997) or none at all (Lakin *et al*, 1997; Watters *et al*, 1997). Chen and Lee (1996)

referred to a large ATM-containing complex and there have been reports of interaction between ATM and other proteins.

4.7: Isolation of the ATM product

4.7.1: Background

As ATM protein was consistently found in the bottom fraction of a sucrose gradient, even when larger proteins were still within the gradient, an experiment was carried out to establish if this feature could be exploited for isolation of the ATM product.

4.7.2: Materials and methods

Protein was extracted from normal lymphoblastoid cells, clarified by centrifugation at 10,000rpm for 20 minutes at 4°C, then divided into samples of 250µl. These samples were centrifuged at 15,000rpm for different lengths of time (10 mins; 20 mins; 40 mins; 60 mins; 120 mins) and the supernatants removed to fresh tubes. The pellets were dissolved in 333µl of 1x SDS loading buffer and 83µl of 4x buffer was added to the each supernatant, and 60µl of each sample was run on a 5% SDS gel.

4.7.3: Results

The results, shown in figure 4.8, clearly illustrate that the ATM product is being pelleted at this speed (lanes 7 and 9), and that after 2hrs most of the ATM protein has been collected (lane 11).

4.7.4: Conclusions

Only a small amount of ATM has been reported in the microsomal fraction of cell extracts. Further, it is unlikely that any membranes could remain intact in the presence of NP40, SDS and deoxycholate (DK Apps, personal communication), so the sedimentation of microsomes cannot explain the rapid pelleting of ATM. A 10% SDS gel was run in parallel and blotted, and the membrane was probed with DA6.147. This gel (figure 4.8) shows that MHC class II protein is not pelleted under the same conditions and so it is unlikely that proteins are incompletely solubilized. However, as the ATM protein (350kDa) is much larger than MHC class II (35kDa), this comparison is not necessarily conclusive.

Intriguingly, when experiments to extract the ATM protein by sedimentation or by binding to Dolichos biflorus were repeated with samples from placenta (in which ATM had previously been detected), the small amount of product present remained in the supernatant (figure 4.2) and the unbound fraction respectively (results not shown). As there was a portion of ATM product in the supernatant and unbound fraction of normal cell extract, it seems possible that there is more than one species of ATM protein. If only one of these is expressed in placenta (ie one which does not pellet at low speed centrifugation and does not bind Dolichos biflorus), this could explain the low levels of ATM detected in crude placental extracts. A future study could determine whether the portion of ATM from a normal cell line remaining in the supernatant after centrifugation and the portion which does not bind to Dolichos biflorus are the same species, and furthermore if this is the same species as placental ATM.

4.8: Immunostaining

4.8.1: Background

An efficient method was sought to detect ATM distribution within the cell. It had been intended to investigate the number of ATM-containing structures in relation to cell cycle, and to investigate the distribution of proteins (such as c-Abl) which bind to ATM. It was also intended to compare ATM staining in unstimulated lymphocytes and lymphocytes stimulated with PHA (section 4.4). Unfortunately, a fault with the fluorescence microscope caused this study to be curtailed.

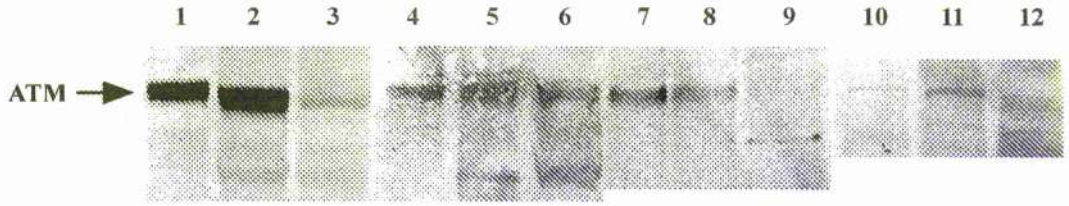
4.8.2: Materials and methods

Normal and mutant fibroblasts were grown overnight on coverslips, then probed with the anti-ATM antibody, followed by an FITC-conjugated secondary antibody.

4.8.3: Reasults

Preliminary experiments to study the distribution of the ATM protein within the cell indicated that the polyclonal antibody FP8 produced the best results. A strong signal was detected in the nucleus of normal cells, while only a faint, non-specific staining of the AT cells was detected (figure 4.9). A punctate staining pattern, as described by by Brown *et al* (1997), was detected, but the number of structures staining with anti-ATM varied from cell to cell (figure 4.10).

Figure 4.1: A comparison of polyclonal anti-ATM antibodies.



Probed with Ab-3:
Lane 1: protein sample from a normal cell line.
Lane 2: protein sample from an AT heterozygous cell line.
Lane 3: protein sample from an AT cell line.

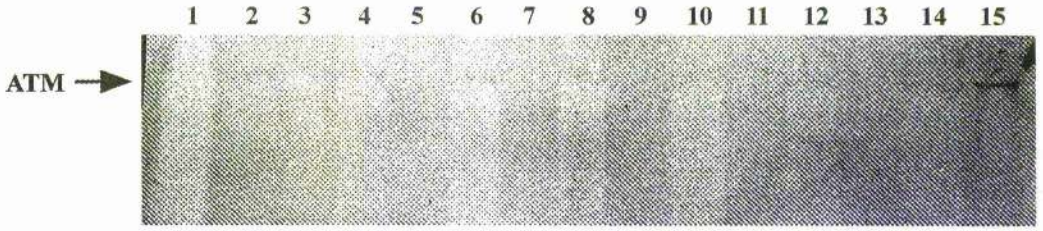
Probed with ATM.B:
Lane 4: protein sample from a normal cell line.
Lane 5: protein sample from an AT heterozygous cell line.
Lane 6: protein sample from an AT heterozygous cell line.

Probed with ATM.V:
Lane 7: protein sample from a normal cell line.
Lane 8: protein sample from an AT heterozygous cell line.
Lane 9: protein sample from an AT cell line.

Probed with FP-8:
Lane 10: protein sample from a normal cell line.
Lane 11: protein sample from an AT heterozygous cell line.
Lane 12: protein sample from an AT cell line.

Shown is a montage of immunoblots each probed with one of the four polyclonal anti-ATM antibdies. Although Ab-3 produces a strong ATM signal, there is a band of similar size in the homozygote sample (lane 3). ATM.V and FP-8 both detect a band which is not present in the homozygote sample but, as with ATM.B, the band is not significantly stronger than the non-specific banding.

Figure 4.2: Placental ATM does not pellet at low speed centrifugation.



Crude placental extract was initially spun at 900rpm for 6 mins to remove solid tissue:
Lane 1: pellet after 6 mins at 900rpm.
Lane 2: supernatant after 6 mins at 900rpm.

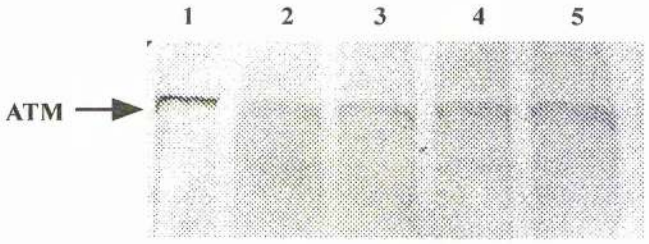
The supernatant was then centrifuged at 10,000rpm for 20 mins to clarify of small particles.
Lane 3: pellet after 20 mins at 10,000rpm.
Lane 4: supernatant after 20 mins at 10,000rpm.

The clarified extract was centrifuged at 23,100g for the following times:
Lane 5: pellet after 10 mins.
Lane 6: supernatant after 10 mins.
Lane 7: pellet after 20 mins.
Lane 8: supernatant after 20 mins.
Lane 9: pellet after 40 mins.
Lane 10: supernatant after 40 mins.
Lane 11: pellet after 60 mins.
Lane 12: supernatant after 60 mins.
Lane 13: pellet after 120 mins.
Lane 14: supernatant after 120 mins.

Lane 15: positive control; protein extract from a normal cell line.

A crude placental protein sample was prepared and clarified of solid material in two stages. The supernatant was then centrifuged for different lengths of time at 23,100g. Pellets were resuspended in a volume of 1x SDS buffer equivalent to the volume of supernatant plus 4x SDS buffer. Aliquots were run on a 5% SDS gel, which was blotted and probed with 2C1 (anti-ATM). Only weak bans are seen, and there is no ATM detected in any pellet, even after 2hrs at 23,100g.

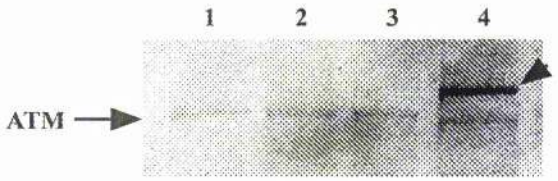
Figure 4.3: An ATM homologue is expressed in ionising radiation sensitive hamster cells.



Lane 1: positive control; protein extract from a normal cell line.
Lane 2: irs1 protein sample.
Lane 3: irs1 protein sample.
Lane 4: irs2 protein sample.
Lane 5: irs2 protein sample.

A fresh normal cell extract was run as a positive control alongside extracts from hamster cells displaying either the ionising radiation sensitive 1 (irs1) or irs2 pheneotype. The hamster extracts had been prepared by another reasearcher and had been stored for over a year. Weak bands of the same size as ATM are seen in both cell types. The intensity of the signal may be due to differences in the extraction protocol or the age of the samples.

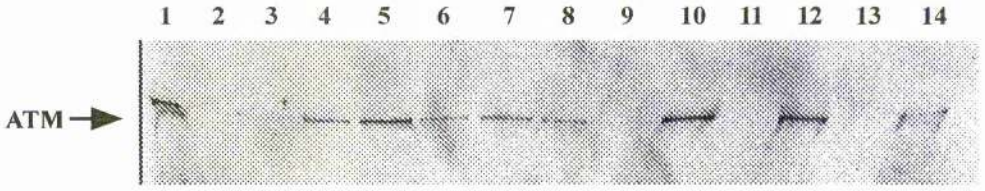
Figure 4.4: The effect of PHA stimulation on the expression of ATM by human T lymphocytes.



Lane 1: unstimulated cells
Lane 2: 24hrs post stimulation
Lane 3: 48hrs post stimulation
Lane 4: 72hrs post stimulation

Total cell extracts were prepared from unstimulated T cells and from T cells at different intervals after stimulation with PHA and run on a 5% SDS gel. The gel was blotted and the membrane probed with 2C1. The arrowhead indicates a band detected by the ATM antibody, which is only present in cells 72hrs after stimulation with PHA.

Figure 4.5: Lectin binding properties of the ATM protein.

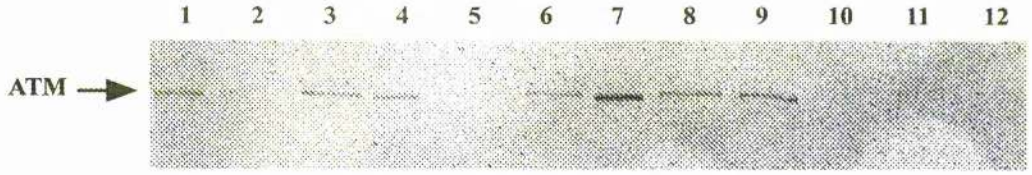


Lane 1: protein extract from AT heterozygote AD
Lane 2: size markers
Lane 3: AD protein, fraction bound to concanavalin A
Lane 4: AD protein, fraction unbound to con A
Lane 5: AD protein, fraction bound to dolichos biflorus
Lane 6: AD protein, fraction unbound to dolichos buiflorus
Lane 7: AD protein, fraction bound to glycine max
Lane 8: AD protein, fraction unbound to glycine max
Lane 9: AD protein, fraction bound to lens culinaris
Lane 10: AD protein, fraction unbound to lens culinaris
Lane 11: AD protein, fraction bound to tetragonolobus purpureas
Lane 12: AD protein, fraction unbound to tetragonolobus purpureas
Lane 13: AD protein, fraction bound to triticum vulgaris
Lane 14: AD protein, fraction unbound to triticum vulgaris

Samples of normal protein were tumbled overnight with one of a selection of agarose-immobilised lectins. The bound proteins were removed by boiling in 1x loading buffer and run on a 5% SDS gel alongside the supernatants. Blotting and probing with 2C1 shows that ATM binds to dolichos biflorus and glycine max.

Figure 4.6: Western blot analysis of whole protein extracts treated with endoglycosidase.

a) protein samples were tumbled overnight with dolichos biflorus beads. The bound and unbound fractions were run on a 5% SDS gel and the blot probed with antibody 2C1. Untreated ATM binds to the lectin (lane 8), while ATM treated with endoglycosidase H does not (lanes 2 and 5).



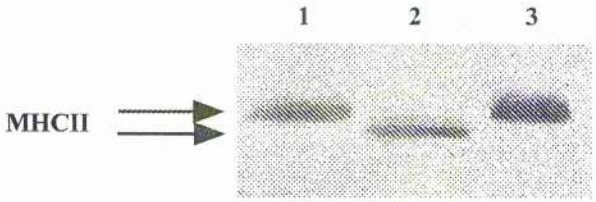
Lane 1: normal protein sample treated with endoglycosidase H
Lane 2: as lane 1, fraction bound to dolichos biflorus
Lane 3: as lane 1, fraction unbound to dolichos biflorus

Lanes 4, 5, 6: as lanes 1, 2, 3, but samples were acidified with NaAc pH5.5 prior to endoglycosidase treatment

Lane 7: normal protein sample
Lane 8: fraction of normal protein sample bound to dolichos biflorus
Lane 9: fraction of normal protein sample unbound to dolichos biflorus

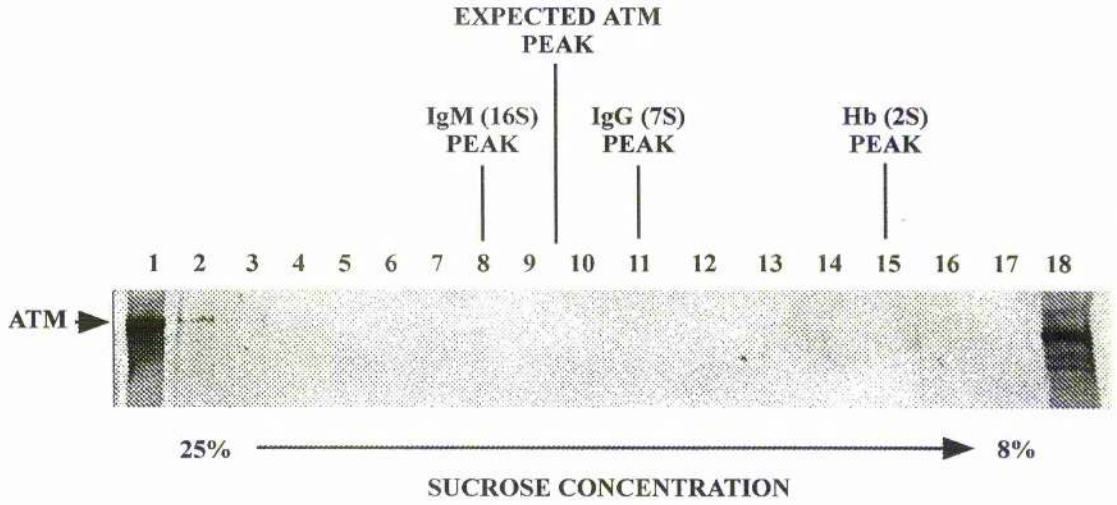
Lane 10: ATM homozygote protein sample
Lane 11: fraction of homozygote sample bound to dolichos biflorus
Lane 12: fraction of homozygous sample unbound to dolichos biflorus

b) a protein sample treated with endoglycosidase H was run on a 10% SDS gel alongside untreated samples. The blot was probed with antibody DA6.147 and the size difference between untreated and treated MHC class II confirms efficient deglycosylation has taken place.



Lanes 1 and 3: protein samples from AT heterozygous lymphoblastoid cell lines
Lane 2: protein sample, treated with endoglycosidase, from an AT heterozygous cell line

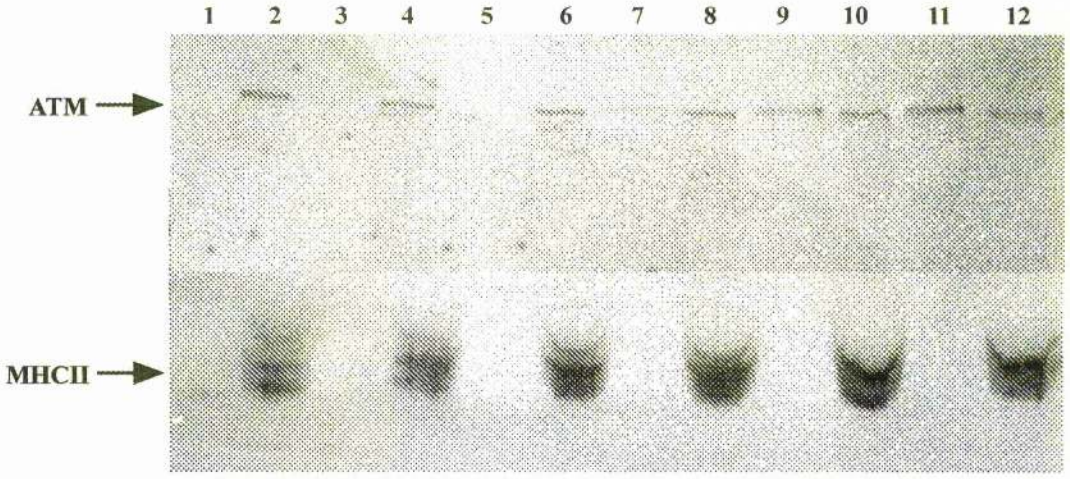
Figure 4.7: Velocity sedimentation of a normal protein extract.



Lanes 1 and 18: whole protein extract from normal lymphoblastoid cells.
Lanes 2 to 17: fractions of normal cell extract separated by velocity sedimentation.

A 5% SDS gel of surose gradient fractions collected after velocity sedimentation of a normal protein sample. After 16hrs centrifugation at 28k rpm in an SW28 rotor. ATM protein can be detected only in the bottom fraction (lane 2). ATM was expected to be found in fractions 9 and 10, based on the positions of IgG, IgM and haemoglobin within a gradient run in parallel.

Figure 4.8: ATM protein is found in the pellet after low speed centrifugation of a protein sample.



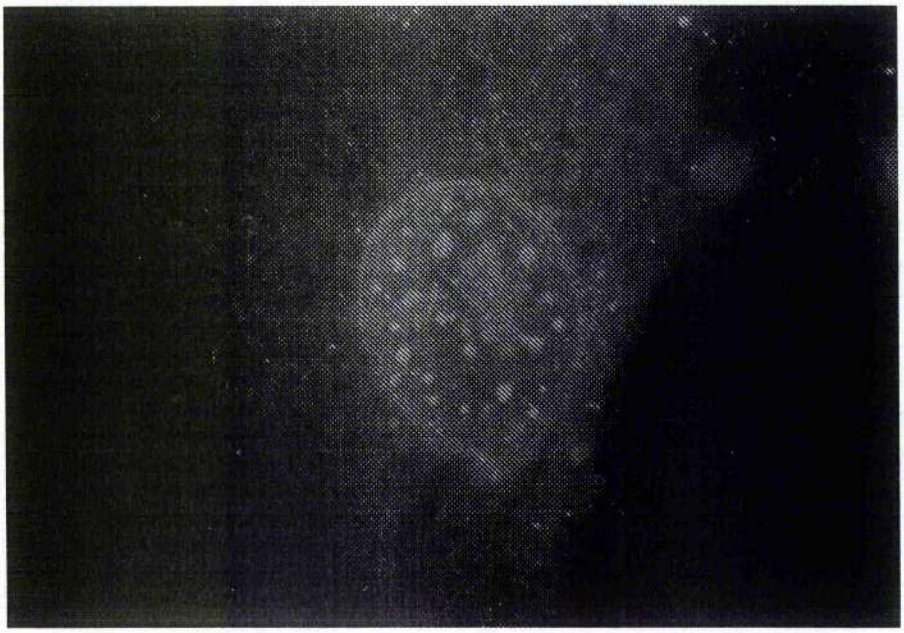
Crude cell extract was initially centrifuged at 10,000rpm for 20 mins to clarify.
Lane 1: pellet after 10,000rpm, 20 mins.
Lane 2: supernatant (clarified extract).

The clarified extract was centrifuged at 23,100g for the following times:
Lane 3: pellet after 10 mins.
Lane 4: supernatant after 10 mins.
Lane 5: pellet after 20 mins.
Lane 6: supernatant after 20 mins.
Lane 7: pellet after 40 mins.
Lane 8: supernatant after 40 mins.
Lane 9: pellet after 60 mins
Lane 10: supernatnt after 60 mins.
Lane 11: pellet after 120 mins.
Lane 12: supernatant after 120 mins.

Samples of normal protein extract were centrifuged for different lengths of time. Each pellets were resuspended in a volume of SDS loading buffer equivalent to the volume of the supernatant plus 4x loading buffer. Aliquots of each were run in duplicate on a 5% and 10% SDS gels which were blotted and probed with 2C1 (anti-ATM) and DA6.147 (anti-MHC class II) respectively. It can clearly be seen that, while MHC class II remains in the supernatant even after the longest spin, ATM is visible in the pellet after only 40 minutes at 15,000rpm (23,100g). Most ATM is detected in the pellet after 2hrs at 23,100g.

Figure 4.9: Nuclear structures in normal fibroblasts stain with anti-ATM while AT cells show no specific staining.

Normal or AT fibroblasts were grown overnight on a coverslip. After probing with FP8 (anti-ATM), cells were stained with an FITC-conjugated secondary antibody and viewed under a fluorescence microscope.

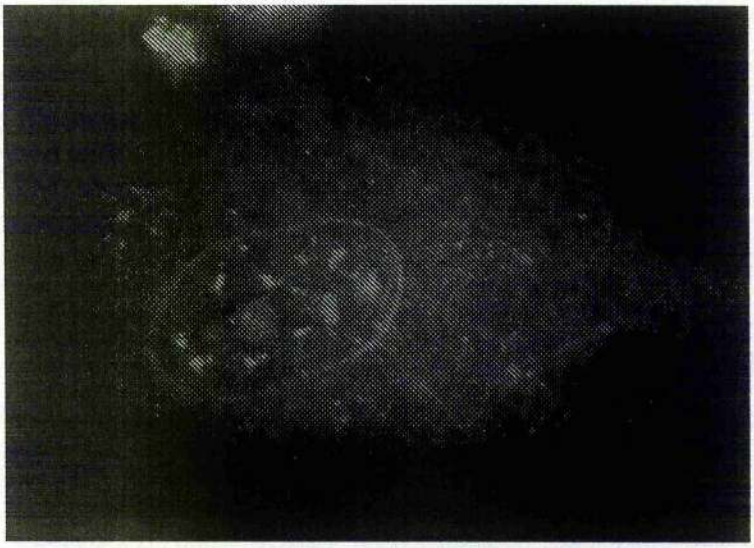


a) a number of specific structures in the nucleus of a normal cell stain with anti-ATM.

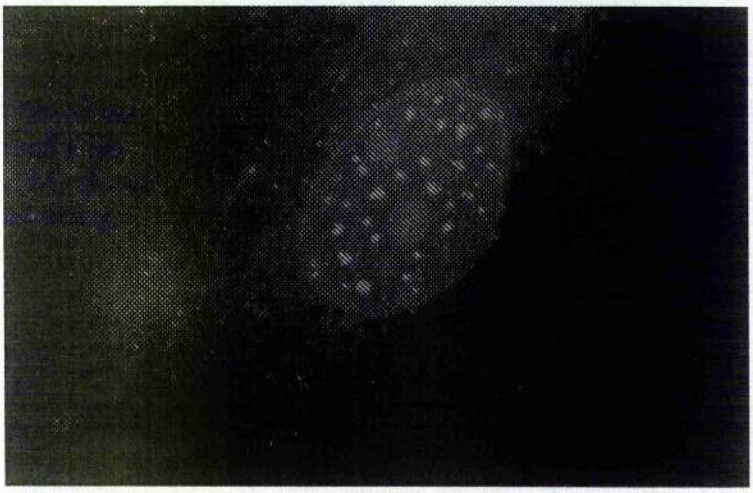
b) the nuclei of AT fibroblasts do not stain strongly with anti-ATM.

Figure 4.10: The number of nuclear structures staining with anti-ATM varies in normal fibroblast cells.

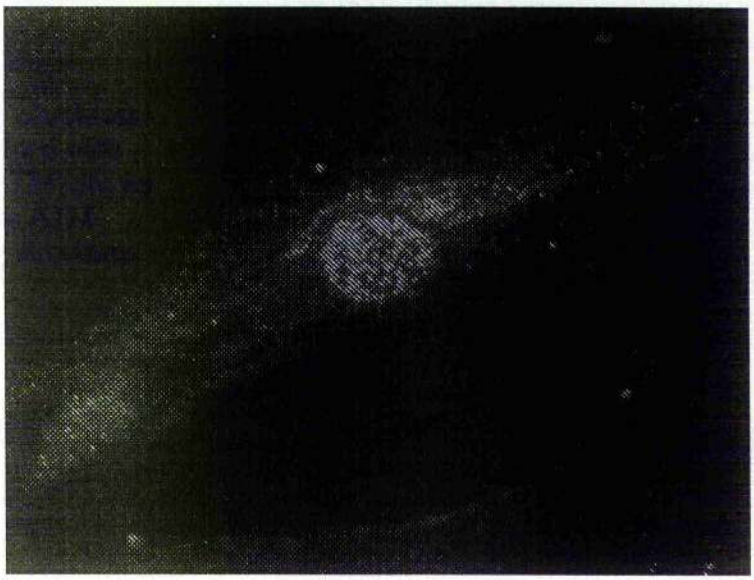
a) a normal fibroblast nucleus stained with FP8 (anti-ATM) shows 16 ATM-containing structures.



b) a normal fibroblast nucleus stained with FP8 (anti-ATM) shows 36 ATM-containing structures.



c) a normal fibroblast nucleus stained with FP8 (anti-ATM) shows innumerable ATM -containing structures.



Chapter 5:

Discussion and Conclusions

5.1: Introduction

There is much dispute about the relative risk of breast cancer in AT heterozygotes, with estimates ranging from no risk to a risk 6.8 times that of the general population (Swift *et al*, 1987). The results reported here provide evidence that the two most common mutations in the British Isles do not contribute appreciably to the breast cancer burden in Scotland. Also presented is evidence that the ATM protein is found in both a glycosylated and an unglycosylated form and that there may be more than one splice variant. These different forms of the protein may be responsible for different functions of the gene.

5.2: The two Celtic ATM mutations do not appear to be found at an elevated frequency among Scottish breast cancer patients

Enzymatic mismatch cleavage method of mutation detection by the endonuclease VII enzyme (Solaro *et al*, 1993) appeared to be an ideal technique for studying ATM mutations. The ability to detect most mutations within a relatively long sequence (as long as the cleavage products can be distinguished from the normal PCR amplimer) would be attractive, given the size (12.8kb, Uziel *et al*, 1996) of the ATM transcript and the fact that many different mutations are found along the length of the gene. Unfortunately, this technique proved to be less efficient than expected, and it was therefore decided to concentrate on the detection of specific mutations.

In order to design a rapid test for the exon-skipping Celtic mutation, the mutations had first to be sequenced at the genomic level. As exon 20 was missing from the

transcript but not from the gene, it was expected the mutation would occur in the splice acceptor site preceding the skipped exon. A comparison of the normal and mutant intron 19 was successfully achieved by sequencing a normal and a heterozygous DNA sample with a reverse primer within exon 20 and forward primers sequentially closer to the intron 19/ exon 20 boundary. The results revealed that, as expected, the genomic lesion responsible was a three base pair deletion within a sequence which corresponds to the general form of a splice acceptor site.

Rapid genomic DNA tests were then designed for the two Celtic mutations. In each case, analysis with the Wisconsin software package predicted the loss of a restriction site close to the mutation which could be detected by digestion with the appropriate enzyme (MseI for the exon-skipping mutation; ApoI for the Δ9bp deletion) and running the fragments on an agarose gel. Once screening was underway, a more time-efficient method of detecting the two mutations was used, with the PCR products being loaded sequentially on a sequencing-type gel, blotted onto nylon which was probed with a radiolabelled primer and viewed by autoradiography. The inclusion of positive controls indicated that both types of test were reliable, with the latter being preferred for ease. These tests were not specific for the Celtic mutations, being able to detect any other difference in amplimer size between normal and mutant sequences, but, had any difference been observed, the appropriate RFLP test would have been performed to confirm this was due to the Celtic mutations.

The results presented in chapter 3 provide strong evidence that the two Celtic ATM mutations (Byrd *et al*, 1996; Wright *et al*, 1996) do not contribute appreciably to the breast cancer burden in Scotland. Based on figures accepted at the time this study

began, it was expected to find at least two Celtic mutations in the 412 samples tested. If these mutations confer upon the carrier a predisposition to breast cancer, it would be expected that this figure would have been even greater. Consistent with material published after this study was complete (Stankovic *et al*, 1998), these data indicate that even the most common ATM mutations in the United Kingdom constitute only a small percentage of all mutations found in the UK. At the time this study began, 12 Celtic mutations (8 of the 9bp deletion; 4 of the splice site mutation) had been identified out of a total of 103 mutations from individuals of a wide variety of ethnic origins, and of these 12, seven were of Celtic descent. Since then, many more ATM mutations have been reported and up to 1998 a total 106 mutations had been found in British patients alone (Teletar *et al*, 1996; Gilad *et al*, 1996; Stankovic *et al*, 1998), but there still remained only the 12 identified Celtic mutations. The initial figures were therefore misleading in terms of the number of Celtic mutations expected in a Scottish population. The results presented in chapter 3 have two possible explanations: the Celtic mutations are found in the general Scottish population at a frequency lower than predicted in 1996; the Celtic mutations do not confer any predisposition to breast cancer.

The published reports on the relationship between breast cancer and the ATM gene are divided between those supporting the case for a strong link (eg Swift *et al*, 1987; Morrell *et al*, 1990) and those which suggest there is no link (eg Vorechovsky *et al*, 1996a, Chen *et al*, 1998). As would be expected in such a situation, review papers suggest an intermediate predisposition to breast cancer among AT heterozygotes (Easton *et al*, 1994). However, few individual papers reach this conclusion, suggesting either that the interpretation of results plays a significant role in the

conclusions of reports or that there is a consistent discrepency in the efficiency of detection methods used. This problem is exacerbated by the relatively small sample sizes in most of these studies and the wide confidence intervals (CI). In most cases the results are consistent with both schools of thought within 95% CI (reviewed in Easton, 1994). It therefore seems likely that the question will not be answered satisfactorily until a large scale study has been performed in which a large number of breast cancer patients are screened for all ATM mutations, using a technique with a high detection efficiency.

AT patients are particularly susceptible to lymphoid malignancies and there is strong evidence that impaired expression of the ATM gene is a feature of many B-cell chronic lymphocytic leukaemias (B-CLL) (Stankovic *et al*, 1999). Of 20 tumours studied, 8 were found to express reduced levels of ATM protein or none at all. Heterozygous germline mutations were found in two patients and *de novo* mutations were found in the tumours of these two patients and six others. There is also evidence of inactivation of the ATM gene by somatic ATM mutations and LOH of the ATM region in T-cell prolymphocytic leukaemia (T-PLL) (Vorechovsky *et al*, 1997; Stilgenbauer *et al*, 1997; Stoppa-Lyonnet *et al*, 1998). These results are consistent with the definition of ATM as a tumour-suppressor gene. It seems plausible that somatic inactivation could also be involved in some breast cancers, and there is evidence of LOH in the region of the ATM gene (Carter *et al*, 1994; Hampton *et al*, 1994; Kerangueven *et al*, 1997). Vorechovsky *et al* (1996a) reported LOH in 47% of 36 breast cancer tumours, but failed to detect any mutations in the remaining alleles, apparently ruling out the ATM gene as the tumour-suppressor involved (though it is, of course, possible that two intact copies of the gene are required for its tumour-

suppressor function). This would suggest that, if AT carriers are at increased risk of breast cancer, the mechanism by which an ATM mutation contributes to tumorgenicity in the breast is different to the manner in which its somatic inactivation may cause T-PLL.

A significant point could be the assertion that some ATM mutations may be associated with carcinogenesis while others are not (Stankovic *et al*, 1998) combined with the evidence that mutant ATM protein has a dominant negative effect over normal protein (Zhang *et al*, 1997; Scott *et al*, 1998). Only a small number of ATM mutations result in expression of a mutant protein (Lakin *et al*, 1996; Watters *et al*, 1997; Brown *et al*, 1997) and these tend to cause a mild AT phenotype in patients. Such a mutation may increase the chance of cancer among carriers by compromising the activity of the normal protein, and yet AT families with a mild phenotype mutation may not be efficiently ascertained if classical AT features are not observed. In such an instance, important information relating breast cancer predisposition to AT carrier status may be overlooked.

5.3: Expression of the ATM protein

The evidence of sequence homology of the C terminus of ATM to the functional domain of the PI3-K gene family (Savitsky *et al*, 1995a; Zakian, 1995) suggests a role for ATM in signal transduction and cell cycle checkpoint control. Its association with p53 (Kastan *et al*, 1992; Khanna and Lavin, 1993; Canman *et al*, 1994) also indicates that ATM may be involved in regulation of apoptosis. It is evident from the high cancer rate among AT patients (Morrell *et al*, 1986) that disruption of the ATM gene

can be a contributory factor in tumorigenesis, and it seems likely that even partial disruption (ie in heterozygotes) of a gene mediating both cell growth and cell death could cause a predisposition to cancer. It is therefore important to understand the mechanisms by which ATM operates.

Five anti-ATM antibodies were acquired for immunological detection of the ATM product and their specificity for detection of ATM in western blot experiments was compared. Although all of the antibodies detected a protein of around 350kDa, experiments showed that the monoclonal antibody, 2C1, provided the most consistent results with the lowest background staining.

Savitsky *et al* (1997) reported the presence of alternative splice varients of the ATM gene, but there has been little discussion of the importance of this. Evidence has been presented here (section 4.4) that T lymphocytes express ATM constitutively, but 72 hours post PHA stimulation, a second protein band was detected by an anti-ATM antibody. The results of this experiment could be repeated, but inconsistently. While the possibility that this is an artefactual result cannot be ruled out, the fact that no general non-specific banding was detected in the 72hr sample, and that the new band was as strong as the original, suggests that this band does represent a form of the ATM protein. This could indicate that normal cells express different species of ATM protein depending on the state of growth stimulation. This is potentially significant as, if a mutation occurs in an alternative exon, levels of ATM protein may appear normal in unstimulated cells, while dividing cells are unable to produce an alternative ATM protein which may play an important role in cell division. It had been intended to investigate the expression of ATM in lymphocytes from breast cancer patients who

had been selected for a high radiation sensitivity as measured by the G_2 assay. It had been arranged that blood samples would be supplied from Ninewells Hospital, Dundee, but unfortunately, due to a change in clinical personnel, the samples were not provided. It would, however, be extremely interesting to investigate ATM expression in response to PHA among breast cancer patients. If a difference between expression in cells of a breast cancer patient and a normal individual was detected, differences in ATM transcripts could also be looked at. The presence or absence of specific domains at particular times during the cell cycle could give clues to the functions of these domains.

When velocity sedimentation experiments were performed on protein samples from normal cells (section 4.6), the ATM protein was consistently found in the bottom fraction, despite the larger protein IgM being found in the middle of the gradient. It was later found (section 4.7) that ATM could easily be pelleted by centrifugation at the relatively low speed of 23,100g for 2hrs. It has been shown that the ATM protein associates constitutively with p53 (Watters *et al*, 1997) and c-Abl (Shaffman *et al*, 1997) and there are unpublished data from a number of labs which suggest that it is part of a large complex (E. Lee, personal communication). If this complex is so large that it cannot be properly solubilised in RIPA buffer, this may explain the rapid sedimentation of the ATM product seen in sections 4.6 and 4.7. Possible candidates for the complex are: that which contains RNA polymerase II (RNAPII) and RPA and is involved in DNA repair (Maldonado *et al*, 1996; Lavin, 1998); the complex containing hMre11 and hRad50, which forms in response to DNA double strand breaks (Maser *et al*, 1997; Dolganov *et al*, 1996).

As a portion of ATM did not sediment in these experiments, there may be a portion of the protein which is not bound in the complex. If this is the case, there are three possible reasons: there is an excess of the ATM protein over the other components of the complex; the ATM protein is present in an activated form and an inactivated form (one being found in the complex, the other being free in the cell), the difference being the state of phosphorylation or glycosylation; differential splicing gives rise to more than one species of ATM protein, and these play different roles within the cell. Another explanation for the two sizes of ATM product could be post-translational processing rather than alternative splicing.

Chen and Lee (1996) have shown that the ATM product can be phosphorylated and evidence presented in section 4.5 shows that it can also be glycosylated. Glycosylation has been shown to affect the state of activation of other proteins (Meynial, Longo and Combes, 1995). Most ATM product was shown to bind to the lectins Dolichos biflorus and Glycine max, indicating the presence of the N-linked sugar groups α-galNAc and galNAc. The observation that the lectin binding properties of ATM are lost after digestion with endoglycosidase H confirms that this is not an artefactual result.

It was unfortunate that placenta failed to provide a reliable source of ATM protein. Experiments using placental extracts gave widely differing results with respect to ATM expression. Most samples showed little ATM expression, while others showed none at all, and one sample had ATM levels similar to a sample from normal lymphoblastoid cells. This variation could be due to differences in the freshness of the placenta, the number of white blood cells in the tissue or the extraction efficiency.

The placental ATM protein did not appear to behave in the same way as ATM from lymphoblastoid cells, neither binding to Dolichos biflorus, nor pelleting at relatively low speed centrifugation. Placenta seems unlikely to be a source of ATM protein for further immunological studies, but the fact that expression of ATM in placenta appears different from other normal cells may be of interest. While it is generally accepted that ATM is ubiquitously expressed (Lakin *et al*, 1996; Chen and Lee, 1996; Watters *et al*, 1997; Brown *et al*, 1997), there is also evidence that elevated levels are expressed in certain developing tissues (Chen and Lee, 1996). It is, therefore, also possible that ATM expression is down-regulated in placenta once it is no longer of use.

The fact that a protein of similar size to ATM was detected in samples from hamster cells displaying the irs1 and irs2 phenotypes suggests that these phenotypes do not correspond to human AT (figure 4.3). The fact that there is a difference in intensity of the band between the control sample and the test samples could be due to a partial disruption of the ATM homologue in the hamster cells, but is more likely to be due to one of the following: a difference in the preparation protocol, as these samples had been prepared earlier by another researcher; the age of the samples, as they had been stored for over one year; minor differences between human ATM protein and the hamster homologue affect the binding efficiency of the antibody. An experiment could be carried out using fresh extracts from irs1 and irs2 cells and from normal hamster cells to make a more direct comparison.

Preliminary experiments illustrated that the immunostaining techniques used could successfully detect the ATM protein within the cell nucleus, and the punctate

distribution described by Lakin *et al* (1996) was confirmed. A wide variation in the number of structures staining with anti-ATM was observed and it had been intended to investigate this feature further. If the ATM protein is bound to different numbers of structures at specific stages of the cell cycle, this may relate to the putative splice variant seen in section 4.4. ATM is known to be a component of meiotic nodules which may check for homology prior to synapsis (Plug *et al*, 1998) and it is concievable that during mitosis ATM plays a different role as part of a different complex. Perhaps ATM follows the paradigm of BRCA1, which localises to discrete nuclear foci during S-phase, with these foci dispersing after DNA damage (Scully *et al*, 1997). It had also been intended to investigate the pattern seen when immunostaining with anti-cAbl antibody. The c-Abl protein should be associated with the same structures as ATM, but the pattern may be disrupted in AT cells. Unfortunately, damage to the microscope prevented further immunostaining experiments.

5.4: Future work

The suspected link between cancer predisposition and ATM heterozygosity has yet to be proved or disproved, but the information gathered so far should allow a more efficient approach to research in this area. A possible study could screen breast cancer patients for mutations which have clearly been associated with a strong relative risk of breast cancer. The 7271T→G mutation (Stankovic *et al*, 1998), for example, conferred an estimated relative breast cancer risk of 12.7 upon carriers.

Alternatively, research could identify those breast cancer patients who have a cellular radiosensitivity within the expected range of AT heterozygotes (Garoff and Schwarz, 1978; West *et al*, 1995; Mitchell and Scott, 1997) and perform a comprehensive screening process (perhaps even sequencing of the whole gene) on these pre-selected cases. There is evidence that G2-phase cells from many cancer prone conditions exhibit higher yields of X-ray induced chromosome damage than those found in the majority of healthy controls (Sanford *et al*, 1989). If this is a causal relationship, and if different AT mutations confer different levels of cancer predisposition upon carriers, then most AT heterozygotes among breast cancer patients would be detected by this method.

As Swift *et al* (1987, 1990) suggested that certain ATM mutations may be site-specific with respect to cancer predisposition, it could be informative to investigate AT carrier status of sufferers of cancers other than that of the breast. For example, in Costa Rica, where six mutations represent over 95% of all AT mutations (Teletar *et al*, 1998a), a comprehensive screen of patients suffering cancers at a number of different sites could be carried out. The resultant data would be more statistically powerful than existing studies due to the high throughput of a system detecting a few specific mutations and due to the high detection rate.

Clarification of the manner in which the ATM protein acts in the cell is vital in order to unravel the pathways in which it is involved. The fact that most ATM in a normal cell extract is pelleted by centrifugation for 2hrs at 23,100g may be explained by the presence of ATM-containing complexes described by Chen and Lee (1996). It should be possible to determine if this is this is the case by resuspending the pellet in a

volume of RIPA buffer equal to the supernatant and performing parallel experiments on the two samples. Anti-ATM immunoprecipitates from the two samples could be analysed by western blotting for the presence of other proteins involved in the DNA damage response, including c-Abl and p53. If these proteins are present in the immunoprecipitate from the pellet, but not the supernatant, this would confirm that it is a complex that is sedimenting and would also suggest that the portion of ATM remaining in the supernatant is not bound in a complex. Similarly, experiments to compare the lectin binding properties of the ATM in the pellet and supernatant could identify differences in glycosylation.

Once the nature of the ATM protein present in a normal sample has been established, comparisons can be made with the protein present in placental samples and in PHA stimulated human T lymphocytes. It could be very important to determine if there is a difference in the species of ATM expressed, or its state of glycosylation, between the placental tissue (which is no longer functioning) and the stimulated T cells. The fact that AT results in cerebellar degeneration (Boder and Sedgwick, 1958) indicates that ATM plays an important role in non-dividing cells, but another form of the protein may be required for activating checkpoints and maintaining genome integrity.

Due to conservation of the pathway and components involved in checkpoint control between mammals and yeast (reviewed by Elledge, 1996; Carr, 1994), homologues of human checkpoint proteins have been found in S. cerevisiae (eg Siede *et al*, 1996) and S. pombe (eg Al-Khodairy *et al*, 1994). It seems likely that these model systems will continue to prove important tools in research into the processes of checkpoint control.

5.5: Conclusions

The experiments in this study show that ATM exits in several forms: as two sizes; in a glycosylated and an unglycosylated state; bound in a large complex and free within the cell. Rather than experimental artefacts, these may all be of biological significance, or perhaps represent the ATM protein at different stages of maturation.

The question of whether heterozygosity for a mutation in the ATM gene can predispose an individual to cancer is an important one, due to the relatively high frequency of AT carriers. However, the weight of evidence seems to suggest that the contribution to the burden of breast cancer of individuals carrying a single germline ATM mutation is not great, and in light of this it may be more efficient to consider ATM heterozygosity in a wider context. Disruption of the DNA damage response pathway (including damage detection, signal transduction, cell cycle arrest and repair) in which ATM is involved is undoubtably a potential cause of tumorgenicity. A more profound understanding of this response should allow the conflicting reports on the involvement of ATM carrier status in breast cancer to be rationalised.

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